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Lignocellulose biomass degradation by marine microorganisms

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ABSTRACT

Lignocellulosic containing plants are those types of biomass that include wood, agricultural residues and paper wastes. They are composite polymeric material containing primarily cellulose, hemicellulose and lignin. Lignin is a complex three dimensional polymer composed of six carbon phenolic rings, with various carbon chains and other chemical functionalities. Lignin is non crystalline in nature and serves to bind the cellulosic fibers, making the lignocellulosic mass resistant to enzymatic degradation. Lignin is hydrolyzed by ligninases, thus releasing cellulose which can be used to produce ethanol. In the present work microorganisms were isolated from coastal areas of Tamilnadu and Karnataka and subjected to screening for lignocellulase activity. The organisms exhibiting maximal degradation were identified using 16s r RNA technique as Bacillus pumilus and Mesorhizzobium sps., two fungal species were identified as Aspergillus niger and Trichoderma viride. These organisms were used to breakdown 18 different lignocellulosic biomasses for a period of sixty days. The organisms exhibited different levels of degradation at different time intervals in various substrates. Maximum degradation was observed in about ten substrates commonly degraded by all the four organisms in the first three weeks. It was observed that Eucalyptus, Maize leaves, Crotalaria, Honge, Mango leaves and Jamun leaves showed a great potential as substrates which were maximally degraded and could be utilized for further work to obtain alternate fuel. Mesorhizobium sps., showed better degradation in all the substrates compared to the other organisms.

Key Words: LignoCellulose, LignoCellulases, *Bacillus pumilus*, *Mesorhizzobium sps.*, *Aspergillus niger* and *Trichoderma viride*.

INTRODUCTION

Energy conversion, utilization and access underlie many of the great challenges of our time, including those associated with sustainability, environmental quality, security and poverty. Biotechnology could give rise to important new energy conversion processes. Resources for biological conversion of energy to forms useful to humanity includes majorly the plant biomass. Among forms of plant biomass, lignocellulosic biomass is particularly well suited for energy applications because of its large scale availability, low cost and environmentally benign production [26].

Efficient ethanol production processes and cheap substrates are needed. Current ethanol production processes using crops such as sugar cane and corn are well established; however utilization of a cheaper substrate such as lignocelluloses could make bio-ethanol more competitive with fossil fuel, the processing and utilization of this substrate is complex and there is a requirement for efficient micro-organisms to ferment a variety of sugars[16].

Utilization of lignocelluloses as a substrate for ethanol production has a barrier in its complex structure, which resists degradation, lignocelluloses is composed of three main fractions: cellulose(45%), hemicelluloses(30%) and lignin(25%)[33]. cellulose is the most abundant polymer and can be hydrolyzed chemically or enzymatically. Hemicelluloses is a highly branched heteropolymer and is more easily hydrolyzed than cellulose.[3] lignin is the most abundant aromatic polymer in nature, is a macromolecule of phenolic character[32] and binds cellulose and hemicelluloses making it resistant to degradation.

Lignin is formed in vascular plant cell walls by the oxidative coupling of several related phenylpropanoid precursors: coniferyl alcohol, sinapyl alcohol, and *p*-hydroxycinnamyl alcohol.

Peroxidases or Iaccases in the plant cell wall oxidize these monomers by one electron, yielding transient resonance-stabilized phenoxy radicals that then polymerize in a variety of configurations. The possible ways that the precursors can couple can be portrayed on paper simply by drawing the conventional resonance forms of the phenoxy radicals, and then by linking the most important of these in various pair wise combinations. This subject has been extensively reviewed [1; 15], and it will suffice here simply to say that lignin consists primarily of the intermonomer linkages. Lignin is covalently associated with hemicelluloses in the cell wall via numerous types of linkage. Among the most important are ether bonds between the benzylic carbon of lignin and the carbohydrate moiety, ester bonds between the benzylic carbon of lignin and uronic acid residues, and lignin-glycosidic bonds. In graminaceous plants, hydroxycinnamic acid residues are frequent in the lignin, and are attached to hemicelluloses via ester linkages. The matrix of lignin and hemicellulose encrusts and protects the cellulose of the plant cell wall [17]. Fungi that degrade lignin are faced with several problems. Since the polymer is extremely large and highly branched, ligninolytic mechanisms must be extracellular. Since it is interconnected by stable ether and carbon–carbon bonds, these mechanisms must be oxidative rather than hydrolytic. Since lignin consists of a mixture of stereoirregular units, fungal ligninolytic agents have to be much less specific than typical biological catalysts. Finally, the fact that lignin is insoluble in water limits its bioavailability to ligninolytic systems and dictates that ligninolysis is a slow process

Lignin peroxidases:

