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Research Article

PRETREATMENT OF AGRICULTURAL WASTE WITH *PLEUROTUS SP.* FOR ETHANOL PRODUCTION

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ABSTRACT: Bioethanol is a liquid fuel obtained from fermentation of sugar/starch crops. Lignocellulosic biomass being less expensive is considered a future alternative for the food crops. One of the main challenges for the use of lignocellulosics is the development of an efficient pre-treatment process. Pretreatments are classified into three - physical, chemical, and biological pretreatment. Chemical process has not been proven suitable so far, due to high costs and production of undesired by-products. Biologically, hydrolysis can be enhanced by microbial or enzymatic pretreatment. Studies show that the edible mushrooms of *Pleurotus sp.* produce several extracellular enzymes which reduce the structural and chemical complexity of fibre. In the present study, *P. ostreatus* and *P. eous* were cultivated on paddy straw. Spent substrate left after mushroom cultivation was powdered and used for ethanol production. *Saccharomyces sp*. was used for fermentation studies. Untreated paddy straw was used as control. Production of ethanol from *P. ostreatus* substrate was 5.5 times more when compared to untreated paddy straw, while the spent substrate of *P. eous* gave 5 times increase in ethanol yield. Assays showed the presence of several extracellular enzymes in the spent substrate of both species, which together contributed to the increase in ethanol yield.
Key words: Bioethanol; lignin; cellulose; lignocellulosic

INTRODUCTION

Ethanol, also called as ethyl alcohol, is a colourless liquid fuel. The fermentation of sugar into ethanol is one of the earliest organic reactions employed by humanity. In modern times, ethanol intended for industrial use is produced either through the hydration of ethylene, or biologically by fermenting sugar or starch with yeast. Ethanol can also be obtained from fermentation of cellulose. Hence, second generation ethanol is derived from lignocellulosic materials [1]. Lignocellulosic biomass refers to plant materials composed of cellulose, hemicellulose and lignin, such as agricultural residues, wood residues, municipal paper waste etc. Since it comes from plants, the combustion of lignocellulosic ethanol produces no net carbon dioxide into the earth's atmosphere.

One barrier for the production of ethanol from biomass is that, in lignocellulosic materials cellulose is tightly bound to hemicelluloses and lignin. Lignin resists degradation and confers hydrolytic stability and structural robustness to the cell walls of the plants. This robustness or "recalcitrance" is attributable to the cross linking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages [2]. To extract the fermentable sugars, one must first disconnect the celluloses from the lignin, and then hydrolyze the newly freed celluloses to break them down into simple monosaccharides. Another challenge to biomass fermentation is the high percentage of pentoses in the hemicellulose, such as xylose, or wood sugar. Unlike hexoses, like glucose, pentoses are difficult to ferment. The problems presented by the lignin and hemicellulose fractions are the foci of much contemporary research.

To overcome lignocellulose recalcitrance, pretreatment is required. The goal of pretreatment is to alter the physical features and chemical composition of lignocellulose to make it more digestible [3, 4]. Specifically, pretreatment improves enzyme access and effectiveness by removing or altering lignin, removing hemicellulose, decrystallising cellulose and reducing the degree of polymerisation in cellulose. A pretreatment that accomplishes all of these goals is likely to be very expensive, so most pretreatments focus on achieving just a few. It is clear, however, that different pretreatments affect biomass in different ways [3, 5]. The desirable characteristics of a pretreatment process are that it limits the formation of degradation products that may inhibit fermentative microorganisms, require minimal energy, effective on multiple lignocellulose feed stocks and minimize capital and operating costs. Pretreatment by physical, chemical or biological means is a well-investigated process for ethanol production from lignocellulosic materials [6]. Biological treatment using various types of rot fungi, a safe and environmentally friendly method, is increasingly being advocated as a process that does not require high energy for lignin removal from a lignocellulosic biomass, despite extensive lignin degradation [7]. In biological pretreatment processes, microorganisms such as brown-, white-, and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials [8]. Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as Lignin peroxidases and laccase [9]. These enzymes are regulated by carbon and nitrogen sources. White-rot fungi are the most effective for biological pretreatment of lignocellulosic materials [10]. Hatakka (1983) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus sp.* in 5 weeks [11]. The present work deals with biological pretreatment of the lignocellulosic agriwaste, paddy straw, using *Pleurotus sp.* for ethanol production.

MATERIALS AND METHODS

Pleurotus sp. such as *P. ostreatus* and *P. eous* were cultivated on paddy straw, as per the procedure outlined by Madhusudhanan et al. (2003) [12]. Spawn to substrate ratio was (1:10 w/w). Mushrooms were harvested on 18th and 21st day. After the harvest, the spent substrate (SS), the growing medium left after mushroom cultivation, was incubated until 35th day at 28˚C and 60% humidity. Quantitative assays were done for the extracellular enzymes present in the SS of *P. eous* and *P. ostreatus* from 21st-35th 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 (LiP), laccase and xylanases were assayed [13, 14, 15, 16]. 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 [16, 17]. Assays and estimations were done in triplicate. SS sampled on 21st, 28th and 35thday, 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 1:20w/v (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 [18, 19].

