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Fungal Pretreatment and Hydrolysis of Sawdust Wastes from Ethiopian Sawmills for Sugar Production

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Abstract: Pretreatment with wood rot fungi has been reported for improving the enzymatic hydrolysis of lignocelluloses into usable sugars. In this research sawdust samples of *Eucalyptus globulus* and *Cupressus lusitanica* were pretreated with three white rot fungi and then hydrolyzed with enzyme extracts from three cellulolytic wood rot fungi. Results indicated that the amounts of lignin, cellulose and hemicellulose losses of the two sawdust types were significantly increased with the increasing incubation days. Pretreating with the white rot fungi resulted in more selective modifications in the lignin content of the sawdust samples. Sugar yield from the hydrolysis of the white rot fungi pretreated sawdust in all cases was significantly higher than that has been obtained from the control indicating the efficiency of the white rot fungi. The effects of the white rot fungi vary with biomass species on delignification efficiency. Results also indicated that with increasing hydrolysis period, sugar yields were found increased. By improving their culture conditions, these wood rot fungi could be used to efficiently reduce recalcitrance of raw sawdust wastes and then efficiently hydrolyze the pretreated lignocellulosic resources into usable sugars. This finding appears to be the first for Ethiopian wood rot fungal isolates in this regard.

Key words: Wood rot fungi · Pretreatment · Hydrolysis · Ethiopia

INTRODUCTION

Lignocellulosic biomass is renewable, widely available and mostly composed of about 10-25 % lignin, 20-30 % hemicellulose and 40-50 % cellulose [1, 2, 3]. The carbohydrate composition of lignocelluloses has been considered high but potentially it is not easily available due to its recalcitrant nature for hydrolysis by microbial actions [4]. Pretreatment techniques can remove the lignin fraction and expose the polysaccharides for hydrolysis [5, 6]. These techniques can be categorized into physical, chemical and biological methods [7, 8]. While physical pretreatment reduces the size of the lignocellulosic chain, chemical pretreatment polymerizes the lignocellulosic biomass [9]. On the other hand, biological pretreatment involves multiple enzymes that work synergistically to modify lignocellulosic substrates [10-12]. It has been reported that compared to the other pretreatment approaches, biological pretreatments are more economical, ecofriendly and less health hazardous [1].

Recently, efforts have been made towards achieving hydrolysis of cellulosic materials under environmentally friendly parameters. Such parameters could be obtained either by utilizing low chemical concentrations which will minimize environmental effects or through biological pretreatment of lignocellulosic substrates [13, 14].

White rot fungi (WRF) are considered as the major lignin degraders. Extensive researches have been conducted on basidiomycetous fungi to isolate new strains with an enormous secretion of ligninolytic enzymes with a potential of industrial applications [15]. Screening for efficient delignifying and hydrolytic WRF fungi from natural environment has been recommended as one of the methods of getting such efficient isolates [5, 16-18]. Ligninolytic enzymes are secreted in different

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combination by different WRF [19, 20]. The WRF pretreated lignocellulosic samples can easily be hydrolyzed with enzymes of the hydrolytic wood rot fungi [21, 22].

Ethiopia is categorized among countries with forest coverage of 10-30% [20]. Oromia regional state has high forest coverage of 3.1 million ha, which is 8.5% of its total land area and accounts for 70% of Ethiopia's total forest coverage [23]. The region state's total forest concession is 1, 752, 488 ha, of which 74215 ha, 1209955 ha and 468318 ha are classified as plantations, natural forests and other lands, respectively [24]. Oromia's forest industry has over 30 sawmills located in different zones and it is the largest supplier of processed wood products in the country. While processing this huge potential of processed wood products for the country, large amount of sawdust generated every day. As a result, it is common to see huge pile up of sawdust by the vicinities of these sawmills [personal observation]. Unfortunately, this resource is not currently being utilized.

