RESEARCH ARTICLE

Bioconversion of brewer's spent grains to bioethanol

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Abstract

Spent grains (SG), the residue remaining after extraction of wort, are a major by-product of brewing. This lignocelluose-rich biomass may provide a source of sugars for fuel ethanol fermentations. Dilute acid and enzyme treatments were developed to convert the hemicellulose and cellulose fractions to glucose, xylose and arabinose. Pretreatment of dried, milled grains with 0.16 N HNO_3 at 121 °C for 15 min was chosen as the most suitable method for solubilizing grains before enzymatic digestion with cellulase and hemicellulase preparations. Solids loading concentrations (10%, 15% and 20% w/v) were compared and reducing sugar between 40 and 48 g $(100\,g\,SG)^{-1}$ was extracted. Hydrolysate, prepared from 20% SG, pretreated with 0.16 N HNO_3, partially neutralized to pH 5–6 and digested with enzymes for 18 h, contained 27 g L^{-1} glucose, 16.7 g L^{-1} xylose and 11.9 g L^{-1} arabinose. Fermentation of this hydrolysate for 48 h by *Pichia stipitis* and *Kluyveromyces marxianus* resulted in 8.3 and 5.9 g L^{-1} ethanol corresponding to ethanol conversion yields of 0.32 and 0.23 g ethanol $(g\,substrate)^{-1}$, respectively. Substrate utilization efficiency was less when compared with glucose/xylose mixtures in synthetic media, suggesting that yeast inhibitory compounds derived from SG were present in the hydrolysate.

Introduction

Industrial production of fuel ethanol is predominantly from agricultural crops. In North America, bioethanol is primarily produced from maize while in Brazil, sugarcane is used (Wheals *et al.*, 1999; Bothast & Schlicher, 2005). This leads to direct conflict with food production and also has consequences for land practice with increasing usage of pesticides and herbicides being reported (Pimentel, 2003; Pimentel & Patzek, 2007). To meet the increasing demand for alternative biofuels, biomass sources, other than those used as food or requiring large changes in land practice, need to be exploited. Waste lignocellulose biomass from agriculture, food processing and forestry represents renewable supplies of fermentable sugars that can provide a feedstock for bioethanol production by microorganisms. We have identified spent grains (SG) from breweries and distilleries in Scotland as a potential biomass source for bioethanol.

SG consist of the residues remaining after starch extraction for wort preparation. Use of SG has mainly been limited to animal feed with farmers often collecting the wet grains free of charge (Pass & Lambert, 2003; Mussatto *et al.*, 2006). However, the declining dairy industry in Scotland and the increased availability of similar feeds from biofuel plants places greater pressure on breweries to explore alternative disposal routes. SG are a rich source of lignocellulose, which may be converted to fermentable sugars for production of bioethanol. In brewing, the spent malted barley grains are *c.* 80% cell wall material, with the remainder being mainly protein (Jay *et al.*, 2008) and consist of 16.8–21.9% cellulose, 28.4–29.6% hemicellulose and 21.7–23.0% klason (acid-insoluble) lignin (Table 1). Novel uses include as an additive in cereal-based foodstuffs, energy production via direct combustion or anaerobic digestion to biogas, as an adsorbent for metals and dyes and for cultivation of mushrooms (Mussatto *et al.*, 2006). The hemicellulose and cellulose in brewer's SG may be hydrolysed for microbial conversion to lactic acid (Shindo & Tachibana, 2004; Cruz *et al.*, 2007), xylitol or arabitol (Duarte *et al.*, 2004; Mussatto & Roberto, 2005) while compounds with antioxidant activity such as ferulic and *p*-coumaric acids may be extracted from the lignin fraction (Cruz *et al.*, 2007; Mussatto *et al.*, 2007). Hydrolysis methods have mainly focused on hemicellulose for production of xylo-oligosaccharides (Carvalheiro *et al.*, 2004a) or complete hydrolysis to pentoses for fermentation

Table 1. Composition of brewer's spent grains (summarized from Carvalheiro *et al.*, 2004a, b and Mussatto & Roberto, 2006 for SG from 100% malt)

Component	g (100 g SG)$^{-1}$
Cellulose	16.8–21.9
Hemicellulose	
Xylan	19.8–20.6
Arabinan	8.5–9.4
Klason lignin	21.7–23.0
Acetyl groups	1.1–1.4
Protein	15.3–24.6
Ash	1.0–4.6

to polyols (Duarte *et al.*, 2004; Mussatto & Roberto, 2005; Carvalheiro *et al.*, 2005, 2006). The hemicellulose fraction is highly susceptible to high temperatures and acid treatment. Autohydrolysis at temperatures up to 190 °C results in conversion of hemicelluloses to soluble xylo-oligosaccharides (Carvalheiro *et al.*, 2004a; Duarte *et al.*, 2004). Dilute sulphuric acid treatment at temperatures between 90 and 130 °C results in near-complete hemicellulose hydrolysis and liberation of pentose sugars (Beldman *et al.*, 1987; Carvalheiro *et al.*, 2004b; Mussatto & Roberto, 2006). The cellulose fraction is not as susceptible to acid or high temperature treatment (Beldman *et al.*, 1987) but may be hydrolysed with enzymes. However, as with other lignocellulosic biomass, direct hydrolysis with cellulase and hemicellulase enzymes is inhibited by the complex structure and crystallinity of lignocellulose with chemical and/or physical pretreatment required for complete hydrolysis (Chandra *et al.*, 2007). For example, release of total sugars from SG by treatment with commercial cellulases was increased from 22 to 40 g (100 g SG)$^{-1}$ by pretreating the grains with 0.5 N H_2SO_4 at 90 °C for 4 h (Beldman *et al.*, 1987). Thus, for complete conversion of the hemicellulose and cellulose to monosaccharides, physical and/or chemical pretreatments coupled with enzyme hydrolysis are required.

