

# Upcycling of brewers' spent grains via solid-state fermentation for the production of protein hydrolysates with antioxidant and techno-functional properties

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## ABSTRACT

Brewers' spent grains (BSG) were fermented with *Rhizopus oligosporus* and up to 15% of original protein was hydrolysed. Fermented BSG was then subjected to an ethanolic-alkali extraction and isolated fractions contained 61–66% protein. An evaluation of functional properties suggested that fermented extracts presented superior emulsifying abilities (15–34 m<sup>2</sup>/g of activity and 16–42 min of stability), foaming properties (16–30% capacity and 7–14% stability), and water/oil binding capacities (0.41 g/g and 0.24 g/g, respectively). They also showed significantly higher ABTS inhibition and stronger reducing power than unfermented ones, indicating that fermented BSG protein extract had greater antioxidant activities. No cytotoxic effect was detected in the range of 2–10 mg/mL. When applied in a mayonnaise formulation, fermented hydrolysates demonstrated better emulsion stability in terms of creaming, microstructure and viscosity. Thus, fermented BSG protein is a potential plant-based emulsifier for food, pharmaceutical and cosmetic applications.

## Introduction

In the brewing industry, a large quantity of waste is produced, of which 85% of the total waste comes from brewers' spent grains (BSG). Annually, an estimated 39 million tons of BSG is generated worldwide (Macias-Garbett et al., 2021). Given the difficulty in preserving these residues due to its high moisture content and complex composition, excess BSG is likely to be disposed of or used as cattle feed. However, due to its abundance of fibre and protein which make up 30–50% and 19–30%, respectively, as well as phenolic compounds such as ferulic acid and p-coumaric acid (Macias-Garbett et al., 2021), recent studies have attempted to valorise BSG for various applications. Some examples include using BSG as a construction material, a solid fuel and for human nutrition as a flour, pasta or bread, amongst others (Jackowski et al., 2020).

Currently, the limited use of BSG proteins in food processing is largely due to the difficulty in extracting proteins in BSG. The lack of solubility of BSG proteins is associated to the entrapment of proteins within the complex carbohydrate structure (Connolly et al., 2019) and the aggregate formation between proteins caused by the mashing

process (Celus et al., 2006). Several approaches have been employed to improve the utility of the BSG protein fraction. One method involves alkaline extraction followed by isoelectric precipitation of the extracted proteins (Celus et al., 2007; Connolly et al., 2013; Vieira et al., 2014). This method has proven effective for solubilizing BSG proteins. However, when using alkalis for protein extraction, hemicellulose and lignin in BSG are also alkali-soluble (Rommi et al., 2018), therefore the isolation of pure BSG protein would be more challenging. Furthermore, using an alkali for extraction implies that the solvent cannot be reused unlike an organic solvent with boiling point which can be recovered by distillation. Previous work by Celus et al. (2006) and Wang et al. (2010) have indicated the potential of using an aqueous alcohol mixture for hordein isolation from BSG and barley. A reducing agent may be added to overcome disulfide bonds present in the aggregates. In this way, the environmental impact of extraction can be minimised. Another solution to solubilise the protein fractions is by using commercial peptidases and obtain protein hydrolysates with bioactive and techno-functional properties, which can be used in food and other applications (Celus et al., 2007; Connolly et al., 2019). Even though enzymes are highly specific and only require mild conditions for protein solubilization, the

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use of proprietary enzymes can be costly, especially in large-scale processing. A cheaper alternative is to utilise microbial fermentation by fungal species such as *Rhizopus oligosporus* and *Aspergillus* species as a biological pre-treatment of BSG. These microorganisms have the ability to secrete extracellular enzymes and degrade the waste material (Bekatorou et al., 2007; Canedo et al., 2016; Cooray & Chen, 2018). The added benefit of solid-state fermentation with fungi is the enrichment of protein in the form of fungal biomass. Therefore, other approaches for protein solubilisation, such as a combination of alkali and enzymatic methods, should be explored.

Although there has been research conducted to valorise BSG through biotransformation, these studies mainly utilise BSG as a substrate for microbial production of enzymes or metabolites of industrial interest (Bianco et al., 2020). Other works focused on utilising the whole fermented product as a functional food for humans or as an enriched animal feed (Canedo et al., 2016; Cooray & Chen, 2018) and neglected the potential of fractionating useful components in fermented BSG for further applications in food. To the best of our knowledge, no work has been performed to compare the proteins from fermented and unfermented BSG in terms of their antioxidative and functional properties. Therefore, the objective of this study was to investigate the combined use of microbial fermentation with an aqueous alcohol mixture for protein isolation and to evaluate the effects of fermentation on the antioxidative and functional properties of BSG protein. In addition, the extracted proteins were applied in a food emulsion system to assess its suitability as a plant-based emulsifier. Results from the current study would provide insights into the direct valorisation of BSG proteins achieved through fermentation.

## Materials and methods

### Materials

BSG (76.8% moisture, 25.2% protein on a dry weight basis) was obtained from Asia Pacific Breweries (Singapore) Pte. Ltd. and stored in polyethylene bags at  $-80^{\circ}\text{C}$ . The BSG was thawed at room temperature and autoclaved (Hirayama HG-80, Japan) prior to fermentation.

### Fermentation of BSG

The fermentation of BSG was adopted from the study described by Cooray & Chen (2018). BSG was fermented with *Rhizopus oligosporus* ATCC 64,063 maintained on the plates of Potato Dextrose Agar (PDA). A BSG:inoculum ratio of 10:1 (w/v) and an inoculum concentration of  $10^7$  spores/mL was used and incubated at  $37^{\circ}\text{C}$  for three days. Both fermented and unfermented BSG were freeze-dried, grinded into a powder and passed through a sieve size of 400  $\mu\text{m}$ .

### Extraction of BSG proteins

BSG and fermented BSG (FBSG) were extracted for proteins using an ethanolic-alkali mixture comprising of ethanol and sodium metabisulfite, alkali-adjusted to pH 9. BSG and FBSG were placed in a shaking water bath at  $60^{\circ}\text{C}$  for 2 h. After extraction, centrifugation (5000 rpm, 10 min) was used to remove solids (Thermo Fisher, USA). The protein-containing supernatant was isoelectrically precipitated using hydrochloric acid and ethanol was removed using a rotary evaporator (Heidolph, Germany). Precipitated proteins were centrifuged and the pellet was washed twice with distilled water before freeze-drying. Reagents used were purchased from Sigma-Aldrich (St. Louis MO, USA). Protein content was estimated from the nitrogen content in dried extracts using a Elementar Vario EL III Elemental Analyser and calculated by using the 5.83\*N ratio.

## Characterisation of protein extracts

### Amino acid profile

The amino acids present in the extract were determined according to Pérez-Palacios et al. (2015) with some modifications. Protein extracts were hydrolysed using 6 M HCl at  $105^{\circ}\text{C}$  for 12–24 h and deproteinised by acetonitrile, before derivatisation using *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCI) and then analysed by a gas chromatography equipment (7890A, Agilent Technologies) coupled to a mass spectrometer (5975C inert MSD with Triple Axis Detector, Agilent Technologies) equipped with a HP-5MS capillary column. Amino acid standard (AAS18) from Sigma-Aldrich was used for quantitation.

