STEAM PRETREATMENT OF PINE (*Pinus patula*) WOOD RESIDUE FOR THE PRODUCTION OF REDUCING SUGARS

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The study explores the production of reducing sugars from *Pinus patula* wood residual chips based on steam pretreatment technology. The severity of the steam pretreatment was altered by using two levels of temperature and time and three levels of sulphur dioxide impregnation. The results show that the efficiency of enzymatic hydrolysis depends on the severity of steam pretreatment. On a given cellulose content, the reducing sugar yield increased from 29% (15.6 g/L) under the mildest steam pretreatment conditions (180 °C, 1.5% SO_2) to 91% (42.3 g/L) under the most severe steam pretreatment conditions (225 °C, 3% SO_2). In all cases, the enzymatic hydrolysis yield is dependent on enzyme accessibility to the cellulose chains, as the steam pretreatment severity strongly affects biomass fragmentation.

Keywords: steam pretreatment, *Pinus patula*, enzymatic hydrolysis, glucose yield

INTRODUCTION

Due to its abundance,[1] woody waste is an attractive feedstock for the production of reducing sugars. In Tanzania, *Pinus patula*, a soft wood grown mainly for timber production, produces substantial amounts of residues with high potential for reducing sugars and subsequent bioenergy production. Sao Hill Industries Ltd, situated in south-eastern Tanzania, in Iringa region, has about 18000 hectares of *P. patula* that produce about 70 m^3 of timber per day, equivalent to 25550 m^3/year. About 55 tons/year of sawdust are generated after timber processing.[2] Out of the generated sawdust, approximately 5% is reported to be used for making charcoal briquettes in cement industry, while the remaining part has no specific use, so that it is often dumped, thus contributing to environmental pollution.[3]

The existence of lignin in wood as a feedstock makes the separation of cellulose and hemicellulose equally difficult and challenging, due to their intricate structures.

Therefore, it requires pretreatment for increasing the efficiency of hydrolysis through reduction of the lignin content and cellulose crystallinity, due to the increase in surface area or to the pore size of the materials.[4,5] Steam explosion is an attractive pretreatment process due to its low use of energy and chemicals.[6] Studies have shown that factors, such as moisture content, temperature, chip size and residence time, have a significant influence on enzymatic hydrolysis, fermentation and downstream processing.[7-10] Acid impregnation in steam pretreated lignocellulosic biomass has been shown to improve the enzymatic hydrolysis reactions and to decrease the production of inhibitors.[7,11] Previous studies[12,13] have revealed that SO_2-impregnated steam explosion is considered to be the most effective pretreatment technique for woods, as due to the rigid structure and high amount of lignin. The amount of acid concentration, the temperature and residence time applied in

steam explosion processes vary with the type of feedstock. The quite large number of studies performed in the field of steam explosion revealed that obtaining of high enzymatic hydrolysis yields for steam pretreated biomass, especially for softwoods, is a challenging undertaking. Moreover, to the best of our knowledge, no study has been done to explore the steam pretreatment of *Pinus patula*. Consequently, the present work investigates the pretreatment of *Pinus patula* by the steam explosion pretreatment method for the production of reducing sugars.

MATERIALS AND METHOD

Raw material

The feedstock used in the pretreatment experiment consisted in residual wood chips from *Pinus patula*. The wood residues were chipped into particles with sizes between 2-15 mm, then dried. The moisture content of the sample was determined prior to carrying out each experiment. The chemical compositions (total carbohydrates, lignin and extractives) of both pretreated and non-treated wood chips were determined according to standard methods.[14]

Steam pretreatment

Prior to the steam pretreatment, the wood chips were impregnated with gaseous sulphur dioxide (SO_2), to improve the accessibility of cellulose and to increase the high reducing sugar yield.[7,10] A maximum of 20 shots, each containing 500 g of wood chips, was carried out.

