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Evaluation of dilute-acid pretreated bagasse, corn cob and rice straw for ethanol fermentation by *Saccharomyces cerevisiae*

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Abstract - Bagasse, corn cob, and rice straw agricultural wastes were found to consist of 37, 39 and 34% cellulose and 24, 41 and 22% hemicellulose, respectively, on a dry solid (w/w) basis and thus have the potential to serve as a low cost feedstock for ethanol production. Hydrolysates produced by dilute-acid pretreatment followed by cellulase digestion were evaluated as substrates for ethanol fermentation by *Saccharomyces cerevisiae*. After pretreatment by 141 mM sulphuric acid, bagasse waste released glucose (134 mg/g) at a higher level than that from corn cob (75 mg/g) and rice straw (8 mg/g). Hydroxymethylfurfural (HMF) levels derived from acid pretreatment of bagasse (1.5 g/l), but not corn cob (0.8 g/l) or rice straw (0.1 g/l) attained levels likely to be toxic (1.5 g/l) for *S. cerevisiae* growth and ethanol fermentation rates. All three agricultural wastes released likely non-toxic levels of furfural (< 0.5 g/l) and lactic acid (negligible for bagasse and rice straw and 0.7 g/l for corn cob). After cellulase saccharification of the dilute-acid pretreated agricultural wastes, the glucose content of corn cob hydrolysates (13 ± 0.17 g/l) was marginally higher than that of bagasse (12 ± 0.27 g/l) or rice straw (11 ± 0.07 g/l), yet the ethanol conversion yield by *S. cerevisiae* on corn cob hydrolysate (0.45 ± 0.006 g/g) was lower than that attained with bagasse hydrolysate (0.49 ± 0.007 g/g). Synergistic adverse effects between furfural and HMF with weak acids, or other lignin derived products in the corn cob hydrolysate are proposed as the effective inhibitor(s) for ethanol fermentation by *S. cerevisiae.*

Key words: dilute-acid pretreatment, corn cob, bagasse, hydroxymethylfurfural, ethanol.

INTRODUCTION

Ethanol, a renewable, sustainable and true-green energy source, has gained increasing interest in recent decades as an alternative energy source, since non-renewable fossil fuels are not environmentally friendly, are relatively quickly running out and the price is rapidly increasing in line with increasing demands. In Thailand, fuel grade ethanol is produced from sugar cane molasses and cassava as a partial gasoline replacement up to 10% (v/v) ethanol (gasohol). The use of gasohol for vehicles significantly reduces the net carbon dioxide (a greenhouse gas) emission (Wang *et al*., 1999) and provides cleaner combustion compared to MTBE (methyl tertiary butyl ether), the most common additive in gasoline (McCarthy and Tiemann, 1998). However, the current production cost of ethanol is relatively high compared to that for fossil fuels. Moreover, these primarily starch or simple sugar sources as substrates leave high oxygen demand waste water as an environmental contaminant, and only reduce greenhouse gas emissions some 20-25% relative to petroleum. In order to be cost competitive, especially without government subsidies (green tax), cheaper but plentiful substrates for ethanol production are required to both increase the production capacity and to reduce the production costs of fuel grade ethanol. Potential raw materials for low-cost ethanol production include lignocellulosic biomasses, and especially those from agricultural wastes since these are not only very low in cost but are also abundant. Moreover, importantly, they are not competitive with food production (in contrast to say sugar cane, beetroot, corn and even cassava) and thus are free from the ethical and economical issues of food shortages and corresponding price increases. In addition, their use in ethanol production has the potential to be more environmentally sound with a reduction in greenhouse gas emissions of potentially around 80% relative to petroleum, and a use of rather than dumping of high oxygen demand wastes.

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Lignocellulosic biomasses are typically composed of approximately 40-50% (w/w) cellulose and 25-33% (w/w) hemicellulose which can both be hydrolysed to the fermentable sugars, glucose and xylose, respectively, as well as L-arabinose. The glucose component can be efficiently fermented to ethanol by the yeast *Saccharomyces cerevisiae* or the bacterium *Zymomonas mobilis*, whereas the xylose can be fermented to ethanol by the yeast *Pichia stipitis* (Nigam, 2002). More complicated fermentations including to form butanol and ethanol can be attained using various species from the bacterial genus *Clostridium*, but these require strict anerobic conditions are, currently, more expensive. Within ethanol production, there have been attempts to co-ferment the glucose and xylose, as well as L-arabinose, by genetically engineered *Saccharomyces* sp. (Ho *et al*., 1998) and *Escherichia coli* (Dien *et al*., 2000), but to date these have failed to yield satisfactory ethanol yields.

