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# ETHANOL PRODUCTION FROM SPENT SUBSTRATE OF *PLEUROTUS EOUS*

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**ABSTRACT:** Spent substrate, the residual material of mushroom cultivation, causes disposal problems for cultivators. Currently the spent substrate of different mushrooms is used mainly for composting. Edible mushrooms of *Pleurotus sp.* can grow on a wide range of lignocellulosic substrates. In the present study, *Pleurotus eous* was grown on paddy straw and the spent substrate was used for the production of ethanol. Lignocellulosic biomass cannot be saccharified by enzymes to high yield of ethanol without pretreatment. The root cause for the recalcitrance of lignocellulosic biomass such as paddy straw is the presence of lignin and hemicelluloses on the surface of cellulose. They form a barrier and prevent cellulase from accessing the cellulose in the substrate. In the untreated paddy straw, the amount of hemicelluloses and lignin (in % dry weight) were 20.30 and 20.34 respectively and the total reducing sugar was estimated to be 5.40 mg/g. Extracellular xylanase and ligninases of *P. eous* could reduce the amount of hemicelluloses and lignin to 16 and 11(% dry weight) respectively, by 21st day of cultivation. Growth of mushroom brought a seven fold increase in the total reducing sugar yield (39.20 mg/g) and six fold increase in the production of ethanol (6.48 g/L) after 48hrs of fermentation, when compared to untreated paddy straw.

**Keywords**: Bioethanol; *Pleurotus*; Spent Substrate; Ligninases; Cellulose

**Abbreviations :** SS - spent substrate

## INTRODUCTION

*Pleurotus*, which has several species such as *P. sajorcaju*, *P. ostreatus, P. florida* and *P. eous*, commonly known as oyster mushrooms, belong to the class Basidiomycetes. *Pleurotus* mushrooms are edible with excellent flavour and taste (Somashekar, et al., 2010). They have nutritional as well as medicinal properties (Mahmood, et al., 2011). They are low in calories, fats, sodium, carbohydrates and cholesterol, while being rich in proteins, minerals, vitamins and fibers (Gupta, et al., 2011). Antioxidant and antitumor activities of *Pleurotus sp*. have also been reported (Liu, et al., 2010).

Oyster mushrooms now rank second among the important cultivated mushrooms in the world (Mohamed, et al., 2011). They have become popular among mushroom cultivators, as they can grow in a wide range of temperatures, on various lignocellulosic substrates such as rice straw, wheat straw and saw dust. The major components of these substrates are cellulose, hemicelluloses and lignin. Growth and fruiting of *Pleurotus* mushrooms on these substrates depend on their ability to release hydrolyzing and oxidizing enzymes to degrade these components into smaller molecules for assimilation.

After mushroom cultivation, a considerable amount of spent substrate (SS) remains as residual material. SS needs heat treatment before being removed from the growing chamber. But being expensive, some mushroom growers discard the contaminated SS far from the farm (Rinker, et al., 2010). Without proper treatment, contaminated SS can cause re-contamination. Conversely, recycling of SS can increase sustainability and also help farm economy. Several studies have shown the potential use of the spent substrate in purification of water and soil, cultivation of other mushroom species, cultivation of vegetables, biological control of pests, vermiculture, as well as its use as animal feed and as a source of degradative enzymes (Castro, et al., 2008). In the present study, the feasibility of using the spent substrate of *Pleurotus eous* in the production of ethanol is evaluated.

Bioethanol is a liquid fuel generated from cellulose, starch or sugar. Of all biofuels, ethanol is already produced on a fair scale (about 14–26 M tonne per annum), and is easily applicable in present day internal combustion engine vehicles (ICEVs), as mixing with gasoline is possible (Hamelinck, et al., 2010). About 90% of bioethanol is derived from sugar or starch crops by fermentation (Hamelinck, et al., 2010). But these crops have a high value for food application, and their sugar yield per hectare is very low. Conversion of lignocellulosic biomass, which is both abundant and renewable, into ethanol is a promising alternative. Lignocellulosic biomass contains sugars, but they are much harder to release than those in starchy biomass. Pretreatment can reduce the lignin content and thereby enhance the digestibility of the biomass for ethanol production. However, the chemicals used in the pretreatments for releasing sugars such as sulphuric acid and sodium hydroxide, produce by-products that inhibit fermentation (NREL). Biological pretreatment with microbes or microbial enzymes is the possible alternative. Consequently, in this study we focused on the efficiency of *Pleurotus eous* for delignification and cellulose biodegradation during solid substrate fermentation of paddy straw.

