Solid state fermentation of Brewer's spent grain using *Rhizopus* sp. to enhance nutritional value

Jone Ibarruri*[a], Marta Cebrián [a], Igor Hernández [a,b]

a: Food Research Division, AZTI. Parque Tecnológico de Bizkaia. Astondo Bidea, Edf.609, 48160 Derio, Bizkaia, Spain.

b: Present address: Universidad del País Vasco/ Euskal Herriko Unibertsitatea, Facultad de Farmacia, Paseo de la Facultad 7, 01006, Vitoria-Gasteiz. Spain.

*Corresponding author: E- mail address: *1 Tel.: +34 94 667 178 722 Fax: + 34 94 657 25 55.

Abstract

Purpose: Our objective was to enhance brewer's spent grain (BSG) nutritional value as feed or food ingredient by Solid State Fermentation (SSF) operational parameters optimization with selected fungal strains.

Methods: SSF conditions were optimized by factorial design and surface response analysis to maximize the value of resulting BSG derived biomass. Two *Rhizopus sp* strains were tested as inoculum (one wild and one mutant strain) and time and temperature were analyzed. Measured response variables include, among others, protein content, soluble protein, degree of hydrolysis, antioxidant activity, total phenolic content and antibacterial activity.

Results: Mutated strain resulted in higher total fungal biomass production and was used, with the wild type, for the model development. Highest protein concentration (31.5 %) and soluble protein (47.4 mg/g DM) were obtained when samples were fermented at 30 ºC for 9 days. Obtained biomass presented a modified amino acid profile resulting in an Essential Amino Acid Index (EAAI) of 1.58 and 1.28 compared to FAO human nutrition and animal feed standards, with high antioxidant capacity (63.8 % DPPH reduction) and 11 times higher total polyphenol content. Visual inspection confirmed BSG as very good candidate for complete fungal growth.

Conclusion: Hereby presented results demonstrated that SSF of BSG with selected fungal strains resulted in significant increase of highly appreciated characteristics for feed or food applications, among others, protein concentration, amino acid availability and antioxidant activity, which could lead to a promising valorization alternative.

Keywords: Brewer's spent grain, solid-state fermentation, *Rhizopus* sp., antioxidant capacity, protein enrichment

1 Introduction

Brewer's spent grain (BSG) is a by-product of the beer brewing industry which corresponds to approximately 85 % of total by-products generated during beer production [1]. Its composition can vary depending on the barley variety, harvesting time and malting and mashing conditions [2] but, in general, it is a fiber and protein rich material, around 70 and 20 % respectively, and with low proportion of lipids and ash, 3.9 and 3.4 % respectively. BSG is considered an unstable product and, up to now, no good preservation technologies have been developed [3]. Hence, several strategies have been studied as an alternative to overcome the environmental problems associated with BSG's disposal and/or combustion. The use of raw BSG has been studied in deep, including their uses in human nutrition [4], energy production [5], as a brick component [6], cellulose source [7] or adsorbent [8]. In most cases, not modified BSG has a low commercial value due to its high content of water (75-80 %) and low digestibility protein [9]. BSG utilization also include enzymatic recovery of carbohydrate fractions [10,11] and enzymatic production of antioxidant and antihypertensive compounds [12-14]. However, the use of commercial enzymes is costly. In this case, microbial fermentation to hydrolyze biomass could be a cost-effective alternative, and reports include the use of BSG for organic acids production [15], anaerobic digestion [16] and as substrate for microbial enzyme production [17,18].
Until now, the most used application has been as animal feed, mainly for ruminant due to its high proportion of fiber [1] and bioactive phenolic compounds. Animal feed producers are interested in high protein, high digestibility and "low-toxin" products, that which allow a correct nutritional balance in the recipe. Also, regular availability, low price and constant characteristics are appreciated. BSG can fulfill all these requirements, but higher commercial value would be obtained if protein and digestibility would be increased in a cost-effective process. Also, some biological activities are important for feed application, including antibacterial and antioxidant activities.

BSG, as other large volumes of food processing by-products, can be transformed from poor-nutrient wastes to animal feeds, food ingredients or enzymes when appropriate technologies are used for their valorization [19]. Production of industrially interesting compounds by microorganism can be performed employing different fermentation strategies such as Solid-State Fermentation (SSF) and Submerged Fermentation (SmF). In recent years, SSF has gained scientific and technological attention because it is considered an efficient process for production of enzymes and other compounds such as phenolics, vitamins and flavor compounds, among others [20,21]. Fungi, the main microorganisms used in SSF, are known to produce enzymes which degrade plant cell walls and could also improve biochemical composition and bioactivity of the employed substrates [22]. Fungal fermentation applied to food by-products improves digestibility and availability of proteins, preventing growth of pathogenic microorganisms and undesirable bacteria, through production of compounds with antibiotic activity [23].

One of the most promising fungal genus for SSF is *Rhizopus* sp. This genus includes several species used for enzyme production (glucoamylases, cellulases, tannases), organic acids production (lactic acid, fumaric acid) and animal feed preparation [24,25]. They are also the traditional inoculum for human food

production (tempeh, peka, ragi and loog-pang) [26]. Simple nutritional requirements, wide growth temperature interval (from 25 to 45 ºC), growth and survival pH range (at least, from 4.5 to 7.5) and board fermentative substrates make *Rhizopus* a good candidate to ferment agro-industrial by-products in order to obtain products with higher protein and vitamin contents [27]. *R. oryzae* is a well-known specie of *Rhizopus* genus. Generally recognized as safe (GRAS) by the FDA, it is employed industrially for its capacity to consume a great range of carbon sources [24], lipase production, protease production [28,29] and it has been considered for by-product valorization [30-32]. Using *Rhizopus oryzae* strain during SSF technology, our research group obtained fruit by-products enriched in free fatty acids, amino acid concentration and with improved amino acid profile [30]. Most critical parameters for highest biomass production (by-product, surface, Tª, time, and nitrogen) have been previously optimized in model substrate [30] and in some real substrates (fruit and vegetable by-products) but not optimized for feed application.

Along this paper, we describe parameters optimization for SSF process of BSG using *Rhziopus* sp. as fermentation agent as an improving strategy of nutritional composition, compound availability and bioactivity of the final product. Fermentation conditions were optimized with a rotatory design model for highest protein concentration, optimal degree of hydrolysis, high total phenolic content (TPC) and looking for antioxidant and antimicrobial activity in order to develop a sustainable process for BSG valorization that allows its use as a valuable ingredient for feed or food applications. Resultant biomass is richer in protein and TPC, shows higher antioxidant activity and degree of hydrolysis (DH) than the unfermented BSG and with a modified amino acid and fatty acid profile.

2 Materials and Methods

2.1 Microorganism, mutagens and culture media

One food-derived *Rhizopus* strain isolated and characterized in our laboratory (ROR004) [30] and a strain derived from ROR004 after mutagenesis experiments (ROR00G) were used as strains.

The wild type strain (ROR004) was treated with UV (Atom A/70 Nº557, Barcelona, Spain), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG, Tokyo chemical industry, Zwijndrecht, Belgium) and ethidium bromide (EB, 15585011 Fischer Scientific) at room temperature to induce mutagenesis. For treatment with UV, 10 ml of the spore suspension (4 Log cfu/ml) was plate on sterile petri dish (90 mm diameter) and exposed to UV irradiation (254 nm) for 60 and 90 min at a distance of 20 cm. For treatment with MNNG, 1 ml of the single spore suspension (4 log cfu/ml) was treated with 5 ml MNNG (0.1 mg/ml) for 30 and 50 minutes. Finally, for mutagenesis with EB, 7.4 ml of spore suspension (4 Log cfu/ml) was mixed with 600 µl of EB solution (1 mg/ml) for 90 or 100 minutes. After treatment with MNNG and EB,

spores were washed twice by centrifugation (1500 RCF, 5 minutes), supernatant elimination and pellet resuspension in 1 ml of sterile water. Finally, pellet was dissolved in 1 ml of sterile water. In all cases, final spore suspension was cultivated on PDA agar plates for 48 h at 20 ºC to obtain isolated colonies.

