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Lignocellulosic Bioethanol Production from Grasses Pre-treated With Acid Mine Drainage: Modeling and Comparison of SHF and SSF

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Acid mine drainage (AMD) was used for the pre-treatment of indigenous South African grass (*Ergrostis curvula*), and compared to H_2SO_4 (1 wt%) pre-treatment. The optimal pre-treatment duration were investigated and found to be 1 day for H_2SO_4 and 3 days for AMD pre-treatment. The optimal biomass solid loadings were found to be 20 wt% for both pre-treatment methods. Additionally, enzymatic hydrolysis and fermentation to produce ethanol were investigated for both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In both SHF and SSF, the H_2SO_4 pre-treatment obtained higher concentrations of glucose/ethanol compared to AMD pre-treatment. The concentration of glucose/ethanol obtained using AMD pre-treatment was 70-80% of that achieved using H_2SO_4 pre-treatment. Empirical equations modeling the glucose/ethanol concentration in all processes were determined using a least squares method. Concentrations predicted by the models were found to have a high correlation (r^2 = 0.87–0.99) to concentrations determined experimentally.

Keywords: Dilute Acid Pre-treatment, Lignocellulosic Bioethanol; Separate Hydrolysis and Fermentation (SHF); Simultaneous Saccharification and Fermentation (SSF)

1 Introduction

The burning of fossil fuels is the cause of recently observed atmospheric and oceanic warming, and associated climate change (IPCC, 2014).Although the production of starch based bioethanol as an environmentally friendly alternative to fossil fuels has been growing (71 billion kg produced in 2015 (E4TECH et al., 2015)), its use has various drawbacks. These include increases in food prices, not being entirely carbon neutral, net energy losses, a reduction of available land for food production and land degradation (Lange, 2007).

To overcome these drawbacks, the production of bioethanol from lignocellulosic biomass (agricultural waste, forestry residue and energy crops) has been investigated (Sun and Cheng, 2002), and found to be more environmentally friendly (Searcy and Flynn, 2008). Lignocellulosic biomass consists of cellulose fibrils that are surrounded by a matrix of hemicellulose and lignin. The presence of the lingo-hemicellulose matrix hinders cellulase enzymes from accessing cellulose fibrils, preventing hydrolysis of cellulose to glucose. To overcome this, lignocellulosic biomass needs to undergo pre-treatment to fragment the ligno-hemicellulosic matrix, making the cellulose accessible to enzymes (Figure 1) (Mosier et al., 2005). Glucose produced in hydrolysis can then be fermented to bioethanol by *Saccharomyces cerevisiae* or other microorganisms (Yu and Zhang, 2003).

Various pre-treatment methods have shown effective, including physical, physico-chemical, chemical and biological (Sindhu et al., 2016). Chemical pre-treatment using dilute sulfuric acid has been shown to be the most favorable method of pre-treatment for industrial applications (Alvira et al., 2010; Burman et al., 2018b; Maurya et al.,

2015; Mosier et al., 2005; Taherzadeh and Karimi, 2008). Recently, Magowo et al (2015) demonstrated the suitability of acid mine drainage (AMD) as a source of dilute sulfuric acid for the pre-treatment of lignocellulosic biomass, producing glucose concentrations of up 4.8 g/L from subsequent enzymatic hydrolysis.

AMD is highly acidic (pH 1-3) water with high concentrations of sulfate (<20 g/L) and heavy metals (iron, aluminum <5 g/L) that is formed through a series of geo-chemical reactions when sulfite rich rocks are exposed to oxygen and water, often as a result of mining activities (Johnson and Hallberg, 2005).

AMD has been found to have a negative effect on aquatic and riparian ecosystems due to the toxicity effects of increased chemical concentration. As the pH increases naturally dissolved heavy metals deposit on the ecosystem floor (Simate and Ndlovu, 2014). There is a high prevalence of AMD in the Witwatersrand area and Mpumalanga and KwaZulu-Natal coalfields of South Africa due to mining of gold and coal respectively (Mccarthy et al., 2010).

