PURPLE GUINEA GRASS: PRETREATMENT AND ETHANOL FERMENTATION

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Treatment with dilute sulfuric acid (H_2SO_4) or calcium hydroxide ($Ca(OH)_2$) at 121°C and 103.4 kPa was used to improve the efficiency of the cellulose digestion of purple guinea grass. Cellulase hydrolysis of the dilute H_2SO_4-pretreated purple guinea grass under optimized conditions (6% (w/v) in 3% (w/v) H_2SO_4 for 30 min) yielded a slightly higher level of reducing sugars than that from the $Ca(OH)_2$ pretreatment under optimized conditions (6% (w/v) in 4% (w/v) $Ca(OH)_2$ for 5 min). However, the level of glucose released from the $Ca(OH)_2$-pretreated purple guinea grass was slightly higher than that from the dilute H_2SO_4 pretreatment. Ethanol fermentation, via the separate hydrolysis and fermentation (SHF) process using *Saccharomyces cerevisiae,* of the $Ca(OH)_2$-pretreated purple guinea grass and then hydrolyzed with commercial cellulase (9 PFU/g, dry wt.) for 6 h yielded ethanol at 0.44 g/g glucose (0.21 g/g cellulose) within 48 h, while that from the simultaneous saccharification and fermentation process yielded 14.3% less ethanol at 0.18 g/g cellulose within 96 h (including the 6 h saccharification time). The ethanol yield from the SHF process increased 1.14-fold to 0.497 g/g glucose (0.24 g/g cellulose) when the fermentation was performed in a 5 L fermentor.

Keywords: Purple guinea grass; Ethanol; Pretreatment

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INTRODUCTION

Thailand currently produces approximately 322 million liters per year of ethanol fuel from molasses and cassava (Bloyd 2009). These raw materials are food crops, so their use for bioethanol production is potentially in direct competition with food crop demands, leading to higher or unstable global and regional prices (Office of Agricultural Economics, Ministry of Agriculture, Thailand, 2011). Therefore, in addition to the utilization of the non-food (waste) parts of food crops, research into ethanol production for biofuel in Thailand has focused upon screening for abundant non-food, low-cost crops, as well as in improving the efficiency of their ethanolic conversion.

Purple guinea grass (*Panicum maximum* cv. TD53) is one of the popular forage plants grown in Thailand that gives a very high yield (9.4 to 25.0 tons /hectare) for 10 years or longer (Ria 2001), is easy to harvest, and self-regenerates after harvesting. In addition, purple guinea grass has a low agrochemical consumption and requires less intensive agricultural management. Moreover, it is resistant to drought and grows well on a wide variety of different types of soil and conditions, including being shade tolerant so that it can be grown under the shade of trees or bushes (Cameron and Lemcke 2008). Plantation of this crop, therefore, can thus be performed, for example, within the empty space of a fully growing agricultural area, such as pararubber plantation ditches. These characteristics make purple guinea grass a potentially ideal non-food, low-cost, and abundant crop that is easy to gather for a volume-controllable lignocellulosic substrate for ethanol production.

The efficient conversion of lignocellulosic biomass to fermentable sugars is restrained by its requirement for an efficient pretreatment to improve the accessibility of the cellulose component to cellulase (Taherzadeh and Karimi 2007). Most of the pretreatment methods currently evaluated are effective only when the mass and heat transfer limitations are overcome by particle size reduction (Sousa *et al.* 2009), which increases the economic and environmental cost of the process. Regardless, it is necessary to reduce the particle size prior to the pretreatment so as to increase the surface area to volume ratio and decrease the required enzyme penetration depth of the non-surface exposed material, as well as to decrease the cellulose crystallinity and degree of polymerization (Sousa *et al.* 2009).

This work deals with a comparison of the two most efficient of the current pretreatment methods, that of acid and alkali pretreatments, on the novel non-food crop lignocellulosic substrate, purple guinea grass. Asides their greater efficiency, acid and alkali pretreatment both use inexpensive chemicals, are simple processes, and require relatively simple equipment only (Sousa *et al.* 2009), Acid pretreatment solubilizes hemicellulose and decreases the cellulose crystallinity, whilst alkali pretreatment breaks the intermolecular interactions between lignin and hemicelluloses and so improves the porosity of the biomass (Keshwani and Cheng 2009). Although acid pretreatment liberates inhibitors of the growth and ethanol fermentation ability of *Saccharomyces cerevisiae* and other fermenting microorganism(s), lower levels of these inhibitors are produced if dilute acid, typically sulfuric acid (H_2SO_4), is used in the pretreatment (Taherzadeh and Karimi 2007; Sousa *et al.* 2009). For the alkali pretreatment, sodium hydroxide is the most popular reagent (Digman *et al.* 2010), but calcium hydroxide ($Ca(OH)_2$) has a lower cost, is less toxic than sodium hydroxide, and is recyclable, so reducing the environmental impact and economic cost (Saha and Cotta 2008). Despite the extra cost, the combination of either acid or alkali pretreatment with heat and/or high pressure enhances the cellulose digestibility even further so as to be typically economy-ically viable (Ramos 2003).

The enzymatic hydrolysis of cellulose by cellulases is under end-product negative feedback, in the form of glucose-mediated inhibition of the enzyme. To reduce this inhibition, the glucose released from the hydrolysis of cellulose can be simultaneously fermented to ethanol in a one-stage ethanol production process known as the simultaneous saccharification and fermentation (SSF) process (Ghosh *et al.* 1982).

Since there is an interdependence between the pretreatment method and the type of lignocellulosic substrate (Sousa *et al*. 2009), then one objective of this work was to evaluate the efficacy of either dilute H_2SO_4- or $Ca(OH)_2$-pretreatment on the subsequent cellulose digestibility of purple guinea grass, and to then compare the resultant ethanol yield from the pretreated purple guinea grass obtained from either a two-stage, separate hydrolysis and fermentation (SHF) process or the one-stage SSF process.