The factors that have been identified as significantly affecting the hydrolysis of lignocellulosic biomasses are the porosity (accessible surface area) of the cellulose, the cellulose fibre crystallinity, and the lignin and hemicellulose contents (McMillan, 1994). The presence of lignin and hemicellulose inhibits the access of cellulases into the cellulose reducing the efficiency of hydrolysis to useable reducing sugars (glucose). Indeed, for example one study estimated that some 30-40% of free reducing sugar ends in cellulose are inaccessible to exoglucanases (Kongruang *et al*., 2004). Dilute-acid pretreatment improves the hydrolysis by efficiently removing the hemicellulose, opening up the cellulose accessibility and also resulting in high xylose release levels. This resulting high level of xylan to xylose conversion favours the overall economics of the process because xylan accounts for up to one third of the total carbohydrate in many lignocellulosic biomasses (Hinman *et al*., 1992). Moreover, the xylose derived from lignocellulosic biomasses is a valuable substrate for xylitol production (Karimi *et al*., 2006).

However, against this is that acidic pretreatment may result in the formation of a relatively high concentration of fermentation inhibitors such as furfural, hydroxymethylfurfural and lactic acid, amongst others, that either must be removed when their concentrations are too high or requires the use of inhibitor-resistant strains for the fermentation process.

Although L-arabinose and xylose are relatively easily hydrolysed by mild acidic conditions, less than 20% of the glucose is recovered. Enhanced glucolysis by prolonged acid hydrolysis time, increased temperature and acid molarity all serve to degrade the xylose and produce fermentation inhibitors more than free extra glucose, and thus the requirement for enzymatic cleavage. The synergistic action of endoglucanase and ß-glucosidase activities is required to completely degrade cellulose to glucose. Endoglucanase degrades the cellulose to cellobiose which is then degraded to glucose by ß-glucosidase. However, both enzymes show strong product feedback inhibition. Glucose inhibits ß-glucosidase activity leading to an accumulation of cellobiose, and cellobiose inhibits endoglucanase activity. Therefore, an appropriate ratio of endoglucanase and ß-glucosidase is a key factor to complete degrading of cellulose (Sun and Cheng, 2002), as is removal of the glucose.

In the present study, hydrolysates of bagasse, corn cob and rice straw obtained by dilute-acid pretreatment were analysed for the level of key fermentation inhibitors. After cellulase digestion, the hydrolysates were then fermented to ethanol by *Saccharomyces cerevisiae* and the efficiencies of each of the hydrolysates as a substrate for ethanol fermentation was compared. We chose *S. cerevisiae* as the fermenting microorganism since it has been shown to be the best yeast for fermentation of glucose in lignocellulosic hydrolysates due to both its high ethanol-producing capacity and its high inhibitor tolerance (Palmqvist and Hahn-Hagerdal, 2000).

MATERIALS AND METHODS

Materials. Agricultural wastes; bagasse, corn cob, and rice straw were dried at 80 °C for 24 h, then milled by a hammer mill to a 20 mesh particle size and subsequently stored in a desiccator at room temperature. Cellulases (industrial grade) from *Trichoderma longibrachiatum* (CYTO CL cellulase) and *Trichoderma reesei* (GC 220 cellulase) were a gift from Genecor Co. Ltd., Finland. Analytical grade sulphuric, acetic and lactic acids, glucose, xylose, hydroxymethylfurfural (HMF), and furfural were purchased from Sigma Chemical Co. Ltd., USA.

Microorganism. *Saccharomyces cerevisiae* isolate TISTR 5596 is a high efficiency ethanol producing strain which was developed by the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. It was grown in YPD broth (yeast extract 10 g/l, peptone 10 g/l, and glucose 20 g/l) at 30 °C, 200 rpm for 24 h and used as starter culture at 10% (v/v) for ethanol fermentation.