Lignin peroxidases (LiPs) were the first ligninolytic enzymes to be discovered [11; 23]. They occur in some frequently studied white rot fungi, e.g. *Phanerochaete chrysosoporium*, *Trametes versicolor* and *Bjerkandera* sp. [24; 18; 27] but are evidently absent in others, e.g. *Dichomitus squalens*, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* [12, 20, 27, 30]. LiPs resemble other peroxidases such as the classical, extensively studied enzyme from horseradish, in that they contain ferric heme and operate via a typical peroxidase catalytic cycle [24, 12]. That is, LiP is oxidized by hydrogen peroxide to a two-electron deficient intermediate, which returns to its resting state by performing two one-electron oxidations of donor substrates. However, LiPs are more powerful oxidants than typical peroxidases are, and consequently oxidize not only the usual peroxidase substrates such as phenols and anilines, but also a variety of non-phenolic lignin structures and other aromatic ethers that resemble the basic structural unit of lignin [21]. The simplest aromatic substrates for LiP are methoxylated benzenes and benzyl alcohols, which have been used extensively by enzymologists to study LiP reaction mechanisms.The hydrogen peroxide-dependent oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde is the basis for the standard assay used to detect LiP in fungal cultures [25].

The LiP-catalysed oxidation of a lignin substructure begins with the abstraction of one electron from the donor substrate's aromatic ring, and the resultingspecies, an aryl cation radical, then undergoes a variety of postenzymatic reactions.[22, 31, 14, 24]. Synthetic polymeric lignins are also cleaved at this position by the enzyme *in vitro*, in a reaction that gives net depolymerization [13]. These results strongly support a ligninolytic role for LiP, because C□$_{\alpha}$□-C□$_{\beta}$cleavage is a major route for ligninolysis in many white rot fungi [24]. Other LiPcatalysed reactions that accord with fungal ligninolysis *in vivo* include aromatic ether cleavage at C_{α} and C_{β}□□-oxidation without cleavage. It has been pointed out that ionization of the aromatic ring to give a cation radical is also what occurs when lignin model substrates are analysed in a mass spectrometer, and indeed the fragmentation pattern obtained by this procedure is similar to that obtained when LiP acts on lignin structures [9].

For lignocelluloses to be amena to fermentation it needs to undergo treatments that release its monomeric sugars which then can be converted to ethanol by a micro-organism. Two main steps are: 1.A pretreatment physical or chemical that releases hexoses and pentoses from hemicelluloses. 2. An enzymatic treatment that generates glucose from cellulose. There is no micro-organism currently available that can utilize lignin monomers for ethanol production [16].

The marine biosphere is one of the earth's richest innumerable habitats. Marine micro-organisms are considered as untapped sources of metabolites and products with novel properties. They have a diverse range of enzymatic and are capable of catalyzing various biochemical reactions. Thus, there is enormous scope for investigations to explore the possibilities of deriving new products of economic importance from potential marine micro-organisms. Considering the fact that marine environment is saline in nature it could provide rare and unique microbial products, particularly the enzymes that could be safely used[28].

The microbial degradation of lignocellulose components is not well characterized in the oceans as it is in terrestrial systems. In the process of searching for useful natural products, it is generally agreed that a diverse and less exploited repertoire of microbes is essential to obtain a variety of novel metabolites. The potential contribution of marine sources to the discovery of new bioactive molecules was recently recognized[10].

MATERIALS AND METHODS

SAMPLING AND ISOLATION

Different marine samples were collected from Tamil nadu and Karnataka sea coast. These included sea water samples, wood scrap, rock scrap, sand, algae/ sea weeds and sediment samples. The samples were collected in sterile polythene bags and were preserved in refrigerator until further studies. Standard microbiological methods were followed for the purpose of isolation. [4] One milliliter of the desired dilution was transferred aseptically into Marine agar/Potato dextrose agar plates. Plates were incubated at 37c/ room temperature for 5-10days. After incubation, the organisms were purified on Marine agar/Potato dextrose agar slants and plates.

CELLULOLYTIC ENZYME ASSAYS:

A single agar disc cut from the actively growing colony margin of a culture was used to inoculate each assay medium.

Dye staining of carboxymethylcellulose agar (CMC agar):

CMC is a substrate for endoglucanase and so can be used as a test for endoglucanase and β-glucosidase activity. This assay is a good indicator of cellulolytic ability since endoglucanase is generally produced in larger titres by microorganisms than cellobiohydrolase [6, 5, 29].

