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ORIGINAL PAPER

Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics

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Abstract The biological delignification of lignocellulosic feedstocks, *Prosopis juliflora* and *Lantana camara* was carried out with *Pycnoporus cinnabarinus,* a white rot fungus, at different scales under solid-state fermentation (SSF) and the fungal treated substrates were evaluated for their acid and enzymatic saccharification. The fungal fermentation at 10.0 g substrate level optimally delignified the *P. juliflora* by 11.89% and *L. camara* by 8.36%, and enriched their holocellulose content by 3.32 and 4.87%, respectively, after 15 days. The fungal delignification when scaled up from 10.0 g to 75.0, 200.0 and 500.0 g substrate level, the fungus degraded about 7.69–10.08% lignin in *P. juliflora* and 6.89–7.31% in *L. camara,* and eventually enhanced the holocellulose content by 2.90–3.97 and 4.25–4.61%, respectively. Furthermore, when the fungal fermented *L. camara* and *P. juliflora* was hydrolysed with dilute sulphuric acid, the sugar release was increased by 21.4-42.4% and the phenolics content in hydrolysate was decreased by 18.46 and 19.88%, as compared to the unfermented substrate acid hydrolysis, respectively. The reduction of phenolics in acid hydrolysates of fungal treated substrates decreased the amount of detoxifying material (activated charcoal) by 25.0–33.0% as compared to the amount required to reduce almost the same level of phenolics from unfermented substrate hydrolysates. Moreover, an increment of 21.1–25.1% sugar release was obtained when fungal treated substrates were enzymatically hydrolysed as compared to the hydrolysis of unfermented substrates. This study clearly shows that fungal delignification holds potential in utilizing plant residues for the production of sugars and biofuels.

Keywords Bioethanol · Lignocellulose · Fungal delignification · Solid-state fermentation · *Pycnoporus cinnabarinus* · Saccharification

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Introduction

Lignocellulosic biomass is a potential source of carbohydrate polymers for fermentation but the conversion of its structural polysaccharides into simple sugars is highly problematic due to the recalcitrancy of lignin. Lignin acts as a cementing material, which together with hemicelluloses forms an amorphous matrix in which the cellulosics fibrils are embedded and protected against chemical or enzymatic degradation (Kuhad et al. 1997; Himmel et al. 2007). Therefore, removal of lignin has become a prerequisite for the efficient utilization of carbohydrate from lignocellulosics.

Pretreatment of lignocellulosics is the most important step required to remove lignin and disrupt the crystallinity of carbohydrate fraction without losing too much of the structural sugars (Mosier et al. 2005; Wyman 2007; Kumar et al. 2009). Physicochemical pretreatments such as acid treatment (Gupta et al. 2009), alkali treatment (Carrillo et al. 2005), steam explosion (Öhgren et al. 2006) and ammonia fiber explosion (Teymouri et al. 2005) are well recognized for enhancing the conversion of cellulosic biomass into monomeric sugars. However, these pretreatment methods require high energy and often generate toxic compounds, which makes the process economically unviable and environment unfriendly (Teymouri et al. 2005; Silverstein et al. 2007). Therefore, environmentally benign pretreatment methods are required to reduce release of pollutants, costs and improve cellulose saccharification.

The biological pretreatments of plant residues to improve the accessibility of cellulosic fraction have been attracting the extensive interest of researchers (Taniguchi et al. 2005; Zhang et al. 2007a; Yu et al. 2009b). The potential of biological pretreatments has been explained by the ability of certain microbes to disrupt the plant cell wall by partial breakdown of the lignin/carbohydrate complex (Keller et al. 2003). The most promising microorganisms for biological pretreatment are basidomycetes, and among them the selective lignin degrading white rot fungi holds immense importance (Akhtar et al. 1997; Kuhad et al. 1997; Kuhad and Singh 2007; Yu et al. 2009a). *Pycnoporus cinnabarinus,* a selective lignin degrading white rot fungus, has been reported to produce laccase, degrade lignin and to transform lignin derived compounds (Eggert et al. 1996; Falconiner et al. 1994; Galhaup et al. 2002). Recently, *P. cinnabarinus, Phanerochaete chrysporium* and *Crinepellis* sp. RCK-1 have been tested for their lignin degradation and improvement in acid hydrolysis of the delignified substrates (Kuhar et al. 2008). However, the application of *P. cinnabarinus* for delignifying the lignocellulosic materials and subsequently its effect on enzymatic hydrolysis of the fermented substrate (mycosubstrate) has scarcely been studied.

