

1 Hydro-mechanical processing of brewer's spent grain as a novel route for separation of protein  
2 products with differentiated techno-functional properties.

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9

10 Abstract

11

12 Hydro-mechanical processing using a colloid mill with a large gap setting leads to the preferential  
13 breakup of the residual aleurone and endosperm tissues of brewer's spent grain, forming a protein  
14 rich fines material with small particle size around 1-10  $\mu\text{m}$ . This fraction can be separated from the  
15 coarser husk fraction by centrifugation, giving a protein product with enhanced techno-functional  
16 properties. The fines have good stability in aqueous suspensions, with potential for stabilising other  
17 particulate materials in food or drink formulations. The fines particles can stabilise oil-water  
18 emulsions, possibly through a Pickering mechanism, which may also support use in food  
19 applications. Fines suspensions have strong shear-thinning behaviour, which may be beneficial from  
20 a textural or transport perspective. Spray drying of fines suspensions is shown to avoid particle  
21 coalescence, which is important for effective resuspension on rehydration. The high surface area of  
22 the fines also leads to more efficient digestion by proteases.

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25 Keywords: Brewer's-spent-grain; milling; protein; particles; properties; functionality

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28 Industrial relevance

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30 A novel hydro-mechanical milling process has been investigated for separation of a protein fine  
31 fraction from brewer's spent grain having enhanced techno-functional properties. The small particle  
32 size of the fines would be a key attribute for formulation in shake or smoothie products, where  
33 sensory attributes of the product would not be compromised and the properties of the fines could  
34 confer stability against settling. Applications may be found for the fines material as an ingredient in  
35 spreads and sauces or infant purees, in-particular where the it might be used to stabilise of products  
36 based on oil-water emulsions. The market for protein-rich ingredients for foods and drinks is already  
37 established in the fitness and well-being market, as derived from other vegetable or cereal sources  
38 such as hemp, pea or rice. This controlled pre-milling step is also shown to lead to greater rate and  
39 extent of protease digestion of spent grain, which may be of value for generation of protein and  
40 peptide products for well-being and cosmetics applications.

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44 1. Introduction

45

46 Spent grain is a major by-product from the brewing industry, which consists of the wet solid material  
47 remaining after the mashing process, when the majority of starch and soluble sugars have been  
48 extracted from the malt prior to fermentation of the wort liquor. Over 500,000 tonnes of brewer's  
49 spent grain (BSG) are produced annually by breweries in the UK alone, where the bi-product is sold  
50 primarily as a low-value ruminant feed [Thomas et al 2010]. A range of useful nutritional  
51 components are present in the residual non-extracted tissues of the grain, which include cellulose  
52 and other insoluble polysaccharides, lipids and protein, as well as a proportion of lignin. The

53 nutritional value for feed applications is therefore well established, [Aliyu and Bala 2011]. The  
54 proximate composition of the BSG used in the work is shown in table 1. However, as-made wet BSG  
55 may consist of only 30% solids, even after dewatering, which not only dilutes the deliverable  
56 nutritional value but also leads to microbial instability resulting in a low storage life. The product  
57 must therefore be shipped immediately from the brewery to the livestock facility for use [Mussatto  
58 et al 2006].

59

60 According to fractionations based on differential extractabilities, the main protein fractions of BSG  
61 consists of a range of hordeins, with other fractions including glutelins, also albumins and globulins,  
62 as described in previous studies [Celus et al 2006]. The relatively high amount of this protein  
63 component raises the possibility of further processing of BSG to separate a protein enriched  
64 product, which would have higher value for both feed and also for human nutrition or wellbeing  
65 supplements or as cosmetics ingredients [McCarthy et al 2013]. Many investigations have focussed  
66 on methods for extracting the proteins present in BSG by application of commercially available  
67 protease enzymes, which are designed to reduce protein molecular weight and therefore increase  
68 solubility. Crude extraction yields in excess of 70% have been quoted, where extracts consist of  
69 lower molecular weight oligomers and peptides, as a result of the hydrolysis of amide linkages  
70 between amino acids. [Celus et al 2007; Treimo et al 2008, Robertson et al 2011]. Although the  
71 process is simple, typically operating at 50-60°C, further process effort is required to recover the  
72 protein concentrate as a final solid, also with the requirement to adjust the pH by alkali addition to  
73 neutralise the additional amino acid functionality resulting from hydrolysis. Actual recoverable yield  
74 may therefore be lowered and the final product may also have a relatively high mineral content in  
75 the form of the amino acid carboxylate counter-ion. Other chemical approaches for protein  
76 extraction have also been extensively reported, either based on solubilisation of the BSG proteins in  
77 aqueous alkali [Tang et al. 2009] or ethanolic alkali [Cookman and Glatz 2009]. Usually sodium  
78 hydroxide is the alkali of choice, with extraction again under relatively mild conditions, for example

79 at 60°C, over a few hours. The solubilised protein is then precipitated by acid addition, which may  
80 cause denaturation, although molecular weight may be preserved [Scopes 1994; Celus et al 2007].  
81 The precipitate is separated, for example by centrifugation, and therefore recoverable yields may  
82 again be reduced. Also, the purity of the resulting concentrate will depend on the extent of co-  
83 extractability of other grain components in the alkaline medium, for example hemicelluloses and  
84 lignin [Vieria et al. 2013]

85

86 The possibility of application of physical methods for separation of a protein concentrate from BSG  
87 has been much less widely reported, which is however a route that could present an alternative to  
88 enzyme or alkali extraction methodologies [Kanauchi and Agata 1997, Schwencke 2006]. Wet roller  
89 milling is a process widely used in the brewing industry. This has been used to abrade and disrupt  
90 the spent grain material, which can then be sieved to allow separation of a fine protein rich fraction  
91 from a coarser protein depleted material [Kishi et al. 1997]. Products are reported which contain  
92 significantly higher protein content than the original spent grain, where dewatering is carried out to  
93 produce a wet cake, which can be dried to give a stable product. This simple concept appears worthy  
94 of a more fundamental investigation, to understand the physical aspects of the process more fully,  
95 and to consider if features of the process might be further modified or improved, and also to  
96 consider whether any novel properties of the enriched fine protein fraction may point to specific  
97 food applications. In support of these aims, a commercially available colloid mill has been used for  
98 hydro-mechanical processing of BSG, to generate protein fine fractions under controlled conditions,  
99 and also for evaluation as a potential alternative process route. A controlled laboratory  
100 centrifugation technique has been used as part of these investigations, to better understand the  
101 separation behaviour of the fines and coarse fractions, which may relate to particle size and surface  
102 chemical properties. Microscopy techniques have been used to characterise the physical features of  
103 the separated particles. Studies of the dispersion and settling behaviour of the protein enriched fines

104 were also carried out in different media. Other post processing methods have also been applied to  
105 the protein enriched fines learn more about potential for scale-up and different applications.

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107

108 2. Materials and methods

109

110 2.1 Grain Materials

111

112 A batch of wet spent grain was recovered from the commercial production process at Molson Coors  
113 UK, Burton-on-Trent brewery. The original malt had been hammer milled prior to mashing and sugar  
114 extraction, where after discharge from the mash tun the spent grain residue was dewatered by filter  
115 press to a solids content of around 27 wt%. Individual portions of this material were frozen at -18°C,  
116 for storage in readiness for experimental investigations.