CBM medium supplemented with 2% w/v low viscosity CMC and 1.6% w/v agar.Aseptical transfer to the Petri dishes.Inoculated with test organism.Incubated at room temperature for 5 to 10 days. Plate flooded with 2% w/v aqueous Congo red (C.I.22120) and is left for 15 mins. Stain is removed and the agar surface is washed with distilled water.Plate is flooded with 1M NaCl to destain for 15mins.CMC degradation around the colonies appear as yellow-opaque area against a red colour for undegraded CMC.[6, 5, 29]

DYE STAINING OF XYLAN AGAR:

XBM medium which is incorporated with 4% w/v xylan and 1.6% w/v agar was used and aseptically transfered to the Petri dishes, Inoculated with test organism and incubated at room temperature /37 oC for 5 to 10 days. Plates were flooded with iodine stain (0.25% w/v aqueous I2 and KI). Stain removed after 5 min , agar surface washed with distilled water.Xylan degradation around the colonies appeared as yellow-opaque area against a blue/reddish purple colour for undegraded.[2, 6]

LIGNIN MODIFING ENZYME ASSAYS:

LBM medium, supplemented with 0.25% w/v lignin and 1.6% w/v agar .1 ml of separate sterilized 20% w/v aqueous glucose solution was added to each 100ml of growth medium prepared.Aseptically transferred to the Petri dishes.Inoculated with test organismIncubated at room temperature/37oC for 5 to 10 days. After 5-10 days growth agar plates were stained with 1% ferric chloride and potassium ferricyanide prepared freshly before use.Clear zones around colonies indicating oxidation of phenolic compounds against blue green undegraded lignin.[5]

SUBSTRATE OPTIMIZATION:

Agrowaste samples were collected from different parts of rural Bangalore, like Rice bran, Wheat bran, Bamboo leaves, Banana stem waste and Peepal leaves .The samples were air dried for 3-5 days depending on the moisture content and then powdered.5 gms of the substrate was mixed with 50ml of distilled water in 0.8% Ammonium nitrate in 250 ml Erlenmeyer flasks and sterilized.[8, 19].

The substrates were inoculated with 1ml of overnight incubated Bacterial cultures, and 2 loopfull of 5 day old fungal cultures. The inoculated substrates were Incubated at RT.lignin degradation assay, was carried out at an interval of 7 days to check for lignin degradation.Enzyme activity was calculated based on the conversion of reducing sugar veratryl alcohol to veratyl aldehyde.

Enzyme Activity:

Lignin peroxidase (LiP) activity was measured using the method described[23]. In this method, the increase in absorbance at 310 nm due to oxidation of the veratryl alcohol to veratryl aldehyde is measured.Briefly, a reaction mixture containing 2.2 ml of sodium tartrate buffer (50 mM, pH 4 at 25oC), 40 µl of veratryl alcohol (2 mM) and 240 µl of the culture supernatantwas prepared. Next, the reaction was initiated by the addition of 20 µl of H_2O_2 (0.2 mM) to the reaction mixture. The absorbance was then measured immediately.[7]

RESULTS

In the present work, marine microorganisms were isolated from soil sediments, rock scraps, wood pieces and water samples from the backwaters of Pondicherry (Puducheri), Cuddalore (Tamil Nadu) and Mangalore (coastal Karnataka), using standard microbiological techniques. Forty three fungal isolates, ninety six bacterial isolates and twelve actinomycete species were obtained from marine source.

Table 1: Potential lignocellulose degraders :

Organism	CELLULASES	XYLANASES	LIGNINASES
Bacteria	42	9	11
Fungi	16	4	5
Actinomycetes	11	2	3

Organisms exhibiting maximum zone of clearance for all three substrates were chosen for further studies and have been identified by molecular analysis (16srRNA studies and sequencing) as:

Table 2: Identified Species

SL NO.	Organisms
I. FUNGAL SPECIES	
1	*Aspergillus niger*
2	*Trichoderma viride*
II. BACTERIA	
3	*Bacillus pumilus*
4	*Mesorhizobium sps.*

Lignin oxidation using hydrogen peroxide observed at 310nm exhibited that each of the chosen organisms had the abilities to oxidize lignin which were varied in each organism and substrate