In the present study, biological delignification of two different weeds (lignocellulosic substrate) viz. *Lantana camara* and *Prosopis juliflora* using *P. cinnabarinus* was attempted under solid-state fermentation (SSF) conditions with pretreatment scalability up to 500.0 g substrate level. The pretreated substrates were further evaluated for acid as well as enzymatic hydrolysis. The acid and enzymatic hydrolysis of fungal fermented material resulted in significant increase in the release of sugars as compared to unfermented ones. Interestingly, the acid hydrolysates of fungal fermented substrates were found to have lesser amount of toxic compounds as compared to hydrolysate from unfermented material.

Materials and methods

Raw material and chemicals

The woods of *L. camara* and *P. juliflora* were collected locally and processed through a combination of chipping and milling to attain a particle size of 1–2 mm using a laboratory knife mill (Metrex Scientific Instrumentation, Delhi, India). The grounded materials washed thoroughly with water and dried overnight at 60°C were used throughout the study.

Cellulase from *Trichoderma reesei* (ATCC 26921), β-glucosidase (Novozyme 188) from *Aspergillus niger,* and 3,5-dinitrosalicylic acid (DNS) were brought from Sigma, St. Louis, Missouri, USA, while, all other chemicals were purchased locally.

Microorganism and inoculum preparation

Pycnoporus cinnabarinus ATCC 2004378, a kind gift from Late Dr. K. E. L. Eriksson, Professor Emeritus, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, USA, was grown at 30°C on malt extract agar (MEA, Dhawan and Kuhad 2003). The medium contained (g l^{-1}): malt extract 20.0, KH_2PO_4 0.5, $MgSO_4$ 0.5, $Ca(NO_3)_2$ 0.5 and agar 20.0 (pH 5.5), while the stock cultures were maintained on MEA slants at 4°C with periodic transfer.

The fungal cultures were grown statically at 30°C in 250 ml Erlenmeyer flasks with 50 ml of malt extract broth (MEB, Dhawan and Kuhad 2003), which were inoculated with 2 fungal discs (8 mm diameter each) from a 6 day old colony grown on MEA. The cultures were harvested and the aseptically collected fungal mat was homogenized and transferred into a fresh 50 ml MEB in 250 ml Erlenmeyer flasks. They were grown at 30°C and 150 rpm in a rotatory incubator shaker (Innova-40, New Brunswick Scientific, USA) for

another 6 days to develop the inoculum for solid state fermentation of lignocellulosic materials.

Fungal treatment of *L. camara* and *P. juliflora* under SSF

The pretreatment was carried out in 500 ml Erlenmeyer flasks with 10.0 g of the oven dried lignocellulosic substrate (*L. camara* or *P. juliflora*), moistened with mineral salt solution (MSS) containing (g l^{-1}): KH_2PO_4 0.5, $MgSO_4$ 0.5, $Ca(NO_3)_2$ 0.5 and pH 5.5 to obtain a substrate to moisture ratio of 1:2.5 and autoclaved (121°C for 30 min). Each flask was then inoculated with 7.5 mg fungal mass (dry weight) per gram dry substrate (gds) and incubated at 30°C for 25 days. The solid state fungal fermentation of both the substrates was also studied at 75.0, 200.0 and 500.0 g substrate levels in enamel trays of different sizes: 21.5 cm × 16.5 cm × 5.0 cm, 31.5 cm × 26.5 cm 6.0 cm and 41.5 cm × 34.5 cm × 8.0 cm, respectively. The samples (mycosubstrate) were harvested periodically, washed thoroughly and dried overnight at 60°C. The dried mycosubstrates were then analysed for biochemical changes, acid and enzymatic saccharification. The flasks containing sterilized uninoculated substrates served as control.

Evaluation of delignified substrates for acid hydrolysis

The fungal pretreated and control (non-fungal treated) substrates were hydrolyzed at 10% (w/v) substrate consistency with dilute sulfuric acid (3.0%, v/v) at 120°C for 45 min (Gupta et al. 2009). The hydrolysate was vacuum filtered and analyzed for reducing sugars and total phenolics. The residual substrate after hydrolysis was washed thoroughly to a neutral pH, dried at 60°C in a hot air oven till constant weight was achieved.

Detoxification of acid hydrolysates

Acid hydrolysates of unfermented and fungal treated substrates were detoxified by adding varied dosages of activated charcoal (0–2.5%, w/v) under constant stirring at room temperature for 30 min. After incubation, the hydrolysate was vacuum filtered and analysed for total sugars and phenolics.