MATERIALS AND METHODS

Purple Guinea Grass

Purple guinea grass (*P. maximum* cv. TD 53) was collected from the Department of Livestock Development, Ministry of Agriculture, Pakchong district, Nakhon Ratchasima province, Thailand. It was oven-dried at 70 to 80 ^oC until at constant weight, then cut and hammer-milled to a fine powder, and sieved to a 20-40 mesh particle size.

Microorganism

Saccharomyces cerevisiae TISTR 5596 was obtained from the Thailand Institute of Scientific and Technological Research (TISTR). A single colony of *S. cerevisiae*, grown on yeast peptone dextrose agar (YPDA; yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, and agar 15 g/L, pH 4.5) at 30 °C for 24 h, was inoculated into YPDB (YPDA without agar) and incubated at 30 ^oC (200 rpm) for 24 h prior to transferring at 1% (v/v) to fresh YPDB, incubating at the same conditions and then being used as the inoculum.

Pretreatment and its Optimization

Pretreatment comprised of suspending the dried, milled, and sieved purple guinea grass particles (20 to 40 mesh size) in the selected concentration of either dilute H_2SO_4 or $Ca(OH)_2$ solution (50 mL in a 250 mL flask) at the indicated loading level and then autoclaving at 121^oC and 103.4 kPa for the indicated time. The partial optimization of the pretreatment process was performed via univariate analysis. First, the concentration of H_2SO_4 (1.0 - 3.5% (w/v)) or $Ca(OH)_2$ (1.5 - 3.0% (w/v)) was varied with a 3% (w/v, dry weight basis (DS)) loading and autoclaving for 30 min. The optimal concentration in each case, determined in terms of the amount of reducing sugar released when subsequently subjected to cellulose hydrolysis, was then selected for all subsequent trials. Next, the substrate loading was varied (3, 6 or 8% (w/v, DS)) with an autoclaving period of 30 min, and likewise the best loading level was then selected by the same criteria as being optimal for all subsequent trails. However, in the case of the $Ca(OH)_2$ pretreatment, variation in the substrate loading was evaluated with a constant substrate: alkali (w/w) ratio. Finally, with the optimally selected H_2SO_4 or $Ca(OH)_2$ concentration and substrate loading level, the autoclave time was varied between 0 to 60 min, and the optimal time selected using the same criteria. Note that in preliminary trials $Ca(OH)_2$ concentrations below 1.5% (w/v) were not optimal (data not shown) and so were excluded from this trial here.

Susceptibility of the pretreated purple guinea grass to cellulase hydrolysis was evaluated in terms of the amount of reducing sugar that was released. The dilute H_2SO_4 pretreatment slurry, which contained the pretreated purple guinea grass suspended in the pretreatment hydrolysate, was adjusted to pH 6.0 (the optimal pH for cellulase activity) by the addition of 10 N NaOH. The $Ca(OH)_2$ pretreatment slurry was filtered through a 40 mesh stainless steel sieve, and the resultant filtrate was centrifuged at 4 ^oC, 10,000 x g for 10 min to completely remove any residual $Ca(OH)_2$ powder from the pretreatment hydrolysate. The $Ca(OH)_2$ pretreated purple guinea grass was then resuspended in the previously separate pretreatment hydrolysate, and the pH was adjusted to 6.0 by the addition of 10 N H_2SO_4. Cellulase GC 220 at 6820 FPU/mL (Genecor International, Inc., USA) was added at 9.2 uL/g DS, equivalent to 63 FPU/g DS, and incubated with shaking (120 rpm) at 40 ^oC (the manufacturer's stated optimal temperature for this enzyme) for 72 h. After cellulose digestion the purple guinea grass residue was removed by filtering and centrifugation. The harvested supernatant was adjusted to pH 7.0 and analyzed for the reducing sugar content.

In addition, at the above determined optimal dilute H_2SO_4 and $Ca(OH)_2$ pretreatment conditions, the level of glucose, xylose and five of the potentially fermentation-inhibitory byproducts (furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde and vanillin) in the pretreatment hydrolysate were also evaluated after adjusting the pH to 7.0.

Cellulase Hydrolysis of the Pretreated Purple Guinea Grass

The pretreatment slurry, at 6% (w/v) substrate loading, was filtered and centrifuged (10,000 x g for 10 min at 4 ^oC) to separate the purple guinea grass particles from the pretreatment hydrolysate and, in the case of $Ca(OH)_2$ pretreatment, from $Ca(OH)_2$ residues. The pretreated purple guinea grass was then suspended in 100 mM sodium citrate buffer pH 5.0 at the same volume as the separated pretreatment hydrolysate. Accellerase^{TM} 1000 (265 FPU/mL, 236 pNPG β-glucosidase units/mL) (Genecor International, Inc., USA), which was used instead of the CG220 cellulase because it contains a higher activity ratio of ß-glucosidase: endoglucanase than GC 220, was added at a dose of 9 FPU/g DS and incubated at 50 ^oC for 6 h. The reaction was then filtered and centrifuged for 10 min at 4 ^oC and 11,292 x g. The harvested supernatant was analyzed for the level of reducing sugars and glucose. The enzyme dose (9 FPU/ g, DS) was selected from the glucose liberation efficiency of the enzyme over the first 6 h of hydrolysis (data not shown).

Ethanol Fermentation at a Small Laboratory Flask Scale (0.05 L)

Separate hydrolysis and fermentation (SHF) method

Purple guinea grass was pretreated by $Ca(OH)_2$ and then hydrolyzed by Accellerase^{TM} 1000 (9 FPU/g; DS) as detailed above. The cellulolytic hydrolysate was then sterilized by autoclaving at 110 ^oC, 68.93 kPa for 10 min and used as the fermentation medium. The *S. cerevisiae* inoculum, prepared as described above, was inoculated at 10% (v/v) into the fermentation medium and incubated at 30 ^oC without shaking for 72 h under oxygen limited conditions, by fermenting 40 mL in a 50 mL flask with a gas-tight cotton plug covered tightly with parafilm. After fermentation and

centrifugation, the obtained supernatant was analyzed for ethanol levels by gas chromatography (GC). The effect of 0-30 mM $(NH_4)_2SO_4$ supplementation of the fermentation broth, and of the presence or absence of the residual glucose in the *S. cerevisiae* inoculum (YPDB or glucose-free YPDB (YPDB-G) media, respectively), on the ethanol yield were also determined.

