Degradation of lignin in agricultural residues by locally isolated fungus *Neurospora discreta*

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Abstract:
Locally isolated fungus, *Neurospora discreta* was evaluated for its ability to degrade lignin in two agricultural residues: cocopeat and sugarcane bagasse with varying lignin concentrations and structures. Using Klason's lignin estimation, high performance liquid chromatography and UV-visible spectroscopy we found that *N. discreta* was able to degrade up to twice as much lignin in sugarcane bagasse as the well-known white rot fungus *Phanerochaete chrysosporium* and produced nearly 1.5 times the amount of lignin degradation products in submerged culture. Based on this data, *N. discreta* is a promising alternative to white rot fungi for a faster microbial pre-treatment of agricultural residues. This fungus can also be explored in the future for complete bioprocessing of lignocellulosic biomass, as it contains the enzymatic machinery required for ethanol fermentation as well. This paper presents the lignin degrading capability of *N. discreta* for the first time, and also discusses the difference in biodegradability of cocopeat and sugarcane bagasse.

Keywords: Lignin biodegradation, *Neurospora discreta*, Agricultural residues, White rot fungi

1. Introduction:

A country such as India generates nearly 350 million tonnes of agricultural residues per annum [1]. A large portion of these residues is disposed of by burning or landfilling, resulting in severe environmental issues such as air pollution and groundwater contamination. However, unlike hazardous waste, agricultural residues essentially consist of biodegradable lignocellulosic biomass, which can potentially be used to produce biofuels and other value added products [2]. It is therefore imperative to develop a viable process that can not only degrade large quantities of recalcitrant agricultural residues but also produce biofuels which will solve the dual problem of waste treatment and energy crisis.

Lignocellulosic biomass typically contains cellulose (35–50% by weight), hemicellulose (20-35%) and lignin (15-50%) with considerable variation in composition depending on the plant species it is derived from as well as the age of the plant [3]. The presence of lignin in the biomass lowers the susceptibility of cellulose and hemicellulose to hydrolysis and fermentation, thereby reducing overall process yields in biofuels production. Furthermore, the structure of lignin - in particular, the concentration of monolignols, infuences its susceptibility to degradation [4]. Syringyl (S) units present in the lignin have lower redox potentials and degree of condensation, and are easier to degrade, while guaiacyl (G) units are involved in more C-C linkages that contribute to condensed recalcitrant lignins [5]. S/G ratio can therefore serve as an indicator of degradability of the biomass species [5–7].

Chemical and thermochemical methods have been employed for lignin removal by the industry [8] but they have drawbacks such as high energy requirements and detrimental impacts on the environment. Microbial degradation of lignin overcomes many of these drawbacks although low yields and long processing times could be potential roadblocks to a successful process [9, 10].

Basidiomycetes, in particular, white rot fungi are known to produce ligninolytic enzymes such as lignin peroxidase (LiPs) , manganese peroxidase (MnPs), versatile peroxidase (VP) and laccases that can effectively breakdown the macrostructure of lignin [11]. However, the use of white rot fungi as a delignification step is still not considered a viable alternative to chemical or thermochemical routes due to the low rates of delignification.

In our lab we have compared the best known white rot fungus, *P. chrysosporium* with a locally isolated strain of *Neurospora discreta*. An extensively studied and well known species, *Neurospora crassa* has served as a model organism for various genetic and molecular studies as well as for its biotechnological applications in production of ethanol [12–14]. Rodriguz et al., studied ligno sulfonate biodegration by *Chrysonilia sitophila*, an anamorph of *Neurospora sitophila* [15]. The same research group also carried out degradation studies on β-O-4 lignin model compounds to study the mechanism of lignin degradation [16]. Lignin degradation on softwood *Pinus radiata* was also studied and it was reported that *C. sitophila* caused 20% weight loss of pine wood in 3 months, with the losses of carbohydrate and lignin being 18% and 25%, respectively [17]. However, Neurospora has not been explored for lignin degradation in agricultural residues thus far. Moreover, the species *N. discreta* has never been tested for its ability to degrade lignin. In fact, this fungal species is usually found in western North America, Europe and Central Africa and easily found growing on trees affected by forest fires [18]. To our knowledge, the occurrence of *N. discreta* is thus far unrecorded in India.

This paper describes the first study on the use of *N. discreta* in the biodegradation of lignin and its performance compared to the well-known white rot fungus *P. chrysosporium*. Owing to its rapid growth, *N. discreta* was found to be the more effective of the two in degrading lignin. Two types of biomass residues were tested: cocopeat or coir pith (50% lignin [19]) and sugarcane bagasse (25%

lignin [2]). These residues were selected for three reasons: (1) abundant availability of these residues in India and other tropical and sub-tropical regions, (2) a wide difference in lignin concentration and (3) difference in syringyl to guaiacyl (S/G) ratios. Coco peat has an S/G ratio of 0.23 [20], while sugarcane bagasse has a ratio of 0.85 [21], making cocopeat harder to degrade compared to sugarcane bagasse, which was confirmed by our data.