In this research work, therefore, sawdust samples of the two most utilized timber species in Ethiopia (*Eucalyptus globulus* and *Cupressus lusitanica*) were pretreated with WRF (*Pholiota squarrose*, *Ganoderma aplanatum* and *Polyporus giganteus*) and hydrolyzed with enzymes of the hydrolytic wood rot fungi (*Phellinus tremulae*, *Pholiota adipose*, *Armilleria mellea*). The effect of each WRF was studied by analyzing the change in compositions of the lignin and structural components and effects of hydrolytic wood rot fungal efficiency by analyzing the digestibility of the pretreated sample in the production of fermentable sugar molecules.

MATERIALS AND METHODS

Composition Analysis: Fresh sawdust samples of *Eucalyptus globulus* and *Cupressus lusitanica* were obtained from the sawmills of Dagaga site, Arsi branch of Oromia forest and wildlife enterprise (OFWE), Ethiopia. The sawdust samples were dried, ground and passed through 5 mm pore size sieve and used for compositional analyses. Total solid, ash and extractive contents of the raw sawdust samples were determined according to NREL [25]. The total amount of solids remaining after heating the sample at 105°C to constant weight was obtained. Klason lignin contents of raw and pretreated sawdust samples were determined following the procedures of ASTM [26]. Cellulose contents of the raw and pretreated sawdust samples were determined according to the procedures of Kurschner and Hoffer method [27]. Finally, hemicellulose contents of the raw and pretreated sawdust samples were determined by weight difference using the following formula.

% Hemicellulose = 100 – (% Lignin + Cellulose + % Ash + %Extractives

Fungal Pretreatment of Sawdust

Selection of Ligninolytic Fungal Isolates: Three WRF (*Pholiota squarrose* 003-2G, *Ganoderma aplanatum* 006-2G and *Polyporus giganteus* 005-1G) were used the pretreatment activity. Selection of the fungal isolates was done based on their ligninolytic enzyme assays [28, 29].

Inoculum Preparation: Inoculum for each of the selected WRF was prepared using the standard medium containing 10.0 g glucose, 3.0 g yeast extract, 3.0 g peptone, 1.0 g KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$ per liter of distilled water [30]. The pH of the medium was adjusted to 6.0 with 2M NaOH. Four disks (Ø 5 mm) of each isolate were inoculated and grown on a rotary shaker at 150 rpm and at room temperature in 250 ml flasks containing 100 ml of the medium. After six days of fungal cultivation, mycelial pellets were homogenized and used as inoculum.

Sawdust Pretreatment: The sawdust samples were dried, ground and passed through 5 mm pore size sieve. 20 g of such sawdust from each species was placed in 100 ml Erlenmeyer flasks in triplicates and conditioned with distilled water to obtain a moisture content of 75%. The flasks were autoclaved at 121°C for 15 minutes, cooled and inoculated with a 10 ml inoculum on the top of the substrate in each flask. The inoculated flasks were incubated at 30°C in static conditions for 30, 45 and 60 days. For each treatment, a triplicate of flasks with sawdust but without fungal inoculum was similarly incubated as a control. At the end each predetermined incubation day, sample was washed with distilled water (30 ml) at 180 rpm for an hour and filtered under vacuum to remove the water-soluble components. The solid fraction of each sample was dried in an oven at 65°C and its lignin and structural polysaccharide (cellulose and hemicellulose) contents were determined [26, 27].

Enzymatic Hydrolysis of the Pretreated Sawdust

Selection of Hydrolytic Fungal Isolates: Three hydrolytic wood rot fungi (*Phellinus tremulae* 030-1D, *Pholiota adipose* 026-2D, *Armilleria mellea* 033-1G) were used for the hydrolysis experiments. These fungal isolates were

selected based on their hydrolytic enzyme assay reported by Megersa and Gure [31]. An inoculum of each of the three hydrolytic fungi was prepared using the standard media [30].

