

Implementation of White rot fungal Pretreated Rice straw for Sustainable Bioethanol Production by *Saccharomyces cerevisiae*

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Abstract

*This study presents the amount of bioethanol produced from rice straw pretreated with white rot Fungi, *Pleurotus ostreatus*, by Separate Hydrolysis and Fermentation (SHF) method using yeast. Rice straw was an attractive lignocellulosic material for bioethanol production since it is one of the most abundant renewable sources in India. The pretreatment of biomass is necessary to remove the surrounding matrix of lignin prior to enzymatic hydrolysis of cellulose to glucose and fermentation of glucose to ethanol. The rice straw was biologically pretreated with white rot fungi, *Pleurotus ostreatus*, for 44 days to degrade the lignin matrix. During the pretreatment, 27.85% of lignin and 24.30% of cellulose was degraded in 24 days. Results were justified from the structural changes observed before and after the pretreatment, evaluated by SEM and FT-IR studies. Maximum amount of glucose was released during hydrolysis at optimum conditions such as pH 4.5, temperature 50°C and incubation time 36hrs. The amount of ethanol produced from white rot fungal pretreated rice straw by Separate Hydrolysis and Fermentation using *saccharomyces cerevisiae* after 3 days of fermentation was 2.3% (v/w).*

Keywords: *Pretreatment, *Pleurotus ostreatus*, Fermentation, *Saccharomyces cerevisiae*, Hydrolysis.*

1. Introduction

In the 20th century, the world economy has been dominated by technologies that depend on fossil energy, such as petroleum, coal, or natural gas to produce fuels, chemicals, materials and power [1]. Utilization of fossil fuels increases due to the huge

population and advanced technologies [2]; it increases environmental pollution, the emission of green house gases and global warming [3]. To solve these problems, many researchers have tried to convert biomass into fuel ethanol for an alternative to fossil fuels. Bioenergy is a form of stored energy from the sun, present in materials such as plant matter and animal waste (biomass). Biomass, a renewable source, is replenished more quickly than the fossil fuels. The demand for biofuels from traditional food crops (corn, sorghum, maize) leads to raise in global food prices as well as threaten to food security. Production of biofuels without impairing food security is possible by investigating non-edible agricultural products, include the cellulosic biomass as well as waste materials which can be obtained throughout the year and are relatively inexpensive [3,4]. India is the second largest producer of rice in the world, accounting for about 22% of worldwide rice production reported by Food and Agriculture Organization (Statistical Database for Crop Production) Rome, Italy. Rice straw, the non edible lignocellulosic by product worldwide used for bioethanol production, and has an estimation of global production of 600–900 million tons per year [5]. Efficient conversion of lignocellulosic biomass to biofuel requires three sequential steps: Pretreatment, Hydrolysis and Fermentation [6,7]. The high crystallinity of cellulose and the presence of lignin matrix make the lignocellulosic complex fairly resistant to enzymatic hydrolysis processes without pretreatment [8, 9]. Chemical pretreatments with either acid or base have serious disadvantages in terms of the requirement of specialized corrosion resistant equipments, extensive washing, proper disposal of chemical wastes and glucose degradation [10]. Physical pretreatments like Liquid hot water pretreatment, Microwave pretreatment, subcritical and supercritical water

treatment processes require expensive special instruments that have substantial energy requirements depending on the complexity of the process [10]. Biological pretreatment is a safe and an environmental friendly method. The most promising microorganisms for biological pretreatment are white-rot fungi, especially *Pleurotus ostreatus* [10]. The most promising organisms for alcoholic fermentation of lignocellulosic sugars are yeasts. These Eukaryotic organisms have much larger cells than bacteria, which facilitates their separation from the fermentation broth. Yeast fermentations are resistant to virus infection and bacterial contamination, and yeasts are often more resistant to ethanol than bacteria [11]. The government of India is aggressively promoting the concept of blending gasoline with ethanol to reduce dependence on gasoline, and about 500 million liters of ethanol would be required every year, even if 10% ethanol is blended with gasoline [12]. The aim of this present study is to optimize the conditions for hydrolysis and fermentation. We also tried to investigate the role of biological pretreatment on structural disintegration of rice straw through Scanning Electron Microscopy (SEM) and functional group changes by Fourier Transform Infra Red Spectroscopy (FT-IR).