117

118

119 2.2 Colloid milling

120

121 A vertical toothed colloid mill was used for processing the BSG material under controlled conditions,  
122 operating at a fixed speed of 3000 rpm, fitted with a 7 litre hopper (MZ50 model, FrymaKoruma AG,  
123 Switzerland). The gap between outer fixed stator and inner rotor was set at 1 mm, which was  
124 considered to be a sufficiently large tolerance to limit the direct cutting of the initial grain particles,  
125 whilst generating high hydro-mechanical shear forces sufficient for particle break-up. The rotor and  
126 stator had a square-tooth cross-helical profile, with depth reducing from the inlet to outlet end.

127

128 A 2.5 l batch of 10 wt% slurry of the spent grain was prepared by appropriate addition of water to  
129 the as-received material. The samples were repeatedly passed through the colloid mill, up to 24

130 cycles in total, each time collecting the full batch of product from the outlet before feeding back into  
131 the inlet hopper. Duplicate samples of 45 ml were collected in 50 ml graduated falcon centrifuge  
132 tubes at selected numbers of mill passes, from 2 up to the 24 cycle limit, ensuring that no settling on  
133 sampling had occurred and that the slurries were representative of the total batch. These were  
134 retained for further processing and analysis.

135

### 136 2.3 Sedimentation and centrifugation

137

138 The duplicate samples of slurries collected after increasing numbers of mill passes were allowed to  
139 stand vertically in the falcon tubes under normal gravity for 1 hour. After standing, the settled  
140 volume of the solids layer below any clear layer was measured using the graduated scale on the side  
141 of the tubes. The extent of solids settling was then expressed as a proportion of the total sample  
142 volume. Both duplicate samples in the falcon tubes were then re-suspended by vigorous shaking  
143 using an orbital shaker and were loaded immediately into a laboratory centrifuge (Heraeus 16,  
144 Thermo-Fisher, UK). Three experiments were carried out at different centrifuge speeds. For the first  
145 experiment the tubes were spun at 28 g for 30 minutes, which was sufficient time at this speed for  
146 the solid material to settle to equilibrium. After spinning, the volumes of the total settled solids and  
147 also any separate upper layer of fines were measured for one duplicate set of samples, using the  
148 graduated scale as before. For the other duplicate set of samples, small amounts of the upper fines  
149 layer were removed using a spatula and retained for further analysis. This sampling arrangement  
150 avoided disturbance of the quantity of measurement of layer volumes of the first duplicate set.  
151 For the second experiment, both duplicate sample sets were re-suspended in the tubes by vigorous  
152 orbital shaking, then re-loaded into the centrifuge and spun at 447 g for 10 minutes. After  
153 centrifugation, the layer volumes of the first duplicate set were measured as before. Further small  
154 amounts of fines were taken from the second duplicate set for analysis as before. In the third  
155 experiment the operation was repeated in full with centrifugation at 2800 g for 5 minutes, with

156 measurement and sampling as before. The small portions of fines taken from the second set of  
157 duplicate tubes were each spread evenly onto plastic petri dishes and allowed to dry under ambient  
158 laboratory conditions overnight, before transferring for storage for further analysis. This gentle  
159 drying regime was chosen to minimise risk of alterations to the particle structure or properties.

160

161 A larger single batch of fines were produced by subjecting a 2 l quantity of 10 wt% slurry of spent  
162 grain to 24 passes through the colloid mill, followed by centrifuging at 2800 g using a floor-standing  
163 centrifuge (J21, Beckman UK.). The upper fines layer was separated and stored in the wet state at  
164 3°C in preparation for further experiments. The moisture content of a small representative quantity  
165 of this as-made fines material was determined by gravimetry, by weighing, drying at 100°C and  
166 reweighing.

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168

#### 169 2.4 Compositional analysis

170

171 A number of different BSG derived samples were acquired separately, including both milled fines  
172 fractions and fractions generated by methods leading to higher concentrations of protein [Tang et al.  
173 2009]. These were required to construct a broad range calibration for a rapid infrared spectroscopic  
174 method for protein determination. The nitrogen contents of these calibration samples were initially  
175 analysed by an automated Dumas method, with values converted to protein concentration using the  
176 relation, % protein = % N x 6.25. The infrared spectroscopy measurements were performed using an  
177 attenuated total reflectance (ATR) sampling accessory, requiring low milligram samples (Tensor FTIR,  
178 Bruker AG). The method was based on the measurement of the intensity of the amide II absorbance  
179 at 1518 cm<sup>-1</sup>, which is related to the amount of peptide linkages and hence the amount of protein.  
180 The measurement of the intensity of the organic C-O absorbance at 1097cm<sup>-1</sup> provided a relation to  
181 the amount of hydroxyl groups present and hence amount of polysaccharide and lignin. The

182 intensities of both these characteristic frequencies were normalised by comparison with the  
183 intensities of frequencies in adjacent non absorbing regions. From the measurement of the  
184 calibration samples an empirical relation was derived for total protein content, as shown in equation  
185 (1), where constants of A = 106 and B = -0.6 were identified by least-squares fitting. The protein  
186 contents of all experimental samples were measured using this rapid technique, with examples of  
187 spectra shown in Figure 1.

188

$$189 \quad \% \text{ protein} = \left[ A \times \frac{(I_{1518} - I_{1800})}{(I_{1518} - I_{1800}) + (I_{1097} - I_{899})} + B \right] \quad (1)$$

190

191 Analysis of the amino acid profile of the original BSG and representative fine fraction was carried by  
192 an external provider using a standard HPLC chromatographic method (Sciantec Analytical Ltd, UK).  
193 Determination of the crude fibre fraction of the original grain was carried out using the neutral  
194 detergent fibre extraction protocol [Van Soest et al. 1991]. Application of this protocol was difficult  
195 as a result of the small particle size of the fines material and an estimate of fibre content was  
196 therefore made by quantitative comparison of the C-O infrared absorbance at 1097 cm<sup>-1</sup> between  
197 the grain and fines spectra, with internal intensity referencing [Marotte et al, 2007]. The lignin  
198 content of whole BSG and separated fines material was determined by the acetyl bromide method  
199 [Hatfield et al. 1999]. Analysis of the lipid content of grain and fines material was determined by  
200 extraction in diethyl ether, followed by ambient drying and weighing of the extract [Thiex et al  
201 2003].

202

## 203 2.5 Microscopy

204

205 Images of particles dispersed in water and oil were obtained under transmitted light conditions, at  
206 low magnification (x4 objective) and also at high magnification (x100 objective) using an oil-  
207 immersion method. Microscopy imaging was performed on water suspensions of the original BSG



208 and BSG which had been subjected to 15 passes through the colloid mill. This was performed under  
209 reflected light conditions, with oblique illumination, through a cover slip. These suspensions were  
210 stained using an aqueous solution of acid Fuschin dye, for selective coloration of the protein rich  
211 component of the material. Dye addition was carried out on a dropwise basis, with examination to  
212 determine the optimum dye concentration [Craeyveld et al. 2009; Niemi et al. 2012].