### Degree of protein hydrolysis

The degree of hydrolysis (DH) was quantified using the trinitrobenzenesulphonic acid (TNBS) method described by Adler-Nissen (1979). Briefly, 5% (w/v) TNBS (Thermo Scientific) was diluted to 0.1% (w/v) with water. Protein extracts and standard solutions were solubilised in 1% SDS. The TNBS reaction was carried out by adding 0.25 mL of sample or test solutions to 2.0 mL of sodium phosphate buffer (0.2125 M, pH 8.2), followed by addition of 2.0 mL of 0.1% (w/v) TNBS reagent. The tubes were shaken and incubated at  $50^{\circ}\text{C}$  for 60 min in a covered water bath. After 60 min, 4.0 mL of 0.1 N HCl is added to terminate the reaction and cooled at room temperature before reading absorbance at 340 nm (Varioskan LUX, Thermo Fisher Scientific). A calibration curve using *L*-leucine (0–2.0 mM) in 1% SDS was constructed for quantitation. DH was calculated using:  $\text{DH}\% = 100\text{AN}_2 - 100\text{AN}_1\text{Npb}$ , where  $\text{AN}_1$  and  $\text{AN}_2$  refer to the amino nitrogen content of the BSG protein before and after hydrolysis (mg/g protein) respectively, and Npb the nitrogen content of the peptide bonds in the BSG protein (mg/g protein). A value of 112.1 was used as the Npb for BSG protein (Connolly et al., 2014).

### Gel electrophoresis of protein fractions

The molecular weight range of protein extracts were estimated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in a vertical Bio-Rad mini-gel electrophoresis unit. Equal volumes of protein extracts (10 mg/ml) were combined with 2x SDS sample buffer and incubated at  $95^{\circ}\text{C}$  for 5 min. After a short centrifugation and cooling to room temperature, 10  $\mu\text{L}$  of the supernatant was loaded into the well. SDS-PAGE was performed using a 12% separating gel with a 4% stacking gel at 75 V for 30 min then increased to 110 V. After electrophoresis, the gel was stained with 0.1% Coomassie Blue Staining Solution containing methanol and acetic acid for 1 h and destained with water, methanol and acetic acid.

### Functional properties

#### Protein solubility

The extracts solubility at different pHs was determined by following the Intarasirisawat et al. (2012) method. Briefly, protein extracts were dispersed in distilled water and the pH was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using either 6 N HCl or 6 N NaOH with the final concentration of 10 mg protein/mL. Then, the mixture was stirred for 90 min followed by centrifugation at 1800 g for 30 min. The supernatant was used for the determination of protein content using the Lowry's method. Protein solubility was calculated as follows:

$$\text{Solubility}(\%) = \frac{\text{Protein content in supernatant}}{\text{Total protein in extract}} \times 100\%$$

#### Emulsifying activity and stability

The emulsifying properties of the extracts were investigated following the method of Klompong et al. (2007) with minor modifications. Peanut oil (5 mL) and 15 mL of 1% protein extract were mixed and

the pH was adjusted to 3, 5 and 8. The mixture was vortexed for 2 min and a 50  $\mu\text{L}$  of aliquot of the emulsion was pipetted from the bottom of the test tube at 0 and 10 min after homogenization and mixed with 2.5 mL of 0.1% sodium dodecyl sulphate solution using a vortex (Model VM-1000, MRC, UK) at speed 10. The absorbances of the mixture ( $A_0$  and  $A_{10}$ ) were measured at 500 nm using a spectrophotometer and the emulsifying activity index (EAI) and emulsion stability index (ESI) were determined using the formulae below:

$$EAI \left( \frac{m^2}{g} \right) = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{proteinweight}(g)}$$

$$ESI(\text{min}) = A_0 \times \Delta t / \Delta A$$

where  $\Delta A = A_0 - A_{10}$  and  $\Delta t = 10$  min.

#### Foaming properties

The foaming capacity and stability of the extracts were investigated based on the method described by [Klompong et al. \(2007\)](#) and [Intar-asirisawat et al. \(2012\)](#) with minor modifications. Briefly, 10 mL of 0.5% protein extract in a 25 mL cylinder was adjusted to pH 3, 5 and 8, and stirred at a speed of 15,000 rpm for 2 min at room temperature to incorporate air. The volume of the whipped sample was recorded after 30 min. The foaming capacity and stability were calculated according to the following equations:

$$\text{Foaming capacity}(\%) = \frac{(A - B)}{B} \times 100$$

$$\text{Foaming stability}(\%) = \frac{A_{30\text{min}} - B}{A_{0\text{min}} - B} \times 100$$

where  $A$  = volume after whipping (mL) and  $B$  = volume before whipping (mL).

#### Water and oil binding capacities

For determining water and oil binding capacities, a solid-liquid ratio of 1:40 (w/v) of distilled water or soybean oil was added to known masses of protein extracts in pre-weighed tubes respectively. Samples were vortexed for 30 sec and left to stand at room temperature for 30 min before being centrifuged at 4000 g for 25 min. The supernatant was decanted. The mass of bound water and oil was determined by difference in mass of samples according to [Yu et al. \(2007\)](#).

#### Antioxidative assays

##### 2,2'-9-azino-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The ABTS radical cation assay based on the method of [Re et al. \(1999\)](#) was used to determine antioxidative capacity. Briefly, ABTS radical cation was prepared by reacting a 7 mM ABTS (Roche Diagnostics) stock solution with a known concentration of potassium persulfate (Sigma-Aldrich) so that the final concentration of potassium persulfate is 2.45 mM. The mixture was left to stand in the dark at room temperature for 16 h before use. A working ABTS<sup>•+</sup> solution with an absorbance of  $0.70 \pm 0.02$  at 734 nm was prepared by diluting with Phosphate-Buffered Saline (PBS) buffer. Ten microlitres of protein extract or standard was added to 190  $\mu\text{L}$  of ABTS<sup>•+</sup> and mixed thoroughly in a microplate well before being read at 734 nm (Varioskan LUX, Thermo Fisher Scientific). The antioxidative capacity was calculated as:  $\% \text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$ , where  $A_{\text{control}}$  is the absorbance of the working ABTS<sup>•+</sup> solution and  $A_{\text{sample}}$  is the absorbance of the samples with ABTS<sup>•+</sup> reagent. Gallic acid was used as a standard.

