Enzymatic Saccharification of Acid/Alkali Pre-treated, Mill-run, and Depithed Sugarcane Bagasse

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In South Africa, approximately 3 × 10^6 tons of sugarcane bagasse is produced annually by 14 factories located on the north coast of KwaZulu-Natal. It is one of the most readily available lignocellulosic materials for ethanol production through enzymatic saccharification and hydrolysis. Pre-treatment enables disruption of the naturally resistant structure of lignocellulosic biomass to make the cellulose accessible to hydrolysis for conversion to biofuels. In this study, pre-treatment of depithed bagasse and mill-run bagasse was done using acid (3% H_2SO_4 v/v) followed by alkali (4% NaOH w/v), and the pre-treated solid was subjected to enzymatic hydrolysis. The effects of different conditions for enzymatic saccharification such as enzyme dose, reaction time, and amount of surfactant were studied in detail. The pre-treated substrate (10% w/v) when hydrolysed using 30 FPU/gds/40 FPU/g dry substrate (gds) with 0.4% (v/v) Tween® 80 for 20 h resulted in 608 mg/gds (depithed bagasse) and 604 mg/gds (mill-run bagasse) total reducing sugars.

Keywords: Bagasse; Pre-treatment; Total reducing sugars; Saccharification

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INTRODUCTION

Globally, there is a drive to use renewable materials for the production of biofuels due to several sustainability concerns, such as the scarcity of fossil fuels, mitigation of green-house gas emissions, economic issues, and environmental concerns (Alam *et al*. 2013). Lignocellulosic materials are renewable feedstock comprising a wide variety of agricultural residues, forest products, and dedicated crops.

Sugarcane bagasse, an agriculture residue, possesses great potential for conversion to biofuels and is one of the most important agro-industrial crops produced in South Africa. Sugarcane bagasse is an extremely nonhomogeneous material made up of around 30% to 40% pith fibre, which is derived from the core of the plant and is mainly parenchyma material. The sugarcane bagasse in which the pith is removed is called depithed bagasse. Sugarcane bagasse consists of 38% to 50% cellulose, 22% to 28% hemicellulose, and 19% to 33% lignin, as illustrated in Table 1. These components of lignocellulosic biomass form a very complex structure that makes it resistant to chemical and/or microbial degradation (Gupta *et al.* 2011). By virtue of this strong packaging of the biomass structural polymers, an effective pre-treatment method is required to break the lignin and hemicellulose seal to expose the cellulose for hydrolysis (Gupta *et al*. 2009, 2011; Manzoor *et al*. 2012).

Table 1. Chemical Composition of Raw Sugarcane Bagasse (% w/w, dry basis) from the Literature

Components (%)	References					
	Rainey (2009)	da Silva *et al*. (2010)	Rocha *et al*. (2011)	Rabelo *et al*. (2011)	Canilha *et al*. (2012)	Chandel *et al*. (2014)
Cellulose	47.0	38.8	45.5	38.4	45.0	39.53
Hemicellulose	27.0	26.0	27.0	23.2	25.8	25.63
Lignin	23.0	32.4	21.1	25	19.1	30.36
Ash	1.0	2.8	2.2	1.5	1.0	1.44
Extractives	-	-	4.6	-	9.1	2.90

Pre-treatment is a critical step in cellulosic bioethanol technology because it has a huge impact on the quality of the carbohydrate-containing streams and the cost of the carbohydrates (Balat *et al*. 2008). Pre-treatment methods can be classified into different categories: physical, physiochemical, chemical, biological, and electrical, and they differ in their mechanisms of action (Kumar *et al*. 2009; Kuhad *et al*. 2011a). The most commonly used acid for pretreatment is sulphuric acid, where its reaction with biomass promotes hemicellulose breakdown to xylose and other sugars (Mosier *et al*. 2005; Kuhad *et al*. 2010). Acid pretreatment is preferred because the operation process takes place at low and medium temperatures and consequently decreases energy costs (Girio *et al*. 2010). However, when there are high concentrations of acid, problems can occur with equipment corrosion and result in expensive maintenance costs (Alvira *et al*. 2010). This is the reason why dilute acid is widely used for acid treatment. After acid pretreatment on the sugarcane bagasse is completed, the solid fraction obtained consists of cellulose and lignin and the liquid fraction contains hemicellulose.

To remove lignin from the solid fraction, alkali pretreatment is used, which causes swelling, leading to an increase in internal surface area, a decrease in the degree of polymerisation and crystallinity, a separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Zheng *et al*. 2009; Gupta *et al.* 2011), making cellulose available for the enzymatic degradation (Gupta *et al.* 2011; Sarkar *et al*. 2012). Hydrolysis using enzymes (cellulases) is an ideal approach to degrade cellulose into reducing sugars because mild reaction conditions (pH between 4.8 to 5.0 and temperatures between 45 to 50 °C) can be used; it does not present corrosion problems in the reactors and results in negligible by-product formation with high sugar yields. However, enzymatic hydrolysis depends on optimised conditions for maximal efficiency (hydrolysis temperature, reaction time, pH, enzyme loading, and substrate concentration) and suffers from end-product inhibition and biomass structural restraints (Canilha *et al.* 2012).

Cellulases are composed of domains or modules, which are structurally and functionally discrete units, hence the term "cellulases module" (Kuhad *et al*. 2011b). Cellulases are inducible enzymes synthesised by a large diversity of microorganisms, including both fungi and bacteria, during their growth on cellulosic materials (Kuhad *et al*. 2011b). Cellulases break down the cellulose molecule into monosaccharides (simple sugars), such as β-glucose, or shorter polysaccharides and oligosaccharides. Enzymatic hydrolysis of cellulosic fractions requires three classes of cellulolytic enzymes (cellulases): (1) endo-β-1,4-glucanases (EG.E.C.3.2.1.4), which attack regions of low crystallinity in the cellulose fibre, creating free chain ends; (2) cellobiohydrolases or exoglucanase (CBH.E.C.3.2.1.91) which degrade the molecule further by removing cellobiose units the

free chains-ends; and (3) β-glucosidases (E.C.3.2.1.21) which hydrolyse cellobiose to produce glucose (Kuhad *et al.* 2011a; Canilha *et al.* 2012).

Due to the high cost of enzymes, the addition of surfactant after biomass pretreatment is another approach used for the conversion of cellulose to reducing sugars to improve enzymatic hydrolysis in a way that the enzymes required may be reduced. Surfactants have been said to enhance enzymatic digestion because surfactants: (i) alter the substrate structure and make it more accessible to enzymes. (ii) stabilize enzymes and prevent their denaturation during hydrolysis. (iii) increase positive interactions between substrates and enzymes, and (iv) reduce non-productive adsorption of enzymes (Qing *et al*. 2010). Another two primary mechanisms of surfactant effects that have been suggested are that surfactants: (i) adsorb to the lignin surface and reduce unproductive enzyme binding and (ii) positively affect cellulase activity and enzymes stabilities (Yang *et al*. 2015). Surfactants consist of both hydrophobic and hydrophilic properties which can decrease the surface tension between two liquid phases to improve the removal of hydrophobic compounds (Qing *et al*. 2010; Cao 2012). Tween and PEG are the mostly-used non-ionic surfactants to enhance cellulose conversion and that is due to their ability to block non-productive adsorption of cellulase enzymes on lignin matrix hence efficiently increasing the enzymatic hydrolysis of pretreated lignocellulosic biomass (Zhou *et al*. 2015; Borjesson *et al*. 2007). Tween 80 (polysorbate 80) is derived from polyethoxylated sorbitan and oleic acid. The hydrophilic-lipophilic balance (HLB) of Tween 80 is 15, which means it is highly water soluble. Surfactants with high HLB values are useful in extracting hydrophobic degradation products from lignin and hemicellulose (Kurakake *et al*. 1994; Cao. 2012). PEGs are expensive when compared to polysorbates; the market prize for PEGs is about U.S. $1.51 kg, where for polysorbates it is about $0.25 kg (Tu and Saddler. 2010). The addition of Tween 80 during enzymatic hydrolysis of biomass can significantly reduce enzyme concentrations and assist with the recycling of enzymes (Tu and Saddler. 2010). For this work Tween 80 was used to study the effect of surfactant on the saccharification of pre-treated sugarcane bagasse.

