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# 1 Brewers' spent yeast and grain as second-generation feedstuff for aquaculture feed

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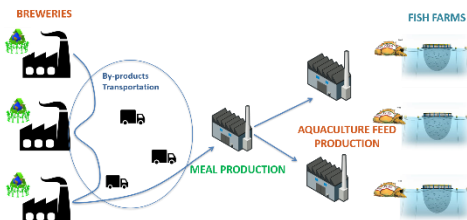
## 9 Abstract

10 Aquafeeds are formulated to contain all the essential nutrients that fishes need to keep healthy. They are highly dependent  
11 on marine ingredients: fish meal and oil. Hence, alternative ingredients which successfully replace these marine  
12 ingredients are required to result in sustainable and economical feeds. In this context, brewers' spent grain and yeast arise  
13 as alternative potential ingredients for aquafeed due their availability and nutritional content. However, reducing  
14 aquaculture's dependence on marine resources depends not only on developing alternative ingredients but also on  
15 improving their feed efficiencies.

16 In this context, Life Brewery project (LIFE16ENV/ES/000160) proposes an enzymatical hydrolysis step prior to the  
17 stabilization process to improve the digestibility of brewers' by-products and, therefore, increases the assimilation of  
18 nutrients by fishes. Hence, optimum hydrolysis conditions for both brewers' spent grain and yeast have been defined by  
19 comparing different enzymes combination and hydrolysis conditions at laboratory scale. Afterwards, selected enzymes  
20 and conditions have been validated at industrial scale. Finally, the digestibility of different experimental diets containing  
21 both hydrolysed and un-hydrolysed ingredients from brewers' waste has been determined with positive results.

22 Based on obtained results, it can be concluded that both hydrolysed and un-hydrolysed brewers' spent grain and yeast are  
23 suitable as alternative ingredients which successfully replace marine ingredients. Nevertheless, hydrolysis step improves  
24 ingredients efficiency and involves higher digestibility than un-hydrolysed ingredients.

## 25 Graphical abstract



26

27 **Keywords:** Brewers' by-products; Valorisation; Hydrolysis; Alternative ingredients; Sustainability; Circular economy.

## 28 1 Introduction

29 Aquaculture sector continues to grow faster than other major food production sectors. The average annual growth rate  
30 during the period 2001–2016 was 5.8 percent [1]. In this context, aquafeeds are specially formulated to contain all the  
31 essential nutrients that farmed fishes need to keep healthy. They are highly dependent on marine ingredients: specially  
32 fish meal (FM) and fish oil (FO). According to IFFO, approximately 69 % of FM and 75 % of FO are utilized in  
33 aquaculture production [2]. However, the global fish meal production remains stable over the years or it does not grow at  
34 the same rate as demand, with small oscillations due to natural phenomenon such as El Niño phenomenon [1].  
35 Consequently, an increased demand of marine origin ingredients coupled with the stagnation of the recent global fish  
36 meal production makes necessary to develop alternative ingredients which successfully replace these marine components  
37 with non-traditional sources.

38 Moreover, Samuel-Fitwi et al. [3] demonstrated that replacing FM by other alternative ingredients, such as soybean or  
39 rapeseed, involves less environmental impact per tonne of aqua-feed in both Acidification potential (AP); Global  
40 Warming potential (GWP); Eutrophication potential (EP) or Land competition (LC) than fish meal based standard aqua-  
41 feed. Thus, in case of the GWP, the fish meal standard trout feed has an impact of 1,797 kg CO<sub>2</sub> equivalent per ton while  
42 the soybean meal and rapeseed meal based aquafeeds has 1,019.65 and 1,037.13 kg CO<sub>2</sub>, respectively. Consequently,  
43 alternative ingredients are also required to result in sustainable feeds.

44 Within this framework, brewers' by-products arise high potential to be reuse as an alternative raw material for aquafeed  
45 due to their availability and their nutritional value. In this sense, European Union (EU) is the 2<sup>nd</sup> largest beer producer in  
46 the world, ahead of USA, Brazil and Russia. According to Eurostat, over 40 billion litres of beer were produced in the  
47 European Union (EU) in 2018 [4]. The largest volume of solid by-products produced by breweries are brewers' spent

48 grains (BSG) (80 % of total solid by-products), followed by brewers' spent yeast (BSY) (10 %). So, given EU beer  
49 production in 2018, about 7 million tons of BSG (14-20 kg per Hl of beer [5, 6]) and 0.9 million tons of BSY (2.0 -4.0 kg  
50 per Hl of beer [7-8]) were generated. BSY and BSG are often conventionally reused as animal feed and, in some cases,  
51 bioethanol production or landfill refuge [9, 10]. This implies the loss of a valuable product. In addition, the use of these  
52 by-products as a direct supply for animal feed without any treatment depends on many factors which can limit significantly  
53 their feasibility and, in many cases, can make it unsustainable. The high moisture content together with its high microbial  
54 load and the high temperature at which they are generated makes their useful life not more than 48 hours [11, 12].

55 Regarding the nutritional value of brewers' by-products, the chemical composition of BSG is characterized by a high-  
56 water content (75–80 %) and a high protein content (18–35.4 %, w/w). Their essential amino acids represent  
57 approximately 30 % of its total protein content. Lysine accounts for 14.3 % of the total protein content. Other amino acids  
58 in significant quantity are leucine, phenylalanine, isoleucine, threonine and tryptophan. Moreover, the presence of several  
59 polysaccharides that are constituents of BSG, such as  $\beta$ -glucans and phenolic compounds, have potential health benefits  
60 [7]. In the case of BSY, its chemical composition is characterized by a high-water content (85–90%) and the presence of  
61 carbohydrates, proteins, free amino acids, ash, vitamins and fatty acids. The main important amino acids of BSY are  
62 leucine, lysine, tyrosine, arginine, cysteine, histidine, isoleucine, methionine, phenylalanine, threonine, tryptophan and  
63 valine. Thus, BSY is an excellent source of high-quality protein. In addition, BSY cell wall contains  $\beta$ -glucans (8%, w/w  
64 dry weight) and the external layer is formed by mannoproteins. These two compounds have immunomodulatory,  
65 antimutagenic and anticarcinogenic activities [7]. Therefore, the nutritional value of BSG and BSY arises high potential  
66 to be alternative ingredients to reduce the high dependence on marine resources of aquafeeds.

67 However, reducing aquaculture's dependence on marine resources depends not only on developing alternative ingredients  
68 but also on improving their feed efficiencies. Thus, proteins of animal origin are considered nutritionally superior to plant  
69 origin ones because they have a better proportion of essential amino acids and do not contain anti-nutritional factors [13, 14].  
70 Moreover, the suitable level of substitution of FM and FO by these new alternative ingredients depends on the target  
71 species [15, 16]. Therefore, increasing the digestibility of these alternative ingredients will increase the assimilation of  
72 nutrients by fishes and, thus, the viability of their inclusion in aquafeeds. In this sense, hydrolysis of ingredients arises  
73 enormous potential to improve their physical and biochemical properties, leading to a better intestinal absorption [15, 17,  
74 18]. The hydrolysis of a protein is the reaction which breaks the peptide bonds of the proteins to obtain peptides and free  
75 amino acids of different molecular weights [19]. This process must be always adapted to the characteristics of both the  
76 initial product to be hydrolysed and the final product to be obtained. In the case of the production of protein hydrolysates  
77 from animal by-products which are rich in keratin chemical hydrolysis in acid medium is normally used. While in the  
78 other case of animal by-products and by-products such as soybeans, cereals or yeast enzymatic hydrolysis is normally  
79 used [20].

80 Finally, obtained hydrolysates are characterized by their high moisture content which makes them rapidly biodegradable  
81 due to the microbial activity [11, 12]. Thus, their stabilization by applying a drying process is of utmost importance.  
82 However, traditional drying processes (rotary drum, fluidized bed, etc.) are energy intensive processes and, subsequently,  
83 most of them economically unfeasible at industrial level. Hence, a low energy consumption drying process is necessary  
84 to guarantee the profitability of the development of brewers' by-products-based ingredients.