Simultaneous saccharification and fermentation (SSF) method

Purple guinea grass was pretreated with $Ca(OH)_2$ and suspended in 100 mM sodium citrate buffer pH 5.0 at the same volume as the separated pretreatment hydrolysate, as detailed above. This was then simultaneously hydrolyzed by Accellerase™ 1000 (9 FPU/g, DS) and fermented to ethanol at 30 °C under oxygen limitation by *S. cerevisiae* (10% (v/v) initial inoculum). At the end of the fermentation period the reaction was filtered and centrifuged, and the harvested supernatant was analyzed for ethanol levels by GC. The effect of varying the reaction temperature (25, 30, 35, 40, and 45 ^oC), pH (4.5, 5.0, and 5.5), reaction time (24, 48, 72, 96, and 120 h), addition of 0-30 mM $(NH_4)_2SO_4$, and the presence or absence of the residual glucose in the *S. cerevisiae* inoculum (YPDB or YPDB-G media, respectively), on the resultant ethanol yield were determined sequentially by univariate analysis with starting values of 30 °C, pH 5.0, 48 h, 0 mM $(NH_4)_2SO_4$, and with the yeast inoculum in YPDB, respectively.

Ethanol Fermentation in a 5 L Fermentor

Purple guinea grass was pretreated and hydrolyzed by Accellerase™ 1000 as described above. The cellulolytic hydrolysate, which was found to contain 12 g/L glucose, was used as fermentation medium in an *in situ* sterilizeable (110 ^oC, 68.93 kPa, 10 min) 5 L fermentor (B. E. Marubishi, model 10 L, Japan). The *S. cerevisiae* inoculum, prepared as described above, was centrifuged, and the harvested cells were resuspended in fresh YPDB-G medium to the same volume as the original centrifugate and was then used as the inoculum at 10% (v/v). Fermentation was performed at 30 ^oC, with agitation (100 rpm) and without aeration. The pH was controlled at 4.5 ± 0.4 by the addition of 4 N HCl or 5 N NaOH, as required. A 10% (w/v) antifoaming agent solution (Adecanol LG-805; Asahi Denka Co. Ltd., Japan) was used for controlling foam. Samples were withdrawn and centrifuged at 4 ^oC, 7227 x g for 10 min and the harvested supernatant was then analyzed for its ethanol content by GC.

Analytical Procedures

Cellulose, hemicellulose and lignin contents were determined by the Technical Association of Pulp and Paper Industry method (TAPPI 1988). Reducing sugars were quantified using the Somogyi-Nelson method (Somogyi 1952). Glucose, xylose and five pretreatment byproducts (furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde and vanillin) were analyzed by HPLC (Agilent 1100 Series) equipped with a quaternary pump, on-line degasser, autoinjector, and on-line thermostat, and controlled with the ChemStation software (Agilent Technology Co. Ltd., USA). Sugars were identified and quantified using an Aminex column HPX-87P (300 x 7.8 mm) with a Carbo-P micro-guard cartridge (Bio-Rad, USA). The column was maintained at 80 ^oC,

and 20 µL of each sample was injected at a time and eluted with Milli-Q filtered water at a flow rate of 0.6 mL/min by using a refractive index detector. Glucose was additionally analyzed with a glucose analyzer (YSI Ltd., England).

The chromatographic separations of the five selected pretreatment byproducts were carried out using a Pinnacle™ II C18 column (5 µm x 250 x 4.6 mm) (Krackeler Scientific, Inc., USA.), and eluted with a 1% (v/v) acetic acid: water mobile phase gradient starting from 85:15 to 60:40 (v/v) in 15 min at a flow rate of 1.0 mL/min. The column was maintained at 40 °C, and 5 µL of each sample was injected at a time, and the elutant was detected using a diode array detector at 275 nm.

Data Analysis

Data are shown as the mean ± one standard deviation (1 SD). To ascertain whether the differences between means or treatments were significant, the data were first confirmed to be normally distributed and of equal variance and were then analyzed by Analysis of Variance (ANOVA) and Holm-Sidak and Tukey ad hoc tests. Significance was accepted at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Raw Material Composition

The purple guinea grass (*P. maximum* cv. TD53) used in this study was found to be composed of 41.7% (w/w, DS) cellulose, 27.1% (w/w, DS) hemicellulose, and 10.4% (w/w, DS) lignin. Note that this cultivar is used as a fodder crop and has not yet been selected for improved cellulose content or ease of bioethanol fermentation.