##### Ferric ion reducing antioxidant power (FRAP)

The FRAP assay was conducted as described previously by [Benzie & Strain \(1999\)](#) with minor modifications. The FRAP working solution was freshly prepared by mixing acetate buffer (300 mM, pH 3.6) with ferric chloride (30 mM in water) and 2,4,6-Tripyridyl-s-Triazine (TPTZ, 40

mM dissolved with 40 mM HCl) following a volumetric ratio of 10:1:1 immediately before use. The FRAP reagent was warmed at 37 °C for 10 min before 950  $\mu\text{L}$  was added to 50  $\mu\text{L}$  of protein extract or standard. The samples were incubated at 37 °C for 30 min and absorbance was read at 593 nm (Varioskan LUX, Thermo Fisher Scientific). Gallic acid was used as a reference standard, with concentrations ranging from 0.1 mg/ml to 0.5 mg/mL.

##### Total phenolic content (TPC)

The Folin-Ciocalteu (F-C) assay was used to determine the phenolic content of the protein extracts. To 50  $\mu\text{L}$  of sample solution (10 mg/mL), 20  $\mu\text{L}$  of F-C reagent (Sigma) followed by 930  $\mu\text{L}$  of 700 mM sodium carbonate solution was added. The samples were carefully mixed and incubated at room temperature for 1 h. Absorbance was read at 765 nm. Gallic acid was used as a reference standard, with concentrations ranging from 0.1 mg/ml to 0.5 mg/mL.

##### Cytotoxic assay of protein extracts

The cytotoxic potential of protein extracts against HepG2 cell line was determined by a MTT (3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) metabolic viability assay. The cells were seeded on a 96-well plate at a density of  $1.0 \times 10^4$  cells/well and incubated overnight at 37 °C in a 5% CO<sub>2</sub> atmosphere. Various concentrations of unfermented and fermented protein extracts (2, 5, 10 mg/mL) were added to each well and cultured for 24 h at 37 °C. Twenty milligrams per millilitre MTT solution was prepared in PBS and diluted with Dulbecco's Modified Eagle Medium (DMEM) (1:10) before adding into each well. The cells were incubated with MTT solution for 3 h at 37 °C. Next, MTT solution was removed by pipetting and 100  $\mu\text{L}$  of DMSO was added to solubilise the insoluble purple crystals. The absorbance of the coloured solution was read at 540 nm (Varioskan LUX, Thermo Fisher Scientific) and compared to the control (protein extract absent). The relative cell viability (%) was calculated according to:  $\% \text{Relative viability} =$

$$\frac{Abs_{\text{sample@540nm}}}{Abs_{\text{control@540nm}}} \times 100\%$$

##### Application as a plant-based emulsifier

##### Emulsion preparation

Mayonnaise, an oil-in-water emulsion, was prepared to examine the potential of unfermented and fermented BSG protein extracts (BSGP and FBSGP) as a plant-based emulsifier in a food system. The emulsion was prepared in accordance with [Rahmati et al. \(2014\)](#) with slight modifications. Briefly, all dry ingredients were added into an immersion blender (Cornell, USA), followed by adding in emulsifier, mustard, vinegar and water. During blending, oil was gradually added into the mixture and blended at high speed in short pulses to avoid over-beating. Pure egg yolk and whole egg were used as emulsifiers in two separate control experiments. The ratio of BSGP or FBSGP to whole egg for a 10% emulsifier was also varied on three levels: 40% (F)BSGP + 60% whole egg (BSGP40), 60% (F)BSGP + 40% whole egg (BSGP60), 100% (F)BSGP. Prepared mayonnaise emulsions were stored in a refrigerator (4 °C) for further characterization.

##### Creaming and thermal creaming

Five grams of each sample was transferred into tubes and centrifuged at 5,000 rpm for 20 min. Creaming at room temperature was determined by measuring the height of the separated cream from emulsion ( $H$ ) and the initial emulsion height in the tube ( $H_0$ ). The percentage of creaming (%H) was determined by the following equation:  $\%H = \frac{H}{H_0} \times 100\%$ . A similar procedure was employed for thermal creaming, but tubes were immersed in an 80 °C water bath for 20 min prior to centrifugation.

##### Viscosity measurement

The viscosity of the emulsions was measured using a rheometer AR

2000 with a 25 mm stainless steel parallel plate (TA Instruments, USA) at 25 °C at a shear rate of 50 s<sup>-1</sup> as described by Rahmati et al. (2014).

#### Optical microscope observation

Samples were observed under a light microscope (Olympus IX71) equipped with a digital camera (DP70, Olympus) to estimate the size of emulsion droplets and to compare the microstructure of the emulsions.

#### Statistical analysis

All experimental data were analysed using Minitab v. 19 Statistical Software (Minitab Inc., Coventry, UK). For comparisons between two groups of data, namely the amino acid profile, water and oil binding capacities, ABTS, FRAP, TPC and MTT assays, the student's *t*-test was used for data analysis. The remaining experimental data were analysed by carrying out One-way ANOVA followed by Tukey's test. The results were then expressed as mean value ± standard deviation at 95% confidence intervals.

## Results and discussion

#### Characterization of BSG protein before and after fermentation

The protein extracts from unfermented and fermented BSG (BSGP and FBSGP) were characterised based on their protein content, degree of hydrolysis from fermentation, amino acid profile and molecular weight.

Results from elemental analysis showed that fermentation resulted in protein enrichment of BSG from 25.2% to 34.1%. A higher protein content in FBSGP is due to the increase in fungal biomass from fermentation and the secretion of extracellular enzymes to break down BSG (Bianco et al., 2020; Canedo et al., 2016). Using an ethanolic-alkali mixture for protein extraction, a protein content of 60.7% and 66.2% was obtained for BSGP and FBSGP respectively, suggesting that protein isolation was effective. These results are accounted for as the extraction solvent was selected based on the major presence of alcohol-soluble hordeins and some alkali-soluble glutelins that are present in BSG. A food-safe reducing agent, sodium metabisulfite, was added to overcome the extensive disulfide bonds that are formed due to mashing. Our findings showed a higher protein content than the study by Celus et al. (2006) using propanol under reducing conditions without pH

adjustments. In contrast, the protein content in this study is lower than that obtained by a sequential alkali extraction as demonstrated by Vieira et al. (2014). It is, however, worth noting that using an organic solvent like ethanol allows solvent recovery and it can be recycled for subsequent protein extractions. The protein content using an ethanolic-alkali mixture can be improved further by optimizing extraction conditions (pH, temperature, time, concentration) that are appropriate for solubilising proteins and minimizing impurities such as hemicellulose and lignin.

After three days of fermentation, proteins were hydrolysed to a degree of 14.6% as proteases from *R. oligosporus* converted BSG proteins to peptides and amino acids. These results are comparable to the highest DH obtained from an enzymatic hydrolysis using Alcalase as reported by Celus et al. (2007). Therefore, microbial fermentation can easily achieve the same DH as commercial enzymes and can be considered to be cost-effective, albeit at the expense of a longer incubation time.