Enzymatic hydrolysis of fermented and non-fermented plant residues

Enzymatic hydrolysis of fermented and control (non-fermented) substrate samples was carried out at 5.0% (w/v) substrate consistency in 50 mM citrate phosphate buffer (pH 5.0). Prior to the enzyme loading, the slurry was incubated at 50°C for 2 h at 150 rpm. Thereafter, cellulase (24 FPU gds^{-1}), β-glucosidase (120 U gds^{-1}) and 1.0% (v/v) Tween 80 were added to the preincubated substrate suspension and the saccharification was carried out at 50°C and 150 rpm for 48 h. Samples were withdrawn after every 6 h, centrifuged at 10,000 rpm for 15 min and the supernatants were analysed for total reducing sugars.

Analytical methods

The total phenolics present in hydrolysates were determined by the method of Singleton et al. (1999) using Vanillin as standard, while, the reducing sugars were estimated by DNS method (Miller 1959).

The plant material was extracted with alcohol-benzene (1:2 v/v) to remove wax and resins etc. The extractive free, oven dried plant material was processed for biochemical analysis following the TAPPI (1992) protocols. The holocellulose to lignin ratio (H/L) was calculated as follows:

$$\mathrm{H/L} = \frac{\text{Amount of holocellulose (H) present in the substrate}}{\text{Amount of lignin (L) present in the substrate}} \times 100$$

The percent loss and gain of different components such as total organic matter (TOM) loss, lignin and holocellulose in fungal treated substrates were calculated using Eqs. 1 and 2, respectively.

$$\mathrm{Loss}(\%) = \frac{M_i - M_f}{M_i} \times 100 \quad (1)$$

$$\mathrm{Gain}(\%) = \frac{M_f - M_i}{M_i} \times 100 \quad (2)$$

where M_i is the amount of component in the control (non-fermented) substrate and M_f is the amount of the component left in the mycosubstrate.

All the experiments and the analysis were carried out in triplicates and the data presented is the mean

value of the triplicates. The standard deviation was calculated using the mean values and remained within the range of ±10.0%.

Results and discussion

Solid state fermentation (SSF) of lignocellulosic substrates

Irrespective of the substrates used, the increase in loss of total organic matter (TOM) correlated positively with the increase in lignin degradation, and holocellulose to lignin (H/L) ratio. While, the holocellulose content increased till 15 days of fungal fermentation and marginally declined thereafter (Table 2). The SSF of *L. camara* and *P. juliflora* by *P. cinnabarinus* after 25 days caused 18.87 and 15.40% loss of TOM, respectively (Table 1). The loss in TOM may be attributed to the CO_2 evolution due to the metabolic activities of fungus on the substrates, however, the weight loss in substrates includes the loss of lignin and holocellulose. The fungus degraded more amount of lignin in *P. juliflora* (13.13%) than in *L. camara* (8.87%). The fungal delignification in both the substrates was higher during the first 15 days, and thereafter no significant improvement in lignin degradation was observed (Table 1). Our results are in accordance with the earlier reports on biological delignification of lignocellulosic materials with white rot fungi (Bustamente et al. 1999; Taniguchi et al. 2005; Meza et al. 2006). *P. cinnabarinus* was reported to degrade 8.5% lignin in sugarcane bagasse (Meza et al. 2006), whereas, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* degraded 9.0 and 17.0% lignin in depithed sugarcane bagasse, respectively (Bustamente et al. 1999). The fungal cultures have been observed to degrade more lignin in wheat straw than in woody material and this low ability of lignin degradation in woody substrate by the fungi could be attributed to the difference in the lignin properties of wood and straw (Taniguchi et al. 2005). Thus lignin structure could be seen as a detrimental factor for economic exploitation of plant residues.

