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Innovative and intensified technology for the biological pretreatment of agro waste for ethanol production

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Abstract−Lignocellulosic biomass is an abundant, renewable resource, but the structural and chemical complexity of biomass acts as a hindrance in its effective utilization for cellulosic ethanol production. Hence, effective pretreatment is always necessary to remove the surrounding matrix of lignin prior to the enzymatic hydrolysis. Pretreatment of rice straw by *Pleurotus florida* was found to be effective and resulted in 49% lignin degradation, whereas fungus along with grape leaves resulted in 99% lignin degradation. This method not only explores a pathway for utilizing the solid agro waste but also results in a value-added product of edible mushrooms that has proved to be the best pretreatment technology for ethanol production. FTIR and SEM analysis confirmed the structural transformation taking place during the pretreatment. The components of grape leaves were also analyzed using GC-MS.

Key words: Lignocellulose, Pretreatment, *Pleurotus* sp., Rice Straw, Grape Leaf

INTRODUCTION

With the growing population, use of fuel for transportation is tremendously increasing, and hence subsequently leading for demand. Ethanol production by biotechnological means has proved to act as an alternative fuel and thus acquired considerable interest. The rise in prices and environmental problems caused by fossil fuels has contributed to this recent interest from economical and ecological perspectives [1,2]. Brazil produces ethanol from the fermentation of cane juice whereas in the US corn is used. In the US, fuel ethanol has been used in gasohol or oxygenated fuels since the 1980s and contains up to 10% ethanol by volume [3]. Bioethanol produced from plants acts as possible alternative to fossil fuels, which would decrease worldwide carbon dioxide emissions. Currently bioethanol is mainly produced from sugar cane, sugar beets, and corn starch, using yeasts. In ethanol fermentation with yeast, sugar cane and sugar beets have an advantage in that they contain directly fermentable sugars: sucrose, glucose, and fructose. However, as the use of these crops for ethanol production would compete with their use as food sources, a non-competitive crop, sweet sorghum, has recently come to be looked upon as a promising source of bioethanol [4].

Biomass can be considered as the mass of organic material from any biological material available for conversion into bioproducts. Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose and cellulose which make them a substrate of enormous biotechnological value [5]. Rice straw is one of the abundant lignocellulosic waste materials in the world which is a by-product of rice production and a great bioresource [6].

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Lignocellulosics are considered as recalcitrant molecules which cannot be degraded easily because of complex chemical nature [7]. Due to the crystalline structure of cellulose fibrils surrounded by hemicellulose and the presence of the lignin seal, penetration by degrading enzymes is prevented, thereby hindering the effective utilization of this renewable resource for ethanol production [8,9].

A number of different methods, such as steam explosion [10,11], steam with dilute sulfuric acid [11] or sulfur dioxide [12], organosolvent extraction [13], and white rot fungi [14] have been extensively investigated. White rot fungi degrade the lignocellulosics in an environmentally friendly way and play a key role in organic matter recycling. Fungi utilize these resources as food substrates for energy. They are expected to be potentially useful as a pretreatment in biorefinery processes, including the production of biofuels and functional chemicals from lignocellulosic materials [15].

An ideal technology is needed to reduce the lignin content and crystallinity of the cellulose and increase the surface area of these materials. To enhance susceptibility to enzymatic hydrolysis and digestibility, or the nutritive value of different lignocellulases, various pretreatments using white-rot fungi have been examined [13]. But these methods fail to express the full potential of the source.

Wood-destroying fungi excrete cellulolytic enzymes into the medium to digest cellulose. This makes the cellulose become degraded, thereby reducing the amount of cellulose available for ethanol production. Mendel et al. [16] have conducted the most extensive work on naturally occurring cellulase inhibitors. A water soluble, thermostable, high-molecular-weight substance, found in grape leaves, inhibits the enzymatic hydrolysis of soluble cellulose [17].

The present work is focused on the study of the low cost biological pretreatment of rice straw for effective lignin degradation. Two ligninolytic fungi, *Pleurotus ostreatus* and *Pleurotus florida*, are used for this purpose. In addition, the effect of grape leaves on availability of cellulose content and lignin degradation of rice straw

is observed to consider its further utilization for ethanol production. The components present in grape leaves were further analyzed using GC-MS and the study of these components forms part of future work.