## MATERIALS AND METHODS

Spawn of *P. eous* used in this study was procured from Kerala Agricultural University, Vellanikkara, Thrissur. The substrate paddy straw was procured locally. Mushroom cultivation was done as per the procedure outlined by Madhusudhanan et al. (2003). Spawn to substrate ratio was (1:10 w/w). Mushrooms were harvested on 18$^{th}$ and 21$^{st}$ day. After the harvest, the SS was incubated until 49$^{th}$ day at 28˚C and 60% humidity. Biological efficiency (BE in %) of *P. eous* was determined, according to the equation:

Biological efficiency = (Fresh weight of mushroom/ dry weight of substrate) × 100

SS sampled on 21$^{st}$, 28$^{th}$, 35$^{th}$, 42$^{nd}$, and 49$^{th}$ day, dried overnight at 50±2°C and powdered to 425µ was used for ethanol production. Untreated substrate was used as control. Fermentation medium consisted of substrate suspended in distilled water at 1:40w/v (2.5%), supplemented with 0.3% ammonium sulphate, 0.15% potassium di-hydrogen phosphate and 0.5% yeast extract. Medium was sterilized and total reducing sugar was estimated. *Saccharomyces sp.* (isolated from coconut toddy) was grown in a medium containing 6.0% sucrose, 0.5% yeast extract and 0.5% peptone for 24hrs at 28˚C. Cells were pelleted out at 8000rpm for 15min and used as inoculum (2.5%) for fermentation. Medium was inoculated and incubated for 48hrs at 28˚C with intermittent shaking. The fermented medium was checked for ethanol content colorimetrically (Bennette, 1971; Pilone, 1985).

Control and dried substrates were used for estimations of cellulose, hemicellulose and lignin. Cellulose content was estimated by anthrone method (Updegroff, 1969). The amount of acid insoluble lignin was estimated by the Klason method (KCL, 1982). Hemicellulose was estimated by the method of Goering and Van Soest (Goering and Van Soest, 1975).

Quantitative assays were done for the extra cellular enzymes present in the SS of *P. eous* from 21st-49th day of cultivation at 7 day intervals. Crude enzyme extract was prepared by mixing the substrate with distilled water (1:1w/v) and 0.05%Tween 80 with intermittent shaking for 30min. The mixture was centrifuged and the supernatant was used for assays. Extracellular enzymes such as cellulase, lignin peroxidase, laccase and xylanase were assayed (Ghose, 1987; Tien and Kirk, 1988; Buswell and Odier, 1987; Miller, 1972). Assays were done at room temperature and at pH 5.4 (the pH of the mushroom bed). Total reducing sugar and total proteins were estimated simultaneously (Miller, 1972; Lowry, et al., 1951). Assays and estimations were done in triplicate.

Scanning Electron Micrographs (SEM) of the control and 21st day SS (SS-21) were recorded using SEM-EDS in SEI mode, under low vacuum resolution (4nm). Fourier Transform Infrared (FTIR) spectroscopy was done for samples using Thermo Nicolet, Avatar 370 model spectroscope of spectral range 4000-400 $cm^{-1}$ and 4 $cm^{-1}$ resolution. Samples were analysed for crystallinity using an X-ray diffractometre (XRD, Bruker Kappa Apex II) at a wavelength of 1.5406Aº.Samples were scanned over the angular range 3-80°, 2θ. The crystalline index of cellulose, $I_c$, was determined based on the empirical method, $Cr_I$ (%) = ($I_{002}$ – $I_{am}$) / $I_{002}$ x 100, were $I_{002}$ is the counter reading at peak intensity at a 2θ angle close to 22.5° corresponding to crystalline cellulose, and $I_{am}$ is the counter reading at peak intensity at a 2θ angle close to 18.7° representing the amorphous fraction of cellulosic fibres (Mwaikambo and Ansell, 2002).

