

Enhancing Enzymatic Digestibility of Alkaline Pretreated Banana Pseudostem for Sugar Production

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This study compares the efficacy of a soaking pretreatment with an alkaline solution for banana pseudostem prior to enzymatic hydrolysis. Banana pseudostem was pretreated by soaking in sodium hydroxide solutions at various concentrations and durations. The pretreatment more than doubled delignification but retained 82.09% of the holocellulose content and 73.74% of the cellulose content. The enzymatic (*Trichoderma reesei*) digestibility of pretreated banana pseudostem was found to have been enhanced by 44.41% as compared to initial biomass. This was evidenced by higher enzymatic activities (endoglucanase, exoglucanase, and β -glucosidase) on the treated sample. Meanwhile, glucose yield showed a proportional relationship with incubation time and enzyme loading throughout the hydrolysis process.

Keywords: *Banana pseudostem; Alkaline pretreatment; Enzymatic hydrolysis; Sugar yield; Trichoderma reesei*

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INTRODUCTION

Banana is a fast-growing crop that is the primary exported fruit and second most cultivated fruit in Malaysia. Banana cultivation covers almost ten percent, or 33,584 ha, of the total fruit area (297,860 ha), with a total production of 535,000 tonnes (Mohamad Roff *et al.* 2012). This produces a large amount of biomass residues (approximately 88% of the banana plant), and the residues turn out to be materials sources for other value-added products (textiles, pulps, chemicals, *etc.*).

The availability of fibres is thus enormous, and various processing methods have been discussed. However, not all the methods are suitable and available to produce high-yield bioethanol that is environmentally sound. Understanding the nature of fibres is crucial, as there are physical and chemical barriers that inhibit the accessibility of substrate for ethanol production. Pretreatment has been suggested to help remove these barriers, especially the lignin and acetyl groups that inhibit the accessibility of enzymes to holocellulose (Kim and Lee 2006). There are various pretreatment methods, such as chemical (sulphuric acid, sodium hydroxide, and hydrogen peroxide), biological (lignolytic fungi), and physiochemical (steam explosion and ammonia explosion). Among these methods, alkaline pretreatment is one of the most applied methods as it is simple and effective. In sodium hydroxide (NaOH) pretreatment, a peeling mechanism is responsible for the loss of hemicellulose sugar molecules. The peeling mechanism reduces

end groups, while hydrolytic cleavage of glycosidic bond occurs much less frequently (Gupta 2008). The peeling reaction unzips carbohydrates by removing the terminal sugar molecules one at a time at the reducing end (aldehyde). Saponification of intermolecular ester bonds that serve as cross links within xylan hemicelluloses and other components, such as lignin and other hemicellulose, also occurs. The drawback of the process includes the environmental friendly issue compared with biological pretreatment, long reaction times (in hours or days), and the fact that some of the solubilized moieties can act as inhibitors during hydrolysis (Balat *et al.* 2008; Qing *et al.* 2010).

The hydrolysis process results in simplification of carbohydrates into reduced sugars either by chemical or biological means. In particular, the hydrolysis by *Trichoderma* has been well established for fermentable sugar production. According to El-Zawawy *et al.* (2011), hydrolysis of lignocellulosic biomass by enzymes provides better yield without generating any side products. Enzymatic hydrolysis of cellulose consists of cellulase adsorption onto the surface of cellulose, which further causes biodegradation into fermentable sugar and desorption from cellulase (Taherzadeh and Karimi 2007). However, there are several factors that may influence hydrolysis yields. The enzyme efficiency actually depends on various factors such as pH, temperature, agitation, enzyme concentration, reaction time, and chemical species, which may influence the reaction rate (Kaya *et al.* 2000).

In this paper, the effect of NaOH pretreatment with various concentrations and soaking times of NaOH on banana pseudostem were investigated. Further processes were carried out on *Trichoderma reesei* cellulase hydrolysis to identify the effectiveness of pretreatment on sugar production with different incubation times and enzyme loading.

EXPERIMENTAL

Materials

Banana pseudostems were collected from the Bukit Changgang banana plantation in Selangor, Malaysia. At harvest, the mature plants were 10 to 12 months old. The pseudostem was cut to a size of 5 cm x 5 cm and oven-dried to a moisture content of 10 to 15%. After that, the sample was ground to the size of 7 to 30 mesh screen (595 to 2830 μm) for alkaline pretreatment. Reagents used in enzymatic assay included DNS (3,5-dinitrosalicylic acid), microcrystalline cellulose (Avicel®), carboxymethyl cellulose (CMC), and *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG).