85 This study is focused on BSG and BY as alternative ingredients for aquafeed which successfully replace marine components.  
86 Specifically, the objective is to determine the optimum hydrolysis process by comparing different enzymes combinations  
87 and hydrolysis conditions, to develop an efficient and sustainable drying process and to assess the increased digestibility  
88 of hydrolysed ingredients with respect to non-hydrolysed in a feed efficiency growth trial with fishes.

## 89 **2 Material and methods**

### 90 **2.1 Analytical methods**

#### 91 **2.1.1 Characterization of hydrolysates for hydrolysis kinetic study**

92 The analytical method for determining the protein content during the hydrolysis kinetic study was Nitrogen  
93 Determination by the Kjeldahl Method [21].

94 The analytical method for determining the molecular profile distribution was Sodium Dodecyl Sulphate Polyacrylamide  
95 Gel Electrophoresis (SDS-PAGE). Thus, in dry and semi-dry samples, equal amounts of 0.5 g of each sample were  
96 collected. After collection, a soft extraction was performed using ACN: H<sub>2</sub>O [1:1] with 0.1 % TFA. Extracted samples  
97 were loaded in SDS-PAGE (12 % acrylamide, 1 mm width, 10 lanes) a left running during 1.5 hours at 125 volts. In the  
98 case of liquid samples, 100  $\mu$ l were collected, and loaded in each corresponding SDS-PAGE. After running, samples gels  
99 were fixed and stained with coomassie blue stain overnight, and finally, destained and stored with water at 4° C. All process  
100 was performed following the Laemmli standardized methodology [22]

101 The analytical method for determining the hydrolysis degree was High Performance Liquid Chromatography (HPLC)  
102 [23].

### 103 2.1.2 Characterization of final ingredients, experimental feeds & faeces for digestibility studio

104 The analytical methods for the characterization of the final ingredients of brewers' by-products-based meals, the  
105 experimental feeds used in the digestibility study and the faeces obtained in the digestibility trial were selected following  
106 the official methods of analysis published by AOAC (2000) [24].

- 107 • Dry matter (%), dry at 105°C for 16-18 h and estimated by gravimetry (AOAC method 925.09)
- 108 • Crude protein (%) by Dumas procedure and analysis of Nitrogen (FP-528 LECO, AOAC method 968.06)
- 109 • Crude fat (%) Büchi extraction system (B-811 and AOAC method 920.39)
- 110 • Ash (%), ashing the sample at 550°C for 16-18h (AOAC method 942.05)
- 111 • Gross energy (cal/g) using an adiabatic calorimeter (DIN 51900)
- 112 • Crude fibre (%) using an Ankon fibre analyser based on filter bag technology (AOAC method 962.09)
- 113 • Carbohydrates and Starch (%) Enzymatic method (AOAC method 996.11)
- 114 • Phosphorus (%) Molibdovanadate spectrophotometric method (AOAC method 965.17)
- 115 • Vitamin B2 (ppm) HPLC followed by UV detection at 445 nm
- 116 • Beta-glucan (g/100 g DM) using a kit Megazyme (Megazyme Int, Ireland) following the method of McCleary  
117 & Codd [25].
- 118 • Total amino acids content (%) Pre-derivatization with o-phthalaldehydet3-mercapto propionic acid (OPA/MPA)  
119 and 9-fluorenyl-methylchloroformate (FMOC) and HPLC separation by DAD/FL detection [26].
- 120 • Yttrium was analysed by inductively coupled plasma mass spectrometry using the method by Garantum-Tjeldsto  
121 et al [27].

### 122 2.2 Hydrolysis kinetic studio and effectiveness determination of each hydrolysis scenario

123 The methodology for the assessment of the hydrolysis kinetic depends on the brewers' by-product aimed of study.

#### 124 2.2.1 Brewers' spent yeast

125 The selection of the commercial enzymes was based on the hydrolysis objectives: on the one hand, to hydrolyse the  
126 protein with the aim of increasing the digestibility of ingredients in fishes [15, 17, 18] and, on the other hand, to increase  
127 the palatability. In this sense, there are some studies that shown the importance of reducing bitterness in the acceptance  
128 of new diets by fishes [28]. Thus, the selected commercial enzymes were:

- 129 • Protamex®: the key enzyme activity is provided by serine endo-protease that hydrolyses internal peptide bonds
- 130 • Flavourzyme®: the key enzyme activity is provided by exopeptidase that liberates amino acids by hydrolysis of  
131 the N-terminal peptide bond.

132 Within this framework, three different scenarios were studied at laboratory scale: enzymes hydrolysis kinetic and  
133 effectiveness when acting alone or when they are combined

- 134 • Scenario 1: supply Protamex® and Flavourzyme® enzymes at the same time  
135 The objective of this scenario was to assess the protease activity of both enzymes at the same time, taking  
136 advantage of the improvement of the reduction of bitterness with the Flavourzyme® enzyme.
- 137 • Scenario 2: supply only Protamex® enzyme  
138 The objective of this scenario was to assess the protease activity of the Protamex®, without the action of  
139 Flavourzyme® for reducing the bitterness of final product.
- 140 • Scenario 3: supply only Flavourzyme® enzyme  
141 The objective of this scenario was to assess if the protease activity of Flavourzyme® was high enough compared  
142 with the scenario 2 and scenario 3.

143 The hydrolysis kinetic of each hydrolysis scenario was studied by analysing the protein content of intermediate  
144 hydrolysates at different stages of the hydrolysis process by Kjeldahl method.

145 The effectiveness of each hydrolysis scenario was assessed by determining the molecular profile distribution of different  
146 hydrolysates by SDS-PAGE to assess the hydrolysis degree. With the aim of simulating the production conditions of the  
147 final ingredients described in section 2.3, obtained hydrolysates from each hydrolysis scenario was mechanical dewatered  
148 to obtain a liquid fraction, which is going to be rejected and discharged, and a semi-solid fraction, which is the objective  
149 fraction and which is going to be dried for the determination of protein digestibility in fishes.

#### 150 2.2.2 Brewers' spent grain

151 The selection of the commercial enzymes was based on the hydrolysis objectives that, in case of BSG, was not only to  
152 hydrolyse the protein but also the fibre. There are some studies that shown that fibre is a potential anti-nutritional for  
153 fishes [13, 14]. Thus, the selected commercial enzymes were:

- 154 • Celluclast ®: the key enzyme activity is provided by a selected strain of fungus called *Trichoderma resei* that  
155 hydrolyses cellulose to glucose, cellobiose and larger polymers of glucose.
- 156 • Protamex®: the key enzyme activity is provided by serine endo-protease that hydrolyses internal peptide bonds

157 Within this framework, three different scenarios were studied at laboratory scale: enzymes hydrolysis kinetic and  
158 effectiveness when acting alone, when they are combined and when they are sequenced or supplied at the same time

159 • Scenario 1: sequenced supply of Celluclast® and Protamex® enzymes.  
160 Celluclast® enzymes is supplied firstly and, once this has made the fibre hydrolysis, the Protamex® enzyme is  
161 supplied.

162 The objective of this scenario was to test the increase of the access to protein of Protamex® enzyme after the  
163 fiber hydrolysis.

164 • Scenario 2: supply of Celluclast® and Protamex® enzymes at the same time

165 The objective was to compare the effectiveness regarding scenario 1.

166 • Scenario 3: supply only Protamex®

167 The objective was to compare the effectiveness of Protamex® enzyme without fiber hydrolysis and regarding  
168 scenario 1 and 2.

169 The hydrolysis kinetic of each hydrolysis scenario was studied by analysing the protein content of intermediate  
170 hydrolysates at different stages of the hydrolysis process by Kjeldahl method.

