

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

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

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21 **Abstract**

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

38

39

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

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## 43 **1. Introduction**

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

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

66 process (Villegas-Torres et al., 2015). According to Han and Liu (2010), around 20% of the total  
67 protein in corn DDGS is contributed by yeast. From a valorisation perspective, the protein in  
68 DDGS can be recovered using chemical extraction methods and could be potentially utilised in  
69 food, feed and agricultural applications (Chatzifragkou et al., 2015; Chatzifragkou et al., 2016).  
70 Cellulose and hemicellulose are the main carbohydrates in DDGS that can be potentially  
71 hydrolysed to monomeric sugars, namely glucose, xylose and arabinose and used as fermentation  
72 feedstock (Bals et al., 2006; Xu and Hanna, 2010). However, plant-derived biomass residues  
73 including DDGS are known for their rigid structure and reluctance to enzymatic breakdown, which  
74 renders the release of fermentable monomeric sugars from such matrices a challenge (Zhao et al.,  
75 2012). The main contributors towards the complex and rigid structure of plant biomass are the  
76 interactions between lignin, cellulose and hemicellulose as well as the crystallinity of cellulose.  
77 Because of the complexity of biomass structure, the hydrolysis of lignocellulosic materials into  
78 fermentable sugars constitutes a major bottleneck in biorefining industries. DDGS has the  
79 advantage over other agri-food biomass residues and by-products that it contains low amounts of  
80 lignin (~ 5% on a dry basis), which renders the fractionation process considerably easier  
81 (Chatzifragkou et al., 2016).

82 A significant amount of work has demonstrated that for the complete hydrolysis of various  
83 agri-food materials, efficient, scalable and cost-effective pretreatment strategies are required to  
84 enhance the enzymatic digestibility of the carbohydrates and thus increase their conversion to  
85 fermentable sugars (Kim et al., 2016). A number of pretreatment strategies have been investigated  
86 for a variety of feedstocks with the focus being on lignocellulosic materials, including dilute acid  
87 hydrolysis (Hsu et al., 2010), ammonium fibre expansion (AFEX) (Dien et al., 2008; Kim et al.,  
88 2008b), hot water extraction (Kim et al., 2009; Yang et al., 2011), steam explosion (Yang et al.,

89 2011) and alkaline treatment (Asghar et al., 2015; Kim et al., 2016; Kim et al., 2008a; McIntosh  
90 and Vancov, 2011; Subhedar and Gogate, 2014). This has been previously demonstrated for a  
91 variety of biomass including soybean straw (Wan et al., 2011), sugarcane bagasse (da Silva et al.,  
92 2016; Zhao et al., 2009), switchgrass (Xu et al., 2010), wheat straw (Asghar et al., 2015; Han et  
93 al., 2012; McIntosh and Vancov, 2011), barley hull (Kim et al., 2008a), corn stover (Yang et al.,  
94 2011) and corn-based DDGS (Dien et al., 2008; Kim et al., 2008b). In this study, the influence of  
95 NaOH concentration, residence time and extraction temperature on the recovery of carbohydrates  
96 from wheat DDGS was investigated.

97         Among these pretreatments, the use of alkaline reagents is promising as it can alter the  
98 degree of polymerization of lignocellulosic components and increase the porosity and surface area  
99 of the biomass by solubilising hemicellulose, thus swelling its structure and potentially rendering  
100 it more susceptible to subsequent enzymatic saccharification (Chatzifragkou et al., 2015; Wan et  
101 al., 2011). Moreover, alkaline pretreatment is relatively simple and scalable, while the method  
102 normally uses chemicals such as ammonia, sodium hydroxide (NaOH), sodium carbonate  
103 ( $\text{Na}_2\text{CO}_3$ ), and calcium hydroxide ( $\text{CaOH}_2$ ). NaOH has attracted more attention as it is one of the  
104 strongest base catalysts and has a long history of being used as a reagent to pretreat the  
105 lignocellulosic material (Kim et al., 2016). The aim of this study was to investigate the effect of  
106 alkaline (NaOH) pretreatment of DDGS on the enzymatic digestibility of its cellulosic and  
107 hemicellulosic components and monitor the chemical and physical changes taking place during  
108 DDGS alkaline pretreatment, fractionation and subsequent enzymatic hydrolysis. This knowledge  
109 is important in order to design effective strategies for the fractionation and hydrolysis of DDGS  
110 targeting the production of nutrient-rich fermentation feedstocks.

111

## 112 2. Materials and Methods

### 113 2.1 Raw materials and enzymes

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

122

### 123 2.2 Composition of DDGS

124 The proximate composition (moisture, crude protein, crude fat and ash) of DDGS was  
125 determined according to the official methods of the Association of Analytical Communities (1996)  
126 (AOAC). The moisture content was determined using the oven-dry method at 105 °C (overnight)  
127 and was expressed as percentage by weight of sample. The dried DDGS was then heated for ash  
128 content determination in a muffle furnace at 530 °C for 4 hours. The protein content was  
129 determined using the Kjeldahl method, where DDGS was digested in concentrated sulfuric acid,  
130 H<sub>2</sub>SO<sub>4</sub>, followed by distillation and titration with 0.1 N H<sub>2</sub>SO<sub>4</sub>. The nitrogen content of DDGS  
131 was then multiplied by a factor of 5.7 (nitrogen conversion factor for wheat) to calculate the  
132 percentage of crude protein. For fat content analysis, petroleum ether was used as the extraction  
133 solvent. The extraction was carried out in a Soxhlet apparatus for 4 hours. The solvent was



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

138         The carbohydrate (cellulose and hemicellulose) content and lignin content of DDGS were  
139 determined according to the method by the National Renewable Energy Laboratory (NREL/TP-  
140 510-42618), which involves a two-step acid hydrolysis process to hydrolyse polysaccharides to  
141 monosaccharides (glucose derived from cellulose, and xylose and arabinose derived from  
142 hemicellulose). Acid-soluble lignin was quantified by ultraviolet spectroscopy at 320 nm  
143 wavelength, while acid-insoluble lignin was determined gravimetrically after acid hydrolysis  
144 (Sluiter et al., 2011). The sugar composition of the acid hydrolysed DDGS samples was determined  
145 by high performance liquid chromatography (HPLC) following the protocol described in section  
146 2.5.4.