In addition to the suitability of AMD for pre-treatment of biomass, it has also been shown that this could be combined with a process to remediate the AMD using sulfate reducing bacteria (SRB) (Magowo et al., 2015; Ramla and Sheridan, 2015). Long stem grasses have been identified as a good source of lignocellulosic biomass due to their prevalence in the Grassland and Savanah biomes in which both the Witwatersrand area and KwaZulu-Natal mines are located (Burman et al., 2018a, 2017; Westensee et al., 2018). However, these studies have yet to determine the optimum conditions of pre-treatment with AMD, or the final ethanol/glucose concentration achievable. There

is also a need for modeling of the reactions in order to perform a full process design and techno-economic evaluation.

Enzymatic hydrolysis and fermentation has been investigated in different reactor configurations including separate hydrolysis and fermentation (SHF) in which hydrolysis and fermentation occur in separate reactors, and simultaneous saccharification and fermentation (SSF), in which hydrolysis occur in the same reactor. Although SSF reduces the product inhibition of glucose on the hydrolysis reaction by keeping glucose concentration low, the compromised of reactor temperature of SSF (38°C) between optimal temperature for hydrolysis (45-50°C) and fermentation (30-37°C) (Olsson et al., 2006; Saha et al., 2005), results in decrease in the rate of both reactions. It is therefore not always clear which reactor configuration is more environmentally and economically favorable (Philippidis and Smith, 1995). Ethanol concentrations ranging from 4.72 – 16.0 g/L for SHF and 1.8 – 28.1 g/L for SSF have been achieved using various species of grass biomass (Mohapatra et al., 2017).

Previous studies have shown that glucose and ethanol concentrations from hydrolysis and fermentation can be predicted using kinetic models (Bansal et al., 2009; Liao et al., 2008; Ye and Berson, 2011; Zheng et al., 2009). Various different types of kinetic models have been developed including, Michaelis-Menton based models, adsorption based models and soluble substrate based models. Although these models predict concentrations over time well, the experimental work required to develop them is time and resource intensive. The use of these models outside of the conditions at which they were developed (biomass, pre-treatment, operating conditions) is also not applicable. It is thus proposed that an empirical model be developed to predict the

concentration of glucose and ethanol over time during both SHF and SSF, for biomass pre-treated with AMD.

This paper presents the optimum conditions for pre-treatment of long stem grass using AMD and H_2SO_4; determines the concentration of glucose and ethanol in SHF and SSF reactor configurations for biomass pre-treated with AMD and H_2SO_4, and develops an empirical mathematical model for the processes of SHF and SSF.

2 Materials and methods

The materials and methods is presented in 5 main sub-sections: Materials (characterization of AMD and grass); Optimization of biomass pre-treatment (determination of optimal pre-treatment for maximized enzymatic hydrolysis sugar recovery); Enzymatic hydrolysis and fermentation (performance of SHF and SSF using optimally pre-treated biomass); Analytical methods; and Empirical model development.

2.1 Materials

AMD was sourced from a coal mine in the eMalahleni district in South Africa. pH was determined using the Ohaus ST2100-F Bench top pH meter. Sulfate concentration was determined using a Merck Sulfate Test Kit #114548 (www.sigmaaldrich.com). Dissolved metal concentrations were determined by ICP-OES by UIS Analytical Services (www.uis-as.co.za) using method UIS-AC-T007. A summary of the results is presented in the results section.

An indigenous South African grass classified as *Ergrostis curvula* (Weeping Lovegrass or Oulandsgrass) was sourced from an agricultural supplier in Gauteng Province, South

Africa. The structural composition of this grass was determined using the National Renewable Energy Laboratory (NREL) method (Sluiter et al., 2008b). This method first determines the extractives through soxhlet extraction then determines the monosaccharide composition by hydrolyzing the grass using a two-stage concentrated/ dilute sulfuric acid, and then analyzing the hydrolysate for monosaccharides by high performance liquid chromatography (HPLC). The only deviation from this method was the use of a single stage soxhlet extraction using acetone as opposed to a two-stage ethanol/ water extraction. The structural composition is presented in the results.