Potato Dextrose Agar (PDA, Oxoid CM0139, Basingstoke, Hampshire, England), Mueller Hilton Broth (MHB, Oxoid CM0405, Basingstoke, Hampshire, England) and Bacteriological Agar (Oxoid LP001, Basingstoke, Hampshire, England) were used for bacterial and fungal propagation and count. Buffered Peptone Water (Oxoid CM1049, Basingstoke, Hampshire, England) was used for sample dilution when required. All media were prepared as recommended by the producer and sterilized at 121 ºC for 15 min. The total microbial and fungal determination was done at 30 ºC for 48 h.

2.2 Brewer's spent grain (BSG)

BSG was provided by Boga Cooperative (an artisanal brewery sited in Mungia, Spain) as by-product after standard barley brewing process. Sample was frozen at -20 ºC until it was used. After defrozen (18 hours, 4 ºC), sample was sterilized at 110 ºC for 15 min and cool down up to room temperature in sterile conditions before inoculation. In all experiments, substrate density was maintained at 0.09 g/cm^2 and no extra nitrogen was added, as previously optimized [30].

2.3 Analytical determinations

2.3.1 Gross composition

Dry matter (DM) was calculated by drying the sample at 60 ºC for 24 hours, until constant weight. Crude protein content of the substrates was determined by Kjeldahl method [33]. Mycelia protein content for mutant strain characterization was determined by biuret method proposed by Satari et al. [34]. Briefly, 100 mg of sample was mixed with 3 mL of 1 M sodium hydroxide and boiled for 10 min. After immediate cooling in an ice bath, required dilutions were prepared with 1 M sodium hydroxide and $CuSO_4$ * $5H_2O$ (2.5 %) (Merck, Darmstadt, Germany) was added to the test tubes (25 % v/v final volume) and mixed gently for 5 minutes. The clear supernatant was collected after centrifugation and the absorbance was read at 555 nm. Different concentrations of bovine serum albumin (BSA, Sigma-Aldrich, Darmstadt, Germany) dissolved in 1 M sodium hydroxide were used as standards.

Total fat was determined as described previously [35]. Briefly, biomass (500-1000 mg) was mixed with 3 ml of methanol, 1.5 ml of chloroform and 1.2 ml of water and homogenized for 1 minute. After this time, 1.5 ml of chloroform and 1.2 ml of water were added, homogenized again and centrifuged (3800 RCF, 15 minutes). Lipids were determined in the organic phase. Fatty acid profile was determined as methyl esters by adding 5 ml of sodium methylate (0.2 %) to previously extracted lipid fraction and boiled for 10 minutes in a reflux system. Samples were cooled in ice, neutralized by adding HCL (5 %) in methanol and again boiled for 10 minutes, cooled in ice and mixed with 5 ml of n-hexane. Saturated NaCl was added and the organic phase analyze by GC-FID. Chromatograph was fitted with a DB-23 column of Agilent Technologies (60 m x 0.25 mm) and programed at 150 º C for 1 minute, a 5 ºC/min gradient up to

200 ºC, a second gradient of 2 ºC/min up to 230 ºC and 20 minutes at 230 ºC. Fatty acid methyl esters were identified by comparing the retention times with standards and were expressed as percentages of total fatty acids.

Amino acid profile was determined by HPLC as referenced before [30]. Protein quality was evaluated by the essential amino acid index (EAAI) as described before [36]. EAAI is based on the content of essential amino acids compared to a reference protein or specific requirements for human nutrition [37] or animal feeding [38], and it is used as a rapid method to evaluate and optimize the amino acid content of food and feed formulations. EAAI equation is described as follow:

$$EAAI = \sqrt[n]{\sum n \frac{mg\ of\ EAA\ in\ 1\ g\ of\ tested\ protein}{mg\ of\ EAA\ in\ 1\ g\ of\ reference\ protein}} \qquad \text{(eq. 1)}$$

n: the number of essential amino acids referenced

2.3.2 Degree of hydrolysis (DH)

The degree of hydrolysis (DH, %) was determined with the phthaldialdehyde (OPA) method [39]. OPA reagent (79765, Sigma Aldrich, Steinheim, Germany) was prepared as follow: 1,907 g Na tetraborate decahydrate (B9876, Sigma Aldrich, Steinheim, Germany) and 0.3 g Na-dodecyl-sulfate (SDS 99 %, 230421000 Fischer Scientific, New jersey, USA) were dissolved in 51.5 mL deionized water. 40 mg OPA was dissolved in 1 mL methanol and added. Finally, 200µl of β-mercaptoethanol (M6250 Sigma Aldrich, Steinheim, Germany) were added to the solution. 250 uM of Nα-Acetyl-L-lysine (J65083, Alfa Aesar) was prepared in deionized water and subsequent dilutions were used as standard. The sample solution was adjusted to 0.05 g protein/l and 60 µl of sample or standard were added to microplate wells. 180 µl of OPA reagent was added to each well and incubated at ambient temperature for 5 minutes. The measurement was done at 360 nm emission wavelength and 460 nm excitation wavelength. Degree of hydrolysis was calculated as follow:

$$DH\ (\%) = \frac{h}{htot} * 100\ \ (eq.2)$$

where *htot* are the total number of peptide bonds per protein equivalent, and *h* is the number of hydrolyzed bonds. As described by Nielsen et al. [39], for general porpoise, htot = 0.8 g eq/kg prot. To calculate *h*, described equation is:

$$h\ (meqv/g\ protein) = \frac{lysine\ NH2 - \beta}{\alpha} \quad (eq.3)$$

As recommended [39], when raw material has not been examined previously, then α and β are estimated to be 1.00 and 0.40, respectively and *Lysine-NH2* is:

$$Lysine\ NH2 = \frac{meqv\ lysine}{P} \qquad (eq.4)$$

where P is the protein (g/l) in the sample (0.05 g/l) and *meqv Lysine* are the lysine milliequivalents estimated in the sample using the standard curve.

2.3.3 Bioactive compounds extraction

Bioactive compounds were extracted using water as solvent. 10 g of sample were mixed with 25 ml of distilled water and the mixture was placed in a rotary shaker for 1 hour at 180 rpm 37 ºC in order to homogenize the flasks. Afterwards, the broth was centrifuged (22500 RCF, 10 minutes, 4 °C) and the supernatant, containing the water soluble metabolites was filtered through a 0.45 µm filter. Those extracts were used for soluble protein, DH, antioxidant activity, TPC, antibacterial activity and reducing sugars determination.

2.3.4 Antibacterial activity

The antibacterial activity of the extracts was determined by agar diffusion method [40]. Two bacterial species were used as test microorganisms, *Salmonella enterica* (CECT 4156) and *Escherichia coli* (CECT 516), and sub-cultured in Mueller-Hinton agar at 37 ºC for 24-48 hours.

Sensible strains were inoculated in a 10 ml of Mueller-Hilton broth until optical density of 0.5 (10^8 cfu/ml) and incubated at 30 ºC for 1 hour. 100 µl of bacterial suspension was added to 4 ml of Mueller Hilton soft agar (7 % bacteriological agar w/v) maintained at 48 ºC, vortexed and quickly cast in a Mueller Hilton petri plate. After solidification, the wells (5 mm diameter Whatman filter nº 1) were prepared and the extracts were added (10 µl) to the wells and incubated at 30 ºC 24-48 hours. Antibacterial activity was measured as the diameter of the clear zone of growth compared to a positive control, (tetracycline, 1 g/l), and a negative control, (sodium phosphate buffer, 0.01 M, pH 7.5).