213

## 214 2.6 Oil and water suspensions and emulsions

215

216 Amounts of wet fines produced from the large 2 l batch were mixed with water in cylindrical glass  
217 vials at differing concentrations from 0.5 to 5 wt% dry basis, at a 40 ml volume, accounting for the  
218 existing moisture content. The suspensions were shaken vigorously and allowed to stand at ambient  
219 temperature, after which the aggregation and settling characteristics of each vial sample were  
220 recorded over time by imaging and measurement of the layer heights. As a confirmation of  
221 behaviour the procedure was repeated by re-suspending the fines by repeated vigorous shaking. A  
222 further batch of the centrifuged fines was repeatedly washed in methanol, followed by re-  
223 centrifugation and decantation of the methanol layer, in order to replace all water present in the  
224 material by organic solvent. The methanol wetted fines were finally air dried, which avoided any  
225 irreversible coalescence of particles. The dried fines were then re-suspended and shaken in 40 ml of  
226 sunflower oil at different concentrations in cylindrical glass vials, as above, again with measurement  
227 of gravitational settling over time. Again for confirmation the procedure was repeated by re-  
228 suspending the fines by repeated vigorous shaking. Microscopy images of representative samples of  
229 the oil and water particle dispersions were also obtained under transmitted light conditions.

230

231 Further amounts of the wet fines were made up in 20 mls of water in cylindrical glass vials to achieve  
232 final total emulsion concentrations from 1 to 10 wt% dry basis, which were shaken vigorously to aid  
233 dispersion. Then 20 mls of sunflower oil were added to each of the vials, which were vigorously

234 shaken again and allowed to stand at ambient temperature. The appearance and stability of the  
235 resulting oil and water emulsions were determined after standing as before, by imaging and  
236 observation of phase heights at increasing time periods. For confirmation of behaviour the  
237 procedure was repeated by re-suspending the emulsions by repeated vigorous shaking

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239

## 240 2.7 Rheology of fines suspensions

241

242 Three samples of wet fines from the large 2 l batch preparation were made up to 5, 10 and 14 wt%  
243 dry basis by appropriate addition of water. The rheological properties of these suspensions were  
244 measured using a rotational viscometer, using a concentric cylinder geometry, with a gap width of 1  
245 mm with a matt surfaced inner cylinder (RheolabQC, Anton-Paar GmbH). An upward and downward  
246 shear-rate sweep was applied from 2 to 50 /sec, measured over 100 points in each direction at 0.5  
247 sec/point. The temperature for all measurements was stabilised at 20°C

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249

## 250 2.8 Wet-milling and enzyme digestion

251

252 Two slurries of 1 l of the initial spent grain were made up at 10 wt% solids content, adjusted to pH 9,  
253 by formulation using a stock of 2M sodium tetraborate/boric acid, to give a final buffer  
254 concentration of 0.1 M. This formulation was required to avoid the reduction in pH during digestion,  
255 which would otherwise occur due to the creation of free amino acid groups due to the hydrolysis of  
256 amide bonds. One batch of slurry was subjected to 24 passes through the colloid mill, as described  
257 above. This and the other un-milled batch of slurry were preheated to 60°C and incubated for 5  
258 hours in the presence of a commercial Alcalase 2.5L enzyme product, dosed at a concentration of  
259 0.1% on dry weight of grain (Sigma-Aldrich Ltd, UK). Small samples from each batch were taken at

260 increasing time intervals during digestion. These were immediately chilled to 3°C to minimise  
261 enzyme action, then centrifuged to allow collection of the supernatant liquor for analysis of protein  
262 concentration. A further 1 l batch of slurry was subjected to the same 24 colloid mill passes but was  
263 incubated without enzyme. The separated supernatant liquors from all samples were immediately  
264 frozen for storage. Each sample set was re-thawed to 3°C as required and 100 µl aliquots were mixed  
265 with a 2 ml quantity of commercial Bradford reagent, then held for 20 minutes for colour  
266 development (Biorad Inc). Absorbances were measured at 595 nm and converted to apparent  
267 protein concentration using a bovine serum albumin calibration curve, with a vegetable protein  
268 factor of 2 applied [Kruger 2002].

269

270

## 271 2.9 Spray drying of fines suspensions

272

273 Fines suspensions at 5% and 10% solids were dried using a Buchi B-190 laboratory spray dryer, fitted  
274 with a 0.7 mm spray nozzle, aspirated with dry compressed air. The inlet temperature was set at  
275 120°C and the pump rate and chamber air flow rate was adjusted to ensure the outlet temperature  
276 did not exceed 60°C. Representative samples of dried powder were examined by transmission optical  
277 microscopy as described above.

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279

## 280 3. Results and discussion

281

282

### 283 3.1 Milling and centrifugation

284

285 The 10 wt% slurry of the original BSG flowed easily through the colloid mill, gaining a smoother  
286 texture at each pass, as observed visually by its behaviour when poured into the feed hopper. The  
287 relatively coarse particles of the unmilled slurry settled under normal gravity after 1 hour to leave a  
288 clear upper liquid layer, with no obvious stratification within the settled particulate material.  
289 However, the visual settling of the slurry reduced as the number of mill passes increased, shown in  
290 Figure 2a, with no upper liquid layer observed after 12 passes. Centrifugation of the unmilled slurry  
291 resulted in greater settling of the particulate material, which reduced in phase volume to 50 % of the  
292 total sample volume after centrifuging at 2800 g, compared to 71% on settling under normal gravity.  
293 No visual stratification within this phase was observed for the unmilled material following  
294 centrifugation. However, after progressive milling centrifugation resulted in a distinct stratification  
295 of the slurry particles into an upper fines fraction and a lower coarser fraction, as illustrated in Figure  
296 2c. The proportion of the upper fines fraction increased with increasing number of passes through  
297 the mill, from Figure 2b, appearing to reach a constant value after 12 mill passes. Also, the figure  
298 shows that proportion of the resulting fines fraction was greater at higher centrifuge speed, reaching  
299 a maximum of approximately 28 % of the total volume of the particulate phase after centrifugation  
300 at 2800 g, with data averaged over 12 to 24 mill passes. Accounting for gravimetric moisture content  
301 of the two particulate layers, this gave a maximum yield of the upper fines fraction at 2800 g of 24%  
302 on dry basis with respect to the original BSG. Sedimentation velocities are theoretically linearly  
303 proportional to the gravitational field, so the improvement in separation of the finer and coarser  
304 particles at higher centrifugal speed may be a result of the increased settling distance, due to the  
305 greater compaction of the particulate material. These results indicate that there is advantage in  
306 centrifugation at high speed as part of an industrial process.

307

308 Reflected light microscopy images of as-received BSG particles from the hammer-milled malt are  
309 shown in Figure 3a. These revealed irregular amorphous particulate structures which have become  
310 pink-red stained due to absorption of the Acid Fuschin dye, likely to be associated with the inner

311 protein containing aleurone tissue of the original grain [Jääskeläinen et al. 2013, Aubert et al. 2018].  
312 These tissue regions are distinct from the more sheaf-like structures presumed to be from the husk  
313 and pericarp tissues, which do not contain protein and remained predominately unstained. Figure 3b  
314 shows that on multiple passes through the colloid mill the protein containing tissues were broken  
315 down into very small pink-stained fragments, which became debonded from the larger  
316 husk/pericarp particles, forming a separated dispersed population, estimated to be around 1 - 10  $\mu\text{m}$   
317 in size. In contrast, the husk/pericarp particles did not appear to be significantly reduced in size,  
318 remaining at 1 - 2 mm dimensions, These smaller protein containing particles would therefore show  
319 slower sedimentation rate under gravitational force, due to the greater influence of Brownian  
320 motion, and would form the upper fine fraction after centrifugation. However, from the settling  
321 experiments under normal gravity it appears that milling reduces the sedimentation rate of whole  
322 slurry, which may therefore suggest the presence of interactions between the smaller fines particles  
323 and the coarser husk/pericarp particles. This appears to be an important feature of the overall  
324 process, where milling allows the total slurry to be retained in a homogenous suspended state prior  
325 to the application of the high centrifugal force, which then allows differential sedimentation of the  
326 fine and coarse fractions.

