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Bagasse pretreatment by some wood-rotting fungi in ethanol production

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Abstract. *Tewolde D, Gessesse A. 2019. Bagasse pretreatment by some wood-rotting fungi in ethanol production. Bioteknologi 16: 21-30.* Biomass from lignocellulosic materials is a sustainable feedstock for ethanol production. During ethanol production, pretreatment modifies the lignin barrier to make cellulose more accessible to enzymatic hydrolysis. The biological pretreatment involves selecting rot fungi that preferentially degrade lignin while retaining a minimum amount of polysaccharides. Despite not being well developed, the enzymatic nature of the reaction is an advantage over other pretreatment methods. These fungi produce ligninolytic enzymes, predominantly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) in different combinations. Biological pretreatments have shown weight loss of lignin and improved yields of ethanol. However, only a few well-characterized white rots have been explored. This study evaluated 15 wood rotting fungi isolated from Ethiopia for pretreatment during 15 days of solid state fermentation using bagasse as a lignocellulosic substrate. The production of ligninolytic enzymes by *Fomitiporia aethiopica*, *F. pseudopunctata*, *Fomitopsis carnea* and *Vanderbylia vicina* were reported for the first time in this study. The white rots, *F. aethiopica*, *Perenniporia tephropora*, *Inonotus* sp. and *Pleurotus sajor-caju*, were highly selective based on maximum and minimum productivity of ligninolytic and polysaccharide degrading enzymes, respectively. The pretreatment by the white rots caused ligninolysis and better cellulose digestibility was obtained with higher lignin loss. Among the selective degraders, *P. tephropora* caused the highest lignin loss (7.71%) and cellulose digestibility (29.44%) after enzyme hydrolysis of the pretreated bagasse. This digestibility showed an improvement of 38.74 % in comparison with untreated bagasse. In addition to high MnP productivity (55.87 U/g), *P. tephropora* also produced high titers of Lac (79.65 U/g) in contrast to the other selective degraders that might have attributed to better lignin loss. The ethanol yield from the fermentation of cellulase enzyme hydrolyzed *P. tephropora* pretreated bagasse was 1.87 g/L, which improved by 27.21 % compared with untreated bagasse (1.47 g/L). Therefore, *P. tephropora* pretreatment enhances ethanol production from bagasse through partial degradation of lignin, which improves the accessibility of cellulose to enzyme hydrolysis.

Keywords: Bagasse, ethanol, ligninolytic enzymes, pretreatment, rot fungi

INTRODUCTION

Ethanol has been used as a biofuel to provide an alternative energy source and thus reduce oil consumption. Simple sugars and starchy biomass serve as raw materials for ethanol production. However, these substrates are in limited quantity and constitute animal feed and human food (Sun and Cheng 2002). Therefore, the use of lignocellulosic biomass as a raw material is attracting growing interest. It is abundant, cheap, and the ethanol production process has very low net CO_2 emissions compared to other feedstocks (Tomas-Pejo et al. 2008). However, process technology for producing ethanol from lignocellulosic biomass is complicated, energy-intensive and under development. The biggest challenge lies in processing the raw material due to the structural arrangement of the fiber components. Cellulose, the main sugar reservoir, is surrounded by a wall of lignin which becomes a barrier to enzymatic hydrolysis. Therefore, biomass must be pretreated in the production process to remove lignin, increase porosity and decrease cellulose crystallinity (Sanchez and Cardona 2008).

There are several pretreatment systems, which are divided into physical, physicochemical, chemical and biological. Biological pretreatment involves mainly selective lignin-degrading fungi capable of removing lignin with minimal loss of polysaccharides (Itoh et al. 2003). Selective lignin degraders preferentially degrade lignin and hemicelluloses and degrade cellulose only in the later stages (Hakala 2007). Biological pretreatment is performed under mild reaction conditions with few side reactions. As a result, the system has very low chemical consumption, lower energy consumption and less susceptibility to pressure and corrosion than other pretreatments (Lee 1997; Samsuri et al. 2008).

Selective degradation is characteristic of some species of white rot fungi. These fungi degrade lignin mainly through the action of extracellular oxidative enzymes, lignin peroxidase, manganese peroxidase and laccase (Hakala 2007). Different fungi produce these enzymes in different combinations (Tuor et al. 1995). The degradation mechanism is the oxidation of lignin to form cationic radicals which initiate a non-enzymatic chain reaction and ultimately mineralize the polymer (Hakala 2007). Biological pretreatment studies of various lignocellulosic biomasses have produced a loss of lignin with a low degradation of polysaccharides with consequent improvement in ethanol production (Itoh et al. 2003; Munoz et al. 2007; Samsuri et al. 2008; Bak et al. 2009). Although these studies show promising results, only a few well-characterized white rot species have been evaluated for pretreatment. Therefore, there is a need to assess the

potential of different putrefactive fungi seeking a better lignin degradation system than bare cellulose without significant self-consumption.

Sugar cane bagasse can be used as a lignocellulosic biomass substrate for ethanol production. Bagasse is the residue of fiber that remains after the mechanical extraction of the sap from the cane stalks. It has a high cellulosic composition, 30-43% (Kadam 2000), making it suitable for ethanol production (Buaban et al. 2010). Sugar mills burn bagasse as fuel to generate steam and continue to produce a 15-25% surplus. This product can be further increased if steam generators are improved to reduce consumption, facilitated by solar energy, and if bagasse is replaced by waste piping (Kadam 2000). In the Ethiopian context, with the development of hydroelectric projects, the electricity supply could be surplus soon. Thus, given the increased demand for liquid fuel, the sugar industry has the opportunity to replace bagasse fuel with electrical energy. With an oversupply of bagasse and the integration of appropriate ethanol processing technology with existing molasses at ethanol fermenters in the sugar industry, ethanol production from bagasse could be possible. However, as with all other lignocellulosic biomasses, the accessibility of polysaccharides in bagasse biomass is limited. Therefore, different pretreatment systems should be studied to develop a process for converting bagasse into ethanol.