Besides delignification, the availability of carbohydrates is also an important criterion for evaluating the biological pretreatment performance because the higher cellulose content in fermented substrates eventually provides higher accessibility of

Table 1 Analysis of compositional changes during the fungal treatment of *L. camara* and *P. juliflora* using *P. cinnabarinus* at different time intervals (5–25 days)

Components	Substrates												
	L. camara					*P. juliflora*							
Time (days):	Control	5	10	15	20	25	Control	5	10	15	20	25	
Weight (g)	10.0	8.91 (10.87)[a]	8.67 (13.27)[a]	8.47 (15.33)[a]	8.37 (16.27)[a]	8.11 (18.87)[a]	10.0	9.23 (7.73)[a]	9.05 (9.47)[a]	8.81 (11.87)[a]	8.63 (13.67)[a]	8.46 (15.40)[a]	
Klason-Lignin (%)	33.18	31.64 (4.63)[a]	30.91 (6.85)[a]	30.41 (8.36)[a]	30.28 (8.74)[a]	30.24 (8.87)[a]	30.71	28.71 (6.51)[a]	27.72 (9.72)[a]	27.06 (11.89)[a]	26.89 (12.44)[a]	26.68 (13.13)[a]	
Holocellulose (%)	59.82	60.94 (1.87)[b]	61.70 (3.14)[b]	62.73 (4.87)[b]	62.45 (4.40)[b]	62.25 (4.06)[b]	63.58	64.44 (1.35)[b]	64.92 (2.11)[b]	65.69 (3.32)[b]	65.42 (2.90)[b]	65.07 (2.34)[b]	
H/L	1.80	1.93 (6.99)[b]	2.00 (10.90)[b]	2.06 (14.62)[b]	2.06 (14.58)[b]	2.06 (14.37)[b]	2.07	2.24 (8.42)[b]	2.34 (13.12)[b]	2.43 (17.28)[b]	2.43 (17.54)[b]	2.44 (17.83)[b]	

[a] Percentage loss in fungal treated substrates with respect to control (untreated) substrates

[b] Percentage gain in fungal treated substrates with respect to control (untreated) substrates

Table 2 Compositional changes during the scale up of biological delignification of *L. camara* and *P. juliflora* using *P. cinnabarinus* at different levels (75.0, 200.0 and 500.0 g)

Compound	Substrates (g)							
	L. camara				*P. juliflora*			
	75	200	500	SD	75	200	500	SD
Weight (%)	61.94 (17.41)[a]	167.04 (16.48)[a]	424.05 (15.19)[a]	1.11	63.33 (15.56)[a]	174.68 (12.66)[a]	439.75 (12.05)[a]	1.88
Klason-Lignin (%)	30.75 (7.31)[a]	30.89 (6.89)[a]	30.85 (7.01)[a]	0.22	28.35 (7.69)[a]	27.61 (10.08)[a]	27.69 (9.84)[a]	1.32
Holocellulose (%)	62.58 (4.61)[b]	62.36 (4.25)[b]	62.45 (4.39)[b]	0.18	65.42 (2.90)[b]	66.10 (3.97)[b]	65.86 (3.58)[b]	0.54
H/L	2.03 (13.04)[b]	2.02 (12.14)[b]	2.03 (12.44)[b]	0.006	2.31 (11.49)[b]	2.39 (15.64)[b]	2.38 (14.90)[b]	0.004

[a] Percentage loss in fungal treated substrates with respect to control (untreated) substrates

[b] Percentage gain in fungal treated substrates with respect to control (untreated) substrates

carbohydrates for acid or enzymatic saccharification (Shi et al. 2008). There are several reports where *P. chrysosporium,* the most widely studied white rot fungus for lignin degradation, resulted in higher amounts of lignin degradation but the fungus was also found to cause a significant loss in carbohydrate fraction (Taniguchi et al. 2005; Kuhar et al. 2008). However our results showed an increase in holocellulose content in both the fungal fermented substrates by 3.32 and 4.87% compared to control (unfermented) substrates after 15 days and thereafter it declined gradually (Table 1). The fungal delignification also increased the H/L ratio by 14.62 and 17.28% in *L. camara* and *P. juliflora*, respectively after 15 days (Table 1). The increase in H/L ratio may be due to the reason that the fungus produces a fairly good amount of laccase with very low activity of carbohydrate hydrolyzing enzymes (Alves et al. 2004), which might have resulted in higher degradation of lignin as compared to holocellulose in the *P. cinnabarinus* treated substrates. Similar results were also obtained during the biological pretreatment of wheat straw using *Streptomyces cyaneus*, which caused 16 and 8% degradation of lignin and holocellulose, respectively, after 15 days of SSF and eventually enhanced the H/L ratio of the fermented wheat straw (Berrocal et al. 2000).