147

### 148 2.3 DDGS alkaline pretreatment process

149         DDGS was treated with sodium hydroxide (NaOH) at different concentrations (0, 1, 3 and  
150 5%, w/v), temperatures (30, 50, 70 and 121 °C) and time (0.25, 0.5 and 6 h) in 250 ml screw cap  
151 glass bottles, using a 1:10 solid to liquid ratio (100 mL of solution). The trials at 30, 50 and 70 °C  
152 were conducted in a water-bath, with the stirring set at 200 rpm, whereas the trial at 121 °C was  
153 carried out in an autoclave (pressure ~16 psi). After the pretreatment, the material was cooled  
154 down to room temperature and centrifuged at 17,105 x g (Heraeus Multifuge X3R, Thermo Fisher,  
155 USA) for 20 minutes at 4 °C. The obtained solids were extensively washed with distilled water

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

159

## 160 2.4 Enzymatic hydrolysis of DDGS

161 Enzymatic hydrolysis of untreated and pretreated DDGS solids was carried out in 250 ml  
162 Duran bottles at 50 °C and 300 rpm for 48 hours. Different ratios of Accellerase® 1500 to cellulose  
163 (1 ml: 3.3, 0.66, 0.33 and 0.22 g) were used to hydrolyse untreated DDGS (30%, w/v). Samples  
164 were collected at several times intervals, heat inactivated at 95 °C for 10 minutes and centrifuged  
165 at 17,105 x g for 20 minutes (15 °C). The supernatant was kept for sugar analysis, which was  
166 conducted by HPLC (see section 2.5.4). The enzyme loading concentration that gave the highest  
167 amount of glucose released was selected and tested against alkaline pretreated DDGS.

168

## 169 2.5 Physicochemical characterisation of DDGS

170

### 171 2.5.1 Fourier transform infrared (FTIR) analysis

172 FTIR analysis of untreated and pretreated DDGS solid samples was performed to  
173 determine the changes in functional groups caused as a result of the pretreatment process. One  
174 gram of dried sample (particle size < 0.85 mm) was uniformly spread on the crystal surface area  
175 and covered by a flat probe tip. The spectra (10 scans per sample) of both DDGS samples were  
176 collected from 4000 to 500 cm<sup>-1</sup> at a 4 cm<sup>-1</sup> resolution using a benchtop FTIR Spectrometer  
177 (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 K $\alpha$  radiation ( $\lambda = 1.54 \text{ \AA}$ ). The scan range was between  $2\theta = 5$  and  $65^\circ$   
185 with a step size of  $0.02^\circ$  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):

$$187 \quad X_c (\%) = F_c / (F_c + F_a) \times 100,$$

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<sub>2</sub>SO<sub>4</sub> as mobile phase. The  
203 temperature of the column was set at 65 °C 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  $\pm$  standard deviation.

219

### 220 3. Results and discussion

#### 221 3.1 Chemical composition of DDGS

222 The composition of wheat DDGS is presented in Table 1. DDGS contained high amounts of protein  
223 and fibre (total of cellulose, hemicellulose and beta-glucans) equal to approximately 28.3 and  
224 31.4% (w/w), respectively, whereas the amounts of lignin, lipids and starch were approximately  
225 2.9, 3.4 and 0.8 % (w/w), respectively. The low starch content was expected as starch is hydrolysed  
226 during the saccharification step during the bioethanol production process. The protein, fibre, fat  
227 and mineral contents (ash) were broadly in line with other works reporting the composition of  
228 wheat DDGS (Chatzifragkou et al., 2016; Cozannet et al., 2010; Pedersen et al., 2014; Widyaratne  
229 and Zijlstra, 2007). In terms of the protein and fat contents, the results obtained in this study are  
230 similar with the data reported by Chatzifragkou et al. (Chatzifragkou et al., 2016), who reported  
231 29% for protein and 3.4% for fat. However, higher protein and fat contents were reported by  
232 Cozannet et al., (Cozannet et al., 2010) (36% and 4.6%, respectively) and Pedersen et al., (Pedersen  
233 et al., 2014) (33.4% and 5.25%, respectively). In terms of the ash content, this study found similar  
234 result (5.6%, w/w) with Cozannet et al., (Cozannet et al., 2010) (5.2%) and Widyaratne and Zijlstra  
235 (2007) (5.3%), whereas other studies reported varied ash contents, i.e. 3.9% (Chatzifragkou et al.,  
236 2016) and 9.1% (w/w) (Pedersen et al., 2014). Cellulose (including  $\beta$ -glucan) and hemicellulose  
237 contents of DDGS were approximately 11.1 and 20.3% (w/w), respectively. These values are lower  
238 than data reported by Chatzifragkou et al., (Chatzifragkou et al., 2016) (15% cellulose and 25%  
239 hemicellulose). However, lower cellulose (7%) and higher hemicellulose (42%) was reported for  
240 wheat DDGS derived from a bioethanol plant in western Canada (Nuez Ortín and Yu, 2009). The  
241 variation in the nutritional composition of DDGS might be associated with differences in the  
242 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**

<b>Component</b>	<b>Composition (% , w/w dry basis)</b>
<b>Moisture</b>	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

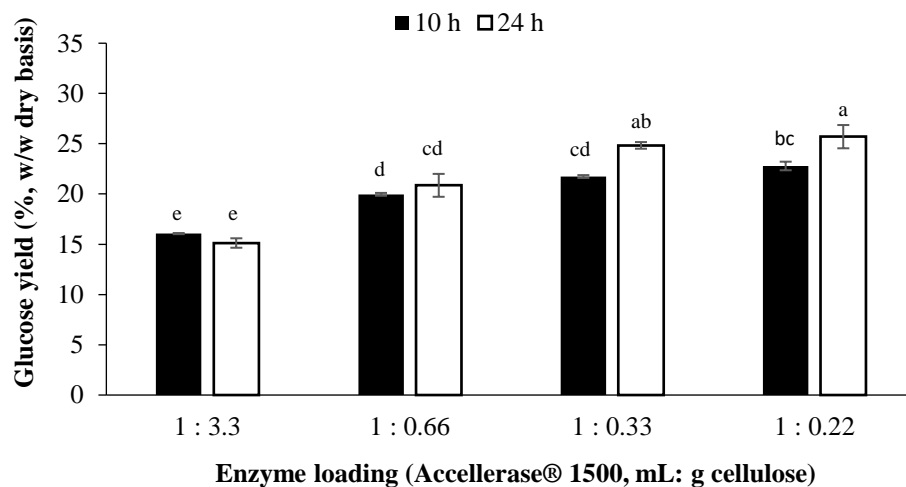
246 \*It is assumed that all glucose comes from cellulose, though  $\beta$ -glucans might be also present at small  
247 amounts  
248 n.d.: not detected

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

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



269  
 270 Figure 1: Effect of different concentrations of Accellerase ® 1500 enzyme on glucose production from  
 271 **untreated** DDGS at 50 °C. Means with different alphabet <sup>abcd</sup> are significantly (P < 0.005) different (Tukey's  
 272 multiple range test).