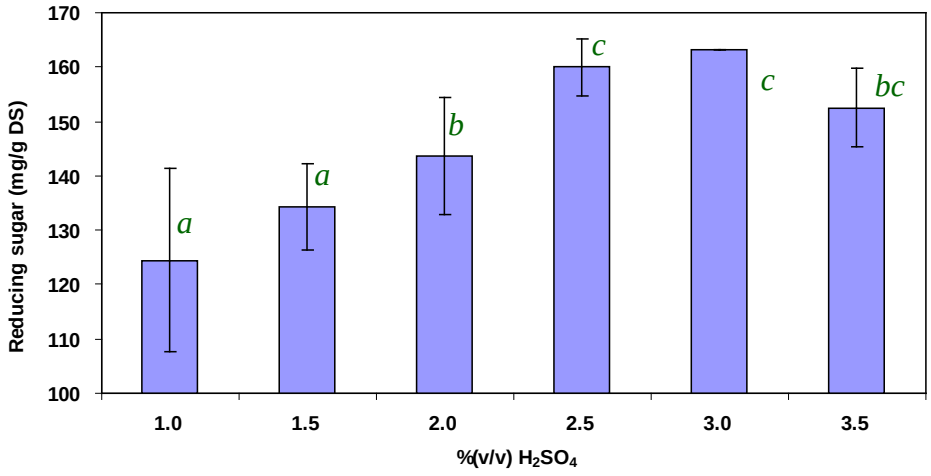

Fig. 1. Effect of the H_2SO_4 pretreatment concentration on the subsequent susceptibility to cellulase hydrolysis. Purple guinea grass (3% (w/v, DS)) was pretreated with the indicated concentration of H_2SO_4 for 30 min at 121 °C, and then subjected to cellulase hydrolysis (63 FPU/g DS for 72 h at 40 °C) prior to determining the reducing sugar level. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$).

Dilute Sulfuric Acid Pretreatment

The susceptibility of pretreated purple guinea grass to cellulase hydrolysis, measured in terms of the amount of released reducing sugars, increased as the H_2SO_4 concentration in the pretreatment increased from 1% (w/v) to a maximal level with 3% (w/v) H_2SO_4, at which point 163 mg/g DS of reducing sugar level was obtained (Fig. 1).

The amount of reducing sugar released per gram of substrate loaded increased when the purple guinea grass loading was increased from 3 to 6% (w/v, DS), but then it declined significantly at a substrate loading of 8% (w/v, DS) to the lowest observed level (Fig. 2).

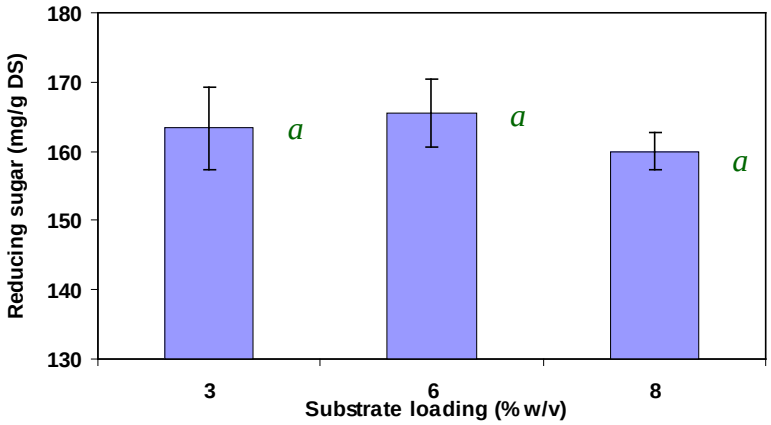

Fig. 2. Effect of the purple guinea grass loading level in the pretreatment process on the subsequent susceptibility to cellulase hydrolysis. Purple guinea grass was loaded at the indicated levels in 3% (w/v) H_2SO_4, autoclaved at 121 ^{o}C for 30 min, and then subjected to cellulase hydrolysis (63 FPU/g DS for 72 h at 40 ^{o}C) prior to determining the reducing sugar level. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$).

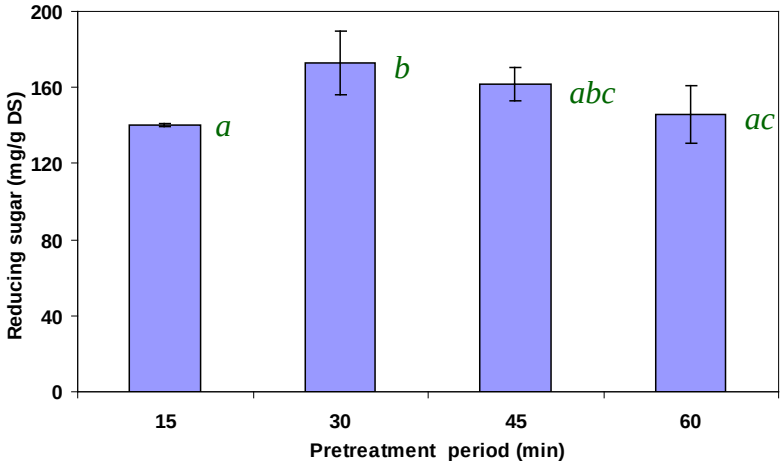

Fig. 3. Effect of the pretreatment period on the subsequent susceptibility to cellulase hydrolysis. Purple guinea grass (6% (w/v, DS)) was pretreated in 3% (w/v) H_2SO_4 at 121 ^{o}C for the indicated times and then subjected to cellulase hydrolysis (63 FPU/g DS for 72 h at 40 ^{o}C) before determining the reducing sugar level. Data are displayed as the mean ± 1SD, and are derived from triplicate tests. Means with a different lower case letter are significantly different ($p < 0.05$).

Increasing the autoclaving time from 15 to 30 minutes increased the yield of released reducing sugar, but further increases in the autoclave period to 45 and 60 min decreased the yield of reducing sugar slightly (Fig. 3).

Thus, from this univariate analysis, the partially optimized acid pretreatment conditions were selected as a purple guinea grass loading of 6% (w/v, DS) suspended in 3% (w/v) H_2SO_4 and heated at 121^{o}C, 103.4 kPa for 30 min. Under these conditions, 173 mg/g DS reducing sugar was subsequentially released after hydrolysis by cellulase GC220 (63 FPU/g, DS) for 72 h at 40 ^{o}C.

Calcium Hydroxide Pretreatment

With respect to the alkali pretreatment, purple guinea grass loaded at 3% (w/v, DS) was most susceptible to cellulase hydrolysis after pretreatment with 2.0% (w/v) $Ca(OH)_2$ (Fig. 4), which represents a substrate: $Ca(OH)_2$ (w/w) ratio of 1.5:1.