MATERIALS AND METHODS

Most experiments were conducted in the Microbial Biotechnology Laboratory, Unit of the Biotechnology Program, Addis Ababa University, Ethiopia.

Culture preparation

The 15 rot fungi in Table 1 were generously provided by Dr. Adane Bitew (Department of Medical Laboratory Technology, School of Medicine, Addis Ababa University, Ethiopia), as slant or plate cultures. These rot fungi were subcultured on 41 g/L potato dextrose agar (PDA) plates and were incubated for 5-7 days at 27°C.

Solid state fermentation

Production of ligninolytic enzyme and pretreatment by the rot fungi were studied during SSF of bagasse substrate. The SSF conditions were prepared as follows.

Substrate preparation

Sugar cane bagasse residues from mechanical crushing of the cane stalks were purchased from a local cane juice shop. The bagasse was dried in the sun, cut into smaller pieces with a cleaver, ground with a blender and passed through a 1 mm sieve (to remove smaller particles). The residual sucrose on the fiber was removed by immersing it in water (approximately 1: 100 w/v) for 1 hour in two turns with regular agitation. This bagasse preparation is dried in the sun and used as a lignocellulose substrate.

Seed culture preparation

With additional carbon and nitrogen supplements, a mineral salt solution was prepared from Basal III medium and trace elements (10: 1 v/v) (Tien and Kirk 1988; Kumar et al. 2006). Basal III medium was prepared from KH_2PO_4, 20 g/L; $MgSO_4$, 5 g/L and $CaCl_2$, 1 g/L. The trace element solution consisted of (g/L) $MgSO_4$, 3; $MnSO_4$, 0.5; NaCl, 1; $FeSO_4.7H_2O$, 0.1; $CoCl_2$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4$, 0.1; AlK (SO_4) $2.12H_2O$, 0.01; H_3BO_3, 0.01 and $Na_2MoO_4.2H_2O$, 0.01 dissolved in a nitrilotriacetate solution, 1.5 g/L adjusted to pH 6.5 with 3% KOH. Glucose (1 g/L) and peptone (10 g/L) were added as a supplement of carbon and nitrogen, respectively. The bagasse preparation (5 g) in a 500 mL Erlenmeyer flask was autoclaved at 121°C for 30 minutes. After cooling, 15 mL of the mineral salt solution with the supplements was membrane filtered (0.2 µm) on the substrate.

Each flask was inoculated with four agar cube blocks of approximately 6 x 5 mm^2 (Samsuri et al. 2008) cut from the peripheral sides of the mycelium on the PDA cultures. The SSF culture was incubated stationary at 27°C for 15 days. The growth characteristics of each fungal mushroom on PDA and SSF media were compared by visual observation of the mycelium.

Enzyme extraction and assay

The SSF substrate was immersed in 50 mL of sodium acetate buffer (25 mM, pH 4.5) on a rotary shaker (120 rpm) at room temperature for 90 minutes. The liquid homogenate was filtered through cotton gauze. The extract was centrifuged at 3000 rpm for 5 minutes to remove solids and kept in aliquots at -20°C.

The crude extract was used directly as an enzyme in the test tubes. The concentration between enzyme and substrate was maintained at 1:10 (v/v). The absorbance of each tube was measured on a 1 mL 1 cm (wide) cuvette using a Jenway 6300 visible spectrophotometer (Jenway Ltd., England). The productivity of the enzyme (U/g) was determined using equation 1, where U (U/mL) is the unit expressing the activity per mL of enzyme used, and (mL) is the volume of the enzyme extract collected from the SSF substrate and s (g) is the weight of the SSF substrate. All enzymatic reactions were performed in triplicate according to the respective protocols.

$$\text{Enzyme productivity} = U \times e / s \qquad [1]$$

Manganese peroxidase assay

MnP activity was determined according to Kuwahara et al. (1984) based on the oxidation of phenol red, characterized by a color change from red to pink-purple, under alkaline conditions. The reaction mixture consisted of phenol red (0.01%), sodium lactate (25 mM), $MnSO_4$ (100 µM) and egg albumin (0.1%) in 20 mM sodium succinate buffer (pH 4 ,5). To start the reaction, H_2O_2 (100 µM, final concentration) was added, and the tubes were incubated at 30°C for 5 minutes. The reaction was terminated with 2N NaOH (40 µL/mL) and the absorbance was measured at 610 nm. One unit (U) was defined as the amount of enzyme required to increase by 0.1 optical density (OD) units per minute.

Laccase assay

Lac activity was detected based on the oxidation of guaiacol (C_6H_4 (OH) (OCH_3)), causing the colorless substrate to turn brown (Coll et al. 1993). The reaction mixture was 1 mM guaiacol in 50 mM sodium acetate buffer (pH 4.5). The tubes were incubated at 37°C for 1 hour and the absorbance was measured at 465 nm. According to Arora and Sandhu (1985), a unit is defined as the amount of enzyme required to increase by 1 OD unit per hour.