There has been little research on conversion of brewer's SG to bioethanol. Steam explosion to liquefy grains, followed by bioethanol production by simultaneous saccharification and fermentation (SSF) has been described (Shindo & Tachibana, 2006). SSF involved saccharification of liquefied grains with enzymes (glucoamylase, cellulase and hemicellulase) with simultaneous yeast fermentation. The highest ethanol concentration, *c.* 48 g L^{-1}, was achieved after 4 days fermentation with coimmobilized *Saccharomyces cerevisiae* and *Pichia stipitis*. This is the only paper, to our knowledge, that deals with ethanol fermentation of sugars originating from both hemicellulose and cellulose fractions of brewer's SG. Research on hydrolysis of SG for ethanolic fermentations has mainly focussed on spent maize (corn) a major by-product of bioethanol plants in North America. This can be either as corn fibre from wet milled processes (Schell *et al.*, 2004) or distiller's dried grains with solubles (DDGS) from the dry-grind process (Belyea *et al.*, 2004). It is estimated that the conversion of residual starch and lignocellulosic components in corn fibre would increase ethanol yields of a corn wet mill by 13% (Grohmann & Bothast, 1997). Fermentation of combined hydrolysis products from hemicellulose and cellulose has been reported although separate treatments are preferred to yield streams rich in either pentose or hexose sugars (Tucker *et al.*, 2004; Dien *et al.*, 2005; Chen *et al.*, 2007).

Combined conversion of all polysaccharide fractions of brewer's SG by acid and enzyme treatment has not been reported. In this work, a process for mild acid pretreatment of brewer's SG, followed by enzyme hydrolysis to maximize recovery of sugars was developed. As the hydrolysate contained hexose and pentose sugars, this represents a challenge to yeast fermentation. *Saccharomyces cerevisiae*, the yeast traditionally used in the alcohol industry, does not utilize pentose sugars. Fermentation of hydrolysate by *P. stipitis* and *Kluyveromyces marxianus* was investigated. The production of ethanol by these yeasts in synthetic media was compared with the fermentation performance in hydrolysate, in order to assess the feasibility of the yeasts' application to bioethanol production from SG cellulose and hemicellulose.

Materials and methods

Source, storage and composition of SG

SG were kindly provided by the Caledonian Brewery, Edinburgh, Scotland. They were from an ale mash, consisting of 96% malted barley and 4% roasted barley. SG were dried at 80 °C and stored in sealed containers until required. Wet grains had a moisture content of 76.8% while dried grains had 3.3% moisture. Dried grains were milled using a hammer mill fitted with a 5-mm-sized grating (Cutting Mill SM 100, Retsch GmBH, Germany) and used in all experiments.

Yeast strains and growth conditions

The yeast strains used were *P. stipitis* NCYC 1540 and *K. marxianus* NCYC 1425 (following preselection of several pentose-fermenting yeasts from culture collections). Strains were maintained on 4% xylose agar slopes consisting of (g L^{-1}): yeast extract, 5; Bacteriological peptone, 5; Technical Agar (No. 3), 12, adjusted to pH 5.5 (all from Oxoid Ltd, Hampshire, UK). For all experiments yeast inocula were prepared in xylose synthetic media consisting of (g L^{-1}): potassium hydrogen phthalate, 3.06; yeast extract, 0.4; $(NH_4)_2SO_4$, 3.4; Yeast Nitrogen Base (Formedium Ltd, Norwich, UK), 6.9; xylose, 40 and adjusted to pH 5.5.

Colonies from agar slopes were transferred to 100 mL xylose synthetic media in 250-mL Erlenmyer flasks and incubated at 30 °C on a rotary shaker at 100 r.p.m. (Electron Incubator, Infors UK Ltd, Surrey, UK). Cells from 48-h cultures were washed with sterile water and used for fermentation studies. Viability of cells was determined by staining with equal volume of methylene violet solution (0.01% methylene violet 3RAX, 2% sodium citrate) for 5 min (Smart *et al.*, 1999). Cells were counted with a haemocytometer, with dead cells stained violet and live cells unstained.

Hydrolysis of SG

Acid pretreatment of SG and the effect on enzyme hydrolysis was investigated. Constant slurry volumes of 40 mL were used throughout, with all parameters assessed in triplicate. Ten per cent (w/v) milled grains were hydrolysed with either 0.08 N H_2SO_4, HCl or HNO_3 by autoclaving at 121 °C for 15 min (Falcon 30 autoclave, LTE Scientific, Oldham, UK). The pH was adjusted to 5–6 using 4 M NaOH. Acid-pretreated grains were either analysed directly or used for further enzyme hydrolysis. For analysis, samples were centrifuged (1500 ***g***, 10 min) and the supernatant retained for sugar profiling.