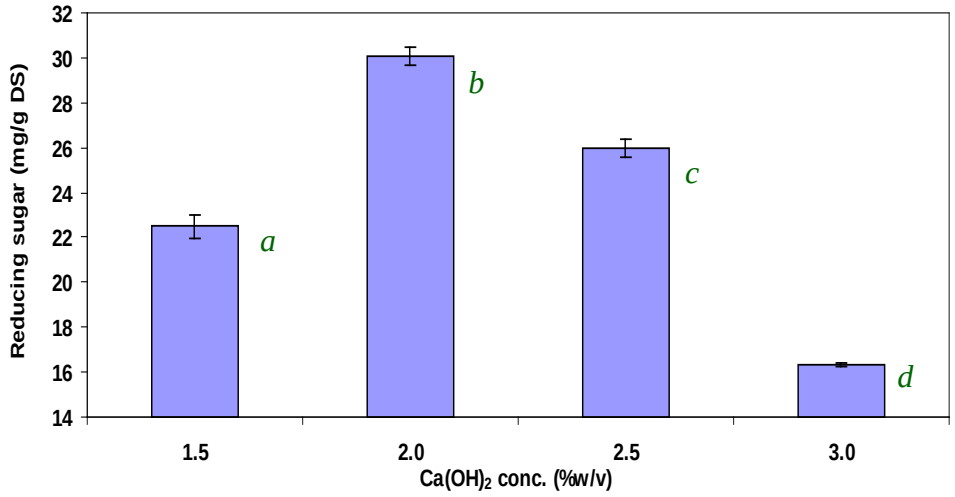

Fig. 4. Effect of the $Ca(OH)_2$ pretreatment concentration on the subsequent susceptibility to cellulase hydrolysis. Purple guinea grass (3% (w/v, DS)) was pretreated with the indicated concentrations of $Ca(OH)_2$ at 121 ^{o}C for 30 min and then subjected to cellulase hydrolysis (63 FPU/g DS for 72 h at 40 ^{o}C) prior to determining the reducing sugar level. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.001$).

Maintaining this 1.5:1 substrate: $Ca(OH)_2$ (w/w) ratio, the apparent susceptibility to cellulase hydrolysis of the pretreated purple guinea grass increased when the loading level increased to 6% (w/v, DS), although that observed at 8% (w/v, DS) was essentially the same and statistically not significantly different to that at 6% (w/v, DS) (Fig. 5).

Finally, at a 6% (w/v, DS) loading and a $Ca(OH)_2$ (w/w) ratio of 1.5:1, the maximum reducing sugar release occurred after 5 min autoclaving, remained essentially the same at 10 and 15 min autoclaving, and then it decreased significantly (~1.3- to ~two-fold) with increasing autoclave times from 30 to 60 minutes (Fig. 6). That autoclave periods of longer than 15 min for the $Ca(OH)_2$ pretreatment lowered the observed saccharification efficiency is likely to be because the increase severity of the pretreat-

ment caused carbohydrate loss and so a lower level of reducing sugars being detected after hydrolysis.

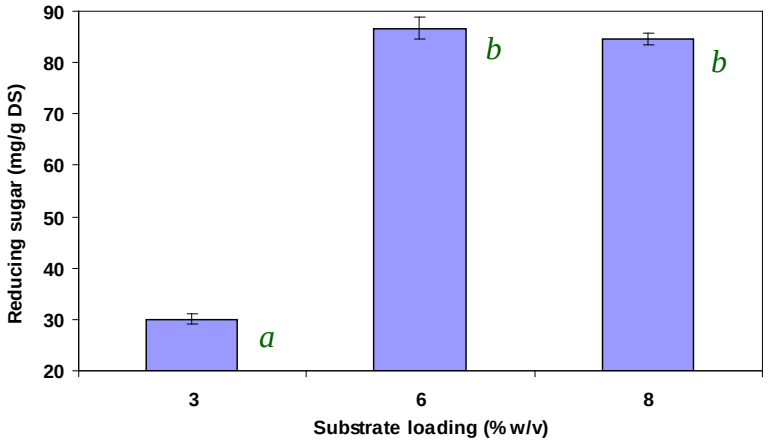

Fig. 5. Effect of the purple guinea grass loading level in the alkali pretreatment process on the subsequent susceptibility to cellulase hydrolysis. Purple guinea grass was pretreated with 1.5 g/g $Ca(OH)_2$ at 121 ^{o}C for 30 min and then subjected to cellulase hydrolysis (63 FPU/g DS for 72 h at 40 ^{o}C) prior to determination of the reducing sugar level. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.001$).

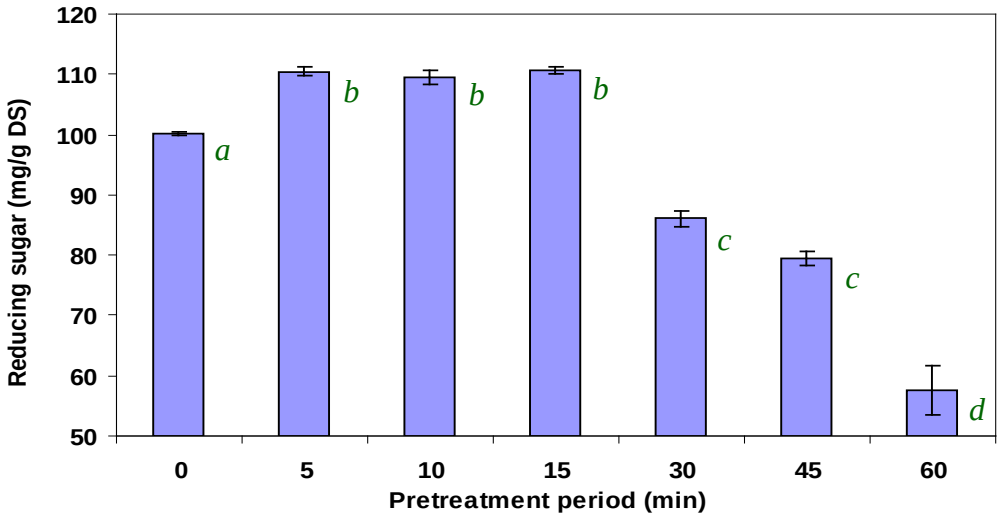

Fig. 6. Effect of the alkali pretreatment period on the subsequent susceptibility to cellulase hydrolysis. Purple guinea grass (6% (w/v, DS)) was pretreated with 1.5 g/g $Ca(OH)_2$ at 121 ^{o}C for the indicated time periods and subsequently subjected to cellulase hydrolysis (63 FPU/g DS for 72 h at 40 ^{o}C) prior to determination of the reducing sugar level. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.001$).