327

328 Samples of the upper fine fraction were examined under transmitted light microscopy, which  
329 revealed the uniform texture of the particles, in Figure 4a, which in this semi-concentrated state in  
330 water appeared to partially aggregate to form an open extended physical network. The interactions  
331 through this network may assist with stabilisation of the husk/pericarp particles in the whole slurry.  
332 Observation at higher magnification shows the irregular shape of the small aleurone and endosperm  
333 particles, in Figure 4c, although this image also show evidence of more spherical structures around 1  
334 - 5  $\mu\text{m}$  in diameter, which may be associated with protein storage vacuoles which have been  
335 liberated from these tissues [Swanson et al. 1998]. These vacuoles are the repositories of proteins as  
336 required on germination to support seed growth [Herman and Larkins 1999].

337

338

### 339 3.2 Composition of the fine fraction

340

341 The protein content of the separated fine material was enhanced compared to the original BSG, as  
342 summarised in table 1. The fines protein content appeared to increase slightly with increased milling  
343 time, in Figure 2b, although this trend was only detected for the highest centrifugation speed and  
344 was within the experimental error. A value of 51 wt% protein content was determined after 24 mill  
345 passes for the fines which compared with 27 wt% for the original spent grain, representing almost a  
346 2 times enhancement. Based on solid content, the weight yield of the fine fraction is estimated to be  
347 20% of total solid material so the protein in the fines represents a yield of around 38% of the total  
348 protein in the BSG, with the remaining 62% still incorporated in the particles of the coarser  
349 centrifuge fraction. This unrecovered protein may be a result of a limit in the efficiency of separation  
350 by differential sedimentation, or alternatively some protein containing tissue may still be intimately  
351 associated with the coarser husk/pericarp particles.

352

353 The fines material contained a mix of protein, carbohydrate, lignin and lipid components, as  
354 summarised in table 1. While the protein content of the fines was enhanced, the amount of  
355 carbohydrate was correspondingly reduced compared to the original BSG, which is consistent with  
356 the removal of husk and pericarp tissue from this fraction. The remaining carbohydrates in the fines  
357 fraction were presumed to originate from the cell wall material of aleurone and endosperm tissues,  
358 carried forward from the original grain. Table 2 summaries the amino acid composition of the  
359 original BSG and fines, which indicated that the materials had the same overall profile. This suggests  
360 that separation of the protein occurred in a non-selective manner, with the fine fraction derived  
361 from all protein containing tissue in the original structure. The analytical data suggested a moderate  
362 increase in the ether extractable lipid content of the fines fraction compared to the original spent

363 grain, which might have some nutritional benefit as a contributor to calorific value [Thomas et al,  
364 2013]. This lipid material would originally be present in oil-bodies distributed within the aleurone  
365 cells and different tissues of the embryo, which also appears to be carried forward during  
366 centrifugation [Neuberger et al. 2008]. One previous study also confirmed the reduction in non-  
367 digestible carbohydrate in the fines, which also indicated that this material contained proportionally  
368 less cellulose, which is a component of the more structural husk and related tissue, which was  
369 separated from the fines [Kanauchi and Agata 1997].

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Table 1: composition of brewer's spent grain materials

373

|              | Whole BSG<br>(%-total dry wt.) | BSG-fines<br>(%-total dry wt.) |
|--------------|--------------------------------|--------------------------------|
| Carbohydrate | 48.5 ±1.1                      | 30.5 ±1.8                      |
| Lignin       | 14.0 ±0.65                     | 5.1 ±0.2                       |
| Protein      | 27.1 ±0.9                      | 51.0 ±1.6                      |
| lipid        | 8.6 ±0.11                      | 12.0 ±0.21                     |

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Table 2: Amino acid proportions in brewers' spent grain materials

|                | Whole BSG<br>(%- of total measured) | separated fines<br>(%-of total measured) |
|----------------|-------------------------------------|--|
| alanine        | 5.0                                 | 5.2                                      |
| arginine       | 5.8                                 | 5.9                                      |
| aspartine      | 7.4                                 | 8.8                                      |
| cystine        | 2.2                                 | 1.7                                      |
| glutamine      | 21.7                                | 19.4                                     |
| glycine        | 4.3                                 | 4.8                                      |
| histine        | 2.4                                 | 2.5                                      |
| iso-leucine    | 4.4                                 | 4.4                                      |
| leucine        | 8.0                                 | 8.0                                      |
| lysine         | 4.4                                 | 4.5                                      |
| methionine     | 2.1                                 | 2.2                                      |
| phenyl-alanine | 5.8                                 | 5.7                                      |
| proline        | 9.7                                 | 9.2                                      |
| serine         | 4.6                                 | 4.6                                      |
| threonine      | 3.8                                 | 4.0                                      |
| tyrosine       | 2.7                                 | 3.2                                      |
| valine         | 5.8                                 | 5.7                                      |

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391

## 392 3.3 Properties of fines suspensions and emulsions

393

394 The micrographs in Figure 3b and 4a-e revealed the relatively small size of the fines particles, around

395 1 - 10 $\mu$ m, which were generated despite the large 1 mm gap size of the mill. This indicates that the

396 grain tissue creating the fines is of low mechanical integrity so can be broken up effectively by the

397 hydro-mechanical shear forces in the mill, without need for direct attrition between fixed and

398 rotating metal surfaces. The observation of settling behaviour of water suspensions of fines under

399 normal gravity revealed that suspension stability increased with solids content, from Figure 5a,

400 where although partial settling occurred at 1 wt% solid content, a single stable dispersed phase was



401 observed at 3 wt%, after 1 hour standing. In a further set of experiments it was found that a stable  
402 suspension could also be formed at a 3 wt% concentration in vegetable oil, also illustrated in Figure  
403 5a. This compatibility with both oil and water media suggests that the fines particles have both  
404 hydrophilic and hydrophobic characteristics. This may stem from the natural hydrophobicity of the  
405 exposed aliphatic and aromatic functional groups of the protein, together with the hydrophilic  
406 character of the residual polysaccharide material, conferred the presence of sugar hydroxyl groups  
407 [Saha and Hayashi 2001]. These characteristics were further demonstrated by the observed textures  
408 of the fines material suspended in water and oil, in Figures 4a and 4b. These micrographs revealed  
409 the appearance of a partially aggregated but open network structure in both media, where particle-  
410 particle and particle-liquid interactions are apparently in balance.

411

412 The potential dual hydrophobic-hydrophilic nature of the BSG fines suggest that the particles may  
413 have the ability to stabilise mixtures of oil and water as an emulsion. This is a property associated  
414 with so-called Pickering particles, which is a term describing a mechanism of stabilisation where  
415 small particles arrange at the interface between two immiscible liquids, stopping droplets from  
416 coalescing together by acting as a physical barrier [Wang et al. 2011; Liu and Tang 2018]. This  
417 mechanism would operate in addition to any stabilisation caused by the increased viscosity due to  
418 particle addition. In addition, a Pickering type mechanism is more effective when the particles are  
419 partially hydrophobic, so are wetted evenly between the two different hydrophobic and hydrophilic  
420 liquids and have contact with both phases. The images of mixtures of water and vegetable oil  
421 provide further evidence of the emulsion stabilisation effect, in Figure 5b and 5c, where in the  
422 absence of the fines the two liquids fully separated immediately on standing, with the boundary  
423 between the oil and water layers marked by an arrow due to lack of contrast. However, emulsion  
424 stability improved even with 1 wt% added fines, although after 10 minutes standing two phases  
425 were observed, where an upper oil majority phase had partly separated as an oil in water emulsion,  
426 stabilised by a proportion of the fines particles, with a continuous water phase below containing a

427 lower settled layer of fines particles. Stability improved at higher fines concentration, where at 3  
428 wt% content and above no gross separation was seen after 10 minutes, where the single phase again  
429 consisted of an oil-in-water emulsion. The mixture with 6 wt% added fines showed no gross phase  
430 separation after 2 hours standing. A micrograph of a spot of the emulsion in Figure 6 showed  
431 droplets of oil in water with the presence of fines particles in both phases, but also with particles at  
432 the surface of the individual oil droplets, providing further evidence of a Pickering type mechanism.