2. Materials and methods:

2.1. Lignocellulosic biomass substrates

Sugarcane bagasse and cocopeat were both obtained from local vendors. They were chopped to 20-30 mm size, thoroughly washed with tap water and then dried at 80^{o}C in a hot air oven till constant weight was achieved. The dried substrates were stored in air tight containers at 2-4^{o}C.

2.2. Fungal strains

Pure culture of *Phanerochaete chrysosporium* (NCIM 1197) was obtained fromNational Collection of Industrial Microorganisms, Pune and stored at 2-4 ^{o}C. The strain was subcultured in potato dextrose agar (PDA) slants and platesby incubating at 28^{o}C for 5 days. The fully sporulated plates were either used immediately or stored at 2-4 ^{o}C. These working stock cultures were used for inoculating the submerged fermentations.

N. discreta was isolated from the bark of a Subabul wood tree. Light orange colored patches of fungus found growing on the bark were scraped using a sterile loop and streaked on a potato dextrose agar plate. The plate was incubated at 28^{o}C and cell growth was observed within 24 hours of incubation. Within 48-72 hours abundant cell growth was observed with the characteristic orange colored filaments. Cells from the parent plate were

subcultured once again into multiple PDA plates and the culture was similarly maintained thereafter.

2.3. Fermentation set-up

Submerged fermentations were set up in 250 ml erlenmeyer flasks. Each flask contained 1 g of substrate (cocopeat or bagasse), 0.5 g of sucrose in 100 ml Vogel's minimal media [22].The flasks were covered with non-absorbent cotton plugs and autoclaved at 120^{o}C for 15 mins. A conidial suspension of each of the fungi was prepared by dislodging the filaments from agar via gentle scraping and adding the cells to a known quantity of minimal media. The fungal suspension was gently mixed and filtered through a sterile muslin cloth to obtain a clear spore suspension. The final spore count was adjusted to approximately 10^{7}cells/ml and 1 ml suspension was used to inoculate each flask. The flasks were incubated at 30^{o}C for 30 days. For each fermentation flask, a corresponding control containing the same quantity of substrate and minimal media was also set up without any cells. Each set was run in duplicate.

2.4. Sample preparation

At each time point, one flask of each fungal strain was removed, the contents were filtered to separate the liquid and solid fractions. The solid fractions were thoroughly washed with de-ionised water, filtered and dried at 105^{o}C till constant weight. The liquid fractions were centrifuged at 10000 rpm for 10 min and the clear supernatant was stored at 4^{o}C for further analysis.

2.5. Reducing sugar content

The concentration of total reducing sugars was measured by dinitro salicyclic acid (DNSA) method [23] in the clear supernatant at regular time intervals. The concentration of reducing sugars in the samples was calculated using a standard curve plotted between the optical density at 540 nm and the concentration of standard glucose solution.

2.6. Percentage lignin degradation

The lignin content in the solid biomass samples were measured using Klason's standard method [24]. Total lignin content was obtained by adding the acid insoluble lignin measured gravimetrically and acid soluble lignin measured spectrophotometrically.

Lignin content was measured before and after the fermentation to calculate percentage lignin degradation.

$$Percentage\ lignin\ degradation = \frac{(initial\ lignin\ content - final\ lignin\ content)}{(initial\ lignin\ content)}$$

2.7. High Performance Liquid Chromatography (HPLC) Analysis

The clear supernatant obtained from fermentation sets were analyzed for lignin degradation products using HPLC method. Chromatographic separation was performed on Thermo scientific BDS Hypersil C-18 column (250 x 4.6 mm ID, 5μm particle size). The flow rate was 1 ml/min, injection volume was 25uL and the column temperature was set at 27^{o}C. The mobile phase used was a mixture of two solvents; A: 1% acetic acid in deionized water and B: 100% acetonitrile and gradient elution with following conditions was used: at 0 min - solvent B concentration is 0, 5 mins - B concentration is 3%, 10 mins- 6% B concentration, 15 mins – 10%B concentration and 20 mins - 20%B concentration. The baseline was monitored for20 min before the next injection and the elute was continuously monitored by PDA detector at 254 nm. The data was integrated and analyzed using the Shimadzu Automated Software system.