Bacillus pumilus **(Fig 1(a) & 1(b)).**

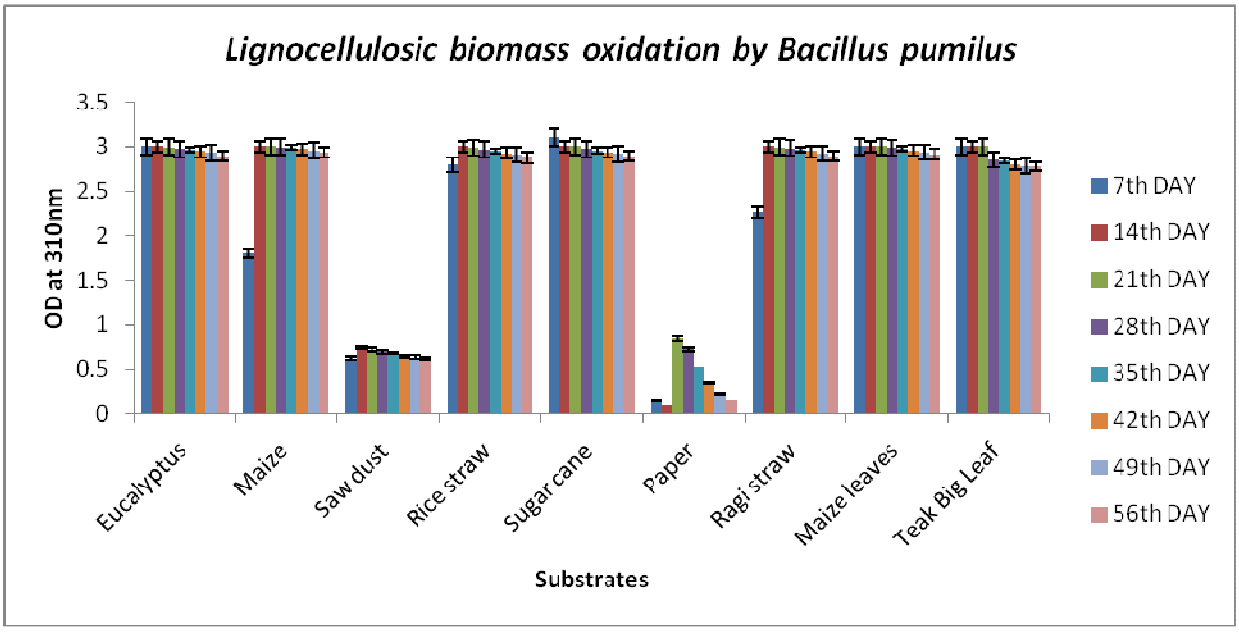

Fig: 1(a)

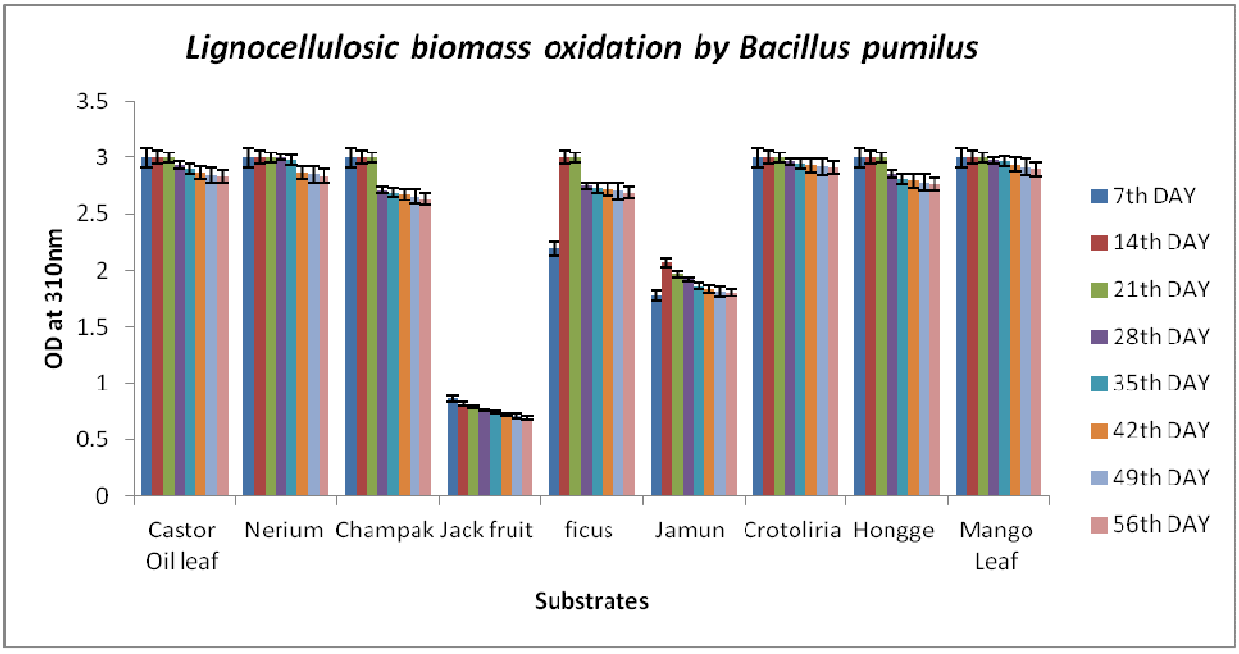

Fig: 1(b)

Bacillus pumilus exhibited maximal oxidation of lignin in 12 substrates, they are: Eucalyptus, Maize, Rice straw, Sugar cane, Maize leaves, Teak big leaves, Castor oil leaf, Nerium, Champak, Crotoliria, Hongge, Mango leaves within the 1st 3 weeks.

