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Alkaline fractionation and enzymatic saccharification of wheat dried distillers grains with solubles (DDGS)

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Abstract

The complete utilisation of Dried Distillers Grains with Soluble (DDGS) requires effective pretreatment strategies aiming to increase the enzymatic digestibility of cellulose and improve its conversion to fermentable sugars. To this end, the effect of different NaOH concentrations (0 – 5%, w/v) and temperature (30 – 121 ^{o}C) on the fractionation of DDGS to its main components (cellulose, hemicellulose, proteins) was evaluated. As the NaOH concentration and temperature increased, the total sugar content of the pretreated DDGS solids progressively increased to a maximum of ~88%. At 121 ^{o}C and 5% NaOH, the DDGS solid residue consisted primarily of glucose (~53%), a 5-fold increase compared to the original DDGS, reflecting the presence of cellulose, and to a lesser extent by xylose (~25%) and arabinose (~10%) reflecting the presence of hemicellulose. Approximately 83% of the initial hemicellulose and 79% of the protein contents were removed into the liquid fraction during alkaline pretreatment. The enzymatic digestibility of the pretreated DDGS solids by the Accellerase® 1500 cellulase enzyme was significantly improved, resulting in a 3.6 fold increase in glucose yield compared to untreated DDGS. Mass balance analysis demonstrated that the proposed process scheme recovers the majority of the key DDGS components (cellulose, hemicellulose, proteins) in an efficient manner with relatively low losses, and provides a viable approach for the valorisation of DDGS.

Keywords: DDGS, alkaline pretreatment, cellulose, hemicellulose, cellulase

1. Introduction

The production of biofuels and platform chemicals such as bioethanol and organic acids from renewable bioresources is attracting significant attention. Dried Distillers Grains with Solubles (DDGS) is a bioresource that is produced in large amounts worldwide as a by-product from bioethanol and distillery industries. With regards to bioethanol production, approximately one third of every kilogram of wheat or corn is converted into ethanol, one third into carbon dioxide and one third into DDGS (Bruynooghe et al., 2013; Chatzifragkou et al., 2015). In the USA, DDGS from bioethanol plants is corn-based (Moreau et al., 2012), whereas, in the United Kingdom, Canada, and most of European countries, it is wheat based (Burton et al., 2014). DDGS is used as animal feed and is an important source of energy, protein, water-soluble vitamins and minerals for livestock. However, the addition of DDGS to livestock feed can only account for up to 30% (on a dry matter basis) of the diet, as higher levels may cause palatability and excessive protein consumption issues. Moreover, the variation in the nutritional composition of DDGS, particularly its protein content, depends on the source and the production processes, and is an obstacle for the extensive utilisation of DDGS as animal feed supplement for ruminants (Belyea et al., 2010). Considering the above, as well as the increased bioethanol-derived DDGS availability in future years, it is important to identify higher value alternative ways for the valorisation of DDGS, which support the circular bioeconomy concept.

The main components of DDGS include protein, fibre, lipids, minerals and vitamins; compared to the original grain composition, they are concentrated approximately 3-fold in DDGS, as a result of the DDGS production process which consists of a series of concentration steps and a final drying step. In the case of wheat DDGS, the protein derives from wheat (gluten, globulins and albumins) and the yeast cells, as the latter are not separated during the DDGS production

process (Villegas-Torres et al., 2015). According to Han and Liu (2010), around 20% of the total protein in corn DDGS is contributed by yeast. From a valorisation perspective, the protein in DDGS can be recovered using chemical extraction methods and could be potentially utilised in food, feed and agricultural applications (Chatzifragkou et al., 2015; Chatzifragkou et al., 2016). Cellulose and hemicellulose are the main carbohydrates in DDGS that can be potentially hydrolysed to monomeric sugars, namely glucose, xylose and arabinose and used as fermentation feedstock (Bals et al., 2006; Xu and Hanna, 2010). However, plant-derived biomass residues including DDGS are known for their rigid structure and reluctance to enzymatic breakdown, which renders the release of fermentable monomeric sugars from such matrices a challenge (Zhao et al., 2012). The main contributors towards the complex and rigid structure of plant biomass are the interactions between lignin, cellulose and hemicellulose as well as the crystallinity of cellulose. Because of the complexity of biomass structure, the hydrolysis of lignocellulosic materials into fermentable sugars constitutes a major bottleneck in biorefining industries. DDGS has the advantage over other agri-food biomass residues and by-products that it contains low amounts of lignin (~ 5% on a dry basis), which renders the fractionation process considerably easier (Chatzifragkou et al., 2016).

A significant amount of work has demonstrated that for the complete hydrolysis of various agri-food materials, efficient, scalable and cost-effective pretreatment strategies are required to enhance the enzymatic digestibility of the carbohydrates and thus increase their conversion to fermentable sugars (Kim et al., 2016). A number of pretreatment strategies have been investigated for a variety of feedstocks with the focus being on lignocellulosic materials, including dilute acid hydrolysis (Hsu et al., 2010), ammonium fibre expansion (AFEX) (Dien et al., 2008; Kim et al., 2008b), hot water extraction (Kim et al., 2009; Yang et al., 2011), steam explosion (Yang et al.,

2011) and alkaline treatment (Asghar et al., 2015; Kim et al., 2016; Kim et al., 2008a; McIntosh and Vancov, 2011; Subhedar and Gogate, 2014). This has been previously demonstrated for a variety of biomass including soybean straw (Wan et al., 2011), sugarcane bagasse (da Silva et al., 2016; Zhao et al., 2009), switchgrass (Xu et al., 2010), wheat straw (Asghar et al., 2015; Han et al., 2012; McIntosh and Vancov, 2011), barley hull (Kim et al., 2008a), corn stover (Yang et al., 2011) and corn-based DDGS (Dien et al., 2008; Kim et al., 2008b). In this study, the influence of NaOH concentration, residence time and extraction temperature on the recovery of carbohydrates from wheat DDGS was investigated.

Among these pretreatments, the use of alkaline regents is promising as it can alter the degree of polymerization of lignocellulosic components and increase the porosity and surface area of the biomass by solubilising hemicellulose, thus swelling its structure and potentially rendering it more susceptible to subsequent enzymatic saccharification (Chatzifragkou et al., 2015; Wan et al., 2011). Moreover, alkaline pretreatment is relatively simple and scalable, while the method normally uses chemicals such as ammonia, sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), and calcium hydroxide ($CaOH_2$). NaOH has attracted more attention as it is one of the strongest base catalysts and has a long history of being used as a reagent to pretreat the lignocellulosic material (Kim et al., 2016). The aim of this study was to investigate the effect of alkaline (NaOH) pretreatment of DDGS on the enzymatic digestibility of its cellulosic and hemicellulosic components and monitor the chemical and physical changes taking place during DDGS alkaline pretreatment, fractionation and subsequent enzymatic hydrolysis. This knowledge is important in order to design effective strategies for the fractionation and hydrolysis of DDGS targeting the production of nutrient-rich fermentation feedstocks.

2. Materials and Methods

2.1 Raw materials and enzymes

Dried Distillers Grains with Solubles (DDGS) was supplied from a UK bioethanol plant (Vivergo, Yorkshire, UK). DDGS was ground into fine powder using a coffee grinder (DeLonghi, Australia), sieved through sieve mesh No. 20 (particle size smaller than 0.85 mm) and stored at room temperature (20 ^{o}C) prior to analysis. The commercial enzyme, Accellerase® 1500 was kindly provided by Danisco US Inc. (Genencor, Leiden, Netherlands) and was stored at 4 ^{o}C until further use. According to the manufacturer's specifications, Accellerase® 1500 exerted endo-glucananase (2200 – 2800 CMC U/g), exoglucanase, hemicellulase and β-glucosidase (450 – 775 *p*NPG U/g) activities.