Following acid pretreatment, partially neutralized samples were incubated with a cocktail of enzymes (cellulase, β-glucosidase, hemicellulase and xylanase) available from the Novozymes Biomass sample kit (courtesy of Novozymes A/S, Denmark). A brief description of the enzymes, activity, optimum pH, temperature and dose concentration is provided in Table 2. A pH between 5–6, temperature of 50 °C and agitation at 130 r.p.m. was chosen for hydrolysis and samples were incubated with all enzymes for *c.* 18 h. After enzyme treatment, the samples were centrifuged and the sugar composition determined.

HNO_3 was chosen as the most suitable acid and SG concentration was further optimized, to produce a hydrolysate with a high concentration of sugars. Pretreatment of 10%, 15% and 20% (w/v) SG with either 0.08 or 0.16 N HNO_3 before enzyme hydrolysis was compared.

Table 2. Enzymes used in this study (data provided by Novozymes A/S)

Enzyme	Activity	pH	Temperature (°C)	Dose (% w/w SG)*
Cellulase complex	700 EGU† g^{-1}	4.5–6.5	45–60	6
β-Glucosidase	250 CbU‡ g^{-1}	2.5–6.5	45–70	0.6
Xylanase	500 FXU§ g^{-1}	4.5–6.0	35–55	0.5
Hemicellulase	750 FXU g^{-1}	5.0–8.0	45–70	0.4

*Dose values were calculated based on 10% SG slurry with the same volume of enzymes used at higher solids loading.
†Endoglucanase units.
‡β-Glucanase units.
§Fungal xylanase units.

Preparation of hydrolysate and yeast fermentations

Enzyme hydrolysis of 20% SG, pretreated with 0.16 N HNO_3 was used for preparing hydrolysate for fermentation. This was done on a larger scale using 1 L quantities of slurry in 2-L baffled flasks rather than 40 mL slurry used previously. Following autoclaving, the pH of the slurry was adjusted between pH 5 and 6 by stepwise addition of 10 M NaOH before addition of enzymes. After enzyme hydrolysis for 18 h at 50 °C, 150 r.p.m., the hydrolysate was separated by centrifugation. Hydrolysate was analysed for sugar content and pH. For fermentations, hydrolysate was sterilized by autoclaving and separated into 50 mL aliquots and inoculated with either *P. stipitis* or *K. marxianus* from 48 h xylose cultures to an initial density of 0.5 g L^{-1}. Cultures were incubated at 30 °C on a rotary shaker at 100 r.p.m. and sampled for 48 h. Samples were centrifuged at 1500 ***g***, 10 min and the supernatant retained for pH, sugar and ethanol analysis. The pellet was resuspended in sterile water and the viability and concentration of cells determined.

The fermentation performance of *P. stipitis* or *K. marxianus* on glucose and xylose in synthetic media was assessed. One hundred millilitres of media containing the appropriate carbon source in 250-mL Erlenmyer flasks were inoculated with cells as described above. Cultures were incubated at 30 °C, 100 r.p.m. and were sampled over a period of 2 days. All fermentations were performed in triplicate. Samples were treated as above. Dry weight (g L^{-1}) calibration was determined by the absorbance of the suspension at a wavelength of 600 nm.

Analysis

The dry matter content of samples was determined by drying triplicate samples for 24 h in an oven at 105 °C. All per cent concentrations are quoted on a dry weight basis unless stated otherwise.

For sugar analysis, samples were further clarified by centrifuging at 13 200 ***g*** for 5 min. The reducing sugar (RS) concentration was determined using the dinitrosalicylic RS assay (Miller, 1959) and sugar concentration expressed in terms of glucose equivalents by reference to glucose standards. The sugar profile was determined by HPLC using a BioRad Aminex HPX-87P column with a refractive index detector. Samples were filtered through a 0.2-μm cellulose acetate syringe filter, with sample pH adjusted to between pH 5 and 9 before analysis.

Ethanol was analysed using a Shimadzu gas chromatography mass spectrometer GCMS-QP2010 fitted with an

Agilet HP blood alcohol capillary column (ID: 0.32 mm, length 7.5 m, film 20 μm).

Results and discussion

A combined acid and enzyme hydrolysis process was developed to extract cellulose and hemicelluloses sugars from brewer's SG, at a concentration suitable for yeast fermentation. The SG were from a mash used to produce ale and were dried to a moisture content of *c.* 3% and milled before hydrolysis. Drying maintains the chemical characteristics of SG for long periods, whereas when stored in the obtained form, SG deteriorate after just 1 week (Mussatto & Roberto, 2006). The grains were also milled to improve ease of handling, enabling higher solids loading to be maintained with constant volumes. Milling may also improve the sugar extraction process, increasing the surface area of the particles, allowing greater contact between enzymes and the grains.

