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Bioconversion of Rice Straw into Ethanol: Fungi and Yeasts are the Backbone Microbiota of the Process

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A B S T R A C T

Biologically treated paddy straw was hydrolysed using commercial cellulase and fermented to ethanol by yeast. Lignin degrading fungus *Pleurotus sajor-caju* removed 35.1 % lignin from paddy straw at 40 days incubation. Hydrolysis of biologically treated paddy straw with cellulase enzyme loaded at 5 FPU/g substrate at 50^0C resulted in about 119 mg/g sugar release. Fermentation of enzymatic hydrolysate by *Saccharomyces cerevisiae* resulted in production of 2.0 % ethanol after 72 h incubation at 30°C.

Introduction

Among cereals, rice is the world s second largest crop after wheat, however, it produces unlimited amounts of residues. The processing of rice yields extraordinary quantities of straw agroresidue. Not less than 20 % is used for paper and fertilizers production as well as fodder and the remaining part is left in the open fields for burning along a period that may extend to > 30 days to get rid of leftover debris. The resulting emission obviously contributes to the air pollution known as the Black Cloud. It is well recognized that, plant cell walls are the most abundant renewable source of fermentable sugars on earth and are the major reservoir of fixed carbon in nature. The main components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant (Yang *et al.*, 2007). Cellulase enzymes can hydrolyze cellulose forming glucose and other commodity chemicals. Cellulases is more interested because of their different applications in industries of starch processing, grain alcohol fermentation, malting and

brewing, extraction of fruit and vegetable juices, pulp and paper industry as well as textile industry (Zhou *et al.,* 2008). One of the potential applications of cellulases is the production of ethanol fuel from lignocellulosic biomass which is a good substitute for gasoline in internal combustion engines. The most promising technology for the conversion of the lignocellulosic biomass to ethanol is based on the enzymatic breakdown of cellulose by cellulase enzymes (Ahamed and Vermette, 2008). Cellulose, hemicellulose and lignin are the largest sources of hexsose and pentose sugars having a great potential for the production of bioethanol (Kuhad and Singh, 1993). The mixture of cellulose and hemicellulose however, is tightly bound to lignin mainly by hydrogen bond and also by some covalent bonds. In order to remove lignin, reduce cellulose crystallinity, increase the porosity of the materials and to make cellulose desirable to hydrolysis for subsequent fermentation, a pretreatment process is essential. Thus, from lignocellulosic materials, ethanol is produced by using three steps:-pre-treatment, hydrolysis and fermentation (Krishna and Chowdhary, 2000). Depending upon the structure of lignocellulosic materials the most effective pretreatment method could be selected. There are different kind of pretreatments and the main categories are: physical, chemical and biological. Physical and chemical processes have not been proven suitable, due to high cost and production of undesirable by-products. Chemical hydrolysis though beneficial by being rapid but is limited by lower sugar recovery efficiency, formation of furfural and other degeradation products poisonous to the fermentation microorganisms and raise environmental concerns due to disposal of acid.

Biological method of pretreatment is cheaper, safer, less energy consuming, highly specific, no degradation products of glucose are formed, ecofriendly and takes place under mild environmental conditions with low energy requirements (Sukumaran *et al.,* 2010). Most of these processes, however, are slow thus limiting their application at industrial level. For biological pretreatment of lignocellulosic materials, white-rot fungi are most effective as they produce ligninases which are helpful in cellulose degradation but their efficiency is low. With the ability to degerade lignin, fungi of the class basidiomycetes can also be used for pretreatment of biomass for ethanol production. Therefore, for efficient pretreatment of paddy straw for ethanol production, there is a need to select suitable fungal strain. Ethanol can be produced from paddy straw after getting free sugars from cellulose and hemicellulose followed by fermentation by using suitable yeast strains.

Materials and Methods

Paddy straw

Paddy straw after harvest of rice was obtained from farmer's field, Hisar. It was dried at 80±2°C, communited to small pieces using wiley grinder.