2.2 Composition of DDGS

The proximate composition (moisture, crude protein, crude fat and ash) of DDGS was determined according to the official methods of the Association of Analytical Communities (1996) (AOAC). The moisture content was determined using the oven-dry method at 105 ^{o}C (overnight) and was expressed as percentage by weight of sample. The dried DDGS was then heated for ash content determination in a muffle furnace at 530 ^{o}C for 4 hours. The protein content was determined using the Kjeldahl method, where DDGS was digested in concentrated sulfuric acid, H_2SO_4, followed by distillation and titration with 0.1 N H_2SO_4. The nitrogen content of DDGS was then multiplied by a factor of 5.7 (nitrogen conversion factor for wheat) to calculate the percentage of crude protein. For fat content analysis, petroleum ether was used as the extraction solvent. The extraction was carried out in a Soxhlet apparatus for 4 hours. The solvent was

removed from the extracted fat by evaporation using a rotary evaporator at 60 ^{o}C, followed by oven drying at 105 ^{o}C for approximately 1 hour. The percentage fat was calculated on a mass basis compared to the initial sample. The total starch content was quantified using the Megazyme determination kit (K-TSTA 09/14, Megazyme, Ireland).

The carbohydrate (cellulose and hemicellulose) content and lignin content of DDGS were determined according to the method by the National Renewable Energy Laboratory (NREL/TP-510-42618), which involves a two-step acid hydrolysis process to hydrolyse polysaccharides to monosaccharides (glucose derived from cellulose, and xylose and arabinose derived from hemicellulose). Acid-soluble lignin was quantified by ultraviolet spectroscopy at 320 nm wavelength, while acid-insoluble lignin was determined gravimetrically after acid hydrolysis (Sluiter et al., 2011). The sugar composition of the acid hydrolysed DDGS samples was determined by high performance liquid chromatography (HPLC) following the protocol described in section 2.5.4.

2.3 DDGS alkaline pretreatment process

DDGS was treated with sodium hydroxide (NaOH) at different concentrations (0, 1, 3 and 5%, w/v), temperatures (30, 50, 70 and 121 ^{o}C) and time (0.25, 0.5 and 6 h) in 250 ml screw cap glass bottles, using a 1:10 solid to liquid ratio (100 mL of solution). The trials at 30, 50 and 70 ^{o}C were conducted in a water-bath, with the stirring set at 200 rpm, whereas the trial at 121 ^{o}C was carried out in an autoclave (pressure ~16 psi). After the pretreatment, the material was cooled down to room temperature and centrifuged at 17,105 x g (Heraeus Multifuge X3R, Thermo Fisher, USA) for 20 minutes at 4 ^{o}C. The obtained solids were extensively washed with distilled water

until the pH reached around 8; the pH was then adjusted between 5 - 5.5 using 6 M HCl. Both insoluble and soluble fractions were frozen (-20 ^{o}C), freeze-dried for approximately 5 days, and stored in a closed container at room temperature until further analysis.

2.4 Enzymatic hydrolysis of DDGS

Enzymatic hydrolysis of untreated and pretreated DDGS solids was carried out in 250 ml Duran bottles at 50 ^{o}C and 300 rpm for 48 hours. Different ratios of Accellerase® 1500 to cellulose (1 ml: 3.3, 0.66, 0.33 and 0.22 g) were used to hydrolyse untreated DDGS (30%, w/v). Samples were collected at several times intervals, heat inactivated at 95 ^{o}C for 10 minutes and centrifuged at 17,105 x g for 20 minutes (15 ^{o}C). The supernatant was kept for sugar analysis, which was conducted by HPLC (see section 2.5.4). The enzyme loading concentration that gave the highest amount of glucose released was selected and tested against alkaline pretreated DDGS.

2.5 Physicochemical characterisation of DDGS

2.5.1 Fourier transform infrared (FTIR) analysis

FTIR analysis of untreated and pretreated DDGS solid samples was performed to determine the changes in functional groups caused as a result of the pretreatment process. One gram of dried sample (particle size < 0.85 mm) was uniformly spread on the crystal surface area and covered by a flat probe tip. The spectra (10 scans per sample) of both DDGS samples were collected from 4000 to 500 cm^{-1} at a 4 cm^{-1} resolution using a benchtop FTIR Spectrometer (Perkin-Elmer Spectrum 100, USA), equipped with a universal attenuated total reflection (ATR)

178 accessory and the Atmospheric Vapor Compensation (AVC) software, which was used to remove
179 spectral interferences caused by water and carbon dioxide.
180
181 2.5.2 X-ray diffraction (XRD) analysis
182 The X-ray diffraction pattern and crystallinity of untreated and pretreated DDGS solid
183 samples was determined by a powdered X-ray diffractometer (Bruker D8 Advance, Germany) at
184 40 kV and 40 mA using Cu Kα radiation (λ = 1.54 Å). The scan range was between 2θ = 5 and 65^{o}
185 with a step size of 0.02° and the scattered ray beam was detected using a Lynxeye XE detector.
186 The degree of crystallinity (X_c) was calculated as (Binod et al., 2012; Zhou et al., 2005):
187 $$X_c\ (\%) = F_c / (F_c + F_a)\ \text{X}\ 100,$$
188 where F_c and F_a are the areas of the crystalline and non-crystalline region, respectively.
189
190 2.5.3 Environmental scanning electron microscopy (ESEM) analysis
191 The surface of untreated and pretreated DDGS solid samples was analysed by Quanta FEG
192 600 Environmental Scanning Electron Microscopy (FEI Co. Inc., Hillsboro, Oregon). Samples
193 were mounted onto SEM stubs using carbon tape and then sputter coated with a thin layer of gold
194 to prevent charging during imaging. The parameters used for imaging were: 20 kV of accelerating
195 voltages, 4.0 spot size and a working distance approximately 10 – 12 mm. Images were recorded
196 under vacuum at 200X magnification.
197

198 2.5.4 Analytical method

199 The sugars composition of DDGS solid samples (untreated, alkaline treated, enzymatically
200 treated) and DDGS hydrolysate were determined by HPLC. An Agilent Infinity 1260 system
201 (Agilent Technologies, USA) was used with an Aminex HPX-87H column (Bio-rad, Hercules,
202 CA). Analysis was performed at 0.6 ml/min flow rate, with 5 mM H_2SO_4 as mobile phase. The
203 temperature of the column was set at 65 ^oC and sugars were detected using a refractive index
204 detector (RID). Quantification of compounds was performed according to external calibration
205 curves using glucose, arabinose, xylose (Sigma Aldrich), cellobiose, xylobiose and xylotriose
206 (Megazyme) as standards.

207 Mass recoveries were calculated as the mass of insoluble solids or mass of soluble material
208 (dried liquid fraction) recovered, and expressed as a percentage of the initial mass. Total mass
209 recovery was calculated by adding up the masses of insoluble solids and soluble material together
210 (Wan et al., 2011). Sugar recovery was calculated by expressing the amount of glucose, xylose or
211 arabinose released in the hydrolysates as a percentage of the amount of each respective sugar in
212 the original DDGS (da Silva et al., 2016).

213

214 2.7 Statistical Analysis

215 Statistical analysis was conducted using the Minitab®16 statistical analysis software. One-
216 way analysis of variance (ANOVA) with a Tukey's multiple comparison test was used to
217 determine significant differences between treatments, at a confidence level of 95% ($P < 0.05$).
218 Results are presented as mean ± standard deviation.