Changes in amino acid composition were also observed between BSGP and FBSGP as shown in Fig. 1. In both BSGP and FBSGP, proline and glutamic acid were present in the largest proportions. This agrees with other research that have established the majority of proteins in BSG as prolamins (hordeins) and some glutelins, with the former being proteins that are rich in proline and glutamine (Wang et al., 2010). During fermentation, proline and glutamine were efficiently utilised and a relative increase in some of the essential amino acids was seen (valine, methionine, lysine, histidine), which was also observed by Treimo et al. (2008) during enzymatic hydrolysis of BSG proteins. This indicates an improvement in nutritional status of the fermented product and hence its extracted proteins. In addition, fermentation did not drastically change the ratio of hydrophobic to hydrophilic amino acids. About 47% of amino acids in both BSGP and FBSGP are considered as hydrophobic amino acids, implying that a good balance between surface hydrophilicity and hydrophobicity may exist, thus suggesting a possible emulsification property.

BSGP and FBSGP were subjected to SDS-PAGE as shown in Fig. S1. Barley hordeins are classified into 5 groups (A, B, C, D and  $\gamma$ -hordeins) on the basis of their electrophoretic mobilities and amino acid compositions. B-hordeins make up 70–80% of the hordein fraction with bands around 35–50 kDa and C-hordeins represent 10–20% of the hordein fraction with their molecular weight ranging around 55–80 kDa. Other hordein fractions make up the minority (Celus et al., 2006). In this study,

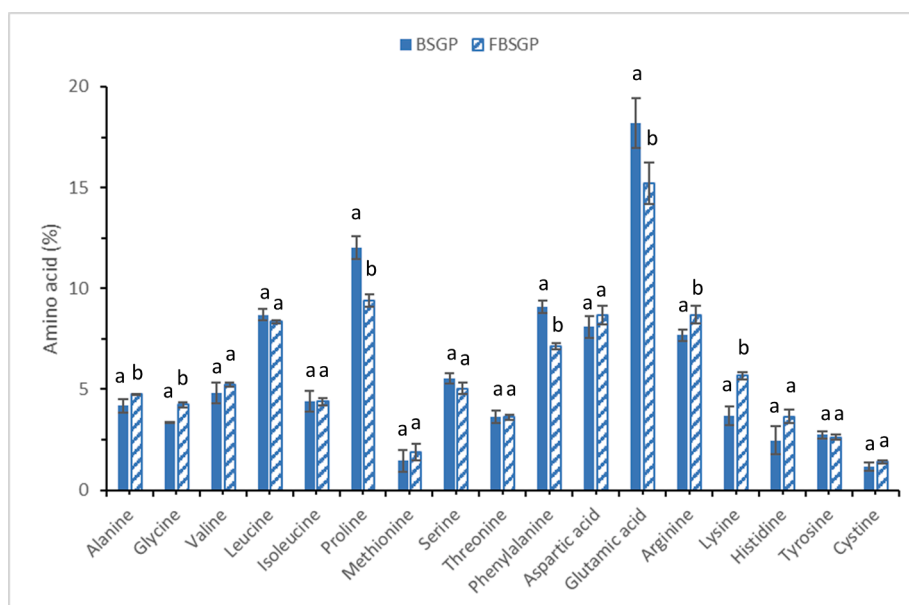


Fig. 1. Changes in amino acid composition for unfermented and fermented BSG after three days. Aspartic acid includes asparagine. Glutamic acid includes glutamine. Results are means of triplicate determinations. Values with different letters denote significant differences ( $p < 0.05$ ).

both protein extracts presented several bands ranging from about 20 kDa to 55 kDa, suggesting that they contain polypeptides of similar molecular weights. The results align well with available literature as the visible bands at higher molecular weights likely represent the B and C-hordein subunits in barley, while the lower molecular weights could be the A-hordeins (Connolly et al., 2013; Wang et al., 2010).

### Functional properties of BSGP and FBSGP

#### Solubility

Functional properties of proteins are dependent on specific properties such as size, shape, amino acid composition, hydrophilicity and hydrophobicity, amongst others. It has been known that solubility is one of the most practical indexes of protein physicochemical properties as it dictates the applicability of proteins in food systems (Malecki et al., 2021). Fig. 2a shows the protein solubilities as a function of pH for BSGP and FBSGP. It can be seen that both BSGP and FBSGP were soluble over a wide range of pH. The solubility of both proteins increased with an increase in pH. Regardless of fermentation process, the results showed that pH had a significant effect on solubility with all samples having highest

solubility between pH 6.0–10.0. Generally, FBSGP (11.72–86.84%) had higher solubility compared to that of BSGP (4.51–54.30%). The lowest solubility for both proteins was found at pH 3. Similar pH dependent solubility profiles have been observed in another study on the protein of processed finger millet flour that had undergone fermentation process (Gowthamraj et al., 2021). The fungal fermentation process adopted in this study increased the solubility of the proteins by hydrolysing the complex structure of proteins to simpler amino acids through the actions of proteases secreted by *R. oligosporus*. This facilitates the proteins to solubilise in the extracting solution. Besides, both nonpolar and some polar amino acid groups that are buried inside the globular protein molecules could be exposed to the surface after unfolding of the proteins. These exposed polar amino acids may interact with water molecules through hydrogen bonds and electrostatic interactions and subsequently increase the protein solubility (Celus et al., 2007). The low solubility of these proteins at pH 3 could possibly be due to the lowest repulsion of proteins at their isoelectric point, and such phenomenon has also been reported in the work by Intarasirisawat et al. (2012). FBSGP with high solubility over a wide pH range could therefore be applied widely in formulated food systems.