Dilute-acid pretreatment. The milled agricultural wastes were slurred at 10% (w/v dry solid basis; DS) in various concentrations (0-376 mM) of sulphuric acid, and autoclaved at 121 °C for 1 h. When cooled to room temperature, the pH was adjusted to 5.0 with 10 M NaOH and clarified by centrifugation at 15000 *x g* for 10 min. Aliquots of the clarified supernatant were then used to analyse the concen-

trations of total reducing sugars, glucose, xylose and fermentation inhibitory compounds.

Enzymatic saccharification. The agricultural wastes at 5% (w/v DS) were pretreated by 141 mM sulphuric acid, the pH adjusted to 5.0 and subjected to enzymatic saccharification by a mixture of CYTO CL and GC 220 cellulases, and shaken slowly (110 rpm) at 50 °C for 48 h. Endoglucanase unit used per 100 g dry weight substrate of CYTO CL and GC 220 were 946 and 138, respectively. The ratio of endoglucanase:ß-glucosidase activity was 3:1.

Ethanol fermentation. Supernatants of the 48 h enzymatic hydrolysate supplemented with 1% (w/v) glucose and 0.4% (w/v) $(NH_4)_2SO_4$ were adjusted to pH 4.5 and subsequently used as substrate for ethanol fermentation by *S. cerevisiae*. Stationary phase cultures of the yeast in YPD (see above) were inoculated at 10% (v/v) and incubated at 30 °C without shaking for 72 h. The addition of 1% (w/v) glucose may seem counterintuitive but was added to minimise the relatively high variances that are seen in the analysis of ethanol concentrations produced from original low glucose concentrations since in this study we wish to assay for inhibitors of fermentation in the supernatant rather than actual fermentability. Ammonium sulphate was added to these nitrogen poor samples as a nitrogen source for optimal yeast growth and fermentation rates.

Analytical procedures. The cellulose and hemicellulose contents of the agricultural wastes were determined by the method described by the Technical Association of Pulp and Paper Industry TAPPI 203, om-88 method (TAPPI, 1988). Total reducing sugar levels were estimated by the dinitrosalicyclic acid method as described by Miller (1959). Carboxymethyl cellulase and ß-glucosidase activities were analysed by the methods described by Ghose (1987) and Sternberg *et al*. (1977), respectively. The reaction mixture (1.0 ml) contained 0.5 ml of appropriately diluted enzyme solution and either 0.5 ml of 2% (w/v) carboxymethyl cellulose in 20 mM citrate buffer pH 4.8, or 0.4% (w/v) D-salicin in 25 mM citrate buffer pH 4.5, respectively. After 30 min incubation at 50 ^{O}C, the amount of reducing sugar liberated in the reaction mixture was measured. One unit (U) of each enzyme activity is defined as the amount of enzyme which liberates 1 µmol of glucose per minute under the above-specific conditions. Glucose, xylose, furfural, HMF, acetic acid, lactic acid and ethanol concentrations were analysed by HPLC (Agilent 1100 Series equipped with quaternary pump, on-line degasser, autoinjector, column thermostat, refractive index detector, and a ChemStation software, Agilent Technology Co. Ltd., USA). Sugars were identified and quantified by Aminex column HPX-87P (300 x 7.8) with a Carbo-P micro-guard cartridge (Bio-Rad, USA). The column was maintained at 80 °C 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. Furfural, HMF, organic acids and ethanol were identified and quantified by Aminex column HPX-87H (300 x 7.8) with a Cation H micro-guard cartridge (Bio-Rad, USA). The column was maintained at 55 °C and 50 µl of each sample was injected at a time and eluted by 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min. Analytical grade glucose, xylose, furfural, HMP, acetic acid, lactic acid and ethanol were used as authentic standards.

RESULTS AND DISCUSSIONS

Agricultural wastes

The cellulose and hemicellulose contents of the three agricultural wastes used in this investigation are shown in Table 1. Across the three types of waste, cellulose and hemicellulose contents ranged from 34-39% (w/w DS) and 22-41% (w/w DS), respectively.

TABLE 1 - Cellulose and hemicellulose contents of the three agricultural wastes

Components	Dry solid (%, w/w)		
	Bagasse	Corn cob	Rice straw
Cellulose	37 ± 3.0	39 ± 2.3	34 ± 3.2
Hemicellulose	24 ± 2.5	41 ± 3.3	22 ± 1.0

The results are displayed as the mean ± SD of derived from triplicate determinations.