Enzyme

A commercial preparation of cellulase enzyme (Palkosoft super 720) was kindly supplied by Maps India Ltd. Ahmedabad, Gujarat.

Fungal cultures

Lignin degrading fungal isolates, namely: *Pleurotus ostreatus* were procured from IMTECH Chandigarh, *Pleurotus sp.* from Department of Microbiology and *Pleurotus sajor-caju* from Department of Plant Pathology CCSHAU, Hisar. The fungal cultures were maintained on potato dextrose agar (PDA) slants by regular sub-culturing and stored at 4^{0}C. For inocula preparation, potato dextrose agar (PDA) medium was used.

Yeast strains

The hexose fermenting yeast strain *Saccharomyces cerevisiae* (HAU-1) and pentose fermenting yeast strain *Pachysolen tannophilus* were procured from the Department of Microbiology, CCSHAU, Hisar. The yeast cultures were maintained on yeast extract peptone dextrose (YEPD) slants by regular sub-culturing and stored at 4^0C. For inocula preparation, yeast extract peptone sucrose (YEPS) medium was used.

Pretreatment of paddy straw

The fungi were grown on potato dextrose agar slants for one week at 30^0C. After one week, fungal growth was transferred in wheat bran (moisture content 60%) and incubated at 30^0C for one week. Fungal mycelium from wheat bran was transferred to paddy straw (autoclaved at 15 psi pressure for 15 minutes) impregnated with mineral salt medium (20 mM and pH= 4.5) at 1:5 ratio and incubated at 30^0C. Fungal growth was removed from paddy straw at different time intervals and remaining paddy straw was dried at 80 ± 2^0C for further use. The cellulose, hemicellulose and lignin content were estimated at different time intervals using standard method (AOAC, 1970).

Hydrolysis

Cellulose and hemicellulose fraction of biologically treated dry paddy straw was hydrolyzed to sugars and then hydrolysate was fermented to ethanol i.e. both the steps (hydrolysis and fermentation) were carried out separately so that each step can operate at its optimum rate. Biologically treated paddy straw was suspended in citrate buffer at 1:10 (solid:liquid) ratio and hydrolyzed enzymatically using commercial cellulase (Palko soft super 720) at 50°C for 2 h at shaking water-bath. Total reducing sugars released were estimated by standard Dinitrosalicylic acid (DNS) method (Miller, 1959) after centrifuging the samples at 5,000 rpm for 10 min.

Fermentation

In order to have maximum ethanol production from hydrolyzed sugars, the hydrolysate of paddy straw was fermented using mono as well as co-culture of hexose fermenting *Saccharomyces cerevisiae* and pentose fermenting yeast *Pachysolen tannophilus* at 30^0C. The yeast inoculum was raised in YEPS at 30^0C. Biomass obtained after 24 h of shaking was centrifuged at 5,000 rpm for 15 min. and inoculated into the hydrolysate at 1.0% (w/v) concentration along with yeast nutrients 0.3% urea or 0.15% ammonium sulphate. The flasks were incubated at 30^0C and ethanol content was estimated by method of Caputi *et al.*, (1968).

Results and Discussion

Pretreatment of paddy straw

For biological treatment fungal mycelium after one week growth on wheat bran was inoculated into paddy straw mixed with mineral salt medium and incubated at 30^0C. Biological treatment resulted decreased in lignin content and lignin removal increased with increase in incubation period. It was found that maximum lignin removal was achieved with *Pleurotus sajor-caju* where only for 4.8% lignin (Table 1) was left after 40 days of incubation compared to 7.4 %lignin content of untreated paddy straw where as lignin content in paddy straw inoculated with *Pleurotus ostreatus* and *Pleurotus sp.* was 4.9 and 5.9, respectively, under similar conditions (Table 2, 3). Similar kin of increase in cellulose with decrease in lignin content of paddy straw was observed by Begum and Alimon (2013) during growth of *Pleurotus*

sajor-caju on paddy straw. Theoretically, it seems increase in cellulose content with decrease in lignin content but practically it happens due to decrease in total solid of paddy straw during growth because the estimation is made on total dry weight basis.