Varying concentrations of standard alkali lignin was taken and HPLC runs were conducted to relate area under the peak with the concentration of the standard. A calibration curve was plotted to relate the areas with standard lignin concentrations. Liquid samples were collected on day 5, day 10, day 20 and day 30. The samples were centrifuged and suitably diluted.

2.8. UV-Visible Spectroscopy

Absorbance value at 420 nm was noted for the clear supernatant samples obtained on day 30 of the fermentation, using HITACHI U-2900 UV Visible spectrophotometer.

3. Results and Discussion:

3.1. Characterization and growth characteristics of the isolate:

For the phylogenetic identification of the isolated fungus, 18srRNA sequencing technique was used. In this technique, DNA was extracted from the sample and the small-subunit rDNA (18S rRNA gene) was subjected to PCR amplifications using primers. The 18srRNA gene sequences were later analyzed using BLAST nucleotide sequence for closest match. Since the presence of this species hasnot been reported in India, the strain was sent to two different laboratories for confirmation of reseults: Microbial Type and Culture Collection and gene bank (MTCC), Chandigarh and Yaazh Xenomics, Chennai, India. Both laboratories reported the fungus to be Neurospora discreta. The ITS/5.8s rRNA gene sequence data from MTTC with 99.47% matchis given in Figure 1.

N.discreta was found to grow very rapidly, as long and thick filaments producing abundant spores in comparison to *P.chrysosporium* (Figure 2). The filaments develop a characteristic orange color over time, due to the accumulation of neurosporaxanthin, a carboxylic

apocarotenoid [25]. The thickness of *N. discreta* filaments was 6-7 times greater than that of *P. chrysosporium* as seen in the microscopic images (Figure 2).

3.2. Carbon utilization:

Figure 3 shows cumulative sugar concentration released from biomass as well as sucrose added in the initial media plotted against time assayed by DNSA method. The total reducing sugar content was measured as 5 mg/ml for cocopeat fermentation setups on day zero, while it was slightly higher (around 6 mg/ml) for sugarcane bagasse fermentations because of the free sugars present in the biomass itself. *N. discreta* rapidly utilized the sugar content in both cocopeat and bagasse fermentations (Figure 3). In the case of *P. chrysosporium*, an initial increase in cumulative sucrose content is seen till day 8 and then a gradual decrease with time is noted, while in *N. discreta*, the sugar content rapidly decreased by day 5 and remained at a minimum value for the rest of the fermentation. This can be attributed to the higher growth rate of *N. discreta* compared to *P. chrysosporium*, which was also observed visually.

The lignin content in the biomass samples at the beginning and end of the fermentations was estimated using Klason's method as mentioned above. Figure 4 shows the percentage lignin degraded by both fungi. In the case of sugarcane bagasse the percent lignin degradation by *N.discreta* was nearly twice that by *P. chrysosporium*. In cocopeat samples, the lignin degradation was around 16% with both the fungal strains after 30 days. A comparison of delignification of cocopeat and bagasse shows that bagasse is easier to degrade compared to coco peat. Lignin in sugarcane bagasse has a higher S/G ratio and is mainly composed of β-O-4 ether bonds, indicating principally linear chains, which makes it easy to degrade. On the other hand, lignin in cocopeat consists predominantly of guaiacyl units (lower S/G ratio)

[20]. The main substructure present in the lignin of cocopeat are the β−O−4′ aryl ether, followed by β−5′ phenylcoumaran substructures and β−β′ resinol substructures. While β−O−4′ aryl ether linkages can be broken down by low redox potential enzymes such as laccases, for breaking the more condensed lignin linkages such as β−5′ phenylcoumaran structures, more evolved ligninases such as lignin peroxidase, manganese peroxidase or versatile peroxidases are required. *P. chrysosporium* and other basidiomycetes are known to produce laccases, lignin peroxidases, manganese peroxidase and versatile peroxidases which would all contribute to the degradation of the various lignin linkages in cocopeat. Although ascomycetes such as Neurospora primarily produce laccases and lignin peroxidases [15, 16, 26, 27], the extremely rapid growth of *N. discreta* seems to compensate, thus resulting in lignin degradation in cocopeat similar to that by *P.chrysosporium.*