Thus, from this univariate analysis, the partially optimized alkali pretreatment conditions were selected as a purple guinea grass loading of 6% (w/v, DS) suspended in 4% (w/v) $Ca(OH)_2$ and heated at 121°C, 103.4 kPa for 5 min. Under these conditions 110.5 mg/g, DS of reducing sugars were released after hydrolysis by cellulase GC220 (63 FPU/g, DS) for 72 h at 40 °C, which is *ca*. 1.56-fold lower than the reducing sugar level obtained by the partially optimized acid-pretreatment.

Pretreatment Sugars and Byproducts

The hydrolysate obtained after pretreatment of purple guinea grass at the optimized alkali or acid pretreatment conditions was analyzed for the level of furfural, hydroxymethylfurfural, 4-hydroxybenzaldehyde, syringaldehyde, and vanillin, which are known to inhibit ethanol fermentation by *S. cerevisiae* (Delgenes *et al*. 1996; Olsson and Hahn-Hagerdal 1996). The concentration of four of these five byproducts was significantly higher in the H_2SO_4-pretreated hydrolysate than in the corresponding $Ca(OH)_2$-pretreatment, with only syringaldehyde being higher in the alkali-pretreated hydrolysate (Table 1). Note that although the lignin-derived phenolic byproducts are more soluble in alkaline conditions, the neutralization of the alkali hydrolysate before analysis may have removed them by precipitation (Chandel *et al*. 2011). In addition, calcium ions crosslink lignin (derivatives) molecules under alkali conditions, and so this substantially decreases the lignin (derivatives) solubilization in the $Ca(OH)_2$-pretreatment hydrolysate (Xu *et al*. 2010). However, in all cases the levels of these five byproducts were much lower than the concentration required for the effective inhibition of *S. cerevisiae* growth and ethanol fermentation (Delgenes *et al*. 1996; Olsson and Hahn-Hagerdal 1996). In contrast to the alkali pretreatment, xylose and glucose, which are thermochemical degradatives of hemicellulose, were detected in the dilute H_2SO_4-pretreatment hydrolysate (Table 1). The xylose is, however, not fermentable to ethanol by the *S. cerevisiae* used in this study (selected for its greater ethanol tolerance than the xylose-utilizing *Pichia stipitis*), and so it remains to be evaluated whether the H_2SO_4-pretreated hydrolysate may be more optimally fermented by the recombinant xylose-utilising *S. cerevisiae* (Matsushika and Sawayama 2008).

Table 1. Pretreatment Byproduct and Sugar Levels in the Pretreatment Hydrolysate Obtained with the Optimized Pretreatment Condition [a]

By products	H_2SO_4 (mg/g DS)	$Ca(OH)_2$ (mg/g DS)	MIC[b] (g/L)
Hydroxymethylfurfural (HMF)	0.124 ± 0.024	ND	1
Furfural	0.303 ± 0.034	ND	1
4-Hydroxybenzaldehyde	0.024 ± 0.003	0.010 ± 0.000	0.75
Vanillin	0.035 ± 0.003	0.008 ± 0.000	0.5
Syringaldehyde	ND	0.017 ± 0.001	0.75
Xylose	71.5 ± 5.9	ND	-
Glucose	23.0 ± 0.1	ND	-

[a] Data are shown as the mean ± 1 SD and are derived from three repeats.
[b] MIC = Minimum reported concentration required to inhibit the growth of and ethanolic fermentation by *S. cerevisiae* (Delgenes *et al*. 1996; Olsson and Hahn-Hagerdal 1996).
ND = Not detected

Cellulase Hydrolysis of the Pretreated Purple Guinea Grass

Dried and milled purple guinea grass particles were pretreated at 6% (w/v, DS) substrate loading with dilute H_2SO_4 or $Ca(OH)_2$ at the respective optimized conditions, and then the particles were hydrolyzed by Accellerase™ 1000 (9 FPU/g, DS) at 50 °C, pH 5.0 for 6 h. The level of glucose released from the $Ca(OH)_2$-pretreated purple guinea grass (11.9 g/L) was slightly (1.18-fold) higher than that released from the H_2SO_4-pretreated samples (10.1 g/L). Increasing the Accellerase™ 1000 level two-fold (to 18 FPU/g, DS) caused the amount of glucose released from the $Ca(OH)_2$-pretreated purple guinea grass to increase slightly (1.05-fold) to 12.5 g/L, but resulted in the glucose released per unit of the enzyme to significantly (1.9-fold) decrease from 11.2 to 5.9 mg/unit. Based on the forgoing consideratoins, alkali-pretreatment and Accellerase™ 1000 at 9 FPU/g DS were chosen for further experiments.

The amount of reducing sugar obtained after cellulase hydrolysis of purple guinea grass (6% w/v, DS) pretreated with dilute H_2SO_4 (3% (v/v), 30 min) while suspended in the pretreatment hydrolysate (173 mg/g) was significantly (1.57-fold) higher than that pretreated with $Ca(OH)_2$ (4% (w/v), 5 min) (110.5 mg/g). Since hemicellulose solubilizes in acid, and the presence of glucose and xylose were only detected in the dilute H_2SO_4-pretreatment hydrolysate (Table 1), then after pretreatment the purple guinea grass was separated from the hydrolysate and subjected to cellulose hydrolysis in 100 mM sodium acetate buffer (pH 5.0). Following cellulase hydrolysis, a slightly higher (1.19-fold) level of glucose was detected in the $Ca(OH)_2$-pretreated purple guinea grass (11.9 g/L) than from the dilute H_2SO_4-pretreated samples (10.1g/L).