Steam pretreatment experimental design

The effect of the 3 main steam explosion parameters (temperature, acid concentration and residence time) was examined by varying their levels, as shown in Table 1. Acidity was altered by varying the sulphur dioxide charge and two pressure levels were applied: a low-pressure level (10 bars, 180 ºC and 10 min), representing the mild steam pretreatment conditions, and a high-steam pressure level (25 bars, 225 ºC and 5 min), representing the extensive steam pretreatment conditions. The severity of pretreatment was designed according to a severity factor, termed Ro, presented by the equation:

$$R_0 = \int_{t_0}^{t} tx\left[\frac{(T-100)}{14.75}\right]dt = tx\exp\left[\frac{(T-100)}{14.75}\right] \quad (1)$$

where t (min) is the residence time at the reaction temperature T (ºC).[15]

After steam explosion, the samples were weighed and filtered. The filtered steam pretreated materials were washed thoroughly by water, to remove the inhibitory materials and also the water-soluble hemicellulose.[16] The washed wet solid was stored at 4°C for subsequent enzymatic hydrolysis. Liquid samples from the treatment and washing processes were also stored for sugar analysis.

Table 1
Steam pretreatment conditions applied

Steam pretreatment (pret.)	Chips in a shot (g)	Number of shots (#)	Exploded material (Kg)	SO_2 charged (% on water)	Steam pretreatment conditions			
					Temp. (ºC)	Pressure (Bar)	Time (min)	Log (Ro)
Mild steam pret.- Low SO_2	500	4	2	0.5%	180	10	10	3.4
Mild steam pret.-Medium SO_2	500	2	1	1.5%	180	10	10	3.4
Mild steam pret.-High SO_2	500	4	2	3%	180	10	10	3.4
Extensive steam pret.- Low SO_2	500	4	2	0.5%	225	25	5	4.4
Extensive steam pret.- Med. SO_2	500	2	1	1.5%	225	25	5	4.4
Extensive steam pret.-High SO_2	500	4	2	3%	225	25	5	4.4

Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated wood was done following the Laboratory analytical procedure by NREL.[17] The washed water-insoluble residue of pretreated *Pinus patula* wood with a sample concentration of 10% w/v dry matter was enzymatically hydrolysed by 0.275 mL of Cellulaclast 1.5 L and 0.14 mL of Novozyme 188 per gram of dry sample weight. The enzymatic activity was of 74 filter paper units (FPU/mL) for Cellulase and of 226 pNPGU (p-nitrophenyl-β-D-galactopyranoside), respectively, for Novozyme 188. The solutions were supplied with 5 mL of citrate buffer (pH 4.8) per gram of dry sample weight, loaded into a 500 mL Erlenmeyer flask. Cellulaclast 1.5 L, a cellulose enzyme from *Trichorderma reesei* and Novozyme 188, a Beta-glucosidase from *Aspergillus niger*, were supplied by Novozyme, Denmark. The experiment was carried out at 50 ºC, for 72 h, on a shaking incubator at 200 rpm (Thermoshake-Gerhardit Laboshake), after which the samples were filtered with Whatman filter paper (Q 110 mm) and their pH adjusted to 5.5, then sterilized at 80 °C for 10 min in the water bath, to stop the reaction. To determine the concentration of glucose, 1 mL of solution was sampled periodically and centrifuged at 14000 rpm for glucose analysis. Control samples of substrate (containing buffer, substrate and water) and enzyme (containing enzymes, water and

buffer) were used parallel to the hydrolysis reaction.