MATERIALS AND METHODS

1. Materials

Rice straw used in the present study was collected from the paddy fields in Sathyamangalam, Erode District, TamilNadu. Grape leaves were obtained from 'TVM Farms' in Dheenampalayam, Coimbatore, TamilNadu. The grape leaves were dried in sunlight for two days and powdered and used for the experiment. The spawn culture of *Pleurotus ostreatus* was obtained from 'Blue Hill Mushrooms' in Coimbatore, Tamilnadu, India and *Pleurotus florida* was obtained from 'Iyer Farms' in Gobichettipalayam, Tamilnadu, India. All chemicals used were of highest purity available and of analytical grade. The instruments used in the present study are UV spectrophotometer (Perkin Elmer Lambda 35, Singapore), scanning electron microscope (SEM) (JEOL 6390, JAPAN), Fourier transform - infra red spectrophotometer (ABB Boomen MB 3000), electronic balance (Mettler Teledo AB 265-S) and gas chromatograph - mass spectrometer (THERMO GC- TRACE ULTRA VER: 5.0, THERMO MS DSQ II).

2. Biological Pretreatment of Lignocellulosics

The biological method of lignin degradation was carried out using two white rot fungi: *P. ostreatus* and *P. florida*. The lignocellulosic material used was rice straw. The effect of grape leaves on the pretreatment process was also assessed.

3. Preparation of Spawn Culture

The spawn culture was prepared by using spore culture method. A leaf of mushroom was collected and the central part alone was taken by removing the top and bottom part of it. The central part was then cut into pieces and inoculated in plates containing potato dextrose media placed under aseptic condition. Sufficient quantity of corn was collected and autoclaved at 30 psi for 1 hour in sterile bottle. After sufficient mycelial growth in PDA media, a portion of it was transferred into sterilized substrate (corn) under aseptic condition plugged with cotton and incubated at a 28 °C. A milky layer formation throughout the substrate signified uniform growth and the grown spores served as the spawn culture required for mushroom cultivation.

4. Pretreatment of Rice Straw Using Spawn Culture with Mushroom Cultivation

The rice straw collected from the paddy field was soaked in sufficient quantity of water for 6-8 hours and autoclaved at 30 psi for 1 hour. The autoclaved rice straw was further air dried and used as substrate for the growth of mushroom in bags. The bags were prepared by placing a layer of rice straw (2 inches thick) followed by the layer of spawn culture (1 cm thick) alternatively into the bag in a compressed condition in a polypropylene bag of size 15′36″. Two such bags were prepared each weighing 1 kg. Two species of spawn culture, *P. ostreatus* and *P. florida* were used. 350 g of spawn culture was used in the preparation of each bag. Adequate number of holes was made in these bags for the initial primordia to come out, which will in turn grow into edible mushrooms. These bags were maintained at room temperature in a dark environment. The bags were sprinkled with water, maintaining moisture content of 70% after 20 days till the final harvest. 5 g rice straw was withdrawn from the bag at an interval of 5 days for carrying out lignin and cellulose estimation.

5. Pretreatment of Rice Straw Using Spawn Culture and Spawn Culture Along with Grape Leaves

In another experiment, the rice straw was treated with spawn cultures of *P. ostreatus* and the species along with grape leaves. The same was conducted with spawn cultures of *P. florida* as well. The rice straw with spawn culture and rice straw with spawn culture along with grape leaves were maintained for 15 days in Erlenmeyer flasks and were used as test samples.

6. Estimation of Lignin Content

Lignin content was estimated for different samples of rice straw. The different samples were untreated rice straw (URS), rice straw treated with spawn culture with mushroom cultivation (TRM), rice straw treated with spawn culture (TRS) for 15 days and rice straw treated with spawn culture along with grape leaves (TRG). The rice straw pretreated with spawn cultures of *P. ostreatus* and *P. florida* were separately used for the test. The spectrophotometric method (Stafford, 1960) [18] was used for the estimation of lignin content. The lignin degradation was further confirmed using Fourier transform infra red (FT-IR) (ABB Boomen MB 3000) analysis and scanning electron microscopy (SEM) analysis (JEOL 6390, JAPAN) after coating the samples with Au/Pd alloy.

