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Fungal pretreatment improves amenability of lignocellulosic material for its saccharification to sugars

Deepa Deswal, Rishi Gupta, Preeti Nandal, Ramesh Chander Kuhad*

Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India

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ABSTRACT

The sugarcane bagasse was biologically pretreated with three white-rot fungi; *Pleurotus florida*, *Coriolopsis caperata* RCK 2011 and *Ganoderma* sp. rckk-02, individually under solid-state fermentation. *P. florida*, *C. caperata* RCK 2011 and *Ganoderma* sp. rckk-02 degraded lignin up to 7.91, 5.48 and 5.58%, respectively. The lignocellulolytic enzymes produced by these fungi were also monitored during solid state fermentation of sugarcane bagasse. The fungal fermented sugarcane bagasse when hydrolyzed with crude cellulases from brown-rot fungus, *Fomitopsis* sp. RCK2010, released comparatively 1.5–2.4 fold higher sugars than in case of untreated sugarcane bagasse. The study demonstrated that white-rot fungal pretreatment improved the amenability of plant material for enzymatic hydrolysis.

1. Introduction

The annual production of lignocellulosic materials has been estimated about 10^{10} metric tons worldwide (Alvira, Tomas-Pejo, Ballesteros, & Negro, 2010). Lignocellulosic biomass chiefly consists of cellulose, a homopolymer of glucose; hemicellulose, a heteropolymer of pentoses and hexoses; and lignin, a polymer of phenyl propanoid units (Kuhad, Singh, & Eriksson, 1997). The polysaccharides (cellulose and hemicellulose) on average accounts for 55–75% on dry weight basis in plant cell wall and can be deconstructed into simple sugars, which further can be fermented to alcohols such as ethanol and butanol (Swana, Yang, Behnam, & Thompson, 2011), organic acids, acetone and glycerol (Celińska and Grajek, 2009; Tran, Cheirsilp, Hodgson, & Umsakul, 2010). Among these, ethanol from lignocellulosics, is one of the valuable energy sources, which could meet the growing energy demand (Lynd et al., 2008).

The lignocellulosic residues which don't compete with food demand, provide a low cost feedstock for production of fuels and commodity chemicals and thereby can offer economic, environmental and strategic advantages (Sukumaran et al., 2010). Sugarcane bagasse (SB) is one of the abundantly available low-cost plant residue, which could be used for production of biofuels (Rocha et al., 2011; Soares, Travassos, Baudel, Benachour, & Abreu, 2011). Nearly, 101.3 million metric tons of SB is annually produced in India (Sukumaran et al., 2010).

The most critical step in conversion of plant material into ethanol is to bring about fractionation of plant material in individual components (Dyk & Pletschke, 2012). The partial or complete removal of lignin is essential because its cross linking with hemicellulose makes it highly recalcitrant and obstructs any chemical or enzymatic pretreatment (Dyk & Pletschke, 2012). Thus to enhance the overall chemical or enzymatic hydrolysis, the crystalline structure of holocellulose needs to be disintegrated and the lignin to be broken down to increase the accessibility of cellulases to the substrates. Therefore, an efficient pretreatment method is necessary to produce cellulose largely free of hemicellulose and lignin. Variety of chemical and physiochemical pretreatment methods; steam explosion, steam treatment with diluted sulfuric acid or alkali, organosolv extraction and ammonia fiber expansion have been largely used to improve the enzymatic hydrolysis of plant biomass (Alvira et al., 2010). However, most of these pretreatments are energy intensive, require costly equipments and often generate toxic compounds, which make the process environment unfriendly and commercially uncompetitive (Alvira et al., 2010).

Recently researchers have started working on biological pretreatment as an alternative method either to partially replace or bring down the severity of chemicals generally used in different pretreatment methods (Gupta, Mehta, Khasa, & Kuhad, 2011; Menon & Rao, 2012). Biological pretreatment is advantageous in improving enzymatic saccharification of plant biomass, because of low energy input, cost, reduction in chemical requirement and mild processing conditions (Saritha, Arora, & Nain, 2012). The

* Corresponding author. Tel.: +91 9871509870; fax: +91 11 24115270.
E-mail address: kuhad85@gmail.com (R.C. Kuhad).