171 The effectiveness of each hydrolysis scenario was assessed by determining the molecular profile distribution of different  
172 hydrolysates by SDS-PAGE to assess the hydrolysis degree. With the aim of simulating the production conditions of the  
173 final ingredients described in section 2.3, obtained hydrolysates from each hydrolysis scenario was mechanical dewatered  
174 to obtain the liquid fraction, which is going to be rejected and discharged, and a semi-solid fraction, which is the objective  
175 fraction and which is going to be dried for the determination of protein digestibility in fishes.

### 176 **2.3 Scaling up of ingredients production**

177 The methodology for scaling up the ingredient production consisted of a first mechanical dehydration to reduce as much  
178 as possible the humidity (less than 60 %), which involves a low energy demand and, therefore, a reduction of the energy  
179 necessary for thermal drying in the second step. The second phase applies a thermal drying to reduce moisture content  
180 below 10 %.

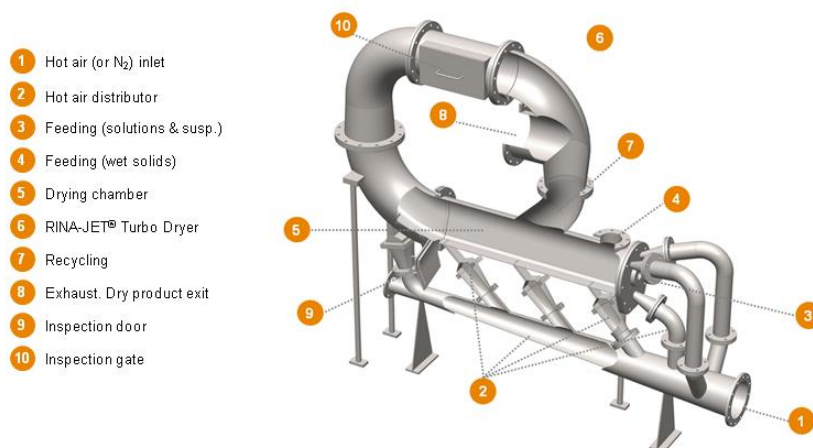
181 Mechanical dehydration technologies selection depends on the physical properties of the targeted products. In case of  
182 BSG, the selected technology was a filter centrifuge whereas in case of BY the selected technology was a decanter  
183 centrifuge:

184 • Filter centrifuge is based on the principle of gravity and centrifugal force. It puts an object in rotation around a  
185 fixed axis and applies a force perpendicular to the axis of spin (outward). The centrifugal force makes denser  
186 particles to move outward in the radial direction and less dense particles to the centre. Therefore, in case of BSG,  
187 the solid particles are trapped in a separation mesh that can have different pore size due to the centrifugal force  
188 and the liquid flows out from the upper side of the separator.

189 • Decanter centrifuge is based on the principle of separation via buoyancy to separate continuously solid materials  
190 from liquids. Considering that a denser particle falls to the bottom, while a less dense particle is suspended above  
191 it, a decanter centrifuge increases the rate of settling using continuous rotation to reduce the settling time of the  
192 particles.

193 Thermal drying technology selection depends fundamentally on its efficiency. Thus, flash drying technology was selected  
194 as the most appropriate thermal drying technology for drying both BSY and BSG.

195 • Flash dryer is based on the instant, self-regulating and continuous drying of wet solids. It is a high thermal  
196 efficiency technology which combines the effect of turbulence with the high-speed movement of wet solid  
197 particles to dry them instantly. The products are broken in the drying chamber (Figure 1) and the surface area of  
198 particles increases significantly, and therefore, decreasing the required energy to dry them. Finally, a minimum  
199 heating during a short time of residence - fractions of a second - makes it suitable for temperature-sensitive  
200 products maintaining the nutritional value and food security [29].



**Fig. 1:** Structure of the RINA-JET flash dryer technology

201

202

203 The different equipment used in this study for the scaling up of the ingredients production were:

204

1. Decanter centrifuge “GEA CA 225-00-33”, owned by GEA Westfalia (Barcelona, Spain).

205

2. Filter centrifuge “RINA 200F 1000 S PI”, owned by Riera Nadeu company (Granollers, Spain).

206

3. Flash dryer “RINA-JET S-1008”, owned by the Riera Nadeu company (Granollers; Spain).

#### 207 **2.4 Determination of protein digestibility in fishes**

208 The selected fish species was gilthead seabream (*Sparus aurata*) as a model of Mediterranean aquaculture.

##### 209 **2.4.1 Experimental diets determination**

210 A commercial-based diet for gilthead seabream using fish meal (Corpesca Super Prime LT, Chile) was formulated and  
 211 extruded at IRTA facilities using an extruder (marca y modelo). To this mixture 20 g / kg or Yttrium oxide (Sigma, Spain)  
 212 was added as an inert marker for the evaluation of the apparent digestibility coefficient (ADC).

213 In addition, four experimental diets were also produced by mixing 700 g / kg of the basal mixture and 300 g / kg of each  
 214 test ingredient (BSY and BSG, hydrolysed and unhydrolyzed). The reference diet contained 209 g / kg of starch to enable  
 215 extrusion of the pellets which was hindered due to its high fibre content.

216 All the diets were formulated to be iso-protein and iso-lipidic and are presented in Tables 1 and 2. The inclusion of spent  
 217 yeast and spent grain resulted in experimental diets that had 393 to 420 g/Kg crude protein, 218 to 224 g/Kg crude fat, 17  
 218 to 19 MJ/Kg gross energy, reflecting the similarity among the diets

219 **Table 1:** Formula of the experimental diets for protein digestibility determination in Gilthead seabream

Ingredients	Control	D-Spent yeast 30 %	H-Spent yeast 30 %	D- Spent grain 20 %	H-Spent grain 20 %
Fish meal 70 LT	60.00	40.00	42.00	50.00	50.00
Wheat starch	20.95	9.45	7.45	10.00	10.00
Spent yeast	-	30.00	-	-	-
Hydrolyzed spent yeast	-	-	30.00	-	-
Spent grain	-	-	-	20.00	-
Hydrolyzed spent grain	-	-	-	-	20.00
Fish oil	18.00	19.50	19.50	16.50	17.00
Vit & Min Premix PV01	1.05	1.05	1.05	1.05	1.05
Yttrium	0.02	0.02	0.02	0.02	0.02

220

221 **Table 2:** Composition of the experimental diets for protein digestibility determination in gilthead seabream

Parameters	Control	D-Yeast 30%	H-Yeast 30%	D- Spent grain 20%	H-Spent grain 20%
Dry matter	978,30 ± 3,09	979,20 ± 2,36	976,60 ± 5,46	980,50 ± 5,50	978,10 ± 8,50

(DM, g/Kg)										
Ash (g/Kg DM)	98,80	± 0,98	83,20	± 0,77	78,70	± 0,76	93,60	± 4,24	100,60	± 1,07
Crude protein (g/Kg DM)	419,80	± 3,39	413,30	± 0,16	418,20	± 2,49	417,70	± 3,51	392,80	± 0,70
Crude fat (g/Kg DM)	218,42	± 3,29	223,94	± 1,45	234,04	± 5,71	219,83	± 2,04	221,40	± 1,59
Carbohydrates (g/Kg DM)	215,10	± 8,42	218,50	± 16,36	197,00	± 4,22	130,20	± 9,07	166,20	± 11,46
Gross energy (MJ/Kg DM)	18,65	± 0,08	18,89	± 0,19	19,04	± 0,32	17,25	± 0,22	17,53	± 0,23

222

223

#### 2.4.2 Digestibility trials with fishes

224

The protein digestibility trials of obtained ingredients with fishes were carried out at IRTA facilities using recirculation (RAS) systems. The fishes were purchased in a commercial farm (Piscimar, Castellón, Spain), transported by road to IRTA facilities, acclimatized for 15 days and then randomly distributed in fifteen 500 L tanks with individual faeces sedimentation columns in the outflow of the tanks. Twenty-five fishes with a body weight of 253.01±27.68g were randomly distributed in the tanks connected to the RAS systems and kept at 20°C under natural light.