7. Estimation of Cellulose Content

Cellulose content was estimated for different samples of rice straw. The different samples were URS, TRM, TRS and TRG. The Anthrone method (Updegroff, 1969) [19] was used for the estimation of cellulose content.

8. Extraction of Components Present in Grape Leaves

Compounds were extracted from grape leaves by using the soxhlet apparatus. Soxhlation was carried out using methanol as a solvent. 10 g of grape leaf powder was packed onto the soxhlation apparatus and extraction process was carried on for 12 cycles. The extraction process was completed in 1 day. The solvent present along with the extract was separated by evaporating it using a rotary evaporator (CYBER LAB US, CR 2000). The separated extract was analyzed using GC-MS (Gas Chromatography - Mass Spectrometry) (THERMO GC- TRACE ULTRA VER: 5.0, THERMO MS DSQ II) equipped with capillary standard non-polar column (DB 35-MS) of 30 m height, internal diameter 0.25 mm and film thickness 0.25 μm. One microliter of extract was injected to analyze by GC-MS at preset conditions. The carrier gas used was He with flow rate of 1.0 mL/min. The temperature program was set such as the initial oven temperature was 40 °C, which was further raised to 270 °C at a rate of 8 °C/min. The analyzed data was compared with the inbuilt standard chemical library system (National Institute of Standards and Technology (NIST) library, NBS75K, 2003).

RESULTS AND DISCUSSION

1. Rice Straw Treated Using Spawn Culture with Mushroom Cultivation

The white rot fungi *P. ostreatus* and *P. florida* were used for lignin degradation with edible mushroom cultivation. These fungi exhibited considerable growth in the bags of rice straw.

Three stages of growth were observed namely, initial primordium

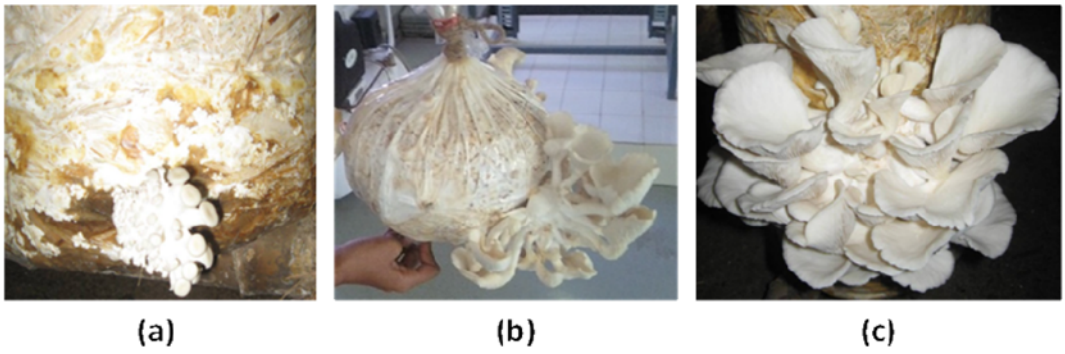

Fig. 1. *P. ostreatus* (a) initial primordium formation (33rd day), (b) moderate growth (35th day), adequate growth (37th day).

Fig. 2. *P. florida* (a) initial primordium formation (30th day), (b) moderate growth (32nd day), adequate growth (34th day).

formation, moderate growth of mushrooms and adequate growth of mushrooms (Fig. 1). From Fig. 1 it was observed that initial primordium formation took place on the 33rd day with the spawn culture of *P. ostreatus*, followed by moderate growth on 35th day and reached an adequate growth in to edible mushroom on 37th day.

Similar growth pattern was observed (Fig. 2) on the bag with the spawn culture of *P. florida*. On the 30th day initial primordium was observed followed by moderate growth on the 32nd day. Adequate growth was observed on the 34th day. On comparison between the various growth stages exhibited by *P. ostreatus* and *P. florida*, it was observed that *P. florida* showed a slightly faster growth.

2. Lignin Degradation

The lignin content was estimated in various samples of rice straw treated using *P. florida* and *P. ostreatus*. The samples were URS, TRM, TRS and TRG.