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potential of biological pretreatment has been explained by the ability of certain microorganisms to disrupt the plant cell wall by partial breakdown of the lignin-carbohydrate complex (Sanchez, 2009; Pinto et al., 2012). Among microorganisms, the white rot fungi are the most promising lignin degraders and thus have potential for biological breakdown of plant materials (Dias et al., 2010). Different white-rot fungi vary greatly in their capabilities of degrading lignin and carbohydrates in plant cell wall. Recently, *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, *Crinipellis* sp. RCK-1, *Pleurotus ostreatus* and *Trametes versicolor* have been tested for their lignin degradation abilities, when grown under solid-state fermentation (SSF) (Gupta, Mehta, Khasa, & Kuhad, 2011; Kuhar, Nair, & Kuhad, 2008; Shrivastava et al., 2011).

The present study was aimed at evaluating the abilities of three white rot fungi; *Pleurotus florida*, *Coriolopsis caperata* RCK2011 and *Ganoderma* sp. rckk-02 to ferment SB for transforming it into a more amenable material for enzymatic hydrolysis. Additionally an attempt has been made to test the efficacy of cellulases from *Fomitopsis* sp. RCK2010, a brown-rot fungus, for saccharifying the fungal treated SB.

2. Materials and methods

2.1. Raw material

Lignocellulosic substrates, wheat bran (WB) and sugarcane bagasse (SB), were obtained locally. SB was 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 Pvt. Ltd., New Delhi, India). The grounded material was used throughout the study.

2.2. Microorganisms and culture conditions

Pleurotus florida, *Coriolopsis caperata* RCK2011, *Ganoderma* sp. rckk-02 and *Fomitopsis* sp. RCK2010 were obtained from the culture collection of Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, New Delhi, India. The fungal cultures were grown on malt extract agar (MEA) composed of (g/L): malt extract, 20.0; $Ca(NO_3)_2{\cdot}4H_2O$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 0.5; and agar, 20.0 (pH 5.5) at 30 °C (Dhawan & Kuhad, 2002; Vasdev, Dhawan, Kapoor, & Kuhad, 2005). The fungal cultures were maintained periodically on MEA at 30 °C and stored at 4 °C.

2.3. Inoculum preparation

Fungal inoculum was prepared by growing each fungus separately in 250 ml Erlenmeyer flasks containing 50 ml of sterile malt extract broth (MEB) having (g/L): malt extract, 20.0; $Ca(NO_3)_2{\cdot}4H_2O$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 0.5 (pH 5.5). Each flask was inoculated with four mycelial discs (0.8 cm diameter each) of the respective fungal culture and incubated at 30 °C under static cultivation conditions for 7 days. The mycelial mat thus obtained was homogenized with pestle and mortar under sterile conditions and used as inoculum for biological pretreatment experiments.

2.4. Solid state fermentation of sugarcane bagasse

The fungal pretreatment was carried out in 500 ml Erlenmeyer flasks, each with 20.0 g of the air dried sugarcane bagasse moistened with mineral salt solution (MSS) containing (g/L): KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $Ca(NO_3)_2$, 0.5 and pH 5.5 to obtain a substrate to moisture ratio of 1:4 and autoclaved at 15 psi for 15 min. Each flask was inoculated with 0.25 g ± 0.01 fungal biomass (dry weight) of the respective fungus (*P. florida*, *C. caperata* RCK 2011, *Ganoderma* sp. rckk-02). The contents of the flasks were mixed well under aseptic conditions for homogenous distribution of inoculum through out the substrate and incubated at 30 °C for 25 days. The samples (myco-substrate) from flasks were harvested periodically at regular time intervals of 5 days and used for compositional analysis and enzyme assays. The non-fungal inoculated flasks containing sterilized substrate served as control.

