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Biological Pretreatment of Six Lignocellulosic Wastes for Bioethanol Production

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Abstract: ***The global energy supply depends heavily on a finite supply of fossil fuels, the use of which produces green house gases and pollutants, causing climate change and other environmental problems. Therefore, developing cost effective and environmentally benign alternative energy resources has been gaining importance. Lignocellulosic resources constitute a major portion of agricultural and forestry wastes and it contains approximately 70-80% carbohydrates. In the present study, an attempt was made to achieve pretreatment method which plays an important role in enhancing the enzymatic saccharification thereby making the whole process economically viable. White – rot fungi are well known for production of ligninolytic enzymes which are capable of removing lignin from lignocellulosic substrates. Agricultural wastes, sugarcane baggasse, sweet sorghum, maize stover, groundnut shell, paddy straw and forestry waste, wood chips were used as sources of bioethanol. Pleurotus florida is cultivated by solid state fermentation on these substrates for 28 days. The fruit bodies formed were harvested and their yield, biological efficiency and nutrient content were estimated. The pretreated substrates were further hydrolysed by the cellulolytic enzymes produced by Trichoderma viride. The components of the lytic enzymes, laccase, xylanase, amylase and cellulase were estimated. The sugar rich hydrolysate was further fermented to bioethanol by fermentation processes. Saccharomyces cerevisiae served as fermenting organism. Simultaneous saccharification and fermentation process yielded maximum ethanol in the substrate sweet sorghum.***

Keywords: Lignocellulosic waste – pretreatment- solid state fermentation- simultaneous saccharification and fermentation- bioethanol production

1. Introduction

The worldwide energy need has been increasing exponentially with decrease in reserves of fossil fuels. Further combustion of fossil fuels has serious negative effects on environment because of carbon di oxide emission. This situation is alarming which needs to be solved which leads to identification of alternative fuel sources. One of the potential alternatives is microbial production of biofuel from lignocellulosic wastes. It has attracted worldwide attention, due to its potential as an inexhaustible, low-cost and renewable source of clean energy (Ren et al. 2009). Lignocellulose is available in bulk as lignocellulosic wastes of agricultural and wood industries. It is the largest renewable sources of hexose and pentose sugars with potential use for industrial fermentation especially for ethanol production (Solomon et al. 2007, Chen et al. 2007, Balusu et al. 2005).Lignocellulosic materials, containing cellulose, hemicelluloses and lignin, are products of photosynthesis, which form the structural component of plant cell wall. The major criteria for the selection of waste materials to be used in bio-hydrogen production are the availability, cost, carbohydrate content and biodegradability (Kapdan and Kargi, 2006).

The objective of our study was to produce bioethanol from lignocellulosic wastes like sugarcane bagasse, paddy straw, wood chips, groundnut shell, sweet sorghum stover, maize stover using *Pleurotus florida* for solid state fermentation, *Trichoderma viride* for saccharification, *and Saccharomyces cerevisiae* for fermentation. In the process, reducing sugars produced in cellulose hydrolysis or saccharification is simultaneously fermented to ethanol, which greatly reduces product inhibition to hydrolysis.

2. Materials and Methods

Source of Microorganism

The spawn of *Pleurotus florida* and the fungal strain *Trichoderma viride* were procured from the Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India. *T.viride* was grown in Czapek – Dox agar medium for 5 days at 27± 2°C in Petri plates. The spore inoculums adjusted to 10^6 spores/mL used for cellulolytic enzyme production. For fermentation, the yeast *Saccharomyces cerevisiae* was employed. The yeast culture was obtained from Sakthi sugars Ltd., Erode, Tamil Nadu, India and maintained on Yeast-Peptone-Dextrose (YPD) agar slants (Tantirungkij 1994). The inoculum concentration of 0.1 % (dry weight w/v) was used for the fermentation process.

Pretreatment of substrate

Agricultural wastes, sugarcane baggasse, sweet sorghum, maize stover, groundnut shell, paddy straw and forestry waste, wood chips were used as substrates for bioethanol production. The substrates were pretreated by solid state fermentation process using *P.florida.* The substrates were cut into small pieces (1-2cm), dipped in water for 90 minutes and then sterilized at 121º C for 15 minutes. These sterilized substrates were filled in polyethylene bags of 40cm width and 60cm length and inoculated with 18 days old spawn cultures (Marimuthu et al.1993). The inoculated beds were incubated at 30ºC and were moistened with water. When the substrates were completely impregnated with mycelial growth, water was sprayed lightly on the bags, twice a day till the end of cropping. After full bud formation, the fruit bodies were harvested by using sterile knife and the fresh weight of the buds were determined. The biological efficiency percentage was calculated from the fresh weight yield of mushroom per kilogram of substrate used. The pretreated substrate was washed

thoroughly with distilled water, dried at room temperature and stored in a dessicator.