Enzyme Production: Hydrolytic enzymes of the selected fungal isolates were produced according to Hussain *et al*. [32]. Erlenmeyer flasks were inoculated with 5 ml of the fungal inoculum and incubated at 30°C for 12 days and then 50 ml of 0.05 M citrate buffer (pH 5.0) was added to each flask and left for an hour shaking on a rotary shaker at 150 rpm. The samples were then filtered through clean muslin cloth and the filtrates were centrifuged at 4000 rpm for 15 min. The supernatants were taken as crude enzyme extracts and stored at 4°C for use during hydrolysis.

Hydrolysis of the Pretreated Sawdust: Twenty grams of the WRF-pretreated sawdust was added to each 100 ml flasks and then autoclaved at 121°C for 15 minutes and then loaded with crude enzyme extracts at 5 % (v/w). A citrate buffer solution of 0.05 M was added to the flasks to achieve and maintain a pH of 5 [33]. Following the addition of the enzymes, the flasks were sealed and placed at 40°C, 150 rpm. The flasks were sampled after 24, 48 and 72 hours of incubation and immediately submerged in a water bath at 100°C for 5 minutes, followed by an ice bath. Finally, the samples were centrifuged at 4000 rpm for 15 minutes and the supernatants were maintained for reducing sugar determination.

Total reducing sugar was determined by the 3, 5-dinitrosalicyclic acid (DNS) method [34]. Absorbance of the samples was measured at 540 nm using spectrophotometer and absorbance readings were then converted into equivalent sugar concentration (g/l) using glucose standard curve.

Statistical Analysis: The effect of WRF pretreatment on lignin, cellulose and hemicellulose losses and the effect of the hydrolytic enzymes on the hydrolyses of the pretreated sawdust samples were evaluated using SPSS software for analysis of variance and significance tests at 95% confidence level. Tukey's simultaneous test was performed to assess statistical differences between treatment means of the triplicate measurements.

RESULTS AND DISCUSSION

Composition Analysis: Composition analysis result of the raw sawdust samples is presented in Table 1. Lignin and structural polysaccharide contents of *E. globulus* reported in this study are similar to reports made by different authors [35-37]. On the other hand, Pereira *et al*. [38], by conducting compositional analysis of six clones of *Eucalyptus* in Brazil and reported lignin (28.8-31.4 %), cellulose (46.1-48.8 %), hemicellulose (21.9-22.5 %), extractive (3.1-5.0 %) and ash (0.10-0.18 %) compositions. Findings in this work had lower lignin, cellulose and hemicellulose compositions, similar ash contents but significantly higher extractive contents.

On the other hand, Santos *et al.* [39] reported 34.13 % of Klason lignin composition for *C. lusitanica* which is, of course, slightly higher than contents reported by this paper. Similarly, Almeida *et al*. [40] reported higher lignin (36.21%) and holocellulose (59.19%) contents, lower ash (0.51 %) and similar extractive (4.08 %) contents for *C. lusitanica*.

Pretreatment Effects: The effects of WRF pretreatments on the *E. globulus* and *C. lusitanica* sawdust samples were determined and presented (Fig. 1, Fig. 2). Results showed increased losses of lignin, cellulose and hemicellulose amounts of by two sawdust types with the increasing incubation days. At 30, 45 and 60 days of incubation, significant amounts ($p<0.05$) of lignin and polysaccharides were degraded under the three fungal pretreatments.

The highest lignin loss (13.47 %) by *E. globulus* sawdust was observed due to degradation by isolate *G. aplanatum* 006-2G on day 60 (Fig. 1). This was followed by the lignin loss due to *P. squarrosa* 003-2G (12.33 %) and *P. giganteus* 005-1G (10.93 %) on the same incubation day. Martín-Sampedro *et al*. [41] observed the highest degradation of *E. globulus* on 48 day of incubation which lower than reported here. The highest amount of cellulose was degraded by isolate *P. giganteus* 005-1G on day 60 (9.25 % loss) and the next two highest cellulose losses were recorded on 45 and 60 days by isolate *P. giganteus* 005-1G and *G. aplanatum* 006-2G, respectively.