## RESULTS

In the present study, in untreated paddy straw, the amount of cellulose, hemicelluloses and lignin (in percentage dry weight) was 10, 20 and 20 respectively and the total reducing sugar was estimated to be 5.40 mg/g (Table.1). *P. eous* showed 75% biological efficiency on paddy straw. Ligninases such as LiP and Laccase produced by *P. eous* (Fig.1) could reduce the lignin content to 11% by 21st day (Table.1). Xylanase activity was seen only on 21st day (Fig.2) which reduced the hemicelluloses content to 16% (Table 1). Cellulase activity could be seen from 14th day (Fig.2). The enzymatic hydrolysis in 21 day old substrate showed a seven fold increase in total reducing sugar yield of paddy straw (39.20 mg/g), when compared to untreated control (Table1). Ethanol yield from 21 day old SS after 48hrs of fermentation was estimated to be 6.48 g/L, and was found to be six times more than the yield from untreated paddy straw (1.12 g/L) (Fig.3)

**Table 1**.Composition of lignin, cellulose, hemicellulose, total reducing sugar, and yield of ethanol (after 48hrs of fermentation) of untreated paddy straw and 21 day old SS of *P. eous*

| Paddy straw | Lignin (%) | Cellulose (%) | Hemicellulose (%) | Red. sugar (mg/g) | Ethanol (g/L) |
|---|---|---|---|---|---|
| Untreated | 20.34 ± 0.06 | 10.04 ± 0.05 | 20.30 ± 0.42 | 5.40 ± 0.11 | 1.12 ± 0.13 |
| SS-21 | 10.91 ± 0.41 | 32.92 ± 0.62 1 | 16.20 ± 0.28 | 39.20 ± 0.15 | 6.48 ± 0.55 |

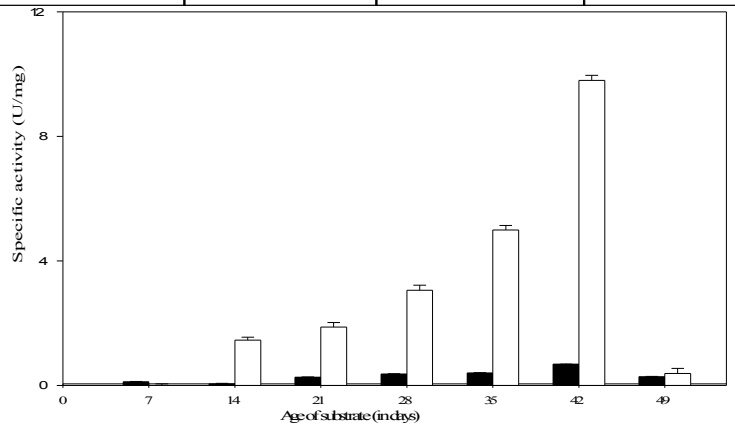


**Figure 1.** Specific activity of extra cellular ligninases in the spent substrate of *P. eous*.
(Bar 1 - LiP, Bar 2 - Laccase)
[Note: Error bars represent ± standard deviation from a triplicate average]

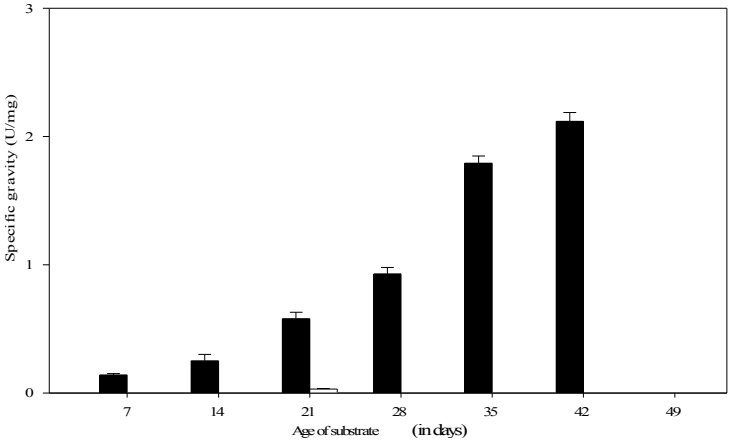


**Figure 2.** Specific activity of extra cellular cellulases and xylanases in the spent substrate of *P. eous*
(Bar 1 - Cellulase, Bar 2 - Xylanase)
[Note: Error bars represent ± standard deviation from a triplicate average]