219

3. Results and discussion

3.1 Chemical composition of DDGS

The composition of wheat DDGS is presented in Table 1. DDGS contained high amounts of protein and fibre (total of cellulose, hemicellulose and beta-glucans) equal to approximately 28.3 and 31.4% (w/w), respectively, whereas the amounts of lignin, lipids and starch were approximately 2.9, 3.4 and 0.8 % (w/w), respectively. The low starch content was expected as starch is hydrolysed during the saccharification step during the bioethanol production process. The protein, fibre, fat and mineral contents (ash) were broadly in line with other works reporting the composition of wheat DDGS (Chatzifragkou et al., 2016; Cozannet et al., 2010; Pedersen et al., 2014; Widyaratne and Zijlstra, 2007). In terms of the protein and fat contents, the results obtained in this study are similar with the data reported by Chatzifragkou et al. (Chatzifragkou et al., 2016), who reported 29% for protein and 3.4% for fat. However, higher protein and fat contents were reported by Cozannet et al., (Cozannet et al., 2010) (36% and 4.6%, respectively) and Pedersen et al., (Pedersen et al., 2014) (33.4% and 5.25%, respectively). In terms of the ash content, this study found similar result (5.6%, w/w) with Cozannet et al., (Cozannet et al., 2010) (5.2%) and Widyaratne and Zijlstra (2007) (5.3%), whereas other studies reported varied ash contents, i.e. 3.9% (Chatzifragkou et al., 2016) and 9.1% (w/w) (Pedersen et al., 2014). Cellulose (including β-glucan) and hemicellulose contents of DDGS were approximately 11.1 and 20.3% (w/w), respectively. These values are lower than data reported by Chatzifragkou et al., (Chatzifragkou et al., 2016) (15% cellulose and 25% hemicellulose). However, lower cellulose (7%) and higher hemicellulose (42%) was reported for wheat DDGS derived from a bioethanol plant in western Canada (Nuez Ortín and Yu, 2009). The variation in the nutritional composition of DDGS might be associated with differences in the production processes used by different plants, the type of wheat cultivar, seasonal variation of

243 harvest and difference in N-to-protein conversion factor (6.25 over 5.7) (Chatzifragkou et al.,
244 2016).

245 Table 1: Chemical composition of wheat DDGS

Component	Composition (%, w/w dry basis)
Moisture	10.8 ± 0.1
Dry matter	89.2 ± 0.1
Crude protein	28.3 ± 0.5
Crude fat	3.40 ± 0.04
Starch	0.8 ± 0.2
*Cellulose (glucose)	11.1 ± 0.4
Hemicellulose	20.3 ± 1.7
Xylose	13.7 ± 1.6
Arabinose	6.6 ± 1.9
Lignin	2.0 ± 0.1
Acid Soluble Lignin	2.9 ± 0.1
Acid Insoluble Lignin	n.d.
Ash	5.64 ± 0.13

246 *It is assumed that all glucose comes from cellulose, though β-glucans might be also present at small
247 amounts
248 n.d.: not detected

249

250 3.2 Enzymatic hydrolysis of untreated DDGS

251 Figure 1 shows the effect of enzyme loading concentration on glucose yield from untreated
252 DDGS. The highest glucose concentration was achieved when DDGS was hydrolysed at a ratio of
253 1 : 0.22 (Accellerase® 1500, ml : g cellulose) for 24 h, corresponding to a yield of ~ 26 % (w/w),
254 although there was no significant difference ($P < 0.05$) with 1 : 0.33 (Accellerase® 1500, ml : g
255 cellulose) ratio. Since cellulose exists in both amorphous and crystalline states, the enzymatically

produced glucose was most likely derived from the amorphous state. At this amorphous state cellulose exists in a disordered arrangement, and Accellerase® 1500 through its endoglucanase and β-glucosidase activities was most likely able to selectively hydrolyse it into glucose and cellulo-oligosaccharides, as also shown previously (Gao et al., 2013). In the case of the crystalline state, the cellulose chains are closely packed together by strong intra- and inter- molecular hydrogen bond linkages. Because of these structural characteristics, cellulose digestibility has been reported to be around 20 % or less without any pretreatment steps for cellulosic biomass residues, such as wheat straw and sugarcane bagasse (Bensah and Mensah, 2013; Mosier et al., 2005; Rabelo et al., 2011). Besides glucose, xylose, arabinose and xylo-oligosaccharides (xylobiose and xylotriose) were also detected (data not shown) at the end of the hydrolysis which are due to the hemicellulase activities of Accellerase® 1500. The results obtained with DDGS in this work are in line with previous observations with lignocellulosic materials, indicating that glucose recovery from DDGS by an enzymatic process is limited by the structural characteristics of DDGS.

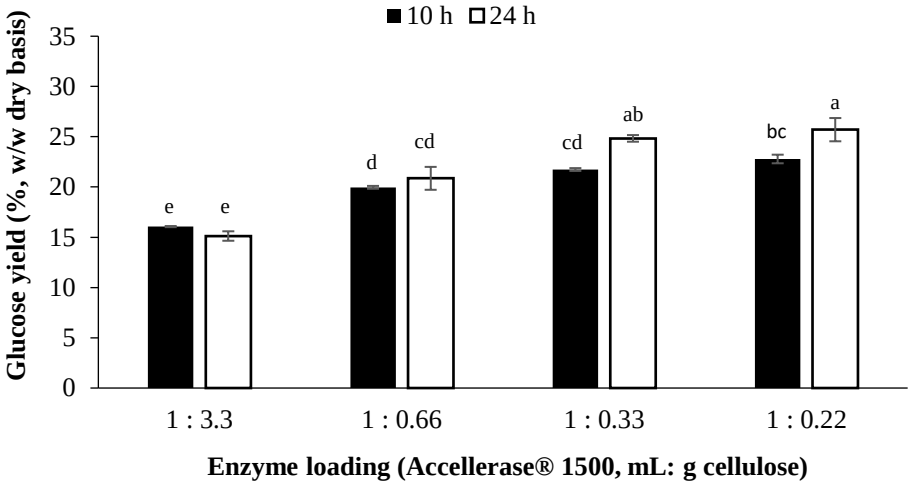

Figure 1: Effect of different concentrations of Accellerase ® 1500 enzyme on glucose production from untreated DDGS at 50 ºC. Means with different alphabet ^{abcd} are significantly ($P < 0.005$) different (Tukey's multiple range test).

3.3 Alkaline pretreatment of DDGS

Table 2 presents the monomeric sugar composition of DDGS solids following pretreatment at various temperatures (30, 50, 70 and 121 ^{o}C) and NaOH concentrations (0, 1, 3 and 5%, w/v) and the recoveries of glucose, xylose and arabinose compared to the original DDGS sample. When DDGS was treated with water at 30, 50, 70 and 121 ^{o}C the total concentrations of glucose, xylose and arabinose in the residual solids ranged from 38 - 42% (w/w) with the recoveries (compared to untreated DDGS) being 70 - 90% (w/w). This finding suggests that the majority of hemicellulose was not extracted from the DDGS solids (reflected by the high recovery of xylose and arabinose in the DDGS solids) and that if water was to be used for the pretreatment of DDGS, temperatures higher than 121 ^{o}C would most likely be needed in order to breakdown the cellulose bonds and solubilise hemicellulose. Pisupati and Tchapda (2015) reported that hemicellulose bonds breakdown at 150 – 230 ^{o}C, while significantly higher temperatures are required for cellulose breakdown (300 to 350 ^{o}C).