Celluclast 1.5L cellulase enzyme (Novozyme), with an activity of 80 FPU/mL, was used for enzymatic hydrolysis at a dosage of 16.5 FPU/g substrate. This was mixed with 0.05M sodium citrate buffer (pH 4.8).

2.2 Optimization of biomass pre-treatment

The effect of pre-treatment time on the yield of enzymatic hydrolysis was evaluated. 20 g of grass in was loaded in 250 mL Erlenmeyer flasks with 200 mL of either 100 wt% AMD or 1 wt% H_2SO_4, incubated at 90°C and 100 RPM (Burman et al., 2018b). Flasks were removed from the incubator at 0, 24, 48, 96, 192 and 288 hrs for the AMD pre-treatment, and 0, 3, 6, 12, 24, 48, and 72 hrs for H_2SO_4 pre-treatment. Pre-treated biomass was washed with de-ionised water filtered and dried.

The effect of biomass solid loading during pre-treatment on the yield of enzymatic hydrolysis was evaluated. 250 mL Erlenmeyer flasks were loaded with 200 mL of either 1 wt% H_2SO_4 or AMD and 5, 10, 15 and 20 wt% grass. These were placed in an shaking incubator at 100 RPM and 90°C. After the optimum pre-treatment time, flasks were removed from the incubator and biomass was washed, filtered and dried.

Enzymatic hydrolysis of pre-treated biomass was then carried out by loading pre-treated biomass (20 wt%) into 250 mL Erlenmeyer flasks containing buffer/ cellulase mixture. These were placed in the shaking incubator at 50°C and 100RPM. Samples were taken after 24 and 48 hours and analysed for glucose.

The optimum pre-treatment time was taken as the time after which there was no longer a rapid increase in glucose produced in enzymatic hydrolysis. This was determined to be 24 hrs for H_2SO_4 pre-treatment and 72 hrs for AMD pre-treatment. The optimal solid loading was taken to be 20 wt%.

All further SHF and SSF experiments were conducted with the optimal pre-treatment conditions determined.

2.3 Enzymatic hydrolysis and fermentation

Many of the methods used in the evaluation of the performance of SHF and SSF were based the NREL LAP "SSF Experimental Protocols — Lignocellulosic Biomass Hydrolysis and Fermentation" (Dowe and Mcmillan, 2008), however as significant modifications to this method were required the method used has been described in full.

2.3.1 Yeast identification

Yeast for fermentation in both SHF and SSF was isolated from a commercially available home distillers' yeast that has been identified for its fast rate of fermentation and high ethanol tolerance (Alcotech Turbo-yeast 48).

The yeast was isolated by inoculating the yeast from packaging into a glucose solution, which was grown at room temperatures for 24 hrs. YPD agar slants (screw topped culture tubes with YPD agar set at an angle) were then produced from the

fermentation broth. Identification of the yeast strain was done using Multi Locus Sequence Typing (MLST) of the following loci ATF1, RPN2, NUP116, STE50 and YBL081W. Extraction of DNA and sequencing of loci was performed by Inqaba Biotech laboratories (http://www.inqababiotec.co.za/) according to the method described by Ayoub et al. (2006). Sequences were then analyzed using BLAST analysis on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) . As there was a lack of allele sequenced for these loci it was not possible to identify the specific strain, however it was confirmed that the micro-organism responsible for fermentation was *Saccharomyces cerevisiae*.

2.3.2 Yeast inoculation preparation

A loopful (10 µL) of *Saccharomyces Cerevisiae* was added to a 250 mL Erlenmeyer Flask, which contained 100 mL sterile medium (5 g/L glucose, 20 g/L peptone and 10 g/L yeast extract). The culture was incubated at 30°C and grown until the OD_{600} was between 0.5 and 0.6 (Dowe and Mcmillan, 2008).

2.3.3 Pre-treatment of biomass

To evaluate the performance of SHF and SSF a standard pre-treated biomass was produced as follows. 500 g of grass underwent pre-treatment at 90°C with either sulfuric acid or AMD at a solid loading ratio of 20 wt%. Sulfuric acid pre-treatment was carried out at 1 wt% H_2SO_4 for 24 hours. AMD pre-treatment was carried out using AMD for 72 hrs. All biomass was pre-treated in the same vessel to ensure the consistent pre-treatment of biomass for all enzymatic hydrolysis and fermentation experiments. Pre-treated biomass was washed, filtered and dried.