In this study, the chemical pretreatment of depithed (DP) and mill-run (MR) sugarcane bagasse with dilute sulphuric acid followed by dilute alkali was performed. The pre-treated substrate was subsequently hydrolysed using cellulases. The final yield of enzymatic hydrolysis was highly dependent on the following factors: composition of biomass, pre-treatment type, enzyme dosage, and efficiency (Alvira *et al*. 2010). Therefore, in the present study, enzymatic hydrolysis of the pre-treated bagasse was done using cellulase from *Trichoderma* species RCKC65 to determine the optimisation of enzyme load, surfactant dosage, and high solid loading for efficient hydrolysis.

EXPERIMENTAL

Materials

Carboxymethyl cellulose, *p*-nitrophenol β-D glucopyranoside, and 3,5-dinitrosalicylic acid (DNSA) were purchased from Sigma (St. Louis, USA), and media components were purchased from Himedia, USA. Other chemicals, *viz.* sulphuric acid (98%), sodium hydroxide, Tween® 80, citric acid, and sodium dihydrogen orthophosphate, of analytical grade, were purchased locally in India. Cellulase was synthesised in-house using *Trichoderma* sp. RCKC65 at the University of Delhi, South Campus, New Delhi, India. Sugarcane bagasse was obtained from a local mill (KwaZulu-Natal, South Africa).

Methods

Preparation of sugarcane bagasse

Depithed and mill-run sugarcane bagasse was dried at 105 °C, milled, and sieved to 40-mesh particle size. The cellulose, hemicellulose, lignin, moisture, and ash contents of raw and pre-treated biomass were determined according to the method described by TAPPI (TAPPI T203 om-83 (1983); TAPPI T222 om-83 (1983); TAPPI T223 hm-84 (1984); TAPPI T208 om-84 (1984); and TAPPI T211om-93 (1996).

Procedure

One gram of sugarcane bagasse and (72%) sulphuric acid (15 mL) were added in a 100 mL beaker for 1 h while stirring at room temperature for lignin determination. The resulted mixture was placed in a 1 L flask, and then 360 mL of water was added. The mixture was boiled at 100 °C for 4 h, filtered, and weighed. For holocellulose determination, in a 500 mL flask, 5 g of sugarcane bagasse and water were added, heated at 100 °C covered with a 100 mL flask for refluxing; 1.5 g of $NaClO_2$ and 0.5 mL of glacial acetic acid were added. The system was set up for refluxing at 100 °C; $NaClO_2$ and acetic acid were added until the bagasse became white. The solids were filtered and washed with water, dried at (100±5 °C) and weighed until the weight was constant. For ash content determination, 1.0 g of sugarcane bagasse was weighed into the silica crucible; it was kept in the muffle furnace for 4 h at 500 °C and weighed. For cellulose content determination, 2.0 g of sugarcane bagasse, 5.0 g of $NaClO_2$, and 100 mL of water were mixed, autoclaved for 30 min, cooled, and then filtered and weighed. Hemicellulose was calculated as a difference of holocellulose and cellulose. All these analyses were done in triplicate.

Depithed and mill-run sugarcane bagasse pre-treatment

Acid: To remove hemicellulose, the substrate was pre-treated with 3% (v/v) sulphuric acid for 30 min in an autoclave at 121 °C. The solution was allowed to cool, and was filtered and washed to neutral pH, and overnight dried at 60 °C in an oven.

Alkali: The dried substrate from acid pre-treatment was further pre-treated with 4% (w/v) sodium hydroxide for 15 min in an autoclave at 121 °C. The solution was allowed to cool, filtered to remove the lignin, washed to a neutral pH, and dried at 60 °C in an oven overnight. The residual pre-treated substrate was stored for further processing.

Production of cellulase

The cellulase was produced by *Trichoderma* sp. RCK65, which was cultivated in solid state fermentation, where wheat bran was used as a substrate. The culture conditions were: pH of 4.5, moisture ratio 1:1 at 30 °C for 3 days (Ghose 1987)

Saccharification of pre-treated biomass

The acid-alkali pre-treated depithed and mill-run sugarcane bagasse was saccharified using crude enzyme extract produced by *Trichoderma* sp. RCKC65 (8 FPU/mL). Enzymatic hydrolysis of the pre-treated material (1.0 g each) was carried out at 10% (w/v) solid loading in 50 mM citrate phosphate buffer (pH 5.0). The substrate with buffer was pre-incubated at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, Germany) at 150 rpm for 1 h in a 100 mL Erlenmeyer flask. The temperature and agitation were the same as the pre-incubation until optimized. Thereafter, to obtain efficient saccharification of the pre-treated biomass, optimisation of cellulase doses (10-40 FPU/g), surfactant, Tween® 80 (0.3% to 0.6%), and solid loading (10% to 16%) was

carried out using One Factor at A Time (OFAT) method (Wahid and Nadir 2013). The reaction continued up to 24 h and the samples were withdrawn at regular intervals, centrifuged at 12,000 rpm for 10 min in a centrifuge (Sigma, Germany), and the supernatant was analysed for total reducing sugars released and saccharification yield.

Analysis of reducing sugars

The DNS method (Miller 1959) was employed in this work, which detects the whole range of reducing sugars. The method consists of reading the absorption at 540 nm in a solution composed of the sample that contains reducing sugars and DNS reagent. The intensity of the color was transformed to mg of sugars using a standard curve. Yao *et al*. (2007); Nasirpour *et al*. (2014); Pandey and Negi. (2015); Das *et al*. (2015); Li *et al*. (2016); Aswathy *et al*. (2010) and Han *et al*. (2012) used DNS method to determine the total reducing sugars. The saccharification yield was calculated using Eq. 1.

$$\text{Saccharification yield} = \frac{\text{total sugar concentration (mg/ml) x volume of saccharified product (ml)}}{\text{Substrate (g)}} \quad (1)$$

Statistical Analysis

All experiments were carried out in triplicate and the data reported were expressed as mean values. Experimental errors, which were calculated as the relative standard deviation, were shown by the error bars in the figures.

Characterization of Untreated and Pre-treated Sugarcane Bagasse

FTIR analysis

The FTIR spectra of the untreated, acid treated and acid/alkali treated sugarcane bagasse samples were recorded on an instrument (Shimadzu FTIR 8400) in the range of 380–4500 cm^{-1} with a resolution of 4 cm^{-1}. This method provides information about the chemical bonds and molecular structure of the material.