Table 2: Sugar composition and recovery in pretreated DDGS solids

Temperature (°C)	Time (h)	NaOH (%, w/v)	Sugar content in pretreated solids (%, w/w dry basis)				Sugar recovery in pretreated solids (%, w/w dry basis) *		
			Glucose	Xylose	Arabinose	Total	**Glucose**	Xylose	Arabinose
30	6	0	**13.4 ± 0.8^{gh}**	19.0 ± 1.4	9.9 ± 0.3	42.3	**73.6 ± 4.2^{a}**	83.9 ± 6.0	90.2 ± 3.0
		1	**14.7 ± 0.2^{fgh}**	18.8 ± 0.1	9.6 ± 0.4	43.1	**63.9 ± 2.6^{b}**	65.9 ± 1.9	69.6 ± 5.1
		3	**17.4 ± 0.4^{fgh}**	18.5 ± 0.1	9.5 ± 0.8	45.4	**53.7 ± 1.3^{c}**	45.6 ± 0.2	48.9 ± 4.3
		5	**20.0 ± 1.4^{f}**	14.4 ± 1.0	9.6 ± 0.2	44.0	**47.7 ± 1.1^{cd}**	27.6 ± 0.7	38.2 ± 2.8
50	6	0	**13.3 ± 0.0^{gh}**	18.7 ± 0.0	9.1 ± 0.0	41.1	**73.3 ± 0.3^{a}**	82.8 ± 0.4	83.3 ± 0.4
		1	**18.0 ± 0.1^{fgh}**	19.4 ± 0.1	8.2 ± 0.1	45.6	**62.5 ± 1.2^{b}**	54.3 ± 0.6	47.7 ± 0.5
		3	**27.6 ± 0.9^{e}**	25.2 ± 0.2	8.9 ± 0.0	61.7	**50.8 ±1.8^{cd}**	37.2 ± 0.4	27.4 ± 0.1
		5	**34.0 ± 0.5^{cd}**	18.9 ± 0.6	9.7 ± 0.6	62.6	**45.6 ± 0.02^{d}**	20.4 ± 0.3	21.7 ± 0.9
70	6	0	**13.2 ± 0.2^{gh}**	17.9 ± 0.4	9.3 ± 0.5	40.4	**72.5 ± 0.04^{a}**	78.7 ± 0.6	84.7 ± 3.1
		1	**28.3 ± 1.3^{de}**	25.2 ± 0.6	9.6 ± 0.5	63.2	**64.9 ± 3.4^{b}**	46.4 ± 0.9	36.6 ± 1.7
		3	**35.3 ± 0.8^{c}**	29.9 ± 0.2	10.2 ± 0.6	75.4	**48.6 ± 1.^{cd}**	33.2 ± 04	23.3 ± 1.4
		5	**44.7 ± 2.2^{b}**	21.8 ± 0.8	10.8 ± 0.3	77.2	**47.1 ± 1.1^{cd}**	18.4 ± 0.2	18.9 ± 0.02
121 (16 psi)	0.25	0	**13.5 ± 0.2^{gh}**	18.3 ± 0.3	10.6 ± 0.1	42.4	**68.4 ± 0.5^{ab}**	74.3 ± 0.8	89.2 ± 0.7
		0.5	**18.6 ± 0.7^{fg}**	21.2 ± 0.2	10.1 ± 0.02	49.9	**62.9 ± 2.2^{b}**	57.4 ± 0.3	56.9 ± 0.4
		1	**29.5 ± 0.4^{cde}**	29.7 ± 0.3	9.7 ± 0.2	69.0	**50.7 ± 0.04^{cd}**	40.9 ± 1.1	28.2 ± 0.1
		3	**43.8 ± 0.8^{b}**	31.5 ± 2.4	10.59 ± 0.3	85.9	**46.8 ± 0.5^{cd}**	27.0 ± 1.9	18.9 ± 0.4
		5	**52.6 ± 0.7^{a}**	25.0 ± 0.2	10.34 ± 0.1	87.9	**44.7 ± 1.1^{d}**	17.1 ± 0.8	14.7 ± 0.7
121 (16 psi)	0.5	0	**12.7 ± 0.2^{h}**	17.0 ± 0.5	8.17 ± 0.04	37.8	**64.1 ± 0.6^{b}**	69.3 ± 2.2	70.0 ± 0.1
		0.5	**17.4 ± 1.6^{fgh}**	19.9 ± 1.4	8.40 ± 0.4	45.7	**62.3 ± 2.6^{b}**	57.4 ± 1.2	50.4 ± 5.1
		1	**27.9 ± 1.0^{e}**	28.7 ± 1.4	7.99 ± 0.5	64.7	**49.0 ± 1.4^{cd}**	40.5 ± 1.6	23.4 ± 1.3
		3	**41.1 ± 1.0^{b}**	30.0 ± 1.8	10.01 ± 0.02	81.2	**47.0 ± 1.5^{cd}**	27.6 ± 1.3	19.1 ± 1.0
		5	**45.4 ± 5.2^{b}**	21.8 ± 1.6	9.47 ± 0.3	76.6	**44.4 ± 1.0^{d}**	17.1 ± 1.1	15.5 ± 1.7

Data reported as an average of two replicates

* Sugar recovery is calculated as the % of a particular sugar compared to its content in untreated DDGS

Means within each vertical line with different alphabet ^{abcdefgh} are significantly ($P < 0.05$) different

As the NaOH increased from 0.5 to 5.0% and the temperature from 30 to 121°C (16 psi) the total sugar content of the pretreated DDGS solids progressively increased, from around ~45% to a maximum of ~88% in the case of 121 °C (~16 psi), with 5% NaOH for 15 minutes. Glucose was in all cases the main sugar component, reflecting the significant presence of cellulose in the pretreated DDGS solids, as opposed to arabinose and xylose, which reflected the hemicellulose

content. In the case of the treatment at 121 ^{o}C with 5% NaOH, the glucose content of the DDGS solids was ~53%, a 5-fold increase compared to the glucose content of the original DDGS, whereas the xylose content was ~25 % and the arabinose ~10%. Alkaline pretreatment causes the solubilisation of hemicelluloses by disrupting the ester and ether bond of hemicellulose with lignin and cellulose microfibrils (Kim et al., 2016; McIntosh and Vancov, 2011). For this reason, and in line with the results in this study, the glucose concentration in the recovered solids has been shown to significantly increase in the case of alkaline pretreatment of lignocellulosic materials such as wheat straw and sugarcane bagasse (Barman et al., 2012; da Silva et al., 2016; McIntosh and Vancov, 2011). Moreover, the increase in cellulose and decrease in the hemicellulose of the pretreated DDGS solids was accompanied by a significant decrease in their protein content, which were solubilised and extracted in the liquid fraction (data not shown). It has been previously shown that under ethanol-alkali conditions (0.1 M), approximately 39.5% and 49.1% of the protein (primarily gliadin and glutenin) was extracted from wheat DDGS and wet distiller's grains, respectively (Chatzifragkou et al., 2016). Similarly, approximately 40% protein was extracted with 1M NaOH from corn wet distillers grains at 70 ^{o}C (Bals et al., 2009).

Taking into account the sugar recovery data presented in Table 2, it can be deduced that alkaline pretreatment selectively removed hemicelluloses over cellulose from DDGS solids. To this end, as the NaOH concentration increased from 0.5% to 5%, in all treatment temperatures, the recovery of xylose and arabinose in the pretreated solids progressively decreased; the lowest hemicellulose recovery was obtained after treatment with 5% NaOH at the highest temperature, 121 ^{o}C, with recoveries of ~17% for xylose and ~15% for arabinose. This is due to the fact that hemicellulose exists in an amorphous state, as heterogeneous and branched polysaccharides, which makes them more susceptible to alkaline action. The alkali reagent solubilises hemicellulose by

disrupting the ester linkage of hydroxycinnamic acid and the arabinose units present in the arabinoxylan molecules as well as and the linkage of the hydroxyl groups in lignin (Dodd and Cann, 2009; Xu et al., 2010). Interestingly, in terms of glucose recovery in the DDGS pretreatment solids, which reflects cellulose recovery, it was observed that as the NaOH increased from 0 to 5% the glucose gradually decreased at all temperatures. More specifically, at 70 ^{o}C, glucose recovery decreased from ~73% (no NaOH) to ~47% (5% NaOH), whereas at 121 ^{o}C, glucose recovery decreased from ~68% (no NaOH) to ~45% (5% NaOH), a phenomenon that will be discussed later on.