Lignin peroxidase assay

The LiP test based on the Azure B bleaching method, according to Archibald (1992) using an absorption spectrophotometer, failed in the present study. The readings of the reagent blank containing the dye were unstable. Vares et al. (1995) have encountered similar problems with the protocol. Therefore, a qualitative test based on blue B discoloration was performed on agar plates by inoculating the rotting fungi on solid media according to Zhao et al. (1996). The medium composition was designed to simulate the SSF condition to predict the actual LiP productivity in the bagasse substrate. SSF simulation medium contained peptone, 10 g/L; glucose, 10 g/L; Azure B, 0.1 g/L and agar, 15 g/L dissolved in a mineral salt solution and the cultures were incubated at 27°C for 10 days. However, MnP could discolor Azure B under certain conditions and produce distortions (Archibald 1992). Thus, the LiP activity of putrefactive fungi showing dye discoloration on SSF simulation medium was confirmed on Mn-deficient agar plates (confirmation medium) supplemented with veratryl alcohol (Zhao et al. 1996; Levin et al 2004). The medium was composed of a mineral salt solution (without $MnSO_4$); glucose, 10 g/L; NH_4NO_3, 26 mM; aspartic acid, 15 mM; thiamine, 1.68 mg/L; sodium succinate, 0.1 M; veratrilic alcohol, 2 mM; agar, 15 g/L and blue B, 0.02 g/L. Cultures were incubated at 27°C for 10 days. All detections of discoloration were confirmed by visual observation of a cubic section removed from areas very close to the growth of the mycelium.

Carboxymethyl cellulase and xylanase assay

The extracellular enzymatic activities of carboxymethylcellulose (CMCase) and xylanase were determined by the DNA method (dinitro salicylic acid, $C_7H_4N_2O_7$), according to Bernfeld (1955). The assay is based on reducing 3,5-DNA (yellow) to 3-amino-5-nitrosalicylic acid (reddish brown) by the respective reducing sugars released from the polysaccharides. DNA reagent was prepared from DNA, 10 g/L; C6H6O, 2g/L; Na2SO3, 0.5 g/L and NaOH, 5 g/L. Substrates contained carboxymethylcellulose (0.5%) and birch xylan (1%) in 50 mM citrate phosphate buffer (pH 5.0) for CMCase and xylanase assays, respectively. The tubes were incubated at 40°C for 15 minutes. The reaction was terminated by adding the DNS reagent to the reaction solution (2: 1 v/v), followed by boiling in a water bath for 5 minutes. The absorbance of each reaction tube was measured at 540 nm after cooling. The activities of CMCase and xylanase were expressed in international units where 1 U is the amount of enzyme required to release 1 µmol of glucose (0.18 mg/mL) (Ghose 1987) and xylose (0.15 mg/mL). respectively per minute. The standard curves were made with known glucose and xylose concentrations (appendices 1A and 1C, respectively).

Screening for selective rot fungi

Highly selective rot fungi were examined, showing maximum and minimum productivity of ligninolytic and polysaccharide degradation enzymes, respectively. The aggregate metric index analysis was used as the screening methodology, similar to the multimetric approach of Barbour et al. (1999) for the environmental data analysis. Productivity of enzymes was used as a measure. Each metric was classified into 4 levels of productivity: high, moderate, low and marginal. The range (score range) between tiers was determined by dividing the range (the difference between maximum and minimum productivity) by 4 (the number of tiers). Unitless scores were then assigned to these levels. Score values range from a maximum of 4 for high to a minimum of 1 for marginal productivity for ligninolytic enzymes. The scoring criterion was reversed for CMCase and xylanase; thus, the score values range from a maximum of 4 for marginal to a minimum of 1 for high productivity. The level of each mushroom was identified based on the metric value (U/g) and scores were assigned accordingly. The aggregate index for each mushroom was determined by summing the scores of all metrics. These indices have been divided into 4 levels of selectivity: high, moderate, low and poor. The interval between levels was determined as described for the metrics and each mushroom was ranked based on its index value.

Minimal xylanase activity was set as a selection criterion in the present study, although residual xylan was not used as a substrate for saccharification and fermentation. This was done to consider its potential as a major component of the bagasse.

Screening for effective ligninolysis

Lignin compositions and cellulose digestibility of bagasse, pretreated by the highly selective rot fungi during the SSF, were compared with untreated bagasse to screen for effective ligninolysis.

Lignin degradation

According to Munoz et al. (2007) by hydrolysis of polysaccharides with concentrated acid. Acid-soluble lignin was also analyzed by UV absorbance (Ferraz et al. 2000; Sluiter et al. 2008) of acid-hydrolyzed filtrate. Before hydrolysis, residual non-structural materials or extractable (since some of their composition was removed during soaking) were removed to facilitate acid penetration. Mineralized lignin components, water-soluble inorganic materials, non-structural sugars and nitrogenous materials were removed by autoclaving in an aqueous suspension at neutral pH to avoid hydrolysis hemicelluloses. According to Sluiter et al. (2005) by Soxhlet extraction with ethanol.

First, the fungal biomass on the pretreated bagasse was removed by soaking twice in 2 L of distilled water for 30 minutes with continuous agitation. The floating biomass of

low-density fungi was decanted and the bagasse was recovered (Kumar et al. 2006). Next, the bagasse was suspended in water (1: 100 w/v), adjusted to pH 7-7.5, and sterilized in an autoclave at 121°C for 15 minutes. The liquid was discarded, and the bagasse was washed with 200 mL of boiling water. Other extractants were removed with ethanol (96%) in a Soxhlet extractor (Glassco Laboratory Equipment Ltd., UK) set to reflux for 9 hours. After extraction, the fiber was washed with 80 mL of fresh ethanol (96%) and air dried.