Scale up of biological delignification

The fungal delignification of lignocellulosic substrates was scaled up under similar conditions such as particle size, substrate to moisture ratio, pH, temperature, tray volume to substrate ratio. The study revealed that irrespective of the scale of treatment, the lignin degradation was found to be comparatively more or less same. The *P. cinnabarinus* when grown on *L. camara* and *P. juliflora* at larger scales (75.0, 200.0 and 500.0 g) caused 15.19–17.41 and 12.05–15.56% weight loss, respectively. Moreover, the fungal pretreatment of *L. camara* and *P. juliflora* removed lignin by 6.89–7.31 and 7.69–10.08% and also enhanced the holocellulose content by 4.25–4.61 and 2.9–3.97%, respectively, after 15 days of incubation. Furthermore, the H/L ratio of fermented *L. camara* and *P. juliflora* remained in the range of 2.02–2.03 and 2.31–2.39%, respectively (Table 2). The insignificant difference in H/L ratios and compositional changes in the fermented substrates at different scale experiments demonstrated the possibility to further scale up of the process.

Evaluation of biologically delignified substrates for acid saccharification

Acid hydrolysis of fungal fermented and the control (unfermented) substrates was evaluated. The acid hydrolysis of fungal fermented *L. camara* and *P. juliflora* resulted in 131.4 and 189.7 mg gds^{-1} sugar yield as compared to sugar release of 108.2 and 133.2 mg sugar gds^{-1} from control (unfermented) substrates, respectively (Fig. 1). The acid saccharification of fungal fermented materials has significantly improved in sugar release (21.4–42.4%) as compared to the unfermented substrates. The increased sugar release in fungal treated substrates might be because of the preferential lignin degradation ability in the

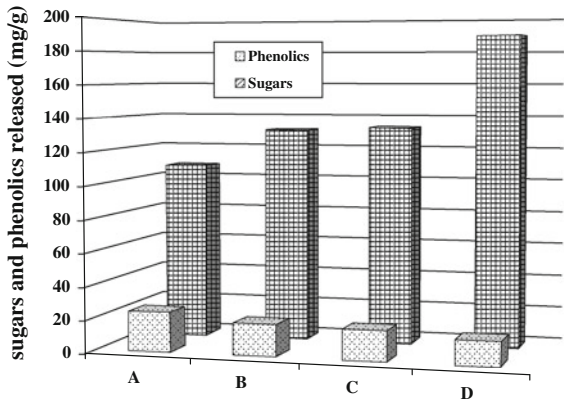

Fig. 1 Release of sugars and phenolics during the acid hydrolysis (3% H_2SO_4, 120°C and 45 min) of *P. cinnabarinus* treated and control (untreated) substrates (where A, untreated *L. camara*; B, SSF-treated *L. camara*; C, untreated *P. juliflora*; D, SSF-treated *P. juliflora*)

substrates by the fungus *P. cinnabarinus,* which in turn would have resulted in enrichment of carbohydrate content in the fermented substrates. Similar results have been reported in fungal treated wheat straw by Kuhar et al. (2008).

Acid hydrolysis not only release the reducing sugars but also release some lignin degradation compounds (phenolics), which are toxic to the fermenting microorganisms (Palmqvist et al. 1999; Mussatto and Roberto 2004; Chandel et al. 2007). The acid hydrolysates obtained from fungal treated *L. camara* and *P. juliflora* were found to have 18.5–19.9% less phenolics as compared to the hydrolysates of unfermented substrates (Fig. 1). This reduction in phenolics can be attributed to the fungal degradation of phenolic compounds (Kuhar et al. 2008).

An attempt has been made to evaluate the effect of fungal pretreatment on reduction in the usage of chemicals in detoxification of acid hydrolysates. Interestingly, the activated charcoal even when used at a lower concentration (1 and 1.5% w/v, 25–33% lower than control) removed more than 90% phenolics from acid hydrolysates of fungal treated substrates as compared to the acid hydrolysates of unfermented ones (Table 3). Our observations clearly indicated that fungal fermentation (delignification) of lignocellulosic material resulted in holocellulosic materials more vulnerable to acid hydrolysis, which in turn gives hydrolysates rich in pentose sugars and lesser phenolics.

Evaluation of biologically delignified substrates for enzymatic saccharification

In comparison to the unfermented substrates, the fungal fermented *L. camara* and *P. juliflora* when hydrolysed with cellulases for 48 h, an increase of 25.1 and 21.1% (w/w) saccharification with release of reducing sugars (389.1 and 402.1 mg gds^{-1}), respectively, was achieved (Fig. 2). The enhanced enzymatic saccharification of fungal fermented substrates may be attributed to the partial degradation of lignin seal responsible for preventing the penetration of cellulase molecules (Taniguchi et al. 2005). Moreover, the improvement in enzymatic hydrolysis could be because the biological treatment increases the initial adsorption of cellulases to the cellulose, which in turn enhances the sugars released during the enzymatic hydrolysis (Yu et al. 2009a).