Methods

Alkaline pretreatment

Various concentrations of NaOH (1, 2, 4, and 7% w/v) were mixed with solid ground particles at a ratio of 1:10 and soaked at room temperature for 4, 8, 12, and 24 h. Treated samples were rinsed with tap water until the pH value reached 7. Samples were then dried in an oven at 60 °C until constant weight and stored at room temperature. The chemical compositions were analysed according to standard methods: Klason lignin (TAPPI T222 om-98), cellulose (TAPPI T203); holocellulose was determined according to Wise *et al.* (1946).

Enzymatic hydrolysis of the pretreated banana pseudostem

Pretreated ground particles (1.0 g) were hydrolysed with a commercial cellulase enzyme (7, 15, 22.5, 30 FPU/g substrate) from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich, St Louis, MO) in 0.05 M citrate buffer (pH 4.8). The mixture was placed in a 50-mL Erlenmeyer flask plugged with cotton and incubated in a shaker agitated at 150 rpm, 50 °C for interval durations of 4, 24, and 48 h. For sampling purposes, 2 mL of the reaction mixture was removed from the hydrolysate. The solids were separated by centrifugation (3000 x g; 15 min) and placed in boiling water for 15 minutes at 4 °C. The supernatants obtained after biomass separations were stored at -4 °C until further analysis.

Glucose analysis

The sugar yield was determined by high performance liquid chromatography (HPLC) (Jasco 1580) with a refractive index detector (RI 1530) and a Merck Purospher STAR LC Columns LC-NH₂ column (4.6 x 250 mm). The mobile phase was 80% acetonitrile, with a flow rate of 1.5 mL/min. Fructose, glucose, sucrose, arabinose, mannose, xylose, and galactose were used as sugar standards. Glucose yield was determined by Eq. (1) according to Chen *et al.* (2007),

$$\text{Glucose yield (\%)} = \frac{\text{Glucose } \left(\frac{\text{g}}{\text{L}}\right) \times 100 \times 0.9}{\text{Cellulose content in the substrate (g)}} \quad (1)$$

where 0.90 (162/180) is the conversion factor due to the uptake of one water molecule during hydrolysis of cellulose to glucose (Mussatto *et al.* 2008).

Enzymatic activity

The activity of β -glucosidases towards *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) was measured by the amount of *p*-nitrophenol (*p*NP) liberated from *p*NPG using a calibration curve at 410 nm (Cai *et al.* 1998). The reaction mixture contained 0.5 mL, 5 M *p*NPG in 50 mM sodium acetate buffer (pH 4.8), and 0.5 mL enzyme solution. After a 15 min incubation at 50 °C, the reaction was stopped by the addition of 2 mL of 1 M Na₂CO₃, and the colour was formed as a result of *p*NP liberation. One unit of β -glucosidases activity was defined as the amount of enzyme required to liberate 1 μ mol of *p*NP per minute under the assay conditions.

Endoglucanase activity was assayed using 50 mM of citrate buffer (pH 4.8), 2% carboxymethyl cellulose (CMC), and 0.5 mL of culture supernatant according to Zhang *et al.* (2009), with some modifications. The mixture was maintained at 50 °C for 30 min, terminated by adding 0.75 mL of 3-5 dinitrosalicylic acid (DNS) reagent, boiled at 100 °C for 5 min, and the absorbance determined at 540 nm. One unit of endoglucanase was expressed by μ mmol of glucose liberated per minute per mL.

Exoglucanase was measured based on the amount of reducing sugars released in the reaction mixtures, which consisted of 0.1 M acetate buffer (pH 4.8), 1.25% microcrystalline cellulose, and 0.4 mL of dilute culture supernatant, and maintained at 50 °C for 30 min according to Zhang *et al.* (2009), with some modification. Meanwhile, 0.75 mL of DNS reagent was added and the mixture boiled at 100 °C for 30 min and the absorbance determined at 540 nm. One unit of endoglucanase was expressed by μ mmol of glucose liberated per minute per mL.