3.3. Analysis of lignin degradation products:

3.3.1. High Performance Liquid Chromatography

The liquid supernatant from the submerged fermentation flasks was subjected to RP-HPLC to analyze the lignin degradation products. Soluble alkali lignin was used as a standard for comparison. A chromatogram of the standard alkali lignin analyzed using RP-HPLC is shown in Fig. 5(e). The RP-HPLC analysis of the standard sample indicated the presence of a major peak at retention time of 2.9 ± 0.2 min along with other minor peaks. In the case of the *N. discreta* a major single peak is seen at retention time of 2.9 ± 0.015 minutes which corresponds to the standard alkali lignin peak, while in *P.chrysosporium* samples three smaller peaks are seen between retention time : 2.0 and 3.2 which changed with time. The additional lignin degrading enzymes (manganese and versatile peroxidases) in *P. chrysosporium* could be the reason for the

more heterogenous profile. From figure 5 (a to d), a clear increase is seen in the peak height and the peak area in the time point samples as the fermentation progresses. The concentration of the solubilzed lignin (in mg/ml) in liquid supernantant samples was calculated from the calibration curve obtained from standard alkali lignin plotted at different dilutions. This data, shown in figure 6 further confirms that sugarcane bagasse is much easier to degrade than cocopeat as it correlates with the Klason's lignin values.

In cocopeat fermentation, a gradual increase in area is seen from day 5 to day 30 with both the fungi. Clearly, *N.discreta* has shown higher concentrations of lignin degraded products on all the days. On an average, at the end of 30 days, a 1.8 fold increase is seen using *N.discreta* over *P.chrysosporium* for cocopeat biomass. In the case of sugarcane bagasse, a 1.3 fold increase in peak areas is seen with *N.discreta* over *P.chrysosporium*. There is no change seen in peak areas from day 10 to day 30, with both the fungi. This further confirms that it is easier to degrade lignin from bagasse which takes about 10 days of fermentation, while cocopeat with more lignin content and different structure takes more than 30 days.

3.3.2. UV-Spectroscopy

The lignin fraction contained in lignocellulosic biomass is hydrophobic and insoluble in water. Fungal action breaks down some of lignin bonds, thereby breaking down the polymer into smaller sub-units and also causing some of these units to become soluble in water. Literature recommends the use of 420 nm to measure soluble lignin degradation products [28]. The liquid supernatant of day 30 samples were used and their average absorbance values are shown in figure 7. The absorbance values of bagasse samples with *N.discreta* were nearly twice that of *P. chrysosporium*. In

cocopeat fermentations, as expected, the difference in absorbance values was lower compared to bagasse although *N. discreta* did result in a significantly higher absorbance compared to *P. chrysosporium.*

4. Conclusion

In this study, we have demonstrated for the first time that the indigenous fungal strain *Neurospora discreta* can degrade lignin effectively in agricultural residues cocopeat and sugarcane bagasse. Its longer and thicker filaments, significantly higher spore count and faster growth rate make it a promising alternative to white rot fungi for microbial pre-treatment of lignocellulosic biomass. Moreoever, as Neurospora is also known to possess the enzymatic machinery for hexose and pentose fermentation, it can be tested for complete bioprocessing of agricultural residues to produce ethanol. We also found that of the two substrates, sugarcane bagasse is much easier to degrade compared to the more recalcitrant cocopeat, owing to the difference in S/G ratios as discussed. Future work will focus on enhancing the effectiveness of *N. discreta* in lignin degradation by addition of laccase mediators as well as evaluating its effectiveness in subsequent production of ethanol.

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6. References:

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Figure Captions

Figure 1: 18s rRNA sequence results for the isolated *N.discreta* strain

Figure 2: Microscopic images of fungi after staining with lactophenol cotton blue (a) *P.chrysosporium* and (b) *N. discreta*

Figure 3: Cumulative sugar concentration measured by DNSA method against time (days) during treatment of cocopeat and sugarcane bagasse with *P. chrysosporium* and *N. discreta*

Figure 4: Percent lignin degradation measured by Klason's lignin content after 30 days of fermentation

Figure 5: Chromatograms of liquid supernatant samples of (a) cocopeat treated with *N. discreta*, (b) cocopeat treated with *P. chrysosporium* (c) sugarcane bagasse treated with *N. discreta* (d) sugarcane bagasse treated with *P. chrysosporium* and (e) standard alkali lignin (0.5 mg/ml).

Figure 6: Concentration of degraded lignin measured by HPLC in liquid supernatant samples of (a) Sugarcane bagasse and (b) Cocopeat treated with *N. discreta* and *P.chrysosporium*

Figure 7: Absorbance values at 420 nm (Dilution factor : 2) of liquid supernatant from sugarcane bagasse and cocopeat submerged fermentations