The extract-free bagasse (0.1 g) was treated with 1.5 mL of H_2SO_4 (72%) in a test tube. The tube was shaken vigorously on a vortex and continuously every 10 minutes for 2 hours. The resulting acid hydrolyzate was diluted in 56 mL of distilled water to bring the concentration to 1.88%. The hydrolyzate was autoclaved at 1210°C for 1 hour and filtered through a 0.45 µm calibrated Whatman glass fiber filter. The residue on the filter (the acid-insoluble lignin) was washed with 100 mL of water and dried in an oven at 105°C to constant weight. The filtrate collected from acid hydrolysis was used to determine acid-soluble lignin. The absorbance of the filtrate was analyzed at 205 nm using a Lambda 19 UV-Vis spectrophotometer (Perkin Elmer Inc., Germany). The acid-soluble lignin concentration (%) was determined using Equation 2, where A is the absorbance, V (L) is the filtrate volume, and ε (105 L/g cm) is the absorbance of soluble lignin, l (~1 cm) is the trough path length, and w1 (g) is the initial weight of bagasse without extract.

$$\text{Acid soluble lignin} = A \text{ x } V\text{x } 100/\text{s x } l \text{ x w1} \qquad [2]$$

The lignin composition of the extract free bagasse was the sum of acid-insoluble and acid-soluble lignin. Therefore, lignin weight losses caused by the selected rot fungi pretreatments were determined from the difference in composition with the lignin content of the untreated extract-free bagasse.

Cellulose digestibility

Cellulose digestibility was defined as the percentage of the theoretical maximum of glucose obtained from the hydrolysis of cellulose (Bak et al. 2009). The glucose released from enzyme hydrolysis and the cellulose composition of untreated bagasse was determined as follows.

Enzyme hydrolysis

The bagasse substrate for enzymatic hydrolysis was prepared by removing the fungal biomass and water-soluble mineralized lignin (to avoid enzyme inhibition), without ethanol extraction. Cellulase enzyme (Endoglucanase, EC 3.2.1.4) extracted from Aspergillus niger (chemical group Sigma, USA) was used to hydrolyze cellulose. The enzyme (60 U/g cellulose) was dissolved in 50 mM citrate-phosphate buffer (pH 5.0) and centrifuged at 3000 rpm for 5 minutes to precipitate the particulate. The bagasse (1 g) was hydrolyzed by the solid-state enzyme (1:10) and sterilized on a filter (0.2 µm) at 37°C for 5 days. Glucose was extracted by immersing the hydrolyzate in 10 mL of water at room temperature on a rotary shaker (120 rpm) for 20 minutes. The amount of glucose was estimated using the standard curve. A small change has been made to the composition of the DNS reagent, with the addition of sodium and potassium tartrate, 200 g/L to increase the stability of the reduced DNS (Miller 1959).

Cellulose composition

The water-soluble and ethanol extracts were removed from untreated bagasse according to Sluiter et al. (2005) by Soxhlet extraction, refluxed for 18 and 9 hours, respectively. Then, the bagasse was air dried and the composition of the remaining extracts was estimated.

The free extractive residue (1 g) was delignified according to Ferraz et al. (2003) in a solution of 0.1 mL of acetic acid and 0.6 g of sodium chlorite in 32 mL of aqueous solution. The reaction was carried out in a water bath at 80°C for 1 hour. Equal amounts of sodium chlorite and acetic acid were added every hour over 4 rounds. The mixture was filtered through a porous glass filter No. 4 and washed with cold water and acetone. The filtered residue was extracted with 95% ethanol in the Soxhlet extractor for 4 hours. Finally, the residue was washed with cold water and dried in an oven at 105 ° C to constant weight.

The oven-dried holocellulose residue (0.5 g) was treated with 2.5 mL NaOH (17.5%) at 20°C. NaOH (5 mL) was added 3 times at 5-minute intervals. The mixture was allowed to stand for 30 minutes, then diluted with 8.25 mL of distilled water and left to stand again at 20°C for 1 hour. The fiber was filtered through a #4 fritted glass crucible and soaked in 50 mL of NaOH (8.3%) for 2 minutes. The crucible was soaked in 3.25 mL of acetic acid (10%) for 3 minutes and washed with 300 mL of water. The residual cellulose was dried in an oven at 105°C to constant weight (Rowell et al. 2005). The cellulose composition C (%) was calculated using equations 3, 4 and 5, where c '(%) is the cellulose composition in the extractive free holocellulose, w2 (g) is the weight of the extractive free holocellulose residue, w3 is (g) the weight of cellulose in the extractive free holocellulose, H (%) is the holocellulose composition in the bagasse, L (g) is the extractive free lignin composition per gram of bagasse and E (g) is the composition of the remaining extracts per gram of bagasse. The digestibility of cellulose (%) was estimated using equation 6 where g (g) is the amount of glucose per gram of substrate.

$$c' = \frac{\text{w3}}{\text{w2}} \text{ x } 100 \qquad (3)$$

$$H = (1- L)\,(1-E) \text{ x } 100 \qquad (4)$$

$$C = c' \text{ x } H \text{ x } 100 \qquad (5)$$

$$\text{Cellulose digestibility} = \frac{g}{c} \text{ x } 100 \qquad (6)$$

Ethanol fermentation and GC analysis

The substrates for the ethanol fermentation were cellulase hydrolyzate from a pretreatment, causing the highest lignin loss and cellulose digestibility; Untreated bagasse cellulose hydrolyzate and standard glucose. Saccharomyces cerevisiae strain TAYI 4-2 was obtained from Amare Gesese (Ph.D.). The yeast stem cells for the inoculum were cultured aerobically in 50 mL of broth consisting of (g/L) glucose, 10; yeast extract, 1; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.1 and $(NH_4)2SO_4$, 0.1 (Samsuri et al. 2008). The fermentation broth was prepared from 5 mL of substrate and additional nutrients (g/L): yeast extract, 1; NaH_2PO_4, 1.2; $MgSO_4.7H_2O$, 0.025 and NH_4HPO_4, 0.5. The pH of the broth was adjusted to 5.5 with NaOH (Vaithanomsat et al. 2011). Finally, a 10% (v/v) yeast inoculum was added and anaerobic fermentation of 5 mL broth was carried out in 7 mL tubes for 72 hours at 30°C.