Dilute-acid pretreatment

The results of different sulphuric acid concentrations on the amount of reducing sugar released from each of the three agricultural wastes (10%, w/v, DS)) are summarised in Fig. 1. The maximal level of reducing sugar released from bagasse and corn cob was obtained by pretreatment with 141-235 mM sulphuric acid, with potential optimum at 188 mM, whilst that for rice straw was 188-376 mM with a potential optimum of 282 mM sulphuric acid. Comparing across the optimal sulphuric acid concentration for each substrate, a higher reducing sugar level was released from corn cob (473 ± 2.5 mg/g) than from bagasse (402 ± 1.2 mg/g) or rice straw (255 ± 4.9 mg/g), (Fig. 1) yet, in contrast, the highest level of glucose was detected from bagasse (145 ± 4.5 mg/g) and not corn cob (86 ± 6.5 mg/g) or rice straw (12 ± 0.3 mg/g). (Table 2). The hemicellulose composition level in corn cob was higher than bagasse and rice straw (Table 1). Xylose liberation was highest in corn cob (206 ± 6.1 mg/g) but significantly lower amounts in bagasse (120 ±

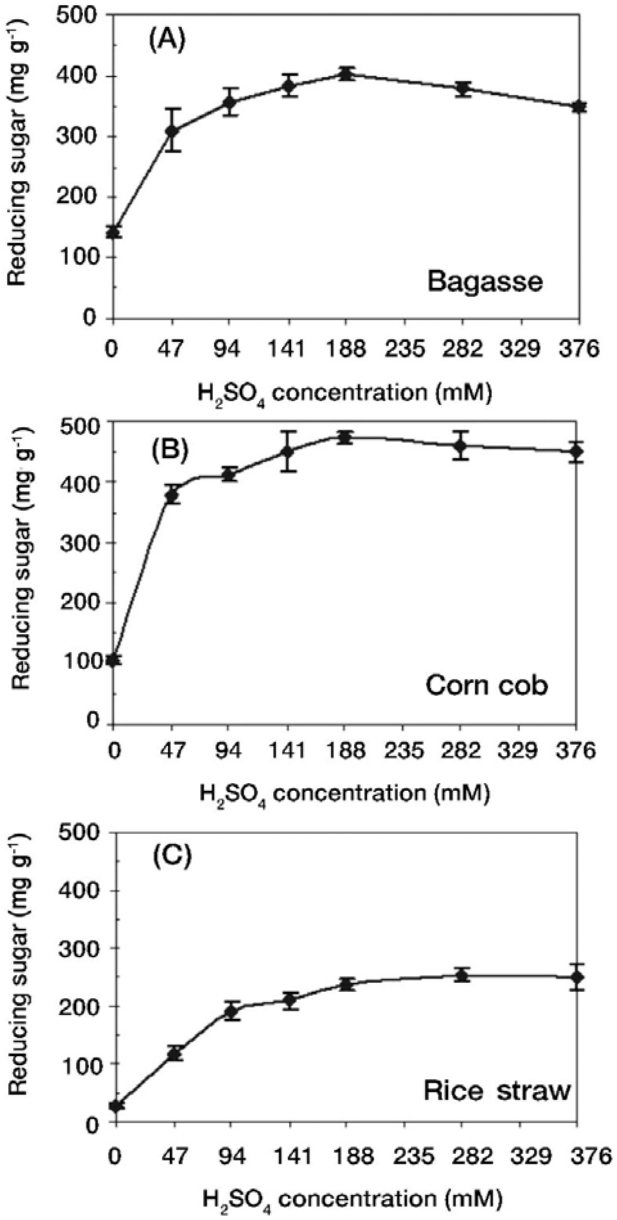

FIG. 1 - The effect of varying sulphuric acid concentrations (121 °C for 1 h) on the amount of total reducing sugars liberated from bagasse (A), corn cob (B) and rice straw (C) agricultural wastes (10%, w/v dry solid basis). The data are displayed as the mean ± 1 SD, and are derived from three independent experiments.

0.5 mg/g) and rice straw (119 ± 0.4 mg/g) (Table 2). Indeed, xylose release from corn cob and rice straw was 2.5 and 10 times higher than glucose, respectively, which is to be expected from the greater resilience of cellulose to weak acid hydrolysis than hemicellulose (Szczodrak and Fiedurek, 1995; Nguyen *et al*., 1998; Davis *et al*., 2005). The greater yield of glucose than xylose that was detected in the acid pretreated bagasse is not unexpected since this is due to the presence of residual sugar cane juice.