225

226

227

228

The experimental diets were randomly assigned to the tanks and fed in triplicate. Fish were fed 100 g of the feeds once daily for 2 weeks before faecal collection. Gilthead seabream faeces were collected in the sedimentation columns for 3 alternate days. Gilthead sea bream were also fed once per day during the collection period and the tanks cleaned to avoid any uneaten feed in the tanks and in the faecal collectors. Faecal samples were freeze dried for 24h and stored at -20°C until chemical analyses.

229

230

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232

233

The apparent digestibility coefficients (ADC) of the experimental diets were calculated according to Maynard et al methodology [30]

234

235

236

$ADC (\%) = 100 \times (1 - (\text{dietary } Y_2O_3 \text{ level} / \text{faeces } Y_2O_3 \text{ level}) \times (\text{faeces nutrient or energy level} / \text{dietary nutrient or energy level}))$

237

238

The ADC of the test ingredients were estimated according to National Research Council method [31]

239

$ADC_{BSG} (\%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) \times ((0.8 \times D_{ref}) / (0.2 \times D_{ing}))]$

240

$ADC_{BSY} (\%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) \times ((0.7 \times D_{ref}) / (0.3 \times D_{ing}))]$

241

Where  $ADC_{test}$  = ADC (%) of the experimental diet

242

$ADC_{ref}$  = ADC (%) of the reference diet

243

$D_{ref}$  = g/Kg nutrient (or MJ/Kg gross energy) of the reference diet (DM basis)

244

$D_{ing}$  = g/Kg of nutrient (or MJ/Kg gross energy) of the test diet (DM basis)

245

### 3 Results and discussion

246

#### 3.1 Hydrolysis kinetic studio and effectiveness determination of each hydrolysis scenario

247

##### 3.1.1 Spent yeast

248

The hydrolysis kinetic studio results show that descripción de cómo se aumenta la proteína en la fase líquida (se solubiliza) y como disminuye en la fase sólida (Figuras)

249

250

MIKEL

251

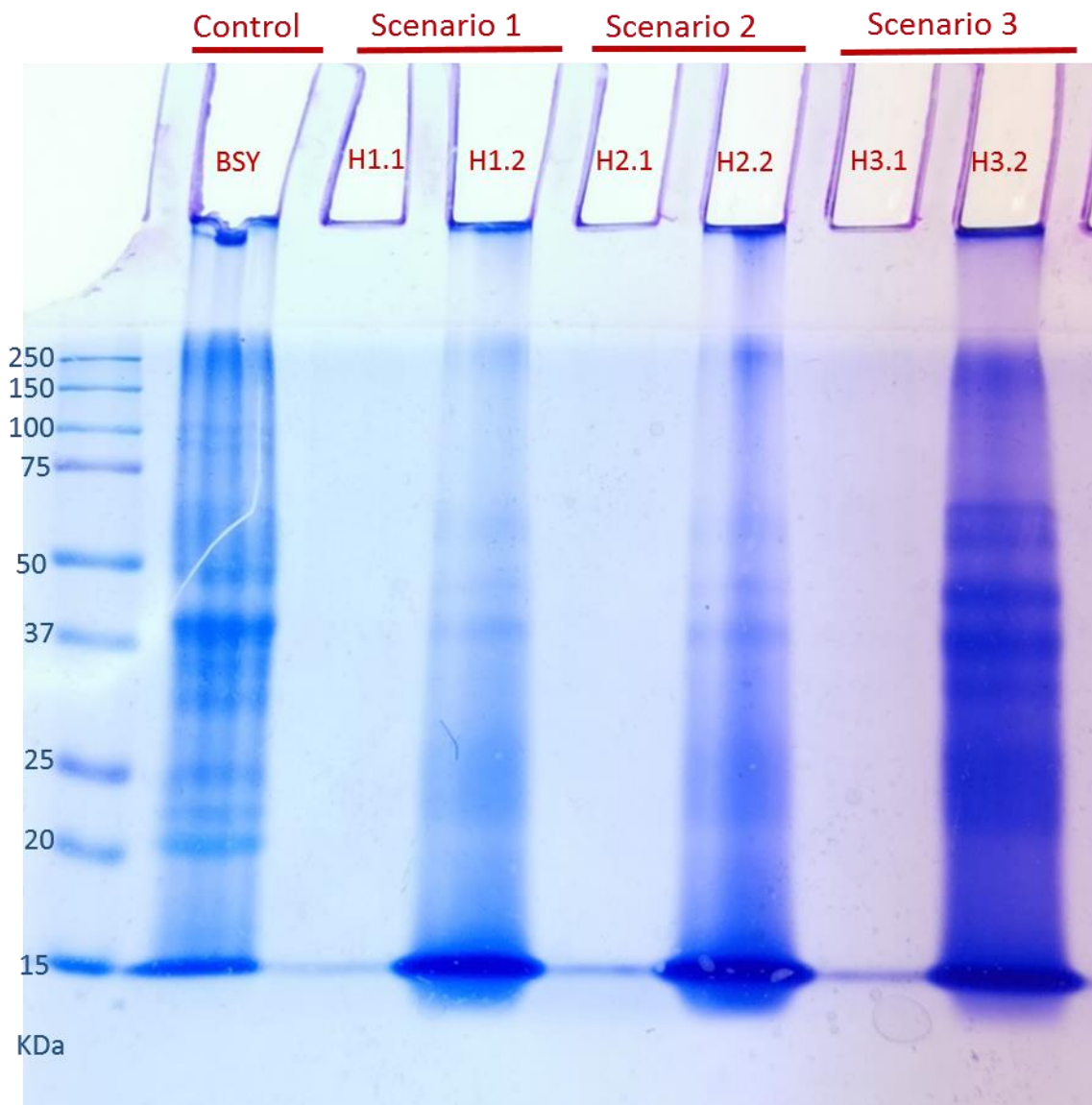
252

The results of effectiveness of each hydrolysis scenario by SDS-PAGE (Figure 2) show that, in H3.2 lane (semi-solid fraction), protease activity has been low, with dense high molecular mass bands (above 25 kDa). On the other hand, H1.2 and H2.2 (semi-solid fractions) look very similar, with apparently higher protease activity, in contrast to H3.2 (semi-solid fraction). In all cases, liquid fractions (H1.1, H2.1 and H3.1 lanes) show very little amount of protein.

253

254

255



**Fig. 2:** Molecular profile distribution of different BSY hydrolysis scenarios by SDS-PAGE method

256

257

258 Considering the obtained results, the scenario 1 (supply Protamex® and Flavourzyme® enzymes at the same time) has  
 259 been selected as the most appropriate for the protein hydrolysis of BSY to produce a new ingredient for aquaculture feed.  
 260 The scenario 3 (supply only Flavourzyme® enzyme) has been rejected due to the low protease activity. In addition,  
 261 although the effectiveness of the scenario 2 (supply only Protamex® enzymes) is quite similar to scenario 2, and therefore  
 262 the expected protein digestibility improvement in fishes should be similar, the expected reduction in the bitterness related  
 263 to scenario 1 due to the activity of Flavourzyme® enzymes has been considered of high importance to ensure the viability  
 264 of this new ingredient for aquaculture feed application.

265

### 3.1.2 Spent grain

266

The hydrolysis kinetic studio results show that descripción de cómo se aumenta la proteína en la fase líquida (se solubiliza)  
 267 y como disminuye en la fase sólida (Figuras)

268

MIKEL

269

270

The results of effectiveness of each hydrolysis scenario by SDS-PAGE (Figure 3), they show that, xxxxx.

271

CIC-BIOGUNE

272

273

**Fig. 3:** Molecular profile distribution of different BSG hydrolysis scenarios by SDS-PAGE method



274 Considering the obtained results, scenario 1 (sequenced supply of Celluclast® and Protamex® enzymes), scenario 2  
 275 (supply of Celluclast® and Protamex® enzymes at the same time) and scenario 3 (supply only Protamex®) are viable for  
 276 the protein hydrolysis of BSG to produce a new ingredient for aquaculture feed.