Biological Efficiency % = Fresh weight of mushroom/Dry weight of substrate X 100

Cellular constituents of substrates

The effect of fungal pretreatment on the substrate was studied by analyzing the cellular constituents of the substrates before and after pretreatment. The cellular constituents of the substrates, lignin (AOAC, 1975), cellulose (Updegroff, 1969), hemi-celluloses (Goering and VAnsoest, 1975), crude fiber (Maynard, 1970) and ash content was estimated before and after solid state fermentation. To determine ash content the weighed sample (500mg) was maintained at dual red heat (600°c) for 30 minutes in a silica crucible and after cooling, the ashed material was reweighed.

Ash content (%) = (Weighed of ash material /Weighed of fresh material) ×100

Production of saccharifying enzymes

For hydrolyzing enzyme production studies, *T.viride* was grown in minimal salt medium (Carter and Bull, 1968). The medium was amended with starch for amylase, cellulose for cellulase and xylan for xylanase and ABTS for laccase enzyme production. The fungal spore suspension (10^6 spores/mL) was inoculated (10% v/v) into the substrate amended medium (Carter and Bull, 1968) and inoculated for 7 days on a rotary shaker (125 rpm) at 27±2°C. On seventh day, the culture broth was filtered on a preweighed Whatman No.1 filter paper. The filtrate was centrifuged at 10,000 rpm for 10 min at 4°C and used as crude enzyme. In amylase, cellulase and xylanase the enzyme activity was estimated by determining reducing sugar (Miller, 1972) released from their respective substrates. Laccase activity was measured by the method of Evans 1985. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1µmol of substrate per minute.

Saccharification

Production of Saccharifying enzyme

The pretreated lignocellulosic substrate (1% w/v) amended medium (Carter and Bull, 1968) was inoculated with *Trichoderma viride* spore suspension (10% w/v) having a concentration of 10^6 spores/ml and incubated on orbital shaker (125 rpm) at 27±2°C for 7 days. After 7 days, the culture broth was filtered and centrifuged at 10,000 rpm for 20 min at 4° C. The clear supernatant was used as saccharifying enzyme and analyzed for amylase, cellulase, xylanase and laccase activity.

Saccharification of substrate

The enzyme preparation was added to the pretreated substrate suspended in 0.01 M phosphate buffer pH 6.8, (1% w/v) at a concentration of 1 IU cellulase activity per mL of suspension and incubated at 37°C for 24 hr. After saccharification, the reducing sugars released was estimated by DNS method (Miller,1972) and the percent saccharification was calculated (Tewari et al. 1988)

Saccahrification % = Reducing sugar formed x 0.9 x 100/cellulose content of the pretreated substrate

Fermentation

Simultaneous saccharification and fermentation (SSF)

In this process, the saccharification and fermentation were performed in the same vessel. The saccharifying enzymes (1 IU cellulase/mL) obtained during saccharification of lignocellulosic wastes and the fermenting organism *Saccharomyces cerevisiae* free cells were inoculated simultaneously into the production medium (Barron et al. 1995). Samples were analyzed for ethanol production in a gas chromatographic column.

Ethanol estimation using gas chromatography

Ethanol concenteration was determined using gas chromatography (Shimadzu 2010 series) under the following operating conditions: To 500 µL of fermented broth 5 µl of *n*-butanol (as internal standard) were added and the tube was vortexed for 30 s at maximum speed. To this, 1 ml of ethyl acetate was added, followed by 5 min of vortexing at maximum speed. Finally, the tubes were centrifuged to facilitate phase separation (5,000 g; 2 min at R.T.), and the organic phase (upper) was subjected to GC analysis with an injection volume of 1 µL for a total run time of 7 minutes in 30 m column (RTX-5, ID 0.25 mm). The carrier gas used was nitrogen and the column temperature was 50ºC.

3. Results

Biological efficiency of *P.florida*

Mushrooms rich in proteins, vitamins and minerals are well known for production of ligninolytic enzymes which are capable of removing lignin from lignocellulosic second generation feedstocks. The fruit bodies of *Pleurotus florida* formed were harvested for determining mushroom yield and biological efficiency (Table 1).The biological efficiency (yield of mushroom/Kg substrate on dry weight basis) was calculated (Chang et al. 1981).In the present study, biological efficiency varied significantly due to the effect of cellular constituents of different substrates. Maximum biological efficiency (45%) was obtained from maize stover and minimum biological efficiency (26%) was obtained from sugarcane bagasse.