Analysis

Chemical composition of pretreated and non-treated wood

The chemical composition of both pretreated and non-treated wood chips was determined according to the standard methods, which include SCAN CM 49:03,[18] Tappi T249cm-00,[19] Tappi T222om-88,[20] and the Phenol sulphuric acid method.[14] Wood extractives were analyzed as acetone extractable compounds (SCAN CM 49:03). By acetone extraction, lipophilic wood components, such as fatty acids, resins, fatty alcohols, sterols and glycerides were extracted. In addition, low molecular phenolic compounds, like lagans, were also extracted. The total carbohydrate composition was analyzed by acid hydrolysis and gas chromatography (Tappi T249cm-00), while polysaccharide composition was estimated from the monosaccharide composition, based on typical glucomannan composition for softwoods, as reported by Sjostrom.[21] The acid-insoluble lignins were analyzed as Klason lignin (Tappi T222om-88), whereas acid-soluble lignin was analyzed by UV spectrometry, at 205 nm. Dissolved wood carbohydrates were analyzed by the phenol sulphuric acid method.

Sugar

The sugars were analyzed on a HPLC equipped with a Biorad HPX-87H Aminex column, RI-detector RID6A and UV detector UV SPD6A; pump (LC9A), oven (CTI6A) and autoinjector (Shimadzu SIL-9A). Prior to injection, the supernatant was filtered through a 0.2 μm filter, an amount of 20 μL being injected into the HPLC column. The column was equilibrated with a mobile phase of 5 mM H_2SO_4 and elution was performed at a flow rate of 0.6 mL/min at a temperature of 45 ºC. Standard samples of known sugar concentration (glucose, xylose, galactose, mannose and arabnose) were prepared and analysed, to obtain the average peak area and retention time. The unknown concentration of sugars in the analysed samples was determined on the basis of the concentration factor, obtained by dividing the known concentration of the standards with their average peak areas, shown in the chromatograms.

RESULTS

Pretreatment

Biomass composition before and after steam explosion

The results on steam pretreated and non-treated biomass are shown in Table 2. Significant amounts of hemicelluloses were degraded from 23.2 to 5.7% in the mild steam pretreatment, and dissolved or degraded to 0.3% after extensive steam pretreatment (Fig. 1). In acid-catalysed steam explosion, the rate of hemicellulose removal highly increased with temperature, where complete hemicellulose removal was obtained at a temperature of 225 ºC (Tables 1 and 2). This was due to the fact that, in the presence of acid, the hemicellulose hydrolysed to monomers (xylose, mannose, arabinose, galactose and glucose), while, on increasing temperature, xylose is further converted by acids to furfural and other hexoses, and sugar to hydroxymethylfurfural, which is further degraded to levulinic acid. Previous studies[6,21-23] found out that sugar degradation products, such as furfural and 5-hydroxymethylfurfural (HMF), levulinic acid and formic acid, are formed by severe acid catalyzing steam pretreatment. The amount of cellulose significantly increased – from 37.1 to 48.3% – for mild steam pretreated wood biomass. The increase of cellulose was attributed to the effect of the pretreatment, which modifies the cellulose microfibre structure and removes the cellulose surrounded matrix (hemicellulose) and lignin, so that cellulose can be easily determined. The remaining portion of wood was represented by non-carbohydrate lignin and extractives, whereby the amount of lignin increased from 28.8 to 37.1%, and that of extractives from 2 to 17.9%, under most extreme pretreatment conditions. The higher content of lignin and extractives was caused by the formation of non-lignin structures (pseudolignin) from the hemicellulose hydrolysate and by the lignin condensate reaction, while some was extracted by acetone and some was detected as acid-insoluble lignin.

Effects of steam pretreatment on lipophilic extractable components

Acetone was employed to extract wood extractives like resin acids, fats, terpenes and a variety of phenolic compounds – such as flavonoids, lignans and stilbenes. The results in Table 3 indicate that the relative amounts of acetone extractable components are significantly higher in steam pretreated wood samples than in the untreated wood. For extensive steam pretreated wood samples, 16-17% of them are extractable with lignin.