The described results agree with the report of Nlewem and Thrash (2010) that switchgrass pretreated with NaOH (0.5% (w/v), 90 ^oC, 1 h) was more susceptible to cellulase hydrolysis than when it was pretreated with dilute H_2SO_4 (0.5% (v/v), 121 ^oC, 103.4 kPa, 1 h). Evidence of pore formation on the surface of the switchgrass fibers was visible when pretreated with NaOH, but no physical changes were apparent after dilute H_2SO_4 pretreatment. The presence of such pores increases the surface area that is accessible by cellulase, whilst the lignin content of the NaOH-pretreated, but not the dilute H_2SO_4-pretreated switchgrass was considerably decreased. However, the improvement in the cellulase hydrolysis of the acid-pretreated switchgrass was attributed to the alteration of the chemical composition (xylan to xylose) and neither by the increased internal surface area for cellulase access nor by lignin removal. The optimal switchgrass (0.4 mm particle size) pretreatment conditions were reported by Kim *et al*. (2011) to vary with the pretreatment, and were: ammonia fiber expansion (AFEX, 2 mm particle size, NH_3 1.5 g/g, 150 ^oC, 30 min), dilute acid (H_2SO_4 0.045 g/g, 160 ^oC, 10 min), liquid hot water (H_2O 6.7 g/g, 200 ^oC, 10 min), alkali ($Ca(OH)_2$ 1 g/g, 120 ^oC, 4 h) and soaking aqueous ammonia (NH_4OH 1.35 g/g, 90 ^oC, 24 h). The glucose yield obtained after cellulase hydrolysis of $Ca(OH)_2$-pretreated switchgrass was higher than that from the dilute H_2SO_4-pretreatment as well as the other pretreatment methods. The effect of $Ca(OH)_2$- and dilute H_2SO_4- pretreatment on the susceptibility to cellulase hydrolysis of purple guinea grass reported here then agrees well with that previously reported for switchgrass and discussed above (Kim *et al*. 2011).

Ethanol Fermentation at Flask Scale

Separate hydrolysis and fermentation (SHF)

The hydrolysate obtained after cellulase hydrolysis, which contained 11.9 g/L glucose, was used as the substrate for ethanol fermentation by *S. cerevisiae* (initial inoculum of 10% (v/v)) for 72 h. The level of ethanol produced in the fermentation broth was analyzed every 12 h, revealing a maximum ethanol level (5.9 g/L) at 48 h (data not shown). Supplementation of the hydrolysate with up to 30 mM $(NH_4)_2SO_4$ did not increase the ethanol yield (data not shown), whilst avoiding the introduction of the residual glucose in the yeast inoculum by exchanging the YPDB for YPDB-G medium, reduced the ethanol yield obtained 1.13-fold to 5.24 g/L (equivalent to 0.44 g ethanol/g glucose or 0.21 g ethanol/g cellulose).

Simultaneous saccharification and fermentation (SSF)

The partial optimization of the SSF of the $Ca(OH)_2$-pretreated purple guinea grass was attained by the sequential univariate analysis of the optimal fermentation temperature, pH and fermentation period. With respect to the temperature and pH, the maximum ethanol yield (3.85 g/L) was produced at 30 ^oC and pH 5.0 (data not shown), and this increased to 4.65 g/L when the fermentation period was prolonged to 96 h onwards (Fig. 7). Supplementation of the hydrolysate with $(NH_4)_2SO_4$ up to 30 mM did not significantly alter the ethanol yield (data not shown), whilst avoidance of the addition of residual glucose from the YPDB media of the yeast inoculum by using yeast resuspended in YPDB-G decreased the ethanol yield ~1.04-fold to 4.45 g/L. However, the cellulase hydrolysis and ethanol fermentation in this SSF process may not have been performed under optimal conditions (Saha and Cotta 2008), and so likely represents a lower than optimal ethanol yield and requires further optimization. Nevertheless, the potential of purple guinea grass as a substrate for bioethanol production remains.

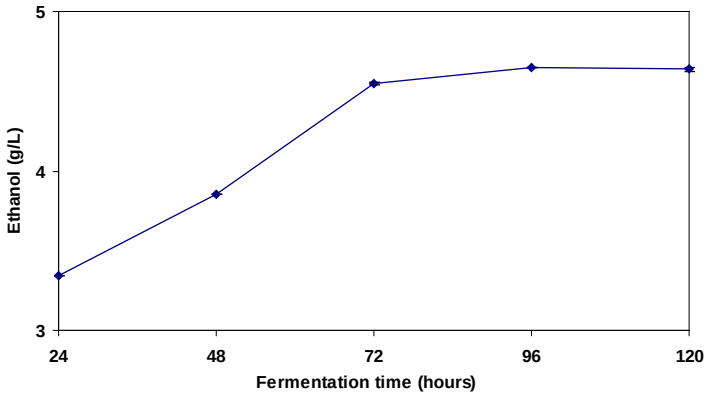

Fig. 7. Time course of ethanol production at 30 °C, pH 5.0 by the simultaneous saccharification and fermentation (SSF) process of $Ca(OH)_2$-pretreated purple guinea grass. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$).