433

434

### 435 3.4 Rheology of fines suspensions

436

437 The network structures of suspensions of fines material visualised by microscopy in Figure 4 are a  
438 result of a balance between particle-particle interactions promoting aggregation of particles and  
439 opposing particle-solvent interactions which encourage particle separation within the liquor  
440 medium. At sufficient concentration the extended network fills the entire bulk volume of the  
441 concentration, leading to longer term stability against gravitational settling. Following these  
442 observations, the ability of the fines particle to form a physical network is expected to lead to  
443 distinct physical properties, which may be apparent from measurements of fluid viscosity. The  
444 behaviour of suspensions measured by rotational viscometry is illustrated by the graphs in Figure 7,  
445 which show on a log-log scale the dependence of viscosity on shear rate for the three suspension  
446 concentrations of 5, 10 and 14 wt%. An inverse log-linear relationship is seen for the two lower  
447 concentrations and the higher concentration up to a shear rate around  $10 \text{ /s}$ , which is an indicator of  
448 shear-thinning behaviour. This can be modelled according to an empirical power law relation,  
449 according to equation (3), where  $\eta$  is the viscosity,  $K$  is a consistency constant,  $\dot{\gamma}$  is the shear rate and  
450  $n$  is the power law index, where for shear thinning behaviour  $0 < n < 1$  [Rao 2014]

451

452 
$$\eta = K\dot{\gamma}^{(n-1)} \quad (3)$$

453

454 The modelled functions for the three concentrations are overlaid as continuous lines over the  
455 experimental data points, where fitting was achieved by iterative adjustment of parameters K and n,  
456 for minimisation of the sum of squared difference between model and experiment, using Microsoft  
457 Excel™. The final fitted parameters for the three concentrations were: for 4 % solids, K = 1.6 and n =  
458 0.1; for 10 % solids, K = 5 and n = 0.24; and for 14 % solids, K = 32 and n = 0.1. The low values for n  
459 confirm the severe shear thinning behaviour, with the parameter K related to the differences in  
460 concentration of particles between the three sample suspensions.

461

462 From a material perspective the shear thinning of these suspensions is explained by the presence at  
463 low shear rates of the previously observed extended physical network formed by particle-particle  
464 interactions, which results in a high resistance to shear. As shear rate increases this weak network  
465 breaks down and particles move past each other more easily, possibly becoming stratified into micro  
466 domains, which flow past each other with low resistance. This behaviour is not untypical of  
467 particulate food suspensions, as used for the formulation of pastes and sauces [Bourne 2002].  
468 Interestingly at the highest concentration of 14 wt%, at high shear rates the fines suspension  
469 entered a Newtonian rheological region, where viscosity became independent of shear rate. This is  
470 presumed to represent the limit of the ability of the particles to reorganise to reduce flow  
471 resistance, which was not encountered at lower concentrations.

472

### 473 3.5 Enzymatic hydrolysis of milled material

474

475 Previous published work has shown that pre-milling can enhance the rate and extent of enzyme  
476 digestion of the polysaccharide fraction of BSG materials, where the use of different wet and dry  
477 mills were evaluated [Niemi et al 2012]. However, in this previous work monomodal size

478 distributions were mostly observed, with all tissues in the grain reduced in size. The requirement to  
479 efficiently grind the polysaccharide containing tissue meant that the preferential breakup of the  
480 weaker protein containing tissue was not studied. As described earlier, the current study has  
481 revealed that the aleurone and endosperm related tissue can be preferentially reduced in size by  
482 hydro-mechanical action, which may then be more digestible in the presence of protease, by virtue  
483 of the increased surface area and the consequent higher accessibility.

484

485 The effect of pre-processing of BSG slurries through the colloid mill on the subsequent enzymatic  
486 solubilisation of protein is shown in Figure 8. In the absence of added enzyme the pre-milling step  
487 liberated a small amount of protein into solution, presumably that which was more loosely  
488 associated with the tissue structure, where solubilisation would be aided by the mildly alkaline  
489 environment. However, limited further release of protein occurred over time, which suggested that  
490 natural protease activity in the grain was not significant, which might potentially have originated  
491 from the original malt. The solubilisation of protein in pre-milled BSG continued at a faster rate in  
492 the presence of added Alcalase enzyme, where the kinetic profile indicated that release continued  
493 beyond the timescale of the experiment. This contrasted with the kinetic profile for the experiment  
494 with added Alcalase but without pre-milling, where no initial liberation of protein occurred and the  
495 overall rate of solubilisation was also slow. Overall, the findings suggested that a hydromechanical or  
496 equivalent technique for achieving preferential breakup of aleurone and endosperm tissue may offer  
497 an efficient approach to improve the rate and extent of enzymatic solubilisation of protein from BSG  
498 material. In this investigation the use of the Bradford reagent for protein assay should not be  
499 considered fully quantitative, as detection becomes less sensitive to molecular weights below 3000  
500 Da. Other published work suggests that enzyme hydrolysis of spent grain leads to a range of  
501 molecular weight peptides so a proportion may be under the threshold for effective dye binding  
502 [Celus et al 2007]. However this was not considered to negate the use of the technique for  
503 comparative purposes.

504

### 505 3.6 Spray drying of fines suspension

506

507 A significant process issue to overcome would be the avoidance of irreversible coalescence of the  
508 fines particles on drying, leaving a hard coarse material with limited functionality. Transporting and  
509 utilising the product as a wet cake would allow easy re-dispersion and formulation in a new food  
510 product, but would increase the risk microbial attack during storage. A post heat treatment or  
511 alternatively the operation of a sterile process, combined with suitable air-tight packaging might  
512 surmount such difficulties. Alternatively, suspensions of fines at moderate concentrations could be  
513 injected through a nozzle system for spray drying, taking advantage of their shear-thinning  
514 behaviour. The results of laboratory experiments conducted as part of the current study revealed  
515 that the powders produced in this way retained their small dimensions, shown in the micrograph in  
516 Figure 4e. The higher magnification image in Figure 4d indicated that the particles became more  
517 spherical in nature, possibly a result of capillary forces acting on the individual soft, water-plasticised  
518 particles during evaporation.