Samples of fermented broth (1 mL) were centrifuged at 10,000 rpm for 5 minutes to precipitate cells and other particles. The supernatant was diluted 10:1 with isopropanol (1 g/L, internal standard). Gas chromatography (GC) was performed on a DANI GC 1000 (DANI Instruments Ltd., Italy) with a flame ionization detector (FID) under optimized operating conditions. An Alltech Econo-Cap ECTM-5 capillary (30 m long, 0.32 mm diameter) coated with 95% methylpolysiloxane (stationary phase) was used as the column. The nitrogen (carrier gas) and hydrogen (FID fuel) flow rates were set at 5 and 0.65 bar, respectively. The GC was conditioned splitless and the inlet, oven and detector temperatures were set to 210, 155 and 250°C, respectively. The starting oven temperature was 50°C, 1 minute hold time at a heating rate of 30°C per minute. After a running time of 4.5 minutes, the peak areas (mV/s) of ethanol and isopropanol were recorded. The data was used to determine the ethanol concentration using the standard curve equation, developed by the same method using a known ethanol concentration. Fermentation broth samples were also analyzed for unfermented glucose composition by the DNA.

RESULTS AND DISCUSSION

Growth characteristics of the rot fungi

The intense growth of 8 rotting fungi was observed on SSF media (Table 1). Only 3 rot had poor growth, as seen on the handheld. Additionally, 11 rotting mushrooms changed the faded yellow color of the bagasse to red or light red.

Production of ligninolytic and polysaccharide degrading enzymes

The productivity of the MnP and Lac enzymes is shown in Figures 1A and 1B, respectively. These enzymes were also distributed in 11 putrefying fungi. However, in terms of productivity, MnP was produced by many putrefying fungi at relatively high titers compared to the Lac (Appendixes 7 and 8). *Laetiporus sulphureus* and *Schizophyllum commune* showed no activity, while *Polyporus* sp. and *Inonotus* sp. produced neither MnP nor Lac. The activities of the MnP and Lac enzymes of *Perenniporia* sp. and *Pleurotus sajor-caju* were also negligible. The highest productivity of MnP and Lac was recorded in *Fomitiporia aethiopica* (55.87 ± 0.37 U/g) and *Perenniporia tephropora* (79.65 ± 1.78 U/g), respectively. The *P. tephropora* also showed high MnP productivity (51.73 ± 1.12 U/g). Marginal productions of MnP and Lac were obtained from *Vanderbylia vicina* (5.56 ± 0.26) and *Lentinus edodes* (2.37 ± 1.11 U/g), respectively. CMCase and xylanase activities were detected from all putrefying fungi (Figures 1C and 1D, respectively). The *P. sajor-caju* had the lowest CMCase productivity (0.3 ± 0.02 U/g), while *Tyromyces* sp. produced the enzyme at the highest titers (8.53 ± 0.19 U/g). The *F. aethiopica* was the least producer of xylanase (2.25 ± 0.14 U/g), while *L. edodes* recorded the highest productivity (19.52 ± 1.12 U/g).

LiP was produced from 7 rotting fungi exhibiting varying intensities of blue B discoloration (Table 2). The discoloration was initially observed in 8 decaying mushrooms grown on SSF simulation media. Among them, Phellinus sp. did not discolor the blue B on the mounting support, excluding the possibility of LiP production. The complete discoloration was observed only in *Polyporus* sp. on both media, while other rot fungi could only fade or partially fade the dye.

Table 1. Growth characteristics of the rot fungi based on visual observation on PDA and SSFmedia

Species	Growth on		Bagasse color
	PDA	SSF	
Fomitiporia pseudopunctata	++++[c]	++++	R[f]
Fomitiporia aethiopica	++[a]	++++	R
Fomitopsis carnea	++++	++++	-[d]
Inonotus sp.	+++[b]	++++	R
Perenniporia tephropora	++	++++	R
Pleurotus sajor-caju	++++	++++	R
Polyporus sp.	++++	++++	-
Vanderbylia vicina	++	++++	R
Lentinus edodes	++++	+++	LiR[e]
Phellinus sp.	++++	+++	R
Schizophyllum commune	++++	+++	
Microporous sp.	+++	+++	LiR
Perenniporia sp.	++	++	LiR
Tyromyces sp.	++	++	LiR
Laetiporus sulphureus	++	++	-

Note: [a]poor, [b]medium, [c]intense, [d]no colour change observed, [e]light red, [f]red

Table 2. The intensity of azure B decolorization on SSF simulating and confirmation media

Species	SSF simulating media	Confirmation media
Polyporus sp.	++++[e]	++++
Fomitiporia aethiopica	+++	+++
Inonotus sp.	+++	+++
Tyromyces sp.	++[c]	+++[d]
Pleurotus sajor-caju	+[b]	+
Microporous sp.	+	+++
Vanderbylia vicina	+	+
Phellinus sp.	++	-[a]

Note: [a]no decolorization, [b]mild fade, [c]fade, [d]partial decolorization, [e]complete decolorization