2.3.5 Antioxidant activity (DPPH radical scavenging activity)

Antioxidant activity of samples was measured using the DPPH radical scavenging activity (DRSA) method. 40 ppm solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl, D9132 Sigma Aldrich, Steinheim, Germany) in methanol was prepared. 280 µl of this solution was added to 20 µl of sample solution. The mixture was incubated at room temperature in the dark for 30 min. Absorbance was measured at 515 nm. The standard comprised of water-methanol (50 % v/v) and different concentrations of Trolox (218940050, Acros Organics, New Jersey, USA). The antioxidant capacity was expressed as Trolox

equivalent capacity (TEAC), µg Trolox equivalents/g DM using the calibration curve. The percentage of reduction of DPPH compared to the blank also was calculated:

$$DPPH\ reduction\ (\%) = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} * 100 \quad (eq.5)$$

2.3.6 Total phenolic content

TPC was measured using the Folin–Ciocalteu method. Initially, 30 µl of Folin–Ciocalteu (J/4100/08, Fischer Scientific, Lougborough, UK) solution was added to 140 µl sample, blank or standard and 140 µl of Na_2CO_3 7 % (w/v). The mixture was incubated at room temperature in the dark for 1 hour and the absorbance was measured at 750 nm. Gallic acid (G7384, Sigma Aldrich, Steinheim, Germany) was used as standard at 1.4-20 ppm and results were expressed as mg of gallic acid equivalent (GAE) per g of DM sample.

2.3.7 Sugar determination

Total glucose was determined by Dinitrosalicylic (DNS) acid reagent method [41] adjusted to microplate assay procedure. Briefly, 25 µl of DNS reagent were mixed with 25 µl of sample, blank or standard (different concentrations of D-glucose) and incubated for 10 minutes at 100 ºC. The microplate was cooled in an ice bath and 250 µl of distilled water added to each well. Absorbance was read at 540 nm.

2.4 Solid State Fermentation (SSF)

All SSF experiments were carried out on 135 mm diameter sterile plate (surface area 143.1 cm^2) at 30 ºC. SSF process was done using ROR004 strain and all variables were monitored from time 0 to 192 hours (n=4 in all cases except for time 0 hours, where n=2).

Factorial design methodology was used for optimal conditions determination. Factorial design consisted on two continuous factors (time and temperature) and one category factor (fungal strain). Considered runs included 2^3 factorial design (8 runs) and central points (4 runs) to determine the best combination between incubation time and temperature and the effect of used strain (category factor) on several response parameters (crude protein, soluble protein, DH, TEAC, DPPH reduction and TPC). Overview of the complete design is described in Table 1 and Table 4.

Table 1 Coded variables screened for three levels (-, 0, +; factors A and B) or two levels (factor C)

Factor	Low (-)	0	High (+)
A: Time (h)	48	144	216
B: Temperature (ºC)	20	25	30

C: Strain	ROR004 (2)	ROR00G (1)

In all cases, substrate density was 0.09 g of dry fruit /cm^2 and 10% of spore suspension (10^7 cfu/ml) was added to the fermentation substrates. Spore suspension was prepared as described before [30].

2.5 Submerged Fermentation (SmF)

During mutant selection, SmF was used to control changes in biomass production. Growth medium includes (g/l): 20 glucose (G/0450/65, Fischer Scientific, Loughborough, UK), 0.75 nitrogen (Protease Peptone, Oxoid LP0085, Basingstoke, Hampshire, England), 3.0 KH_2PO_4, 0.5 $MgSO_4$ * $7H_2O$, 0.5 $FeSO_4$ and 0.5 KCl (all from Panreac Química SA, Barcelona, España). SmF was carried out on sterile 150 ml Erlenmeyer flasks (100 ml medium) at 170 rpm, incubated at 30 ºC for 72 hours in dark conditions. Fungal spore suspensions were prepared as described before [30], adjusted to 10^7 cfu/ml just before inoculation and used to inoculate the medium with 1 % (v/v). Obtained biomass was determined as described in 2.3

2.6 Statistical analysis

All the experiments and analyses were done, at least, duplicated and obtained results were analyzed using the software package Statgraphics Centurion XVI. Factors were considered significant when their probability (p-value) was less than 0.05 and were analyzed with one-way ANOVA (analysis of variance) models. Differences between fermented and unfermented BSG Amino acid and FA profile were determined by T-student statistics. When results were not adjusted to a normal distribution non-parametrical statistic were used, Kruskal-Wallis and Mann-Whitney U test. For factorial design analysis, the F test was used to analyze the statistical significance of proposed equations for each interesting parameter and the analysis of variance (ANOVA) for the response surface quadratic model.

3 Results and Discussion

3.1 Wild strain modification

Rhizopus ROR004 is a wild strain obtained from spoiled fruit samples. This strain presents good technological properties and has been described before [30] but looking for an increase in the growth rate and final biomass production, several procedures for genetical modifications were considered in this work. Treatment with UV resulted in 98.8 % of spore's mortality and similar mortality was obtained with MNNG and EB, 98.7 and 88.4 % respectively.

The success of our modifications was determined considering the growth rate of the obtained strains in PDA and comparing them with the wild strain. During initial selection round, strains with similar (or higher) growth rate than the wild strain were selected and isolated. These results were confirmed in successive re-growth steps in PDA. Finally, 4 strains presented consistently a higher growth rate than ROR004 and were selected for further selection.

Due the difficulty of fungal growth estimation in SSF, all possible mutants (4) were growth in SmF at optimal conditions (material and methods) and results were compared to wild type ROR004 [34]. After 72 hours, growth, biomass, protein, and glucose consumption were analyzed. ROR00G was the strain with significantly highest biomass production (g/l), biomass productivity (g DM/ g consumed glucose) and protein production (g/l) than original strain, (Table 2), and, therefore, was used together with ROR004 during SSF optimization.

Table 2 Comparison of SmF (30ºC, 72 h, 170 rpm) of wild and mutant strains

Strain	Consumed glucose (%)	Biomass (g DM/l)	Biomass productivity (g DM/g consumed glucose)	Protein (% DM)*	Protein production (g/l)
ROR004	88.1 ± 8.2^{a}	3.3 ± 0.2^{a}	0.19 ± 0.02^{a}	25.1 ± 9.6^{a}	0.8 ± 0.3^{a}
ROR00A	83.5 ± 9.5^{a}	3.51 ± 0.6^{a}	0.21 ± 0.02^{a}	24.6 ± 3.8^{a}	0.9 ± 0.2^{a}
ROR00B	97.1 ± 5.0^{a}	4.2 ± 0.6^{ab}	0.22 ± 0.04^{a}	28.5 ± 4.7^{a}	1.2 ± 0.1^{ab}
ROR00G	85.9 ± 4.8^{a}	5.1 ± 0.3^{b}	0.30 ± 0.03^{b}	28.7 ± 3.3^{a}	1.5 ± 0.3^{b}
ROR00J	93.1 ± 5.7^{a}	4.0 ± 0.2^{a}	0.21 ± 0.02^{a}	28.6 ± 2.3^{a}	1.1 ± 0.1^{ab}

Same letters in the same row mean no significant difference between the means at 95 % confidence; * Kruskall Wallis non-parametric test.

3.2 BSG fermentation with *Rhizopus sp.*

In order to determine the variables that change along the SSF, initial experiments were performed and several characteristics, like protein concentration, biological activities and technological properties, were analyzed.

The evolution of interesting characteristics in fermented BSG is very dependent on the considered incubation periods (up to 192 hours) (Fig 1-3). Clearly, SSF with *Rhizopus* sp. changes BSG composition and its TEAC, TPC and DH. Crude protein content increased up to 150 % of initial value, until a final concentration of 31.5 ± 3.7 % after 192 hours of incubation (Fig 1). Other authors have required urea supplementation to obtain similar crude protein (32.9 ± 0.6 % after 168 hours) on fermented BSG using *Rhizopus oligosporus* as fermentation agent [42] but our ROR004 seems to have lower nitrogen requirements (3.3 % in our samples compared with 3.9 % in the supplemented one, DM basis). In other nitrogen rich substrates, fermentation with *Rhizopus sp.* resulted in similar protein increase, such as rice bran (2.4 % nitrogen DM basis) [20,43] and nitrogen supplemented fruit wastes (1.6 % nitrogen DM basis) [30] or vegetable mixture (3.5 % nitrogen DM basis) using *Aspergillus niger* as fermentation agent [44]. When low nitrogen substrates are used, otherwise, protein increase is not quite significant [30].