TGA/ DSC analysis

A Simultaneous Differential Scanning Calorimeter and Thermo-Gravimetric Analyser (SDT) manufactured by TA instruments, model SDT Q600 were used to characterize the thermal stability of the untreated, acid treated and acid/alkali treated sugarcane bagasse samples. Approximately 3.0 mg of each sample was heated from 20 to 1500 °C at a heating rate of up to 50 °C/min.

SEM analysis

A scanning electron microscope manufactured by Carl Zeiss, model EVO 15 HD was used to visualize the difference between untreated, acid and acid/alkali treated bagasse. The samples were coated with gold to avoid charging effects (sputter-coating).

RESULTS AND DISCUSSION

Pretreatment

The compositional analysis of raw samples revealed that the sugarcane bagasse contains cellulose (52.5% depithed, 46.1% mill-run); hemicellulose (20.6% depithed, 23.2% mill-run), and lignin (25.0% depithed, 28.7% mill-run). When comparing these

values with the literature values (Rainey 2009; da Silva *et al*. 2010; Rocha *et al*. 2011; Rabelo *et al*. 2011; Canilha *et al*. 2012 and Chandel *et al*. 2014), it can be observed that there is not much difference. The pre-treatment of substrate with 3% H_2SO_4 decreased the proportion of hemicellulose and lignin and increased the cellulose content for both depithed and mill-run bagasse, which was predictable since the acid pretreatment was used to remove hemicellulose. The pre-treatment of substrate with 4% NaOH increased the proportion of cellulose for both depithed and mill-run bagasse; and reduced the lignin content and hemicellulose for both depithed and mill-run bagasse, which was also expected because alkali pre-treatment was used to remove lignin (Table 2). The compositional analysis of raw, acid pre-treated, and acid/alkali pre-treated bagasse shows that the acid/alkali pre-treatment was able to disrupt the sugarcane bagasse and extract cellulose, which was the compound of interest.

A 10% biomass loading was used in this study; the starting mass was 100 g before pre-treatment. After acid pre-treatment, the mass decreased considerably to 67.6 g, which means that there was a 32% mass loss, mostly because of the removal of hemicellulose as reducing sugars. The acid-pre-treated substrate was then subjected to alkali pre-treatment and the amount left was 29.6 g, which means there was a 56% mass loss, which may be attributed mostly to the loss of lignin, leaving cellulose behind.

Table 2. Compositional Analysis of Raw and Pre-treated Sugarcane Bagasse

Bagasse Sample		Components (wt. %)			
		Cellulose	Hemicellulose	Lignin	Ash
Depithed	Raw	52.5	20.6	25.0	1.9
	Acid Pre-treated	65.7	8.1	24.6	1.6
	Acid/Alkali Pre-treated	83.1	2.7	12.7	1.2
MR	Raw	46.1	23.2	28.7	2.0
	Acid Pre-treated	60.8	10.0	27.0	1.5
	Acid/Alkali Pre-treated	80.6	2.4	14.6	1.4

Depithed = Depithed sugarcane bagasse
MR = Mill-run sugarcane bagasse

Optimisation of enzyme load and time for saccharification of pre-treated depithed and MR sugarcane bagasse

Figures 1 and 2 illustrate the action of cellulase enzyme on acid-alkali-pre-treated, depithed, and mill-run sugarcane bagasse samples, respectively. In order to determine the effect of enzyme load and time on both acid/alkali pretreated mill-run and depithed bagasse, the pretreatment were carried out for 24 h, sampled after 4 h at 50 °C.

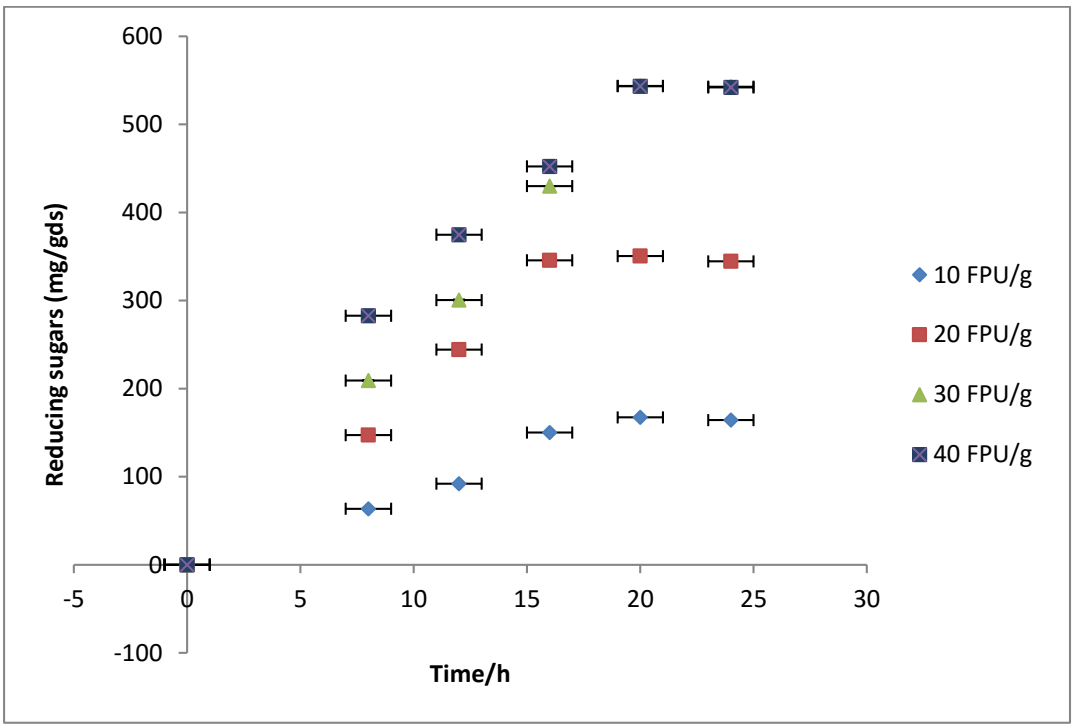

Fig. 1. Optimisation of enzyme load on the hydrolysis of depithed sugarcane bagasse

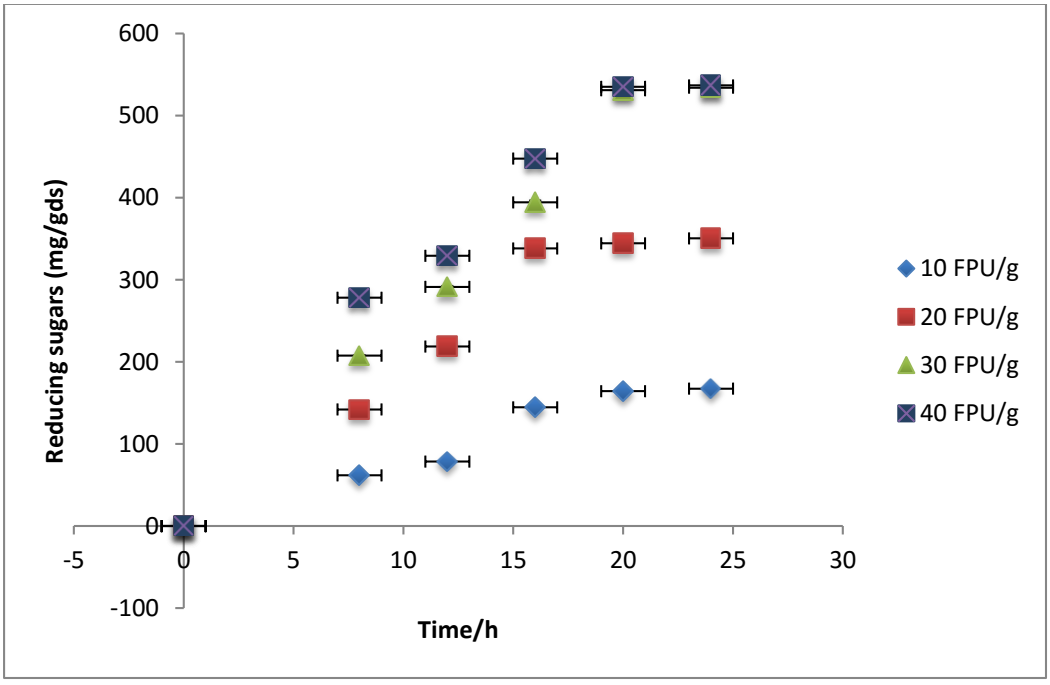

Fig. 2. Optimisation of enzyme load on the hydrolysis of mill-run sugarcane bagasse