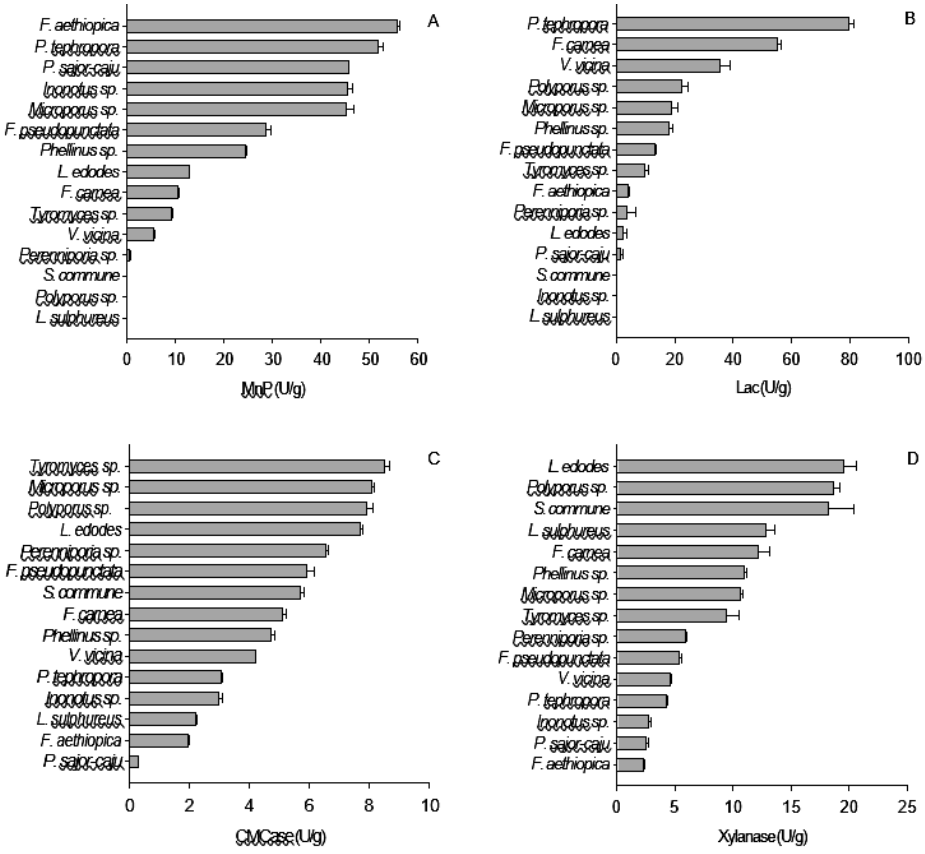

Figure 1. Productivity of ligninolytic and polysaccharide degrading enzymes by the rot fungi. A: MnP, B: Lac, C: CMCase and D: Xylanase

Categorization by ligninolytic enzyme production profile

Based on the production of ligninolytic enzymes, 13 rot fungi were categorized into 5 groups: LiP, MnP and Lac; LiP and MnP; LiP and Lac; MnP and Lac and only Lac producers (Table 3). There were 5 rot fungi grouped under MnP and Lac producers and 4 were LiP, MnP and Lac producers. At least two of the ligninolytic enzymes were simultaneously produced by 12 rot fungi.

Screening for selective rot fungi

The *F. aethiopica*, *P. tephropora*, *Inonotus* sp. and *P. sajor-caju* were highly selective among the rot fungi (Table 4). These fungi belonged to the white rot group. They showed high MnP, marginal xylanase and either low or marginal CMC productivity.

Screening selective rot fungi for effective ligninolysis

Lignin degradation analysis

The lignin composition of the untreated extract-free bagasse was determined to be 25.93% (acid-insoluble, 24% and acid-soluble, 1.93%). The residual lignin compositions in the 4 pretreated bagasse were determined and weight losses of lignin were observed in 3 pretreatments (Table 5). The major loss of lignin (7.71%) was due to pretreatment with *P. tephropora*. Degradation of *P. tephropora* was impossible to determine *P. sajor-caju* as the lignin composition after pretreatment (32.62%) was higher than that obtained in the untreated bagasse.

Cellulose digestibility

The untreated washed bagasse comprised 8.58% residual extractives, 67.72% holocellulose and 42.42% cellulose. All pretreatments increased glucose yields from enzymatic hydrolysis and thus cellulose digestibility (Table 6). Pretreatment with *P. tephropora* resulted in the maximum: glucose yield (7.345 g/L), cellulose digestibility (29.44%), and bagasse saccharification (12.49%, w / w). Compared to untreated bagasse, digestibility was improved by 38.74%. Pretreatment with *P. sajor-caju* had the lowest improvement in digestibility (1.7%). Therefore, *P. tephropora* was chosen as the selective degrader, displaying the most effective ligninolysis among the rot fungi based on lignin loss and cellulose digestibility results.

Ethanol yield

The ethanol yield from the fermentation of the enzyme hydrolysate of *P. tephropora* pretreated bagasse was 1.87 g/L (Table 7). This showed an improvement by 27.21% compared with the untreated bagasse (1.47 g/L). The *P. tephropora* pretreatment hydrolysate had a relatively higher content of unfermented glucose than the untreated bagasse. However, the glucose conversion efficiency (50.87%) was lower than the untreated bagasse (55.56%). The highest conversion efficiency (83.53%) was obtained from fermentation of the standard glucose under the same conditions.

Table 3. Categorization of the rot fungi by ligninolytic enzyme production

Group	Enzymes	
I	LiP, MnP and Lac	*Fomitiporia aethiopica, Microporus* sp., *Tyromyces* sp., *Vanderbylia vicina*
II	LiP and MnP	*Inonotus* sp., *Pleurotus sajor-caju*
III	LiP and Lac	*Polyporus* sp.
IV	MnP and Lac	*Fomitiporia pseudopunctata, Fomitopsis carnea, Lentinus edodes, Perenniporia tephropora, Phellinus* sp.
V	Lac only	*Perenniporia* sp.