2.3.4 Separate hydrolysis and fermentation (SHF)

Pre-treated biomass was loaded into 250 mL Erlenmeyer flasks with 200 mL of buffer/ cellulase mixture at the following solid loading fractions 5, 10, 15 and 20 wt%. Experiments were carried out in duplicate. Flasks were placed in the shaking incubator operating at 50°C and 100 RPM. Samples were taken after, 0, 3, 6, 12, 24, 48, 72, and 168 hrs and analyze. At 168 hrs the slurry was filtered using Whatman 54 filter paper. The biomass was washed and dried.

The filtrate from the hydrolysis experiments was added to 250 mL Erlenmeyer flasks, and inoculated with 10 % v/v *Saccharomyces Cerevisiae* inoculum substituted with yeast extract (10 g/L) and peptone (20g/L). Flasks were placed in the shaking incubator at 35°C and 100 RPM. Samples were taken at 0, 6, 12, 24, 48, 72 and 96 hrs and analyzed.

2.3.5 Simultaneous saccharification and fermentation (SSF)

Pre-treated biomass was loaded into 250mL Erlenmeyer flasks with 200 mL of buffer/ cellulase mixture at solid loading fractions of 5-, 10-, 15- and 20 wt%. Experiments were carried out in duplicate. The flasks were inoculated with 10 % v/v *Saccharomyces Cerevisiae* inoculum substituted with the following nutrients Yeast extract (10 g/L), peptone (20g/L). Flasks were placed in the shaking incubator operating at 37°C and 100 RPM. Liquid samples were taken at 0, 8, 24, 48, 72, 96, 120, 144 and 168 hrs and analyzed.

2.4 Analytical methods

All liquid samples were centrifuged at 2348g for 10 minutes, then filtered through 0.45 µm syringe filters and stored for analysis. All samples were analyzed by HPLC for

xylose, glucose, cellubiose and ethanol using the Biorad-Aminex HPX-87H Column operating at 65°C using 0.05 M H_2SO_4 as the mobile phase at a flow rate of 0.8 mL/min and 20µL injection volume. The run time was set to 10 min. (Sluiter et al., 2008a)

The results from the SHF/SSF experiments were then used to develop an empirical model of the reactions taking place.

2.5 SHF empirical model development

Empirical equations were determined using a least squares method for fitting empirical equations. Different forms of equations were used for hydrolysis in SHF, fermentation in SHF and SSF due to the different nature of the data received.

2.5.1 Separate hydrolysis and fermentation (SHF)

The glucose concentration during hydrolysis in SHF was found to follow a logarithmic equation of the form.

$$G = M_1 ln(t_e) + C_1 \quad (1)$$

where G is the glucose concentration (g/L), t_e is the time of enzymatic hydrolysis (days), and M_1 and C_1 are constants.

Values for M_1 and C_1 were determined using a least square fitting function in Excel (LINEST). Values for M_1 and C_1 were determined for each wt% investigated. These were then plotted against the wt%. Both M_1 and C_1 were found to increase linearly with the increasing wt%. Equations for M_1 and C_1 as a function of wt% were determined, using the least squares function (LINEST) as shown below for M_1

$$M_1 = m_{1,M} \times W + c_{1,M} \quad (2)$$

where W is the biomass solid loading hydrolysis (wt%) and $m_{1,M}$ and $c_{1,M}$ are empirical constants.

Substituting the equations for M_1 and C_1 into Equation (1) we get an empirical equation that can predict the concentration of glucose during hydrolysis under the conditions mentioned above for a range of different solid loadings (5-20 wt%). The final form of the equation is presented in Table 2.

The rate of fermentation was found to have two distinct phases during fermentation *i.e.* a growth phase and a stationary phase. The rate of fermentation was found to be very different during the different phases, with the rate of reaction in the growth phase (r_g) being much slower than the rate of reaction in the stationary (r_s) phase. Both phases were found to last 6 hours. After 12 hours all glucose had been consumed, and thus no more ethanol was produced.