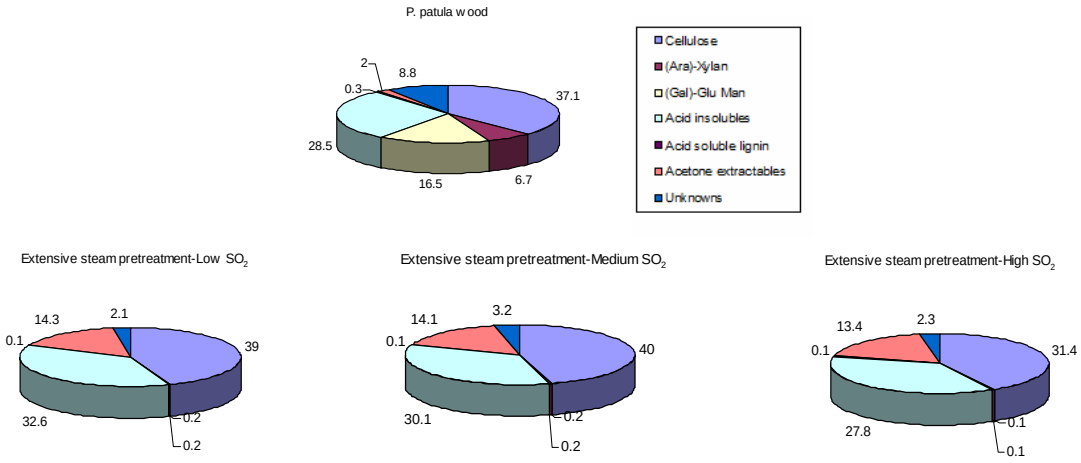

Figure 1: Yield balance of wood component after extensive acid catalysed steam pretreatment of *Pinus patula* wood

Table 2
Chemical composition of acid catalysed, mild and extensive steam pretreatment *Pinus patula* wood samples

Biomass	Wood component, wt%						
	Cellulose	(Ara)-Xylan	(Gal)-Glucomannan	Acid-insoluble (Klason Lignin)	Acid-soluble Lignin	Extractives	Unknown
Pinus patula wood	37.1	6.7	16.5	28.5	0.3	2.0	8.8
Extensive steam pret.-High SO_2	41.7	0.1	0.2	37.0	0.1	17.9	3.0
Mild steam pret.-High SO_2	46.6	1.8	3.9	34.9	0.2	7.4	5.3
Extensive steam pret.-Medium SO_2	45.5	0.3	0.2	34.2	0.1	16.0	3.6
Mild steam pret.-Medium SO_2	48.3	2.2	3.8	36.3	0.2	4.4	4.8
Extensive steam pret.-Low SO_2	44.0	0.3	0.2	36.9	0.1	16.2	2.4
Mild steam pret.-Low SO_2	46.4	2.1	4.1	35.2	0.1	7.0	5.1

Table 3
Lipophilic extractable compounds in steam pretreated *Pinus patula* wood

Wood compounds	*Pinus patula* wood	Mild steam pretreatment, Low SO_2	Mild steam pretreatment, Medium SO_2	Mild steam pretreatment, High SO_2	Extensive steam pretreatment, Low SO_2	Extensive steam pretreatment, Medium SO_2	Extensive steam pretreatment, High SO_2
Lipophilic acetone extractable components, wt%	2.0	7.0	4.4	7.4	16.2	16.0	17.9

A study[24] conducted at PFI evidenced that non-lignin structures (pseudolignin) are formed during steam pretreatment, by lignin condensation reactions with carbohydrates. This has been reported to contribute to the amount of acid-insoluble lignin in the pretreatment materials.[10] NMR spectroscopy indicated that condensed structures were formed between lignin and furfural and/or hydroxylmethylfurfural. In addition, the

results imply that a significant amount of the condensed pseudolignin structures were dissolved under acidic conditions, which means that they will not be analysed as "Klason lignin".[15] Most likely, the significant increase in acetone extractable compounds is due to the formation of such pseudo-lignin condensation products.