277 However, provided fibre is considered as anti-nutritional parameter for fishes [13, 14], the fibre hydrolysis related to the  
 278 activity of Celluclast® enzymes has been considered of high importance to ensure the viability of this new ingredient for  
 279 aquaculture feed application. Thus, the scenario 3 has been rejected for this proposal.

280 Regarding Celluclast® enzyme activity for fibre hydrolysis, this could be inactivated by the Protamex® enzyme activity  
 281 if they act simultaneously (scenario 2). Thus, the scenario 1 (sequenced supply of Celluclast® and Protamex® enzymes)  
 282 has been selected as the most appropriate for the hydrolysis of BSG to produce a new ingredient for aquaculture feed  
 283 because Celluclast® enzyme acts firstly and, once the fibre hydrolysis has been carried out, Protamex® enzyme is  
 284 supplied, so the inactivation of Celluclast® enzyme by Protamex® enzyme has been avoided. In addition, there is a light  
 285 tendency to a higher proteolytic activity in the band of 50 - 150 kDa related to scenario 1.

## 286 3.2 Scaling up of ingredients production

### 287 3.2.1 Spent yeast

288 The scaling up of 2 different ingredients from BSY: hydrolysed (scenario 1) and un-hydrolysed consisted of a first  
 289 mechanical dehydration to reduce as much as possible the humidity (less than 60 %) and a second thermal drying to  
 290 reduce moisture content below 10 %. Obtained ingredients have been used in the determination of protein digestibility in  
 291 fishes in section 3.3.1. The biochemical composition of these ingredients are shown in the Table 3.

292 **Table 3:** Brewers' spent yeast-based ingredients nutritional value

Parameter	Un-hydrolysed Spent yeast	Hydrolysed Spent yeast
Dry matter (%)	94.19	89.05
Crude protein (%)	45.07	41.24
Ether extract (%)	0.35	0.45
Ash (%)	3.99	3.87
Gros energy (cal/g)	4477	4238
Crude fibre (%)	0.64	0.62
Starch (%)	20.59	20.05
Phosphorus (%)	0.91	0.87
Vitamin B2 (ppm)	2.60	5.00
Total a.a. content (%)	40.60	36.63
<i>Aspartic acid</i>	4.51	4.08
<i>Glutamic acid</i>	5.68	5.19
<i>Serine</i>	2.39	2.16
<i>Histidine</i>	1.14	1.02
<i>Glycine</i>	1.78	1.63
<i>Threonine</i>	2.29	2.09
<i>Arginine</i>	2.37	2.12
<i>Alanine</i>	2.77	2.52
<i>Tyrosine</i>	1.62	1.40
<i>Valine</i>	2.50	2.25
<i>Methionine</i>	0.77	0.69
<i>Phenylalanine</i>	2.17	2.00
<i>Isoleucine</i>	2.21	2.02
<i>Leucine</i>	3.31	3.00
<i>Lysine</i>	2.99	2.57

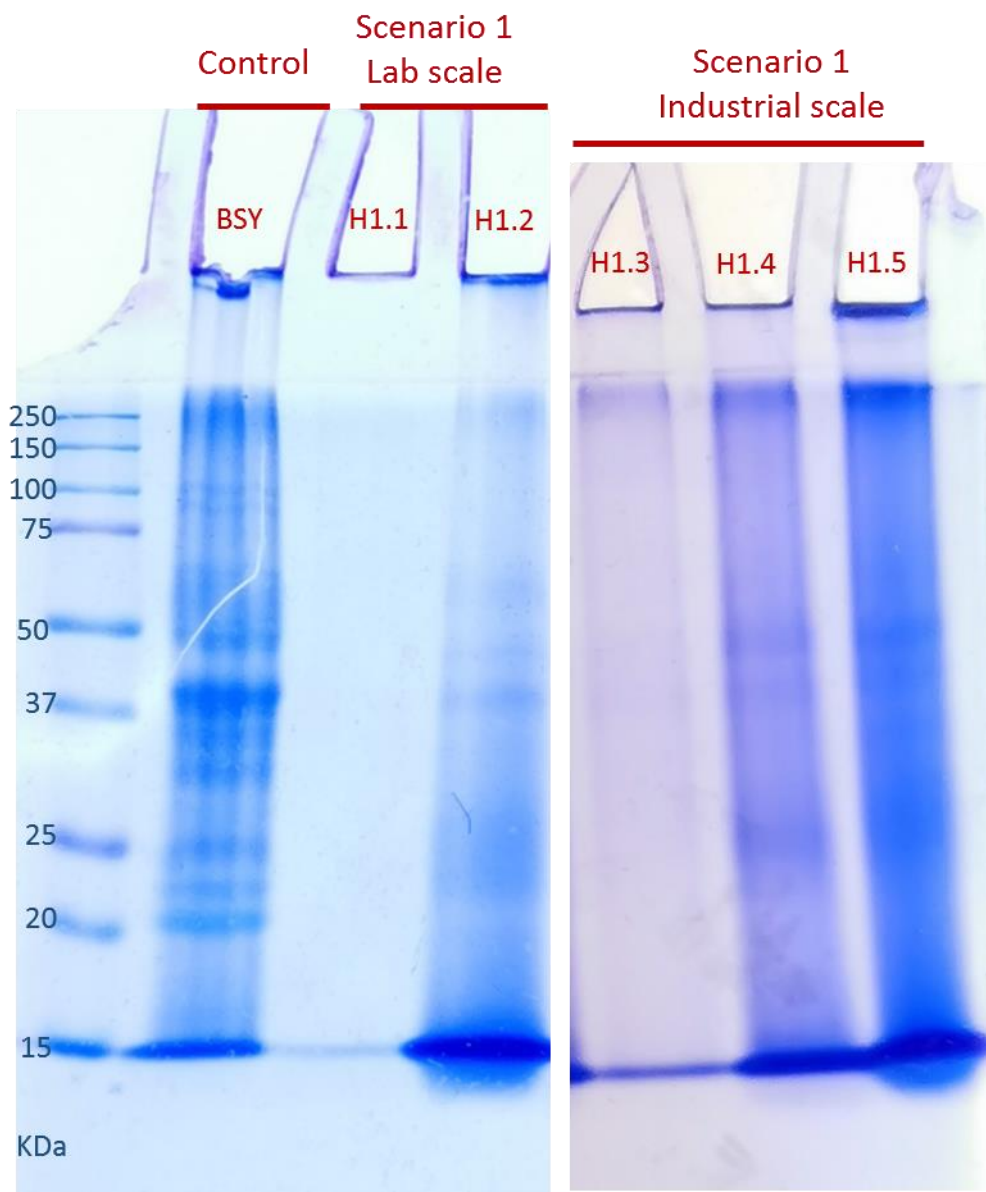
<i>Hydroxyproline</i>	<0.03	<0.03
<i>Proline</i>	2.10	1.89

293

294 Figure 4, related to the SDS-PAGE analysis of the protein hydrolysis effectiveness of the results at laboratory scale (H1.1  
 295 and H1.2) comparing with the industrial scale ones (H1.3, H1.4 and H1.5), shown very similar SDS-PAGE patterns,  
 296 hence, similar levels of protease activity. The amount of hydrolysis seems the same, with similar composition of  
 297 remaining high molecular mass bands. This shows that the scaling up of the scenario 1 at semi-industrial scale was  
 298 performed correctly.

299 In addition, the stabilization process of the semi-solid fraction (H1.4) obtained by performing the hydrolysis scenario 1  
 300 to obtain the dried ingredient (H1.5) show no differences. So, the stabilization process doesn't affect to the protein content  
 301 and its molecular profile distribution.