Soluble protein is 6.5 times higher in fermented BSG compared to non-fermented one, probably due to high fungal protease activity (Fig 1). Other authors have reported lower increase (4 times) compared to the non-fermented BSG although total soluble protein content was higher [42], which depends on the extraction system used. Highest soluble protein value is obtained after 192 hours (29.5 ± 6.2 mg/g DM), while the highest increase was observed in initial 96 hours.

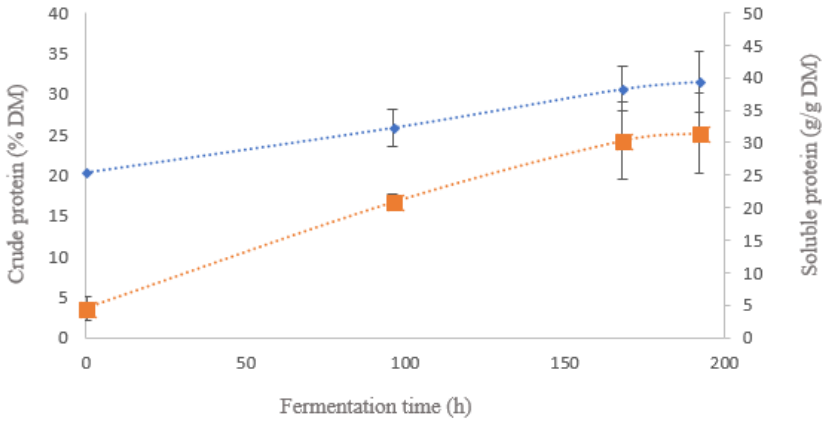


Fig. 1 Crude protein (% DM) ◆ and soluble protein (mg/g DM) ■ during SSF of BSG (from time 0 h to 196 h).

The DH (%) is significantly higher after 96 hours (59.0 ± 5.4 %) compared with other fermentation periods (Fig 2). This could be due to maximal peptides are released at that moment and then, as fermentation continued, those peptides and amino acids are used for fungal protein formation. In any case, fermentation increased dramatically the free amino acid content of the product, what could improve the protein digestibility in feed formulation.

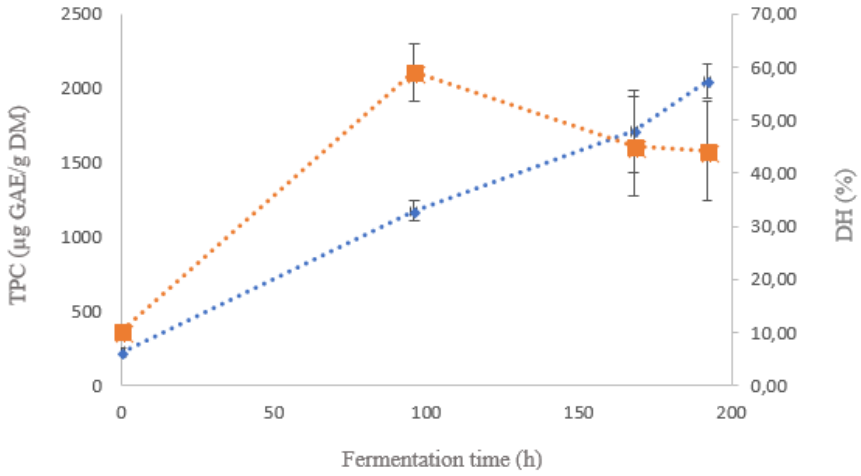


Fig. 2 Degree of hydrolysis (%)◆ and TPC (µg GAE g DM)■ during SSF of BSG (from time 0 h to 196 h).

TEAC and DPPH reduction also were significantly higher in the final extracts. The highest values are obtained at longest fermentation time, with a DPPH reduction of 51.8 ± 4.2 % and TEAC of 965 ± 84 µg/g DM (Fig 3). TPC also increases while fermentation time increases until a final value of 2043 ± 114 µg GAE/g DM (Fig 2) Results show significant lineal relation between TPC and TEAC ($p < 0.05$), with a regression coefficient of 0.984 and R^2 of 96.8. Buenrostro-Figueroa et al. [45] also found a positive correlation between antioxidant activity and TPC release during SSF of fig by-products. Our values show 9 times higher TPC in the final extracts compared to unfermented BSG (from 224 ± 30 to 2043 ± 114), higher than the values reported previously. This release could be related to carbohydrate-cleaving enzymes (β-glucosidase) activity of the fungi, which hydrolyze β-glucosidic linkages [46] and a release of free aglycones with potential high antioxidant activity. DPPH reduction, otherwise, was lower compared to those studies. TPC and antioxidant activity are closely related, using polyphenol containing diets in ruminants has been reported to have promising effects in animal well-being and could be a future strategy for producing meat and milk with antioxidant properties [47-49].

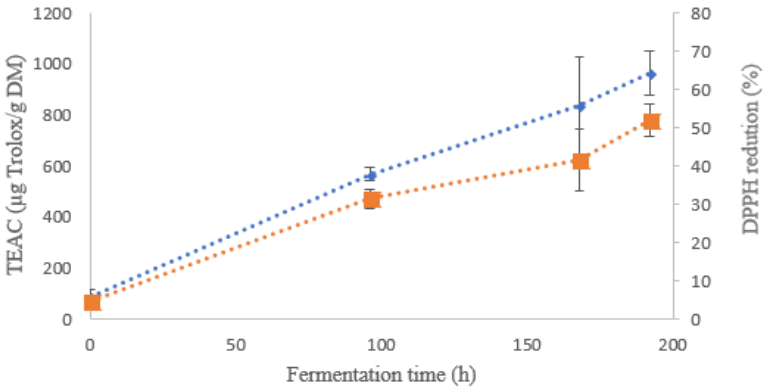


Fig. 3 TEAC (µg Trolox/g DM) ◆ and DPPH reduction (%) ■ during SFF (from time 0 h to time 196 h)

Antibacterial activity was not detected in the extracts obtained from fermented BSG, while positive inhibition was obtained with Tetracycline (1 mg/ml) for *Salmonella enterica* (1 mm) and for *Escherichia coli* (2 mm). Therefore, antibacterial activity was not taken into account for the optimization process. These agree with the results obtained by Almeida et al. [12], who reported a negative antibacterial activity of BSG extracts. Similar situation was observed with reducing sugars concentration (result not shown), that was discarded for further studies.

Finally, visual inspection of the obtained product (see Supplementary material with plates photographed during fermentation evolution) corroborated that BSG is an ideal substrate for SSF with filamentous

fungi, due to its composition and its particle size [9]. Small particles with rigid internal structure allow a complete fungal growth in short time period and a complete use of the inter-particle space [50]. This result in a homogenous fermented material and avoid the presence of under-fermented areas (usually the inner parts of the solids substrate).

3.3 Optimization of fermentation conditions

Statistical analysis of obtained results demonstrated that proposed models are significant for the response factors considered (Table 3). As overview, results demonstrated that equations resulting with proposed variables can predict positively protein proportion, soluble protein concentration, DH, TEAC, DPPH reduction and TPC. In all cases, p-values were lower than 0.05 (Table 3). The percentage of variation (R^2-value) that can be attributed to the independent variables indicates that those significant variables can explain in around 80-90 % of the final value (65.5 for total protein, 98.4 for soluble protein, 77.7 for DH, 78.4 for TEAC, 82.9 for DPPH reduction and 92.6 for TPC).