Mesorhizobium **(Fig 2(a) & 2(b))**

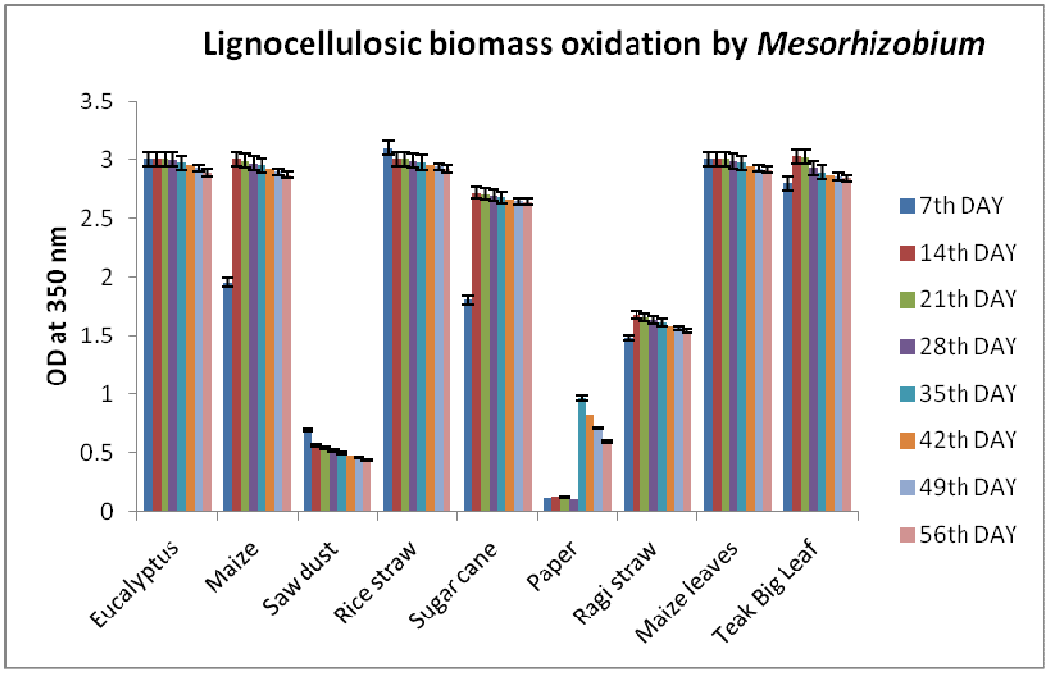

Fig: 2(a)

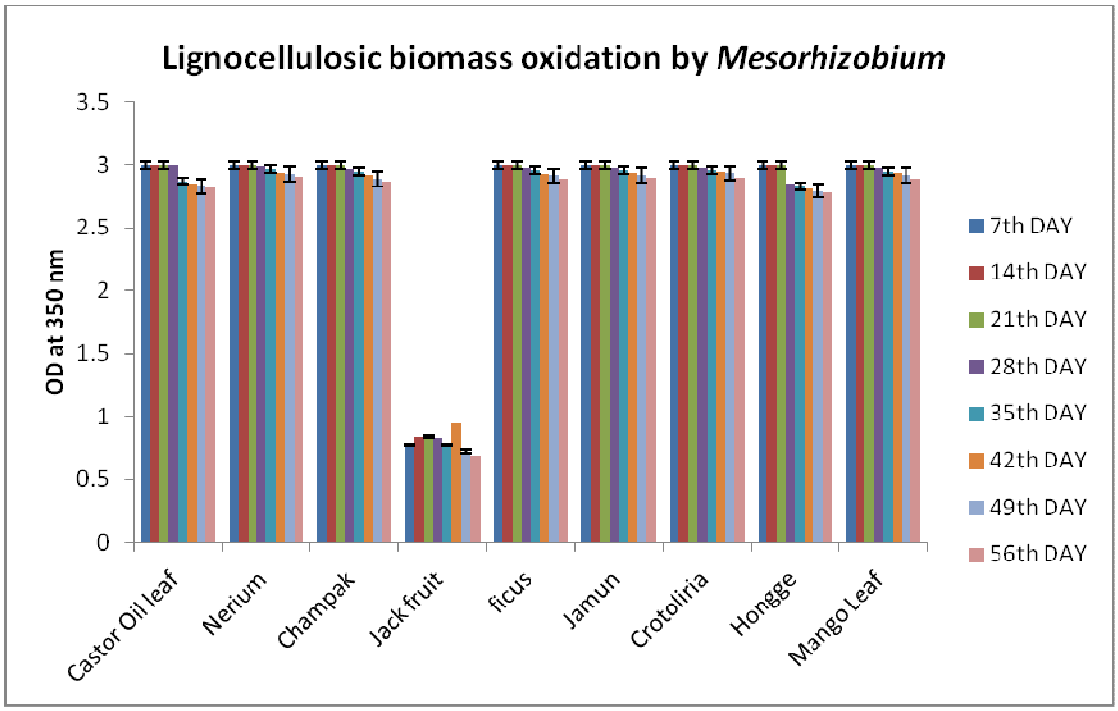

Fig: 2(b)