Due to ligninolytic nature of *Pleurotus sajor-caju*, *Pleurotus ostreatus* and *Pleurotus sp.* lignin and hemicellulose content of paddy straw decreased with fungal inoculation as a result cellulose content seemed to increase. The Cellulose and hemicellulose content of *Pleurotus ostreatus* treated straw was 42.3 and 20.1 respectively, while respective value for *Pleurotus sp.* were 42.0 and 23.5 and *Pleurotus sajor-caju* 43.6 and 19.3 under similar conditions.

To study the effect of mineral salt concentration on delignification by *Pleurotus sajor-caju*. Mineral salt medium was added initially and after 20 days to retain the moisture but it was found that there was not so much difference in delignification of paddy straw on addition of mineral salt once or twice (Table 4).

Comparision of delignification of paddy straw by different fungal cultures after 40 days of time intervals indicated that *Pleurotus sajor-caju* removed maximum 35.1% lignin, while 33.7 and 20.2 % lignin was removed by *Pleurotus ostreatus* and *Pleurotus sp. Pleurotus sajor-caju* respectively, (Fig. 1).

A comparison of untreated paddy straw and biologically treated paddy straw by *Pleurotus sajor-caju* is shown in Fig. 2. After biological pretreatment there was increase in cellulose content from 38.0 to 43.6%, on the other hand there was decrease in lignin and hemicellulose content from 7.4 to 4.8% and 26.8 to 18.9%, respectively. It was found that after 10 days of fungal grown on paddy straw 98.9% total solids were recovered while solid recovery after 40 days of incubation was 97.6% (Table 5). Based upon efficiency of delignification, *Pleurotus sajor-caju* treated paddy straw was used for fuel ethanol production by hydrolysis and fermentation.

Hydrolysis

Prior to ethanolic fermentation by yeast, cellulose and hemicellulose components of paddy straw need to be processed by saccharification technology in order to release fermentable sugars. Hyrolysis of biologically treated paddy straw was carried out at 50^0C for 2 h in shaking water-bath by using commercial cellulase (5 FPU/g) and citrate buffer (0.2 M) at 1:10 ratio. Hydrolysis of *Pleurotus sajor-caju* treated paddy straw resulted in maximum 119 mg/g total reducing sugars released after 2 h at 50^0C while from untreated paddy straw 81.6 mg/g total reducing sugars were released (Table 6). Wati *et al.*, (2007) reported the release of 65% total reducing sugars by enzymatic hydrolysis of alkali treated paddy straw at 50^0C after 2 h incubation. Saccharification efficiency depends upon the available carbohydrates and reaction conditions. Optimum conditions were provided forenzyme activity so that the residual unreacted substrate may be acted upon during fermentation also.

Fermentation

Sugars produced as a result of hydrolysis were fermented to ethanol by yeast. Hexose sugars are considered to be easily fermented to ethanol whereas pentose sugars are not fermented by most alcohol producing yeasts.

Table.1 Analysis of *Pleurotus sajor-caju* treated paddy straw at different time intervals

Component	Incubation period (days)			
	10	**20**	**30**	**40**
Cellulose (%)	39.5 (3.9↑)	42.4 (11.5↑)	42.9 (12.8↑)	43.6 (14.7↑)
Hemicellulose (%)	24.4 (8.9↓)	21 (21.2↓)	20.4 (23.8↓)	19.3 (27.9↓)
Lignin (%)	6.0 (18.9↓)	5.5 (25.6↓)	5.1 (31.0↓)	4.8 (35.1↓)

Table.2 Analysis of *Pleurotus ostreatus* treated paddy straw at different time intervals

Component	Incubation period (days)			
	10	**20**	**30**	**40**
Cellulose (%)	39.1 (2.8↑)	41.2 (8.4↑)	41.9 (10.2↑)	42.3 (11.3↑)
Hemicellulose (%)	24.5 (8.5↓)	22.2 (17.1↓)	20.8 (22.3↓)	20.1 (25.0↓)
Lignin (%)	5.9 (20.2↓)	5.2 (29.7↓)	5.0 (32.4↓)	4.9 (33.7↓)