Model	Protein (% DM)	Soluble protein (mg/g DM)	DH (%)	TEAC (µg Trolox equivalent /g DM)	DPPH reduction (%)	TPC (µg GAE/ g DM)
Transformation	none	none	none	none	none	none
Model d.f.	3	4	4	4	4	4
P-value	0.0297*	0.0000*	0.0196*	0.0177*	0.0080*	0.0005*
Error d.f.	8	7	7	7	7	7
Stnd. Error	2.98977	2.50646	9.60358	131.34	9.49405	200.804
R2	65.4	96.8	77.7	78.4	82.9	92.6

Table 3 Statistical parameters (ANOVA) of the equations in each studied variable

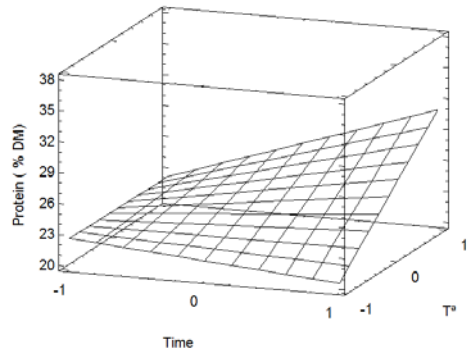
*Statistically significant ($p < 0.05$)

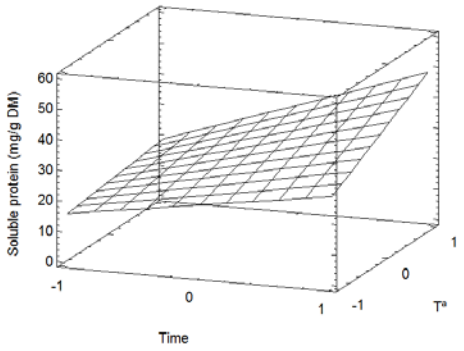
Protein production (% DM) is dependent of the temperature. Higher temperature leads to higher protein content and interaction between time and temperature is also significant. When fermentation time is lower there are no differences between samples, otherwise when fermentation time increases the interaction is significant and higher temperature increases protein content (Fig 4a). Fermented BSG is composed of 31.7 ± 7.6 % of protein (Table 4).

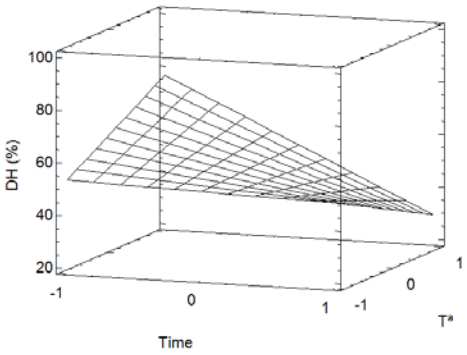
Soluble protein concentration significantly depends on time, temperature and their interaction (Fig 4b). While time increases, soluble protein increases, and high temperature and fermentation time have a significant effect. Our model describes the maximal soluble protein (47.4 ± 3.8 mg/g DM) after 9 days of fermentation (Table 4). Other authors [42] have reported a maximum soluble protein after 6 days of fermentation.

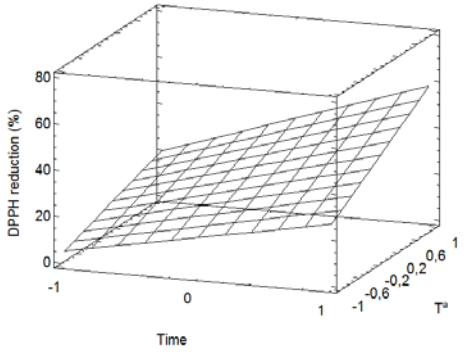
Significant parameters for DH are time and the interaction between time and temperature (Fig 4c). The DH is higher at middle fermentation time (96 hours). When time is lower, higher temperature increases the DH, but when fermentation time is longer, lower temperature leads to higher DH. As mentioned above, fungal metabolism is higher at 30 ºC and as fermentation time increases, this activity would result in amino acid assimilation for biomass production and as consequence a decrease in the DH of soluble proteins. However, DH is higher at any fermentation time compared to the unfermented BSG, what could improve the digestibility of the BSG protein.

TEAC and DPPH reduction depends on time and temperature (Fig 4d and 4e). Higher fermentation time and temperature provide the highest antioxidant activity and DPPH reduction, 795 ± 102 µg Trolox equivalents/g DM and 63.8 ± 1.9 % respectively (Table 4). There is no interaction between time and









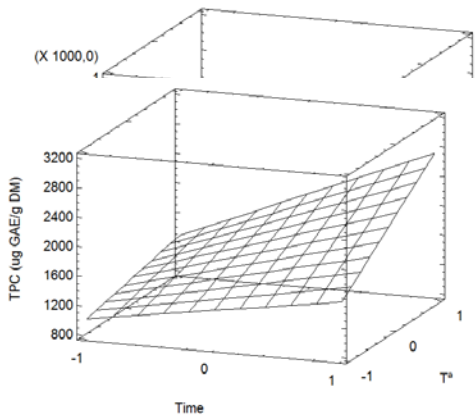


Fig. 4 SRM analysis of variables (a: SRM analysis for protein concentration; b: SRM analysis for soluble protein; c: SRM analysis for DH; d: SRM analysis for TEAC; e: SRM analysis for DPPH reduction; f: SRM analysis for TPC)

Table 4 Observed and adjusted results of factorial design during BSG fermentation

Time	Strain	T[a]	Protein (% DM)		Soluble protein (mg/g DM)		DH (%)		TEAC (µg Trolox equivalent/g DM)		DPPH reduction (%)		TPC (µg GAE/g DM)	
			Observed	Adjsuted	Observed	Adjusted	Observed	Adjusted	Observed	Adjusted	Observed	Adjusted	Observed	Adjusted
1	2	1	37.02	31.05	44.7	45.7	32.4	30.3	723	709	6.,5	59.1	2668	2564
-1	1	-1	23.21	22.50	14.2	15.5	55.5	53.2	138	97	9.6	4.6	1012	1005
0	2	0	22.87	24.07	21.8	25.1	55.4	53.0	255	355	20.0	28.1	1120	1475
0	1	0	23.26	24.07	26.2	28.0	54.4	52.6	227	392	16.5	27.5	1491	1646
-1	2	1	22.15	22.34	19.0	16.5	66.0	77.5	362	291	26.3	21.3	1263	1144
-1	2	-1	23.00	22.50	14.2	12.5	46.0	53.6	154	60	10.2	5.2	1020	834
1	1	-1	21.87	20.39	28.6	28.4	48.3	50.3	443	396	28.7	26.1	1671	1528
1	2	-1	20.12	20.39	25.8	25.5	47.3	50.7	446	359	34.2	26.8	1406	1357
1	1	1	26.30	31.05	50.0	48.6	22.5	29.9	867	746	65.1	58.5	2825	2736
0	1	0	23.65	24.07	31.5	28.0	51.7	52.6	282	392	19.2	27.5	1486	1646
-1	1	1	23.74	22.34	17.4	19.4	83.3	77.1	392	328	25.7	20.7	1391	1316
0	2	0	21.61	24.07	24.8	25.1	71.1	53.0	188	355	15.5	28.1	1372	1475

temperature. TPC also depends on time and temperature (Fig 4f). Higher fermentation time and temperature are the best combination for highest TPC (2747 ± 112 µg GAE/g DM). Temperature and time interaction is also a significant factor, while at lower fermentation periods temperature effect is lower, at higher fermentation time this effect is increased. Other authors have also reported a release of phenolic compounds and an increase of antioxidant activity during rice bran and plum fruit fermentation using *R. oryzae* and *R. oligosporus* as fermentation agents [20,51,52]. During the SSF processes, the production of enzymes (amylases, pectinases, xylanases, proteases, β-glucosidase, tannase, and ellagitannase) and their synergic activity would increase the TPC release, enhancing the TEAC of the obtained extracts.

Strain effect is not significant for any of the studied variables, what could be because mutagens did not have any significant effect on the protein synthesis, TPC release and other studied variables.

Optimal conditions for all variables, except for DH, are highest temperature (30 ºC) and longest fermentation time (216 hours) (Table 5). For DH, optimal conditions are high temperature but short fermentation time. Although there are not statistical differences between strains, protein concentration, DH and DPPH reduction are maximal using strain 2 (ROR004) and soluble protein, TEAC and TPC using strain 1 (ROR00G). As an overall strategy for BSG valorization, the attempt is to optimize all responses at a time, in that case the optimal conditions are 30 ºC, 216 h of fermentation time and strain 1. These conditions would lead to a fermented BSG with 31.1 % protein, 48.6 mg soluble protein/g DM, 29.9 % DH, 746 µg Trolox equivalent /g DM, 58.5 % DPPH reduction and 2736 µg GAE /g DM.