A gradual decrease in the lignin content was observed from the URS to TRS to TRM. TRG exhibited a significant decrease in lignin content (Fig. 3). It can be inferred that grape leaves are enhancing the lignin degradability of the fungi. The lignin content value decreased from 1.124 to 0.0104 for *P. florida* and 1.124 to 0.0405 for *P. ostreatus*, which was due to the metabolic action of the fungus during its growth.

P. florida was observed to have an increased effect on lignin degradation when compared to *P. ostreatus* (Figs. 3 & 4). Hence it can be inferred that *P. florida* exhibits better lignin degradation of up

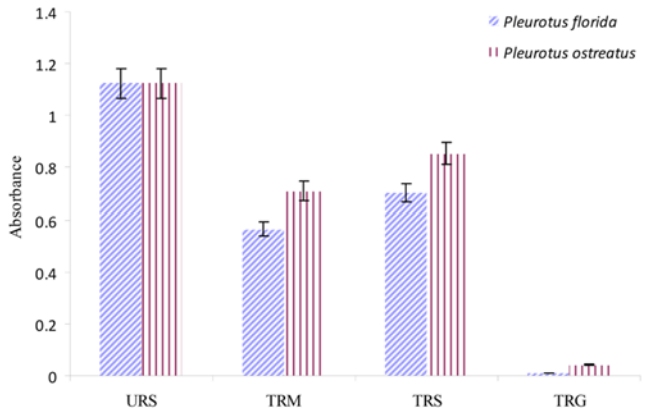

Fig. 3. Lignin content in various treated sample using two spawn cultures of *Pleurotus* sp.

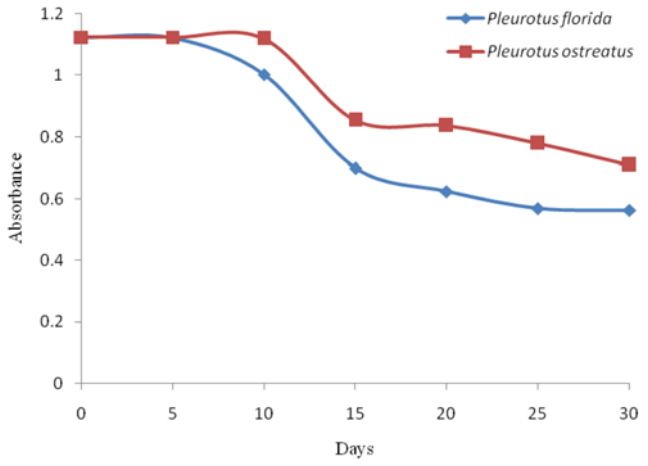

Fig. 4. Profile of lignin degradation of rice straw treated using spawn cultures of *Pleurotus* sp. with mushroom cultivation.

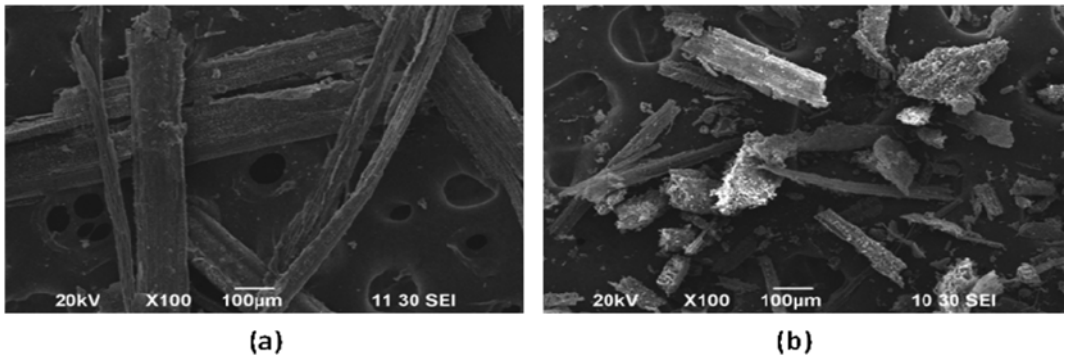

Fig. 5. SEM images: (a) Rice straw treated with *P. florida*, (b) Rice straw treated with *P. florida* along with grape leaves.

to 99%, and thus it can be used as an effective biological pretreatment of rice straw. The studies carried out on *Pleurotus* sp. by Singh et al. [20] indicated a gradual decrease in the lignin up to harvest of crop during basidiome compared to spawn run stage. A report showed that lignin loss of 80.16% at 10 N sodium hydroxide held in steam for 10 min in case of rice husk [21].