The samples were hydrolyzed with enzyme load of 10 FPU/gds (grams dry substrate), 20 FPU/gds, 30 FPU/gds, and 40 FPU/gds. It was found that when the mill-run bagasse was hydrolysed with 40 FPU/gds (cellulase) for 24 h, the maximum amount of total reducing sugars was released (536 mg/gds), whereas the depithed bagasse released a maximum amount of total reducing sugars when it was hydrolysed with 30 FPU/gds (cellulase) for 20 h. 10 FPU/gds gave the lowest total reducing sugars for both pretreated depithed and mill-run bagasse. It was observed that as the enzyme load increases and time increases, more reducing sugars are released from the pretreated bagasse.

Ramachandriyaa *et al*. (2013) performed experiments using 46 FPU/g of Accelerase® 1500/g glucan (0.5 mL/g glucan) to study the effect of enzyme dosage on glucose yield. Various enzyme loadings of 46 FPU/g glucan (0.5 19 mL/g glucan), 34.5 FPU/g glucan (0.375 mL/g glucan), 23 FPU/g glucan (0.25 mL/g glucan), and 11.5 FPU/g glucan (0.125 mL/g glucan) were used to hydrolyse pretreated biomass. Their findings also showed that increasing the enzyme dosage also increased the glucose concentration or total reducing sugars and that is because the low enzyme dosage lowers enzyme binding to cellulose which decreases the glucose yield or total reducing sugars. 46 FPU/g glucan gave the maximum yield of glucose (82.7 g/L) at 672 h using 16% solid loading.

Optimisation of surfactant dose and time for the saccharification of pre-treated depithed and MR sugarcane bagasse

Zhou *et al*. (2015) reported that the enzymatic hydrolysis of pure cellulose was not consistently improved by surfactants, meaning that surfactants can increase or decrease the enzymatic hydrolysis reaction. Wen *et al*. (2013) showed that one of the reasons that can cause surfactant to decrease the enzymatic hydrolysis reaction is the application of a high concentration surfactant (Tween 80), which could inhibit the enzyme activity and thus hinder the enzymatic hydrolysis process.

Figures 3 and 4 illustrate the action of Tween 80 surfactant on acid/alkali-pre-treated depithed and mill-run sugarcane bagasse samples, respectively. The acid/alkali-pre-treated bagasse was used as a substrate with the enzyme dose that released the maximum amount of total reducing sugars in Figs. 1 and 2. The samples were hydrolysed with an enzyme dosage of 30 FPU/gds (depithed bagasse)/ 40 FPU/gds (mill-run bagasse) with the addition of surfactant (Tween 80) from 0.3 to 0.6% for 20 h. Among the various dosages of Tween 80 tested, 0.4% (v/v) after 20 h of incubation resulted in the maximum increase in saccharification efficiency with a release of 608 and 605 mg/gds total reducing sugars from depithed and mill-run sugarcane bagasse samples, respectively. The addition of a surfactant to the enzymatic hydrolysis process increased the total reducing sugars yield by 12% for depithed bagasse and 13% for mill-run bagasse. For this study, Tween® 80 was selected because it does not denature the cellulolytic enzyme that brings about saccharification (Nystrom and Allen 1976; Wilke 1978). Wen *et al*. (2013) studied the effects surfactant on enzymatic hydrolysis using a different type of biomass called sweet sorghum bagasse (SSB), which showed that Tween 80 can increase the enzymatic hydrolytic efficiency of SSB by 21.3% but they also discovered that high concentration of Tween 80 would inhibit the enzyme activity, and thus hinder the enzymatic hydrolysing process. This effect was also observed in the present work; when the concentration of surfactants increased from 0.5% to 0.6%, the total reducing sugars produced decreased. Wen *et al*. (2013) also studied adsorption, which showed that there was limited adsorption of Tween 80 and cellulase on SSB and that Tween 80 could reduce the adsorption of cellulase on SSB, interfering with the reaction between the substrate and enzyme.

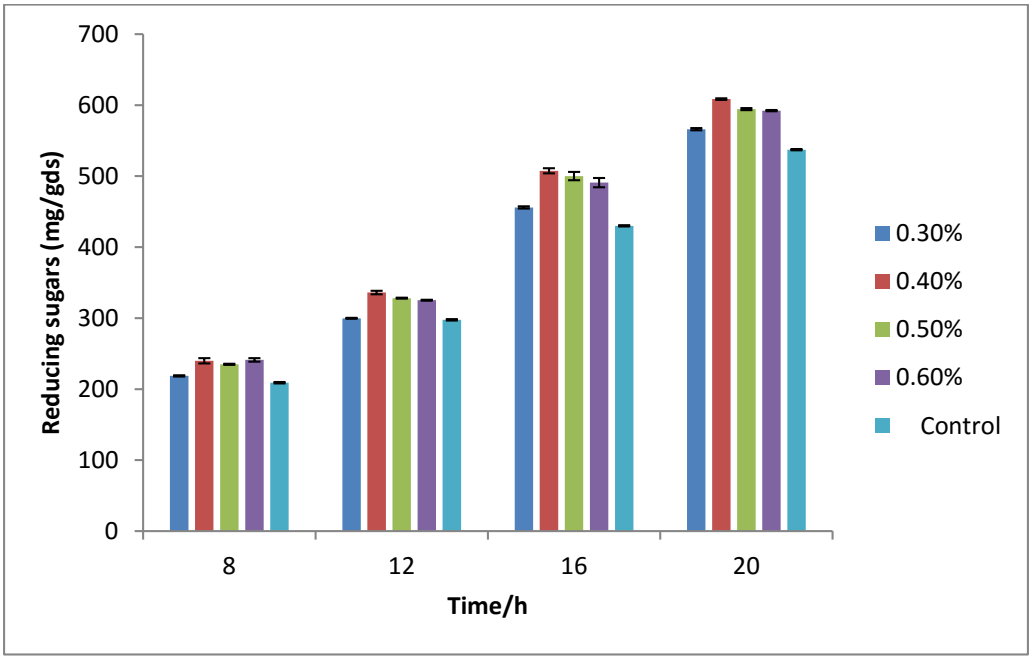

Fig. 3. Optimisation of surfactant dosages on the hydrolysis of depithed sugarcane bagasse