RESULTS AND DISCUSSION

Like other basidiomycetes, *Pleurotus sp.* exhibit two main phases during its life cycle. The first phase is the complete colonization of the substrate followed by a second phase of fruiting. In the local mushroom farms, *Pleurotus sp.* is cultivated in plastic bags and it takes about 13-15 days for complete colonization of the substrate by its mycelium. After colonization, *Pleurotus sp.* starts the fruiting phase which lasts for 3-5 days. A maximum of three flushes of oyster mushrooms are obtained from each bags and afterwards the spent substrate is discarded.

During different phases of growth, *Pleurotus sp.* has different patterns of production of hydrolytic and oxidizing enzymes. In the present study, to better understand the production of enzymes by *Pleurotus sp.*, a comparison of enzyme profiles of *P. eous* and *P. ostreatus*, during their growth on paddy straw, was performed. Among the two mushrooms, *P. ostreatus* showed faster mycelial growth than *P. eous*. Assays showed the presence of the extracellular enzymes such as cellulases, xylanases and ligninases in the spent substrate of both species, which contributed to the increase in ethanol yield. In the substrate of *P. eous*, cellulase activity was recorded only from 14th day, but in the substrate of *P. ostreatus* cellulase activity could be seen from 7th day (Fig.1). *P. ostreatus* gave more cellulase and xylanase activity when compared to *P. eous* (Fig. 1 and 2). Production of xylanases by *P. eous* was negligible in comparison to the xylanase profile of *P. ostreatus*. *P. ostreatus* showed a wave like pattern in the production of cellulases and xylanases with a sharp decrease in their activity on 21st day.

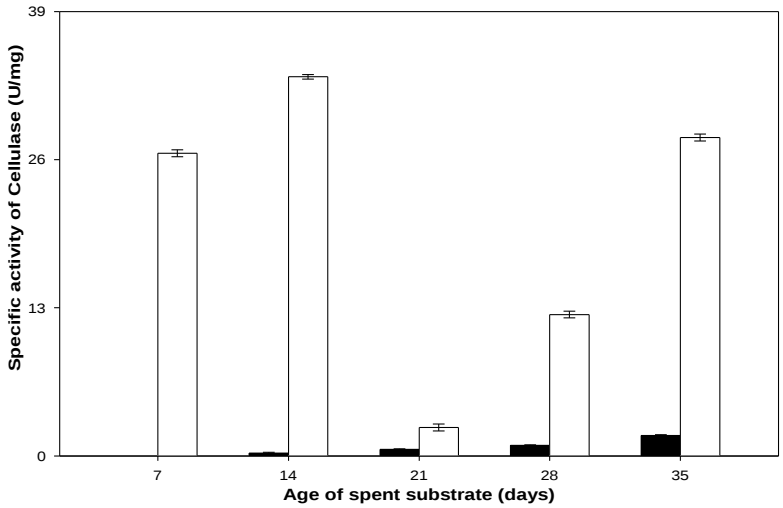

Fig 1 Specific activity of Cellulase in the spent substrate of *P. eous* (bar 1) and *P. ostreatus* [Note: Error bars represent ± standard deviation from a triplicate average]

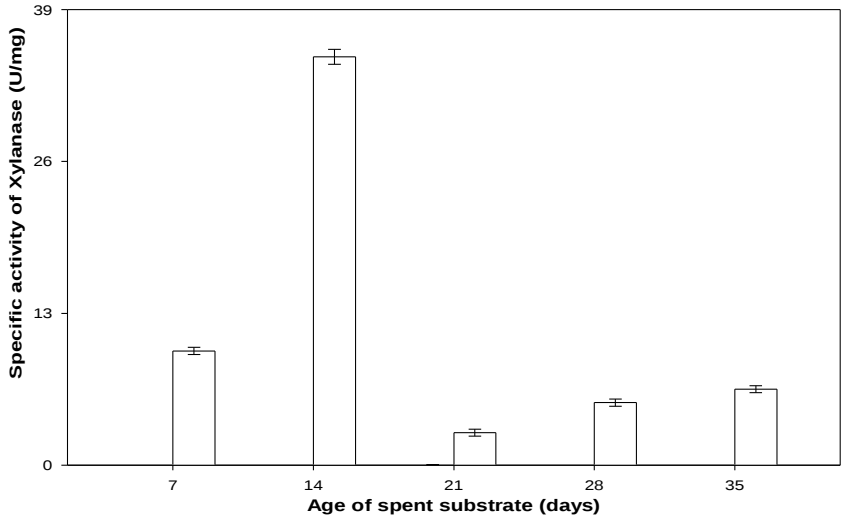

Fig 2 Specific activity of Xylanases in the spent substrate of *P. eous* (bar 1) and *P. ostreatus* [Note: Error bars represent ± standard deviation from a triplicate average]