3. SEM and FT-IR Analysis

SEM and FT-IR analysis were performed for various samples of rice straw treated with *Pleurotus* sp. at different conditions, i.e., *Pleurotus* sp. alone and *Pleurotus* sp. along with grape leaves.

The SEM images indicated that rice straw treated with *P. florida* (Fig. 5(a)) showed relatively intact structure when compared to that of the rice straw treated with *P. florida* along with grape leaves (Fig. 5(b)). In Fig. 5(a) the rice straw treated with *P. florida* seemed to be lengthy and streaky, whereas it was reduced to fine ground particles when treated with *P. florida* along with grape leaves (Fig. 5(b)), thus creating particles with large surface area.

Akihiro et al. [22] obtained similar results during the removal of lignin from rice straw with hot-compressed water (HCW) and dry ball mill (DBM) treatment. SEM examination of rice husk (RH) before and after chemical treatments by Emiliano et al. [23] gave further insight into the RH morphology and its modification. The

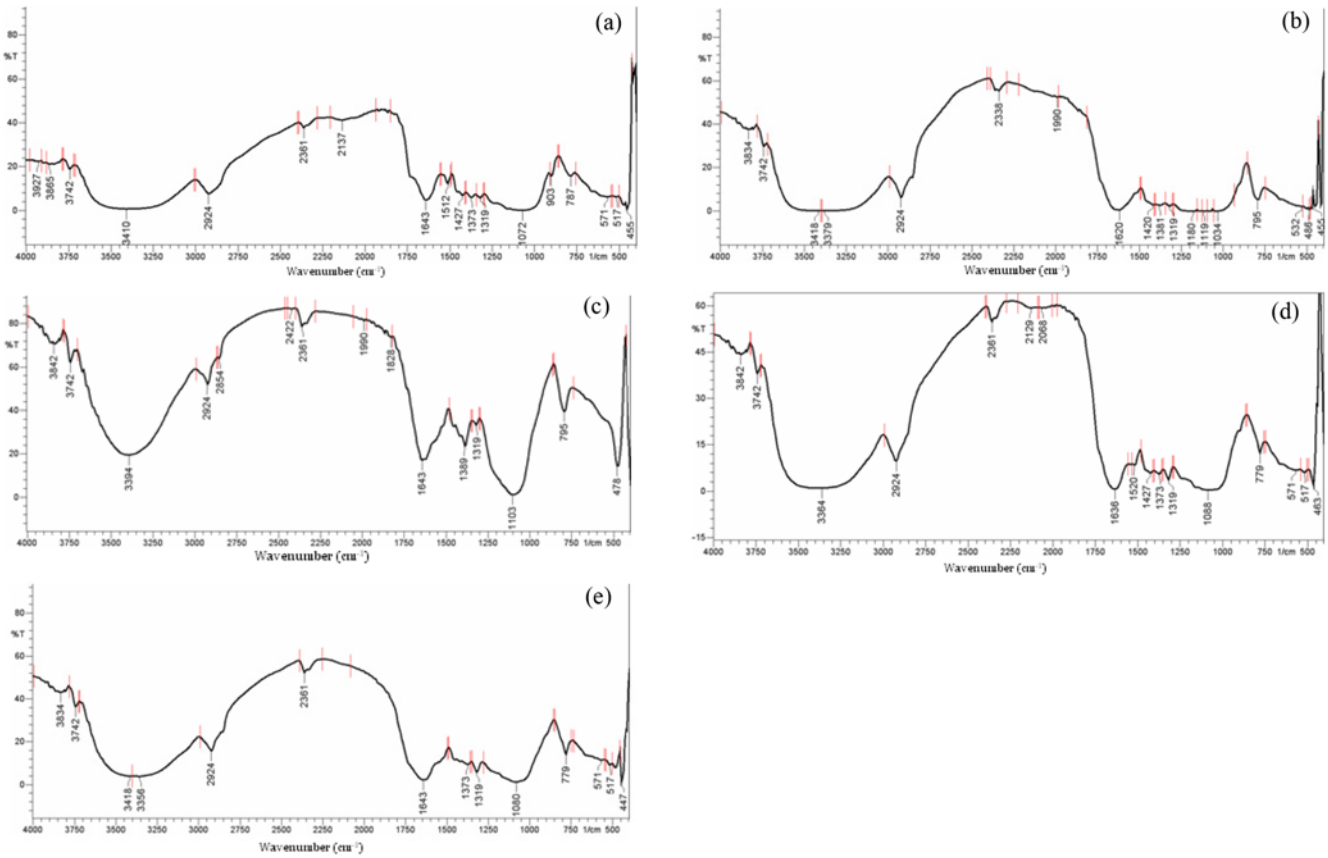

Fig. 6. (a) FT-IR spectra for URS-Untreated rice straw. (b) FT-IR spectra for TRS *P. ostreatus* - rice straw treated with *P. ostreatus*. (c) FT-IR spectra for TRS *P. florida* - rice straw treated with *P. florida*. (d) FT-IR spectra for TRG *P. ostreatus* - rice straw treated with *P. ostreatus* along with grape leaves. (e) FT-IR spectra for TRG *P. florida* - rice straw treated with *P. florida* along with grape leaves.

results indicated that chemical treatments change the RH morphology (inner and outer surface), eliminate hemicelluloses and lignin components, reduce silica content and probably decrease the crystallinity of cellulose fraction. In Fig. 5(b) globular structures are observed as a result of degradation. In a similar study during SEM analysis, clear cellulose microfibrils and lignin globules were not observed in milled rice straw particles, but the appearance of globules, which are characteristic of lignin deposition around the cellulose layer, was evident in acid-treated rice straw [24]. This shows that treatment by biological means degrades the lignin and reduces the crystallinity of cellulose fraction.