Conversion of SG to fermentable sugars

The polysaccharide content of SG is *c.* 50% of the dry weight (Table 1), so effective hydrolysis should yield over 50 g RS per 100 g SG. Acid pretreatment followed by enzyme hydrolysis of the partially neutralized slurry was used to release sugars from brewer's SG. Initially, hydrolysis was assessed in terms of glucose RS equivalents and the RS concentration expressed as a percentage of the initial dry weight of the SG. In this case, the higher RS concentrations corresponded to greater conversion of structural carbohydrate polymers to monosaccharides. Pretreatment with distilled water and 0.08 N H_2SO_4, HCl and HNO_3 was compared, corresponding to acid concentrations of 3.9%, 2.9% and 5.0% (w/w SG), respectively, for a 10% SG slurry (Fig. 1). In the absence of acid, hydrolysis was minimal, with 9.2 g RS $(100\,g\,SG)^{-1}$ released after thermal treatment at 121 °C. For effective autohydrolysis, higher temperatures are required, with hemicelluloses being converted to soluble oligosaccharides after treatment in water at temperatures up to 190 °C (Carvalheiro *et al.*, 2004a; Duarte *et al.*, 2004). Incubation of temperature pretreated SG with enzymes doubled the amount of RS released, although this was still less than expected for complete hydrolysis, confirming that a chemical pretreatment step was required. Sulphuric acid is most commonly used for hemicellulose hydrolysis of both brewer's (Beldman *et al.*, 1987; Carvalheiro *et al.*, 2004b; Mussatto & Roberto, 2005, 2006) and corn SG (Schell *et al.*, 2004; Tucker *et al.*, 2004; Chen *et al.*, 2007). The concentration of acid is often reported as either per cent based on weight of SG or overall slurry. Here, normal concentrations were used so that the contribution of protons from each acid was consistent. In dilute acid hydrolysis, it is the protons that break the heterocyclic ether bonds between the sugar

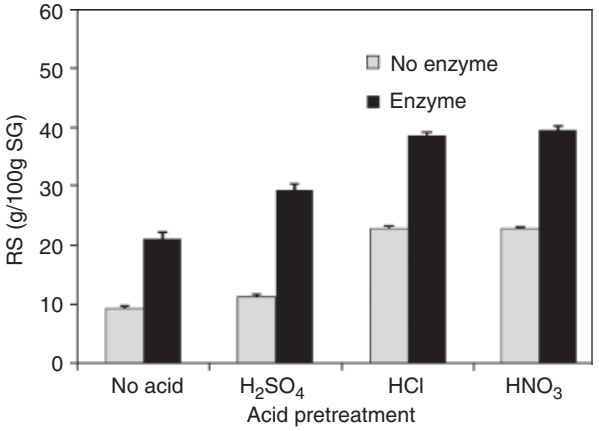

Fig. 1. Effect of acid pretreatment on release of RSs from SG. Ten per cent (w/v) SG were pretreated with either water or 0.08 N acid and neutralized with 10 N NaOH before enzyme treatment at 50 °C for 18 h. The RS is expressed as a percentage of the initial dry weight of the sample and was measured before and after enzyme treatment. Mean values ± SD from triplicate determinations are shown.

monomers in the polymeric chains formed by hemicelluloses and cellulose (Mussatto & Roberto, 2006). HCl and HNO_3 were more effective than H_2SO_4 with treatment resulting in liberation of twice the amount of RS. HCl and HNO_3 pretreatment also improved enzyme action, with 38.4 and 39.4 g RS $(100\,g\,SG)^{-1}$ released, respectively compared with 29.2 g RS $(100\,g\,SG)^{-1}$ released following H_2SO_4 pretreatment (Fig. 1). It is possible that the nucleophile activity of Cl ions from HCl and oxidative action from HNO_3 enhanced the decrystallization of lignocellulose.

High initial solids concentration is required to produce a hydrolysate with a suitable sugar concentration for fermentation. The sugar concentration may also be increased by evaporation of a low sugar solution, but in addition to the added cost, this may also increase the concentration of inhibitors in the hydrolysate, such as hydroxymethylfurfural and furfural (Mussatto *et al.*, 2005). In this case, a further treatment step is required to remove fermentation inhibitors. The SG concentration was increased with the aim of producing hydrolysate with high sugar content. HNO_3 pretreatment and enzyme hydrolysis of 10%, 15% and 20% solids was compared. The efficiency of enzymatic hydrolysis was reduced at the higher solids loading with 29.5 g of RS released per 100 g SG for 20% solids ratio, compared with 39.4 and 37.5 g at 10% and 15% solids, respectively (Fig. 2a). However, in terms of RS concentration in the hydrolysate, this corresponded to 38.1, 54.5 and 57.0 g RS L^{-1} for 10%, 15% and 20% solids, respectively. Sugar recovery increased by *c.* 10 g RS $(100\,g\,SG)^{-1}$ in all cases when the acid concentration was doubled before enzyme digestion, although hydrolysis of sugars from grains at 20% solids loading was still *c.* 0.8 times that at lower SG concentrations