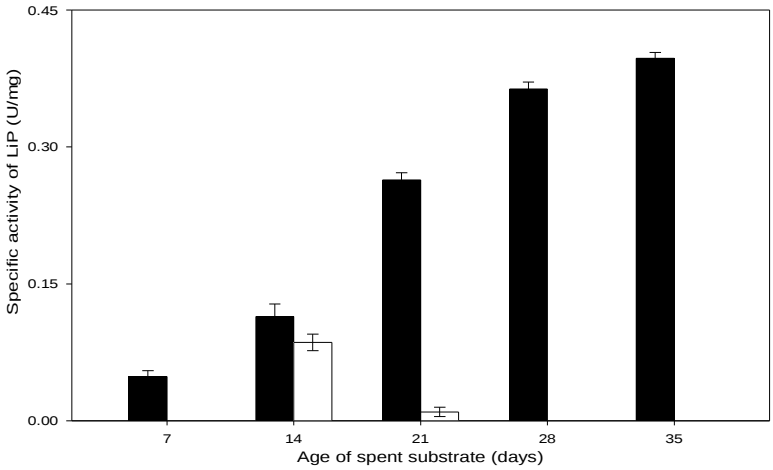

Fig 3 Specific activity of Lignin Peroxidase in the spent substrate of *P. eous* (bar 1) and *P. ostreatus*
[Note: Error bars represent ± standard deviation from a triplicate average]

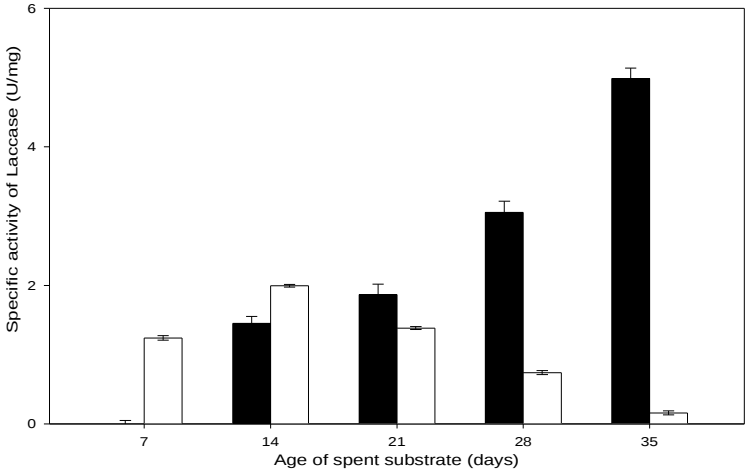

Fig 4 Specific activity of Laccase in the spent substrate of *P. eous* (bar 1) and *P. ostreatus* [Note: Error bars represent ± standard deviation from a triplicate average]

For both species, production of cellulase was more before 21st day of cultivation, while ligninases production continued to increase even after 21st day. Many authors reported that substrate lignin content was negatively correlated with saccharification of cellulose [20, 21]. In the present study we observed that the LiP and laccase activity increased gradually throughout the cultivation period of both mushrooms (Fig. 3 and 4). *P. eous* produced more ligninases when compared to *P. ostreatus*. Both mushrooms could produce significant level of laccase during the cultivation period which was comparatively higher than LiP activity.

Yield of total reducing sugar and ethanol from 21day old spent substrate of *P. eous* and *P. ostreatus* was evaluated. Total reducing sugar from untreated paddy straw was estimated to be 5.4 mg/g. But the enzymatic hydrolysis till 21st day gave a six fold increase in total reducing sugar yield from the SS of *P. ostreatus* (30.80 mg/g), when compared to the sugar yield (26.8 mg/g) from the SS of *P. eous* (Table1). However, in spite of increase in cellulase activity with age of substrate, total reducing sugar was highest in 21 day old SS of both mushrooms possibly because of the increased accessibility of cellulase to cellulose, due to the partial removal of lignin and hemicellulose. Yield of ethanol from *P. ostreatus* substrate was 5.5 times more (243.20mg/g) when compared to untreated paddy straw(44.80mg/g), while the spent substrate of *P. eous* gave 5 times increase (227.20mg/g) in ethanol yield (Table 1).

Table 1 Yield of total reducing sugar and ethanol (after 48hrs of fermentation) from untreated paddy straw and 21 day old spent substrate of *P. eous* and *P. ostreatus*

Paddy straw	Red. sugar (mg/g)	Ethanol (mg/g)
Untreated	5.4 ± 0.1	44.80 ± 3.00
SS of *P. eous*	26.80 ± 0.21	227.20 ± 5.00
SS of *P. ostreatus*	30.80 ± 0.22	243.20 ± 5.02

CONCLUSION

Present work highlights a pretreatment process which provides economic feasibility to harness the renewable materials, and at the same time cleaning up the environment. In recent years mushroom industry has faced increasing challenges regarding an environment friendly disposal of spent substrate. An obvious solution to the disposal problem is to increase the demand of spent substrate through the exploration of new applications for usage. The above results indicate that, *Pleurotus* cultivation can be an effective pretreatment for lignocellulosic substrates such as paddy straw, for ethanol production, and at the same time this study provides new information for recycling mushroom spent substrate.

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