The FT-IR spectra of the samples are shown in the Fig. 6(a)-(e). The aromatic absorption region of the spectra contains bands assigned to lignin. Of the whole wave number range (500-4,000 cm^{-1}), only the subrange of 500-1,850 cm^{-1} is known to encompass lignin related information [25]. Aromatic skeletal vibrations in lignin are assigned at 1,425-1,430 cm^{-1}, 1,505-1 515 cm^{-1} [26]. A disappearance in the aromatic stretches is observed from untreated rice straw to the rice straw treated with fungi to the rice straw treated with rice straw along with grape leaves. The aromatic rings present in URS at 1,512 cm^{-1} and 1,427 cm^{-1} reduce to 1,420 cm^{-1} in TRS *P. ostreatus* and it completely disappears in the samples TRS *P. florida* and TRG *P. florida*. However, in the sample TRG *P. ostreatus* (Fig. 6(d)) shows minor peaks around 1,430 and 1,500 cm^{-1} which reveals that lignin was not much degraded using *P. ostreatus* compared to *P. florida.* A loss in the aromatic moieties in the lignin polymer portion of the straw is imparted by the fungal degradation. The broad band at 3,350 cm^{-1} was associated with O-H stretching of the hydrogen bonds of cellulose.

4. Cellulose Content

The cellulose content was estimated in various samples of rice straw treated using *P. florida* and *P. ostreatus*. The samples were URS, TRM, TRS and TRG.

A gradual decrease in the cellulose content is observed from the untreated rice straw to rice straw with spawn culture incubated for 15 days to rice straw treated with the fungi and incubated for 30 days (Fig. 7). During the growth of mushroom, cellulose content of substrates was reduced with maximum rate at basidiome stage compared to spawn run stage due to increase in the activity of the cellulase enzyme produced by the end of spawn run period [27].

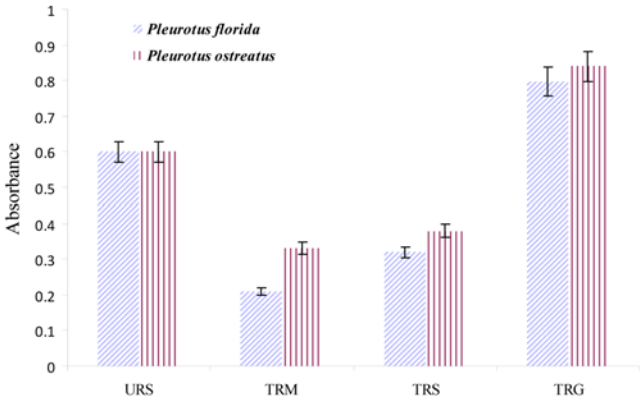

Fig. 7. Cellulose content in various treated sample using two spawn cultures of *Pleurotus* sp.