Ethanol Fermentation in a 5 L Fermentor

Purple guinea grass loaded at 6% (w/v, DS) was pretreated by $Ca(OH)_2$ under the partially optimized conditions and then further hydrolyzed with Accellerase ™1000 at 9 FPU/g DS for 6 h. The hydrolysate obtained after cellulase hydrolysis, which was found to contain 12.0 g/L glucose, was used as the substrate for ethanol fermentation in an *in situ* sterilizeable 5 L fermentor. *S. cerevisiae*, resuspended in YPDB-G, was used as the inoculum at 10% (v/v). Under these conditions the maximum ethanol yield (5.92 g/L; equivalent to 0.497 g ethanol/g glucose or 0.24 g ethanol/g cellulose) was produced after 9 h of fermentation, and declined slightly to an almost stable level at 12 h onwards (Fig. 8).

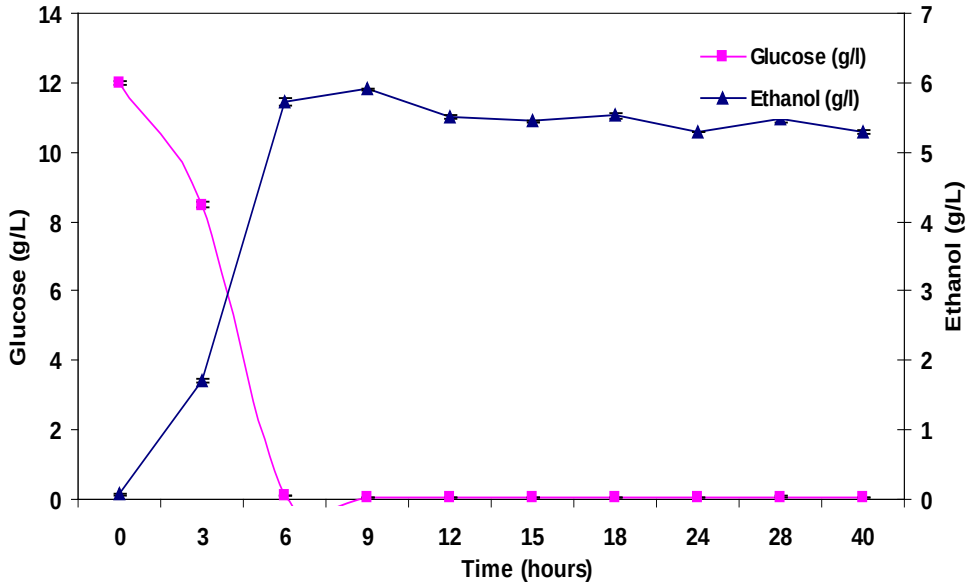

Fig. 8. Separate hydrolysis and ethanol fermentation (SHF) of $Ca(OH)_2$-pretreated purple guinea grass in a 5 L fermentor. The data are displayed as the mean ± 1SD, and are derived from triplicate experiments. Means with a different lower case letter are significantly different ($p < 0.05$).

The theoretical ethanol production yield from glucose is 0.51 g ethanol/g glucose. Depending on the feedstock and process, the actual yield obtained can vary from 60% to 90% of the theoretical yield (Onsoy *et al.* 2007). The ethanol production yields (g ethanol/g glucose) in this report were 0.44 (flask scale) and 0.49 (5 L fermentor), which are 86% and 96% of the theoretical yields, respectively.

A low ethanol production yield (0.37 g/g glucose) was reported from the SSF of $Ca(OH_2)$-pretreated switchgrass, which was suggested to be the result of a low cellulose digestibility (Chang *et al*. 2001). Dilute acid-pretreated switchgrass gave a slightly higher ethanol production yield when fermented by the SHF process (0.47 g/g glucose) than by the SSF method (0.46 g/ g glucose) (Chung *et al*. 2005). Li *et al*. (2009) reported an ethanol production yield of 0.48 g/g glucose from phosphoric acid-acetone-pretreated Burmuda grass by the SSF method (25 FPU/ g DS cellulose, pH 5.0, 38 ^{o}C, 150 rpm, 96

h using *S. cerevisiae*). In contrast, lower ethanol production yields from switchgrass pretreated with dilute H_2SO_4 (0.24 g/g glucose) and $Ca(OH)_2$ (0.28 g/g glucose) at ambient temperature and pressure by the SSF method were reported by Digman *et al*. (2010). The SSF conditions used in this case were a 10% (w/w, DS) substrate loading, 5 FPU/g DS of Cellulase, plus 15 IU/g DS of β-glucosidase with the hydrolysate set to pH 4.5, supplemented with 10 g/L yeast extract and 20 g/L peptone and fermented by *S. cerevisiae* at 35 ^{o}C for 72 h. However, the low ethanol production yield obtained was likely to be due to the use of ambient temperature and pressure in the pretreatment.

The low ethanol concentration obtained from the purple guinea grass in this work is due to the fact that the cellulose content of purple guinea grass was not fully utilized. Bearing in mind the economic concerns of commercial production, a low substrate and cellulase loading was used. Therefore, it is likely that the ethanol concentration could be improved by maximizing the amount of glucose liberated through increasing the substrate loading in the pretreatment step and the cellulase loading in the cellulase hydrolysis step. Optimization of the β-glucosidase and endoglucanase ratio of the cellulase used will also likely improve the amount of glucose liberated during the cellulase hydrolysis step.

CONCLUSIONS

$Ca(OH_2)$-pretreated purple guinea grass was more susceptible to cellulase digestion than that from dilute H_2SO_4 pretreatment. Fermentation of the $Ca(OH_2)$-pretreated purple guinea grass (100 g containing 41.7 g of cellulose) by the SHF and SSF methods yielded 8.76 and 7.51 g of ethanol per 100 g pretreated purple guinea grass, respectively, at a 0.05 L scale, but this was increased to 10.01 g of ethanol by using the SHF system in a 5 L fermentor. The high ethanol production yield (96% of theoretical), including a high cellulose content (41.7% w/w), along with the high productivity, ease of harvesting and self-regeneration after harvesting, a low agrochemical consumption, and low agricultural management requirement, means that purple guinea grass, currently farmed as a forage crop, is also a promising herbaceous energy crop.

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