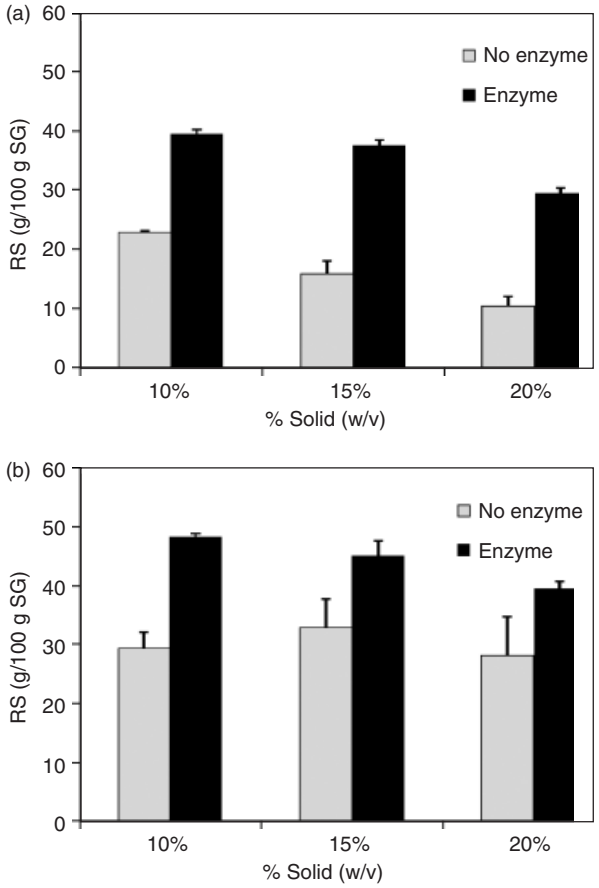

Fig. 2. Hydrolysis of SG at different solids loading concentrations (10%, 15% or 20% w/v SG). SG were pretreated with either (a) 0.08 N or (b) 0.16 N HNO_3, and pH adjusted between pH 5 and 6 with 10 N NaOH before enzyme digestion. The RS is expressed as a percentage of the initial dry weight of the sample and was measured before and after enzyme treatment. Mean values ± SD from triplicate determinations are shown.

(Fig. 2b). In this case, the concentration of sugars in the hydrolysate following enzyme treatment was 46.7, 65.6 and 76.4 g RS L^{-1} for 10%, 15% and 20% solids, respectively (Fig. 2b).

The monosaccharides released by enzyme hydrolysis of 0.16 N HNO_3-pretreated grains were profiled using HPLC (Fig. 3). The amount of monosaccharides recovered as a percentage of SG was similar irrespective of the initial solids loading. Approximately 15, 14 and 7 g of glucose, xylose and arabinose, respectively, were recovered per 100 g SG. The glucose and arabinose concentrations were equivalent to that expected for near-complete hydrolysis of structural carbohydrates. However, the xylose concentration was less than that expected, considering that 20% of SG are composed of xylan (Table 1). Also, at the highest solids concentration there was a reduction in 'other RS' i.e. the RSs concentration less the sum of the monosaccharides. It may

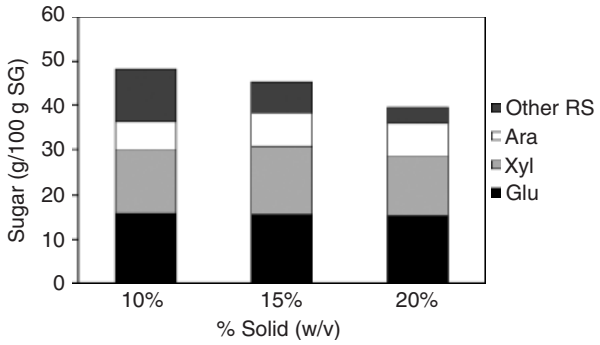

Fig. 3. Sugar composition of SGH following enzyme hydrolysis of 10%, 15% or 20% (w/v) solids pretreated with 0.16 N HNO_3 and neutralized with 10 N NaOH. The monosaccharides (arabinose, xylose and glucose) were determined using HPLC while other RSs was calculated as the difference between the total RS concentration less the monosaccharide components and appeared as a broad peak with a lower retention time than the monosaccharides on the HPLC scans.

be possible that this residual fraction consists of a dilute acid and/or enzyme resistant xylan fraction. It appears that for lower solid loading, i.e., greater ratio of acid to SG, more of this fraction was extracted and partially hydrolysed although not completely converted to monosaccharides.

Hydrolysate for fermentation was prepared from 20% (w/v) SG, pretreated with 0.16 N HNO_3. NaOH was used to partially neutralize the hydrolysate before enzyme hydrolysis. $Ca(OH)_2$ is often the preferred base but a precipitate of $CaSO_4$ is formed which must be removed. In addition to adding an extra process, it may also clog pipes (Schell *et al.*, 2004) and large quantities of insoluble residue have to be disposed of. The RS concentration of the hydrolysate was 66.6 g L^{-1} and with a pH of 5.2, it was deemed suitable to use directly for fermentation. The concentrations of monosaccharides were determined using HPLC with glucose, xylose and arabinose concentrations of 27.4, 16.7 and 11.9 g L^{-1}, respectively. The hydrolysate was not converted completely to monosaccharides, with oligomers eluting as a broad peak between 5 and 12 min (Fig. 4). As discussed previously, these oligomers may originate from acid-solubilized xylan fractions that are not hydrolysed completely to xylose monomers. A complex enzyme mixture is required for complete enzymatic conversion of arabinoxylan. These include endo-1,4-β-xylanases which attack the main chain, β-xylosidases which hydrolyse xylo-oligosaccharide to xylose and accessory enzymes such as acetylxylan esterases, α-glucuronidases and α-arabinofuranosidases (Saha, 2003). The xylanase and hemicellulase preparations used here were principally endo-xylanases, with the former having high specificity for the soluble fraction and the latter reacting with both soluble and insoluble arabinoxylans and were originally selected for effectiveness against wheat-based substrates (Novozymes, pers. commun.). The structure of hemicellulose in brewer's

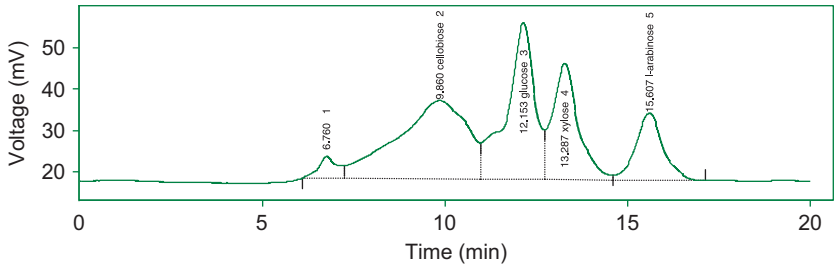

Fig. 4. HPLC analysis of sugars extracted from SG. Hydrolysate was prepared for fermentation studies from 20% (w/v) SG solution, pretreated with 0.16 N HNO_3, neutralized with 10 N NaOH and hydrolysed with enzymes.