302 Finally, as in Figure 2, liquid fractions (H1.1 and H1.3) show very little amount of protein. This involves that the  
 303 stabilization process proposed for drying BSY in an efficient way is compatible with the hydrolysis process since the loss  
 304 of soluble protein in the liquid fraction, which is rejected and discharged, is minimal.



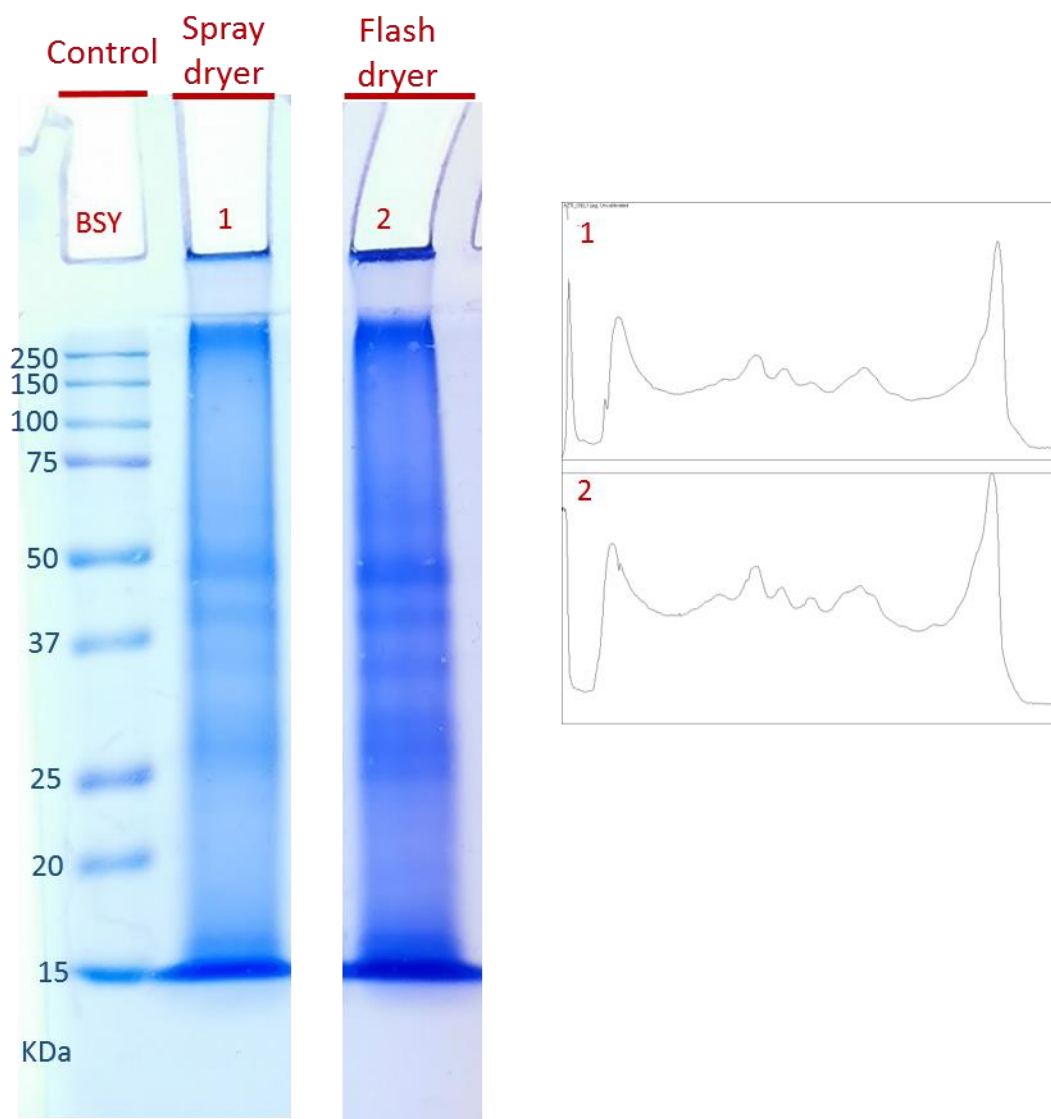
305

**Fig. 4:** Molecular profile distribution of the BSY hydrolysis scenario 1 at lab and industrial scale by SDS-PAGE method

306

308 Finally, Figure 5 compares two different stabilization process: one based on Spry dryer technology (1) and other based  
 309 on Flash dryer technology (2), shows the same SDS-PAGE pattern. The same results can be seen in the corresponding

310 densitograms. So, at this level, this involves that no significative differences related to dried hydrolysates are founded  
 311 between both drying techniques.



312  
 313 **Fig. 5:** Molecular profile distribution of un-hydrolysed BSY dried by spray dryer and flash dryer by SDS-PAGE  
 314 method

312

313

314  
 315 **3.2.2 Spent grain**

316 The scaling up of 2 different ingredients from BSG: hydrolysed (scenario 1) and un-hydrolysed consisted of a first  
 317 mechanical dehydration to reduce as much as possible the humidity (less than 60 %) and a second thermal drying to  
 318 reduce moisture content below 10 %. Obtained ingredients have been used in the determination of protein digestibility in  
 319 fishes in section 3.3.2. The biochemical composition of these ingredients are shown in Table 4

320

**Table 4:** Brewers' spent grain -based ingredients nutritional value

Parameters	Un-hydrolysed Spent grain	Hydrolysed Spent grain
Dry matter (%)	92.00	98.11
Crude protein (%)	22.73	21.38
Ether extract (%)	7.75	11.46
Ash (%)	3.60	5.99
Gros energy (cal/g)	4766	4838
Crude fibre (%)	17.28	16.28
Starch (%)	3.59	3.43

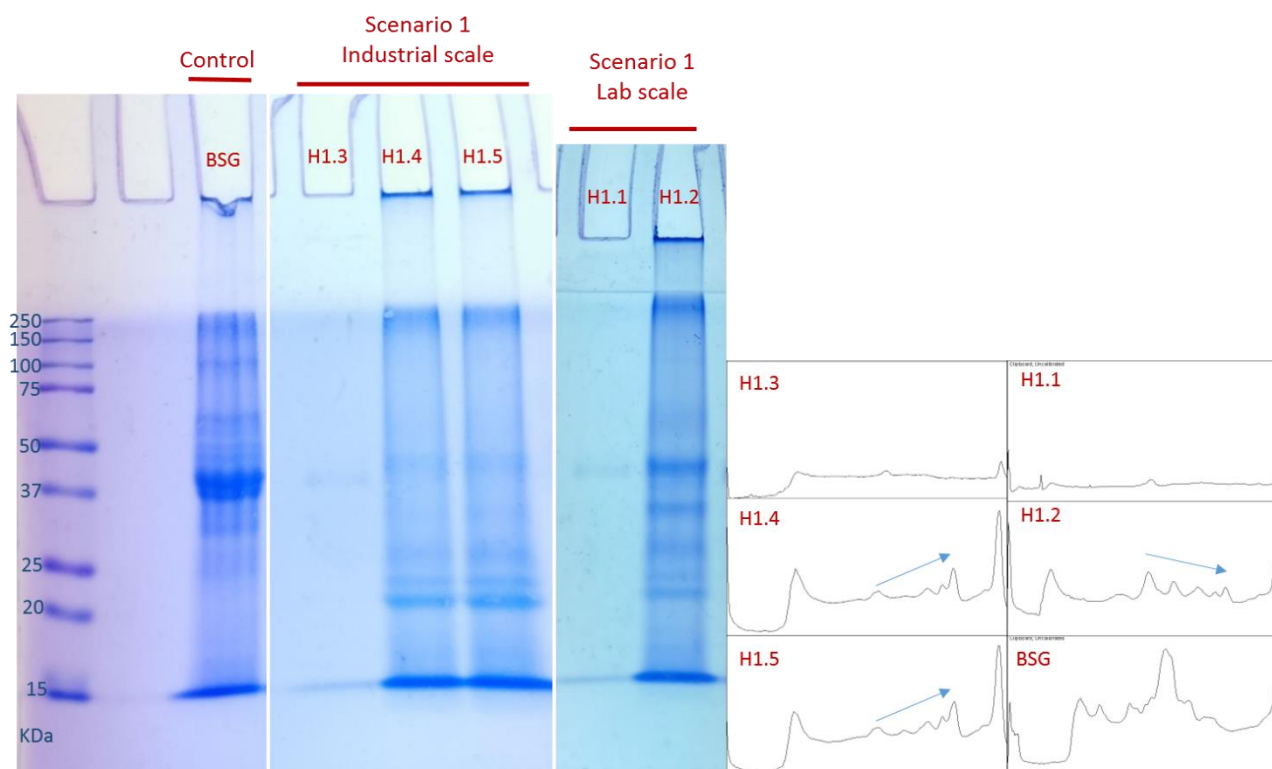
Phosphorus (%)	0.49	0.34
Vitamin B2 (ppm)	0.40	1.40
Total a.a. content (%)	22.51	18.67
<i>Aspartic acid</i>	1.58	1.42
<i>Glutamic acid</i>	4.82	3.62
<i>Serine</i>	1.03	0.86
<i>Histidine</i>	0.60	0.55
<i>Glycine</i>	0.81	0.78
<i>Threonine</i>	0.84	0.79
<i>Arginine</i>	1.18	1.05
<i>Alanine</i>	1.39	1.15
<i>Tyrosine</i>	0.88	0.79
<i>Valine</i>	1.17	1.03
<i>Methionine</i>	0.47	0.38
<i>Phenylalanine</i>	1.33	1.11
<i>Isoleucine</i>	0.94	0.79
<i>Leucine</i>	2.28	1.81
<i>Lysine</i>	0.88	0.72
<i>Hydroxyproline</i>	<0.03	0.03
<i>Proline</i>	2.31	1.79