519

## 520 4. Conclusions

521

522 This work has demonstrated that wet milling using a commercial colloid mill leads to the formation  
523 of a protein rich fines material with particle size range around 1 - 10  $\mu\text{m}$ , derived from the aleurone  
524 and endosperm tissue of the original grain. This fine protein rich fraction can be separated from the  
525 coarser husk and pericarp fraction by centrifugation of the milled slurry to give a protein enhanced  
526 product with useful technofunctional properties. The fines material has good stability in aqueous  
527 suspensions partly as a result of the formation an extended physical network of particle-particle  
528 interactions. This may lead to the potential for stabilisation of food or drink formulations. The fines  
529 material has dual hydrophilic-hydrophobic character and as a consequence may be useful for

530 stabilisation of oil-water food formulations, possibly through a Pickering type emulsification  
531 mechanism. Also, the high surface area of the fines has been shown to lead to faster and more  
532 efficient digestion by proteases, which may be beneficial for the production of protein and peptide  
533 isolates, of value in cosmetics and health formulations. The rheology of the aqueous mixtures of the  
534 fines material has also been studied, which revealed strong shear-thinning behaviour which can be  
535 modelled using an empirical power-law relation, as is often seen in food systems. Shear thinning  
536 may be beneficial in food applications and for pumping and flow of process concentrates. Spray  
537 drying of fines suspensions was shown to avoid coalescence of particles retaining individual particle  
538 identity and conserving technical properties.

539

540

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542

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664

665

666 Figure captions

667

668

669 Figure 1. Infrared spectra of selected BSG materials., a = whole BSG (homogenised for sampling by  
670 dry milling) ; b = protein rich fines material obtained following colloid milling and centrifugation of  
671 BSG ; c = reference high concentration protein isolate obtained by alkali extraction and dialysis.

672

673 Figure 2. a: Gravitational settling of 10 wt% slurry of BSG, measured on standing for 1 hour, after  
674 increasing number of passes through a colloid mill. b: Dependence of volume proportion of fine  
675 fraction of BSG on number of mill passes; after centrifugation at 28 g (●), at 447 g (▲), 2800 g (■) ;  
676 protein concentration of fines obtained at 2800 g (◆). c: example of stratification of fine and coarse  
677 fraction of milled BSG after centrifugation, with upper fine layer indicated by horizontal lines.

678

679 Figure 3. Optical reflectance microscopy images of BSG materials, where pink/red Acid Fuschin stain  
680 has been preferentially absorbed by protein containing tissue. a: as-received dry hammer milled  
681 BSG, b: after further wet milling in a colloid mill.

682

683 Figure 4. Optical transmission microscopy images of suspensions of fine particle fraction separated  
684 from BSG. a: fines dispersed in water, b: fines dispersed in vegetable oil, c: high magnification image

685 of fines particles as created, dispersed in water. Fines particles dispersed in water after spray drying,  
686 at high magnification (d), overall suspension characteristics (e).

687

688 Figure 5. Images of settling behaviour of fines suspensions. a: 1 wt% in water - A, 3 wt% in water - B,  
689 3 wt% in vegetable oil - C. b: 50:50 mixtures of water and vegetable oil, with 0, 1, 2,3, 6, and 10 wt%  
690 total of BSG fines added; (b) after vigorous shaking and standing for 10 minutes. (c) standing for 2  
691 hours.

692

693 Figure 6. Optical transmission micrograph of oil droplets in the continuous water phase of an oil in  
694 water emulsion containing BSG fines particles, showing fines particles distributed within the water  
695 phase and at the oil-water droplet interface.

696

697 Figure 7. Log-log flow curve determined by rotational viscometry for BSG fines suspended in water at  
698 different concentrations; 5 wt% ( $\blacktriangle$ ), 10 wt% ( $\blacksquare$ ) 14 wt% ( $\bullet$ ). Continuous lines through each data  
699 set are fits to power law model; 4 wt% -  $K=1.6$ ,  $n=0.1$ ; 10 wt% -  $K=5$ ,  $n=0.24$ ; 14 wt% -  $K=32$ ,  $n=0.1$ .

700

701 Figure 8. Release of protein over time following enzyme digestion of BSG slurry, prepared at 10 wt%  
702 concentration, with 0.1 wt% Alcalase on solid, incubation at 60°C, pH 9. With enzyme addition with  
703 pre-milling through the colloid mill ( $\blacksquare$ ), with enzyme addition but without pre-milling ( $\blacktriangle$ ), with pre-  
704 milling but without enzyme addition ( $\bullet$ ).

705

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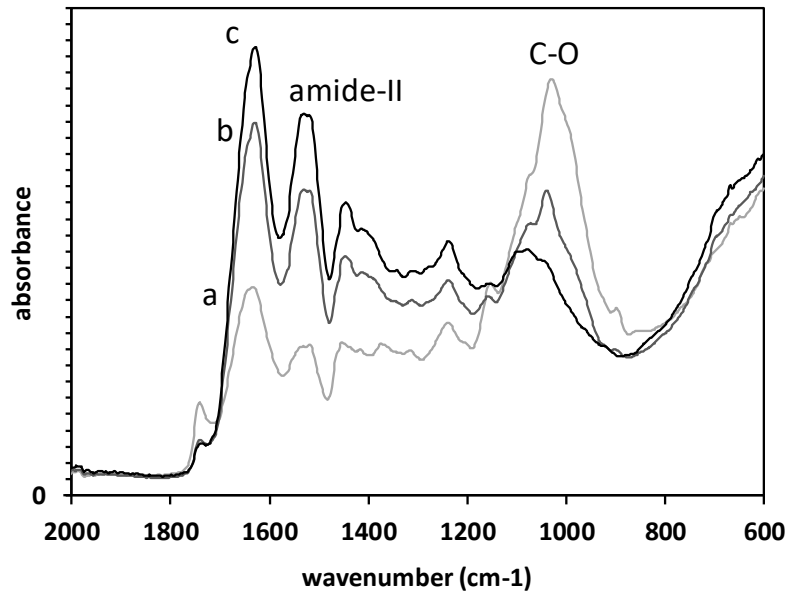
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Fig 1