273

### 274 3.3 Alkaline pretreatment of DDGS

275

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

288

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

296 Data reported as an average of two replicates

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

298 Means within each vertical line with different alphabet <sup>abcde fgh</sup> are significantly (P < 0.05) different

299

300 As the NaOH increased from 0.5 to 5.0% and the temperature from 30 to 121°C (16 psi)

301 the total sugar content of the pretreated DDGS solids progressively increased, from around ~45%

302 to a maximum of ~88% in the case of 121 °C (~16 psi), with 5% NaOH for 15 minutes. Glucose

303 was in all cases the main sugar component, reflecting the significant presence of cellulose in the

304 pretreated DDGS solids, as opposed to arabinose and xylose, which reflected the hemicellulose

305 content. In the case of the treatment at 121 °C with 5% NaOH, the glucose content of the DDGS  
306 solids was ~53%, a 5-fold increase compared to the glucose content of the original DDGS, whereas  
307 the xylose content was ~25 % and the arabinose ~10%. Alkaline pretreatment causes the  
308 solubilisation of hemicelluloses by disrupting the ester and ether bond of hemicellulose with lignin  
309 and cellulose microfibrils (Kim et al., 2016; McIntosh and Vancov, 2011). For this reason, and in  
310 line with the results in this study, the glucose concentration in the recovered solids has been shown  
311 to significantly increase in the case of alkaline pretreatment of lignocellulosic materials such as  
312 wheat straw and sugarcane bagasse (Barman et al., 2012; da Silva et al., 2016; McIntosh and  
313 Vancov, 2011). Moreover, the increase in cellulose and decrease in the hemicellulose of the  
314 pretreated DDGS solids was accompanied by a significant decrease in their protein content, which  
315 were solubilised and extracted in the liquid fraction (data not shown). It has been previously shown  
316 that under ethanol-alkali conditions (0.1 M), approximately 39.5% and 49.1% of the protein  
317 (primarily gliadin and glutenin) was extracted from wheat DDGS and wet distiller's grains,  
318 respectively (Chatzifragkou et al., 2016). Similarly, approximately 40% protein was extracted with  
319 1M NaOH from corn wet distillers grains at 70 °C (Bals et al., 2009).

320 Taking into account the sugar recovery data presented in Table 2, it can be deduced that  
321 alkaline pretreatment selectively removed hemicelluloses over cellulose from DDGS solids. To  
322 this end, as the NaOH concentration increased from 0.5% to 5%, in all treatment temperatures, the  
323 recovery of xylose and arabinose in the pretreated solids progressively decreased; the lowest  
324 hemicellulose recovery was obtained after treatment with 5% NaOH at the highest temperature,  
325 121 °C, with recoveries of ~17% for xylose and ~15% for arabinose. This is due to the fact that  
326 hemicellulose exists in an amorphous state, as heterogeneous and branched polysaccharides, which  
327 makes them more susceptible to alkaline action. The alkali reagent solubilises hemicellulose by

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

336           In order to investigate in more detail the effect of the process on the recoveries of  
337 cellulose and hemicellulose, a total mass and sugar recovery balance was conducted, as shown in  
338 Table 3. Even with no NaOH being added, the extraction process carried out at temperatures from  
339 30 to 121 °C, extracted between 23 to 30% of soluble material from DDGS. This probably denotes  
340 the solubilisation of readily water soluble compounds that are found in DDGS, which are derived  
341 from the condensed distillers soluble (CDS) fraction that is commonly mixed with wet solids prior  
342 to drum drying during DDGS production process, and may include soluble proteins, organic acids  
343 and minerals (Bruynooghe et al., 2013; Liu, 2011). As the NaOH concentration increased, the mass  
344 of the liquid fraction increased due to the solubilisation of hemicelluloses and protein. However,  
345 the total mass balance, for both solid and liquid fractions, was not 100%, which could be attributed  
346 partly to differences in the moisture content of the dried solid (~ 2%) and dried liquid fractions  
347 (~2%), compared to the initial moisture content of untreated DDGS (~11%). Moreover, there is  
348 also a possibility that some organic compounds such as hydroxymethyl furfural (HMF), aldehydes,  
349 glycerol or short chain fatty acids such as lactic acid, which were originally present in DDGS,

350 decomposed to their gas state during alkaline pretreatment at high temperatures (Yin and Tan,  
 351 2012).

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

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

353 \* Data reported as an average of two replicate

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

356 Means within each vertical line with different alphabet <sup>abcdef</sup> are significantly (P < 0.05) different

357

358

359 The total xylose and arabinose recovery in both solid and liquid fractions was in most

360 cases very close to 100% (with some discrepancies found in the case of arabinose), indicating no

361 obvious losses for these compounds during the alkaline pretreatment process. The inconsistency  
362 in arabinose and xylose recovery (> 100%) might due to the variations in the moisture content of  
363 dry solid samples, compared to the initial moisture content of untreated DDGS, as previously  
364 mentioned. In contrast, considerable glucose losses were noted, especially as the temperature  
365 progressively increased from 30 °C to 121 °C. The highest losses were detected when DDGS was  
366 treated with 5% NaOH at 121 °C, and ranged from 30% to 40%. A comprehensive review by Knill  
367 and Kennedy (2003) suggested that the degradation of glucose during exposure to alkaline reagents  
368 such as NaOH, CaCO<sub>3</sub> and KOH leads to the production of various compounds including formic  
369 acid, acetic acid, hydroxyacetic acid, 2-hydroxy-propanoic acid, butyric acid, 2-hydroxybutanoic  
370 acid, 3-deoxy-D-pentonic acid, β-D-glucoisaccharinic acid, D-gluconic acid and D-mannonic acid.  
371 End-wise degradation and alkaline scissions are the main mechanisms contributing to glucose  
372 losses during alkaline pretreatment. In end-wise degradation (or peeling), glucose loses are due to  
373 the dissolution of short chain material, which detaches from the reducing end of cellulose, and  
374 results in the formation of 3-deoxy-2-C-(hydroxymethyl)-erythro and thereo-pentonic acids (D-  
375 glucoisosaccharinic acids). This mechanism normally occurs at temperatures less than 170 °C. On  
376 the other hand, alkaline scission (or hydrolysis) normally occurs at higher temperatures (> 170 °C),  
377 where random hydrolysis of the glycosidic linkages takes place, and results in significant weight  
378 losses and decreases in the degree of polymerisation of cellulose (Knill and Kennedy, 2003). These  
379 reactions have also been suggested in other works, where it was shown that during alkaline  
380 pretreatment of lignocellulosic biomass at high temperatures, cellulose was converted into  
381 dissolved organic compounds such as dihydroxy and dicarboxylic acids, aldehydes, furfural or  
382 1,2,3-benzenetriol (Jönsson and Martín, 2016; Yin and Tan, 2012).