GTAAGGGGTACCTGCGGAGGGATCATTACAGAGTTGCAAAACTCCCACAAACCATCGCGAATCTTACC
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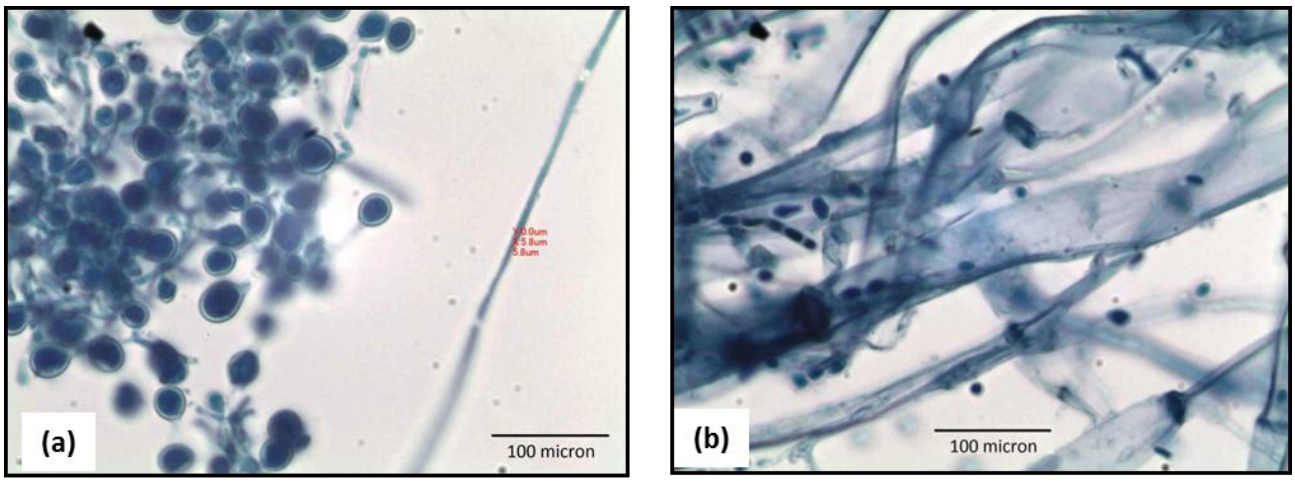
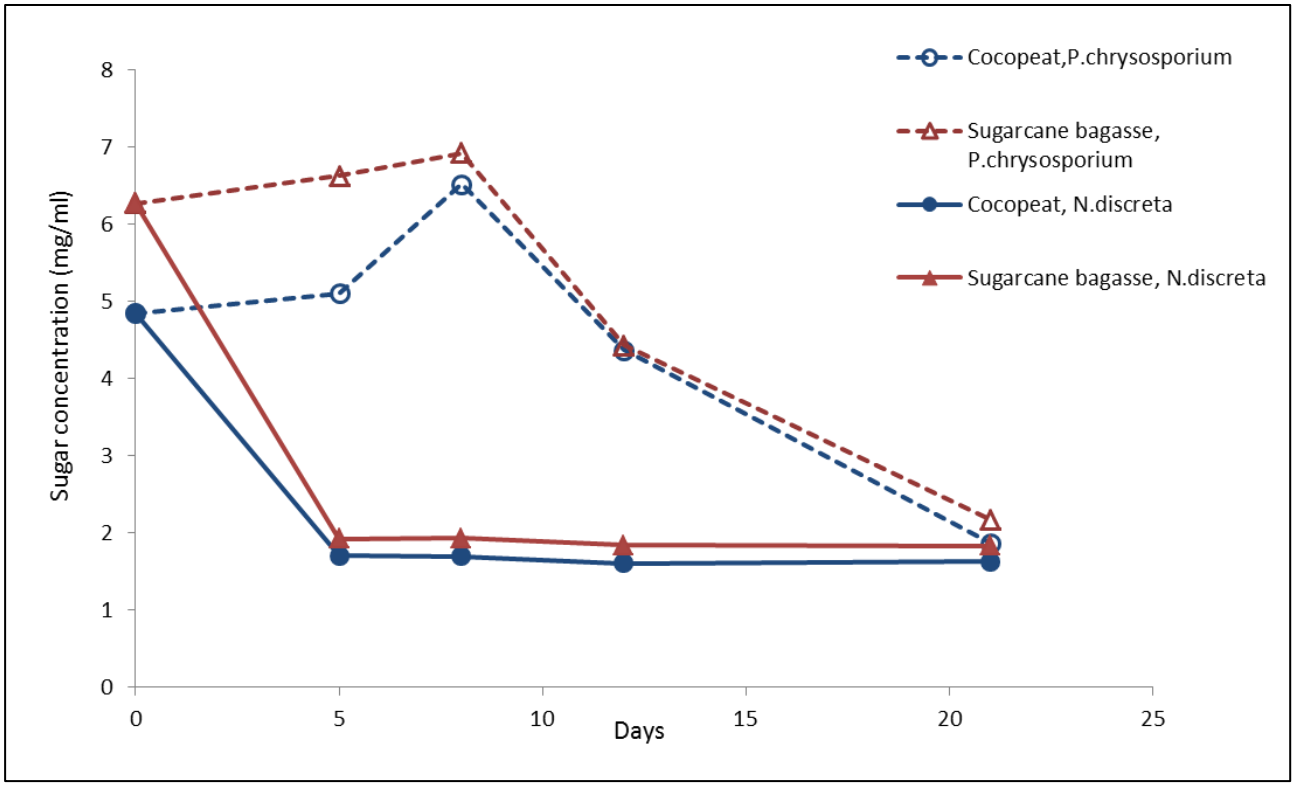