Mesorhizobium exhibited maximal oxidation of lignin in 13 substrates, they are: Eucalyptus, Maize, Rice straw, Maize leaves,Teak big leaves, Castor oil leaf, Nerium, Champak, Crotoliria, Ficus, Jamun, Hongge, Mango leaves within the 1st 3 weeks.

Trichoderma **(Fig 3(a) & 3(b))**

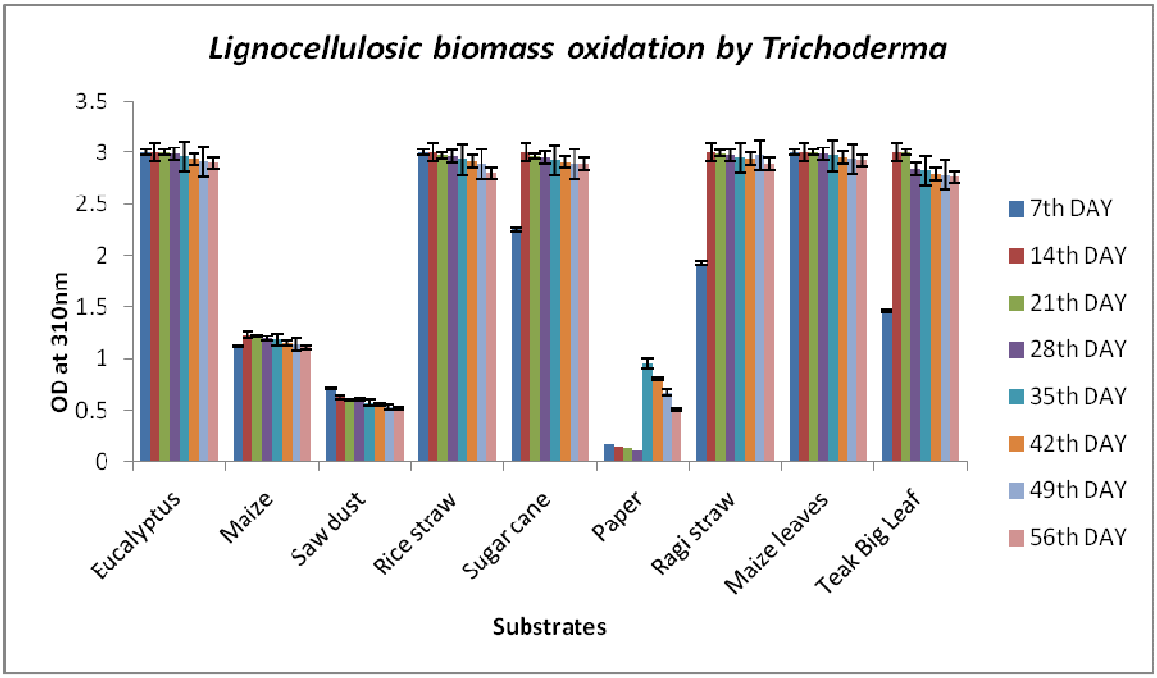

Fig 3(a)