383

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 °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°C**

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

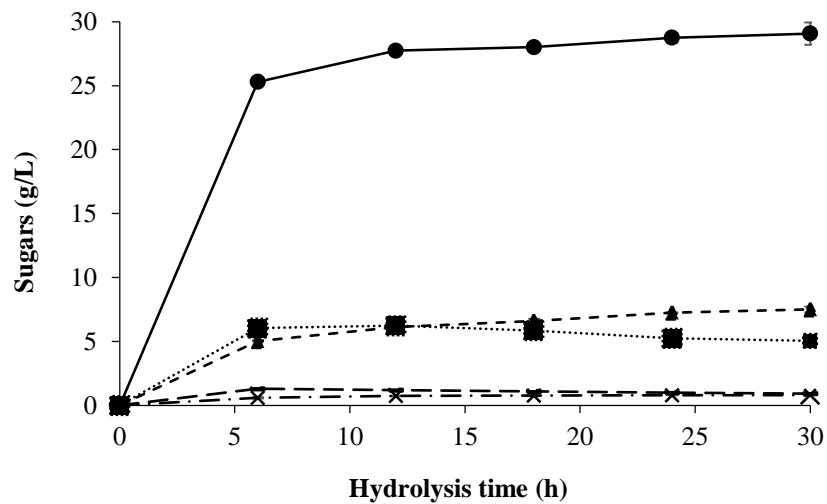
400

401 Accellerase® 1500 has side activities, including hemicellulase and  $\beta$ -glucosidase activities,  
402 as demonstrated by the considerable amounts of xylose and arabinose released into the  
403 hydrolysate. The concentration of xylose and arabinose in the hydrolysate after alkaline  
404 pretreatment was lower than in the case of untreated DDGS solids due to the significant removal  
405 of hemicelluloses during alkaline treatment. However, the pretreated DDGS solids still contained  
406 ~25% of xylose and ~10% of arabinose (Table 2), which are important to be released during  
407 enzyme hydrolysis through the action of hemicellulases in order to enhance glucose release.  
408 Enzyme cocktails that have, in addition to cellulase, hemicellulase activity, can facilitate glucose  
409 release from DDGS and lignocellulosic biomass, as shown for pretreated corn stover (Hu et al.,  
410 2011). Hemicellulases remove the xylan coat at the surface of the pretreated fibre and thus increase  
411 the accessibility of cellulase to the cellulosic fibre (Kumar and Wyman, 2009). Interestingly,  
412 hemicellulases such as xylanases, were also shown to significantly improve the cellulose  
413 hydrolysis of steam pretreated softwood by cellulases, regardless of the fact that this material did  
414 not contain xylan; this was due to the synergistic interaction between cellulase and xylanase, which  
415 changed the gross fibre characteristics of softwood (Hu et al., 2011).

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

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

431



432

433 Figure 2: Time course of sugars released during enzymatic hydrolysis of pretreated DDGS solids (121 °C,  
 434 ~16 psi, 15 minutes, 5% NaOH) at 1 : 0.33 (Accelerase® 1500, mL : g cellulose) ratio, (-■-) xylotriose,  
 435 (···■···) xylobiose, (—●—) glucose, (-▲-) xylose and (-×-) arabinose. No cellobiose was detected.

436

437

438

439



## 440 3.5 Physicochemical characterisation of untreated and pretreated DDGS

441

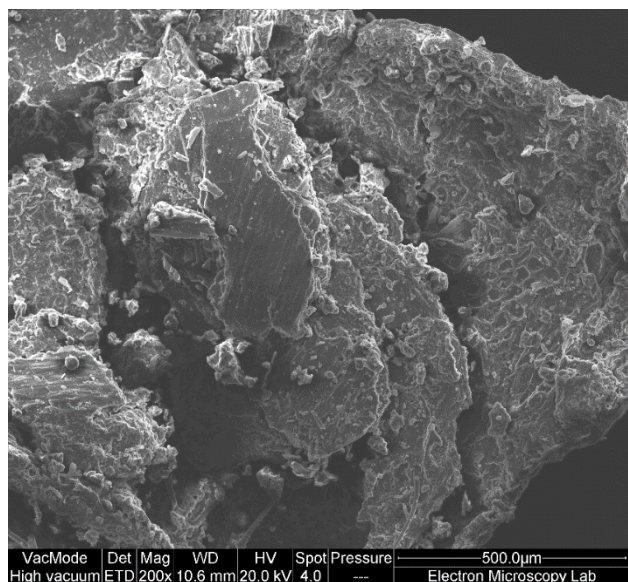
### 442 3.5.1 Morphological surface of DDGS

443

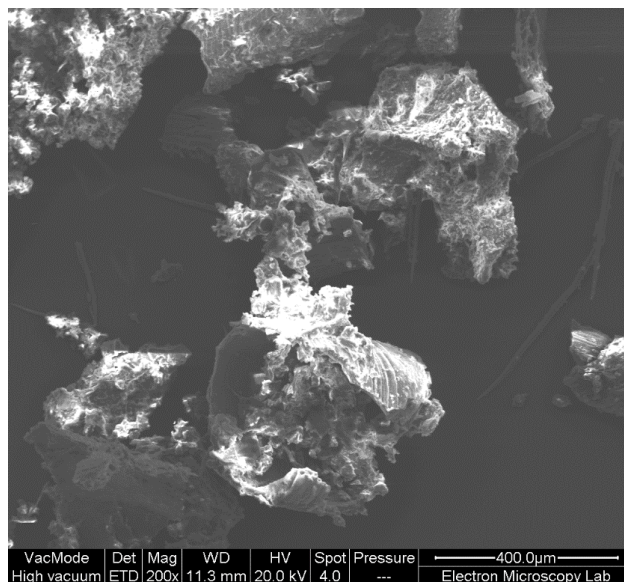
444 The surface of untreated and alkali treated DDGS was observed using ESEM to identify  
445 possible structural changes. The intact, compact and rigid surface structure of untreated DDGS  
446 (Figure 3a) changed into a fully exposed, separated and peeled-off surface in the alkali treated  
447 DDGS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

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

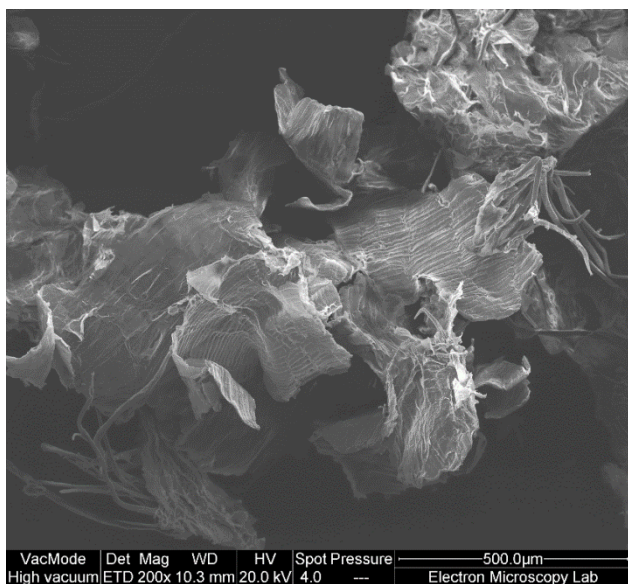
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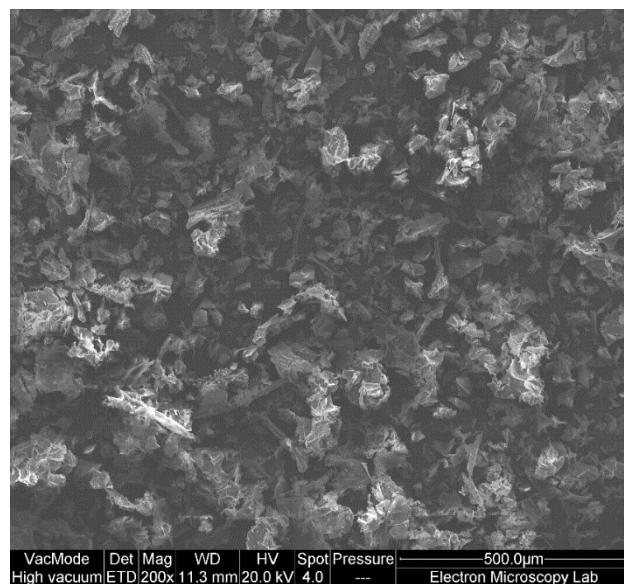
(a)