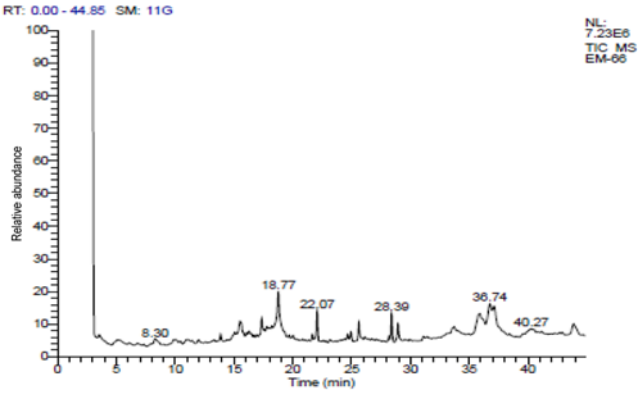

Fig. 8. GC chromatogram of grape leaf extract.

Addition of grape leaves was seen to result in a tremendous increase in the cellulose content by acting as an inhibitor of cellulolytic enzymes secreted by the fungus and by also contributing to the cellulose content. Thus, addition of grape leaves decreases the cellulose degradation. It was reported that during the pretreatment of rice straw with the fungi, the cellulose content degraded along with lignin degradation. This reduces the availability of cellulose for bioconversion to ethanol, which is also evident from the present study. But, this problem was rectified by the addition of grape leaves along with the culture containing rice straw. This was because certain compounds present in the grape leaves act as an inhibitor for the cellulolytic enzymes and also as an additional substrate. On the other hand, a slight reduction in the basidiome production was observed, which may be due to inhibition of cellulose activity.

5. GC-MS Study for Analysis of Components in Grape Leaves

Grape leaf extract obtained using solvent extraction was analyzed using GC-MS. 30 constitutes were identified. The chromatogram (Fig. 8) obtained from GC analysis of grape leaf extract gave various peaks at different retention time suggesting the presence of different volatile compounds in it. Each peak represents a unique compound. The most probable compounds are listed in the Table 1.

The GC-MS analysis of grape leaf extract was identified to contain 30 different constituents. The principal constituents were identified to be 3-hydroxyhexacyclo tridecane-6-one at a retention time of 18.77 min with molecular weight of 202 followed by 4-methylchloride-hex-4-ene-1-ol (retention time: 22.07 min and molecular weight of 148) and 1-hexadecene (CAS) (retention time: 28.39 min and molecular weight of 224) (Table 1). In a study on *Rhododendron* species by Deniz et al. [28] a total of 200 compounds were detected by GC-MS analysis. It was observed that *R. x sochadzeae* leaves contained only phenylethyl alcohol. Khalil et al. [29] report the use of GC-MS to investigate volatile compounds in leaves of *Juniperus excelsa* native to Syria.

The components present in the grape leaves will be further studied for application and optimization for effective pretreatment, which forms part of future work.

CONCLUSIONS

The thrust for fuel resources demands researchers to search up on non-food sources for production of an alternative fuel. Finding sources alone is not important, but effective conversion of sources

Table 1. List of probable compounds in grape leaves identified using MS suggested by NIST library