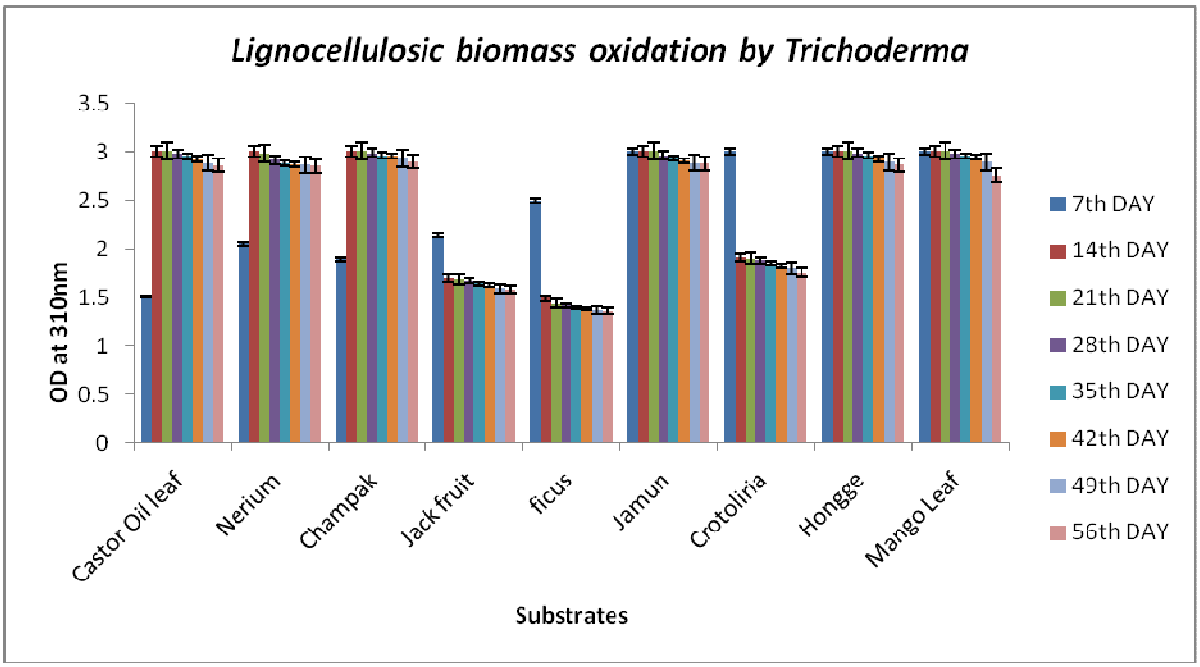

Fig 3(b)

Trichoderma exhibited maximal oxidation of lignin in 11 substrates, they are: Eucalyptus, Rice straw, Sugar cane, Maize leaves, Teak big leaves, Castor oil leaf, Nerium, Champak, Jamun, Hongge, Mango leaves within the 1st 3 weeks.

Aspergillus niger **(Fig 4(a) & 4(b))**

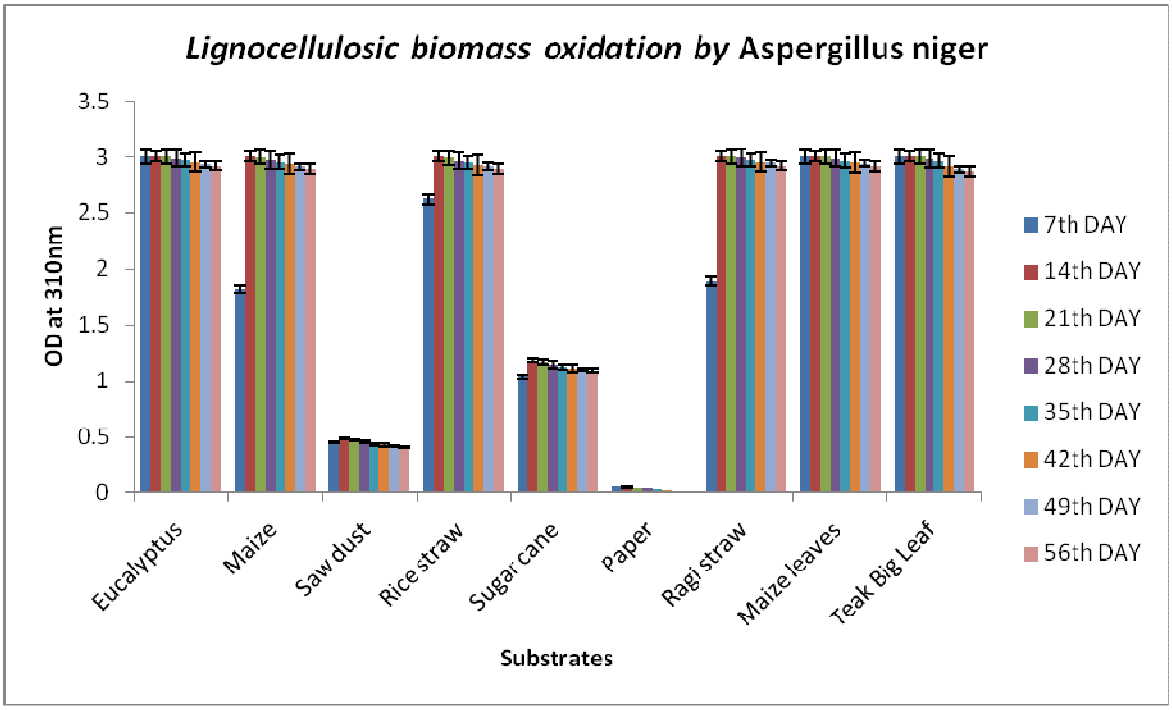

Fig 4(a)