Figure 2: Microscopic images of fungi after staining with lactophenol cotton blue (a) *P.chrysosporium* and (b) *N. discreta*



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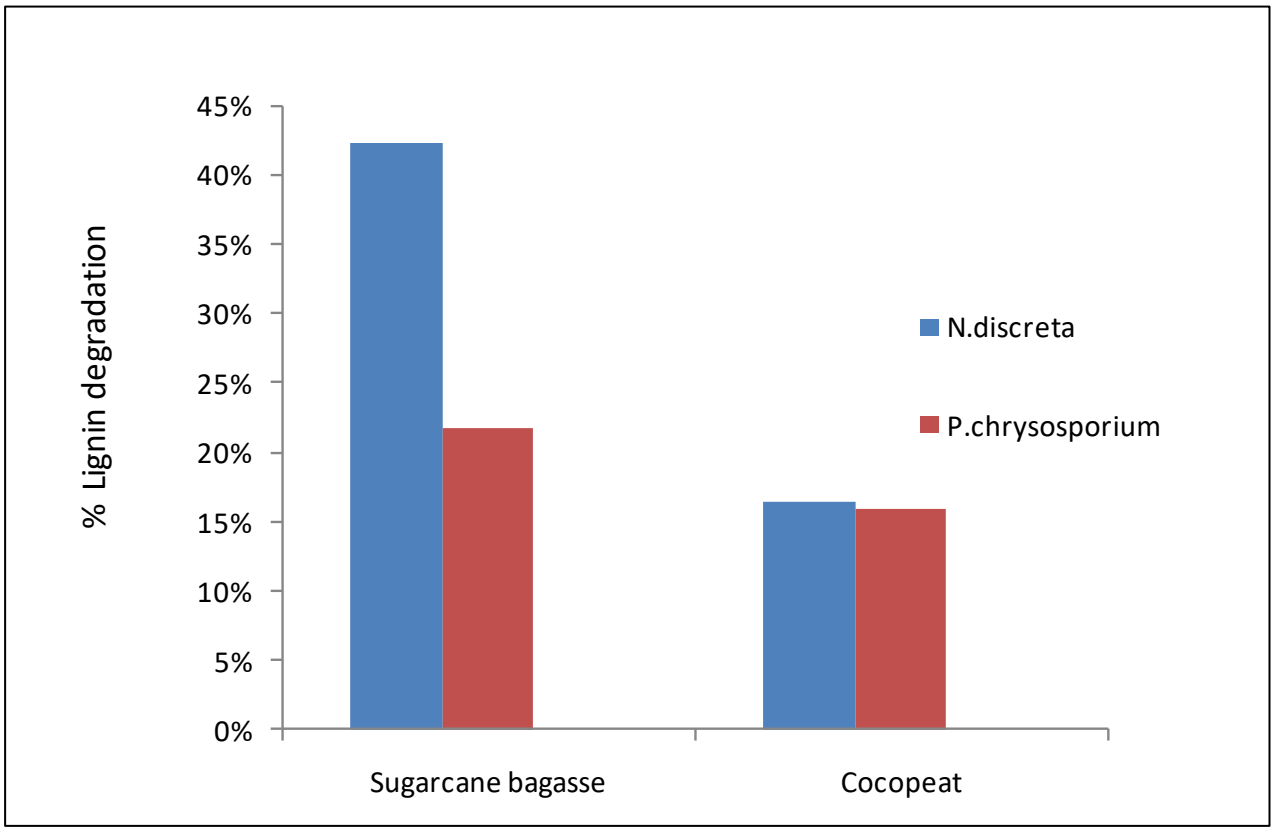

Figure 4: Percent lignin degradation measured by Klason's lignin content after 30 days of fermentation