Degradation of *C. lusitanica* sawdust by the three WRF showed similar effect pattern but the three fungi had no significant difference in amount of lignin lost on 60 days of incubation (Fig. 2). Wood rot fungi of this paper were found be more efficient delignifiers than *C. subvermispora* had on Japanese beech wood [42] within 56 incubation days. Kang *et al*. [43] exposed wood blocks to the WRF *P. chrysosporium*, *C. subvermispora* and *T. versicolor* for 60 days and reported holocellulose loss of 7.9, 10 and 12.1 %, respectively, for pine and 12.1, 12.9 and 14.8 %, respectively, for popular which is found

Table 1: Chemical composition of raw sawdust samples

Parameter	Composition (%)[a]	
	E. globulus	*C. lusitanica*
Moisture content	9.80±1.58	11.8±2.34
Dry matter content	88.21±1.02	87.65±1.09
Klason lignin	23.53±0.72	27.68±1.35
Cellulose	40.47±1.92	42.53±1.11
Hemicellulose	26.82±1.74	25.27±2.01
Extractives	1.23±0.23	4.04±1.08
Ash	1.50±0.31	1.00±0.22

[a] Average values of three measurements

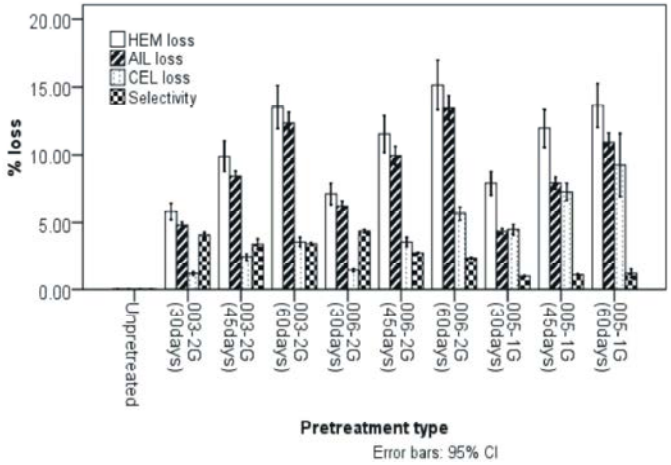

Fig. 1: Composition losses of *E. globulus* sawdust while pretreating with WRF

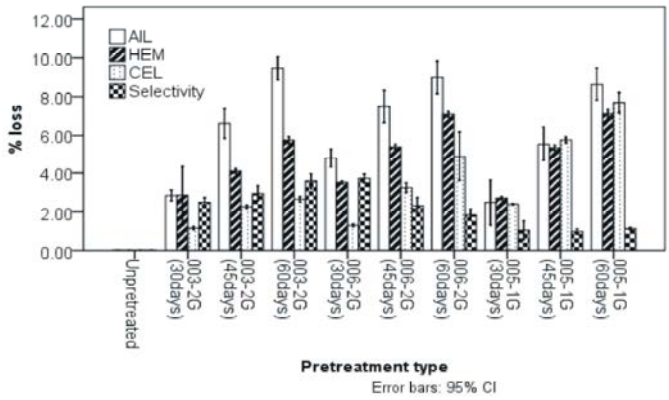

Fig. 2: Composition losses of *C. lusitanica* sawdust while pretreating with WRF

to be similar to the activity of fungal isolates reported in this paper. It has been reported that reduction in lignin content of a biomass facilitates access by hydrolytic enzymes and increases sugar yields [44].