In order to investigate in more detail the effect of the process on the recoveries of cellulose and hemicellulose, a total mass and sugar recovery balance was conducted, as shown in Table 3. Even with no NaOH being added, the extraction process carried out at temperatures from 30 to 121 ^{o}C, extracted between 23 to 30% of soluble material from DDGS. This probably denotes the solubilisation of readily water soluble compounds that are found in DDGS, which are derived from the condensed distillers soluble (CDS) fraction that is commonly mixed with wet solids prior to drum drying during DDGS production process, and may include soluble proteins, organic acids and minerals (Bruynooghe et al., 2013; Liu, 2011). As the NaOH concentration increased, the mass of the liquid fraction increased due to the solubilisation of hemicelluloses and protein. However, the total mass balance, for both solid and liquid fractions, was not 100%, which could be attributed partly to differences in the moisture content of the dried solid (~ 2%) and dried liquid fractions (~2%), compared to the initial moisture content of untreated DDGS (~11%). Moreover, there is also a possibility that some organic compounds such as hydroxymethyl furfural (HMF), aldehydes, glycerol or short chain fatty acids such as lactic acid, which were originally present in DDGS,

decomposed to their gas state during alkaline pretreatment at high temperatures (Yin and Tan, 2012).

Table 3: Overall mass recovery and total sugar in pretreated DDGS solids *

Temperature (ºC)	Time (h)	NaOH (%, w/v)	Mass recovery of pretreated DDGS (% of initial weight)			Total sugar recovery (%)**		
			Solid	Liquid	Total	Glucose	Xylose	Arabinose
30	6	0	60.4 ± 0.1	23.6 ± 0.1	83.9	86.8 ± 3.8^{ab}	104.3 ± 5.5	113.4 ± 2.9
		1	48.0 ± 1.5	33.7 ± 0.3	81.7	82.2 ± 2.4^{abc}	101.9 ± 1.2	112.9 ± 5.7
		3	33.9 ± 0.1	39.1 ± 0.7	73.0	80.4 ± 2.6^{bcd}	98.5 ± 0.2	107.6 ± 5.7
		5	26.4 ± 2.5	49.4 ± 1.5	75.7	82.0 ± 2.1^{abc}	99.4 ± 2.4	115.9 ± 7.1
50	6	0	60.6 ± 0.3	26.5 ± 0.1	87.1	87.3 ± 0.02^{a}	101.9 ± 0.3	109.1 ± 1.2
		1	38.3 ± 0.6	46.2 ± 2.1	84.4	77.0 ± 1.3^{cd}	97.2 ± 1.1	110.5 ± 1.9
		3	20.3 ±0.1	58.0 ± 0.1	78.2	75.2 ± 0.1^{d}	99.8 ± 2.5	119.8 ± 3.9
		5	14.8 ± 0.2	67.9 ± 0.1	82.7	78.8 ± 1.8^{cd}	103.4 ± 0.9	132.7 ± 6.2
70	6	0	60.3 ±0.9	26.9 ± 0.6	87.2	87.6 ± 0.4^{a}	99.1 ± 1.0	109.7 ± 2.6
		1	25.2 ± 0.1	56.9 ± 0.1	82.1	76.6 ± 2.8^{cd}	91.3 ± 0.6	105.5 ± 3.9
		3	15.2 ± 0.1	67.3 ± 0.0	82.5	66.5 ± 2.3^{ef}	91.4 ± 3.5	99.4 ± 5.2
		5	11.6 ± 0.3	77.7 ± 0.5	89.3	67.1 ± 1.2^{e}	88.7 ± 4.8	100.9 ± 5.0
121 (16 psi)	0.25	0	55.7 ± 0.2	27.0 ± 0.1	82.6	87.1 ± 0.7^{a}	102.4 ± 1.2	117.9 ± 1.5
		0.5	37.1 ± 2.1	43.9 ± 0.2	81.0	77.0 ± 1.8^{cd}	101.1 ± 0.2	114.6 ± 0.7
		1	18.9 ± 0.3	56.3 ± 1.7	75.2	61.5 ± 0.1^{ef}	90.0 ± 2.4	110.3 ± 1.6
		3	11.8 ± 0.1	75.9 ± 1.0	87.6	66.1 ± 0.5^{ef}	96.3 ± 0.04	114.7 ± 1.2
		5	9.4 ± 0.4	82.0 ± 0.1	91.3	66.2 ± 1.2^{ef}	94.0 ± 6.8	116.6 ± 2.4
121 (16 psi)	0.5	0	55.8 ± 0.2	30.6 ± 0.4	86.4	87.1 ± 0.6^{a}	104.9 ± 3.9	110.6 ± 6.1
		0.5	39.6 ± 2.1	41.8 ± 1.7	81.4	77.4 ± 1.1^{cd}	97.4 ± 2.5	104.2 ± 2.8
		1	19.3 ± 0.1	59.3 ± 0.3	78.6	60.0 ± 1.0^{f}	88.4 ± 1.0	99.4 ± 0.1
		3	12.6 ± 0.7	72.6 ± 3.1	85.1	64.1 ± 0.9^{ef}	90.3 ± 5.9	104.9 ± 9.3
		5	10.9 ± 1.5	72.6 ± 0.3	83.5	65.8 ± 0.1^{ef}	91.6 ± 1.5	110.1 ± 3.0

* Data reported as an average of two replicate

** Total sugar recovery is calculated as the % of a particular sugar in both the solid and liquid fractions compared to its content in untreated DDGS

Means within each vertical line with different alphabet ^{abcdef} are significantly ($P < 0.05$) different

The total xylose and arabinose recovery in both solid and liquid fractions was in most cases very close to 100% (with some discrepancies found in the case of arabinose), indicating no

obvious losses for these compounds during the alkaline pretreatment process. The inconsistency in arabinose and xylose recovery (> 100%) might due to the variations in the moisture content of dry solid samples, compared to the initial moisture content of untreated DDGS, as previously mentioned. In contrast, considerable glucose losses were noted, especially as the temperature progressively increased from 30 ^{o}C to 121 ^{o}C. The highest losses were detected when DDGS was treated with 5% NaOH at 121 ^{o}C, and ranged from 30% to 40%. A comprehensive review by Knill and Kennedy (2003) suggested that the degradation of glucose during exposure to alkaline reagents such as NaOH, $CaCO_3$ and KOH leads to the production of various compounds including formic acid, acetic acid, hydroxyactic acid, 2-hydroxy-propanoic acid, butyric acid, 2-hydroxybutanoic acid, 3-deoxy-D-pentonic acid, β-D-glucoisaccharinic acid, D-gluconic acid and D-mannoic acid. End-wise degradation and alkaline scissions are the main mechanisms contributing to glucose losses during alkaline pretreatment. In end-wise degradation (or peeling), glucose loses are due to the dissolution of short chain material, which detaches from the reducing end of cellulose, and results in the formation of 3-deoxy-2-*C*-(hydroxymethyl)-erythro and thereo-pentonic acids (D-glucoisosaccharinic acids). This mechanism normally occurs at temperatures less than 170 ^{o}C. On the other hand, alkaline scission (or hydrolysis) normally occurs at higher temperatures (> 170 ^{o}C), where random hydrolysis of the glycosidic linkages takes place, and results in significant weight losses and decreases in the degree of polymerisation of cellulose (Knill and Kennedy, 2003). These reactions have also been suggested in other works, where it was shown that during alkaline pretreatment of lignocellulosic biomass at high temperatures, cellulose was converted into dissolved organic compounds such as dihydroxy and dicarboxylic acids, aldehydes, furfural or 1,2,3-benzenetriol (Jönsson and Martín, 2016; Yin and Tan, 2012).

384 3.4 Enzymatic hydrolysis of pretreated DDGS solids

385 The DDGS solid residues obtained after the alkaline pretreatment conditions which
386 demonstrated the highest total sugar content (5% NaOH, 121 ^{o}C, 15 min) were then subjected to
387 enzymatic hydrolysis using Accellerase® 1500 (

388). It was noted that glucose release was significantly higher when the pretreated DDGS
389 solids were hydrolysed with the enzyme compared to untreated DDGS (86% vs 25%, respectively).
390 This study demonstrated that pretreatment is an important step in improving cellulose digestibility
391 of DDGS. Similar findings were also reported by Xu et al., (Xu et al., 2010) who compared the
392 effect of alkaline pretreatment on switchgrass and showed that the yield of total reducing sugars
393 increased 3.78 times compared to untreated switchgrass. Alkaline pretreatment is known to cause
394 a swelling effect due to the solvation and saponification of hemicelluloses, thus it results in
395 increased porosity and loosening of the structure of DDGS. Therefore, the surface area of cellulose
396 is increased and is more exposed to enzymatic hydrolysis (Han et al., 2012; Kim and Han, 2012).