Data analysis

The data were analysed with SPSS statistic 22. Duncan multiple range tests and mean comparison analysis were with three replicate, at a confidence level of 95%.

RESULTS AND DISCUSSION

Chemical Composition Alternation

The biomass properties were altered in pretreatment. The pretreatment may help in removing or dissolving lignin and hemicellulose, and in reducing the crystallinity of cellulose for easy hydrolysis at a later stage. In a plant cell wall, lignin linked by carbon-carbon and ether bonds forms a tri-dimensional network associated with hemicelluloses polysaccharides (Ibrahim *et al.* 2005). The delignification reactions involve cleavage of non-phenolic β -O-4 linkages and phenolic a-O-4 linkages and release the lignin from association with polysaccharides (Misson *et al.* 2009). In general, lignin content decreased with both concentration and soaking time of NaOH to a limit where lignin degradation did not improve further even with prolonged soaking time and increased NaOH concentration. Figure 1a reveals the reduction in lignin content in banana pseudostem samples after the treatment. At 4% NaOH, greater delignification was achieved compared to other concentrations in its respective time. As for time, 12 h at 4 hour shows the highest delignification with a reduction of 60% compared to the untreated sample.

In Table 1, in NaOH hydrolysis, the alkali hydrolysed the end groups of polysaccharides and thus promoted degradation and decomposition. This is because it leads to delignification, disruption of structural linkages, decrystallization of cellulose, and depolymerization of the carbohydrates (Esposito *et al.* 1993; Mosier *et al.* 2005). Sodium hydroxide acts as a nucleophile during lignin degradation, which fragments and dissolves lignin. First, inter-unit linkages are simplified into smaller units, followed by introduction of hydrophilic groups into polymer and cleaving fragments, making the lignin more soluble (Misson *et al.* 2009).

In the holocellulose analysis, the NaOH treatment gradually increased the accessibility of the carbohydrate. Figure 1b shows that at a 4% NaOH treatment, banana pseudostem samples had the highest holocellulose content regardless of the treatment duration, and a 7% NaOH concentration reduced the amount of holocellulose content. Higher NaOH concentrations did not necessary help in getting a higher amount of holocellulose.

A higher concentration of NaOH is favorable to achieve the maximum removal of lignin but solubilization of the carbohydrates was also carried out, which caused a lesser yield of holocellulose (carbohydrates). Hemicellulose may be solubilized during the process and hence, appropriate concentration and soaking duration are important.

Table 1 shows no significant difference at 95% confidence level between the soaking time of NaOH solutions for holocellulose. Sodium hydroxide pretreatment largely affects hemicelluloses (xylan) solubilization. The mechanism of NaOH pretreatment is assumed to be by saponification of intermolecular ester bonds crosslinking xylan hemicellulose and other polymeric materials such as lignin or other hemicelluloses. Saponification of the uronic ester linkages in 4-O-methyl-D-glucuronic acid pendant along the xylan chain readily occurred in the presence of alkali (Sun and Cheng 2002). The hetero-structures of hemicellulose branched with short lateral chains (Hendriks and Zeeman 2009), and contributed to the higher hemicelluloses degradation at a higher NaOH concentration.