Table 5 Optimal conditions and maximal value for each variable

Factor	Protein (% DM)	Soluble protein (mg/g DM)	DH (%)	TEAC (µg Trolox /g DM)	DPPH reduction (%)	TPC (µg GAE/ g DM)
A: Time (h)	216	216	48	216	216	216
B: Temp. (ºC)	30	30	30	30	30	30
C: Strain	2	1	2	1	2	1
Desirability	0.65	0.96	0.91	0.85	0.9	0.95
Maximal	31.1	48.6	60.8	746	59.1	2736

As described in Table 1, Strain ROR00G is coded as strain 1 and strain ROR004 is coded as strain

3.4 Fermented BSG Amino acid and Fatty acid profile

The amino acid profile of the fermented BSG is dominated by Gln, Asn, Ala, Leu and Lys (Table 6), with high proportion of essential amino acids (up to 43.1 %).

Fermented BSG amino acid profile differs from the unfermented sample in few of the amino acids: an increase in Asn and Met proportion and a decrease of Gln percentage. Although the general profile does not differ significantly, the total amount of almost all amino acids significantly increases in the fermented BSG (Table 6). Compared to unfermented BSG, the fermented BSG Asn, Ser, Gln, His, Thr, Ala, Cys,

Val, Met, Lys, Ile, Leu, and Phe total content (g AA/100 g BSG) are significantly higher. Furthermore, overall

essential amino acid content in fermented BSG is 1.5 times higher than in the unfermented one. Amino acid composition of fermented BSG was evaluated for the essential amino acid index (EAAI). Higher EAAI indicates the presence of higher concentration of essential amino acids. EAAI for fermented BSG is 1.58 compared to human nutrition FAO standard [37] and 1.28 compared to animal feeding FAO standard [38], what means that AA fulfills both requirements. Other commercial protein sources such as soya protein has lower EAAI compared to FAO standards, casein otherwise has a higher EAAI [36].

Table 6 Amino acid profile (%) and total amino acid content (g /100g protein) of the unfermented and fermented BSG at optimal conditions (30 ºC, 216 h)

	AA profile		AA content	
	Unfermented BSG	Fermented BSG	Unfermented BSG	Fermented BSG
Essential AA				
His	2.9 ± 0.1^{a}	2.7 ± 0.5^{a}	0.6 ± 0.0^{a}	0.8 ± 0.1^{b}
Ile	3.7 ± 0.2^{a}	3.7 ± 0.2^{a}	0.7 ± 0.0^{a}	1.2 ± 0.1^{b}
Leu	8.2 ± 0.1^{a}	7.6 ± 0.3^{a}	1.7 ± 0.0^{a}	2.4 ± 0.1^{b}
Lys	6.6 ± 0.2^{a}	6.8 ± 0.4^{a}	1.3 ± 0.0^{a}	2.1 ± 0.1^{b}
Met	1.1 ± 0.0^{a}	1.6 ± 0.2^{b}	0.2 ± 0.0^{a}	0.5 ± 0.1^{b}
Cys	0.9 ± 0.2^{a}	1.3 ± 0.3^{a}	0.2 ± 0.1^{a}	0.4 ± 0.1^{b}
Phe	5.5 ± 0.0^{a}	4.7 ± 0.4^{a}	1.1 ± 0.0^{a}	1.5 ± 0.1^{b}
Tyr	3.7 ± 0.2^{a}	4.4 ± 1.1^{a}	0.7 ± 0.0^{a}	1.4 ± 0.3^{a}
Thr	4.0 ± 0.3^{a}	4.6 ± 0.3^{a}	0.8 ± 0.1^{a}	1.4 ± 0.1^{b}
Val	5.8 ± 0.2^{a}	5.9 ± 0.5^{a}	1.2 ± 0.0^{a}	1.8 ± 0.1^{b}
Total essential	42.4 ± 0.2^{a}	43.1 ± 2.3^{a}	8.6 ± 0.0^{a}	13.6 ± 0.7^{b}
EAAI FAO[1]	1.52 ± 0.03	1.58 ± 0.08		
EAAI FAO[2]	1.20 ± 0.02	1.28 ± 0.05		
Non essential				
Arg	6.4 ± 0.5^{a}	5.0 ± 0.7^{a}	1.3 ± 0.1^{a}	1.6 ± 0.2^{a}
Asn	5.7 ± 0.1^{a}	10.1 ± 0.5^{b}	1.2 ± 0.0^{a}	3.2 ± 0.2^{b}
Ser	5.0 ± 0.1^{a}	5.3 ± 0.3^{a}	1.0 ± 0.0^{a}	1.7 ± 0.1^{b}
Gln	20.6 ± 0.2^{a}	16.0 ± 0.8^{b}	4.2 ± 0.0^{a}	5.0 ± 0.3^{b}
Gly	4.3 ± 0.4^{a}	5.6 ± 0.7^{a}	0.9 ± 0.1^{a}	1.8 ± 0.2^{a}
Ala	6.7 ± 0.2^{a}	9.6 ± 1.7^{a}	1.4 ± 0.0^{a}	3.0 ± 0.5^{b}
Pro*	8.8 ± 0.0^{a}	5.4 ± 1.2^{a}	1.8 ± 0.0^{a}	1.7 ± 0.4^{a}
Total non essential	57.6 ± 0.2^{a}	56.9 ± 2.3^{a}	11.7 ± 0.0^{a}	17.9 ± 0.7^{b}

Same letters in the same row mean no significant difference between the means at 95 % confidence. *: W de Mann-Whitney non-parametric test. 1: [37] 2: [38]

The essential AA profile of fermented BSG is comparable to fishmeal and soybean meal [53] except for Met and Trp. Other essential amino acids otherwise, including Leu, Thr and Val, are in double proportion in the fermented BSG. Lysine is considered the most critical amino acid in aquatic feed and often the most limiting amino acid in feed ingredients [54]. The slightly higher lysine percentage obtained in fermented BSG AA profile (6.8 ± 0.4 %) compared to fishmeal and soybean meal (4.7 and 3.1 % respectively), makes it an interesting alternative protein source, what could contribute greatly towards protecting the surrounding environment and promoting a sustainable protein source for feed and food applications.

Fermentation modifies the fat fraction of the BSG and decreases significantly the total lipid content from 4.2 % in the not fermented samples to 0.7 % after complete fungal growth (Table 7). Other authors have reported also a decrease in total lipids of fermented rice bran [55] due fungal lipase activity [23]. For raw or fermented BSG, predominant FFA include palmitic (C16:0), oleic (C18:1n9) and linoleic (C18:2n6), but FFA profile have significant changes with fermentation. Fungal growth resulted in a significant increase of C16:0, C16:1, C18:0, C18:3n6, C20:0, C201n9, C22:0 and C24:0, leading to a significant increase in the SFA proportion and a tendency of MUFA accumulation. C:182n6 decreases significantly in the fermented BSG leading to an overall decrease of PUFA proportion. C18:3n6, a characteristic FA of *Rhizopus* sp. biomass [55] is only detected in fermented BSG as expected.