Higher selectivity value (lignin lost/cellulose lost) is an indication of better delignification which better preserves cellulose for hydrolysis [11]. The selectivity values of all WRF isolates on *E. globulus* were decreased with the increasing incubation days (Fig. 1). The same was true for *C. lusitanica* except in isolate *P. squarosa* 003-2G (Fig. 2). Similarly, Nazarpour *et al*. [11] reported decreased selectivity values for increased incubation days for the white rot fungus *C. subvermispora*. Isolate *P. squarosa* 003-2G displayed the highest selectivity values and isolate *P. giganteus* 005-1G displayed the least values for *E. globulus* sawdust degradation. But for *C. lusitanica* sawdust, *G. aplanatum* 006-2G showed the highest and *P. giganteus* 005-1G the

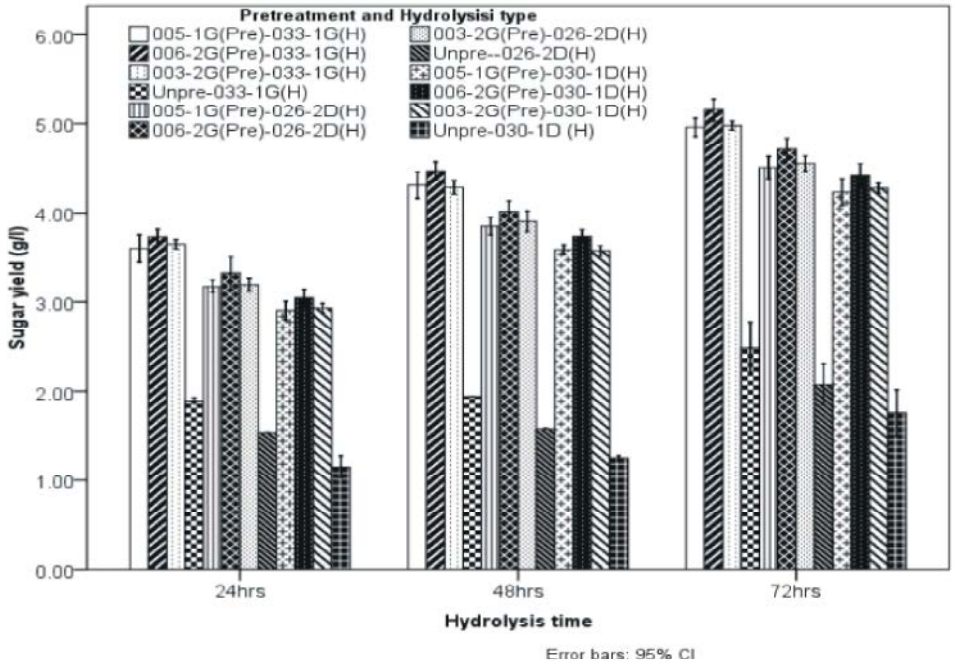

Fig. 3: Sugar yield from enzymatic hydrolysis of WRF pretreated *E. globulus* sawdust