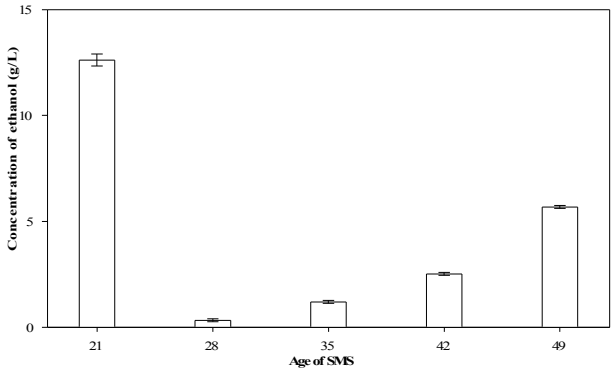


**Figure 3.** Concentration of ethanol (g/L) obtained from the spent substrate of *P. eous*.
[Note: Error bars represent ± standard deviation from a triplicate average]

The SEM images (Fig.4) showed that the surface of untreated (control) sample is rough while the surface of the 21 day old SS sample is relatively smooth. The FTIR spectra of untreated (control) paddy straw as well as that obtained after 21 days of pretreatment with *P. eous* (Fig.5) showed two major sets of absorbance peaks – the first in the range of 4000-2900 $cm^{-1}$, and the second in the range of 1800-700 $cm^{-1}$. The cellulose crystallinity of control and SS-21 observed using XRD, is shown in Fig.5. Two peaks were observed at $2\theta$ of 22.0° and 18.0° for both the untreated and treated rice straws.

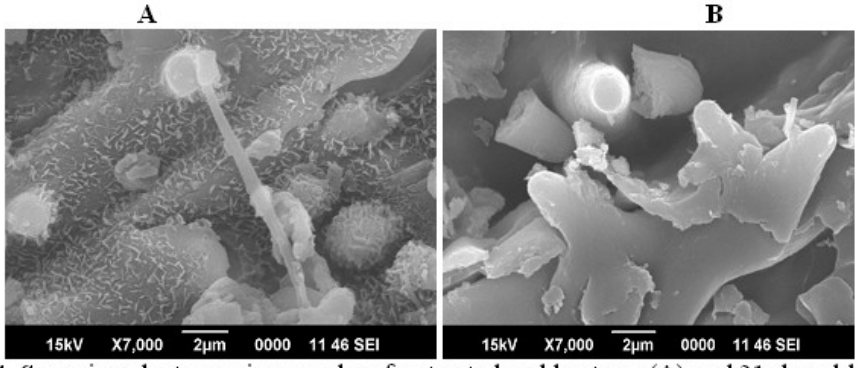


**Figure 4.** Scanning electron micrographs of untreated paddy straw (A) and 21 day old spent substrate (B) of *P. eous*

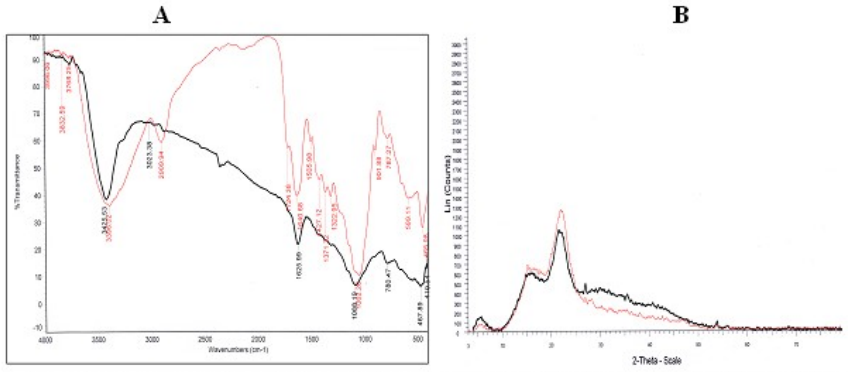


**Figure 5.** FTIR spectrograph (A) and XRD pattern (B) of untreated paddy straw (black) and 21 day old spent substrate of *P. eous*