The concentration of glucose was thus equal to

$$G = G_0 - r_g \times t_f \qquad for\ t_f \in 0{:}\ 6\ hr \qquad (3)$$

$$G = G_0 - r_g \times 6 - r_s \times (t_f - 6) \qquad for\ t_f \in 6{:}\ 12\ hr \qquad (4)$$

Where G_0 is the initial glucose concentration and t_f is the fermentation time.

The ethanol concentration, $E\ (g/L)$ was determined based on the yield of ethanol produced from glucose consumed, $Y_{\frac{A}{P}}, (g/g)$. Note the stoichiometric maximum for this is 0.5111 g/g.

2.5.2 Simultaneous saccharification and fermentation (SSF)

The concentration of ethanol during SSF was found to follow a logarithmic equation of the form

$$E = M_2 ln(t_s) + C_2 \quad (5)$$

where E is the ethanol concentration; t_s is the time of SSF; and M_2 and C_2 are constants.

Values for M_2 and C_2 were determined using a least square fitting function in Excel (LINEST). Values for M_2 and C_2 were determined for each wt% investigated. With increasing solid loading, M_2 was found to increase linearly (similar to equation (2)), whereas C_2 was found to increase logarithmically according to

$$C_2 = m_{2,C} ln(W) + c_{2,C} \quad (6)$$

where W is the biomass solid loading in SSF (wt%) and $m_{2,M}$, $c_{2,M}$, $m_{2,C}$ and $c_{2,C}$ are empirical constants. The final form of the equation is presented in Table 2 in the results section. All empirical constants determined are presented in Table 3 in the results section.

The correlation between the experimentally determined data and the results that the model predicts was determined using excels CORREL function that determines the coefficient of determination (r^2).

3 Results and discussion

3.1 Grass composition

The composition of the biomass is presented in Table 1 along with the standard deviation (SD). Results from previous studies are also presented for weeping love grass (Torget et al., 1990); switch grass (Lee et al., 2007); elephant grass (Menegol et al., 2014) and dwarf napier grass (Wongwatanapaiboon et al., 2012). Switch grass, dwarf napier grass and elephant grass were chosen for comparison as they are grasses that have been found suitable feedstocks for bioethanol production.

The composition of weeping love grass determined in this study is similar to that reported by Torget et al. (1990). The cellulose content was found to be similar for all different grasses presented (35.3 – 38.0 %). The comparable cellulose content of weeping lovegrass to other grass species, indicates that it would be a suitable feedstock for the production of bioethanol from the C6 sugar platform. There was, however, significant variation in the hemicellulose content in the grasses presented. The hemicellulose content was much higher in switchgrass (28.5 %) and dwarf napier grass (34.2 %) than in weeping love grass (20.6 – 21.9 %) and elephant grass (20.5 %). The comparatively low hemicellulose content of weeping love grass indicates it would not be a good feedstock for the production of bioethanol through co-fermentation of bothC5 and C6 sugars.

3.2 AMD composition

The AMD was found to be highly acidic (pH 2.4) with a high concentration of sulfate (12.5 g/L). There was also a high concentration of dissolved iron (4.29 g/L) and low concentrations of dissolved magnesium (0.474 g/L) and aluminum (0.396 g/L). There

were trace concentrations (<0.1g/L) of various other metal such as manganese, silicone, sodium and zinc. Trace concentration of other elements were found, however elements with a concentration of less than 10 mg/L were not listed.

The quality of the AMD used in this investigation was relatively strong, with previous studies reporting AMD pH of 2 – 3, and sulfate concentrations of 3,5 g/L (McCarthy, 2011).The low pH of AMD should enable effective pre-treatment of biomass.

3.3 Optimum pre-treatment of grass

The results for the experiment to determine the optimum pre-treatment time (T) is presented in Figure 2 for both H_2SO_4 (a) and AMD (b). The results of the optimum pre-treatment solid loading ratio is presented in Figure 3 for both H_2SO_4 (a) and AMD (b).