(b)



(c)



(d)

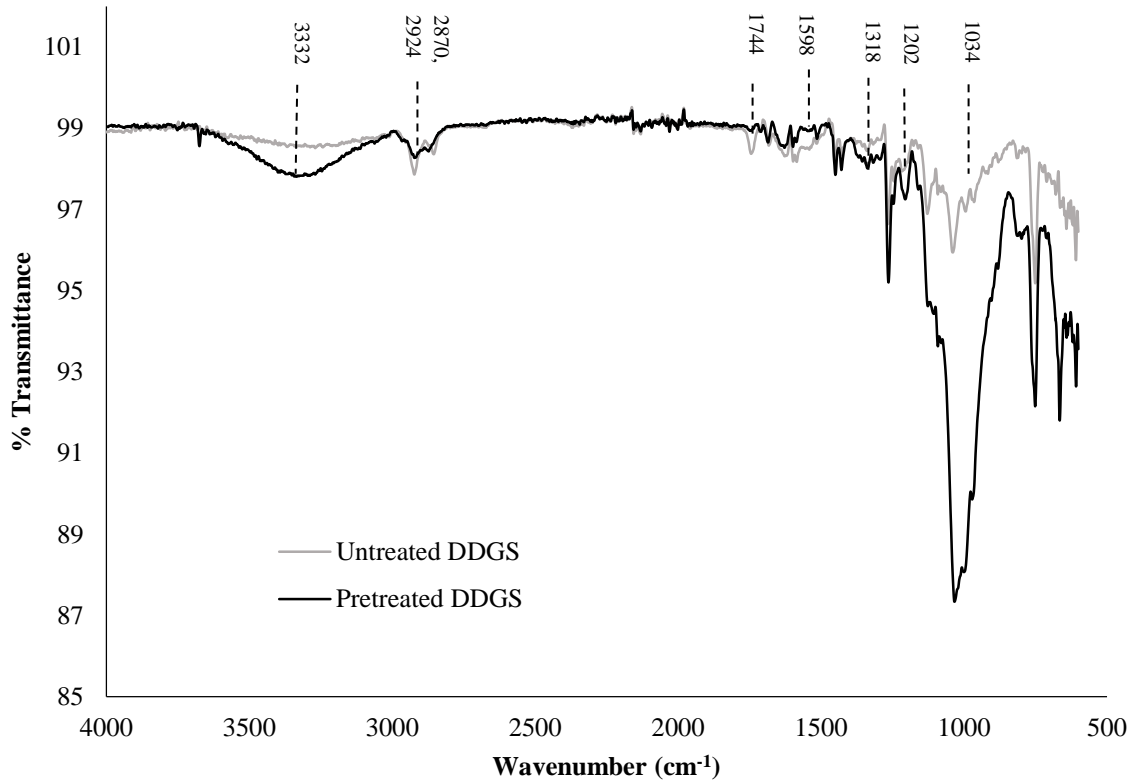
461 Figure 3: SEM images of: (a) untreated DDGS, (b) untreated DDGS hydrolysed with Accellerase® 1500  
 462 at 1 : 0.33 (mL enzyme : g cellulose), (c) alkaline pretreated (121°C ~16 psi, 15 minutes, 5% NaOH) DDGS  
 463 and (d) alkaline pretreated (121°C ~16 psi, 15 minutes, 5% NaOH) DDGS hydrolysed with Accellerase®  
 464 1500 at 1 : 0.33 (mL enzyme : g cellulose).

465

### 466 3.5.2 Spectral characterisation

467         The changes in functional groups caused by alkaline pretreatment on DDGS were  
468 evaluated by Fourier Transform Infrared (FTIR) spectroscopy (Figure 4). A prominent broad peak  
469 was observed at a wavelength of  $1034\text{ cm}^{-1}$  in the case of pretreated DDGS solids as opposed to  
470 untreated DDGS. A numbers of previous research works assigned this peak to C-O, C=C and C-  
471 C-O stretching of  $\beta$ -(1,4) glycoside bonds in cellulose, hemicellulose and lignin (Maryana et al.,  
472 2014; Schwanninger et al., 2004; Sills and Gossett, 2012; Xu et al., 2013). This suggests that the  
473 increased peak intensity was due to the higher content of cellulose and hemicellulose in pretreated  
474 DDGS solids (~88% total cellulose and hemicellulose), compared to untreated DDGS (~31% total  
475 cellulose and hemicellulose). Similar FTIR data were reported for alkaline treated wheat straw  
476 (Asghar et al., 2015) and sugarcane bagasse (Zhang et al., 2013). Moreover, the broad peak centred  
477 at  $3332\text{ cm}^{-1}$  in pretreated DDGS solids, which is absent in untreated DDGS, most likely reflects  
478 an increased intermolecular hydrogen bonding between the  $\beta$ -(1,4) glucan chains of cellulose in  
479 the sample (Hishikawa et al., 2017); this is line with the higher cellulose content of pretrated DDGS  
480 (~53%) (Table 2) compared to untreated DDGS (Table 1). In addition, the very small peaks that  
481 were present in untreated DDGS at  $1598$  and  $1744\text{ cm}^{-1}$  are assigned to C=O stretching and most  
482 likely correspond to the hemicellulose present in DDGS. However, these bands could also be  
483 related to the uronic esters and acetyl groups of the ferulic and p-coumaric acids present in lignin  
484 (Barman et al., 2012; Schwanninger et al., 2004). The peaks disappeared after pretreatment  
485 suggesting that some of hemicellulose or lignin-related compounds were removed during the  
486 pretreatment; this coincides with the results in Table 2, where only 17% xylose and 15% arabinose  
487 were recovered in pretreated DDGS solids.

488



489  
 490 Figure 4: FTIR spectra of untreated DDGS and pretreated (121 °C, ~16 psi, 15 minutes, 5% NaOH) DDGS  
 491 solids.