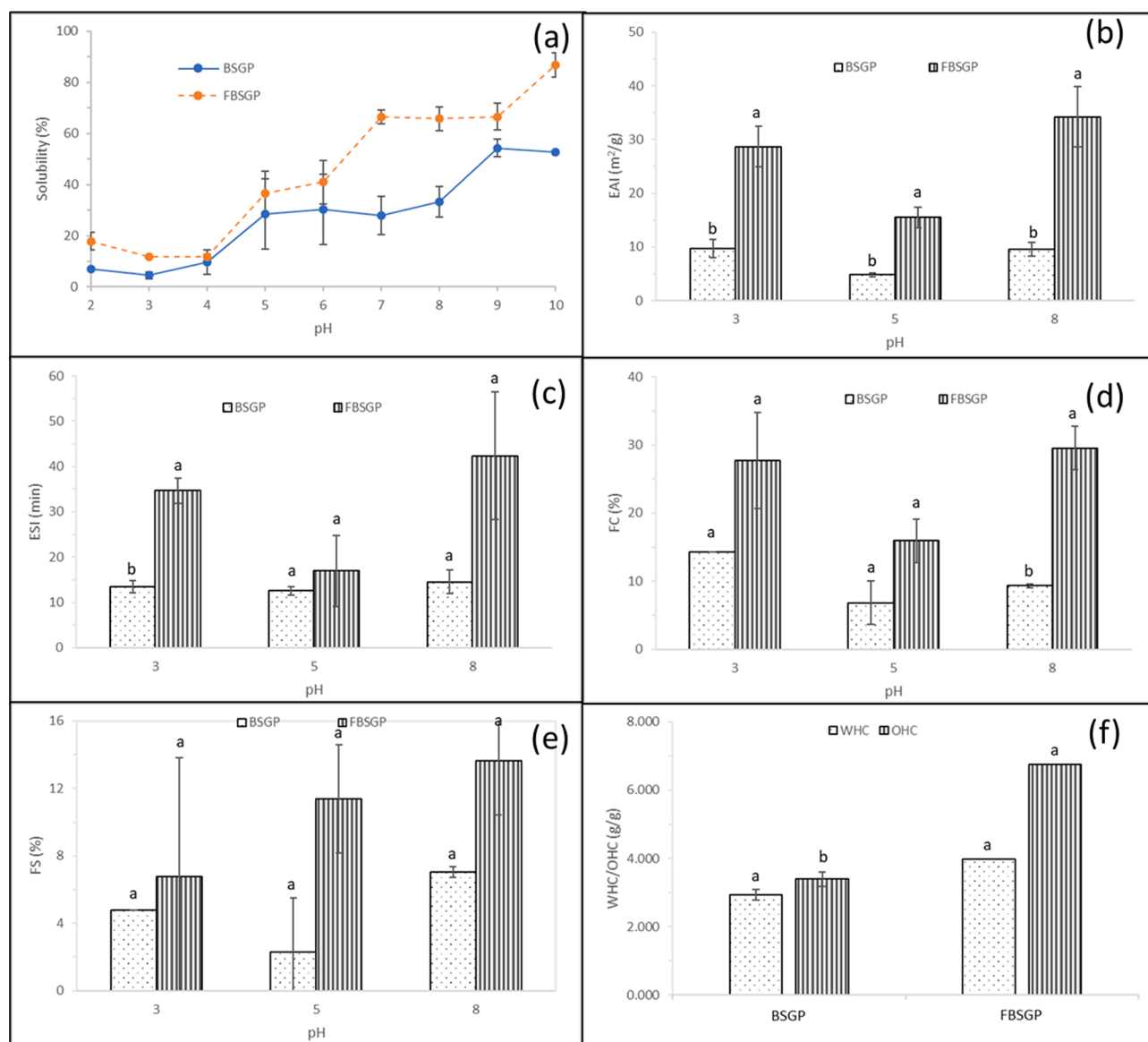


Fig. 2. Solubility (a), emulsifying activity index (EAI) (b), emulsion stability index (ESI) (c), foaming capacity (FC) (d), and foaming stability (FS) (e) water holding capacity (WHC) and oil holding capacity (OHC) (f) of BSGP and FBSGP as influenced by pHs. Values with different letters denote significant differences ( $p < 0.05$ ).

### Emulsifying properties

Since proteins are amphoteric polyelectrolytes, their emulsifying and foaming behaviours are expected to vary with different pHs of the solution. Hence, the effect of pH on these two functional properties was investigated for BSGP and FBSGP. The emulsifying activity index (EAI) and emulsion stability index (ESI) of both BSGP and FBSGP at different pHs are depicted in Fig. 2b and Fig. 2c, respectively. There was a significant difference ( $p < 0.05$ ) in EAI between BSGP and FBSGP where FBSGP (15–34  $m^2/g$ ) had higher EAI values compared to those of BSGP (5–10  $m^2/g$ ). It can be noticed that both types of BSG proteins exhibited the lowest EAI at pH 5 (Fig. 2b). When the pH was decreased to pH 3 or increased to pH 8, the EAI of both proteins increased. A similar trend could be observed in the ESI of both proteins (Fig. 2c). Better emulsifying properties were shown by FBSGP largely due to the structural modification after fermentation in which low-molecular-weight peptides were liberated from their inert globular parent proteins upon microbial fermentation (Saadi et al., 2015). This corroborates with Park et al. (2019) who also demonstrated that the protein hydrolysates obtained from soybean meal after fermentation contained greater amounts of shorter chain peptides and amino acids and thus showed better emulsifying properties. The high EAI values observed for the FBSGP indicates that it has smaller oil droplet size, highlighting the potential of the hydrolysates to act as emulsifiers in formulated food products such as mayonnaise, soups and sauces. Besides, a relatively similar EAI and ESI values were observed for both proteins at pHs 3 and 8 with FBSGP had significantly higher values, indicating that FBSGP stabilised emulsions have an excellent stability. Higher EAI and ESI values were observed at higher pH, showing that there appeared to be an association between increased solubility and EAI and ESI. The results from this study are in parallel to those reported by Negi & Naik (2017) and Connolly et al. (2014) who worked on BSG proteins, and Wang et al. (2010) who worked on barley proteins.

### Foaming properties

The pH dependence of foaming capacity (FC) and foaming stability (FS) of BSGP and FBSGP are shown in Fig. 2d and Fig. 2e, respectively. Regardless of pH, FBSGP (16–30% of FC and 7–14% of FS) had higher FC and FS compared to those of BSGP (7–14% of FC and 2–7% of FS). Celus et al. (2007) had shown also that FC of BSG protein increased after hydrolysis using pepsin. Again, the better foaming properties of FBSGP are due to the presence of low-molecular-weight peptides that have different structural properties and amino acid composition and sequences (Chai et al., 2020). Schlegel et al. (2021) has also demonstrated that fermented lupin hydrolysates showed greater foaming properties compared to the unfermented samples. In this study, both types of protein exhibited the lowest FC at pH 5. When the pH decreased to pH 3 or increased to pH 8, the FC values of FBSGP increased by 74% and 86%, respectively, while the FC values of BSGP increased by 109% and 37%, respectively. These results are similar to those reported by Wang et al. (2010) where the FC values of barley protein fractions increased when it deviated from pH 5 to either pH 3 or pH 8. Similar trend was also observed in the FS for BSGP under study, but not the FBSGP (Fig. 2e). The FS of FBSGP increased with the increase in pH. Connolly et al. (2014) also reported the similar results for their BSG hydrolysates where the FS of their hydrolysates increased as pH increased. This happened due to the protein had an increase net charge at high pH and consequently, a decrease in attractive hydrophobic forces which may increase protein flexibility and thus, enabling the protein to diffuse more rapidly to the air–water interface to encapsulate air molecules (Connolly et al., 2014).