397

398 Table 4: Enzymatic hydrolysis of untreated and pretreated DDGS solids at 1 : 0.33 (Accellerase®
399 1500, mL : g cellulose) ratio for 24 h at 50^{o}C

Conditions	**Monosaccharides (g/L) in the hydrolysate**			**Glucose Yield (%)**
	Glucose	Xylose	Arabinose	
No pretreatment	8.2 ± 0.1	10.8 ± 0.0	1.7 ± 0.0	24.8
5% NaOH (121^{o}C, ~16 psi, 15 min)	28.5 ± 0.6	6.2 ± 0.2	0.6 ± 0.0	86.5

400

Accellerase® 1500 has side activities, including hemicellulase and β-glucosidase activities, as demonstrated by the considerable amounts of xylose and arabinose released into the hydrolysate. The concentration of xylose and arabinose in the hydrolysate after alkaline pretreatment was lower than in the case of untreated DDGS solids due to the significant removal of hemicelluloses during alkaline treatment. However, the pretreated DDGS solids still contained ~25% of xylose and ~10% of arabinose (Table 2), which are important to be released during enzyme hydrolysis through the action of hemicellulases in order to enhance glucose release. Enzyme cocktails that have, in addition to cellulase, hemicellulase activity, can facilitate glucose release from DDGS and lignocellulosic biomass, as shown for pretreated corn stover (Hu et al., 2011). Hemicellulases remove the xylan coat at the surface of the pretreated fibre and thus increase the accessibility of cellulase to the cellulosic fibre (Kumar and Wyman, 2009). Interestingly, hemicellulases such as xylanases, were also shown to significantly improve the cellulose hydrolysis of steam pretreated softwood by cellulases, regardless of the fact that this material did not contain xylan; this was due to the synergistic interaction between cellulase and xylanase, which changed the gross fibre characteristics of softwood (Hu et al., 2011).

Figure 1 shows the time course of the enzymatic hydrolysis of the alkaline pretreated DDGS solids, at 50 ^{o}C. In addition to glucose, xylose and arabinose, some oligosaccharides were also detected, including xylobiose and xylotriose. Interestingly, cellobiose was not detected. The absence of cellobiose suggests that the activity of exo-glucanase and/or β-glucosidase in Accellerase® 1500 was high, which resulted in the conversion of cellobiose and possibly other gluco-oligosaccharides to glucose. Borges et al., (Borges et al., 2014) reported a value of 228 U/mL of β-glucosidase activity in Accellerase® 1500 when cellobiose was used as a substrate. As shown in Figure 2, within 6 hours of hydrolysis, approximately 25 g/L of glucose, 6 g/L of

xylobiose, 5 g/L of xylose, 1.3 g/L of xylotriose and ~0.6 g/L of arabinose were produced. Further increase in hydrolysis time resulted in a slow increase in glucose concertation, reaching a maximum of ~29 g/L after 30 h of hydrolysis. During the same period, the concentration of xylotriose and xylobiose decreased with a concomitant increase in xylose, which reached 7.5 g/L after 30 h. It is most likely that xylobiose and xylotriose were produced through the activities of endo-xylanases present in Accelerase® 1500, whereas xylose, through the activity of beta-xylosidase, which hydrolyses xylobiose to xylose (Badhan et al., 2014).

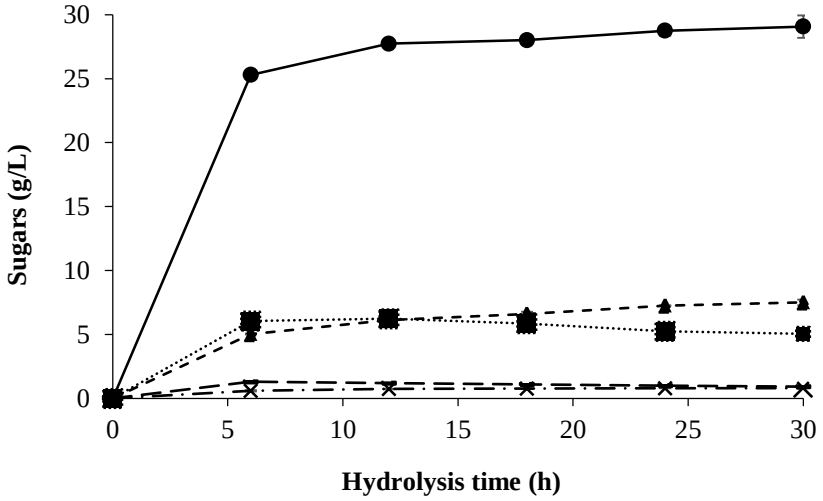

Figure 2: Time course of sugars released during enzymatic hydrolysis of pretreated DDGS solids (121 °C, ~16 psi, 15 minutes, 5% NaOH) at 1 : 0.33 (Accellerase® 1500, mL : g cellulose) ratio, (– ■ –) xylotriose, (····■····) xylobiose, (—●—) glucose, (· –▲– ·) xylose and (–×—) arabinose. No cellobiose was detected.

3.5 Physicochemical characterisation of untreated and pretreated DDGS

3.5.1 Morphological surface of DDGS

The surface of untreated and alkali treated DDGS was observed using ESEM to identify possible structural changes. The intact, compact and rigid surface structure of untreated DDGS (Figure 3a) changed into a fully exposed, separated and peeled-off surface in the alkali treated DDGS (Figure 3c). According to Bensah and Mensah (2013) alkaline pretreatment loosens the structure of biomass, hydrolyses lignin and carbohydrate bonds and decreases the degree of polymerisation and crystallinity of cellulose. Moreover, it also causes the enlargement of the internal surface area of biomass, thus it increases the access of cellulase to the cellulose present in the biomass (Chen et al., 2013; Xu et al., 2010). Previous studies with sugarcane bagasse (Binod et al., 2012) and wheat straw (Asghar et al., 2015) reported that alkaline pretreatment caused the formation of pores on the biomass surface, an effect that was not observed in our study though.

When untreated DDGS solids were hydrolysed with Accellerase® 1500, the changes to the structure were not profound (Figure 3b). It seems that the enzyme was not able to penetrate the rigid structure of the untreated DDGS cell wall, a fact that can further explain the low enzymatic release of glucose (Table 1). In contrast, the Accellerase® 1500 pretreated (with alkali) DDGS solids demonstrated a less rigid structure that was broken into small pieces, indicating the disruption of biomass (Figure 3d).