For enzyme assays, the myco-substrate harvested aseptically from flasks after various intervals, was suspended in 50 ml of citrate phosphate buffer (pH 5.0) and vortexed for 45 min at room temperature. The extrudates were squeezed through muslin cloth and centrifuged at 10,733 × *g* at 4 °C for 10 min (Deswal, Khasa, & Kuhad, 2011; Shrivastava et al., 2011). The supernatant thus obtained was used to assay ligninolytic (laccase, MnP, LiP) and cellulolytic (CMCase and FPase) enzymes.

2.5. Cellulase production from Fomitopsis sp. RCK2010 under SSF

Cellulase production under SSF by *Fomitopsis* sp. RCK2010 was carried out in 250 ml Erlenmeyer flasks as described else where (Deswal et al., 2011).

2.6. Enzymatic saccharification of fungal treated substrate

The untreated SB served as negative control, while SB treated with H_2SO_4 (0.5%, w/v) and NaOH (0.5%, w/v) separately were kept as positive controls. Enzymatic saccharification of fungal pretreated and controls were carried out at 2% (w/v) consistency in citrate phosphate buffer (pH 4.8) containing 0.005% (w/v) sodium azide. Crude enzyme solution equivalent to cellulase activity (FPase) of 20 IU/g substrate was added to each flask and incubated at 50 °C and 150 rpm for 24 h. Samples were withdrawn at regular intervals, centrifuged at 8000 rpm for 10 min and the supernatants were analyzed for release of reducing sugars.

2.7. Analytical methods

The chemical composition (holocellulose, klason lignin) of the control and fungal treated SB was determined following standard TAPPI (1992) protocols.

The saccharification rate was calculated as

$$= \frac{\text{Amount of sugar released}}{\text{Total carbohydrate in pretreated substrate} \times \text{time of incubation}}$$

CMCase and FPase activities were determined following the IUPAC method (Ghose, 1987). The laccase activity was determined as described previously (Dhawan & Kuhad, 2002). The manganese peroxidase (MnP) and lignin peroxidase (LiP) activities were estimated as reported by Martínez, Ruiz-Dueñas, Guillen, and Martínez (1996) and Heinfling, Martinez, Bergbauer, and Szewzyk (1998), respectively. The reducing sugars were quantified by DNSA method (Miller, 1959).

All the experiments were carried out in triplicates and data presented is the mean value ± standard deviation.

3. Results and discussion

3.1. Diversity of lignocellulolytic enzymes during fungal pretreatment

The oxidative (laccase, LiP and MnP) and cellulolytic enzymes (CMCase and FPase) produced by the three fungi tested for biological pretreatment of lignocellulosic substrates were studied. It was observed that irrespective of the fungus used, the maximum laccase production was observed on 15th day and decreased thereafter

Table 1
Enzyme production during fungal cultivation of sugarcane bagasse by *P.florida*, *C.caperata* RCK2011 and *Ganoderma* sp. rckk-02.