### Water and oil holding capacities

Fig. 2f shows the water and oil holding capacities of both BSGP and FBSGP. WHC is a measure of the water-binding of the proteins by polar side chains while OHC is measuring the fat-binding capacity of proteins via nonpolar side chains. In general, FBSGP had higher WHC and OHC

values compared to those of BSGP and the OHC of FBSGP was significantly ( $p < 0.05$ ) higher by 2-fold compared to that of BSGP. Similar results were observed in the study reported by Wasswa et al. (2007) and Kanu et al. (2009) who showed that the WHC of grass carp skin protein hydrolysate and WHC and OHC of defatted sesame flour protein hydrolysates increased with increased hydrolysis, respectively. The occurrence of this phenomenon is attributed to the increased numbers of polar groups such as COOH and  $NH_2$  in the hydrolysates during fermentation process which has a substantial effect on the amount of adsorbed water (Wasswa et al., 2007). Besides, the higher OHC value of FBSGP is largely due to the fact that the proteases from *R. oligosporus* degraded the original protein structure of BSG to various smaller peptides and amino acids and thus, exposing more hydrophobic groups to the oil interface. Oil seed protein isolates are known to have high OHC and their values are usually  $< 4$  g/g (Wang et al., 2010). The FBSGP under study possessed a relatively balanced water and oil holding capacities and its OHC is especially high compared to that of oil seed protein isolates. These properties allow FBSGP to be an excellent water or oil binding ingredient in various food applications such as serving as an ingredient in bakery products, or improving structure and reducing yield losses of extended meat products.

### Antioxidative properties of BSGP and FBSGP

Bioactive properties, specifically the antioxidative capacities, of BSGP and FBSGP were evaluated. Based on ABTS and FRAP assays, both samples possessed antioxidant activities (Table 1), which can be explained by the contribution from phenolic compounds and amino acids present in the extract.

Antioxidants are often associated with the presence of phenolic compounds as they possess the ability to scavenge free radicals and chelate metal ions (Pereira et al., 2009). As confirmed by the F-C assay, phenolic compounds were present in the extracts of both BSG and FBSG, with a greater amount present in the latter. The presence of phenolics could be attributed to the use of a mixture of aqueous ethanol and alkali for extraction. Previous studies have successfully shown that phenolic compounds can be extracted using alkalis (Meneses et al., 2013; Qin et al., 2019) and aqueous ethanol (Meneses et al., 2013; Qin et al., 2019), and the majority of phenolic acids in BSG are ferulic acid and p-coumaric acid (Macias-Garbett et al., 2021). The greater amount of phenolics in the fermented extract is supported by earlier literature since fermentation breaks down the cell walls and enzymatic activities liberate the bound phenolic compounds (Adebo & Gabriela Medina-Meza, 2020; Cooray & Chen, 2018).

In addition to phenolic compounds, the proteins and peptides after hydrolysis, including their bound amino acids, play a part in contributing to the antioxidant activity (Chai et al., 2020; Connolly et al., 2019; Vieira et al., 2017). In the ABTS assay, antioxidants present in solution would donate a hydrogen atom to the synthesised  $ABTS^{\bullet+}$ , resulting in a more stable and less reactive radical, therefore shielding biomolecules from subsequent damage. To demonstrate good radical scavenging activity (translating to higher inhibition), there needs to be an optimal balance of diffusivity and hydrophobicity, with the former depending on the DH and subsequent peptide size, and the latter based on the amino acid composition (Bamdad et al., 2011). Generally, amino acids such as tyrosine, methionine, histidine, lysine and tryptophan are considered to

**Table 1**  
Antioxidant activity and total phenolic content of BSGP and FBSGP.

	ABTS (mg GAE/mg protein DW)	FRAP ( $\mu$ g GAE/mg protein DW)	Total phenolic content ( $\mu$ g GAE/mg protein DW)
BSGP	42.0 $\pm$ 17.7 <sup>a</sup>	129.40 $\pm$ 2.98 <sup>a</sup>	52.34 $\pm$ 0.45 <sup>a</sup>
FBSGP	84.5 $\pm$ 29.4 <sup>b</sup>	140.31 $\pm$ 3.86 <sup>b</sup>	53.75 $\pm$ 0.29 <sup>b</sup>

Results are means of triplicate determinations. Values with different letters denote significant differences ( $p < 0.05$ ).

possess antioxidant activity and demonstrate higher antioxidative activity as part of a peptide (Saito et al., 2003; Sanjukta et al., 2021). These amino acids are present in both extracts, but a significantly greater inhibition level ( $p < 0.05$ ) is seen in FBSGP than BSGP. This can be supported by the greater amount of antioxidative amino acids in FBSGP (Fig. 1) as well as a smaller peptide size of the hydrolysed protein after fermentation (Fig. S1), leading to a high diffusivity for scavenging free radicals. In terms of reducing power, FBSGP also demonstrated a stronger reducing power than BSGP based on the FRAP assay. This is likely associated with the exposure of electron-dense amino acid side chain groups, such as polar or charged moieties after hydrolysis or an increase in free amino acids that act as an additional source of protons and electrons during hydrolysis (Bamdad et al., 2011). Therefore, a stronger antioxidant activity is observed in the fermented extract.

Furthermore, as reported by Saito et al. (2003), antioxidative peptides may exert a strong synergistic effect with other antioxidants, such as phenolic compounds. As such, our results suggest that fermentation is responsible for an increased antioxidant activity due to an improved total phenolic content and enhanced amount of antioxidative peptides present.

#### Cytotoxicity of BSGP and FBSGP

The cytotoxic effects of BSGP and FBSGP were investigated using MTT assay against human liver hepatocarcinoma cells HepG2. This cell line was chosen as it is commonly used for screening the cytotoxic potential of novel chemical entities at early phases (Sarwar et al., 2018). Depending on the type of hydrolysis used, a previous study showed that more than 75% of HepG2 cells were viable at a BSG protein hydrolysate concentration of 1 mg/ml (Vieira et al., 2017). Therefore, a higher concentration range of BSGP and FBSGP from 2 to 10 mg/ml was selected to evaluate the cytotoxic effect in this study. Based on our results (Fig. S2), at the concentrations of 2, 5 and 10 mg/ml, cell viability (relative to the control) was reduced slightly after adding BSGP and FBSGP, which is similar to the work published by Vieira et al. (2017). This may be due to the presence of polyphenols in the samples, as proven in a previous study that phenolic compounds inhibit the proliferation of cancer cells and cause its apoptosis (Qin et al., 2019). Nonetheless, all levels of BSGP and FBSGP (up to 10 mg/ml) showed a relative cell viability greater than 90%, therefore implying that both samples are probably not cytotoxic against HepG2 cells, and they can potentially be used in a food, pharmaceutical or cosmetic application.

#### BSGP and FBSGP as a plant-based emulsifier

From the results obtained in previous sections, FBSGP possessed good emulsifying properties and is non-toxic to cells based on MTT assay. Thus, efforts were expanded to investigate its possibility in food application as a plant-based emulsifier by producing oil-in-water mayonnaise. Various ratios of BSGP-to-whole egg and FBSGP-to-whole egg were used to examine the possibility of BSGP and FBSGP in substituting whole egg as the emulsifier in the production of traditional mayonnaise. Fig. 3 shows the mayonnaise produced using various concentrations of BSGP and FSGP with yolk and whole egg as the controls.