The concentration of glucose initially increases rapidly with increasing pre-treatment time, followed by a steady increase (Figure 2). For H_2SO_4 pre-treatment, the glucose increases rapidly up to 1 day of pre-treatment (~42 g/L) after which the concentration does not increase significantly. For AMD pre-treatment the glucose concentration increases rapidly up to 3 days of pre-treatment (~25 g/L) after which it increases steadily until the 12^{th} day of pre-treatment (~35 g/L). The glucose yield was found to range between 30% - 45% for H_2SO_4 pre-treatment, although there was no obvious correlation between it and pre-treatment time. The Yield for AMD pre-treatment was seen to increase from 25% with no pre-treatment to 35% for 3 days of pre-treatment after which it does not increase significantly. For all following experiments the optimal pre-treatment time was taken to be 1 day for H_2SO_4 pre-treatment and 3 days for AMD pre-treatment, although further investigations should be carried out so as to more accurately determine the optimal pre-treatment time.

There is no correlation between the biomass solid loading in H_2SO_4 pre-treatment and the enzymatic hydrolysis glucose concentration, however the glucose yield was found to increase slightly with increasing biomass solid loading (Figure 3(a)). With AMD pre-treatment both glucose yield and concentration in enzymatic hydrolysis are seen to decrease slightly with increasing pre-treatment solid loading (Figure 3(b)). The low correlation between pre-treatment solid loading and enzymatic hydrolysis sugar concentration is similar to what was reported previously (Lu et al., 2009). Operating pre-treatment at high solid loadings is economically favorable due to the reduced reactor volume and chemical costs, however biomass was found to not be fully wetted at solid loading ratios above 20 wt%. The optimal pre-treatment solid loading was thus taken to be 20 wt% for both AMD and H_2SO_4 pre-treatment.

As can be seen in Figure 2 and Figure 3 there is only a slight increase in the glucose concentration and yield from 1 day of enzymatic hydrolysis to 2 days of enzymatic hydrolysis.

3.4 SHF & SSF experiments

The concentration of glucose during hydrolysis in SHF is presented in Figure 4

Increasing the solid loading results in an increase in final glucose concentration. This was found for both H_2SO_4 pre-treatment (Figure 4(a)), ranging from 14.41 g/L at 5 wt% to 50.56 g/L at 20 wt%, and AMD pre-treatment (Figure 4 (b)), ranging from 9.89 g/L at 5 wt% to 35.76 g/L at 20 wt%. An increase of glucose inhibition at higher solid loadings was not apparent.

A previous study that investigated the production of glucose from grass pre-treated with AMD achieved a maximum glucose concentration of 4.8 g/L (Magowo et al.,

2015). The final glucose concentration achieved in this study are significantly better than this at all biomass solid loading ratios.

It was found that in hydrolysis the final concentration of glucose produced from biomass pre-treated with AMD was 71.9 ± 2.9%% of that achieved in biomass pre-treated with H_2SO_4.

As can be seen in Figure 4, the proposed equations (Equations 1 – 2) fit the experimentally determined glucose concentration. The correlation between the measured and predicted glucose concentration ranges between 0.87 and 0.93 for H_2SO_4 pre-treatment, and 0.90 to 0.93 for AMD pre-treatment of the biomass. The predicted concentration is generally within the standard deviation of the measured concentrations. Notwithstanding this, the model appears to overpredict the glucose concentration initially (before 1 day) and thereafter predicts slightly less than that measured.

The concentration of ethanol and glucose during fermentation is presented in Figure 5, along with the model.

As can be seen in Figure 5, the proposed equations (Equations 3-4) fit the experimentally determined glucose and ethanol concentrations. The correlation between the measured and predicted glucose concentration ranges between 0.96 and 0.99 for H_2SO_4 pre-treatment, and 0.87 to 0.99 for AMD pre-treatment of the biomass.

Likewise the correlation between measured and predicted ethanol concentration ranges between 0.95 and 0.99 for H_2SO_4 pre-treatment, and 0.93 to 0.99 for AMD pre-

treatment of the biomass. This correlation indicates that these equations can be used to accurately model the glucose and ethanol concentrations in this process.