Fungi	Time (day)	Enzyme activity (U/g)					
		Laccase	MnP	LiP	Xylanase	CMCase	FPase
P.florida	5	16.871 ± 0.231	0.0	0.018 ± 0.002	112.251 ± 9.029	0.0	0.028 ± 0.001
	10	77.631 ± 0.197	0.096 ± 0.004	0.033 ± 0.015	145.681 ± 6.031	0.002 ± 0.001	0.044 ± 0.002
	15	102.234 ± 0.314	0.234 ± 0.017	0.095 ± 0.004	190.371 ± 11.074	0.003 ± 0.001	0.061 ± 0.003
	20	81.363 ± 0.171	0.156 ± 0.006	0.122 ± 0.008	181.337 ± 16.112	0.031 ± 0.002	0.074 ± 0.005
	25	52.212 ± 0.114	0.077 ± 0.002	0.083 ± 0.002	173.219 ± 7.202	0.016 ± 0.003	0.049 ± 0.002
C.caperata RCK2011	5	17.543 ± 0.110	0.0	0.027 ± 0.009	195.255 ± 10.020	0.131 ± 0.002	0.832 ± 0.004
	10	20.910 ± 0.211	0.043 ± 0.001	0.043 ± 0.001	248.046 ± 14.104	0.359 ± 0.004	0.901 ± 0.002
	15	27.662 ± 0.173	0.0	0.087 ± 0.001	276.638 ± 11.044	0.677 ± 0.002	1.842 ± 0.101
	20	24.531 ± 0.342	0.0	0.112 ± 0.005	256.223 ± 8.139	0.591 ± 0.017	0.912 ± 0.057
	25	19.227 ± 0.213	0.0	0.061 ± 0.003	231.539 ± 1.167	0.455 ± 0.021	0.771 ± 0.064
Ganoderma sp.rckk-02	5	15.234 ± 0.155	0.015 ± 0.001	0.021 ± 0.009	79.526 ± 2.211	0.021 ± 0.001	0.014 ± 0.008
	10	40.221 ± 0.098	0.063 ± 0.002	0.053 ± 0.002	102.325 ± 3.158	0.053 ± 0.001	0.031 ± 0.001
	15	72.372 ± 0.956	0.121 ± 0.019	0.133 ± 0.028	164.162 ± 11.263	0.125 ± 0.013	0.086 ± 0.004
	20	51.238 ± 0.175	0.239 ± 0.025	0.176 ± 0.045	153.471 ± 9.204	0.098 ± 0.004	0.054 ± 0.002
	25	36.181 ± 0.414	0.142 ± 0.008	0.155 ± 0.011	128.409 ± 5.003	0.042 ± 0.001	0.039 ± 0.005

(Table 1). *P. florida* and *Ganoderma* sp. rckk-02 exhibited maximum MnP activity of 0.234 (15th day) and 0.239 U/g (20th day), however, no detectable level of MnP was observed in SB treated with *C. caperata* RCK 2011. While all the fungi produces LiP in a range 0f 0.11–0.17 U/g substrate. Interestingly, none of the fungus produced detectable levels of either CMCase or FPase (Table 1). Our results are consistent with previous reports on enzyme profile of selective lignin degrading white rot fungi grown on lignocellulosic residues (Carabajal, Levin, Albertó, & Lechner, 2002; Dinis et al., 2009; Shrivastava et al., 2011).

3.2. Fungal delignification of sugarcane bagasse

Lignin is the main component in plant cell wall that limits chemical/biological/enzymatic breakdown of the carbohydrates of the plant material. The control SB used in the study was composed of (w/w) 24.36% lignin and 61.89% holocellulose and others (e.g. extractives, ash). The results obtained here are well within the range of composition reported in earlier studies, where lignin and holocellulose were found to be 17–22% and 59–61% respectively (Lee, Chung, & Day, 2009; Velmurugan & Muthukumar, 2011). Among the three fungi tested, *P. florida* brought about maximum delignification, 7.91% with respect to lignin content of SB after 15 days of fermentation, which was followed by *Ganoderma* sp. rckk-02 (5.58%) and *C. caperata* RCK 2011 (5.48%). The higher lignin degradation after 15 days of incubation may be attributed to the maximum production of ligninolytic enzymes on 15th day (Table 1). However, irrespective of the fungus used, no significant degradation of lignin was observed when the fermentation period was extended to 25 days (Table 2). Our results are in accordance with the earlier reports on biological pretreatment of lignocellulosic materials with white-rot fungi (Meza, Sigoillot, Lomascolo, Navarro, & Auria, 2006; Taniguchi et al., 2005). *Pleurotus* sp. was reported to degrade 6.8% lignin in water hyacinth after 22 days (Mukherjee & Nandi, 2004), while *P. cinnabarinus* was reported to degrade 8.5% lignin in sugarcane bagasse (Meza et al., 2006). Gupta et al. (2011) reported the biological delignification in *Prosopis juliflora* and *Lantana camara* by white rot fungus, *P. cinnabarinus* by 13.13 and 8.87%, respectively. The degradation of SB (i.e. in terms of substrate weight loss, lignin and cellulose degradation) increased with increase in incubation time and this could directly be correlated with the enzyme production profile by these three fungi (Tables 1 and 2).