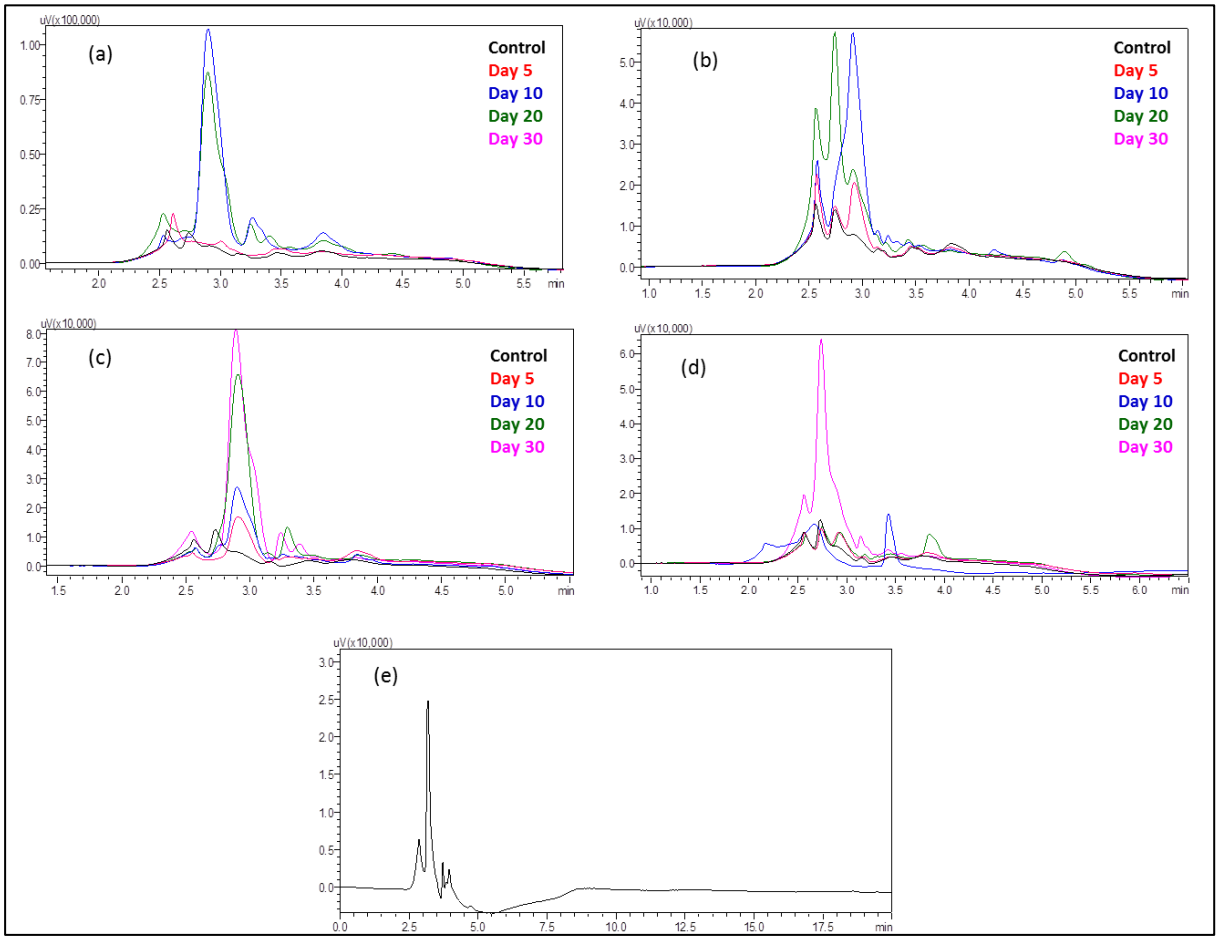

Figure 5: Chromatograms of liquid supernatant samples of (a) cocopeat treated with *N. discreta*, (b) cocopeat treated with *P. chrysosporium* (c) sugarcane bagasse treated with *N. discreta* (d) sugarcane bagasse treated with *P. chrysosporium* and (e) standard alkali lignin (0.5 mg/ml).