#### Creaming and thermal creaming of mayonnaise emulsions

Creaming is similar to sedimentation process, but in the opposite direction where buoyant emulsion droplets tend to rise to the top of a container because of the differences in density between the dispersed and the continuous phases (Rahmati et al., 2014). Thermal creaming, on the other hand, is the accelerated flocculation that causes more creaming due to the high temperature that samples are placed at (Rahmati et al., 2014). Both creaming and thermal creaming of mayonnaise prepared using either yolk, whole egg or different ratios of BSGP-to-whole egg or FBSGP-to-whole egg as the emulsifier are shown in Fig. 4a. It can be noticed that using different concentrations of BSGP and FBSGP as the emulsifier in producing mayonnaise had no significant difference ( $p$  greater than 0.05) in both creaming and thermal creaming tests when comparing to the controls (mayonnaise with only egg yolk or whole egg as the emulsifier), except for 100% of BSGP, indicating that BSGP alone could not be used to substitute egg yolk or whole egg in the production of mayonnaise. This is in line with the EAI and ESI results reported in earlier section of this study where BSGP had lower EAI and ESI compared to those of FBSGP. Hence, it is assumed that the mayonnaise with 100% BSGP was resistant to droplet coalescence which accelerates the creaming process. It has been known that electrostatic repulsion is the main mechanism in avoiding droplet flocculation in protein stabilised emulsions. It occurs between emulsion droplets with electrically charged surfaces that could be observed in emulsions due to the adsorption of ionic or ionisable components such as proteins, phospholipids, polysaccharides and surfactants (Rahmati et al., 2014). It has been reported that the isoelectric point of BSG hydrolysate is positive (Kotlar et al., 2013) and thus, FBSGP is positively charged in mayonnaise which leads to an electrostatic stability of the emulsion.

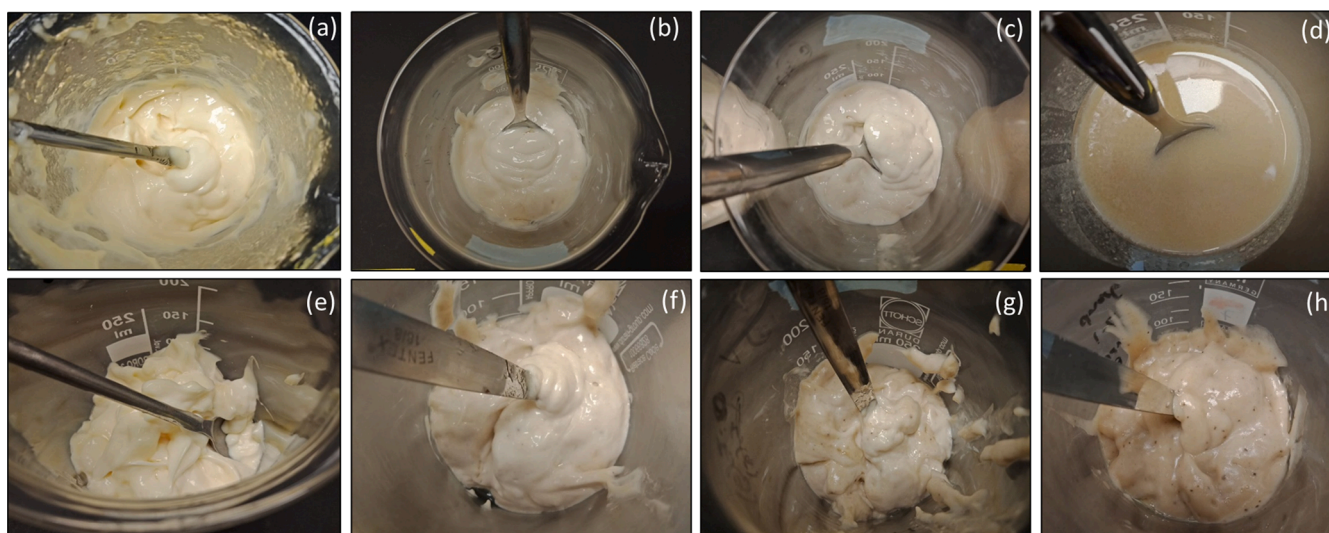
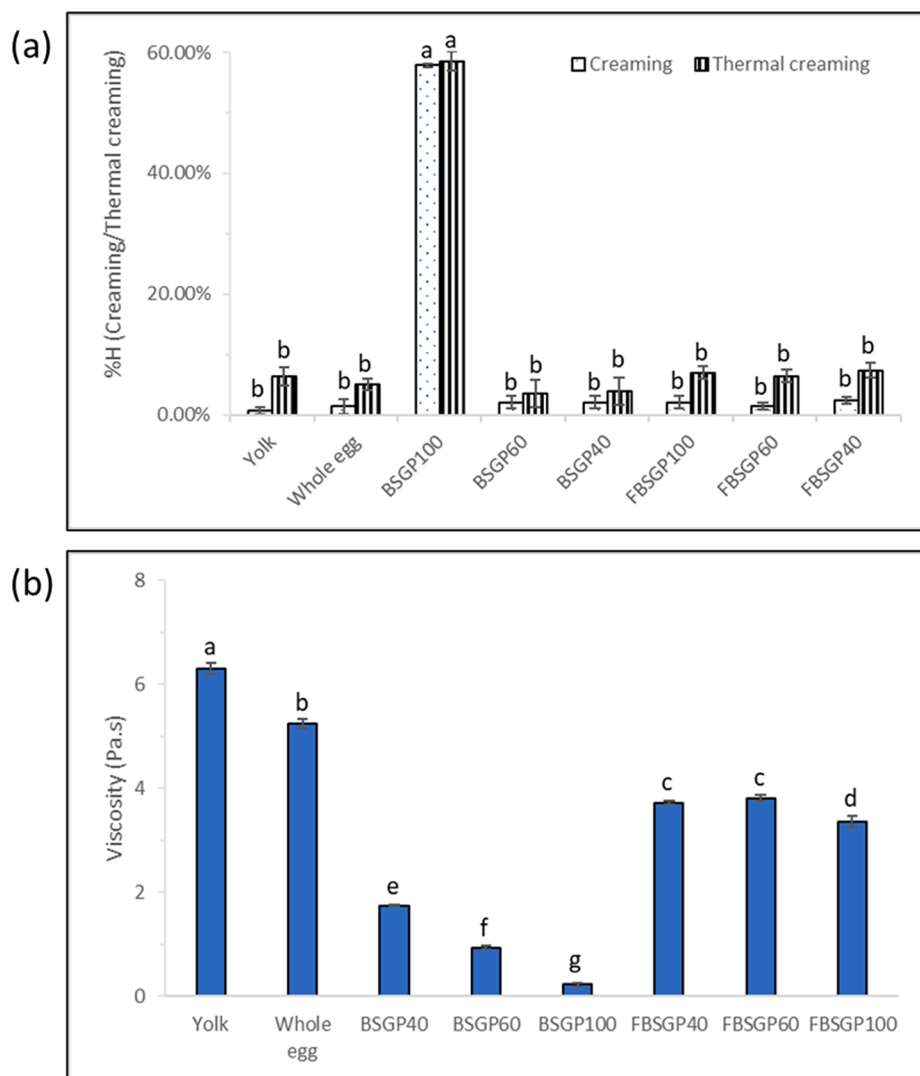
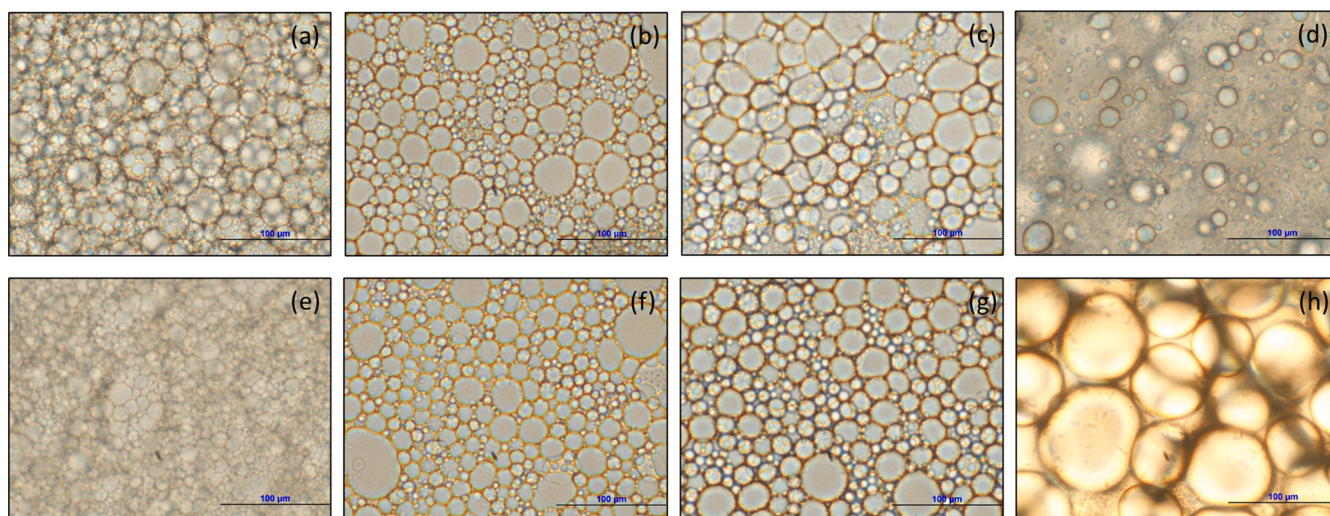