No.	Retention time (min)	Compound	Molecular weight	Molecular formula	Area %
1	3.55	4-chlorobutylacetate	148	$C_6H_9ClO_2$	1.27
2	5.08	24-hydroxyimino-29-norcycloart-3-ol	443	$C_{29}H_{49}NO_2$	4.09
3	8.30	Pentanoic acid, 4-methyl-(CAS)	116	$C_6H_{12}O_2$	2.56
4	9.85	2-benzyl-2-methylthio-3-methylbutanoic acid	238	$C_{13}H_{18}O_2S$	1.02
5	10.95	Butyl-pyridin-2-ylmethyl-amine	164	$C_{10}H_{16}N_2$	1.46
6	11.97	6-chloro-2-(2-thienyl)selenazolo[4,5-6] pyridine	300	$C_{10}H_5ClN_2SSe$	1.16
7	13.86	Ethylenediamin	60	$C_2H_8N_2$	1.85
8	15.06	(4″-ethoxycarbonyl-3″-butenyl) ester of (3″E)-cyclopent-2-en-1-yliden-carboxylic acid	250	$C_{14}H_{18}O_4$	1.42
9	15.51	Ethyl[2-methyl-1-(benzylamino) propane]phenylphosphinate	331	$C_{19}H_{26}NO_2P$	4.54
10	16.29	1-butanol, 4-butoxy-(CAS)	146	$C_8H_{18}O_2$	3.93
11	17.35	(R)-1,7-diphenyl-3-heptanol	268	$C_{19}H_{24}O$	3.67
12	17.79	(4-bromo-1-butynyl) cyclopropane	172	C_7H_9Br	1.03
13	18.77	3-hydroxyhexacyclo[6.5.0.0(3,7).0(4,12).0(5,10).0(9,13)] tridecane-6-one	**202**	**$C_{13}H_{14}O_2$**	**8.98**
14	20.01	2-ethylbutanol-2-13C	100	$C_6H_{12}O$	0.95
15	21.66	3,5-trans-3-hydroxy-5-[(E)-1-pentenyl]-4,5-dihydro-2(3H)-furanone	170	$C_9H_{14}O_3$	1.29
16	22.07	4-methylchloride-hex-4-ene-1-ol	**148**	**$C_7H_{13}ClO$**	**7.93**
17	24.66	N-cyano-3-methylbut-2-enamine	110	$C_6H_{10}N_2$	1.29
18	24.95	Tetradecanoic acid, methyl ester (CAS)	242	$C_{15}H_{30}O_2$	2.33
19	25.62	Decanoic acid (CAS)	172	$C_{10}H_{20}O_2$	4.74
20	28.18	3-cyclohexycyclohexene	164	$C_{12}H_{20}$	0.98
21	28.39	1-hexadecene (CAS)	**224**	**$C_{16}H_{32}$**	**7.19**
22	28.94	Glyceryl tridocasahexaenoate	1022	$C_{69}H_{98}O_6$	4.90
23	31.10	Benzene, 2-propenyl- (CAS)	118	C_9H_{10}	0.89
24	33.71	Salpen-(t-bu) [BC12]2	666	$C_{33}H_{48}B_2C_{14}$	3.11
25	35.78	2-[[2 bromo-4-(1-methylethyl) phenyl]amino]-5-[6-3-pyridinyl)hexyl]pyridine	451	$C_{25}H_{30}BrN_3$	6.64
26	36.21	Bis[1,2-(bis(methoxycarbonyl)-3,5,6-trimethyl phenyl]methane	484	$C_{27}H_{32}O_8$	0.67
27	36.74	p-tolyl[1-phenyl-3-(p-tolyl)-1,2,4-triazol-5yl]ketone-phenylhydrazone	443	$C_{29}H_{25}N_5$	4.78
28	37.13	5-hydroxy-4-(hydroxymethyl)-6-(2-propenyl)-1,3-benzodioxole	208	$C_{11}H_{12}O_4$	4.58
29	40.27	Tetraethyl meso-2,3-bis(4-iso-butyl phenyl)butane-1,1,4,4-tetracarboxylate	610	$C_{36}H_{50}O_8$	5.02
30	43.87	5-bromo-2ethyl-1-trimethylsilyloxy-1-pentene	264	$C_{10}H_{21}BrOSi$	5.72

to ethanol is very important. The lignin in rice straw acts as a hindrance in effective utilization for cellulosic ethanol production. This lignin can be effectively degraded using the white rot fungus. *P. florida* was observed to have an increased effect on lignin degradation when compared to *P. ostreatus.* It in turn implies that rice straw acts as a good substrate for the cultivation of mushroom, giving a value added product. Addition of grape leaves to the rice straw has a significant effect on the degradation of lignin and cellulose content in the rice straw. They are capable of enhancing the process of lignin degradation and reducing the uptake of cellulose. Hence, addition of grape leaves along with *P. florida* could be an innovative and intensified technology for the pretreatment of lignocellulosics for ethanol production. We feel the present study could help for better biological pre-treatment strategy, subsequently leading to increased ethanol production.

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