Following acid treatment, the growth and ethanol producing inhibitor furfural (Banerjee *et al*., 1981), was detected at between 0.1 and 0.2 g/l in all three agricultural waste products which, although higher than that reported for acid treated wheat straw (Saha *et al*., 2005), are potentially below that required to cause significant inhibition of *S. cerevisiae* growth and ethanol fermentation rates. Indeed, 0.5 g/l furfural was required to attain 41 and 43% inhibition of growth and ethanol production, respectively (Delgenes *et al*., 1996). This level of produced furfural in these three agricultural wastes, although higher than that seen in wheat straw, is actually relatively rather low. Moreover, lactic acid was only detected at low amounts in corn cob (0.7 ± 0.05 g/l), and although acetic acid was detected in all three substrates, at 2.3-2.5 g/l (corn cob), 1.8 g/l (bagasse) and 0.8-1.0 g/l (rice straw), it is unlikely, that alone these concentrations are inhibitory at this pH (e.g. Pampulha and Loureiro, 1989). Indeed, even at acetate levels as high as 5 g/l, ethanol production by *S. cerevisiae* was reported to be decreased by only 1% (Delgenes *et al*., 1996).

In contrast, HMF was detected in significant amounts in both bagasse (1.5 to 1.8 g/l) and corn cob (0.8 to 1.0 g/l), in agreement with that reported for sugar cane bagasse before (Alves *et al*., 1998), and at these levels is likely to be inhibitory to yeast growth and ethanol fermentation. For example, Delgenes *et al*. (1996) reported that HMF at just 1 g/l inhibited growth (65%) and ethanol fermentation (71%) of *S. cerevisiae*. In contrast, a

TABLE 2 - The concentration of sugars and fermentation inhibitory compounds in dilute-acid hydrolysates of agricultural waste

Sulphuric acid conc. (mM)	Agricultural wastes	Glucose (mg/g)	Xylose (mg/g)	Inhibitors (g/l)			
				HMF	Furfural	Acetic acid	Lactic acid
188	Bagasse	145 ± 4.5	120 ± 0.5	1.8 ± 0.24	0.1 ± 0.01	1.8 ± 0.19	NT
	Corn cob	86 ± 6.5	206 ± 6.1	1.0 ± 0.07	0.2 ± 0.01	2.5 ± 0.17	0.7 ± 0.05
	Rice straw	12 ± 0.3	119 ± 0.4	0.1 ± 0.01	0.1 ± 0.00	1.0 ± 0.03	NT
141	Bagasse	134 ± 5.3	110 ± 1.8	1.5 ± 0.49	0.1 ± 0.02	1.8 ± 0.59	NT
	Corn cob	75 ± 3.0	184 ± 1.6	0.8 ± 0.08	0.1 ± 0.01	2.3 ± 0.26	0.7 ± 0.05
	Rice straw	8 ± 2.3	78 ± 1.6	0.1 ± 0.01	0.1 ± 0.00	0.8 ± 0.03	NT

Agricultural wastes (10%, w/v dry solid basis) were pretreated at 121 °C for 1 h. The results are displayed as the mean ± SD of triplicate determinations. HMF (hydroxymethylfurfural). NT (not detectable).