2. Methodology

2.1. Composition Analysis of Rice straw

The selected agro material, rice straw, was collected from the agricultural fields nearby Gobiccetipalayam, Erode district, Tamil Nadu, India. The moisture content of biomass was measured by dry weight method. The biomass was dried in oven at 105°C for 24 hrs and reweighed it. The difference in the biomass weight with respect to the initial weight of the biomass gave the moisture content [13]. Cellulose content was determined at 620nm using cold anthrone reagent [14]. Lignin content was analyzed by acetyl bromide method at 280nm [15].

2.2. Biological Pretreatment

The white rot fungi, *Pleurotus ostreatus*, degrade the lignin effectively than other *Pleurotus* species [10]. The spawns of white rot fungi, *Pleurotus ostreatus*, were purchased from Tamil Nadu agricultural university, Coimbatore, Tamil Nadu, India. Initially the air dried rice straw were cut into 5 cm pieces and soaked in water for 16 hrs. The excess water was drained out and the straw was sterilized at 120°C,

15 psi for 1 hr to minimize the contamination. Layer spawning was done using polythene bags of size (20 x 30 cm), fill the substrate in bag, press it to a depth of 8-10 cm and spread a handful of spawn above till it reached a required level and then bags were closed. Specified orifice holes were made on the tightly packed bag and 70% humidity was maintained by sprinkling water throughout the process [16, 17].

2.3. Analysis of Structural and Functional group changes

The morphological changes induced by pretreatment of rice straw with *Pleurotus ostreatus* were examined by Scanning Electron Microscope (SEM) after 24 hrs of drying in hot air oven [10]. The samples coated with gold using a fine coater were observed under a scanning electron microscope at an accelerating voltage at 20kv and the images were taken with magnification of 250X and 3000X for native and pretreated rice straw respectively [10]. Fourier Transform Infrared (FTIR) analysis was done to investigate changes occurred in the functional groups by pretreatment using Perkin Elmer instrument model RX1. The analysis was done for the sample collected on 44th day of pretreatment to find the delignification using native straw as reference. Samples were dried for 24hrs in hot air oven, ground and sieved into fine powder. All the samples (each 3g) were dispersed in spectroscopic grade KBr (300g) at a ratio of 1:100 and pressed at 10Mpa for 3 min to form pellets. The infrared spectral range 4000 - 400cm⁻¹ was obtained with scan rate of 32/sec and 2cm⁻¹ resolution [18].

2.4. Optimization of Enzymatic Hydrolysis parameters with Cellulase

The cellulase enzyme was obtained from Rossari biotech, Tiruppur, Tamil Nadu, India. To maximize the efficiency of hydrolysis of pretreated rice straw with cellulase enzyme, the optimum pH and the temperature were determined by incubating 100g of pretreated rice straw in 500ml Erlenmeyer flask with 10 ml of cellulase at various pH such as 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 in room temperature for 30 hrs [19]. During the batch experiment, in order to optimize the temperature, the flasks were kept at various temperatures such as 30°C, 35°C, 40°C, 45°C, 50°C, 55°C at optimized pH for 30 hrs [20]. To optimize the incubation time, 100 g of pretreated rice straw was added with 10 ml of cellulase enzyme and incubated for 12, 24, 36, 48 hrs at optimized temperature and pH at 100rpm [20]. All

these optimizations were done based on estimating total amount of reducing sugar released by Dinitrosalicylic acid (DNS) method using glucose as standard[21].

2.5. Enzymatic Hydrolysis

Enzyme activity was determined by Filter paper assay for enzyme loading and the activity was expressed in Filter paper units (FPU) [22]. The maximum glucose yield from cellulose was obtained using higher enzyme loading, 25 FPU per gram of biomass. Batch experiment was conducted in a 500 ml conical flask. Hydrolysis of 100 gram of 44 days pretreated rice straw was carried out by adding 25 FPU per gram of substrate and adjusted the pH to optimum pH using citrate buffer. The experiment was carried out at optimum temperature and incubation time at 100rpm [23]. After enzymatic hydrolysis, the solid and liquid portion of hydrolysate was separated by filtration, and the filtrate was taken to fermentation for ethanol production.