492  
 493 3.5.3 X-ray diffraction (XRD)

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

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

502

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

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

505 Data generated from TOPAS 2.1 software (Rietveld refinement method)

506

### 507 3.6 Overall mass balance of valorisation process scheme

508 Figure 5 shows the overall process design based on a alkaline pretreatment process of DDGS  
509 with 5% NaOH at 121°C (~16 psi) for 15 minutes (the best pretreatment condition for obtaining  
510 maximum carbohydrate content in the solid fraction), followed by enzymatic hydrolysis of the  
511 residual solids using Accelerase® 1500, and the mass balances of the key components (sugar  
512 monomers reflecting cellulose and hemicellulose, proteins) in the solid and liquid fractions.  
513 Untreated DDGS contained ~11% glucose reflecting the presence of cellulose (the assumption is  
514 that the level of  $\beta$ -glucan is zero), and ~13.7% xylose and ~6.6% from arabinose, reflecting the  
515 presence of ~20.3% hemicellulose. DDGS was then subjected to alkaline pretreatment after which  
516 ~45% of glucose, ~17% of xylose and ~15% of arabinose remained in the recovered solids  
517 compared to the starting DDGS material. The majority of the hemicellulose, i.e. ~77% of xylose  
518 and ~100% of arabinose (although the discrepancy in the arabinose mass balance is noted) and the

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

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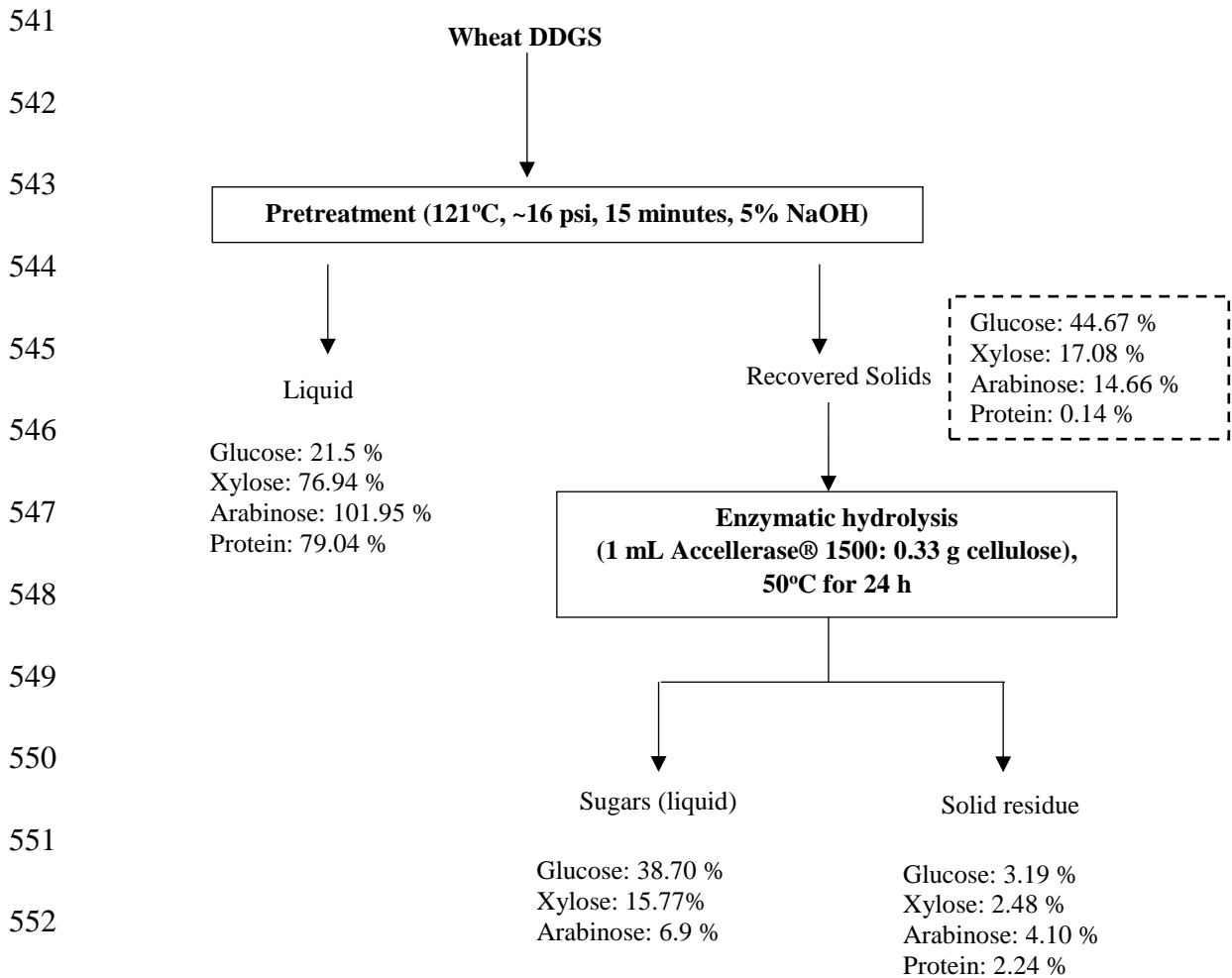
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555 Figure 5: Process scheme and mass balances for the valorisation of DDGS.

557 **4. Conclusions**

558 A cellulose-rich solid material was generated from DDGS by alkaline treatment, containing  
 559 ~88% (w/w) of sugars with the majority attributed to the presence of cellulose (~53%). The  
 560 pretreated DDGS solids exhibited significant enzymatic digestibility, leading to 3.6 fold higher  
 561 glucose concentration compared to untreated DDGS. Approximately 83% of the hemicellulose and

562 79% of the protein present in untreated DDGS were removed during alkaline pretreatment into the  
563 liquid fraction. Mass balance analysis of the proposed DDGS valorisation scheme demonstrated  
564 that the major DDGS components (cellulose, hemicellulose, proteins) were recovered in the solid  
565 and liquid process fractions in an efficient manner.

566

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## 575 **References**

576 Asghar, U., Irfan, M., Iram, M., Huma, Z., Nelofer, R., Nadeem, M., Syed, Q., 2015. Effect of  
577 alkaline pretreatment on delignification of wheat straw. *Nat. Prod. Res.* 29, 125-131.  
578 Association of Analytical Communities, 1996. *Official Methods of Analysis of AOAC*  
579 *International*, 16th ed. AOAC International, Maryland, USA.  
580 Badhan, A., Wang, Y., Gruninger, R., Patton, D., Powlowski, J., Tsang, A., McAllister, T., 2014.  
581 Formulation of enzyme blends to maximize the hydrolysis of alkaline peroxide pretreated alfalfa  
582 hay and barley straw by rumen enzymes and commercial cellulases. *BMC Biotechnology* 14, 31-  
583 31.



584 Bals, B., Balan, V., Dale, B., 2009. Integrating alkaline extraction of proteins with enzymatic  
585 hydrolysis of cellulose from wet distiller's grains and solubles. *Bioresour. Technol.* 100, 5876-  
586 5883.

587 Bals, B., Dale, B., Balan, V., 2006. Enzymatic hydrolysis of distiller's dry grain and solubles  
588 (DDGS) using ammonia fiber expansion pretreatment. *Energy & Fuels* 20, 2732-2736.