Ethanol produced from hydrolysates with a higher initial glucose concentration produced a higher final ethanol concentration. This was found for both hydrolysates produced from H_2SO_4 pre-treated biomass (Figure 5(a)), ranging from 5.29 g/L to 22.25 g/L, and AMD pretreated biomass (Figure 5(b)), ranging from 4.11 g/L to 14.83 g/L. As the ethanol concentration was relatively low in all experiments the effects of ethanol toxicity were not apparent. As increasing the solid loading did not result in an increase of either glucose inhibition or ethanol toxicity it is recommended that SHF to be conducted at 20 wt% solid loading.

It was found that in fermentation the final concentration of ethanol produced from biomass pre-treated with AMD was 73.5 ± 5.7% of that achieved in biomass pre-treated with H_2SO_4.

A review of bioethanol production from grass reported a final ethanol concentrations ranging between 4.72 – 16.0 g/L using SHF (Mohapatra et al., 2017). The final ethanol concentration achieved at 20 wt% in this study are towards the higher end of this range for AMD pre-treatment (14.83 g/L), and better than previously reported for H_2SO_4 pre-treatment (22.25 g/L). Although the final ethanol concentration achieved in this study was relatively good, the enzymatic hydrolysis used in this study was longer than in previous studies.

The concentration of ethanol produced during SSF is presented in Figure 6. The glucose concertation measured during SSF was found to be negligible at all times.

As can be seen in Figure 6, the proposed equations (Equations 5 – 6) fits the experimentally determined ethanol concentrations. The correlation between the measured and predicted ethanol concentration ranges between 0.87 and 0.93 for H_2SO_4 pre-treatment, and 0.87 to 0.94 for AMD pre-treatment of the biomass. This indicates these equations can be used to accurately model the ethanol concentration.

Increasing the solid loading results in an increase in final ethanol concentration for both H_2SO_4 pre-treatment (Figure 6(a)), ranging from 4.65 g/L at 5 wt% to 19.20 g/L at 20 wt%, and AMD pre-treatment (Figure 6(b)), ranging from 4.14 g/L at 5 wt% to 14.43 g/L at 20 wt%. As the ethanol concentration was relatively low in all experiments no ethanol toxicity was experienced, it is hence recommended that SSF be conducted at 20 wt% solid loading.

It was found that in SSF the final ethanol concentration produced from biomass pre-treated with AMD was 78.6 ± 6.9 % of that achieved with biomass pre-treated with H_2SO_4.

A review of bioethanol production from grass reported a final ethanol concentration ranging between 1.8 – 28.1 g/L using SSF (insert reference). The final ethanol concentration achieved at 20 wt% in this study are in the middle of this range for both AMD pre-treatment (14.43 g/L), H_2SO_4 pre-treatment (19.20 g/L). Although the use of different operating conditions in different studies makes direct comparison between studies less meaning full, the final ethanol concentration is a good indicator as operating conditions are often optimized to achieve a higher final ethanol concentration so as to reduce product separation cost.

Although the final ethanol concentration achieved in this study are comparable to previous studies for SSF and SHF using grasses, the final concentration of ethanol is still significantly lower than what is assumed to be achievable (53 g/L) using the most advanced technology and suitable feedstocks (Humbird et al., 2011).

A summary of the empirical equations used is presented Table 2, and the values for the different empirical constants are presented in Table 3.

The concentration of glucose and ethanol achieved from biomass pre-treated with AMD ranged between 67-89% of that produced from biomass pre-treated with H_2SO_4. The average was 74.8 ± 5.2%. The lower concentrations of glucose/ethanol obtained from AMD pre-treatment indicates a that it is a less effective pre-treatment. This may be due to a lower concentration of H^+ ions in the AMD compared to the 1% H_2SO_4 solution. Deposition of metals onto the grass surface during AMD pre-treatment may also hinder enzymatic hydrolysis.