2.6. Fermentation

The baker's yeast was purchased from local bakery and stored in refrigerator. Baker's yeast (*Saccharomyces cerevisiae*) was cultivated aerobically in yeast peptone dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) for 20 hrs at 30°C. The cells were collected by centrifugation at 5000 rpm for 5 min at 4°C, washed with sterilized water twice [24]. The 10% (v/v) inoculum of optical density of 0.2 at 620 nm (OD_{620}) was added into 100ml hydrolysate for anaerobic fermentation [25]. Fermentation was carried out at 30°C at for 1 to 6 days in six conical flasks to optimize the fermentation time [24]. The fermented medium was filtered and centrifuged to remove yeast debris and the supernatant was distilled at 78°C and the ethanol content in the distillate was estimated by chromic acid assay [26].

3. Results and Discussion

3.1. Composition of rice straw before and after the pretreatment

The moisture, cellulose and lignin content of rice straw before the pretreatment were estimated as 6.4%, 40.56%, and 20.14% respectively. Jin *et al* [27] obtained the average moisture content of 6.9% and cellulose content of 34.8% by Near Infra Red Analysis

and Bak *et al* [28] reported 19.70% of lignin. After 24 days of pretreatment, the cellulose and the lignin content were estimated as 30.70% and 14.53% respectively. It was observed that during the pretreatment process the white rot fungi, *Pleurotus ostreatus*, were unable to remove the lignin component alone; it degraded 24.30% of cellulose along with 27.85% of lignin in the rice straw. Taniguchi *et al* [10] reported that 30% of lignin as well as 32% of cellulose degradation with *Pleurotus ostreatus* pretreatment. The lignin removal was significantly less in the samples collected before 44 days and so the minimum period required for pretreatment was found to be 44 days.

3.2. Pretreatment with White rot fungi

The bag was found to be full of mycelia growth after 10 days of incubation and the mushroom started to sprout out after 20 days of incubation. The first and second harvest was done on 23rd day, 43rd day of pretreatment respectively. The growth on 20th, 22nd and 23rd days are shown in the Figure.1 (a,b,c).



Figure.1 (a).Growth on 20th day

Figure.1 (b). Growth on 22nd day



Figure.1 (c).Growth on 23rd day

The total weight of mushroom obtained in the second harvest was significantly less compared to first harvest because the nutrient content of rice straw started to decrease gradually during the progress of pretreatment duration [16, 17].

3.3. SEM and FTIR Analysis

The SEM observations of native and pretreated rice straw showed that partial degradation of lignin deposition around the cellulose layer by pretreatment with the white rot fungi, *Pleurotus ostreatus* (Figure.2 a,b). The pretreatment improves the susceptibility of rice straw to enzymatic hydrolysis due to partial degradation of the lignin that is responsible for preventing penetration of cellulase. These structural analyses proved that biological pretreatment of rice straw degraded the lignin and reduced the crystallinity of cellulose microfibrils [10]. FT-IR spectra showed the structural differences between 44 days pretreated rice straw and the control sample, native straw (Figure.3 a, b). The change observed at 1458cm^{-1} and 1508cm^{-1} was due to C=C stretching of aromatic ring of lignin and asymmetric bending in CH_3 of lignin respectively [18]. The absorption at 3370cm^{-1} was related H bonded OH groups, and 1081cm^{-1} was indicative of C-O stretching at C-3, and C-C stretching and C-O stretching at C-6 [29].