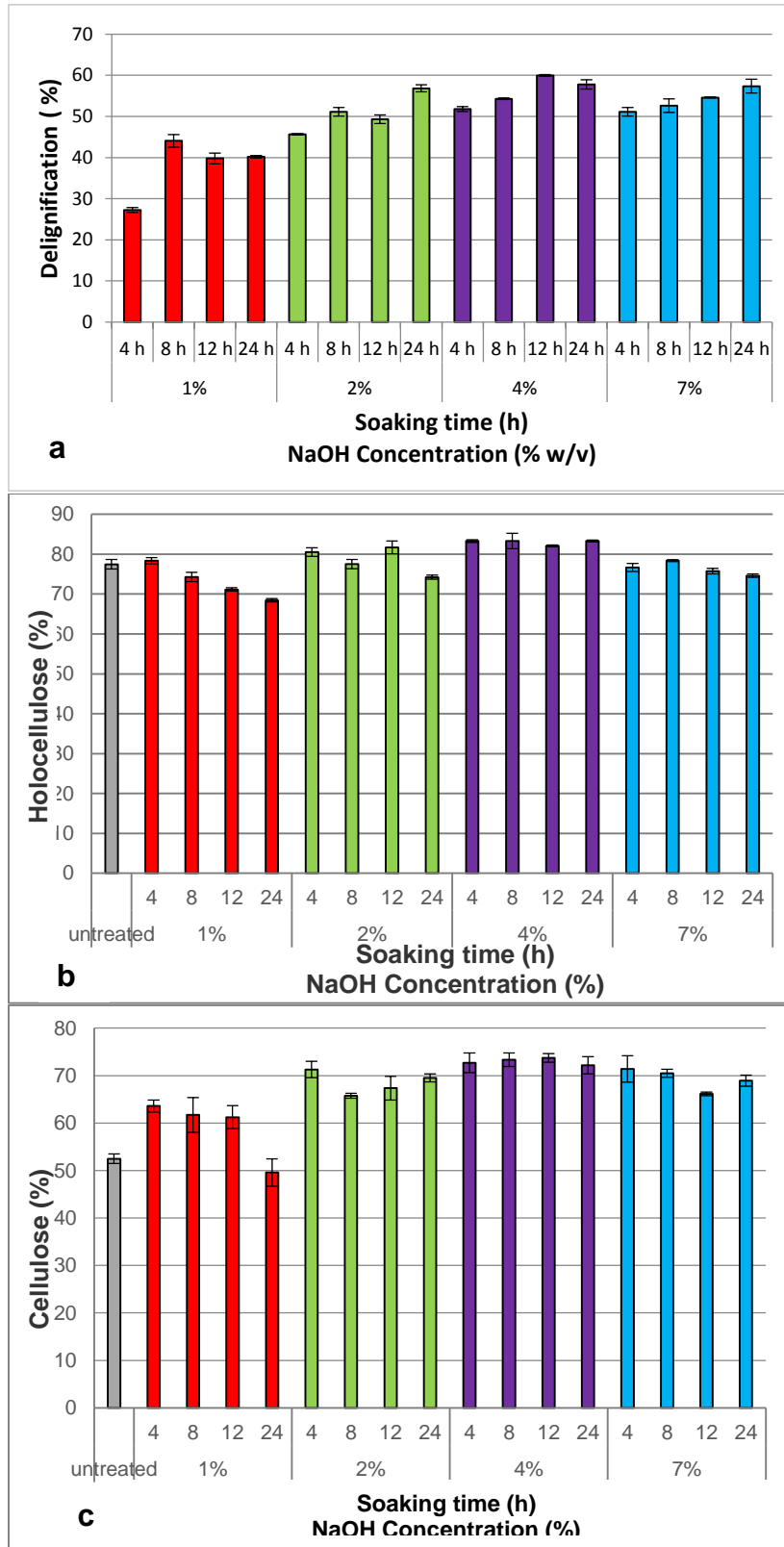


Fig. 1. Percentage of (a) delignification, (b) holocellulose, and (c) cellulose content of substrate after treatment with different NaOH concentrations and treatment durations

The NaOH treatment successfully opens up the accessibility of cellulose. In the work of Siqueira *et al.* (2013), NaClO₂ pretreatment of sugar bagasse was found to improve cellulose content with longer treatment time. Meanwhile, the study of Liong *et al.* (2012) showed that diluted alkali of 7% is most suitable for napier grass, and it can achieve a high level of holocellulose (88.46%), cellulose (82.21%), and low lignin (9.77%). The NaOH treatment on napier grass increased the cellulose content by more than double compared to the untreated sample (Liong *et al.* 2012). In this study, the cellulose content was found to improve with soaking time and NaOH concentration until 4% NaOH treatment, as shown in Fig. 1c. The 4% NaOH pretreatment had the highest cellulose content, but the results showed no significant difference for the soaking time tested at this concentration (Table 1).