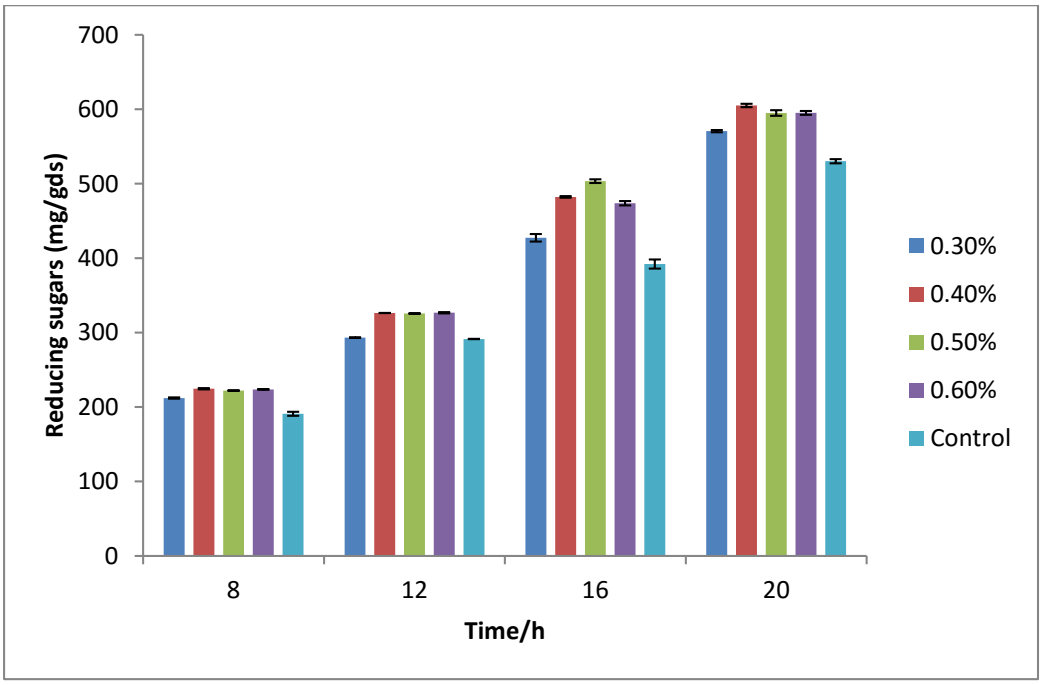

Fig. 4. Optimisation of surfactant dosages on the hydrolysis of mill-run sugarcane bagasse

Optimisation of high solid loading for the saccharification of pre-treated depith and MR sugarcane bagasse

Figures 5 and 6 illustrate the high solid loading effect on acid/alkali pre-treated depithed and mill-run sugarcane bagasse samples, respectively. Various amounts of substrate were used, ranging from 10 to 16%, hydrolysed with enzyme dosage of 30 FPU/gds (depithed bagasse)/ 40 FPU/gds (mill-run bagasse), 0.4% of Tween 80 for 20 h. The maximum total reducing sugars was obtained at 10% solid loading for both depithed and mill-run bagasse, with a sugar release of 609 and 604 mg/gds, respectively. A solid loading of 16% gave the lowest total reducing sugars; this shows that as the solid loading increases, the total reducing sugars released decreases. Also, previous studies have shown that as the substrate concentration increases, the feedback inhibition by cellobiose and glucose is enhanced, leading to the reduced production of reducing sugars in the enzymatic reaction (Han *et al*. 2012).

Han *et al*. (2012) studied the effect of substrate concentration on the enzymatic hydrolysis by using a different type of biomass in a range of 1% to 10% of wheat straw pretreated with alkaline solution (1% sodium hydroxide); they discovered that 3% of wheat straw gave maximum total reducing sugars of 350.18 mg/g. Whereas in this work 10% to 16% of sugarcane bagasse was used and 10% gave the maximum total reducing sugars of 609 mg/gds, 10% in their work gave a low reducing sugars less than 300 mg/g. The huge difference observed in our results may be due to the difference in biomass, organisms for cellulose, and also the enzyme loading. Han *et al*. (2012) used a wheat straw, cellulase produce by *Penicillium* and enzyme loading of 25 FPU/gds, whereas in this work sugarcane pretreated with both acid and alkaline was used, cellulase produced by *Trichoderma sp.* RCKC 65 and enzyme loading of 30 FPU/gds was used. The difference in conditions and the type of biomass may have cause the difference in total reducing sugars produced by 10% substrate.

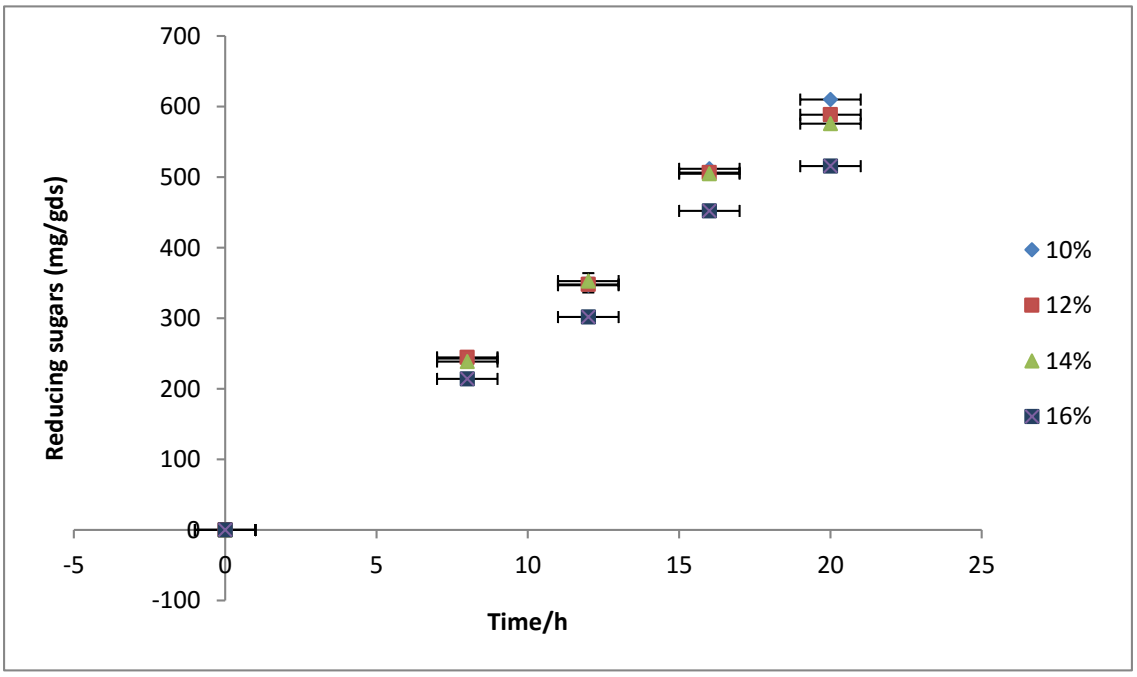

Fig. 5. Optimisation of high solid loading on the hydrolysis of depithed sugarcane bagasse