589 Barman, D.N., Haque, M.A., Kang, T.H., Kim, M.K., Kim, J., Kim, H., Yun, H.D., 2012. Alkali  
590 pretreatment of wheat straw (*Triticum aestivum*) at boiling temperature for producing a bioethanol  
591 precursor. *Biosci. Biotechnol. Biochem.* 76, 2201-2207.

592 Belyea, R.L., Rausch, K.D., Clevenger, T.E., Singh, V., Johnston, D.B., Tumbleson, M.E., 2010.  
593 Sources of variation in composition of DDGS. *Anim. Feed. Sci. Technol.* 159, 122-130.

594 Bensah, E.C., Mensah, M., 2013. Chemical pretreatment methods for the production of cellulosic  
595 ethanol: technologies and innovations. *Int. J. Chem. Eng.* 2013.

596 Binod, P., Satyanagalakshmi, K., Sindhu, R., Janu, K.U., Sukumaran, R.K., Pandey, A., 2012.  
597 Short duration microwave assisted pretreatment enhances the enzymatic saccharification and  
598 fermentable sugar yield from sugarcane bagasse. *Renewable Energy* 37, 109-116.

599 Borges, D.G., Baraldo Junior, A., Farinas, C.S., de Lima Camargo Giordano, R., Tardioli, P.W.,  
600 2014. Enhanced saccharification of sugarcane bagasse using soluble cellulase supplemented with  
601 immobilized  $\beta$ -glucosidase. *Bioresour. Technol.* 167, 206-213.

602 Bruynooghe, J., Mackenzi, J., Russell, S., Swift, M.-L., 2013. *Wheat DDGS Feed Guide*, 1.1 ed.  
603 Canadian International Grains Institute (Cigi), Canadian International Grains Institute (Cigi).

604 Burton, E.J., Scholey, D.V., Williams, P.E.V., 2014. Types, properties and processing of bio-based  
605 animal feed, in: Waldron, K.W. (Ed.), *Advances in Biorefineries: Biomass and Waste Supply*  
606 *Chain Exploitation*, First Edition ed. Woodhead Publishing, Cambridge, UK, pp. 771–802.

607 Chatzifragkou, A., Kosik, O., Prabhakumari, P.C., Lovegrove, A., Frazier, R.A., Shewry, P.R.,  
608 Charalampopoulos, D., 2015. Biorefinery strategies for upgrading Distillers' Dried Grains with  
609 Solubles (DDGS). *Process Biochem.* 50, 2194-2207.

610 Chatzifragkou, A., Prabhakumari, P.C., Kosik, O., Lovegrove, A., Shewry, P.R.,  
611 Charalampopoulos, D., 2016. Extractability and characteristics of proteins deriving from wheat  
612 DDGS. *Food Chem.* 198, 12-19.

613 Chen, Y., Stevens, M.A., Zhu, Y., Holmes, J., Xu, H., 2013. Understanding of alkaline  
614 pretreatment parameters for corn stover enzymatic saccharification. *Biotechnol. Biofuels.* 6, 8.

615 Cozannet, P., Primot, Y., Gady, C., Métayer, J.P., Callu, P., Lessire, M., Skiba, F., Noblet, J., 2010.  
616 Ileal digestibility of amino acids in wheat distillers dried grains with solubles for pigs. *Anim. Feed.*  
617 *Sci. Technol.* 158, 177-186.

618 da Silva, G.T., Chiarello, L.M., Lima, E.M., Ramos, L.P., 2016. Sono-assisted alkaline  
619 pretreatment of sugarcane bagasse for cellulosic ethanol production. *Catalysis Today* 269, 21-28.

620 Dien, B.S., Ximenes, E.A., O'Bryan, P.J., Moniruzzaman, M., Li, X.-L., Balan, V., Dale, B., Cotta,  
621 M.A., 2008. Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated  
622 distillers' grains and their conversion to ethanol. *Bioresour. Technol.* 99, 5216-5225.

623 Dodd, D., Cann, I.K.O., 2009. Enzymatic deconstruction of xylan for biofuel production. *Global*  
624 *change biology. Bioenergy* 1, 2-17.

625 Gao, D., Chundawat, S.P.S., Sethi, A., Balan, V., Gnanakaran, S., Dale, B.E., 2013. Increased  
626 enzyme binding to substrate is not necessary for more efficient cellulose hydrolysis. *Proceedings*  
627 *of the National Academy of Sciences of the United States of America* 110, 10922-10927.

628 Han, J., Liu, K., 2010. Changes in Composition and Amino Acid Profile during Dry Grind Ethanol  
629 Processing from Corn and Estimation of Yeast Contribution toward DDGS Proteins. *J. Agric. Food*  
630 *Chem.* 58, 3430-3437.

631 Han, L., Feng, J., Zhang, S., Ma, Z., Wang, Y., Zhang, X., 2012. Alkali pretreated of wheat straw  
632 and its enzymatic hydrolysis. *Braz. J. Microbiol.* 43, 53-61.

633 Hansen, N.M.L., Plackett, D., 2008. Sustainable Films and Coatings from Hemicelluloses: A  
634 Review. *Biomacromolecules.* 9, 1493-1505.

635 Hishikawa, Y., Togawa, E., Kondo, T., 2017. Characterization of Individual Hydrogen Bonds in  
636 Crystalline Regenerated Cellulose Using Resolved Polarized FTIR Spectra. *ACS Omega* 2, 1469-  
637 1476.

638 Hsu, T.C., Guo, G.L., Chen, W.H., Hwang, W.S., 2010. Effect of dilute acid pretreatment of rice  
639 straw on structural properties and enzymatic hydrolysis. *Bioresour. Technol.* 101.

640 Hu, J., Arantes, V., Saddler, J., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic  
641 substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic  
642 effect? *Biotechnol. Biofuels.* 4, 1-14.

643 Jones, A., Mandal, A., Sharma, S., 2015. Protein-based bioplastics and their antibacterial potential.  
644 *J. Appl. Polym. Sci.* 132.

645 Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory by-  
646 products and strategies for minimizing their effects. *Bioresour. Technol.* 199, 103-112.

647 Kim, I., Han, J.-I., 2012. Optimization of alkaline pretreatment conditions for enhancing glucose  
648 yield of rice straw by response surface methodology. *Biomass Bioenergy* 46, 210-217.

649 Kim, J.S., Lee, Y.Y., Kim, T.H., 2016. A review on alkaline pretreatment technology for  
650 bioconversion of lignocellulosic biomass. *Bioresour. Technol.* 199, 42-48.

651 Kim, T.H., Taylor, F., Hicks, K.B., 2008a. Bioethanol production from barley hull using SAA  
652 (soaking in aqueous ammonia) pretreatment. *Bioresour. Technol.* 99, 5694-5702.

653 Kim, Y., Hendrickson, R., Mosier, N., Ladisch, M., 2009. Liquid Hot Water Pretreatment of  
654 Cellulosic Biomass, in: Mielenz, J.R. (Ed.), *Biofuels*. Humana Press, pp. 93-102.