Table 7 Fatty Acid profile (% of total fatty acids) of the unfermented and fermented BSG at optimal conditions (30 ºC, 216 h)

	Unfermented BSG	Fermented BSG
12:00	ND	ND
14:0	0.6 (0.5-0.7)[a]	0.5 (0.5-0.6)[a]
14:1	ND	ND
15:0	ND	ND
15:1	ND	ND
16:0	25.9 (25.5-26.4)[a]	30.8 (30.5-31.6)[b]
16:1	0.3 (0.3-0.3)[a]	0.4 (0.4-0.6)[b]
17:0	ND	ND
17:1	ND	ND
18:0	2.2 (2.0-2.4)[a]	6.2 (5.0-7.2)[b]
18:1n9	12.1 (11.8-12.2)[a]	18.7 (17.9-21.5)[a]
18.2n-6	57.1 (56.6-57.8)[a]	34.6 (27.9-36.8)[b]
18:3n-6	ND[a]	2.0 (0.9-3.4)[b]
18:3n-3	ND	ND
18:4n-3	ND	ND
20:0	0.3 (0.3-0.3)[a]	1.3 (1.2-1.5)[b]
20:1n-9	0.8 (0.7-0.9)[a]	2.3 (2.2-2.7)[b]
22:0	0.4 (0.4-0.4)[a]	1.5 (1.3-1.7)[b]
20:5n-3	ND	ND
22:1	ND	ND
24:0	0.3 (0.3-0.3)[a]	2.9 (2.0-3.1)[b]

22:5n-3	ND	ND
22:6n-3	ND	ND
SFA	29.7 (29.0-30.5)[a]	43.4 (40.8-45.9)[b]
MUFA	13.3 (12.8-13.3)[a]	21.3 (20.5-24.5)[a]
PUFA	57.1 (56.6-57.8)[a]	36.8 (30.6-38.0)[b]
Total lipids	4.1 (3.9-4.4)[a]	0.7 (0.5-0.8)[b]

Same letters in the same line mean no significant differences between the medians (Q1-Q3) at 95 % confidence determined by W de Mann-Whitney no parametric test. ND: not detected

Biomass composition is a critical aspect for using fungi biomass protein (FBP) for animal feeding or human consumption purposes. Using filamentous fungi as bio-fermentation of BSG leads to a protein rich substrate with higher essential amino acid content and hydrolysis degree. SSF also increases the antioxidant activity of the fermented BSG related to the release of phenolic compounds, what makes SSF a promising alternative to revalorize this agro-industrial by-product as ingredient for feed and food applications. Furthermore, BSG is also known for containing peptides related to antihypertensive activity [13], for its beneficial nutritional composition (high fiber, protein and β-glucans content) [3] and for specific phenolic compounds (ferulic, caffeic and p-coumaric acid) related to anti-cancer, anti-atherogenic and anti-inflammatory effects [56]. Further research is needed to evaluate the effect of SSF by *Rhizopus* sp. in the liberation of those specific peptides and phenolic compounds.

Conclusion

Hereby fungal SSF is a successful strategy for BSG valorization for production of a valuable, sustainable, non-climate dependent protein source for animal feed or human food formulations. Comparing with original BSG, fermented product has higher total protein content, degree of hydrolysis and total soluble protein. Furthermore, TPC of final products is 11 times higher leading to an increase of its TEAC.

Aknowlegment

Authors thank to Boga for providing the BSG. This work was funded by the Basque Government (Department of the Environment, Regional Planning, Agriculture and Fisheries).

References

1. Mussatto, S.I., Dragone, G., Roberto, I.C.: Brewers' spent grain: generation, characteristics and potential applications. Journal of Cereal Science **43**(1), 1-14 (2006).
2. Santos, M., Jiménez, J.J., Bartolomé, B., Gómez-Cordovés, C., del Nozal, M.J.: Variability of brewer's spent grain within a brewery. Food Chemistry **80**(1), 17-21 (2003).
3. Ikram, S., Huang, L.Y., Zhang, H.J., Wang, J., Yin, M.: Composition and nutrient value proposition of brewers spent grain. J. Food Sci. **82**(10), 2232-2242 (2017).
4. Ozturk, S., Ozboy, O., Cavidoglu, I., Koksel, H.: Effects of brewer's spent grain on the quality and dietary fibre content of cookies. J. Inst. Brew. **108**(1), 23-27 (2002).
5. Weger, A., Jung, R., Stenzel, F., Hornung, A.: Optimized energetic usage of brewers ' spent grains. Chemical Engineering & Technology **40**(2), 306-312 (2017).
6. Russ, W., Mörtel, H., Meyer-Pittroff, R.: Application of spent grains to increase porosity in bricks. Construction and Building Materials **19**(2), 117-126 (2005).
7. Mishra, P.K., Gregor, T., Wimmer, R.: Utilising brewer's spent grain as a source of cellulose nanofibres following separation of protein-based biomass. BioResources **12**(1), 107-116 (2017).
8. Chiang, P.C., Chang, P., You, J.H.: Innovative technology for controlling voc emissions. J. Hazard. Mater. **31**(1), 19-28 (1992).
9. Xiros, C., Christakopoulos, P.: Biotechnological potential of brewers spent grain and its recent applications. Waste and Biomass Valorization **3**(2), 213-232 (2012).
10. Carvalheiro, F., Esteves, M.P., Parajó, J.C., Pereira, H., Gírio, F.M.: Production of oligosaccharides by autohydrolysis of brewery's spent grain. Bioresource Technology **91**(1), 93-100 (2004).
11. Steiner, J., Procopio, S., Becker, T.: Brewer's spent grain: source of value-added polysaccharides for the food industry in reference to the health claims. European Food Research and Technology **241**(3), 303-315 (2015).
12. Almeida, A.D., Geraldo, M.R.F., Ribeiro, L.F., Silva, M.V., Maciel, M., Haminiuk, C.W.I.: Bioactive compounds from brewer's spent grain: phenolic compounds, fatty acids and in vitro antioxidant capacity. Acta Sci.-Technol. **39**(3), 269-277 (2017).
13. Connolly, A., O'Keeffe, M.B., Piggott, C.O., Nongonierma, A.B., FitzGerald, R.J.: Generation and identification of angiotensin converting enzyme (ACE) inhibitory peptides from a brewers' spent grain protein isolate. Food Chemistry **176**, 64-71 (2015).
14. Vieira, E., Teixeira, J., Ferreira, I.: Valorization of brewers' spent grain and spent yeast through protein hydrolysates with antioxidant properties. European Food Research and Technology **242**(11), 1975-1984 (2016).
15. Radosavljevic, M., Pejin, J., Kocic-Tanackov, S., Mladenovic, D., Djukic-Vukovic, A., Mojovic, L.: Brewers' spent grain and thin stillage as raw materials in l-(+)-lactic acid fermentation. J. Inst. Brew. **124**(1), 23-30 (2018).
16. Sezun, M., Grilc, V., Zupancic, G.D., Logar, R.M.: Anaerobic digestion of brewery spent grain in a semi-continuous bioreactor: Inhibition by phenolic degradation products. Acta Chimica Slovenica **58**(1), 158-166 (2011).
17. Gregori, A., Švagelj, M., Pahor, B., Berovič, M., Pohleven, F.: The use of spent brewery grains for *Pleurotus ostreatus* cultivation and enzyme production. New biotechnology **25**(2), 157-161 (2008).
18. Sandhya, C., Sumantha, A., Szakacs, G., Pandey, A.: Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. Process Biochemistry **40**(8), 2689-2694 (2005).
19. Nigam, P., Pandey, A.: Biotechnology for agro-industrial residues utilization. Springer (2009).
20. Kupski, L., Cipolatti, E., da Rocha, M., Oliveira, M.D., Souza-Soares, L.D., Badiale-Furlong, E.: Solid-state fermentation for the enrichment and extraction of proteins and antioxidant compounds in rice bran by *Rhizopus oryzae*. Braz. Arch. Biol. Technol. **55**(6), 937-942 (2012).
21. Lizardi-Jimenez, M.A., Hernandez-Martinez, R.: Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. 3 Biotech **7**(1), 44 (2017).
22. Abd Razak, D.L., Abd Rashid, N.Y., Jamaluddin, A., Sharifudin, S.A., Abd Kahar, A., Long, K.: Cosmeceutical potentials and bioactive compounds of rice bran fermented with single and mix culture of *Aspergillus oryzae* and *Rhizopus oryzae*. Journal of the Saudi Society of Agricultural Sciences **16**(2), 127-134 (2017).
23. Cooray, S.T., Chen, W.N.: Valorization of brewer's spent grain using fungi solid-state fermentation to enhance nutritional value. Journal of Functional Foods **42**, 85-94 (2018).