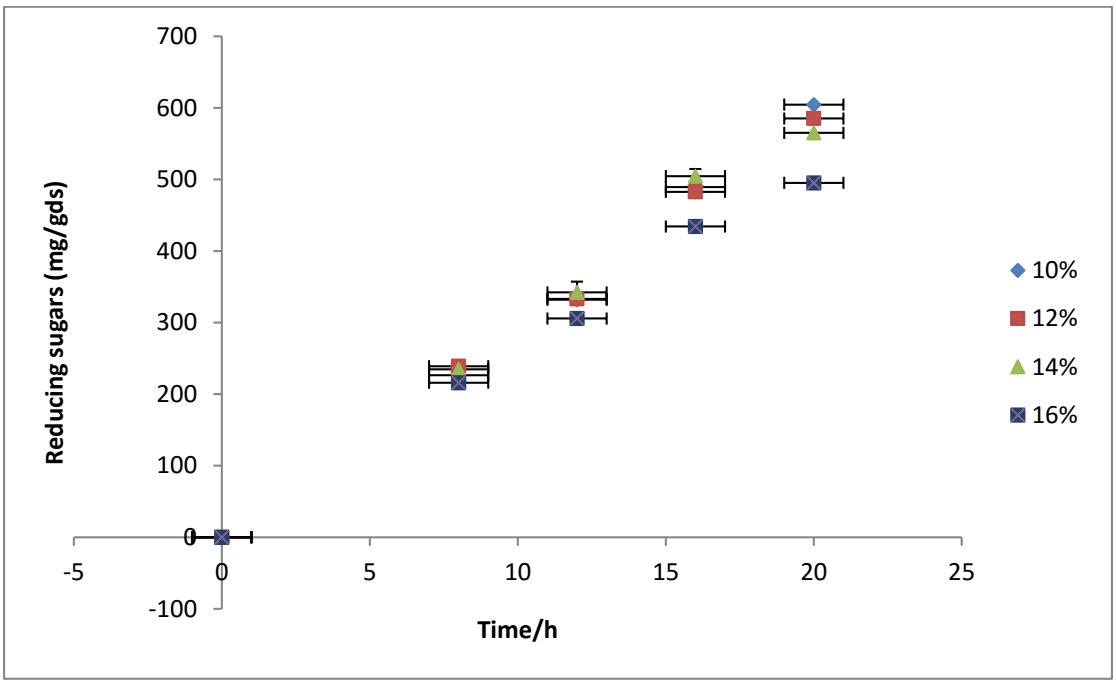

Fig. 6. Optimisation of high solid loading on the hydrolysis of mill-run sugarcane bagasse

Characterisation of Raw and Pre-treated Bagasse

Fourier transform infrared spectroscopy (FTIR)

FTIR was performed to detect changes in functional groups and molecular conformation that were caused by the pre-treatment process. Figures 7 and 8 illustrate the FTIR spectra of the raw and regenerated depithed and mill-run material after acid and alkali pre-treatment. The main functional groups of the biomass components are cellulose, hemicellulose, and lignin. It is notable that these components mostly consist of alkenes, esters, aromatics, ketone, and alcohol, with different oxygen-containing functional groups: for example, OH (3500 to 3200 cm^{-1}), C=O (1770 to 1710 cm^{-1}), C-O-C (1280 cm^{-1}), and C-O-H (1055 cm^{-1}) (Demibras 2000). In both mill-run and depithed sugarcane bagasse, acid/alkali-pre-treated bagasse showed the lowest OH absorption and raw bagasse showed the highest OH absorption. In comparison with raw and acid pre-treated bagasse, a big difference is noticeable in the fingerprint region (1830 to 730 cm^{-1}) for acid/alkali-pre-treated bagasse. This revealed that the pre-treatment specifically removed hemicellulose and lignin fractions and the residual pre-treated material was rich in cellulose. The band at 898 cm^{-1} is characteristic of the glycosidic bond β-(1 → 4) cellulose, corresponding to the C-H deformation in cellulose (Pandey and Pitman 2003; Oh *et al*. 2005; Chandel *et al*. 2014). The region between 1,200 and 1,100 cm^{-1} is a large contribution of hemicellulose and cellulose, which exhibits a maximum value around 1,035 cm^{-1} due to C-O stretching and 1,164 cm^{-1} for the asymmetrical stretching of C-O-C (Pandey 1999; Colom *et al*. 2003; Pandey 2005; Chandel *et al*. 2014). The region around 1,247 cm^{-1} was due to the stretching of C-O, which is characteristic of hemicellulose and lignin (Pandey and Pitman 2003; Chandel *et al*. 2014). A band around 1,458 cm^{-1} is reported to be a deformation of lignin CH_2 and CH_3, and 1,604 cm^{-1} is reported to be stretching of the C=C and C=O lignin aromatic ring. The band around 1,515 cm^{-1} is because of the C=C stretching of the aromatic ring in lignin (Colom *et al*. 2003; Pandey 2005; Chandel *et al*. 2014).

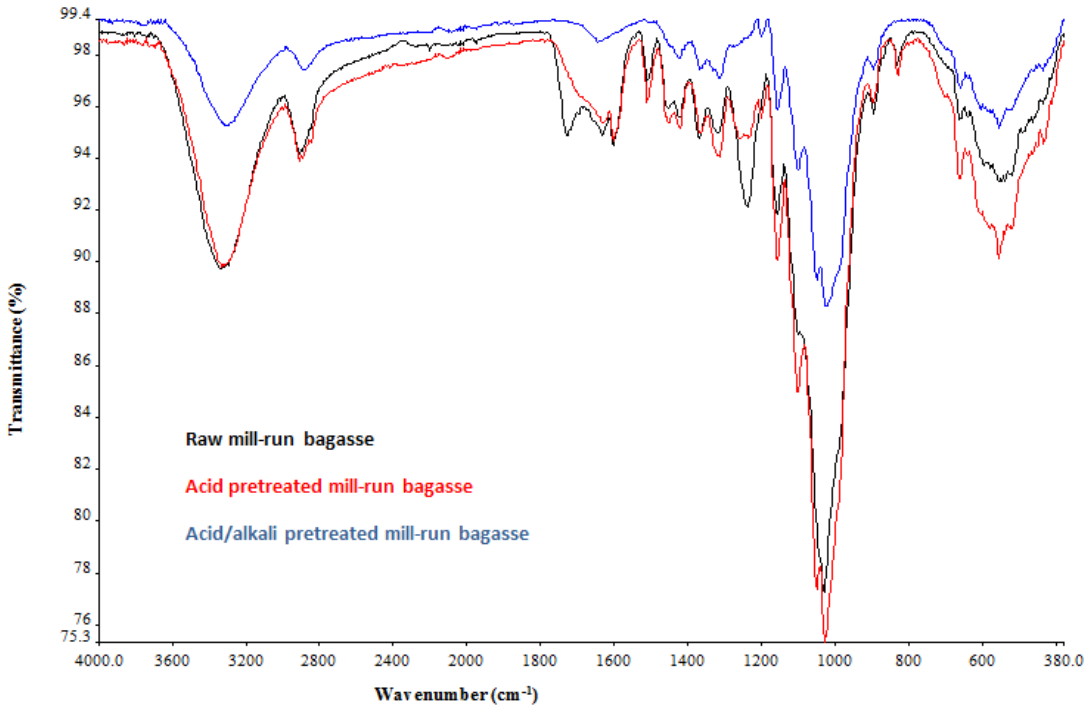

Fig. 7. FTIR spectra of mill-run bagasse (raw, acid-pre-treated, and acid-alkali-pre-treated)