655 Kim, Y., Hendrickson, R., Mosier, N.S., Ladisch, M.R., Bals, B., Balan, V., Dale, B.E., 2008b.  
656 Enzyme hydrolysis and ethanol fermentation of liquid hot water and AFEX pretreated distillers'  
657 grains at high-solids loadings. *Bioresour. Technol.* 99, 5206-5215.

658 Knill, C.J., Kennedy, J.F., 2003. Degradation of cellulose under alkaline conditions. *Carbohydrate*  
659 *Polymers* 51, 281-300.

660 Kumar, R., Wyman, C.E., 2009. Effect of xylanase supplementation of cellulase on digestion of  
661 corn stover solids prepared by leading pretreatment technologies. *Bioresource Technol* 100.

662 Liu, K., 2011. Chemical composition of distillers grains, a review. *J. Agric. Food Chem.* 59, 1508-  
663 1526.

664 Maryana, R., Ma'rifatun, D., Wheni, A., Satriyo, K., Rizal, W.A., 2014. Alkaline pretreatment on  
665 sugarcane bagasse for bioethanol production. *Energy Procedia* 47, 250-254.

666 McIntosh, S., Vancov, T., 2011. Optimisation of dilute alkaline pretreatment for enzymatic  
667 saccharification of wheat straw. *Biomass Bioenergy* 35, 3094-3103.

668 Moreau, R.A., Nghiem, N.P., Rosentrater, K.A., Johnston, D.B., Hicks, K.B., 2012. Ethanol  
669 Production from Starch-Rich Crops Other than Corn and the Composition and Value of Resulting  
670 DDGS, in: Liu, K., Rosentrater, K.A. (Eds.), *Distillers Grain: Production, Properties, and*  
671 *Utilization*. CRC Press: Taylor & Francis Group, Boca Rato, FL, pp. 103 - 117.

672 Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005.  
673 Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource*  
674 *Technol* 96.

675 Nuez Ortín, W.G., Yu, P., 2009. Nutrient variation and availability of wheat DDGS, corn DDGS  
676 and blend DDGS from bioethanol plants. *J. Sci. Food Agric.* 89, 1754-1761.

677 Pedersen, M.B., Dalsgaard, S., Knudsen, K.E.B., Yu, S., Lærke, H.N., 2014. Compositional profile  
678 and variation of Distillers Dried Grains with Solubles from various origins with focus on non-  
679 starch polysaccharides. *Anim. Feed. Sci. Technol.* 197, 130-141.

680 Pisupati, S.V., Tchapda, A.H., 2015. Thermochemical Processing of Biomass, in: Ravindra, V.  
681 (Ed.), *Advances in Bioprocess Technology*. Springer International Publishing, pp. 277-314.

682 Rabelo, S.C., Amezquita Fonseca, N.A., Andrade, R.R., Maciel Filho, R., Costa, A.C., 2011.  
683 Ethanol production from enzymatic hydrolysis of sugarcane bagasse pretreated with lime and  
684 alkaline hydrogen peroxide. *Biomass Bioenergy* 35, 2600-2607.

685 Schwanninger, M., Rodrigues, J., Pereira, H., Hinterstoisser, B., 2004. Effects of short-time  
686 vibratory ball milling on the shape of FT-IR spectra of wood and cellulose. *Vibrational*  
687 *Spectroscopy* 36, 23-40.

688 Sills, D.L., Gossett, J.M., 2012. Using FTIR to predict saccharification from enzymatic hydrolysis  
689 of alkali-pretreated biomasses. *Biotechnol. Bioeng.* 109, 353-362.

690 Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2011.  
691 Determination of Structural Carbohydrates and Lignin in Biomass, Laboratory Analytical  
692 Procedures (LAP) for Standard Biomass Analysis. National Renewable Energy Laboratory  
693 (NREL).

694 Subhedar, P.B., Gogate, P.R., 2014. Alkaline and ultrasound assisted alkaline pretreatment for  
695 intensification of delignification process from sustainable raw-material. *Ultrason. Sonochem.* 21,  
696 216-225.

697 Villegas-Torres, M.F., Ward, J.M., Lye, G.J., 2015. The protein fraction from wheat-based dried  
698 distiller's grain with solubles (DDGS): extraction and valorization. *N. Biotechnol.* 32, 606-611.

699 Wan, C., Zhou, Y., Li, Y., 2011. Liquid hot water and alkaline pretreatment of soybean straw for  
700 improving cellulose digestibility. *Bioresour. Technol.* 102, 6254-6259.

701 Widyaratne, G.P., Zijlstra, R.T., 2007. Nutritional value of wheat and corn distiller's dried grain  
702 with solubles: Digestibility and digestible contents of energy, amino acids and phosphorus,  
703 nutrient excretion and growth performance of grower-finisher pigs. *Can. J. Anim. Sci.* 87, 103-  
704 114.

705 Xiang, Z., Watson, J., Tobimatsu, Y., Runge, T., 2014. Film-forming polymers from distillers'  
706 grains: structural and material properties. *Ind. Crops Prod.* 59, 282-289.

707 Xu, F., Yu, J., Tesso, T., Dowell, F., Wang, D., 2013. Qualitative and quantitative analysis of  
708 lignocellulosic biomass using infrared techniques: A mini-review. *Appl. Energy* 104, 801-809.

709 Xu, J., Cheng, J.J., Sharma-Shivappa, R.R., Burns, J.C., 2010. Sodium Hydroxide Pretreatment of  
710 Switchgrass for Ethanol Production. *Energy & Fuels* 24, 2113-2119.

711 Xu, Y., Hanna, M.A., 2010. Optimum conditions for dilute acid hydrolysis of hemicellulose in  
712 dried distillers grains with solubles. *Ind. Crops Prod.* 32, 511-517.

713 Yang, J., Zhang, X., Yong, Q., Yu, S., 2011. Three-stage enzymatic hydrolysis of steam-exploded  
714 corn stover at high substrate concentration. *Bioresour. Technol.* 102, 4905-4908.

715 Yin, S., Tan, Z., 2012. Hydrothermal liquefaction of cellulose to bio-oil under acidic, neutral and  
716 alkaline conditions. *Appl. Energy* 92, 234-239.

717 Zhang, A.-P., Liu, C.-F., Sun, R.-C., Xie, J., 2013. Extraction, purification, and characterization  
718 of lignin fractions from sugarcane bagasse. *BioResources* 8, 1604-1614.

719 Zhao, X., Peng, F., Cheng, K., Liu, D., 2009. Enhancement of the enzymatic digestibility of  
720 sugarcane bagasse by alkali-peracetic acid pretreatment. *Enzyme. Microb. Technol.* 44, 17-23.

721 Zhao, X., Zhang, L., Liu, D., 2012. Biomass recalcitrance. Part I: the chemical compositions and  
722 physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioprod.*  
723 *Biorefin.* 6, 465-482.

724 Zhou, D., Zhang, L., Guo, S., 2005. Mechanisms of lead biosorption on cellulose/chitin beads.  
725 *Water Research* 39, 3755-3762.

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728

729

730