Yield balance

The yield balance values were only reported for severe steam pretreatment samples, since, owing to their homogeneity, it was difficult to measure accurately the yield of solids in mild pretreated samples. The yield balance of the various wood components is shown in Figure 1 and Table 4. The yield balance reveals that all cellulose is retained after severe acid catalysed steam pretreatment of *Pinus patula*, when a moderate sulphur dioxide charge (0.5-1.5%) is applied, indicating that no cellulose hydrolysis occurred during the pretreatment. However, under more acidic conditions, some of the cellulose was hydrolysed by acids. In addition, the results obtained revealed that no glucomannan and xylan remained after severe steam pretreatment. Some of the hemicelluloses have been hydrolysed and dissolved, but the yield balance shows that a significant amount of the hemicellulose degradation products remained as solids. Hence, as a result of the steam pretreatment, carbohydrate degradation products must have formed condensed structures, which do not dissolve in water. This is in agreement with previous findings, as it has been shown that degradation products, such as furfural and/or hydroxymethylfurfural, form condensed structures with lignin during steam pretreatment.[23]

The yield balance also reveals that a significant amount of the pseudo-lignin and condensed structures formed is extractable by organic solvents. This could be an advantage, as the presence of such compounds may inhibit the subsequent enzymatic hydrolysis and fermentation. Lignin removal may also increase the enzymatic hydrolysis yield by improving cellulose accessibility by enzymes.

Table 4
Yield balance of wood component after extensive acid catalysed steam pretreatment of *Pinus patula* wood

Marking	Composition, % on wood							
	Yield of solids, %	Cellulose	(Ara)-Xylan	(Gal)-Glu Man	Acid-insolubles	Acid-soluble lignin	Acetone extractables	Unknown
P. patula wood	100	37.1	6.7	16.5	28.5	0.3	2.0	8.8
Extensive steam pret.-Low SO_2	89	39.0	0.2	0.2	32.6	0.1	14.3	2.1
Extensive steam pret.-Med. SO_2	88	40.0	0.2	0.2	30.1	0.1	14.1	3.2
Extensive steam pret.-High SO_2	75	31.4	0.1	0.1	27.8	0.1	13.4	2.3

Enzymatic hydrolysis

Enzymatic hydrolysis was only performed on the solid fraction of the steam pretreated wood biomass, which contains lignin and cellulose. Enzymatic hydrolysis was not performed on the liquid fraction, which contains sugars derived from hemicellulose hydrolysis. Unlike glucose, the analysis indicates the presence of xylose, galactose, mannose and arabnose in low concentration (less than 1 g per L, or non-detectable). Glucose concentration was determined over different time intervals, during a 72 h period of enzymatic action, the results revealing an increase in glucose concentration with hydrolysis time (Fig. 2). Hydrolysis yield increases exponentially at the beginning of the experiment, becoming stable after 20 h. This implies that, when exposed to the substrate, the enzymes (celluloclast 1.5 L and Novozyme 188) were very active, and that the cellulose chain was available on the surface to be bound by the enzymes, hence the hydrolysis reaction takes place early, once the binding rate reaches equilibrium.[27] The overall highest hydrolysability was obtained from severe acid catalyzing steam pretreatment, when the substrate released 42.3 g/L glucose after 72 h. Even when the same enzymatic hydrolysis parameters were applied under the mildest and under severe acid catalysed steam pretreatment (3% SO_2), the substrate from severe steam pretreatment conditions yielded

a significantly higher glucose concentration (42.3 g/L) than under the mildest conditions (17.4 g/L). Thus, the difference in yield could be attributed to the different temperature and residence time values applied during pretreatment.