SG differs to that of wheat (Kabel *et al.*, 2002) and as such, the enzymes may not be as effective at converting soluble xylan polymers from barley to its monomeric form. A higher acid concentration could also be used to degrade this portion. However, this may also lead to degradation of pentose sugars, resulting in inhibitory concentrations of furfural in the media.

Overall, the acid concentration used is in line with that previously reported for dilute acid hydrolysis (Macheiner *et al.*, 2003; Carvalheiro *et al.*, 2004b; Mussatto & Roberto, 2005, 2006). However, these reports were mainly concerned with hemicellulose hydrolysis, with low concentrations of glucose recovered. For example, treatment of grains with 10% H_2SO_4 (w/w SG) at 120 °C for 17 min, at a ratio of 8 g liquid g^{-1} of grains, yielded a hydrolysate consisting of 21.9 g L^{-1} xylose, 10.7 g L^{-1} arabinose and 1.2 g L^{-1} glucose (Mussatto & Roberto, 2005). Here, using higher solids loading and enzyme treatment following acid hydrolysis, a hydrolysate consisting of 16.7 g L^{-1} xylose, 11.9 g L^{-1} arabinose and 27.4 g L^{-1} glucose was produced for ethanol fermentations.

Ethanol fermentation

The hydrolysate prepared from SG is complex and may contain fermentation inhibitors. Thus, one of the overall aims of this work was to test the fermentability of the spent grain hydrolysate (SGH). Fermentation performance of *P. stipitis* NCYC 1540 and *K. marxianus* NCYC 1425 in SGH and synthetic media containing different carbon sources (4% glucose, 4% xylose or 2% glucose with 2% xylose) was compared. The *P. stipitis* strain was selected based on its superior performance compared with several xylose-utilizing strains (*Candida*, *Cryptococcus*, *Kluyveromyces*, *Pichia* and *Pachysolen* species), which were initially screened in our laboratory (data not shown). The *K. marxianus* strain was included as it showed excellent activity on glucose, is known to utilize xylose (Yablochkova *et al.*, 2003) and due to its high temperature tolerance (Hughes *et al.*, 1994), has been proposed for use in simultaneous saccharification and fermentation of cellulosic substrates (Barron *et al.*, 1995; Ballesteros *et al.*, 2003). Also, in preliminary experiments, this strain performed better than a distilling strain of *S. cerevisiae* on glucose synthetic media (data not shown). Under the fermentation conditions detailed here, *S. cerevisiae* DCLM, a strain traditionally used in the Scotch whisky industry, produced 8.9 g L^{-1} ethanol after 48 h, compared with 14.5 g L^{-1} by *K. marxianus*. Also, the viability of the whisky distilling strain was $< 50\%$ after 48 h at 30 °C and as such it was deemed unsuitable for investigating the toxicity of the SGH.

A summary of the fermentation parameters, including cell concentration, sugar uptake and ethanol produced after 48 h is provided in Fig. 5. The initial cell inoculum was 0.5 g L^{-1}, corresponding to *c.* 7.5 and 4.5×10^7 cells mL^{-1} for *P. stipitis* and *K. marxianus*, respectively. The yeast grew in all media and cell viability was maintained at >98% over the course of the fermentation. The exception to this was *K. marxianus* in 4% glucose, where viability was reduced to 89% after 48 h incubation. For *P. stipitis*, the cell growth on SGH was comparable to that on glucose, while cell generation was lower on xylose-containing media. Growth of *K. marxianus* was also highest after 48 h on glucose media, with growth on the SGH similar to the mixed glucose/xylose media. This is reflected in sugar utilization. In synthetic media, glucose was completely utilized by both yeasts. When xylose was the sole carbon source, uptake was less than that of glucose, with 87% of the xylose removed by *P. stipitis*, compared with 18% by *K. marxianus*. In glucose/xylose media, *P. stipitis* utilized both carbon sources while xylose utilization by *K. marxianus* was incomplete, with only 29% removed. In the SGH, glucose at an initial concentration of 27.4 g L^{-1} was preferentially utilized and was not detected in the hydrolysate after fermentation. In contrast, xylose removal by *P. stipitis* was less than expected; from an initial concentration of 16.7 g L^{-1}, 6.1 g L^{-1} was consumed. This is in contrast to xylose uptake from synthetic media, where all the sugars were consumed. *Kluyveromyces marxianus* showed less xylose utilization, with 4.6 g L^{-1} removed from the SGH. Arabinose utilization was minimal, with 0.4 and 0.9 g L^{-1} uptake by *P. stipitis* and *K. marxianus*, respectively. This is not surprising considering that very few arabinose-utilizing yeast have been identified (Dien *et al.*, 1996). The hydrolysate showed good buffering capacity, with a mean pH of 5.6 and 4.8 after 48 h fermentation with *P. stipitis* and *K. marxianus*, respectively. This is in contrast to the