24. Ghosh, B., Ray, R.R.: Current commercial perspective of *Rhizopus oryzae*: a review. Journal of Applied Science **11**(14), 2470-2486 (2011).
25. Meussen, B.J., de Graaff, L.H., Sanders, J.P., Weusthuis, R.A.: Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals. Appl Microbiol Biotechnol **94**(4), 875-886 (2012).
26. Cantabrana, I., Perise, R., Hernández, I.: Uses of *Rhizopus oryzae* in the kitchen. International Journal of Gastronomy and Food Science **2**(2), 103-111 (2015).
27. Villas-Boas, S.G., Esposito, E., Mitchell, D.A.: Microbial conversion of lignocellulosic residues for production of animal feeds. Animal Feed Science and Technology **98**(1-2), 1-12 (2002).
28. Lopez, E., Deive, F.J., Longo, M.A., Sanroman, M.A.: Strategies for utilisation of food-processing wastes to produce lipases in solid-state cultures of *Rhizopus oryzae*. Bioprocess Biosyst Eng **33**(8), 929-935 (2010).
29. Hsiao, N.-W., Chen, Y., Kuan, Y.-C., Lee, Y.-C., Lee, S.-K., Chan, H.-H., Kao, C.-H.: Purification and characterization of an aspartic protease from the *Rhizopus oryzae* protease extract, Peptidase R. Electronic Journal of Biotechnology **17**(2), 89-94 (2014).
30. Ibarruri, J., Hernández, I.: *Rhizopus oryzae* as fermentation agent in food derived sub-products. Waste and Biomass Valorization (2017). doi:10.1007/s12649-017-0017-8
31. Ferreira, J.A., Lennartsson, P.R., Niklasson, C., Lundin, M., Edebo, L., Taherzadeh, M.J.: Spent sulphite liquor for cultivation of an edible *Rhizopus* sp. BioResources **7**(1), 173-188 (2012).
32. FazeliNejad, S., Ferreira, J.A., Brandberg, T., Lennartsson, P.R., Taherzadeh, M.J.: Fungal protein and ethanol from lignocelluloses using *Rhizopus* pellets under simultaneous saccharification, filtration and fermentation (SSFF). Biofuel Research Journal **3**(1), 372-378 (2016).
33. Análisis de alimentos: métodos oficiales y recomendados por el Centro de Investigación y Control de la Calidad. Ministerio de Sanidad y Consumo, (1985).
34. Satari, B., Karimi, K., Taherzadeh, M.J., Zamani, A.: Co-production of fungal biomass derived constituents and ethanol from citrus wastes free sugars without auxiliary nutrients in airlift bioreactor. International journal of molecular sciences **17**(3), 302 (2016).
35. Bligh, E.G., Dyer, W.J.: A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37**(8), 911-917 (1959).
36. Waghmare, A.G., Salve, M.K., LeBlanc, J.G., Arya, S.S.: Concentration and characterization of microalgae proteins from *Chlorella pyrenoidosa*. Bioresources and Bioprocessing **3**(1) (2016).
37. FAO/WHO: Protein and amino acid requirements in human nutrition. Expert Consultation, vol. 935. WHO Technical Report Series, pp. 1-7. World Health Organization, Geneva, (2007).
38. FAO/WHO: Nutritional and safety aspects of novel sources of protein for animal feeding. Protein advisory group guidelines no. 15. World Health Organization, Rome, (1974).
39. Nielsen, P.M., Petersen, D., Dambmann, C.: Improved method for determining food protein degree of hydrolysis. J. Food Sci. **66**(5), 642-646 (2001).
40. Bougherra, F., Dilmi-Bouras, A., Balti, R., Przybylski, R., Adoui, F., Elhameur, H., Chevalier, M., Flahaut, C., Dhulster, P., Naima, N.: Antibacterial activity of new peptide from bovine casein hydrolyzed by a serine metalloprotease of *Lactococcus lactis* subsp *lactis* BR16. Journal of Functional Foods **32** (Supplement C), 112-122 (2017).
41. Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry **31**(3), 426-428 (1959
42. Canedo, M.S., de Paula, F.G., da Silva, F.A., Vendruscolo, F.: Protein enrichment of brewery spent grain from *Rhizopus oligospor*us by solid-state fermentation. Bioprocess and Biosystems Engineering **39**(7), 1105-1113 (2016).
43. Oliveira, M.d.S., Feddern, V., Kupski, L., Cipolatti, E.P., Badiale-Furlong, E., de Souza-Soares, L.A.: Physico-chemical characterization of fermented rice bran biomass Caracterización fisico-química de la biomasa del salvado de arroz fermentado. CyTA - Journal of Food **8**(3), 229-236 (2010).
44. Rajesh, N., Imelda, J., Raj, R.P.: Value addition of vegetable wastes by solid-state fermentation using *Aspergillus niger* for use in aquafeed industry. Waste Manag **30**(11), 2223-2227 (2010).
45. Buenrostro-Figueroa, J.J., Velázquez, M., Flores-Ortega, O., Ascacio-Valdés, J.A., Huerta-Ochoa, S., Aguilar, C.N., Prado-Barragán, L.A.: Solid state fermentation of fig (*Ficus carica* L.) by-products using fungi to obtain phenolic compounds with antioxidant activity and qualitative evaluation of phenolics obtained. Process Biochemistry **62**, 16-23 (2017).
46. Ajila, C.M., Gassara, F., Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R.: Polyphenolic antioxidant mobilization in apple pomace by different methods of solid-state fermentation and evaluation of its antioxidant activity. Food and Bioprocess Technology **5**(7), 2697-2707 (2012).

47. Fruet, A.P.B., Stefanello, F.S., Rosado Júnior, A.G., Souza, A.N.M.d., Tonetto, C.J., Nörnberg, J.L.: Whole grains in the finishing of culled ewes in pasture or feedlot: Performance, carcass characteristics and meat quality. Meat Science **113**, 97-103 (2016).
48. Paraskevakis, N.: Effects of dietary dried Greek Oregano (*Origanum vulgare* ssp. *hirtum*) supplementation on blood and milk enzymatic antioxidant indices, on milk total antioxidant capacity and on productivity in goats. Animal Feed Science and Technology **209**, 90-97 (2015).
49. Castillo, C., Pereira, V., Abuelo, A., Hernandez, J.: Effect of supplementation with antioxidants on the quality of bovine milk and meat production. TheScientificWorldJournal **2013**, 616098 (2013).
50. Wang, D., Sakoda, A., Suzuki, M.: Biological efficiency and nutritional value of *Pleurotus ostreatus* cultivated on spent beer grain. Bioresource Technology **78**(3), 293-300 (2001).
51. Dulf, F.V., Vodnar, D.C., Socaciu, C.: Effects of solid-state fermentation with two filamentous fungi on the total phenolic contents, flavonoids, antioxidant activities and lipid fractions of plum fruit (*Prunus domestica* L.) by-products. Food Chem **209**, 27-36 (2016).
52. Correia, R.T.P., McCue, P., Magalhães, M.M.A., Macêdo, G.R., Shetty, K.: Production of phenolic antioxidants by the solid-state bioconversion of pineapple waste mixed with soy flour using *Rhizopus oligosporus*. Process Biochemistry **39**(12), 2167-2172 (2004).
53. Miles, R.D., Chapman, F.A.: The benefits of fish meal in aquaculture diets. In. Institute of Food and Agricultural Sciences, University of Florida, Florida, (2006).
54. Nitayavardhana, S., Issarapayup, K., Pavasant, P., Khanal, S.K.: Production of protein-rich fungal biomass in an airlift bioreactor using vinasse as substrate. Bioresource Technology **133**, 301-306 (2013).
55. Oliveira, M.D., Feddern, V., Kupski, L., Cipolatti, E.P., Badiale-Furlong, E., de Souza-Soares, L.A.: Changes in lipid, fatty acids and phospholipids composition of whole rice bran after solid-state fungal fermentation. Bioresource Technology **102**(17), 8335-8338 (2011).
56. McCarthy, A.L., O'Callaghan, Y.C., Piggott, C.O., FitzGerald, R.J., O'Brien, N.M.: Brewers' spent grain; bioactivity of phenolic component, its role in animal nutrition and potential for incorporation in functional foods: a review. The Proceedings of the Nutrition Society **72**(1), 117-125 (2013).