Although lower final ethanol concentrations require more energy for product separation (distillation), the possible benefit of partial remediation of AMD or incorporation of the two process into one, may make pre-treatment with AMD advantageous. The potential for cheap/ free AMD may also provide added economic incentive for its use. It is recommended that further research be carried out so as to try achieved higher glucose/ ethanol concentrations from biomass pre-treated with AMD that are comparable to that of biomass pre-treated with H_2SO_4.

The high correlation between the experimentally determined and model predicted concentrations (both glucose and ethanol) indicates that the proposed equations

accurately model the glucose and ethanol concentration over time. The proposed equations are well suited to initial process design as determining the equation coefficients is less demanding of time and resources than more complicated models.

4 Conclusion

It was found that the optimum conditions for pre-treatment at low temperature (90°C) were 20 wt% solid loading for both AMD and H_2SO_4, with a pre-treatment time of 3 days for AMD pre-treatment and 1 day for H_2SO_4 pre-treatment. The AMD pre-treatment was found to achieve glucose and ethanol concentrations of 70-80% of the glucose and ethanol concentrations achieved with H_2SO_4 pre-treatment. Simple equations were proposed that were able to model the glucose and ethanol concentration in SHF and SSF based only on the solid loading of biomass and empirically determined coefficients.

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Table 1: Experimentally determined structural composition of Weeping Lovegrass, compared to results presented in literature for Weeping Lovegrass and Switchgrass .

	This study		(Torget et al., 1990)	(Lee et al., 2007)		(Menegol et al., 2014)		(Wongwatanapaiboon et al., 2012)	
Biomass Type	Weeping Lovegrass		Weeping Lovegrass	Switchgrass		Elephant Grass		Dwarf Napier[3]	
Structural Composition	Mean (%)	SD (%)	Mean (%)[1]	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
Hemicellulose	20.6	3.0	21.9%	28.5	3.5	20.51	0.023	34.19	1.24
Cellulose	35.3	3.0	36.7%	37.3	4.4	37.95	1.06	35.64	0.21
Acid Soluble Lignin	2.46	0.15	21.8%[2]	3.5	0.3	1.12	0.02	3.66	0.20
Acid Insoluble Lignin	26.1	5.19		16.2	0.5	19.65	0.78		
Ash	3.08	0.34	5.6%	5.9	1.0	8.53	0.23	0.13	0.12
Extractives	2.33	0.99	6.3%	3.1	0.7	8.84	0.83		
Other								26.38	1.38

[1]The standard deviation was not reported by this study.

[2]The lignin was not reported as acid soluble and acid insoluble lignin just as Klason lignin which was taken to be the total lignin content.

[3]The acid insoluble lignin and extractives were not reported in this study, however an “other” category was reported.

Table 2: Summary of empirical equations

Reaction	Equation	
SHF Hydrolysis	$G = (m_{1,M}W + c_{1,M})ln(t) + (m_{1,C}W + C_{1,C})$	
SHF Fermentation	$G = G_0 - r_g \times t_f$	$for\ t_f \in 0{:}\ 6\ hr$
	$G = G_0 - r_{lg}\ \times\ 6 -\ r_s\ \times\ (t_f - 6)$	$for\ t_f \in 6{:}\ 12\ hr$
SSF	$E = (m_{2,M}W + c_{2,M})ln(t) + (m_{2,C}ln(W) + c_{2,C})$	

Table 3: Empirically determined constants for equations modeling concentration of glucose and ethanol over time in SHF and SSF.

	Constant	Value for biomass pre-treated with H_2SO_4	Value for biomass pre-treated with AMD
Hydrolysis	$m_{1,M}$	0.2224	0.2432
	$c_{1,M}$	0.4858	0.3535
	$m_{1,C}$	1.0071	0.3625
	$C_{1,C}$	1.4986	1.2735
Fermentation	r_g	1.0282	0.7723
	r_s	7.1231	5.4772
	$Y_{\frac{P}{S}}$	0.4352	0.4043
	Yield (%)	85.15	79.10
SSF	$m_{2,M}$	0.1437	0.0973
	$c_{2,M}$	0.0135	0.0865
	$m_{2,C}$	1.8872	1.3983
	$c_{2,C}$	-1.4286	-0.8637