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# Recovery of antioxidative protein hydrolysates with functional properties from fermented brewer's spent grain via microwave-assisted three phase partitioning

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ABSTRACT

The protein shortage for the world's population in the near future has prompted scientists to develop novel methods for the sustainable production of dietary proteins from various alternative sources. In this study, the application of the bioseparation technique known as microwave-assisted three phase partitioning (MATPP) was explored to simultaneously extract and separate proteins from brewer's spent grain (BSG) after fungal fermentation. The results of the study indicated that MATPP (82.2%) recovered twice the amounts of proteins from fermented BSG compared to three phase partitioning (TPP) (41.8%). Besides, no significant differences ($p > 0.05$) were observed in terms of amino acid composition, protein pattern, and some functional properties between the fermented BSG proteins (FBPs) obtained via TPP and MATPP. Additionally, MATPP was found to increase the antioxidant activities of FBPs. These findings suggest that MATPP holds great potential for industrial-scale protein recovery attributed to its effectiveness, simplicity, and speed.

*Industrial relevance:* Microwave-assisted three phase partitioning (MATPP) is an emerging bioseparation technique that could simultaneously extract, separate, and partially purify proteins from protein-rich plant materials effectively within a relatively short timeframe. The fact that the microwave used does not significantly alter most of the physicochemical properties of the plant protein hydrolysates certainly supports the adoption of the technique in industry settings. MATPP demonstrates significant potential for industrial-scale utilization due to its efficiency, simplicity and potential cost reduction in production.

## 1. Introduction

An apparent major issue faced by humanity is the lack of proteins to feed the world in the near future. Consumption of animal proteins contributes 40% to the total global protein intake and is expected to increase by 73% by 2050 due to the growing population (Bhat, Morton, Mason, Bekhit, & Bhat, 2019; Melzener, Verzijden, Buijs, Post, & Flack, 2021; Samarasiri, Chai, & Chen, 2023). Over the past four decades, the global production of meat has tripled and will be projected by 16% greater in 2025 than in 2015 (Bhat et al., 2019). It is obvious that the conventional meat production is no longer practical to meet the demand of 10 billion world population in 2050. This pressure has triggered the development of sustainable proteins from various alternative sources such as plants, microorganisms and insects.

Upcycling by-products from food processing for the production of dietary proteins is a novel way to obtain sustainable dietary proteins to feed the growing world population while reducing food waste and environmental footprint. Brewer's spent grain (BSG) is the major by-product in the brewing industry and contains a relatively high level of protein (19–30%) (Macias-Garbett, Serna-Hernández, Sosa-Hernández, & Parra-Saldívar, 2021). However, due to its poor solubility characteristic that causes difficulties in extracting the BSG proteins, their use has thus been limited in food processing (Celus, Brijs, & Delcour, 2007; Connolly et al., 2019). Several approaches have been explored to extract BSG proteins, including the use of alkaline extraction method (Connolly, Piggott, & Fitzgerald, 2013) and the use of salt or alcohol solutions (Wang et al., 2010). Efforts have also been made by using commercial peptidases to hydrolyze the BSG proteins with an aim to increase the solubility of the protein fractions and subsequently, increasing the protein yield (Celus et al., 2007; Connolly et al., 2019). In our previous

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study, we demonstrated to use a more economical approach i.e., using *Rhizopus oligosporus* to ferment BSG prior to ethanolic-alkali extraction (Chin, Chai, & Chen, 2022). Fungal fermentation is a cost-effective alternative to enzymes for improving the extractability of BSG proteins. Fungi are able to secrete extracellular enzymes that hydrolyze the complex structure of proteins into simpler amino acids (Chai, Voo, & Chen, 2020). This process facilitates the solubilization of proteins in the treatment solutions, thereby enhancing their extractability (Gowthamraj, Raasmika, & Narayanasamy, 2021). Although a considerably amount of proteins could be obtained from BSG using microbial fermentation followed by ethanolic-alkali extraction (Chin et al., 2022), it is good to consider developing an extraction method that requires lower costs, lower energy input, simpler steps, shorter processing time, non-toxic and possesses environmentally friendly characteristics, contributing to the better success in scaling up the process of fermented BSG proteins (FBPs) extraction.

In this study, we attempt to simultaneously extract and separate proteins from BSG post-fermentation via microwave-assisted three phase partitioning (MATPP). In contrast to conventional extraction methods, three phase partitioning (TPP) is an emerging non-chromatographic bioseparation technique that could overcome various associated limitations of the conventional methods. TPP comprises three phases formed from two liquid mixtures that consist of an aqueous salt solution and a water-miscible aliphatic alcohol (Chew et al., 2018). The addition of alcohol to aqueous salt solution precipitates the proteins from the solution, leading to the formation of an interfacial precipitate situated between the lower aqueous and upper organic layers as the alcohol binds to the proteins (Chew et al., 2018).

Although TPP has demonstrated its effectiveness as a simple and fast method for the extraction, purification, and concentration of diverse protein biomolecules as reported by Rajagopalan and Sukumaran (2018), Patil and Yadav (2018) and Yuzugullu Karakus, Acemi, Işık, and Duman (2017) who obtained proteases from *Wrightia tinctoria*, laccase from *Trametes hirsute* and peroxidase from *Amsonia orientalis*, respectively, their works were mainly on extracting particular enzymes. Recently, Patil and Rathod (2022) managed to use ultrasound-assisted TPP to obtain proteins from spent turmeric powder. Considering little work has been done to extract dietary proteins from by-products of food processing using TPP, the objectives of this study were, thus, to employ MATPP to extract dietary proteins from fermented BSG, and to characterize the extracted proteins. The findings of this study offer valuable insights into the potential scalability of the FBPs extraction process using TPP. These results pave the way for further exploration and optimization of large-scale FBPs extraction using this innovative technique.