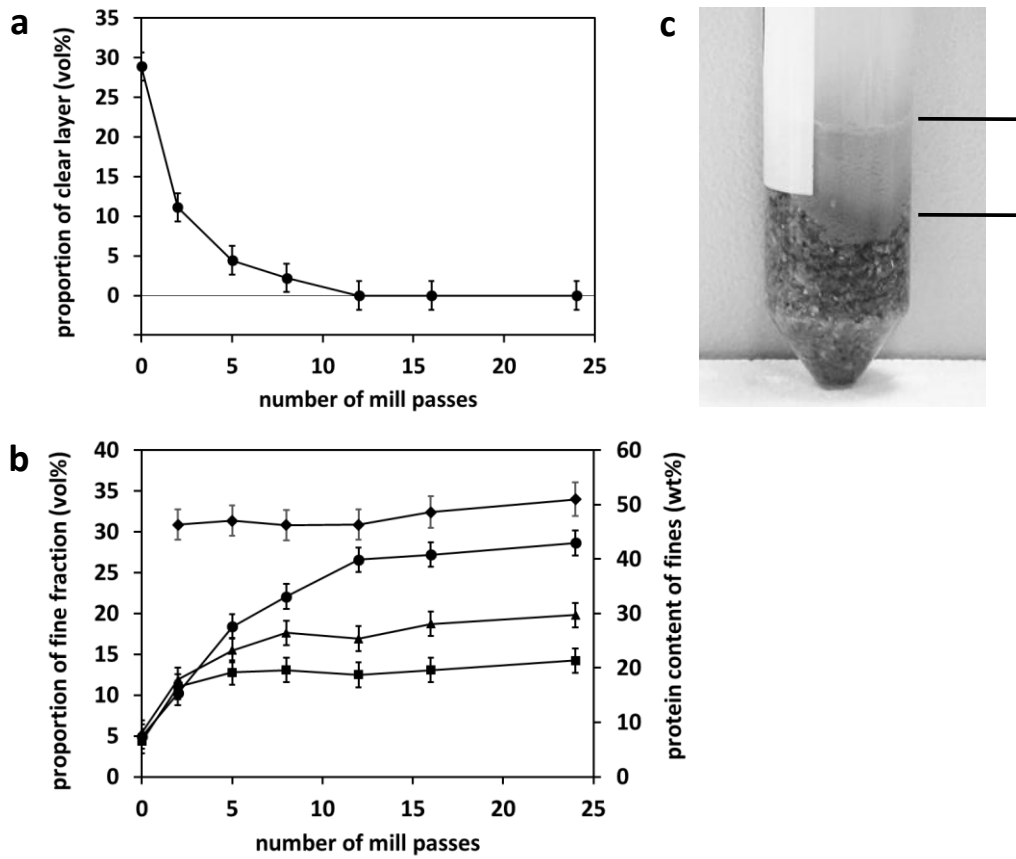


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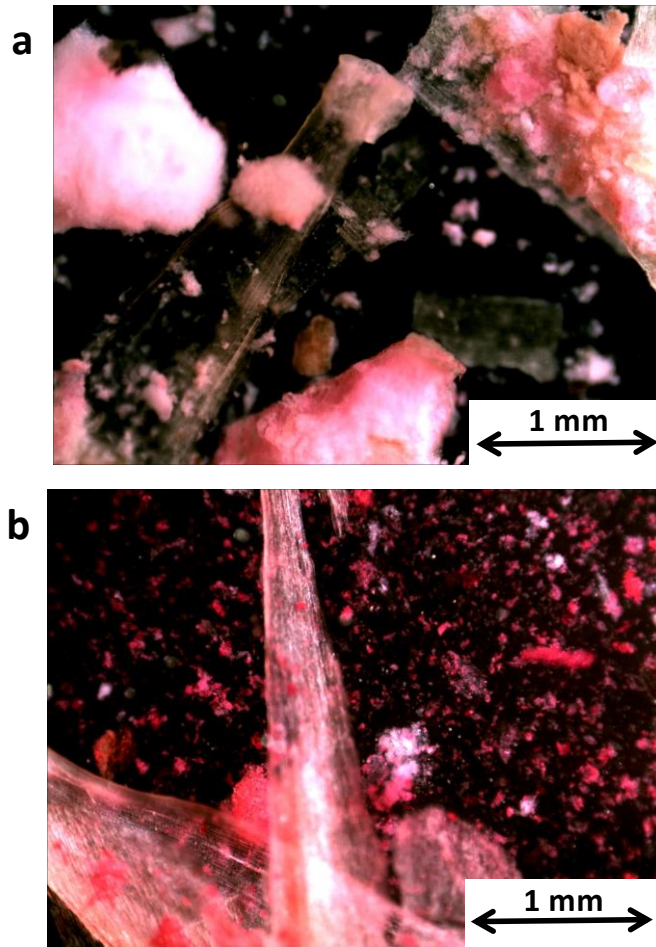
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Fig 2



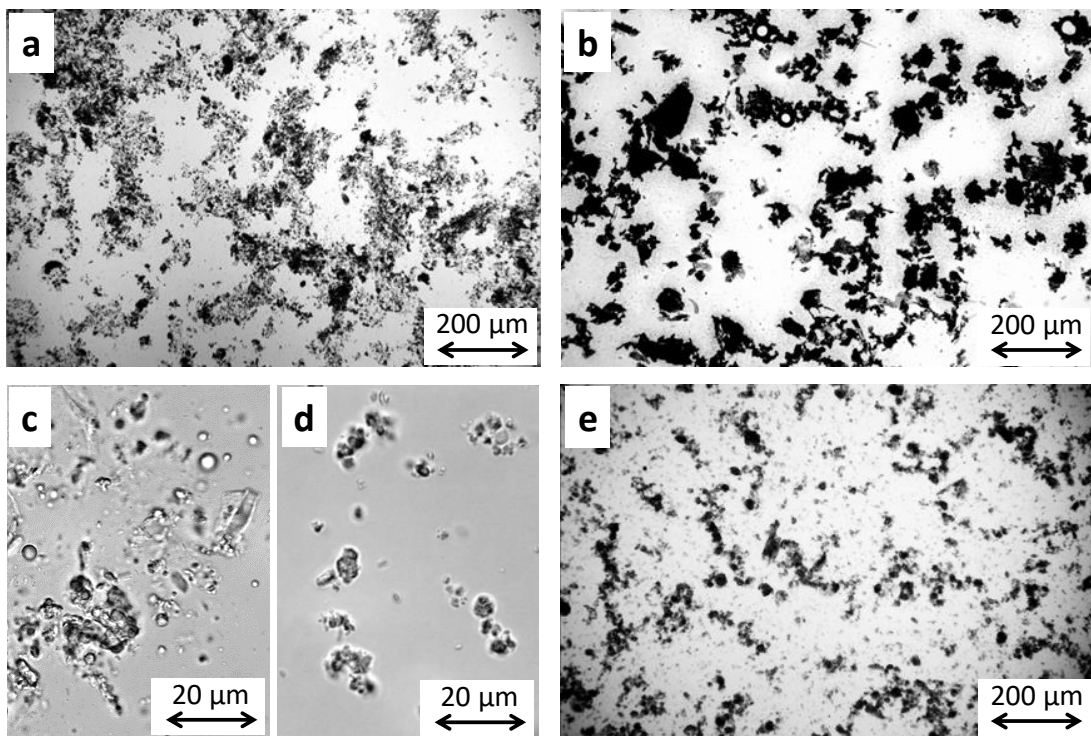
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Fig 3



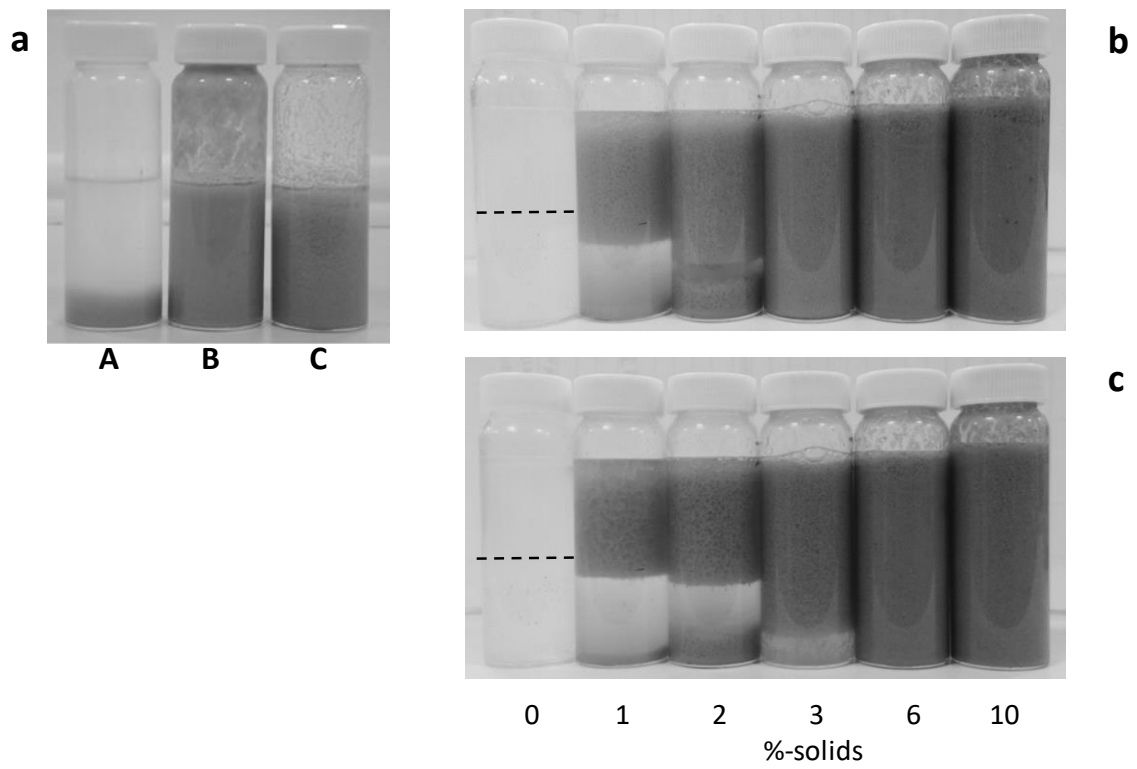
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Fig 4