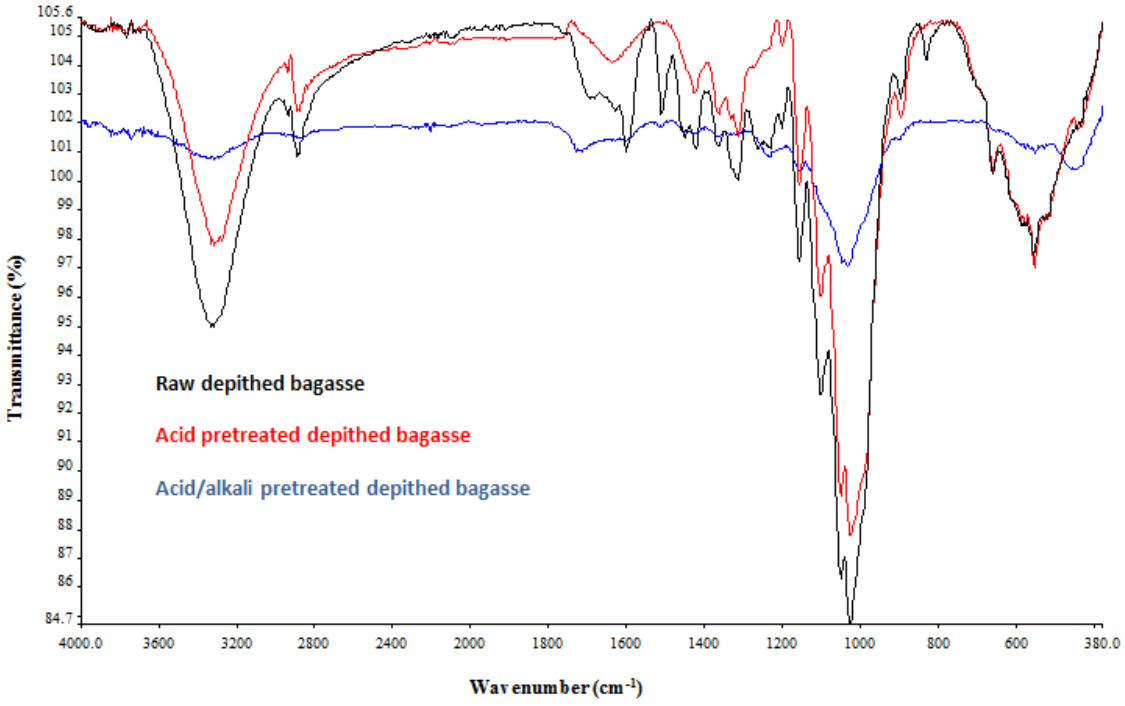

Fig. 8. FTIR spectra of depithed bagasse (raw, acid-pre-treated, and acid/alkali-pre-treated)

A band around 1,733 cm^{-1} is characteristic of C=O stretching of unconjugated hemicellulose. The peak around 2,850 cm^{-1} is reportedly due to the symmetric stretch of

CH and CH_2, while the peak at 2,918 cm^{-1} is due to asymmetrical stretching of CH_2 and CH. Both denote the characteristics of cellulose (Ivanova and Korolok 1989; Chandel *et al*. 2014). The region between 3,800 and 3,000 cm^{-1} covers the related crystalline structure of cellulose. This region represents the sum of the vibration of valence bands of the hydrogen bond of the OH group and the bands of intra-molecular and intermolecular hydrogen bonds (Hinterstoisser and Salmen 1999; Chandel *et al*. 2014).

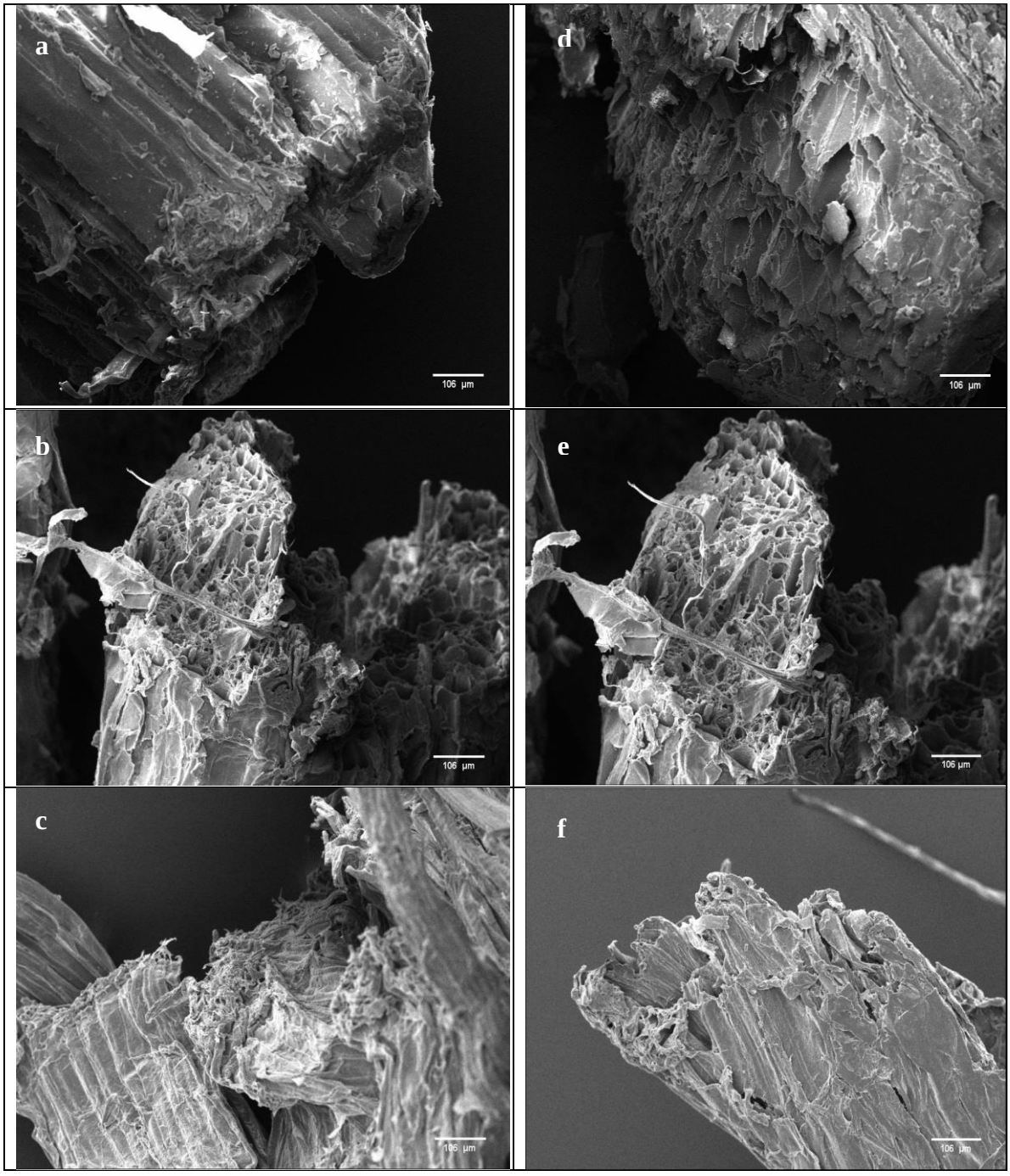

Fig. 9. SEM images of mill-run bagasse (a) raw, (b) acid-pre-treated, (c) acid/alkali-pre-treated; and depithed bagasse (d) raw, (e) acid-pre-treated, (f) acid/alkali-pre-treated

Scanning electron microscopy (SEM)

SEM was used to study the morphology of raw and regenerated sugarcane bagasse after acid/alkali pre-treatment. Figure 9 shows the SEM images at 800× magnification. The images show that the surfaces of the raw fibres (Fig. 9a, d) appear smooth and the surfaces of the pre-treated fibres appear rougher with more porosity (Fig. 9b, c, e, and f). The changes may be attributed to the disruption of the structure and reduction in crystallinity. One of the main objectives of pre-treatment is to increase the surface area and pore size for enzymatic hydrolysis (Alvira *et al.* 2010); therefore, this objective was met, as a remarkable increase in porosity was observed after pre-treatment for both mill-run and depithed bagasse.