Table.3 Analysis of *Pleurotus sp.* treated paddy straw at different time intervals

Component	Incubation period (days)			
	10	**20**	**30**	**40**
Cellulose (%)	39.5 (3.9↑)	40.5 (6.5↑)	41.3 (8.6↑)	42.0 (10.5↑)
Hemicellulose (%)	25.2 (5.9↓)	25.0 (6.7↓)	24.0 (10.4↓)	23.5 (12.3↓)
Lignin (%)	7.0 (5.4↓)	6.2 (16.2↓)	6.1 (17.5↓)	5.9 (20.2↓)

Table.4 Effect of mineral salt concentration on delignification of paddy straw by *P. sajor-caju*

Component	Control	*MS	*MS	**MS	**MS
Cellulose (%)	**38.0**	42.9 (12.8↑)	43.6 (14.7↑)	43.8 (15.2↑)	44.1 (16.0↑)
Hemicellulose (%)	**26.8**	20.4 (23.8↓)	19.3 (27.9↓)	19.1 (8.7↓)	18.9 (29.4↓)
Lignin (%)	**7.4**	5.1 (31.0↓)	4.8 (35.1↓)	4.6 (37.8↓)	4.4 (40.5↓)

*Addition of MS at 0 day

** Addition of MS at 0 and 20th day

Table.5 Solid recovery after growth of *P. sajor-caju* on paddy straw at different time intervals

Time in days	Solid recovery (%)
10	98.9
20	98.3
30	97.6
40	97.6

Table.6 Total reducing sugar content after hydrolysis of biologically treated paddy straw

Time intervals of biological treatment (days)	Total reducing sugars (mg/g)
10	87.3+1.2
20	97.4+0.8
30	118+0.67
40	119+0.74
Control	**81.6+0.3**

Table.7 Ethanol production from biologically pretreated paddy straw with mono and co-culture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus*

Yeast strain	*Ethanol (%v/v)	
	**Urea	***Ammonium sulphate
S. cerevisiae	2.0	1.9
P. tannophilus	1.9	1.7
S. cerevisiae* + *P. tannophilus	2.3	2.1