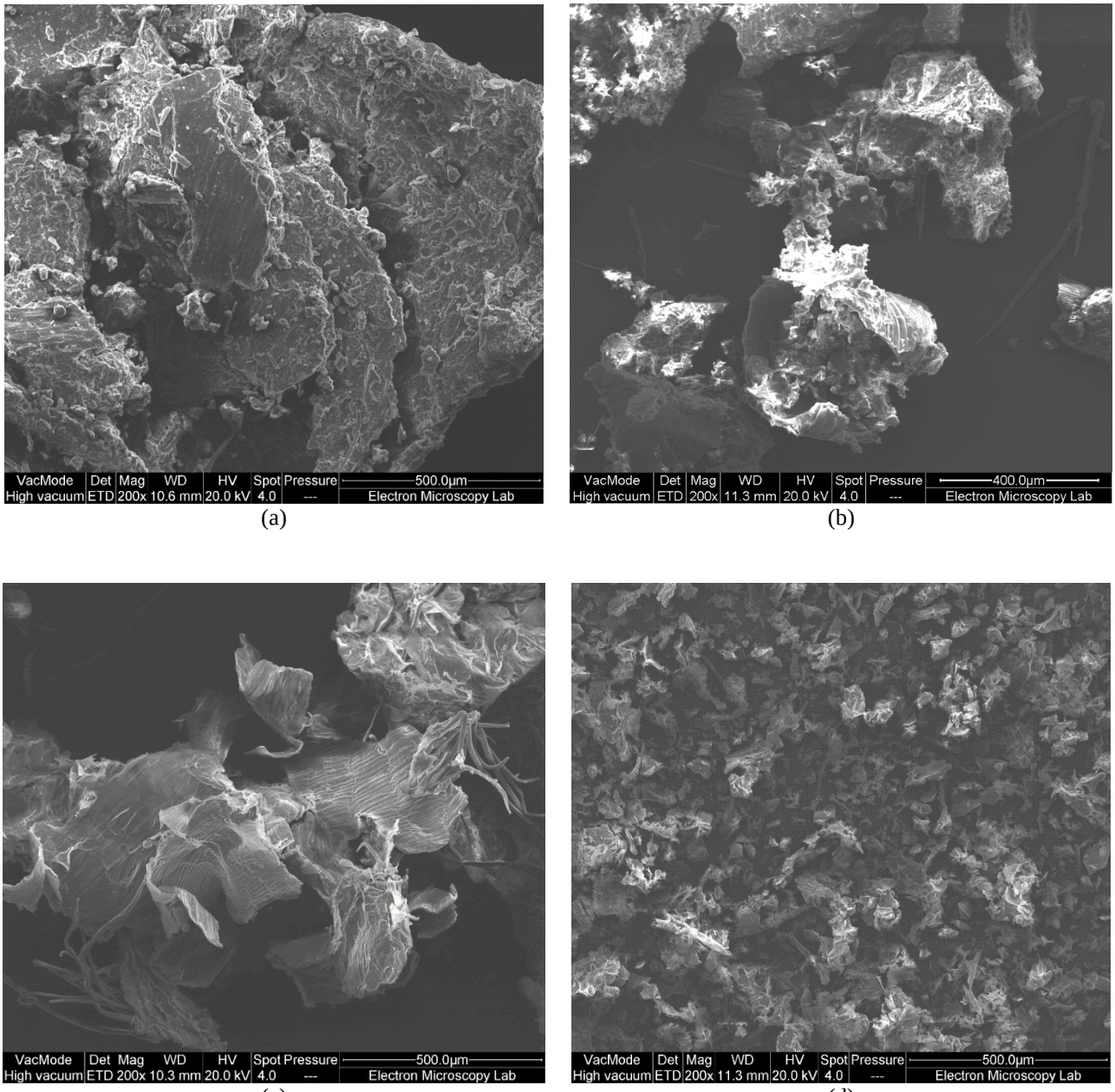

Figure 3: SEM images of: (a) untreated DDGS, (b) untreated DDGS hydrolysed with Accellerase® 1500 at 1 : 0.33 (mL enzyme : g cellulose), (c) alkaline pretreated (121ºC ~16 psi, 15 minutes, 5% NaOH) DDGS and (d) alkaline pretreated (121ºC ~16 psi, 15 minutes, 5% NaOH) DDGS hydrolysed with Accellerase® 1500 at 1 : 0.33 (mL enzyme : g cellulose).

3.5.2 Spectral characterisation

The changes in functional groups caused by alkaline pretreatment on DDGS were evaluated by Fourier Transform Infrared (FTIR) spectroscopy (Figure 4). A prominent broad peak was observed at a wavelength of 1034 cm^{-1} in the case of pretreated DDGS solids as opposed to untreated DDGS. A numbers of previous research works assigned this peak to C-O, C=C and C-C-O stretching of β-(1,4) glycoside bonds in cellulose, hemicellulose and lignin (Maryana et al., 2014; Schwanninger et al., 2004; Sills and Gossett, 2012; Xu et al., 2013). This suggests that the increased peak intensity was due to the higher content of cellulose and hemicellulose in pretreated DDGS solids (~88% total cellulose and hemicellulose), compared to untreated DDGS (~31% total cellulose and hemicellulose). Similar FTIR data were reported for alkaline treated wheat straw (Asghar et al., 2015) and sugarcane bagasse (Zhang et al., 2013). Moreover, the broad peak centred at 3332 cm^{-1} in pretreated DDGS solids, which is absent in untreated DDGS, most likely reflects an increased intermolecular hydrogen bonding between the β-(1,4) glucan chains of cellulose in the sample (Hishikawa et al., 2017); this is line with the higher cellulose content of pretrated DDGS (~53%) (Table 2) compared to untreated DDGS (Table 1). In addition, the very small peaks that were present in untreated DDGS at 1598 and 1744 cm^{-1} are assigned to C=O stretching and most likely correspond to the hemicellulose present in DDGS. However, these bands could also be related to the uronic esters and acetyl groups of the ferulic and p-coumaric acids present in lignin (Barman et al., 2012; Schwanninger et al., 2004). The peaks disappeared after pretreatment suggesting that some of hemicellulose or lignin-related compounds were removed during the pretreatment; this coincides with the results in Table 2, where only 17% xylose and 15% arabinose were recovered in pretreated DDGS solids.

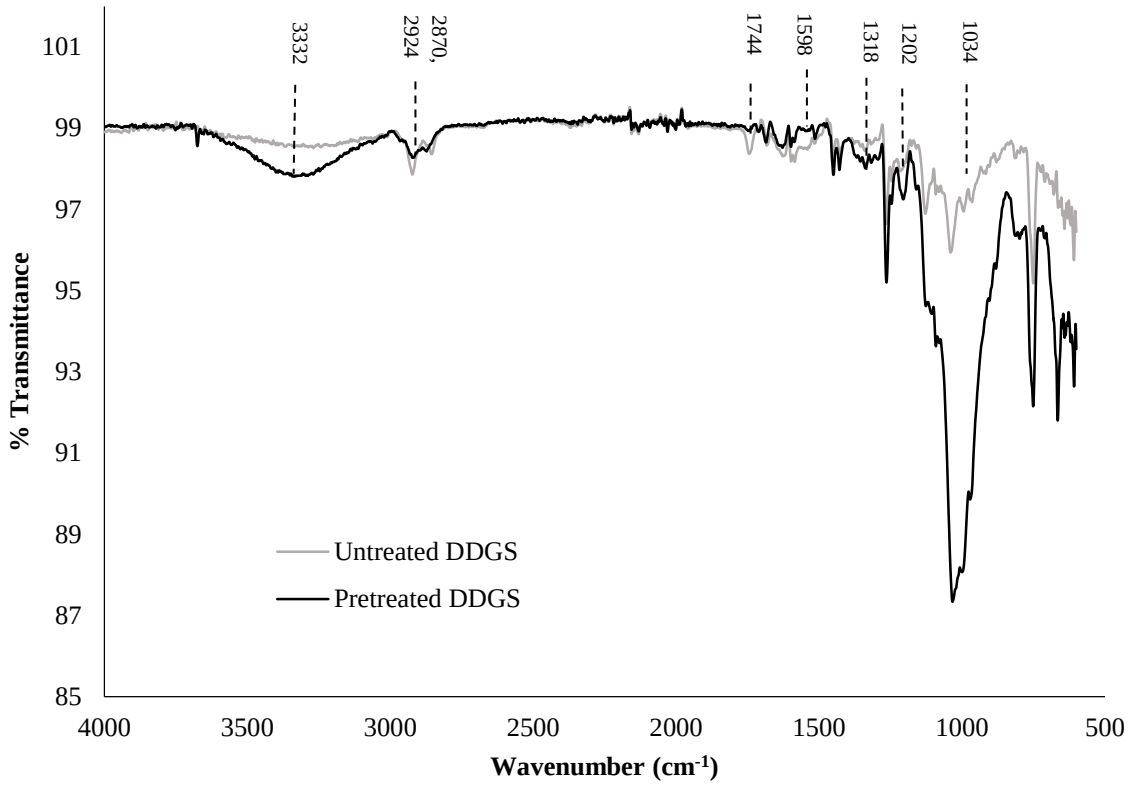

Figure 4: FTIR spectra of untreated DDGS and pretreated (121 °C, ~16 psi, 15 minutes, 5% NaOH) DDGS solids.