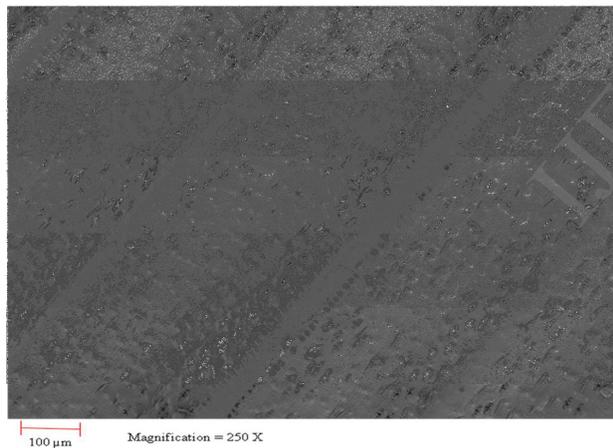


Figure.2(a). SEM image of native rice straw

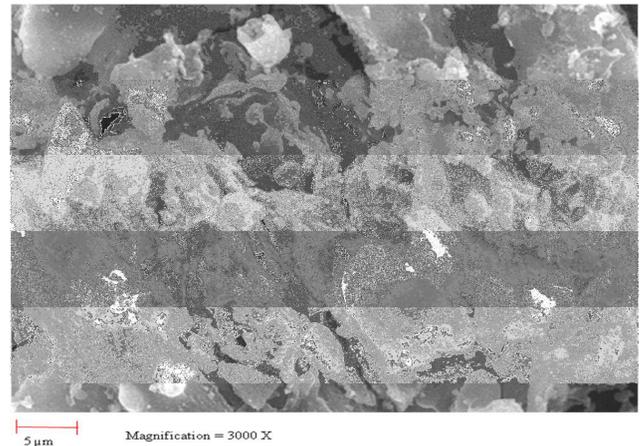


Figure.2 (b). SEM image of rice straw pretreated (44 days) with *Pleurotus ostreatus*

The change observed at 1644cm^{-1} was for bending mode of absorbed water [30]. The change at 1325cm^{-1} was assigned to symmetric CH_2 bending and wagging [18]. The reduction observed in the intensity of the peak around 1540cm^{-1} indicating the structural changes in lignin. The change observed at 874cm^{-1} attributed to structural changes in cellulose [18].

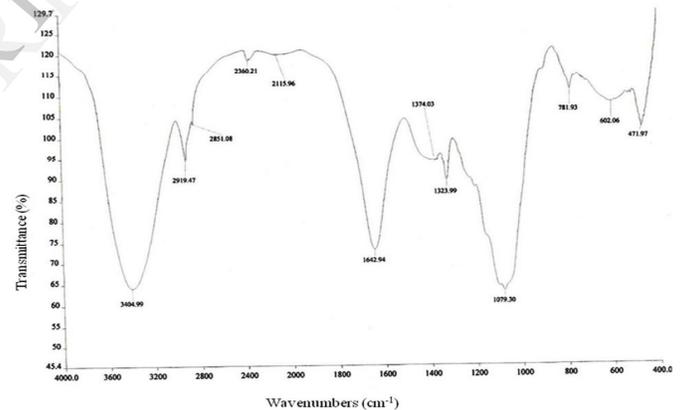


Figure.3 (a). FTIR spectrum of native rice straw

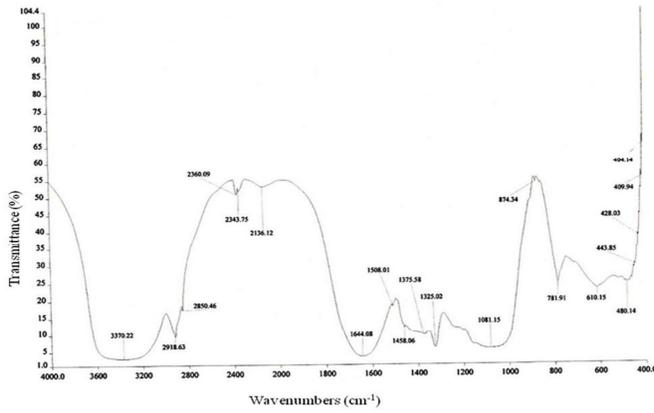


Figure.3 (b). FTIR spectrum of pretreated rice straw (44 days) with *Pleurotus ostreatus*

3.4. Optimum conditions for Enzymatic hydrolysis

The maximum amount of glucose released at pH 4.5 (Figure.4), 50°C (Figure.5) for 36hrs (Figure.6) was 10.11g, 9.99g, 10.10g from 100g of substrate respectively. These parameters were considered as optimum pH, temperature and optimum incubation time.