Table 4. Comparison based on aggregate metrics index analysis

Species	Scores of metrics					Aggregate index	Relative selectivity
	MnP	Lac	LiP	CMC	Xylanase		
Fomitiporia aethiopica	4	1	3	4	4	16	High
Perenniporia tephropora	4	4	1	3	4	16	High
Inonotus sp.	4	1	3	3	4	15	High
Pleurotus sajor-caju	4	1	1	4	4	14	High
Fomitiporia pseudopunctata	3	1	1	2	4	11	Moderate
Vanderbylia vicina	1	2	1	3	4	11	Moderate
Microporous sp.	4	1	1	1	3	10	Low
Fomitopsis carnea	1	3	1	2	2	9	Low
Laetiporus sulphureus	1	1	1	4	2	9	Low
Polyporus sp.	1	2	4	1	1	9	Low
Perenniporia sp.	1	1	1	1	4	8	Low
Phellinus sp.	2	1	1	2	2	8	Low
Tyromyces sp.	1	1	2	1	3	8	Low
Schizophyllum commune	1	1	1	2	1	6	Poor
Lentinus edodes	1	1	1	1	1	5	Poor

Table 5. Residual lignin compositions and weight losses of bagasse after pretreatments

	Acid insoluble lignin (%)	Acid soluble lignin (%)	Total lignin (%)	Lignin loss (%)
Perenniporia tephropora	21.90	2.03	23.93	7.71
Fomitiporia aethiopica	22.45	1.74	24.19	6.71
Inonotus sp.	23.50	1.92	25.42	1.97
Pleurotus sajor-caju	30.80	1.82	32.62	ND[a]

Note: [a]not determined

Table 6. Comparison of glucose yield and cellulose digestibility among pretreatments andwith untreated bagasse

	Glucose yield		Cellulose digestibility	Improvement in digestibility
	g/L	g/g of substrate	(%)	(%)
Untreated	52.950	0.0900	21.22	
Perenniporia tephropora	73.450	0.1249	29.44	38.74
Fomitiporia aethiopica	71.700	0.1219	28.73	35.39
Inonotus sp.	65.250	0.1109	26.15	23.23
Pleurotus sajor-caju	53.850	0.0915	21.58	1.70

Table 7. The *S. cerevisiae* fermentation profile of pretreated and untreated bagasse enzymehydrolysates and standard glucose

Fermentation substrate	Ethanol yield (g/L)[a]	Glucose conversion efficiency (%)	Unfermented glucose (g/L)
Perenniporia tephropora pretreated bagasse hydrolysate	1.87	50.87	2.36
Untreated bagasse hydrolysate	1.47	55.56	1.82
Standard glucose	4.18	83.53	0.97

Discussion

Pretreatment during SSF can be attributed to favorable conditions for the growth of fungi and the production of ligninolytic enzymes. Factors that may have contributed include the similarity of the solid state to the natural environment (Lee 1997), the stimulation of secondary metabolism by the peptone (Kimura et al. 1990; Kaal et al. 1995; Martinez et al. 1996) and a small entity, glucose supplement (Lee 1997) to promote the production of ligninolytic enzymes. Production of MnP and Lac enzymes was more common than LiP in the present study. MnP and Lac producers are common in white rot (Hatakka 2001). MnP is produced by most white rot (Kaal et al. 1995) and is the most studied enzyme from natural substrate degradation (Vares et al. 1995).

Conversely, LiP is less common in white rot (Hakala 2007) and several factors in SSF conditions may have affected production. LiP activity can be regulated by Mn^{2+} in SSF media. Mn reduces the accumulation of veratril alcohol (Hamman et al. 1999), an important redox mediator of the LiP-catalyzed reaction (Hakala 2007). Bonnarme and Jeffries (1990) reported a 2.5-fold increase in LiP activity in an Mn-free medium. Poor LiP productivity makes detecting enzymatic activity difficult (Kaal et al. 1995) and may have influenced the low intensity of the blue B discoloration in the present study.

The production of all predominant ligninolytic enzymes by *F. aethiopica* is the first report of this activity by this redescribed taxa species. The ability to produce high titers of MnP arouses interest to characterize further and study the potential application of the enzyme. The other elevated MnP producer, *P. tephropora*, has previously been studied for its MnP activity (Ralph et al. 1996); however, the enzyme has not been well characterized. MnP activity has also been studied previously in *P. sajor-caju* (Boyle et al. 1992; Tuor et al. 1995; Martinez et al. 1996), *Inonotus* sp. (Palma et al. 2011) and *Microporus* sp. (Song 1997). However, for *Microporus* sp. the role of MnP is not related to the degradation of lignin.

The high productivity of the Lac enzyme by *P. tephropora* has been reported by Ralph et al. (1996). Although Lac's productivity was moderate and low due to *Fomitopsis carnea* and nearby *V. vicina*, this is the earliest report of ligninolytic enzyme activity. Several studies have identified LiP activities from several species of the genus *Polyporus*. LiP activity was detected by the white rot species *P. tulipiferae* (Rothschild et al. 2002), *P. platensis*, *P. brumalis*, *P. pinsitus* and *P. varius* (Tuor et al. 1995) and the brown rot species, *P. ostreiformis* (Dey et al. 1991). Although the productivity was low or marginal, *Microporus* sp., *Tyromyces* sp. and *V. vicina* showed LiP activity which has not been reported before. *Tyromyces* sp. will be added to the few LiP-producing brown rot fungi (Dey et al. 1991; Mtui and Masalu 2008). LiP activities have already been reported by *P. sajor-caju* (Tuor et al. 1995) and *Inonotus* sp. (Risna and Suhirman 2002).

The ligninolytic enzyme production profiles of *F. aethiopica*, *F. pseudopunctata*, *F. carnea*, and *V. vicina* have not been previously reported. Adults of *Inonotus* sp., *Phellinus* sp., *Perenniporia* sp., and *P. sajorcaju* and the absence of any *L. sulphureus* and *S. commune* activity were not entirely consistent with previous studies (Table 1). This could be due to variations in the species or strains of fungi responsible for the rotting and fermentation conditions in the present study. In the case of *L. sulphureus* and *S. commune*, enzymatic activities have been reported by Mtui and Masalu (2008) and Asgher et al. (2008) in liquid cultures, according to Tien and Kirk (1984, 1988). These media also contained a limited source of inorganic N and veratryl alcohol as a lignin substrate. The effect of media on the production of ligninolytic enzymes was examined by Kimura et al. (1990). They achieved high LiP productivity by white rot P. chrysosporium only in crops consisting of limited inorganic nitrogen; while on the contrary *Bjerkandra* sp. produces high titers in the presence of nitrogen-rich organic supplements.