Fig. 3. Mayonnaise emulsions produced using egg yolk (a), 40% BSGP/whole egg (b), 60% BSGP/whole egg (c) 100% BSGP (d), whole egg (e), 40% FBSGP/whole egg (f), 60% FBSGP/whole egg (g) and 100% FBSGP (h) as the emulsifier.



**Fig. 4.** Creaming/thermal creaming (a) and viscosity (b) values of different samples with yolk, whole egg, different ratios of whole egg to BSGP or whole egg to FBSGP as the emulsifier in the mayonnaise. Values with different letters denote significant differences ( $p < 0.05$ ). BSGP100, BSGP60, BSGP40, FBSGP100, FBSGP60 and FBSGP40 denote the ratios of BSGP-to-whole egg 100:0, BSGP-to-whole egg 60:40, BSGP-to-whole egg 40:60, FBSGP-to-whole egg 100:0, FBSGP-to-whole egg 60:40 and FBSGP-to-whole egg 40:60.



**Fig. 5.** Optical microscope micrographs of mayonnaise samples, magnification was  $\times 60$ , bar was 100  $\mu\text{m}$ . Yolk control (a), 40% BSGP/whole egg (b), 60% BSGP/whole egg (c), 100% BSGP (d), whole egg control (e), 40% FBSGP/whole egg (f), 60% FBSGP/whole egg (g) and 100% FBSGP (h) as the emulsifier of the emulsions.



### Viscosity of emulsions

The viscosity of the emulsions prepared using different types of emulsifiers was tested and the results are presented in Fig. 4b. It can be noticed that there was a significant difference ( $p < 0.05$ ) in viscosity among the samples. Emulsion prepared from egg yolk was the most viscous, followed by those prepared from whole egg, FBSGP and BSGP. Both controls under study have comparable viscosity to that reported by Rahmati et al. (2014). Also, the viscosity values of the emulsions prepared using 40%, 60% and 100% of FBSGP (3.4–3.8 Pa·s) are comparable to those of mayonnaise prepared using 25%, 50% and 75% of soy milk (~3.5–4.0 Pa·s) which were said to be feasible to replace conventional emulsifier and could be employed in mayonnaise formulation (Rahmati et al., 2014). Thus, BSG proteins after fermentation could be used as an emulsifier in the production of mayonnaise, largely due to their high emulsifying properties that could result in smaller and homogenous particle size, improved friction force between drops and surface contact area in the emulsions. Low viscosity was noticed in all emulsions with BSGP (72–97% lower compared to that of yolk control which had a viscosity of 6.3 Pa·s) probably due to the large particle size, non-homogenous and scattered microstructures of the emulsions.

### Microstructure of emulsions

The microstructure of mayonnaise emulsions prepared using different types of emulsifiers (yolk, whole egg and different ratios of BSGP or FBSGP-to-whole egg) is demonstrated in Fig. 5. In general, all samples had homogenous and packed microstructures, except for the emulsion with 100% BSGP. The microstructures of emulsions with FBSGP were more homogenous compared to those with BSGP probably due to the smaller oil droplet found in the emulsions which shows the potential of the BSG hydrolysates to act as emulsifiers in formulated food products. As can be observed, although emulsions with 40%, 60% and 100% FBSGP had less amounts of whole egg, their microstructures were homogenous and packed, resembling that of controls with yolk and egg white. This indicates again that FBSGP is suitable to be used as an emulsifier in producing traditional mayonnaise.

### Conclusion

The valorisation of BSG was achieved through microbial fermentation and a subsequent ethanolic-alkali extraction of proteins and phenolics. Proteins were hydrolysed via fungal fermentation and the hydrolysates showed an improvement in emulsifying, foaming and oil/water binding capacities as compared to the unfermented BSG proteins. Moreover, an increase in antioxidative capacity was evident in the fermented sample, partly due to the simultaneous extraction of phenolic compounds in BSG. Given that the proteins showed no cytotoxic effects on HepG2 cells, and its production employed food-safe solvents, BSGP and FBSGP were applied in the formulation of a mayonnaise as an emulsifier. The prepared mayonnaise using FBSGP showed better stability than BSGP and could potentially be used as a substitute for eggs in mayonnaise. From these results, we conclude that FBSGP can be used as a plant-based emulsifier with strong antioxidative properties and is safe for human consumption. However, more studies are needed to optimise the protein extraction conditions so as to improve the protein content and extraction yield. Overall, the elimination of a BSG drying step and the cost of enzymes on top of solvent recovery makes this a cost-effective and scalable method of upcycling BSG.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2021.100184>.

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