Beside delignification, the availability of the cellulose is also an important criterion for evaluating the efficiency of biological pretreatment; which in turn could provide higher amount of cellulose (carbohydrate) for enzymatic saccharification. The holocellulose content in fungal fermented SB was observed to be increased compared to control SB after 15 days of fermentation and thereafter it declined gradually (Table 2).

Table 2
Compositional analysis of sugarcane bagasse during solid state fermentation by white rot fungi.

Fungi	Days	Wt. left (g)	Holocellulose (%)	Lignin (%)	H/L
Control	–	20.00 ± 0.78	24.361 ± 0.15	61.892 ± 0.24	2.540
P.florida	5	18.35 ± 0.83	23.439 ± 0.23	63.561 ± 0.96	2.711
	10	18.01 ± 0.26	22.530 ± 0.17	64.226 ± 0.39	2.850
	15	17.76 ± 0.45	21.581 ± 0.32	65.202 ± 0.44	3.021
	20	16.64 ± 0.66	21.352 ± 0.66	63.647 ± 0.36	2.981
	25	15.52 ± 0.72	21.215 ± 0.35	62.788 ± 0.21	2.959
C.caperata RCK2011	5	18.33 ± 0.33	23.573 ± 0.41	63.110 ± 0.74	2.677
	10	18.14 ± 0.48	22.764 ± 0.26	63.979 ± 0.91	2.810
	15	17.88 ± 0.52	21.978 ± 0.19	64.510 ± 0.32	2.935
	20	17.65 ± 0.77	21.943 ± 0.47	63.872 ± 0.48	2.912
	25	17.48 ± 0.25	21.899 ± 0.53	63.011 ± 0.52	2.877
Ganoderma sp. rckk-02	5	18.40 ± 0.87	23.475 ± 0.56	63.527 ± 0.63	2.706
	10	18.03 ± 0.91	22.583 ± 0.38	64.421 ± 0.32	2.852
	15	17.77 ± 0.47	21.672 ± 0.43	65.328 ± 0.81	3.014
	20	17.56 ± 0.72	21.621 ± 0.29	64.102 ± 0.73	2.964
	25	17.39 ± 0.81	21.598 ± 0.37	63.407 ± 0.45	2.935

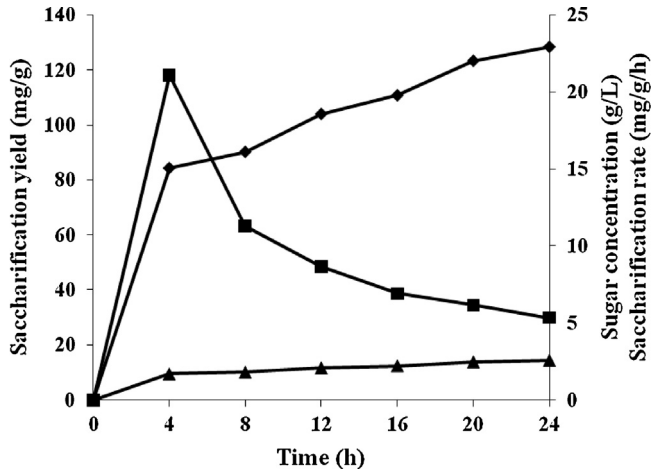

Fig. 1. Enzymatic hydrolysis profile of untreated Sugarcane bagasse. Saccharification yield (♦), sugar concentration (■), saccharification rate (▲).

Table 3
Sugar released on different treatments of sugarcane bagasse.

S.No.	Treatment		Sugar released (mg/g)
1.	Untreated		128.39 ± 6.32
2.	Fungal treated		
		P. florida	303.33 ± 11.13
		C. caperata RCK2011	192.52 ± 8.71
		Ganoderma sp.rckk-02	220.86 ± 9.36
3.	Chemical		
		Acid (H_2SO_4)	98.01 ± 5.36
		Alkali (NaOH)	193.55 ± 7.32

Interestingly it was observed that the fungal pretreatment by *P. florida*, *C.caperata* RCK 2011 and *Ganoderma* sp. rckk-02 gave highest Holocellulose/Lignin (H/L) ratio of 3.021, 2.935 and 3.014, respectively, after 15 days of fermentation. Basu, Gaur, Gomes, Sreekrishnan, and Bisaria (2002) found the highest lignin and lowest cellulose degradation in wheat straw on 6th day of solid state fermentation.