Percent biodegradation of cellular constituents

The biodegradation of the substrates by *P.florida* revealed that degradation in pretreated substrates was more than in untreated substrates. The biologically pretreated substrates consisted of 3 to 28% lignin, 23 to 38% hemicelluloses, 20.5 to 56.2%cellulose.Comparing with the raw substrates,

biological pretreatment resulted in the percent degradation of various substrates ranging from 7 to 58.5% for cellulose, 11.6 to 32.5% for hemicelluloses, 12.5 to 32.5% degradation of lignin. Maximum cellular constituent's degradation was observed in sugarcane bagasse in which enzyme production was also found to be high.

Enzymatic hydrolysis of substrates

In Carter and Bull amended medium, the fungus, *T.viride* produced hydrolyzing enzymes laccase, amylase, cellulase and xylanase on all the substrates. Pretreated ground nut shell supported maximum xylanase (0.951 IU/ml) production, while pretreated sugarcane bagasse maximum amount of cellulase(0.832 IU/ml) was produced, pretreated maize stover yielded maximum amount of laccase (0.853 IU/ml) and amylase (0.602 IU/ml) (Fig.2). Even though the percent biodegradation of cellular constituents and enzyme activity is less in sweet sorghum compared to other substrates, the reducing sugar (77.3%) and total sugar yield (26.6%) was found to be high.

Bioethanol production of substrates

When the sugar rich syrup was further fermented with free cells of *Saccharomyces cerevisiae*, simultaneous saccharification and fermentation resulted in highest ethanol production of 20.9g/Kg of pretreated sweet sorghum substrate after 72 h of incubation. On the contrary, maize straw produced only 14.9 g/kg of substrate. The maximum being from pretreated sweet sorghum hydrolysate which also contained high levels of sugars.

4. Discussion

P.florida produced maximum yield of 690g on wheat straw substrate (Saurabh Deep Singh and Ganesh Prasad, 2012). Our findings correlated with those reported by other studies, combination of coir waste and paddy straw could increase *P.ostreatus* yield by 36% in fresh weight and biological efficiency by 41% (Eyini et al.1995).

The degradation of cellular constituents by ligninolytic enzymes might have exposed the cellulose and hemicelluloses for enzymatic attack. As the white-rot fungus *P.florida* consumed the substrates during its multiplication, it resulted in the decline of solid yield in the solid state fermentation system. These results indicated that biological pretreatment could efficiently fractionate lignocellulosic substrates and allow for the enhancement of enzymatic saccharification.

The enzyme digestibility of the biomass for production of biofuels and bioproducts depends mainly on its lignin content. (Adler, 1977). Laccase plays a role in lignin biodegradation of wood in white-rot fungi. Laccase generates phenoxy radicals from phenols which then spontaneously polymerase, and the enzyme may play a role in detoxification of inhibitory phenolic compounds which are often present in lignocellulosic materials. Conferring to this fungus has an ability to produce extracellular enzymes which are able to degrade lignin and to detoxify inhibitory phenolic compounds may lead to increased substrate colonization and improves fruit body yield during cultivation.

Simultaneous saccharification and fermentation yielded fairly good amount of enzymes, this may be due to the fact that in SSF, the sugar formed were immediately converted to ethanol thus avoiding any feedback inhibition due to the sugars formed. Barron *et al.,* (1995) reported that SSF of the cellulose by the yeast strain *Kluveromyces marxianus* yielded 10g/L ethanol after 60h incubation and 11.8g/L ethanol in the wheat straw hydrolysates by *Pachysolen trannophylus* yeast strain. Ahmad et al (2011)., reported that sago and sweet sorghum substrates produced high amount of ethanol at the end of fermentation which is at 72 h. This happened due to the fact that *S.cerevisiae* yeast cell growth in ethanol fermentation is considered to be a non-associated growth since the ethanol was excreted extracellularly by the yeast.

Pretreated sweet sorghum substrate has potential application due to its high sugar content and efficiency in ethanol production (20.9g/Kg substrate) by simultaneous saccharification fermentation process. From the results found in this study on bioethanol production potential of various lignocellulosic wastes, it can be concluded that sweet sorghum is a very promising raw material for bioethanol production. Sweet sorghum has a high reducing sugar and total sugar content compared to other lignocellulosic materials that have been examined for bioethanol production by pretreatment of the substrates. Biological pretreatment along with the microbial fermentation releases the simple sugars from lignocellulosic wastes for effective bioethanol production.