It has been observed that fungal fermentation with *P. cinnabarinus* has considerable potential in improving enzymatic saccharification of cellulosics into sugar rich syrup. Taniguchi et al. (2005) have reported 330 mg gds^{-1} sugar released from rice straw fermented with *P. ostreatus* after 60 days. Zhang et al. (2007b) when enzymatically hydrolysed

Table 3 Detoxification of phenolics from the acid hydrolysates obtained from different substrates (untreated and fungal treated *L. camara* and *P. juliflora*) using varied dosage of activated charcoal

Amount of charcoal used (%, w/v)	Amount of phenolics (mg g^{-1})			
	L. camara		*P. juliflora*	
	Untreated	SSF-treated	Untreated	SSF-treated
0.00	24.00 (0.00)	19.23 (0.00)	18.26 (0.00)[a]	14.84 (0.00)
0.50	18.69 (22.13)	14.20 (26.15)	13.25 (27.44)	7.13 (51.97)
1.00	12.51 (47.88)	6.04 (68.60)	5.74 (68.57)	0.73 (95.11)
1.50	4.67 (80.54)	0.95 (95.04)	0.85 (95.37)	0.06 (99.59)
2.00	0.09 (99.62)	0.08 (99.56)	0.05 (99.72)	0.05 (99.64)
2.50	0.03 (99.87)	0.04 (99.78)	0.05 (99.74)	0.05 (99.64)

[a] The values in parenthesis is the percentage amount of phenolics removed

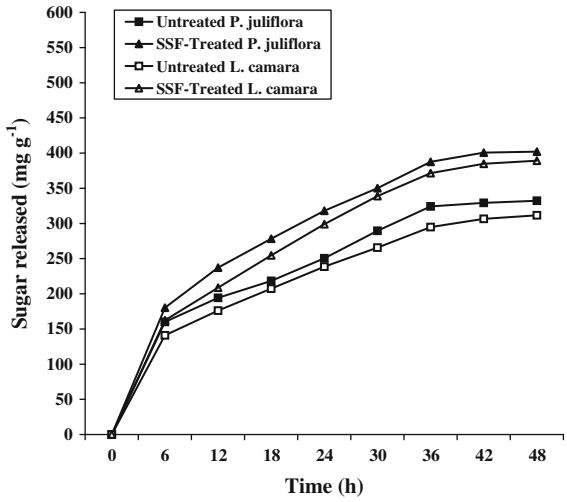

Fig. 2 Enzymatic hydrolysis profile of *P. cinnabarinus* treated and untreated *L. camara* and *P. juliflora*

Trametes versicolor and *Echinodontium taxodii* fermented bamboo culms for 120 days, the sugar released was approximately 360 and 200 mg gds^{-1}, respectively. Recently Yu et al. (2009a) have also reported 255.75 and 70.4 mg gds^{-1} sugar yield from *E. taxodii* fermented chinese willow and china fir after 120 days, as calculated from their data. Thus the sugars released in our study from both the fungal fermented substrates after 15 days was higher (389.1 and 402.1 mg gds^{-1}) than the above cited studies. Although it's difficult to make exact comparison in studies where lignocellulosic substrates are different and moreover fermentation period is variable. But comparatively it seems that the fungus used in this study holds considerably more potentials.

Conclusion

The conventional non-biological pretreatment methods require high energy, often generate toxic substances inhibitory to fermentation, which makes the processes uneconomical and environmentally inimical. The results of this fungal fermentation based study indicate that in order to increase the acid or enzymatic saccharification of hemicellulose and cellulose, it is prerequisite to degrade the lignin in the plant material to be used for producing value added compounds. The fungal fermentation have led to significant improvement in acid hydrolysis of hemicellulosic content and enzymatic hydrolysis of cellulose content in *L. camara* and *P. juliflora.* Moreover, fungal fermentation of plant material has resulted into material, which on acid hydrolysis produces hydrolysates with lesser amount of toxic compounds. Thus, the fungal delignification if optimized can be used as a potential alternative pretreatment method for improving the enzymatic saccharification of lignocellulosic residues and eventually for economizing the ethanol production as biofuel.

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