Table 1. Alkaline Pretreated Samples at Different Concentrations and Treatment Times (Mean ± SD)

Concentration NaOH (% w/v)		Lignin	Holocellulose	α-cellulose
1	MS	10.23	5.57	27.93
	F-ratio	9.29**	97.87***	7.35**
	4 h	14.12±0.59 ^a	78.37±0.75 ^d	63.58±1.26 ^b
	8 h	18.36±0.52 ^b	74.26±1.15 ^c	61.70±0.65 ^b
	12 h	15.20±1.29 ^a	71.14±0.41 ^b	61.24±0.41 ^b
	24 h	15.09±0.28 ^a	68.45±0.43 ^a	56.42±0.89 ^a
2	MS	4.18	62.28	47.11
	F-ratio	5.62±*	46.10***	27.63***
	4 h	12.34±0.16 ^{ab}	84.57±1.08 ^d	74.83±0.72 ^c
	8 h	13.72±1.07 ^b	77.52±1.16 ^b	65.74±0.52 ^a
	12 h	12.79±1.05 ^b	81.73±0.62 ^c	67.35±0.49 ^{ab}
	24 h	10.89±0.85 ^a	74.24±0.49 ^a	69.52±0.86 ^b
4	MS	19.17	1.12	1.45
	F-ratio	133.49***	1.11 ^{ns}	0.41 ^{ns}
	4 h	19.17±0.58 ^c	83.32±0.34 ^a	72.69±1.07 ^a
	8 h	11.53±0.20 ^b	83.31±0.96 ^a	73.35±0.45 ^a
	12 h	10.10±0.16 ^a	82.09±0.22 ^a	73.74±0.91 ^a
	24 h	10.65±1.10 ^{ab}	83.32±0.25 ^a	72.17±0.81 ^a
7	MS	7.13	7.52	15.81
	F-ratio	4.24*	17.00***	10.82**
	4 h	12.33±1.05 ^{ab}	76.65±1.00 ^b	71.40±0.79 ^c
	8 h	11.95±0.64 ^{ab}	78.37±0.24 ^c	70.48±0.86 ^{bc}
	12 h	14.58±0.12 ^b	75.74±0.72 ^{ab}	66.16±0.39 ^a
	24 h	10.76±0.71 ^a	74.62±0.44 ^a	68.93±1.15 ^b
Concentration x Time	MS	21.12	68.79	83.24
	F-ratio	21.484**	81.648***	31.764***

* Statistically significant at 0.05 confidence level

** Statistically significant at 0.01 confidence level

*** Statistically significant at 0.001 confidence level

^{ns} Not statistically significant

The F-ratio is the ratio of the variance between groups to the variance within groups

Superscript within a column bearing the same letter are not significantly different (p<0.05)

All in all, 4% NaOH with 12 h of alkaline pretreatment was chosen, followed by the hydrolysis process. This is due to the favourable characteristic it has shown with its high lignin degradation and the retention of more cellulose after pretreatment. The weight loss during pretreatment was varied in different concentrations ranging from 10% to 20%.

Glucose Analysis

The glucose yield depends on the hydrolysis performance of the enzyme with respect to the substrate. Figure 2 illustrates higher glucose yields with NaOH treated banana pseudostem (about 1.75 times higher) than with untreated banana pseudostem. Once the protective lignin is removed, it opens the accessibility of cellulose for hydrolysis to take place. Immersing the substrate in alkaline solution at a low temperature retains the hemicelluloses in the biomass by minimizing chemical reactions on hemicellulose chains. In this reaction, lignin and hemicelluloses are partially removed by breaking the ester bonds and increasing the porosity of the biomass (Xu *et al.* 2010; Zhang *et al.* 2011). The alkalis promote the enzymatic hydrolysis by removing the acetyl groups of hemicellulose and reducing the hindrance of hydrolytic enzymes (Kong *et al.* 1992). Thus, NaOH can penetrate into the amorphous region of cellulose and started to intrude the neighbouring crystalline areas (Wang *et al.* 2008). The difference between NaOH treated (delignified) and untreated banana pseudostem substrate increased with time. There was 99% confidence of a statistically significant difference between the treated and untreated substrates for their glucose yield.