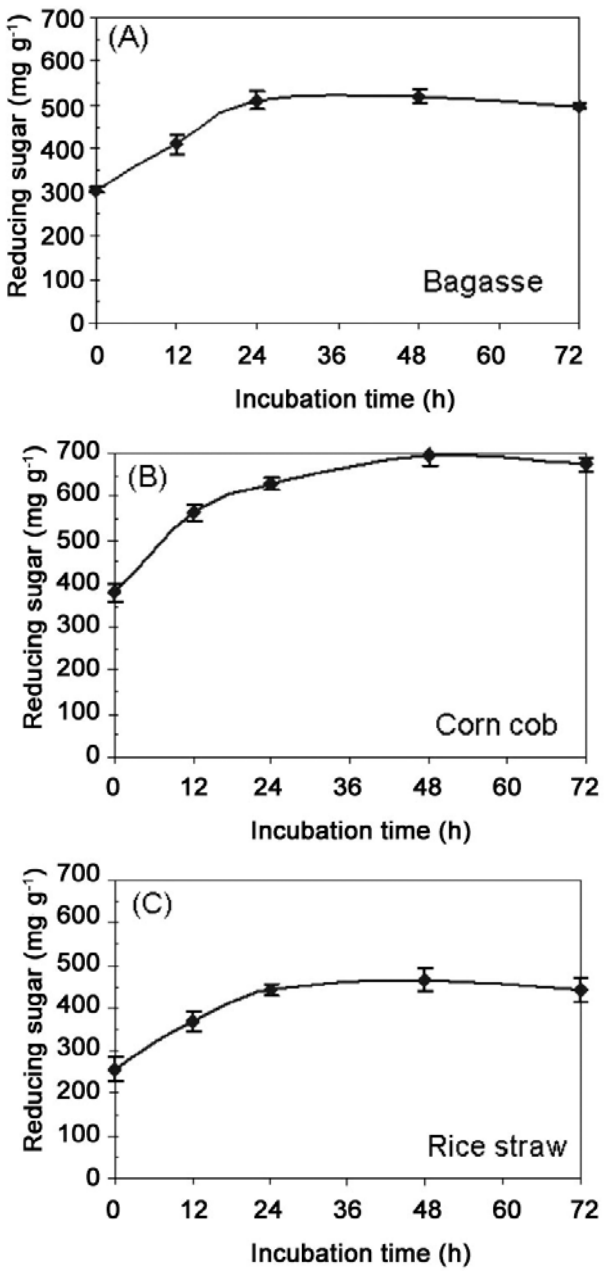

FIG. 2 - The amount of total reducing sugars liberated after dilute-acid pretreatment and enzymatic saccharification from bagasse (A), corn cob (B) and rice straw (C). The dilute-acid pretreated agricultural wastes (5%, w/v dry solid basis) were saccharified by 81 endoglucanase units of a CYTO CL and GC 220 cellulase mixture for 48 h as described in the text, and assayed at the indicated intermediate time points. The data are displayed as the mean ± 1 SD, and are derived from three independent experiments.

much lower and probably not inhibitory level of HMF was observed in rice straw (0.1 g/l), in good agreement with that reported for wheat straw (Saha *et al*., 2005).

Acid pretreatment of agricultural wastes was performed using 141 mM sulphuric acid in all subsequent experiments to reduce the amount of derived HMF.

Enzyme saccharification

Enzymatic release of glucose from cellulose using economically viable levels of enzyme has frequently proven difficult (e.g. Davis *et al*., 2005), and this may in part be due to substrate feedback inhibition. We, therefore, attempted to increase the endoglucanase to ß-glucosidase enzyme activity ratio by mixing CYTO CL with GC 220 cellulases to derive a 3:1 endoglucanase: ß-glucosidase ratio. At 50 ^{O}C and pH 4.5, carboxymethyl cellulase (CMCase) and ß-glucosidase activities of the CYTO CL cellulase were found to be 473 ± 1.3 U/ml and 53 ± 2.2 U/ml, respectively, whilst for the GC 220 cellulase they were 69 ± 2.1 U/ml and 137 ± 2.5 U/ml, respectively. The residual enzyme activity after 72 h incubation at 50 $^{\circ}$C and pH 4.5 of CYTO CL CMCase and GC 220 ß-glucosidase were 93 ± 3.2% and 87 ± 1.6%, respectively. The three agricultural wastes (2.5 g each) were individually pretreated with 141 mM sulphuric acid at 5% (w/v, DS) and saccharified with 81 endoglucanase units of the CYTO CL and GC 220 cellulase mixture as detailed in the methods. The results are summarised in Fig. 2, where the largest amount of reducing sugar released after 48 h was achieved from corn cob (694 ± 2.6 mg/g), followed by bagasse (520 ± 1.6 mg/g) and rice straw (466 ± 4.2 mg/g). Analysis of the specific glucose and xylose composition revealed that the corn cob hydrolysate contained the highest level of total reducing sugars, glucose and xylose (Table 3). Direct quantitative comparisons between the levels of total reducing sugars, glucose and xylose released in acid treated samples (Fig. 1 and Table 2) with those in acid and cellulase treated samples (Fig. 2 and Table 3) are not valid since different initial amounts of substrates were used between the two assays (5 and 2.5 g, respectively). .