3.5.3 X-ray diffraction (XRD)

X-ray diffraction analysis was conducted to assess the effect of crystallinity on the digestibility of DDGS. The obtained XRD spectra were analysed and the results are presented in Table 5. The degrees of crystallinity (X_c) for untreated and pretreated DDGS were different. For untreated DDGS solids, the X_c was estimated at 39.7%, while pretreated DDGS solids had 3 times lower degree of crystallinity, estimated at 13.1%. This was most likely due to the structural changes caused by alkaline pretreatment, more specifically the fact that NaOH cleaves the ester linkages

between lignin and hemicellulose, which reduces the degree of polymerisation of cellulose and consequently the crystallinity of DDGS (Barman et al., 2012).

Table 5: Crystalline and amorphous area of untreated and pretreated solids (121 °C, ~16 psi, 15 minutes, 5% NaOH) DDGS

Substrate	Untreated DDGS	Pretreated DDGS
Crystalline Area	9409.63	1338.27
Amorphous Area	14272.53	8846.83
Degree of crystallinity (X_c), %	39.73	13.14

Data generated from TOPAS 2.1 software (Rietveld refinement method)

3.6 Overall mass balance of valorisation process scheme

Figure 5 shows the overall process design based on a alkaline pretreatment process of DDGS with 5% NaOH at 121°C (~16 psi) for 15 minutes (the best pretreatment condition for obtaining maximum carbohydrate content in the solid fraction), followed by enzymatic hydrolysis of the residual solids using Accelerase® 1500, and the mass balances of the key components (sugar monomers reflecting cellulose and hemicellulose, proteins) in the solid and liquid fractions. Untreated DDGS contained ~11% glucose reflecting the presence of cellulose (the assumption is that the level of β-glucan is zero), and ~13.7% xylose and ~6.6% from arabinose, reflecting the presence of ~20.3% hemicellulose. DDGS was then subjected to alkaline pretreatment after which ~45% of glucose, ~17% of xylose and ~15% of arabinose remained in the recovered solids compared to the starting DDGS material. The majority of the hemicellulose, i.e. ~77% of xylose and ~100% of arabinose (although the discrepancy in the arabinose mass balance is noted) and the

majority of the protein (~79%) were extracted into the liquid fraction. The enzymatic hydrolysis (using Accelerase® 1500) of the pretreated DDGS solids led to the recovery of ~87% of glucose and ~92% of xylose, indicating the production of a glucose-rich medium. Such medium can be used as a fermentation feedstock for the production of a range of platform or speciality chemicals with high market potential, such as lactic acid and succinic acid, in various industrial sectors (e.g. food, plastics, packaging and chemical sectors). The hemicellulose and protein rich liquid fraction could be further explored. For instance, the liquid fraction can be subjected to ultrafiltration and the isolated protein can be used as starting material for biodegradable films and bioplastics production or as precursor for chemical synthesis (Chatzifragkou et al., 2016; Jones et al., 2015). On the other hand, research on the use of hemicelluloses as materials for edible coating, films or food packaging applications has been reported (Hansen and Plackett, 2008; Xiang et al., 2014) and has led to commercial applications. For example, Xylophane AB have successfully marketed their xylan-based packaging material (Chatzifragkou et al., 2015). Overall, the proposed process scheme recovers the majority of the key DDGS components (cellulose, hemicellulose, proteins) in an efficient manner with relatively low losses, and provides a viable approach for the valorisation of DDGS.

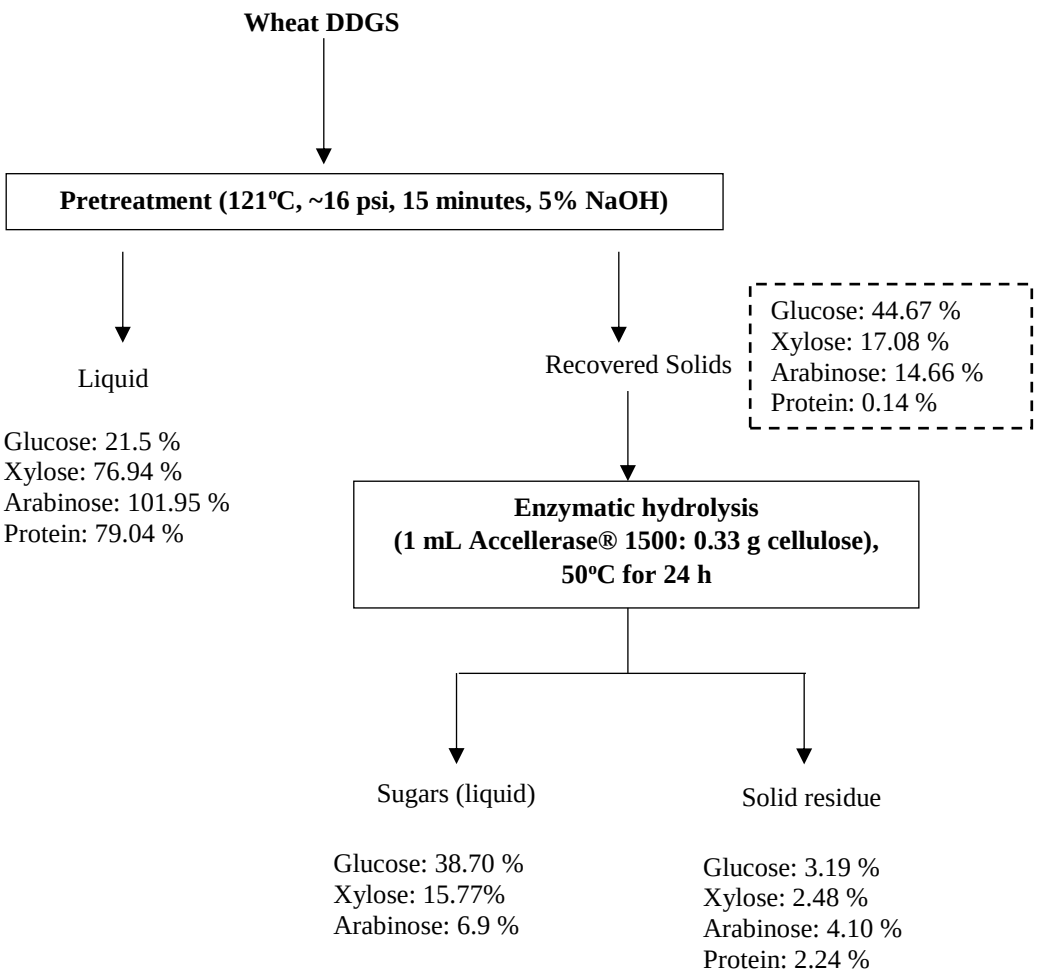

Figure 5: Process scheme and mass balances for the valorisation of DDGS.

4. Conclusions

A cellulose-rich solid material was generated from DDGS by alkaline treatment, containing ~88% (w/w) of sugars with the majority attributed to the presence of cellulose (~53%). The pretreated DDGS solids exhibited significant enzymatic digestibility, leading to 3.6 fold higher glucose concentration compared to untreated DDGS. Approximately 83% of the hemicellulose and

79% of the protein present in untreated DDGS were removed during alkaline pretreatment into the liquid fraction. Mass balance analysis of the proposed DDGS valorisation scheme demonstrated that the major DDGS components (cellulose, hemicellulose, proteins) were recovered in the solid and liquid process fractions in an efficient manner.

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