*after 72h of incubation
**0.3%
***0.15%

Fig. 1 Delignification of paddy straw by different fungi

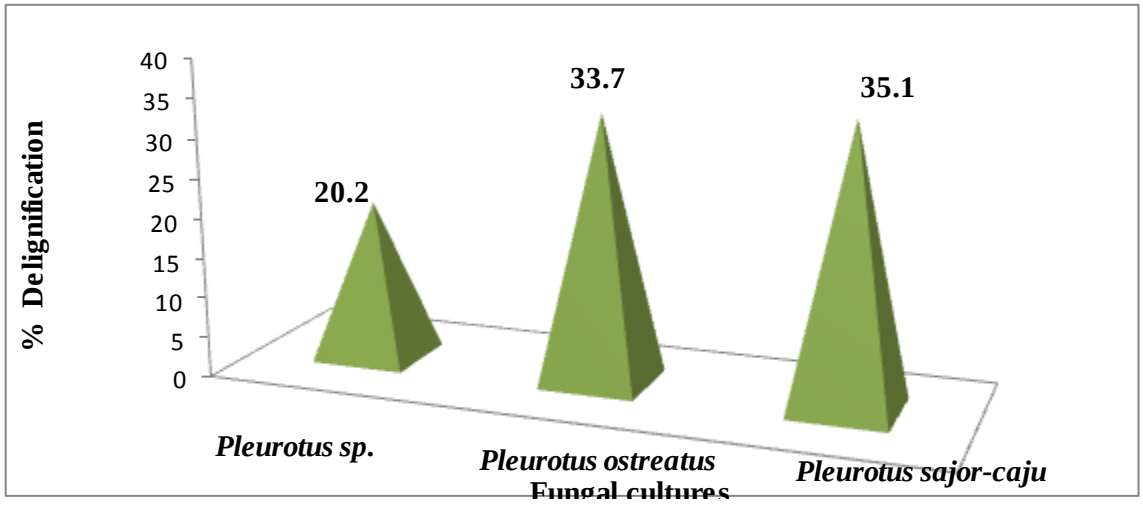
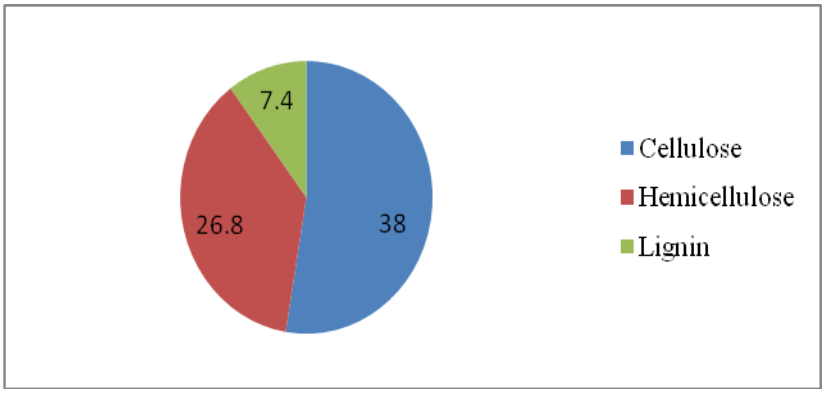
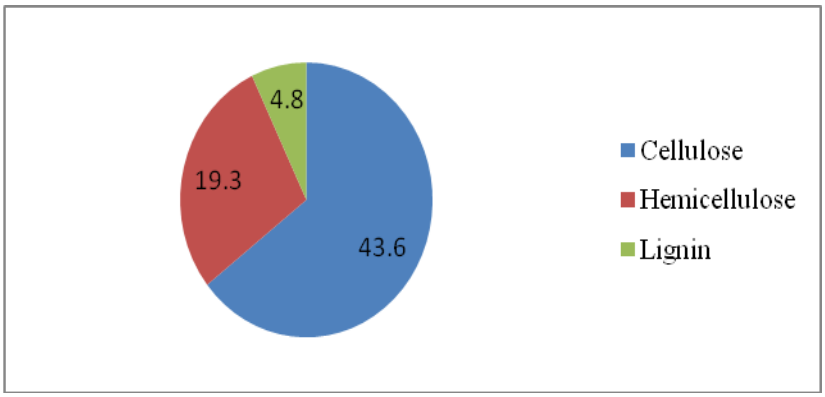


Fig. 2 Composition of paddy straw (a) untreated (b) biologically treated



(a)



(b)

Ethanol production from hydrolysate of *Pleurotus sajor-caju* treated paddy straw using hexose fermenting yeast strain *Saccharomyces cerevisiae* HAU-1 at 30^0C for 72 h revealed that maximum 2.0% (v/v) ethanol was produced from hydrolysate of 40 days treated paddy straw (Table 7).

Goel and Wati (2013) reported release of 75% total reducing sugars by enzymatic hydrolysis of paddy straw with ethanol yield of 20.83 g/l on fermentation of paddy straw by *Candida sp*. at 35^0C after 72 h. Li *et al.,* (2011) reported 21.1 g/l ethanol production within 80 h by SSF of rice straw from 10% w/w of lime pretreated and CO_2 neutralized paddy straw by sequential use of *S. cerevisiae* and *Pichia stipitis* with heat inactivation of *S. cerevisiae* cells prior to xylose fermentation. Srivastava *et al.,* (2014) reported 10.02 ± 1.18 g/l ethanol yield from rice straw with *Zymomonas mobilis* by enzymatic saccharification and fermentation.

In conclusion the current work shows ethanol production from paddy straw by microbial delignification and hydrolysis. Different fungal cultures have significant impact on delignification of paddy straw where *Pleurotus sajor-caju* treated paddy straw resulted in effective removal of lignin. The study opens a way for utilization of spent straw after harvest of *Pleurotus sajor-caju* for ethanol production.

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