321

322 Figure 6, related to the SDS-PAGE analysis of the protein hydrolysis effectiveness of the results at laboratory scale (lane  
323 H1.1 and H1.2) comparing with the industrial scale ones (H1.3, H1.4 and H1.5) shown that a clear hydrolysis has been  
324 performed, in comparison to control (BSG). But, at industrial scale (H1.3 to H1.5) the hydrolysis seems to be higher than  
325 at laboratory scale (H1.1 and H1.2). H1.2 shows higher intensity bands above 25 kDa than those in H1.4 and H1.5. This  
326 is clearly visible by showing the corresponding densitograms. This shows that the scaling up of the scenario 1 at semi-  
327 industrial scale was performed correctly.

328 In addition, the stabilization process of the semi-solid fraction (H1.4) obtained by performing the hydrolysis scenario 1  
329 to obtain the dried ingredient (H1.5) show no differences. So, the stabilization process doesn't affect to the protein content  
330 and its molecular profile distribution.

331 Finally, as in Figure 3, liquid fractions (H1.1 and H1.3) show very little amount of protein. This involves that the  
332 stabilization process proposed for drying BSG in an efficient way is compatible with the hydrolysis process since the loss  
333 of soluble protein in the liquid fraction, which is rejected and discharged, is minimal.



334  
335 **Fig. 6:** Molecular profile distribution of the BSG hydrolysis scenario 1 at lab and industrial scale by SDS-PAGE  
336 method

337 **3.3 Determination of protein digestibility in fishes**

338 **3.3.1 Spent yeast**

339 No mortality was observed during the trial. The apparent digestibility coefficients (ADC) of the experimental diets  
340 formulated with spent yeast (BSY) and used for seabream are presented in Table 5. Digestibility of protein was high in  
341 all the diets (71%-90%).

342 **Table 5:** Apparent Digestibility Coefficients (ACD) of BSY based experimental diets in gilthead seabream

Diet	Protein faeces	Protein diet	ADC	SD
Control	198.1 ± 0.40	419.80 ± 3.39	90.26	0.11
D-Spent yeast 30 %	262.4 ± 1.59	413.30 ± 1.16	71.76	2.73
H-Spent yeast 30 %	223.1 ± 2.79	418.20 ± 2.49	75.01	1.27

343  
344 The ADC of an ingredient reflects the capability of a certain species of fish to utilize its nutrients, predicting its potential  
345 as a feedstuff. In this case the results show acceptable digestibility of BSY for gilthead seabream indicating that BSY  
346 ingredients (protein but also lipids –not shown-) are suitable for aquaculture nutrition showing the hydrolysed prototypes  
347 a higher digestibility than non-hydrolysed.

348  
349 **3.3.2 Spent grain**

350 The apparent digestibility coefficients (ADC) of the experimental diets formulated with spent grain (BSG) and used for  
351 gilthead seabream are shown in the Table 6. Digestibility of protein was high in all the diets (84-90%)

352 **Table 6:** Apparent Digestibility Coefficients (ACD) of BSG based experimental diets in Gilt seabream

Diet	Protein faeces	Protein diet	ADC	SD
Control	198.1 ± 0.40	419.80 ± 3.39	90.26	0.11
D-Spent grain 20 %	118.2 ± 3.41	417.70 ± 3.51	84.01	0.54
H-Spent grain 20 %	87.8 ± 0.90	392.80 ± 0.70	85.22	0.31

354 As in the case of BSY, the results of BSG can be considered acceptable for gilthead seabream on-growing. Thus, brewers'  
355 spent grain (BSG) ingredients can be considered suitable for aquaculture nutrition. As in the case of BSY, hydrolysed  
356 prototypes have shown higher digestibility than non-hydrolysed.

357 In previous studies carried out with Mediterranean species like seabass (Metailler & Huelvan, 1993; Campos et al, 2018)  
358 brewer's yeast have shown good digestibility results, much better when the product was previously hydrolysed and a  
359 peptide fraction higher than 3000 was used (Campos et al, 2018) giving values of 87.9 ADC.

#### 360 4 Conclusions

361 The first conclusion is that the production of alternative ingredients for aquaculture feed based on brewers' by-products  
362 is feasible from the technical point of view. The enzymatic hydrolysis as pre-processing prior to dehydration has been  
363 demonstrated as an appropriate technique to increasing of the protein digestibility of ingredients, leading to a better  
364 intestinal absorption. Subsequently, the stabilization process consisted of a first mechanical dehydration, to reduce as  
365 much as possible the humidity (less than 60 %), and a second thermal drying, to reduce moisture content below 10 %, has  
366 been demonstrated appropriate for brewers'-based ingredient productions. Mechanical dewatering involves less energy  
367 demand and, therefore, a reduction of the energy necessary for thermal drying in the second step.

368 Moreover, brewers' by-products stand as a potential alternative for replacing fish meal in aquaculture feed due to their  
369 availability in Europe (over 40 billion litres of beer were produced in the European Union (EU) in 2018 [4]), their  
370 nutritional characteristics (high protein content), and the protein digestibility results obtained in the trials with fishes  
371 which have shown acceptable digestibility coefficients in the case of gilthead seabream (*Sparus aurata*) used as a model  
372 for Mediterranean aquaculture.

373 Finally, proposed solution involves an increase of the sustainability of aquaculture by providing two new, economically  
374 advantageous, protein sources that could replace fish meal. Thus, the reduction of aquaculture production costs will  
375 contribute to achieve the objectives established by the new European Common Fisheries Policy. In addition, the  
376 replacement of marine origin ingredients (fishmeal) will contribute to reduce significantly wild catches, contributing to  
377 achieve the goals defined in the Marine Strategy Framework Directive.

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382 financing projects with European added value are among its main priorities.

383 All the brewers by-products samples that have been used in this study have been provided by Mahou San – Miguel  
384 company ([www.mahou-sanmiguel.com](http://www.mahou-sanmiguel.com)).