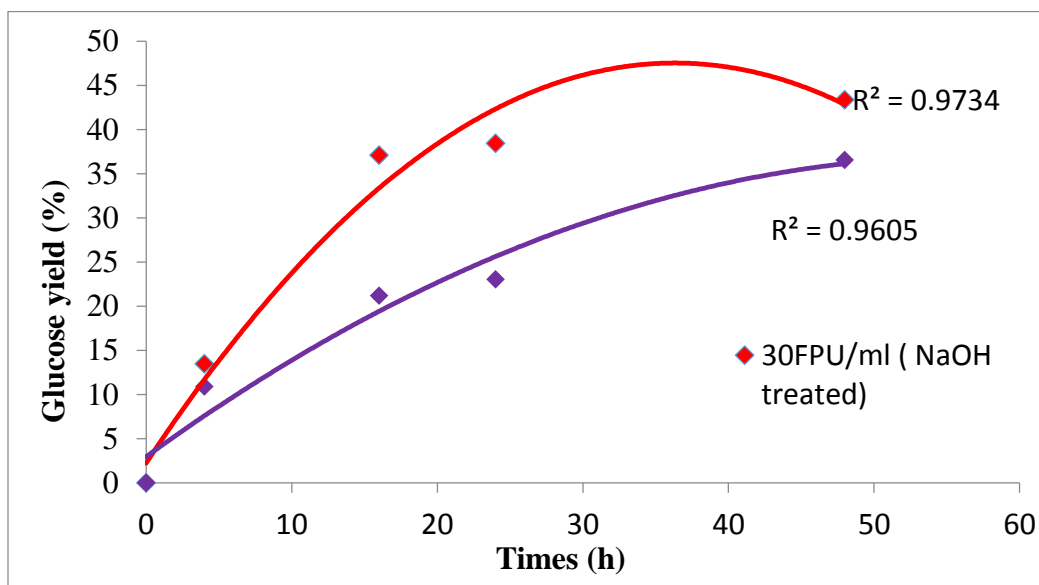


Fig. 2. The percentages of glucose yields for the NaOH pretreated and untreated samples

Table 2 shows the effects of different enzyme loadings and incubation times on glucose yield. In the work of (Mussatto *et al.* 2008), the enzyme loading influence the hydrolysis of cellulose in which the more enzyme loaded, the better the hydrolysis resulted. Hence, more glucose yield can be attained with a higher enzyme loading and incubation time before the optimum condition is reached in which substrate becomes saturated. Increase in enzyme loading resulted in significant difference of glucose yield

especially when the extended incubation time. The highest glucose yield (43.38%) was achieved with the highest enzyme loading of 30 FPU/mL incubated for 48 h.

Table 2. Effects of Different Enzyme Loadings and Incubation Times on Glucose Yield (Mean \pm SD)

Time (h)	Enzyme loading (FPU/g substrate)			
	7.5	15	22.5	30
4 h	7.47 \pm 1.32 ^a	9.28 \pm 1.49 ^a	12.18 \pm 1.41 ^b	13.46 \pm 1.52 ^b
24 h	22.44 \pm 0.48 ^a	29.97 \pm 1.30 ^b	35.41 \pm 1.32 ^c	38.45 \pm 1.74 ^d
48 h	18.43 \pm 1.32 ^a	30.31 \pm 1.85 ^b	38.89 \pm 0.78 ^c	43.38 \pm 1.32 ^d

Superscript within a row bearing the same letter means not significantly different ($p < 0.05$).

Enzymatic Activity

Enzymes such as endoglucanase, exoglucanase, and β -glucosidase are involved in a synergistic reaction of the cellulase component in a heterogeneous reaction system (Gan *et al.* 2003). In cellulose, the β -1,4-glycosidic bond is responsible for linking β -D-glucopyranose units together. The β -1-4-endoglucanase enzymes specifically cleave this internal bond of the cellulose chain, while exoglucanases and β -glucosidases are needed to completely break down cellulose into glucose monomers (Kumar *et al.* 2008); *i.e.*, endoglucanase acts as a pioneer in cleaving cellulose into simple forms. Figure 3 demonstrates the endoglucanase activity of *T. reesei* in the treated and untreated samples. An independent T-test demonstrated a significant difference (at the 99% level) of the two treatments, with alkaline treated samples having higher endoglucanase activity during 24 h of treatment. The alkaline treatment successfully opens the accessibility of cellulose and allows the endoglucanase to more easily cleave the linked glycosidic bonds. Meanwhile, endoglucanase activities are depleted after 48 h of incubation time in the alkaline pretreated sample. Samples with no pretreatment show an increase in endoglucanase activity, even after a 72 h incubation. This may be because it is difficult for the enzyme to penetrate cellulose chains, hence slowing down the reaction and requiring more time for the reaction.