5. Acknowledgement

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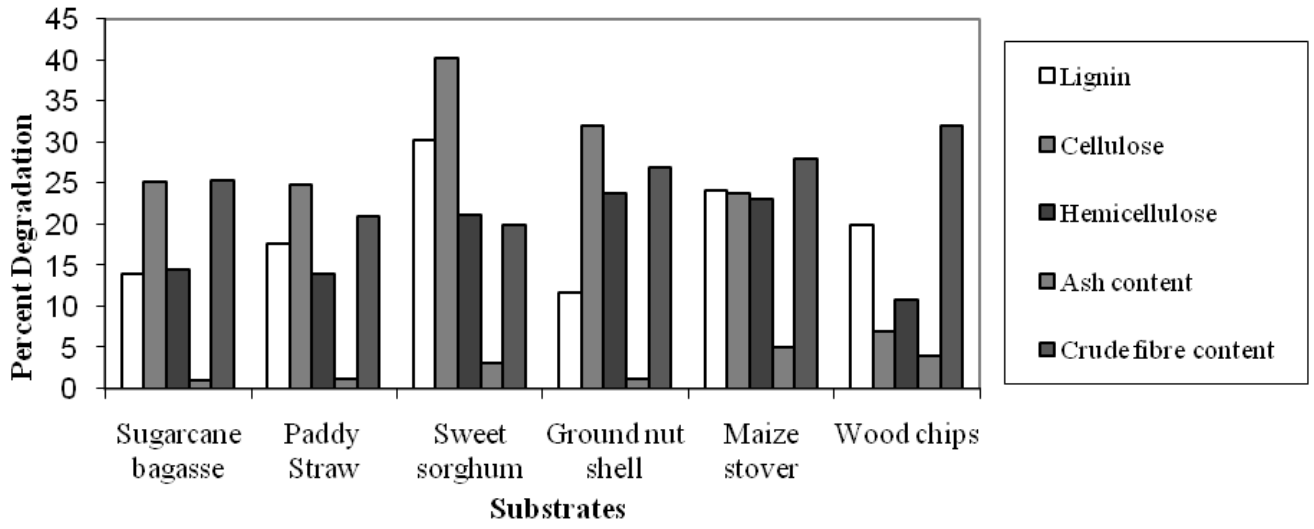

Figure 1: Impact of solid state fermentation by *P. florida* on cellular constituents of substrates

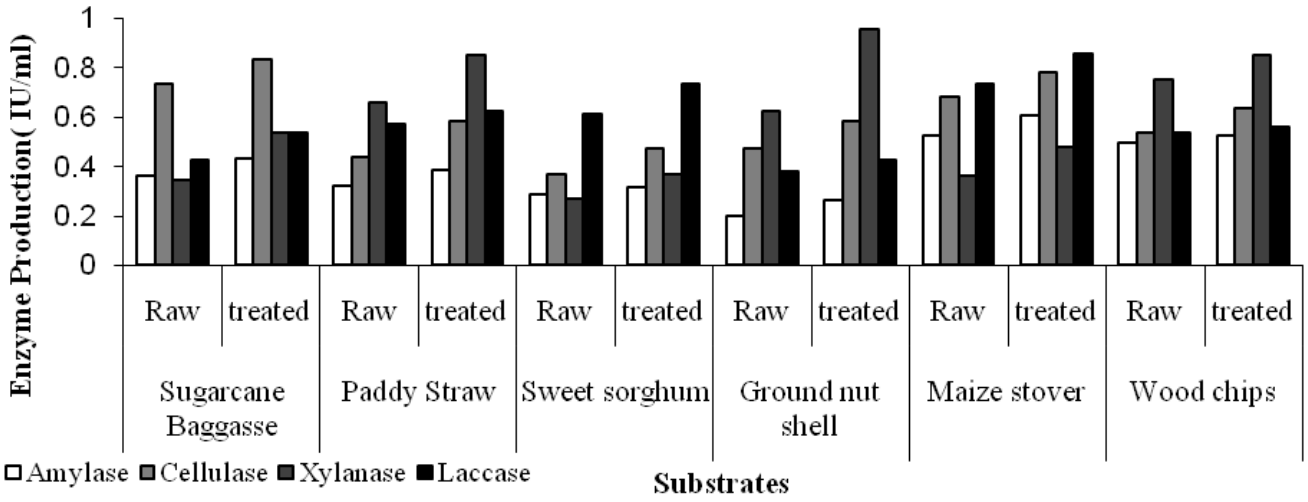

Figure 2: Production of lytic enzymes by *T.viride* on the substrates

Table 1: Yield of Pleurotus florida on different substrates

S. No.	*Substrates*	*Yield(kg/Kg substrate)*	*Biological efficiency (%)*
1	Sugarcane bagasse	0.265 ±0.0021	26
2	Paddy Straw	0.430 ±0.0016	43
3	Sweet sorghum	0.437 ±0.003	43
4	Ground nut shell	0.300 ±0.027	30
5	Maize stover	0.450 ±0.039	45
6	Wood chips	0.275 ±0.018	28