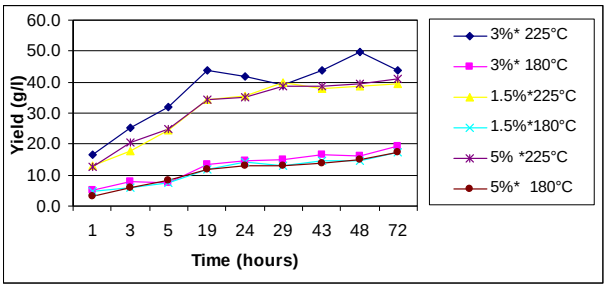

Figure 2: Glucose yield from hydrolysis of *P. patula* wood waste (* sulphur dioxide)

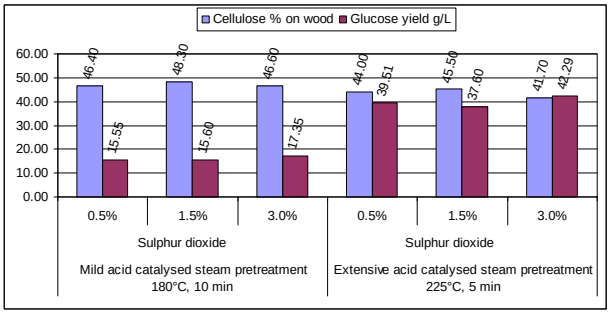

Figure 3: Glucose yield under different pretreatment conditions

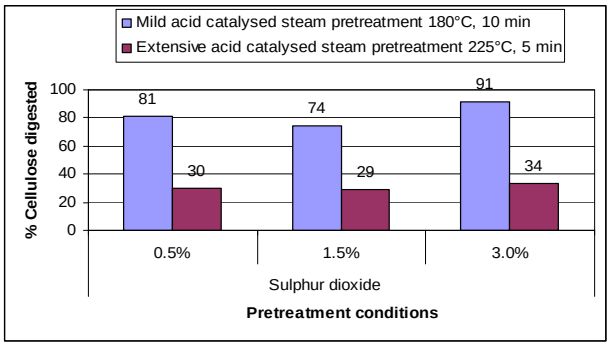

Figure 4: Percentage of cellulose digested in enzymatic hydrolysis

Effect of steam explosion on enzymatic hydrolysis of biomass

The enzymatic hydrolysis experiment indicates different yields for different pretreatment conditions, thus suggesting that the yields are strongly dependent on the pretreatment conditions.[25] As shown in Figure 4, the reducing sugar yield increased from 29% under the mildest steam pretreatment conditions (180 °C, 1.5% SO_2) to 91% under the most severe steam pretreatment conditions (225 °C, 3% SO_2). The main purpose of the pretreatment was to break and/or remove lignin, to hydrolyse the hemicellulose and to turn crystalline cellulose into an amorphous form, thus increasing the cellulose enzymatic hydrolysis yield. This objective has been achieved[26] in the present study as the enzymatic yield exceeded 90%, unlike that of untreated lignocellulosic biomass – reported to be less than 20%. Although Figure 3 shows a high amount of cellulose under mild pretreatment conditions (46.4-48.3%), the hydrolysis yield was rather small compared to that of the severe pretreatment conditions (Fig. 3). The difference could be attributed to poor biomass fragmentation during steam

pretreatment, resulting in a slight decrease in the cellulose crystalline structure.

CONCLUSIONS

Steam explosion pretreatment facilitates the removal of lignin, the hydrolysis of hemicellulose and the increase in the surface area for cellulose hydrolysis. The recovery of hemicellulose sugars depends mostly on the severity of the steam explosion pretreatment. This study evidences significant hemicellulose removal and an increased amount of extractives, due to the formation of condensate between sugars and lignin. Moreover, the glucose yield is determined by enzyme accessibility to the cellulose chains, as the steam pretreatment severity strongly affects biomass fragmentation. This suggests, therefore, that crystallinity decreases, under most extreme steam explosion pretreatment conditions, as a result of the high glucose yield. The amount of glucose obtained (42.3 g/L) due to the higher percent (91%) of cellulose digestion can yield a maximum of 21.57 g/L of theoretical ethanol yield.

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