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Fig 5

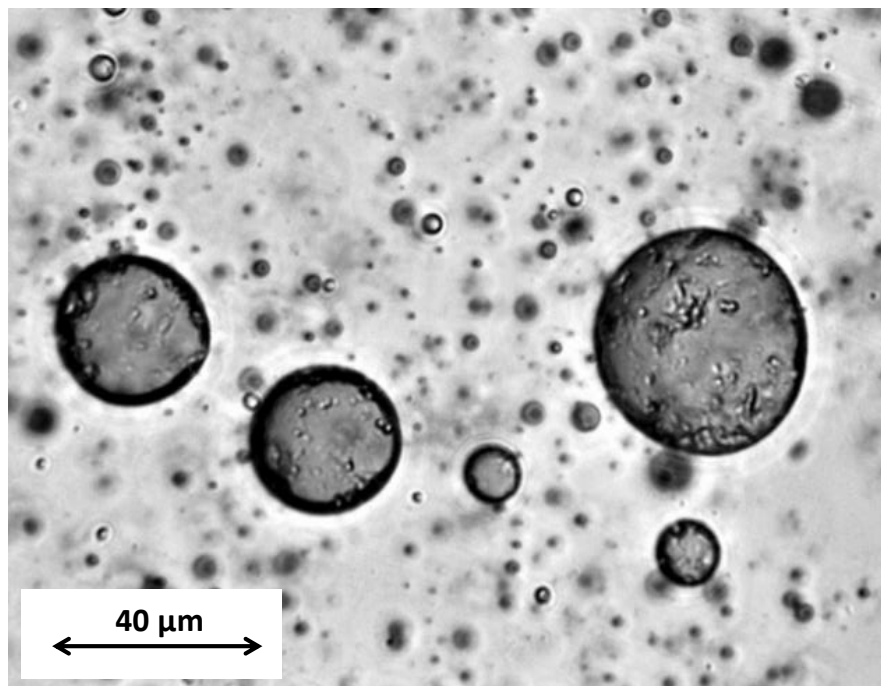


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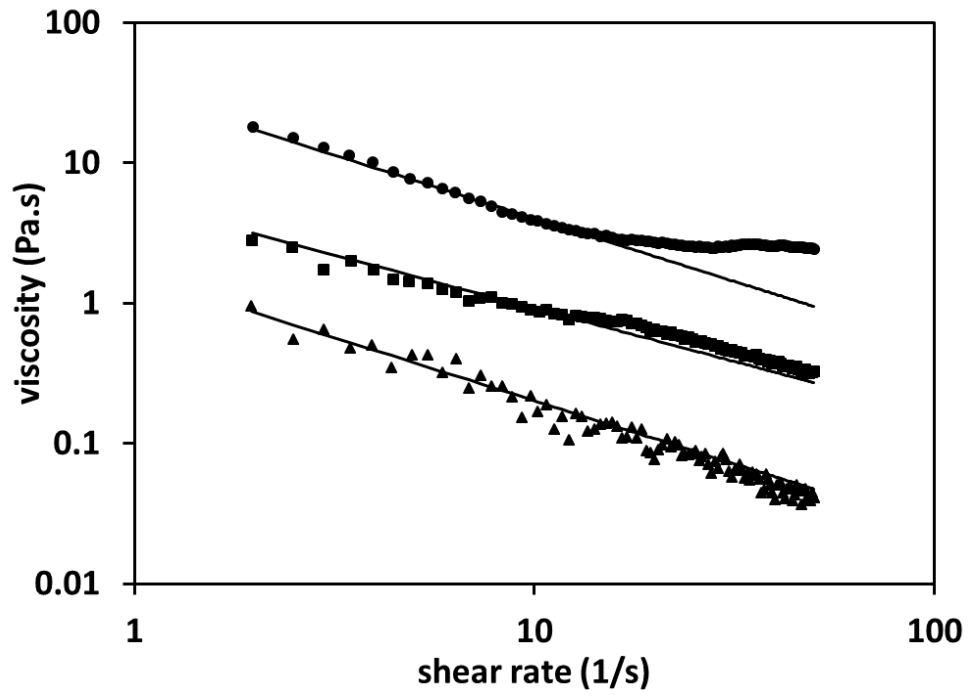
Fig 6



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Fig 7



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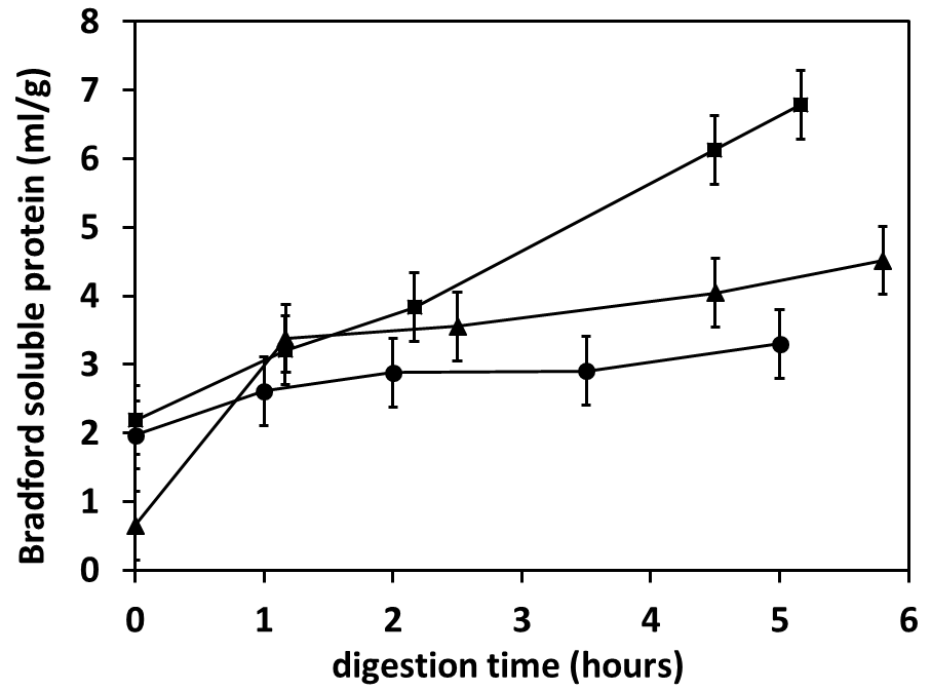
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Fig 8



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