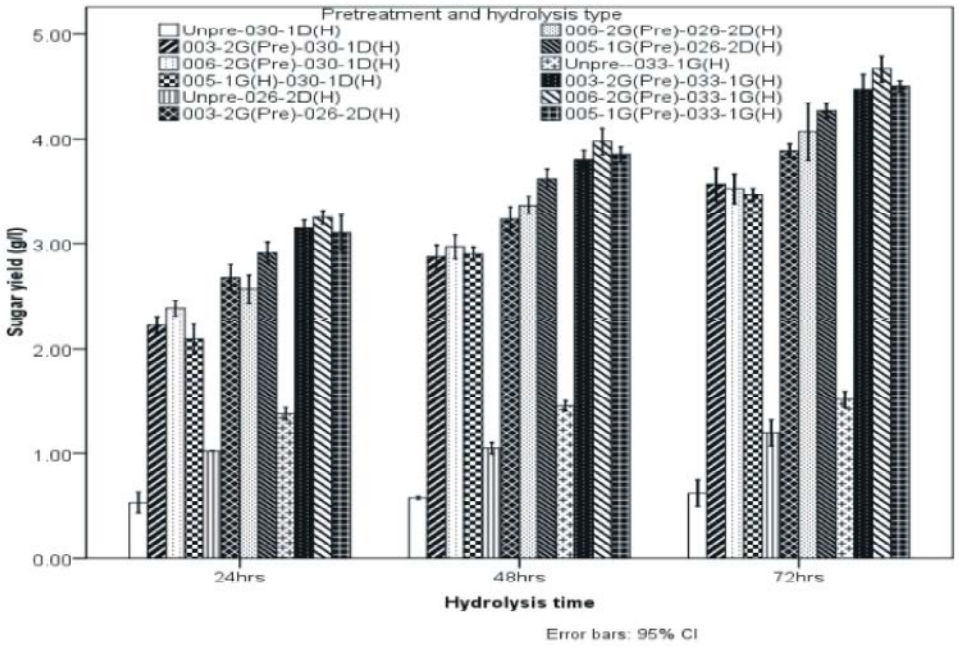

Fig. 4: Sugar yield from enzymatic hydrolysis of WRF pretreated *C. lusitanica* sawdust

least selectivity values. This shows that effect of WRF on delignification and degradation of the carbohydrate depend also on the biomass species. When the early and late incubation days compared, it was found that selectivity values of all isolates decreased with increasing incubation days indicating higher utilization of cellulose with increasing incubation days.

Enzymatic Hydrolysis Effects: Conversion yield of hydrolysis reaction reveals the accessibility of pre-treated lignocelluloses to an enzyme system [45]. Sugar yields of the WRF pretreated sawdust in all cases was significantly higher than the yield obtained from the unpretreated sawdust (Fig. 3, Fig. 4). This indicates that WRF pretreatment efficiently reduced the recalcitrance effects

of the sawdust samples. With increasing hydrolysis period, sugar yields were also increased. The best sugar yield (5.15 g/l) was obtained from the *G. aplanatum* 006-2G pretreated and *A. mellea* 033-1G hydrolyzed *E. globulus* sawdust (Fig. 3). An increase in sugar yield with an increase of hydrolysis duration expressed as a result of breaking of lignin-carbohydrate complex linkage which increase access to the carbohydrate structure and absence of the hydrolyzed sugar molecules conversion to hydroxymethyl furfural or furfural from the hexose and pentose sugar molecules, respectively [7]. In sawdust of *C. lusitanica*, a similar pattern of sugar yields was obtained but the yields were significantly lower than that was obtained from *E. globulus* [Fig. 4]. Both efficiency of the hydrolytic enzymes and total sugar lost during the pretreatment affect the final sugar yield of a substrate [46]. Zhang *et al*. [47] pretreating poplar wood with WRF *T. versicolor* C6915 for eight weeks found an enzymatic hydrolysis 1.8 g/l which was 2.7-fold of the untreated sample. Waghmare *et al*. [48] pretreated and hydrolyzed lignocellulosic substrate with *P. chrysosporium* enzyme extract and reported the maximum reducing sugar yield of 0.3 g/l during the incubation period up to 48 hrs.

CONCLUSION

The present study aimed at obtaining higher sugar yields from sawdust by pretreating with ligninolytic WRF and by hydrolyzing with enzymes from hydrolytic WRF. Ligninolytic WRF pretreatment of sawdust samples from *E. globulus* and *C. lusitanica* significantly improved the hydrolysis of the sawdust samples and higher sugar yields were obtained compared to the control. Results suggest that the enzymatic hydrolysis of the sawdust can be enhanced by biological pretreatment with the wood rot fungi. The fungal pretreatment exhibited higher lignin and hemcicellulose losses over the control. The highest lignin loss of 13.47 % was obtained when pretreated with *Ganoderma aplanatum* for 60 days of incubation. The results also indicated the preferential nature of this white rot fungi showing higher selectivity which shows selective degradation of lignin by the fungi. Similarly, the higher lignin loss was the better sugar yields of sawdust samples. An all cases, fungal pretreatment for 60 days and enzymatic hydrolysis for 72 hours resulted in the highest sugar yields. The sawdust waste accumulated around the Ethiopian sawmills could potentially be converted into sugars that could be further used for different industrial applications. Hence, the biological conversion of sawdust could contribute to serving as a sustainable solid waste management strategy.

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