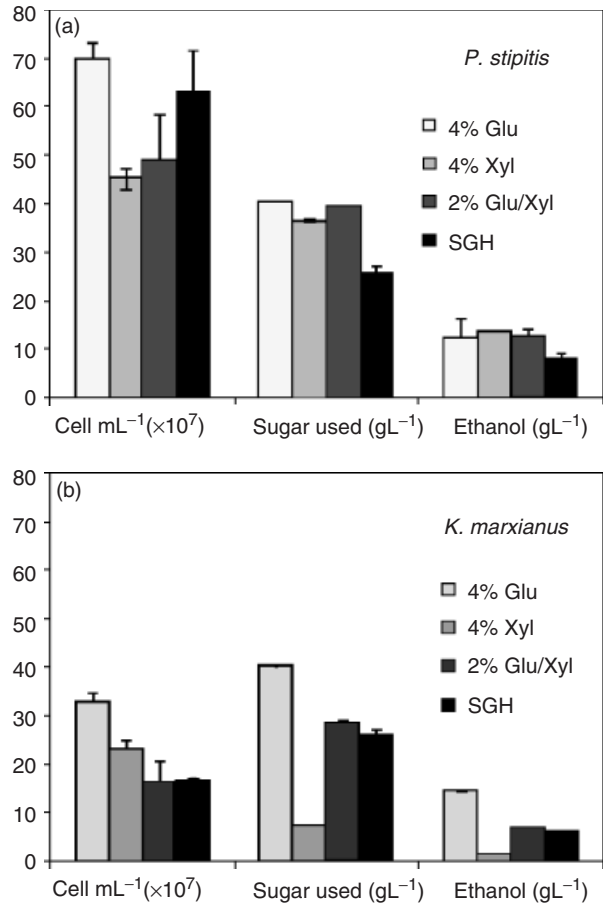

Fig. 5. Comparison of fermentation performance of (a) *Pichia stipitis* and (b) *Kluyveromyces marxianus* in synthetic media containing either 4% glucose (□), 4% xylose, (▨) or 2% glucose and 2% xylose, (■) or in the SGH, (■). Cell concentration, sugar utilization in terms of RS and ethanol produced at 48 h are shown. Mean values ± SD from triplicate determinations were calculated.

synthetic media where pH decreased from pH 5.5 to 3.0–3.3. High buffering capacity of the SGH is desirable from an industrial viewpoint, as rudimentary pH control would be anticipated during the course of a large-scale bioethanol fermentation.

Ethanol production by *P. stipitis* was better on xylose than on glucose, with 13.8 and 12.5 g ethanol L^{-1}, respectively (Fig. 5). This corresponded to a theoretical conversion of substrate to ethanol of 74.5% for xylose and 60.8% for glucose (Table 3). Calculations were based on a theoretical conversion of 0.51 g ethanol g^{-1} of hexose and pentose sugars. The reverse was true for *K. marxianus*; ethanol concentration was around 10-fold less on xylose compared than on glucose with 1.2 and 14.5 g L^{-1} produced after 48 h in xylose and glucose synthetic media (Fig. 5). This was not solely due to the lower xylose utilization as the conversion of xylose to ethanol was reduced considerably with conversion

Table 3. Comparison of yeast fermentation performance on SGH and synthetic media containing glucose and/or xylose

Yeast	Substrate	$Y_{P/S}$ (g g^{-1})*	% Theoretical conversion†	Yield (g ethanol per 100 g SG)‡
P. stipitis	4% Glu	0.31	60.8	
	4% Xyl	0.38	74.5	
	2% Glu/Xyl	0.33	64.7	
	SGH	0.32	62.7	4.2
K. marxianus	4% Glu	0.36	70.6	
	4% Xyl	0.17	33.3	
	2% Glu/Xyl	0.23	45.1	
	SGH	0.23	45.1	3.0

Calculations were based on the 48 h fermentation values from Fig. 5.
*$Y_{P/S}$ calculated as the g ethanol produced per g of substrate consumed.
†% Theoretical conversion calculated based on the stoichiometric conversion of xylose and glucose to ethanol with 1 g of sugar yielding 0.51 g ethanol and calculated as $(Y_{P/S} \div 0.51) \times 100$.
‡Yield of ethanol per 100 g of SG was calculated based on 200 g of spent grain originally used to prepare 1 L of hydrolysate.

efficiencies of 31.5% and 70.1% for xylose and glucose to ethanol, respectively. In this case, it is likely that xylose utilized by *K. marxianus* was directed towards cell division, with cell concentration increasing approximately fivefold after 48 h.

The conversion of utilized substrate to ethanol by the yeasts tested was similar on the SGH compared with 2% glucose/xylose synthetic media (Table 3). For *P. stipitis*, ethanol yield was 0.33 g g^{-1} on glucose/xylose synthetic media and 0.32 g g^{-1} on the SGH. Ethanol yields by *K. marxianus* were 0.23 g g^{-1} for both fermentations. However, the overall sugar consumption was less than expected, resulting in lower ethanol concentrations of 8.3 and 5.9 g L^{-1} for *P. stipitis* and *K. marxianus*, respectively. In particular, the low consumption of xylose on the SGH by *P. stipitis* was unexpected, especially compared with the complete carbon utilization on 2% glucose/2% xylose synthetic media. It is possible that fermentation inhibitors were present in the hydrolysate or that further nutritional supplementation was required, resulting in reduced sugar consumption and ethanol production.