The red color formed on the bagasse substrate during growth may indicate lignin degradation during SSF. Fackler et al. (2007) observed red discoloration of wood, during short-term treatment, due to the release of free phenoxy radicals and resulting quinoid structures due to lignin degradation. In this study, this evidence was seen throughout highly selective white rot.

The productivity of the ligninolytic and polysaccharide degrading enzymes was inversely related to the highly selective degraders. As white rot, these fungi could follow a particular degradation pattern with a higher preference for lignin degradation (Hakala 2007). The SSF conditions may have further influenced this physiology in this study. In addition to stimulating secondary metabolism by dietary supplements, glucose can suppress the production of polysaccharide-degrading enzymes (Levonen-Munoz and Bone 1985). This white rot did not use the polysaccharides despite a better accessibility probably after modification of the lignin. Therefore, the supplements made lignin more likely to be metabolized as a carbon and energy source. This metabolic pathway was suitable for the pretreatment system.

The better degradation of lignin by *P. tephropora* can be attributed to the high productivity of Lac. White rot had no LiP activity, and MnP productivity was comparable to other selective lignin degraders. Arora et al. (2002) noted that white rot with high Lac productivity showed better lignin degradation and that combining one of the ligninolytic enzymes with Lac led to greater lignin loss.

The lignin loss by *P. tephropora* (7.71%) was comparable with *Coriolus versicolor* (6-7%) and *P. chrysosporium* (9%) pretreatments of hardwood lignin (Kashino et al. 1993). In otherstudies, lignin losses of up to 15.7% (Samsuri et al. 2008), 21% (Bak et al. 2009) and 21.7% (Itoh et al. 2003) have been reported. The relatively lower lignin degradation in the present study could be due to incomplete pretreatment from the short SSF period (15 days). This was evident in the red color formation of the bagasse substrate, a characteristic of short-term pretreatment (Fackler et al. 2007). Other factors might include culture conditions and or simply the natural lignin degradation capability of *P. tephropora*.

The lignin composition of bagasse after pretreatment with *P. sajor-caju* was higher than that of untreated

bagasse. This indicated a loss of weight of cellulose and hemicelluloses by degradation, which was compensated by the accumulation of unmodified lignin. However, white rot had only marginal CMCase and xylanase productivity. It is therefore suspected that white rot may have cell-bound CMCase and/or xylanase activity that has not been detected. In a study by Valaskova and Valerian (2006), 66% of the activities of P. ostreatus cellulose, xylan and mannan degrading enzymes were cell-bound. Although weight loss could not be determined, lignin degradation by *P. sajor-caju* was still evident due to the red coloring of the bagasse substrate and high MnP productivity.

A pretreatment that resulted in higher lignin loss showed better cellulose digestibility. Samsuri et al. (2008) and Bak et al. (2009) noted that as lignin weight loss increased, glucose yield was increased by enzymatic hydrolysis. Therefore, the modification of the lignin barrier increased the accessibility of the cellulose for enzymatic hydrolysis.

In previous studies, ethanol yield has been improved while sustaining 2.94% (Samsuri et al. 2008) and 5% (Itoh et al. 2003) cellulose weight losses from pretreatments. The present study also observed improvements in glucose yield, despite CMCase activities. The highest improvement in digestibility was observed from *P. tephropora* pretreatment while having low CMCase productivity, which was higher than only marginal productivity by the other highly selective degraders. Thus, the impact of lignin modification has overshadowed the effect of cellulose degradation by the enzyme.

The saccharification of bagasse pretreated with *P. tephropora* (12.49%) was superior to that of beech wood (9.5%) pretreated with P. chrysosporium (Sawada et al. 1995). The digestibility of cellulose (29.44%) was also higher than that of P. chrysosporium from corn stalks (0.3-12%) and was comparable to Cyathus stercoreus pretreatment (8.3-35.7%) (Keller et al. 2003). In other studies, the digestibility of cellulose reached around 44% (Samsuri et al. 2008) and even 64.9% (Bak et al. 2009). However, these results were obtained from white rot pretreatments which caused greater lignin losses.

Pretreatment with *P. sajor-caju* showed a slight improvement in the digestibility of cellulose (1.7%), despite the impossibility of determining the loss of lignin. The relatively low improvement indicates some form of modification of the lignin. It also implies that the suggested cell-bound polysaccharide-degrading enzyme activity might be dominated by xylanase. However, this suggestion needs to be confirmed by further analysis.

The increase in glucose yield due to *P. tephropora* pretreatment consequently enhanced ethanol production compared with the untreated bagasse. However, the glucose conversion efficiency was slightly reduced due to the pretreatment. As a general rule, the fermentation was highly affected by factors in the biomass saccharification process, as evidenced by the large difference in conversion efficiency between fermentations of standard glucose.

In conclusion, there is a greater prevalence of MnP and Lac enzymes among the studied rot fungi, while LiP is less frequent and exhibits low activity. The white rots, *F. aethiopica*, *P. sajor-caju*, *Inonotus* sp. and *P. tephropora* are relatively highly selective lignin degraders. CMCase and xylanase activity are low, but high quantities of ligninolytic enzymes are produced. The pretreatment of lignin with these selective degraders results in lignin weight losses, and lignin degradation results in an increase in cellulose accessibility. Minimal polysaccharide consumption under these pretreatment conditions does not highly affect the glucose yield (*P. sajor-caju* pretreatment is an exception). The *P. tephropora* is the most effective lignin degrader among these selective white rots. Better ligninolysis is attributed to the high productivity of Lac enzyme in combination with MnP. The pretreatment of bagasse with *P. tephropora* partially degrades lignin, thereby increasing cellulose accessibility to enzyme hydrolysis.

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