#### 385 References

- 386 [1] FAO: The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. FAO.  
387 <http://www.fao.org/3/i9540en/i9540en.pdf> (2018). Accessed 13 November 2019
- 388 [2] Bachis, E.: The Marine Ingredients Organization. Fishmeal and fish oil. A summary of global trends. 57<sup>th</sup> IFFO Annual  
389 Conference. [http://www.iffoevents.com/files/iffo/2.IFFO%20Washington%202017\\_1.pdf](http://www.iffoevents.com/files/iffo/2.IFFO%20Washington%202017_1.pdf) (2017). Accessed 13  
390 November 2019
- 391 [3] Samuel-Fitwi, B., Meyer, S., Reckmann, K., Schroeder, J.P., Schulz, C.: Aspiring for environmentally conscious  
392 aquafeed: comparative LCA of aquafeed manufacturing using different protein sources. J. Clean. Prod. 52, 225-233  
393 (2013)
- 394 [4] EUROSTAT: Happy International Beer Day! EUROSTAT. [https://ec.europa.eu/eurostat/web/products-eurostat-  
395 news/-/EDN-20190802-1](https://ec.europa.eu/eurostat/web/products-eurostat-news/-/EDN-20190802-1) (2019). Accessed 13 November 2019
- 396 [5] Rocha dos Santos, T., Fontes, V., Christe, M., Moretzsohn, P.P., Camporese, E.F.: Characterization and determination  
397 of brewer's solid wastes composition. J I Brewing (2015). <https://doi.org/10.1002/jib.229>
- 398 [6] Mussatto, S.: Brewer's spent grain: A valuable feedstock for industrial applications. J Sci Food Agric (2014).  
399 <https://doi.org/10.1002/jsfa.6486>
- 400 [7] Fărcaș, A.C., Socaci, A.S., Mudura, E., Dulf, F.V., Vodnar, D.C., Tofană, M., Salanță, L.C.: Exploitation of Brewing  
401 Industry Wastes to Produce Functional Ingredients. In: Kanauchi, M. (ed) Brewing technologies, pp. 137-156. Intech  
402 open (2017)
- 403 [8] Hellborg, L., Piscur, J.: Yeast diversity in the brewing industry. In: Preddy, V.R. (ed) Beer in Health and Disease  
404 Prevention, pp. 1068-1073. New York, US: Elsevier (2009)
- 405 [9] Djuragic, O., Levic, J., Serdanovic, S.: Use of new feed from brewery by-products for breeding layers. Rom Biotech  
406 Lett. 15, 5559–5565 (2010)



- 407 [10] Buffington, J.: The Economic Potential of Brewer's Spent Grain (BSG) as a Biomass Feedstock. *Advances in*  
408 *Chemical Engineering and Science* (2014). <http://dx.doi.org/10.4236/aces.2014.43034>
- 409 [11] Tang, Z., Cenkowski, S., Muir, W.E.: Modelling the superheated steam drying of a fixed bed of brewer's spent grain.  
410 *Biosyst Eng.* 87, 67-77 (2004). DOI: 10.1016/j.biosystemseng.2003.09.008
- 411 [12] Thiago, R., Pedro, P., Servulo, E.: Solid wastes in brewing process: A review. *J. Brew. Distilling* (2014).  
412 <https://doi.org/10.5897/JBD2014.0043>
- 413 [13] Kristinsson, H.G., Rasco, B.A.: Fish protein hydrolysates: Production, biochemical, and functional properties. *Crit.*  
414 *Rev. Food Sci. Nutr.* 40, 43-81 (2000)
- 415 [14] Friedman, M.: Nutritional Value of Proteins from Different Food Sources. A Review. *J. Agric. Food Chem.* 44, 6-  
416 29 (1996)
- 417 [15] Stone, D., Wilson, R., Wurtele, E.: Expanding the utilization of sustainable plant products in aquafeeds: A review.  
418 *Aquac. Res.* 38, 551-579 (2007)
- 419 [16] Ng, W.K., Gibon, V.: Fish oil replacement and alternative lipid sources in aquaculture feeds. In: Turchini, G.M., Ng,  
420 W.K., Tocher, D.R. (eds) *Palm oil and Saturated Fatty Acid Rick Vegetable Oils.*, pp. 99-132. CRC Press, Taylor &  
421 Francis Group (2012)
- 422 [17] Hou, H., Li, B., Zhao, X., Zhang, Z., Li, P.: Optimization of enzymatic hydrolysis of Alaska pollock frame for  
423 preparing protein hydrolysates with low-bitterness. *LWT - Food Sci. Technol.* (2011).  
424 <https://doi.org/10.1016/j.lwt.2010.09.009>
- 425 [18] Campos, I., Matos, E., Aragão, C., Pintado, M., Valente, L.: Apparent digestibility coefficients of processed agro-  
426 food by-products in European seabass (*Dicentrarchus labrax*) juveniles. *Aquacult Nutr.* 24 (4), 1274-1286 (2018)
- 427 [19] Ho, Y., Lin, C., Wu, M.C.: Evaluation of the prebiotic effects of citrus pectin hydrolysate. *J. Food Drug Anal.* 25,  
428 550-558 (2017)
- 429 [20] Hou, Y., Wu, Z., Dai, Z., Wang, G., y Wu, G.: Protein hydrolysates in animal nutrition: Industrial production,  
430 bioactive peptides, and functional significance. *J. Anim. Sci. Biotechnol.* 8, (2017)
- 431 [21] Nitrogen Determination by the Kjeldahl Method
- 432 [22] Laemmli, U.: Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature.* 227,  
433 680-685 (1970). <https://doi.org/10.1038/227680a0>
- 434 [23] High Performance Liquid Chromatography (HPLC)
- 435 [24] George W., Latimer, J.R.: *Official Methods of Analysis.* 17<sup>th</sup> edition. The Association of Official Analytical Chemists  
436 AOAC. Gaithersburg, Maryland, USA (2000)
- 437 [25] McCleary, B.V., Codd, R.: Measurement of (I-3) (I-4)- $\beta$ -D-glucan in barley and oats: A streamlined enzymic  
438 procedure. *J. Sci. Fd. Agric.* 55, 303-312 (1991)
- 439 [26] Btitikofer, U., Fuchs, D., Bosset, J.O., Gmtir, W.: Automated HPLC-Amino Acid Determination of Protein  
440 Hydrolysates by Precolumn Derivatization with OPA and FMOC and Comparison with Classical Ion Exchange  
441 Chromatography. *Chromatographia.* 31, 441-447 (1991)
- 442 [27] Garantun-Tjeldsto, O., Ottera, H., Julshamn, K. & Austrend, E.: Food ingestion in juvenile cod estimated by inert  
443 lanthanide markers –effects of food particle size. *ICES J MAR SCI.* 63, 311-319 (2006)
- 444 [28] Importance of savouriness in the acceptance of diets by fishes
- 445 [29] Sagar, V.R., Suresh Kumar, P.: Recent advances in drying and dehydration of fruits and vegetables: a review. *J Food*  
446 *Sci Technol.* 47, 15-26 (2009)
- 447 [30] Maynard, L.A., Loosli, J.K., Hintz, H.F., Warner, R.G.: *Animal nutrition.* New York, NY: McGraw-Hill (1979)
- 448 [31] National Research Council: *Nutrient Requirements of Fish and Shrimp.* Washington, DC. The National Academies  
449 Press (2011). <https://doi.org/10.17226/13039>
- 450 [Campos, I, Matos, E., Aragao, C., Pintado, M., Valente, L.M.P. Apparent digestibility coefficients of processed agro-](#)  
451 [food by-products in European sea bass \(\*Dicentrarchus labrax\*\) juveniles. \*Aquaculture Nutrition\*, 24, 1274-1286 \( 2018\).](#)
- 452 [Metailler, R., Huelvan, C. : Utilisation de levures dans l'alimentation du juvenile de bar \(\*Dicentrarchus labrax\*\). \*Fish\*](#)  
453 [Nutrition in Practice](#) pp945-948. INRA, Paris, France (1993)