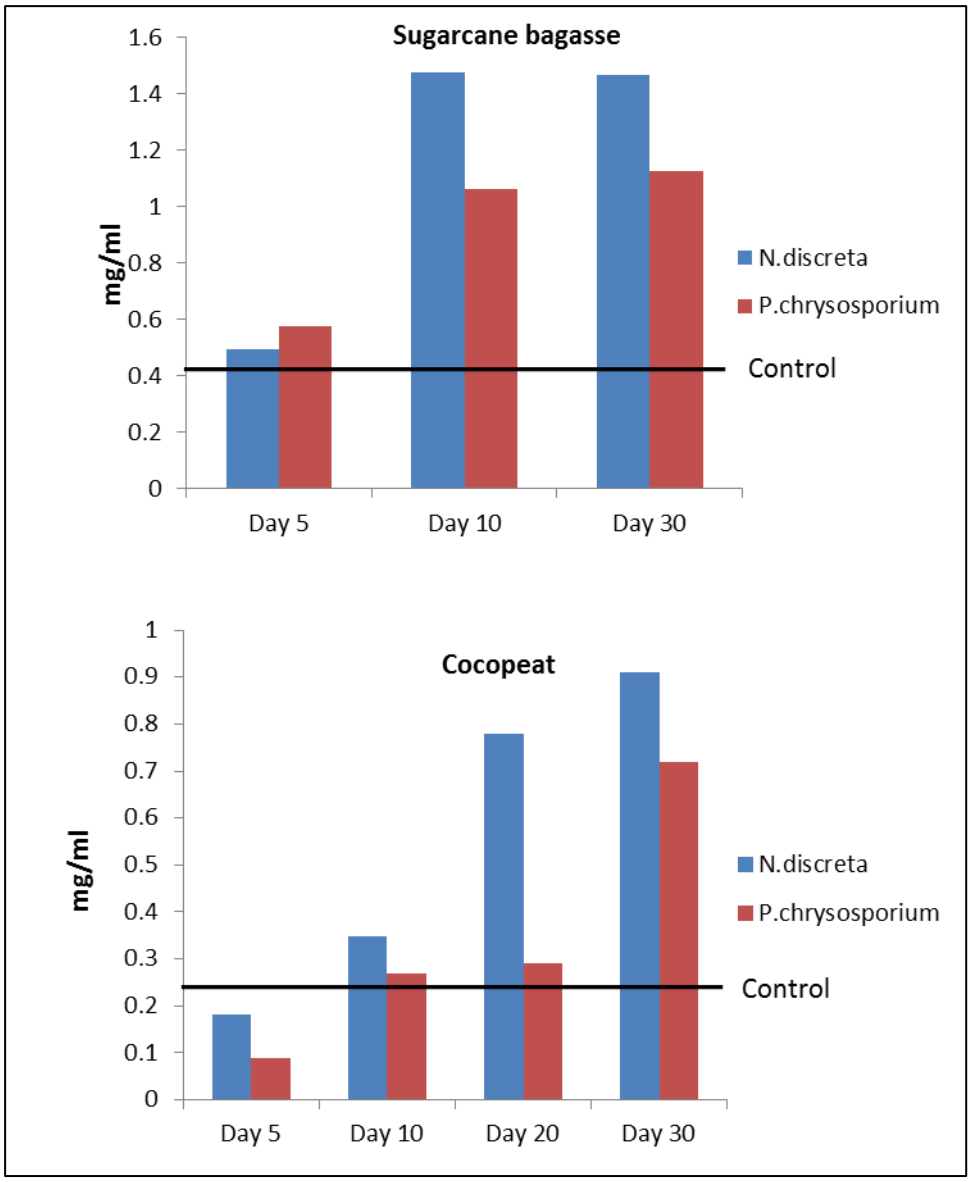

Figure 6: Concentration of degraded lignin measured by HPLC in liquid supernatant samples of (a) Sugarcane bagasse and (b) Cocopeat treated with *N. discreta* and *P.chrysosporium*

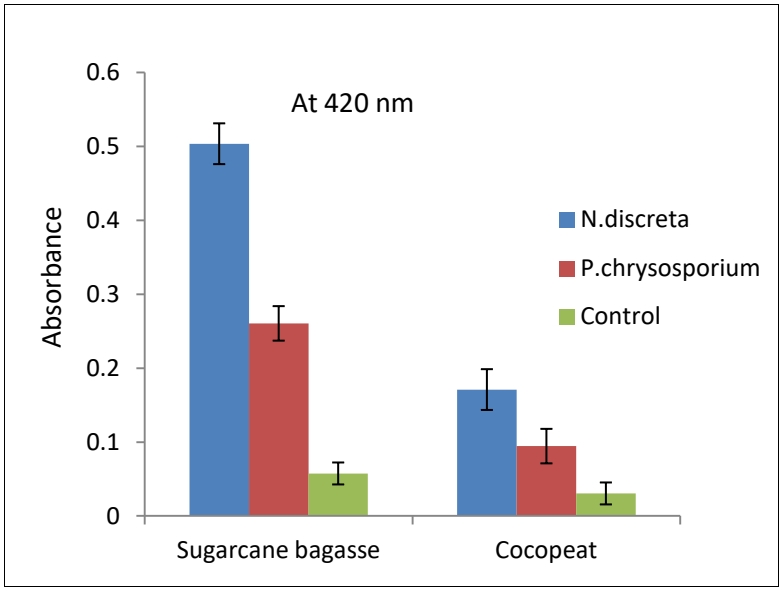

Figure 7: Absorbance values at 420 nm (Dilution factor : 2) of liquid supernatant from sugarcane bagasse and cocopeat submerged fermentations