3.3. Enzymatic saccharification of fungal pretreated sugarcane bagasse

The total reducing sugars obtained after the enzymatic saccharification of untreated SB was 128.39 ± 6.32 mg/g of dry substrate after 24 h of incubation (Fig. 1). Among the fungal pretreated substrates, the maximum cellulose saccharification vis-à-vis sugar release was observed in SB fermented by *P. florida* (303.33 ± 11.13 mg/g of substrate) followed by *Ganoderma* sp.rckk-02 (220.86 ± 9.36 mg/g of substrate) and *C. caperata* RCK 2011 (192.52 ± 8.71 mg/g of substrate) fermented materials (Fig. 2a–c). Irrespective of the fungal pretreated substrates when enzymatically hydrolysed, larger amount of sugars were released compared to the negative (untreated SB) and positive control (acid and alkali treated SB) substrates as well (Table 3). The enhanced enzymatic saccharification in fungal fermented substrate may be attributed to the partial lignin degradation, which may be responsible for preventing the penetration of cellulases (Dias et al., 2010). Moreover, the improvement in enzymatic hydrolysis could be due to the improvement in porosity of plant material because of biological pretreatment with white-rot fungi leading to an increase in the initial adsorption of cellulase to cellulose (Yu, Guo, Zhang, Yan, & Xu, 2009). Dias and co-workers (2010) have reported a release of 120 mg/g substrate sugar from wheat straw fermented with *Irpex lacteus* for 46 days. Similar to this, Shi, Chinn, and Sharma-Shivappa (2009) when pretreated the cotton stalks with *P.chrysosporium* have reported 55.6 mg/g substrate sugar release. However, compared to these studies the sugars released during our study from the 15 days fungal fermented substrate were comparatively higher (Table 4).