Exoglucanase is the intermediate enzyme for hydrolysis which reacts after endoglucanase but before β -glucosidases. This is a good indicator of the availability of the appropriate substrate, which allows enzyme hydrolysis. Enzyme activity decreased after 72 h of treatment duration. (Fig. 3) There were significant differences (99% confidence) for exoglucanase activity on the banana substrate between the alkaline treated and untreated samples. Exoglucanase activities of 4.2 U/mL and 7.2 U/mL were recorded for *Trichoderma harzianum* and *Penicillium funiculosum* (Maeda *et al.* 2011). This is lower than the activities of *T. reesei* in the present study.

The β -glucosidases help in hydrolysing cellobiose into two molecules of glucose. In this study, the enzymatic activity of β -glucosidase was low in this hydrolysis process. The results indicate that the difference in the β -glucosidase activities between the treated (0.39 U/mL) and untreated samples (0.14 U/mL) was highly significant at confidence level of 95% at 24 hours of treatment; both hydrolyses showed that the activities decreased after a 24 h reaction. As compared to the work of Maeda *et al.* (2011), the hydrolysis of bagasse recorded 26.6 U/mL β -glucosidase activity for *Penicillium funiculosum* and 31.4

U/mL for *Trichoderma harzianum*. Hence, the β -glucosidases activities observed in this study were much lower, as *T. reesei* is known to have low β -glucosidases activities.

All in all, the investigation of enzymatic activities supports an understanding that the enzyme reacts vigorously in its first 24 h and the activities become slower towards the end of hydrolysis. There was a huge gap between the hydrolysis rates of pretreated and untreated samples, indicating differences in their enzymatic activities. Hence, more enzyme loading would be required in the hydrolysis of untreated samples.

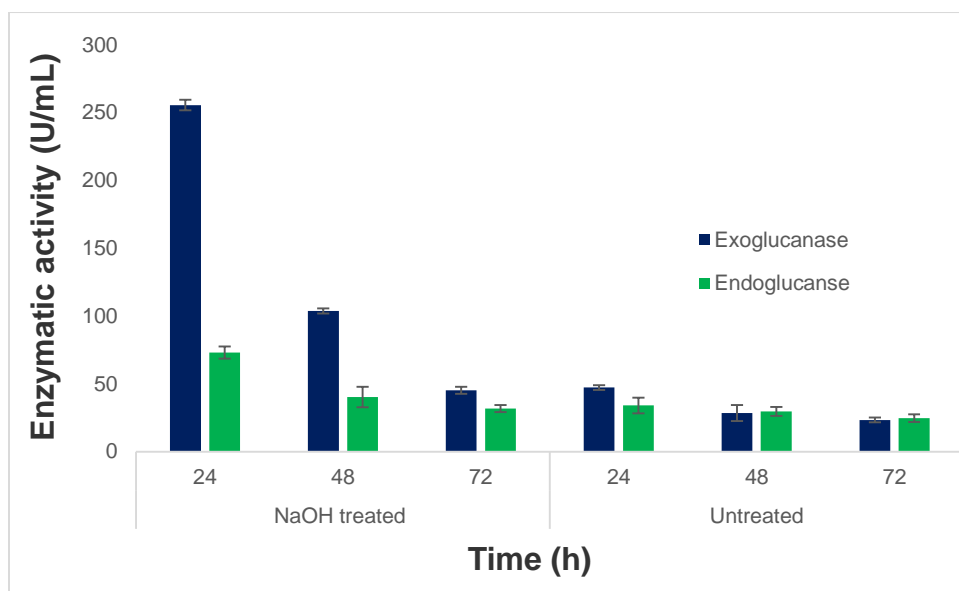


Fig. 3. Endoglucanase and exoglucanase activities of the pretreated and untreated banana pseudostem hydrolysed at 150 rpm, 30 FPU/g substrate cellulase loading

CONCLUSIONS

1. A 4% NaOH concentration with 12 h of soaking time successfully removed almost 60% of lignin and retained as much as 73.74% of cellulose compared to untreated banana pseudostem.
2. The glucose yield for banana pseudostem increased proportionally when the enzyme dosage and incubation time were increased.
3. The glucose yield increased as much as 1.75 times and the enzymatic activities of the treated banana pseudostem were higher than the samples without delignification.

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