Considering the cellulose to hemicellulose ratios of each of the substrates (Table 1) the slight increase in glucose to xylose ratio for bagasse (1.2 to 2; 1.7 fold increase) and corn cob (0.42 to 1.1; 2.6 fold increase), compared to rice straw (0.1 to 1.4; nominal 14 fold increase) is at first sight low and appears to suggest far from complete (poor) glucose release from cellulose under these conditions. However, especially for bagassse with residual sugar cane juice present, much of the glucose is present from phloem and thus artefactually raises the acid treatment non cellulose digested glucose to xylose ratio. In this light, cellulose digestion to liberate glucose may have proceeded more efficiently and the modified endoglucanase:ß-glucosidase activities in an attempt to optimise this aspect may be worthwhile.

Regardless, with the high xylose levels unless more efficient glucose release can be obtained, it may prove optimal to use xylose and glucose co-fermentation, especially for rice straw.

Ethanol fermentation

Enzymatic hydrolysates, supplemented with 1% (w/v) glucose and 0.4% (w/v) $(NH_4)_2SO_4$, were fermented to ethanol by *S. cerevisiae* for 72 h. Although the enzymatic hydrolysate of corn cob contained the highest glucose content (and would still do so after supplementation), the actual ethanol conversion yield on glucose (YP/S) attained with corn cob (0.45 ± 0.006 g/g) was lower than bagasse (0.49 ± 0.007 g/g) with a slightly lower level attained with rice straw (0.42 ± 0.007 g/g).

Mussatto and Roberto (2004) reported that there was a synergistic adverse effect when hydroxymethylfurfural and furfural were combined with a variety of phenolic and aromatic compounds derived from lignin degradation and several types of acids. In a similar vein, Parajo *et al*. (1998) reported that lignin degradation products are more toxic to microorganisms than furfural and hydroxymethylfurfural, whilst vanillin (0.5 g/l) was also noted to decrease ethanol production in *S. cerevisiae* by 30% (Delgenes *et al*., 1996). Thus, although the inhibitors we investigated did not individually appear to reach likely inhibitory levels acting together or with other undefined inhibitors they may additively or, perhaps more likely, synergistically attain significant inhibitory roles. In order to optimise the fermentation of these hydrolysates, and especially corn cob, to improve ethanol yields to economically feasible and industrially reliable levels, the identification of the inhibitors, selection of suitable detoxification methods and or microorganisms of enhanced tolerance to the inhibitors are important and necessary steps for further work.

TABLE 3 - The amount of reducing sugars liberated after dilute-acid pretreatment and enzymatic saccharification

Agricultural wastes	Reducing sugar (g/l)	Glucose (g/l)	Xylose (g/l)
Bagasse	26 ± 0.15	12 ± 0.27	6 ± 0.14
Corn cob	35 ± 0.31	13 ± 0.17	12 ± 0.67
Rice straw	23 ± 0. 53	11 ± 0.07	8 ± 0.91

The results are displayed as the mean ± 1 SD of triplicate determinations.

CONCLUSIONS

Based on the results presented here we conclude that under a combined dilute sulphuric acid (141 mM) pretreatment (1 h at 121 °C) and cellulase driven (81 units of endoglucanase activity) saccharification at 50 °C, pH 5.0 for 48 h, corn cob (5% (w/v, DS)) gave the maximum yield of glucose (268 mg/g) compared to bagasse (234 mg/g) and rice straw (214 mg/g), respectively. However, optimal glucose release under economically viable levels of enzyme addition may not have been attained in bagasse and corn cob substrates. After dilute-acid pretreatment, likely non toxic levels of furfural were detected in both corn cob and bagasse hydrolysates. Hydroxymethylfurfural levels detected in bagasse were almost two fold higher than those in corn cob yet, under these conditions, the ethanol conversion yield of corn cob hydrolysate by *S. cerevisiae* was lower than that attained in the bagasse hydrolysate. However, given the higher lactic acid levels in corn cob hydrolysate it remains plausible that synergistic effects of furfural and hydroxymethylfurfural with weak acids, such as lactic acid, or other lignin degradation products in the corn cob hydrolysate are the key factors causing the reduced ethanol yield in corn cob hydrolysates.

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