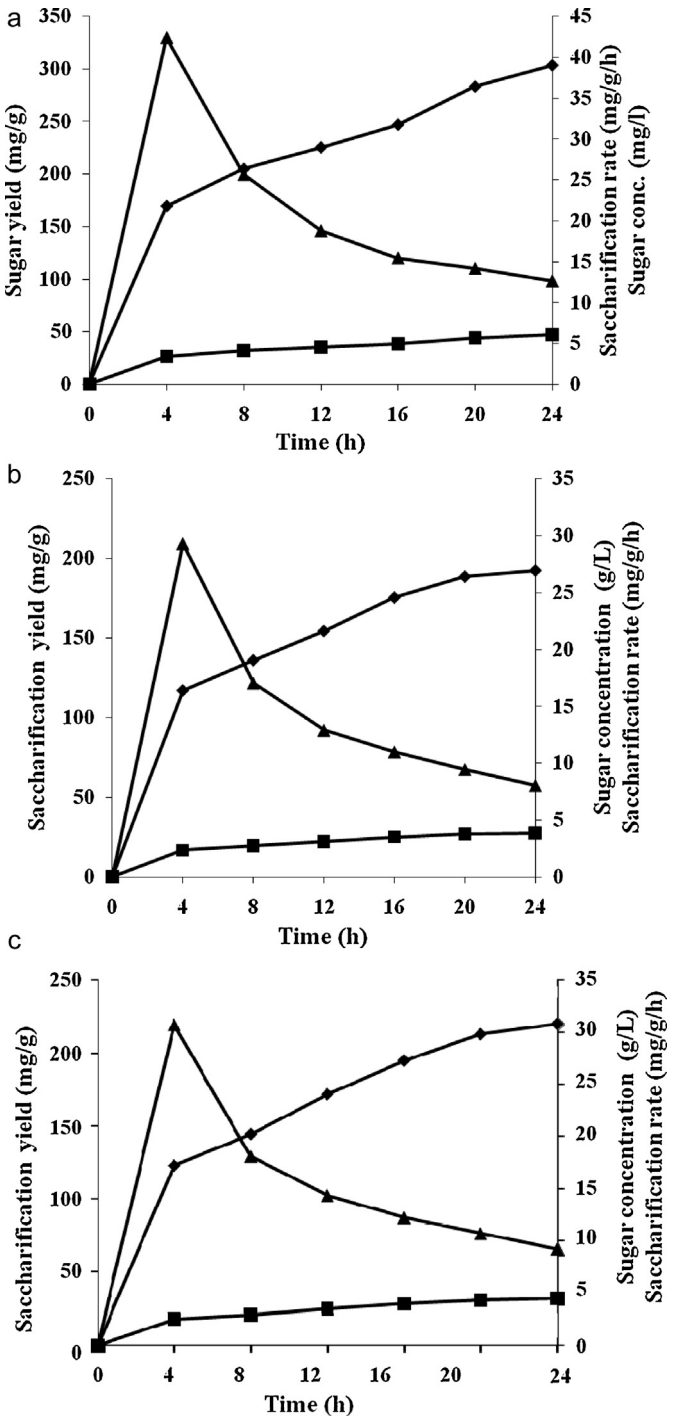

Fig. 2. (a) Enzymatic hydrolysis profile of *Pleurotus florida* treated Sugarcane bagasse. Saccharification yield (♦), sugar concentration (■), saccharification rate (▲). (b) Enzymatic hydrolysis profile of *Coriolopsis caperata* treated Sugarcane bagasse. Saccharification yield (♦), sugar concentration (■), saccharification rate (▲). (c) Enzymatic hydrolysis profile of *Ganoderma* sp.rckk-02 treated Sugarcane bagasse. Saccharification yield (♦), sugar concentration (■), saccharification rate (▲).

Table 4
Comparison of enzymatic saccharification of various fungal pretreated substrates by cellulase enzyme.

Fungal strain	Substrate	Source of cellulase enzyme	Reducing sugar (mg/g)	Reference
Phanerochaete chrysosporium	Cotton stalks	Celluclast 1.5 L (Sigma) + Novozyme 188 (Sigma)	55.6	Shi et al. (2009)
Iprex lacteus	Wheat straw	Commercial Cellulase (Onozuka R-10)	120	Dias et al. (2010)
Ceriporiopsis subvermispora	Corn stover	Commercial cellulase (Spenzyme CP, Genencor)	233	Wan and Li (2010)
Echinodontium taxodii 2538	Chinese willow	Cellulase (Sigma)	175	Yu et al. (2009)
Echinodontium taxodii 2538	China- fir	Cellulase (Sigma)	60	Yu et al. (2009)
Pleurotus ostreatus	Rice straw	Commercial cellulase (Yakult industry)	118.5	Taniguchi et al. (2005)
Pleurotus ostreatus	Rice hull	Commercial cellulase	80	Yu et al. (2009)
Trametes versicolor	Bamboo culms	Cellulase (Sigma)	98	Zhang, Yu, Huang, and Liu (2007)
Echinodontium taxodii	Bamboo culms	Cellulase (Sigma)	100	Zhang et al. (2007)
Pleurotus florida	Sugarcane bagasse	Crude cellulase	303.33	Present study
Coriolopsis caperata RCK2011	Sugarcane bagasse	Crude cellulase	192.52	Present study
Ganoderma sp.rckk-02	Sugarcane bagasse	Crude cellulase	194.86	Present study

4. Conclusion

The white-rot fungi; *P.florida*, *C.caperata* RCK 2011 and *Ganoderma* sp.rckk-02 were able to degrade lignin of SB and exhibited potential in delignification of plant materials. The cellulases from brown-rot fungus *Fomitopsis* sp. RCK2010 were effective in hydrolysis of partially delignified materials into reducing sugars, which could be fermented to either alcohols or organic acids.

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