Thermal analysis

Differential scanning calorimetry (DSC) and thermo-gravimetric analysis (TGA) were used to study the thermal behaviour of the raw and pre-treated substrates from ambient temperature to 800 °C at a heating rate of 10 °C/min under a nitrogen flow. Figures 10 and 11 show the DSC and TGA curves for depithed (raw, acid-, and acid/alkali-pre-treated bagasse) and mill-run (raw, acid-, and acid/alkali-pre-treated bagasse). The DSC curves (Fig. 10) show an exothermic peak at approximately 100 °C, which was due to the removal of moisture when the sample was heated. The TGA curves (Fig. 11) exhibited three degradation steps.

The initial weight loss can be observed between 30 and 100 °C in all scans, which may be attributed to the evaporation of loosely bound moisture on the surfaces of the samples. The second step was observed between 250 and 350 °C, and the third step occurred between 350 and 500 °C. The exothermic peaks that appeared at 350 and 400 °C can be attributed to charring (Yang *et al*. 2007). Table 4 shows the weight loss and degradation temperatures of bagasse fibres.

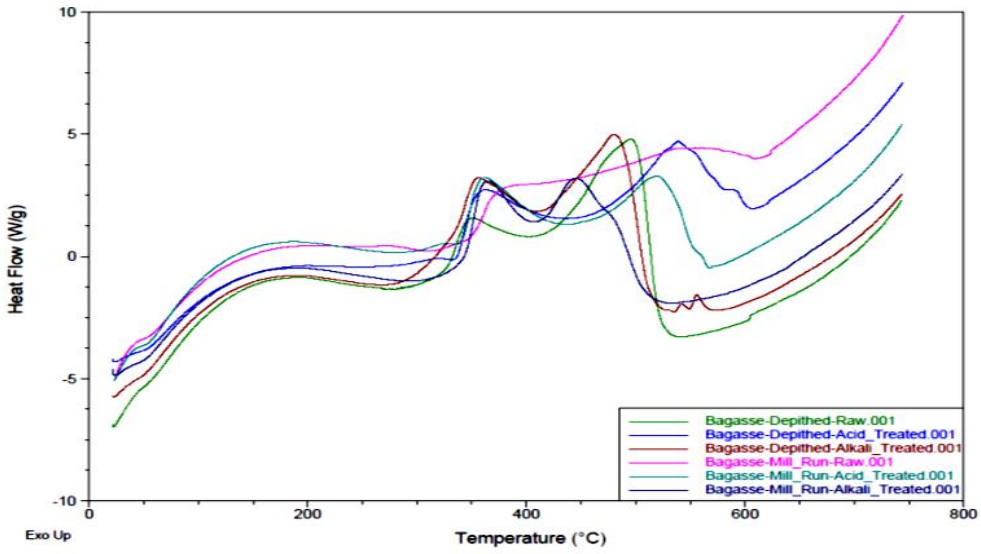

Fig. 10. DSC thermograms for raw and pre-treated mill-run and depithed bagasse

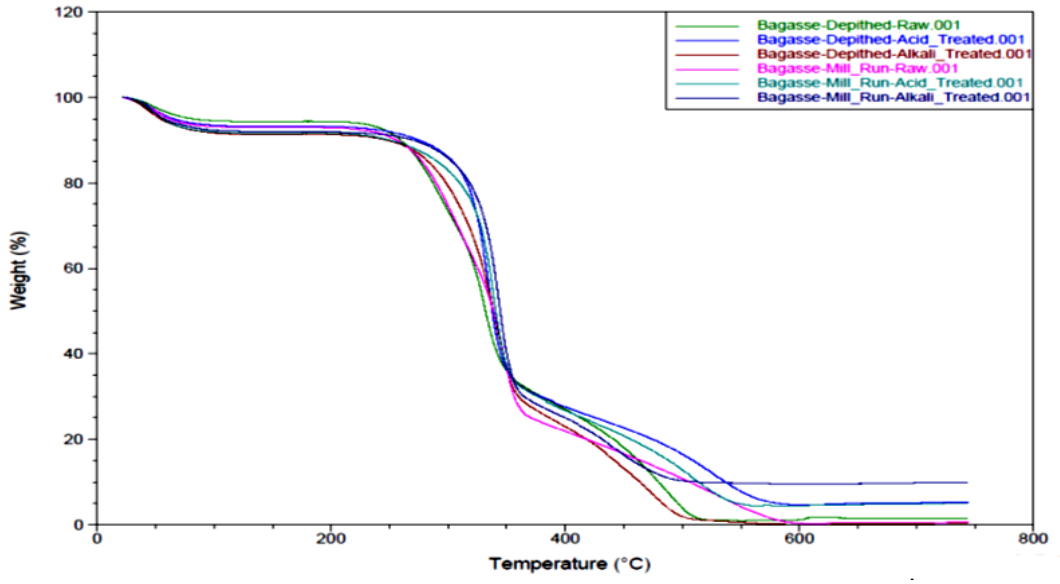

Fig. 11. TGA thermograms for raw and pre-treated mill-run and depithed bagasse

Table 3. Thermal Properties of Bagasse Fibres

Bagasse sample	Pre-treatment	Weight Loss (%)			Degradation temperature (°C)		
		100 °C	350 °C	400 °C	100 °C	350 °C	400 °C
Depith	Raw	5.7	60.7	31.9	192.1	351.0	495.0
	Acid	6.8	59.2	28.1	200.3	362.7	539.3
	Acid/alkali	8.7	62.8	28.1	190.9	356.2	480.2
MR	Raw	6.9	66.6	25.8	201.1	374.6	561.4
	Acid	8.4	59.6	27.4	186.8	362.1	519.6
	Acid/alkali	8.1	62.2	20.2	190.9	364.7	445.1

Depith - Depithed sugarcane bagasse
MR - Mill run sugarcane bagasse

CONCLUSIONS

1. The pre-treatment of the substrates with dilute sulfuric acid followed by sodium hydroxide proved to be successful, as the pore size of the regenerated pulps increased, which can be seen in the SEM images and the compositional analysis also showed the amount of hemicellulose and lignin that was removed from the sugarcane bagasse by the acid-alkali pre-treatment. This led to increased accessibility of the substrate to cellulase enzyme for hydrolysis. Other physical changes on the treated bagasse were determined by FTIR and TGA/DSC analysis.

2. The pre-treated substrate (10% w/v) when hydrolysed using an enzyme dosage of 30 FPU/gds (depithed bagasse) /40 FPU/gds (mill-run bagasse) with a surfactant of 0.4% Tween® 80 for 20 h resulted in 609 mg/gds (depithed bagasse) and 604 mg/gds (mill-run bagasse) total reducing sugars. These were the optimised conditions.

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