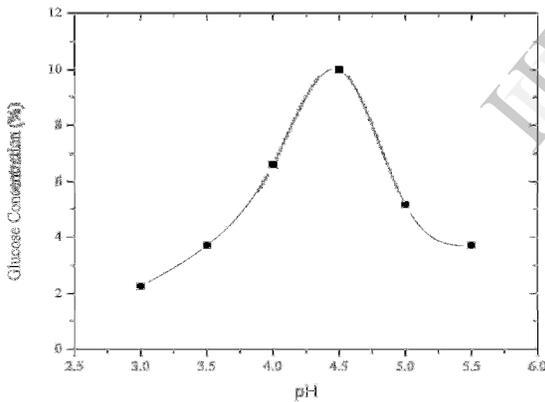


Figure.4. Effect of pH on Enzymatic Hydrolysis of pretreated rice straw

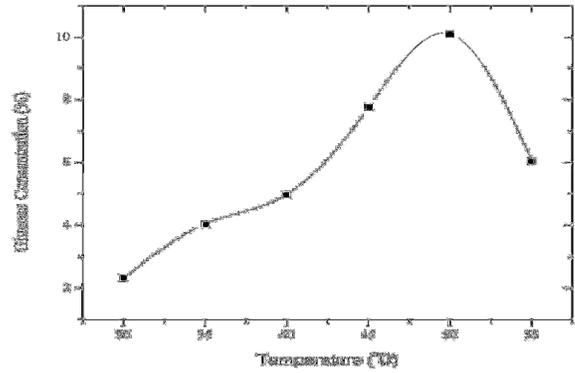


Figure.5. Effect of Temperature on Enzymatic Hydrolysis of pretreated rice straw

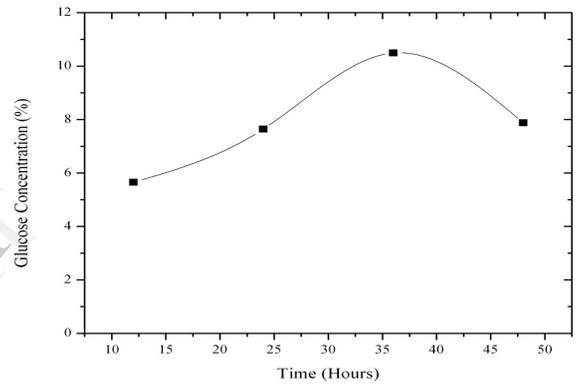


Figure.6. Effect of Incubation Time on Enzymatic Hydrolysis of pretreated rice straw

Except pH 4.5 and temperature 50°C, the amount of glucose produced was comparatively less because cellulase enzyme might be less active in remaining all other pH and temperatures. The amount of glucose released beyond 36hrs was decreased due to following reasons with respect to time: decreased susceptibility of substrate to enzyme attack; enzyme inactivation; enzyme inhibition by glucose and so the optimum time for hydrolysis was 36hrs. Similar optimum conditions were obtained by Rodhe *et al* [20].

3.5. Ethanol Estimation

The maximum amount of ethanol production of 2.3 ml (1.81 g) was observed after 3 days (Figure.7) of fermentation and it was the optimum fermentation period. Ethanol production decreased after 3 days due to reduction of sugar concentration with respect to time and the efficiency of the yeast cell in conversion of sugar to ethanol might be reduced. The volume of ethanol in 12ml of distillate was 2.3 ml. Under these optimum conditions, maximum amount of ethanol produced from 100g of substrate was 2.3 % (v/w) or 1.81%(w/w).The ethanol yield was less for 10% glucose present in the fermentation media. Sukumaran *et al* [31] obtained 9.3% (w/w) ethanol using alkali pretreatment. The amount of ethanol produced in this study was comparatively less because the cellulase enzymes produced by white rot fungi might have degraded the available cellulose for ethanol production during the pretreatment in the rice straw.

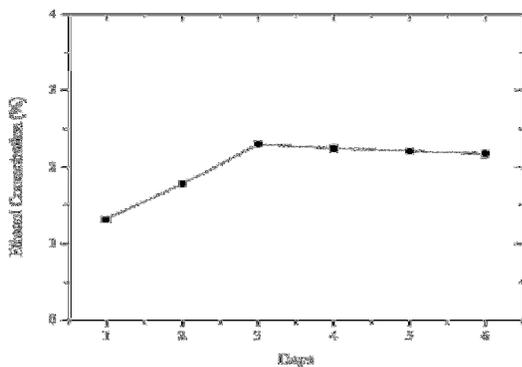


Figure. 7. Optimum Fermentation period for maximum ethanol Production

4. Conclusions

The pretreatment of rice with white rot fungi, *Pleurotus ostreatus*, was an efficient method for lignin degradation. The SEM observations showed that partial degradation of the lignin seal with *Pleurotus ostreatus* resulted in increased susceptibility of rice straw to enzymatic hydrolysis. Hydrolysis process was efficient with cellulase at optimized temperature 50°C and pH

4.5 for 36hrs. This investigation revealed that the fungal pretreatment was environmental friendly because pretreatment was done without using chemicals and other energy required physical methods. This pretreatment provided the edible mushrooms, employability to rural people; but time consuming method. A further study of balanced co- cultivation of cellulase producing organism with *Saccharomyces cerevisiae* is cost-effective by reducing cost for cellulase enzyme.

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6. References

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