Fermentation of lignocellulosic hydrolysate may be inhibited by a range of toxic compounds including weak acids, furan derivatives and phenolic compounds (Palmqvist & Hahn-Hägerdal, 2000). Furfural only presents a problem at levels above 1 g L^{-1} (Roberto *et al.*, 1991) and based on furfural concentrations reported for similar dilute acid processes, it was not expected to be present at a significant level (Carvalheiro *et al.*, 2004b; Duarte *et al.*, 2004). Inhibitors present in dilute acid hydrolysate may also be due to acetic acid, which is produced from hemicelluloses during the hydrolysis process. *Pichia stipitis* fermentation is

inhibited by acetic acid, with the effect being pH dependent (van Zyl *et al.*, 1991). At pH of 4–5, the pH usually associated with yeast fermentations, acetic acid is largely undissociated, which permits diffusion into the cell cytoplasm, where it dissociates and causes intracellular pH to decrease. For this reason, fermentation of hydrolysate above pH 5.5 minimizes inhibitory effects (Telli-Okur & Eken-Saraçoğlu, 2006). The hydrolysate prepared here was at pH 5.3 after enzyme treatment and this was not adjusted before fermentation so inhibition by acetic acid could not be discounted.

Direct comparison of the fermentation results with other reports are hindered by the fact that studies with SG are mainly concerned with polyol production from hemicellulose sugars (Duarte *et al.*, 2004; Mussatto & Roberto, 2005; Carvalheiro *et al.*, 2005, 2006). However, removal of inhibitors and media supplementation has been demonstrated to improve polyol production following dilute acid hydrolysis of SG. Xylitol and arabitol fermentation by *Debaryomyces hansenii* was improved by hydrolysate concentration and supplementation of hydrolysate with casamino acids (Carvalheiro *et al.*, 2005, 2006). Fuel ethanol production by *P. stipitis* fermentation of dilute acid hydrolysate prepared from different lignocellulosic sources has been demonstrated. These hydrolysates primarily consisted of xylose with low glucose levels, with a detoxification step improving ethanol production. Hydrolysate prepared from water hyacinth (*Eichlornia crassipes*) was converted to ethanol with a yield of 0.19 g ethanol (g sugar)$^{-1}$ (Nigam, 2002), which is less than the ethanol yield on SG presented here (0.32 and 0.23 g g^{-1} for *P. stipitis* and *K. marxianus*, respectively). In this case, sugar utilization was increased with a higher ethanol yield of 0.35 g g^{-1} when hydrolysate was detoxified by a combination of boiling and overliming (Nigam, 2002). *Pichia stipitis* fermentation of bagasse hemicellulosic hydrolysate was completely inhibited unless it was treated with activated charcoal, when ethanol yield was 0.35 g g^{-1} (Roberto *et al.*, 1991), while treatment with an anion exchange resin increased the yield from 0.27 to 0.37 g g^{-1} (van Zyl *et al.*, 1991).

Complete conversion of sugars is one of the prerequisites for economically favourable bioethanol production from lignocellulosic biomass (Galbe & Zacchi, 2002). Neither yeast strains assimilated L-arabinose to a significant level. L-Arabinose-fermenting yeasts have been identified, however, the ethanol yield is low (Dien *et al.*, 1996). Improvements in L-arabinose-fermenting strains are necessary before complete yeast fermentation of lignocellulosic hydrolysate is realized (Becker & Boles, 2003). It is possible that a further clean-up step to remove inhibitors from the SGH may additionally improve yeast fermentation. Also, the superior performance of *K. marxianus* on glucose compared with *P. stipitis* suggests that cofermentation with these two yeasts may improve the overall performance with a more rapid removal of glucose by *K. marxianus* allowing the sequential fermentation of xylose by *P. stipitis*.

While the ethanol produced from the SGH was less than expected when compared with synthetic media, the ethanol is nevertheless significant. Under the current nonoptimized fermentation conditions, *P. stipitis* and *K. marxianus* produced 8.3 and 5.9 g L^{-1} ethanol, respectively from a hydrolysate containing 66.6 g RS L^{-1}. This hydrolysate was prepared from a 20% (w/v) SG solution and the ethanol yield may be expressed as 4.2 and 3.0 g ethanol per 100 g of SG, for *P. stipitis* and *K. marxianus*, respectively. In the United Kingdom, *c.* 644 000 tonnes of malt is used by breweries per annum (BBPA, 2006). Assuming that the moisture content of malt is 16% and all the starch (61% of the malt) is extracted during mashing, *c.* 211 000 tonnes of brewery SG is produced in the United Kingdom on a yearly basis. Under the combined acid/enzyme hydrolysis conditions presented here, the nonoptimized fermentation by *P. stipitis* has the potential to convert this SG into 11.4 million litres of anhydrous ethanol. Further improvements on the hydrolysis and fermentation aspects of this process have the potential to increase the ethanol yield from brewery SG by up to fivefold. In Scotland, a similar co-product is SG from Scotch malt and grain whisky distilleries. It is estimated that 345 000 tonnes of distillery SG is produced annually (The Scotch Whisky Research Institute, pers. commun.) and if this can be converted to ethanol by the process outlined here, it has the potential to yield 18.5 million litres of ethanol. We therefore believe that bioconversion of brewer's and distiller's SG represents a valuable contribution to second generation biofuel production.

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