## DISCUSSION

Studies show that the root cause for the recalcitrance of lignocellulosic biomass such as paddy straw is the presence of lignin and hemicelluloses on the surface of cellulose (Zhang, 2008). They form a barrier and prevent cellulase from accessing the cellulose in the substrate. Lignocellulosic biomass cannot be saccharified by enzymes to high yield without a pretreatment. If the pretreatment is very harsh, liberated sugars can be degraded to enzyme- and/or yeast-inhibiting compounds, lowering the overall yields. But, if the pretreatment is too weak it will result in low enzyme accessibility and the same drawbacks as above (Cardona, 2010). Results show that, *P. eous* cultivation on paddy straw could reduce lignin and hemicellulose content. Seven fold increase in total reducing sugar yield of paddy straw, in comparison to untreated control (Table1) was possibly due to the increased accessibility of cellulase to cellulose, due to the partial removal of lignin and hemicellulose. However, in spite of increase in cellulase activity with age of substrate, total reducing sugar was highest in SS-21 and hence the maximum yield of ethanol.

The SEM morphology studies (Fig.4) showed surface roughness of the paddy straw as an indicative of the presence of lignin in the untreated (control) samples. After 21 days of pretreatment with *P. eous*, the residual lignin was apparently eliminated and the surface of the fibres becomes relatively smoother. Similar morphological changes effected by pretreatment with ligninolytic agents, such as per acetic acid, have been observed in sugarcane bagasse (Zhao, et al., 2008).

FTIR spectroscopic studies were done in order to determine changes in functional group that may have been caused by the growth of *P. eous* on paddy straw. The absorbance peaks given by untreated (control) paddy straw as well as that obtained after 21 days of pretreatment with *P. eous* (Fig.5) in the range of 4000-2900 $cm^{-1}$, corresponds to the –OH and aliphatic –CH functional groups present in cellulose, hemicellulose and lignin, and the peaks in the range of 1800-700 $cm^{-1}$ corresponds to the –CH (aromatic hydrogen), C=C (aromatic skeleton) and C=O of ketones in lignin and hemicellulose (Sun, 2003). The absorbance peaks in the 3400-3000 $cm^{-1}$ region have been attributed to the stretching of –OH groups, whereas those around 2900-2800 $cm^{-1}$ to stretching of –CH groups (Khalil, 2001). Both these peaks were higher in the pretreated sample compared to the control, indicating an increase in the –OH and –CH functional groups (acidic or methanolic constituents) as a result of mushroom growth. An absorbance peak at 1625 $cm^{-1}$ in control and 1640 $cm^{-1}$ in the pretreated sample is possibly due to the –OH bending of adsorbed water as reported by Lojewska et al, Nacos et al and Troedec et al. (Lojewska, et al., 2005; Nacos, et al., 2006; Troedec, 2008). Another major peak was observed at 1063 $cm^{-1}$ in control and 1062 $cm^{-1}$ in treated sample, which is possibly due to stretching of –CO and –OH as reported by Nacos et al, to be observed as peaks around 1010-1070 $cm^{-1}$ (Nacos, et al., 2006). Lignin has characteristic peaks between 1600 and 1500 $cm^{-1}$ corresponding to aromatic skeletal vibrations (Moran, 2008). In the present study, absorbance peaks characteristic of lignin were lower in the pretreated sample compared to control.

Cellulose is a complex polymer with crystalline and amorphous areas. The cellulose crystallinity of control and SS-21 observed using XRD shows that the diffraction pattern changed after microbial growth. It was found that the crystallinity of cellulose increased from 46% in the control paddy straw to 50% in 21 day old SS. For lignocellulosic biomass, crystallinity measures the relative amount of crystalline cellulose in the total solid. The crystallinity of the treated paddy straw increased possibly due to removal of lignin and hemicellulose (both of which are amorphous), by the mushrooms (Zhao, 2008).

**Conclusion**

Production of ethanol from the spent substrate of *Pleurotus eous* was evaluated. Based on the results discussed it can be concluded that growing *Pleurotus sp*. can be an effective pretreatment for lignocellulosic substrates such as paddy straw, for bioethanol production. In further research the selection of lignocellulosic substrate and the optimization of fermentation conditions need to be addressed. Ethanol that is produced from lignocellulosic biomass can solve the current conflict between food and fuel production that emerged with the first generation biofuels and can make a contribution to renewable energy production. This work could make the recycling of spent mushroom substrate commercially attractive.

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