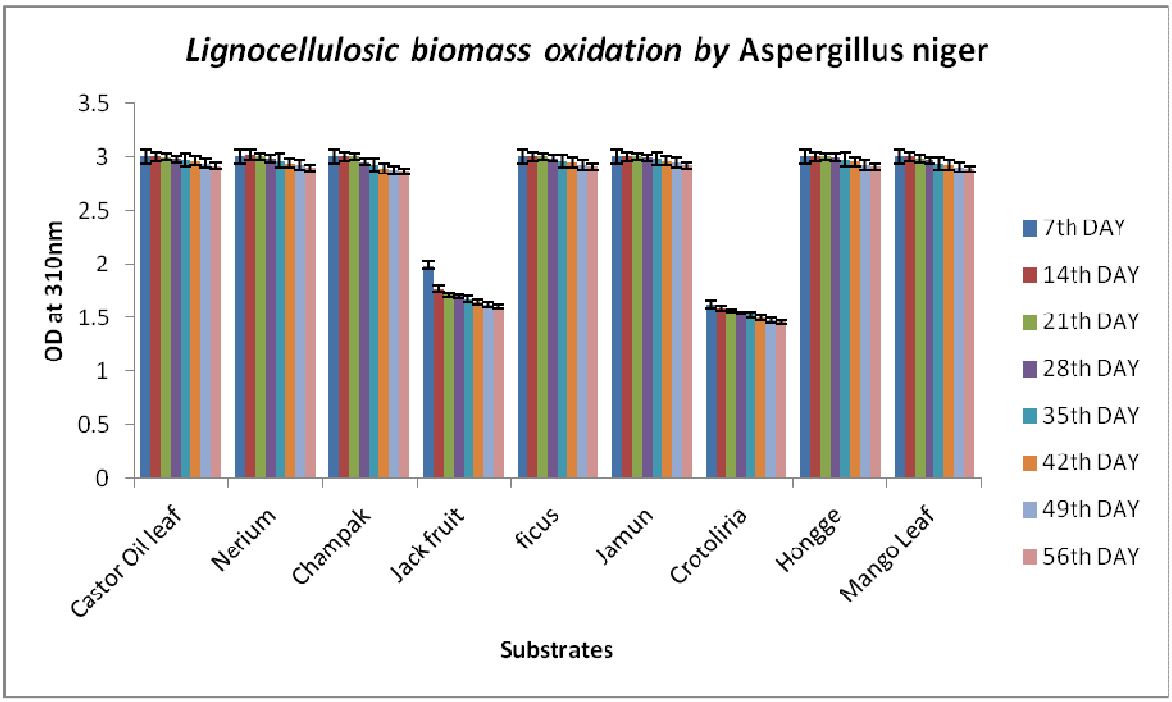

Fig 4(b)

Aspergillus niger exhibited maximal oxidation of lignin in 12 substrates, they are: Eucalyptus, Rice straw, Ragi straw, Maize leaves, Teak big leaves, Castor oil leaf, Nerium, Champak, Ficus, Jamum, Hongge, Mango leaves within the 1st 3 weeks.

DISCUSSION AND CONCLUSION

The present work carried out of lignocellulosic biomass exhibited enzymatic degradation of lignin by oxidizing the lignin polymer into its monomers which could be utilized by industries as food and perfumes, binder/glue, dispersant, emulsifier, sequestrant etc.. maximal oxidation was commonly observed by all the four organisms in seven substrates: Eucalyptus, Maize leaves, Castor oil leaf, Nerium, Champak, Hongge and Mango leaves.

The enzymatic removal of lignin from the lignocellulosic biomass facilitates the removal of lignin and makes the cellulose available to degradation by cellulose producing organisms. In the present study the organisms used have already been found to posses an extensive cellulose producing ability, there by indicating that the organisms can carry out the complex breakdown of the lignocellulosic biomass with its multi enzyme component system ie., lignocellulases. This leads to removal of lignin by oxidation, availability of cellulose and hemicelluloses to be hydrolysed by cellulases and hemicellulases produced by the same organism to release simple sugars which can be used to produce ethanol.

According to the results of Larry et al., (1982) indicated that a temporal relationship existed between the appearance of H2O2 production activity and ligninolytic activity. Since 13-14-day-old cultures of Phanerochaete chrysosporium actively ligninolytic and contained high levels of H202 production activity.

According to the study of Tomati et al. (1995), 70% of lignin was degraded during 35 days when the temperature of the compost was kept at 50C. During the later maturating phase, lignin was again degraded with the degrading ratio increasing from 20.25% (day 28) to 24.89% (day 45).

The organism obtained in the present work indicate the maximal oxidation of lignin without altering the temperature in 12 substrates within a period of 7 to 21 days exhibiting a high potential to lignocellulosic waste degradation which can serve as a source of obtaining ethanol and other related products.

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