## 2. Materials and methods

### 2.1. Materials

The brewery waste, BSG with a moisture content of 76% and a 25% (*w*/w) protein on dry basis, was acquired from a brewery in Singapore. The fresh BSG was placed in polyethylene bags and promptly transported to the lab from the brewery immediately after brewing. It was then stored at −80 °C. Thawing of the BSG was done at room temperature, followed by autoclaving prior to undergoing fungal fermentation. Unless stated otherwise, all standards and chemicals used, predominantly sourced from Sigma-Aldrich (St. Louis, MO, USA), were of analytical-grade purity.

### 2.2. Fungal fermentation of BSG

*Rhizopus oligosporus* ATCC 64063 was employed to ferment the BSG, following the procedure outlined in the study conducted by Chin et al. (2022). Briefly, autoclaved BSG was inoculated with *R. oligosporus* with an inoculum concentration of $10^7$ spores/mL at a ratio of 10:1 (*w*/*v*). The mixture was then incubated at 37 °C in an incubator (LM-570RD, China) for 72 h. After fermentation, the fermented BSG was subjected to drying in an oven (Binder ED 115, Germany) set at 60 °C for a duration of 48 h. Subsequently, the dried fermented BSG was ground into a powder and sieved through a 400 μm mesh.

### 2.3. Extraction of FBPs using MATPP system

FBPs were extracted using a MATPP system. Briefly, BSG powder was blended with water using a commercial kitchen blender (Tefal, Singapore). The mixture was then transferred to a beaker, wrapped with a cling film, and microwaved (Cornell, Singapore). A muslin cloth was used to strain the mixture to obtain the crude BSG extract (CBE). Finally, the CBE was subjected to TPP system. The CBE was saturated with ammonium sulphate; then, t-butanol was added to the saturated CBE. After vortexing the mixture for a duration of 3 min, it was left undisturbed at room temperature for 30 min to facilitate phase separation. Subsequently, centrifugation was performed at a speed of 1000 ×g for a period of 15 min. The upper organic layer and lower aqueous phase were carefully removed by using a pipette. The solid intermediate layer was collected and freeze dried for further analysis. FBPs extracted using TPP without the assistance of microwave was used as control.

### 2.4. Determination of protein content in fermented BSG

The protein content of fermented BSG was assessed following the protein assay done by Geisslitz, Longin, Scherf, and Koehler (2019). The absorbance of the solutions was measured at a wavelength of 595 nm employing a spectrophotometer (Nano Drop 2000, Thermo Scientific, USA).

### 2.5. Characterisation of FBPs

#### 2.5.1. Amino acid composition

The amino acids of FBPs were analyzed using a gas chromatograph (7890 A, Agilent Technologies) coupled with a mass spectrometer (5975C inert MSD with Triple Axis Detector, Agilent Technologies) according to Chin et al. (2022). The analysis was conducted using an HP-5MS capillary column. Before analysis, FBPs was hydrolyzed with 6 M HCl and then deproteinized using acetonitrile. Subsequently, derivatization was carried out using N-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-Butyldimethylchlorosilane.

#### 2.5.2. Molecular weight of FBPs

To determine the molecular weight of FBPs under reducing conditions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed using a vertical Bio-Rad mini-gel electrophoresis unit, as described by Chin et al. (2022). In summary, 10 mg/mL of FBPs was mixed with an equal volume of 2× SDS sample buffer and incubated at 95 °C for 5 min. The mixture was then subjected to centrifugation, and 10 μL of the resulting supernatant was loaded into the well. A 12% separating gel with a 4% stacking gel was used, and the electrophoresis process started at 75 V for 30 min. The voltage was subsequently increased to 110 V for 15 min and finally to 120 V for an additional 15 min. After the completion of the electrophoresis, the gel was stained with a 0.1% Coomassie Blue Staining Solution containing methanol and acetic acid for 1 h. The gel was then de-stained using water, methanol, and acetic acid.

#### 2.5.3. Antioxidant properties

*2.5.3.1. Total phenolic content (TPC).* The total phenolic content (TPC) of FBPs was assessed using the Folin-Ciocalteu assay, following the procedure outlined by Chai et al. (2019). In summary, 20 μL of F—C reagent and 930 μL of 700 mM sodium carbonate solution were added to 50 μL of the FBPs sample (10 mg/mL). The mixture was thoroughly

mixed and left to incubate at room temperature for 1 h. Subsequently, the absorbance of the mixture was measured at a wavelength of 765 nm. To generate the standard curve, gallic acid was used at various concentrations.

*2.5.3.2. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).* The ABTS radical cation assay was conducted following the protocol described by Re et al. (1999). A stock solution of 7 mM ABTS was prepared, and potassium persulfate was added to achieve a final concentration of 2.45 mM. The mixture was kept in the dark at room temperature for 16 h. Phosphate-Buffered Saline (PBS) buffer was diluted to prepare a working solution of $ABTS^{\bullet+}$ with an absorbance of 0.70 ± 0.02 at 734 nm. To measure the antioxidant capacity, 10 μL of FBPs or a standard compound (gallic acid) was added to 190 μL of the $ABTS^{\bullet+}$ solution and thoroughly mixed. The absorbance of the mixture was then measured at 734 nm using a Varioskan LUX spectrophotometer from Thermo Fisher Scientific. The antioxidant capacity of FBPs was calculated using the provided formula.

$$\%Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where $A_{control}$ is the absorbance of the working $ABTS^{\bullet+}$ solution and $A_{sample}$ is the absorbance of the samples with $ABTS^{\bullet+}$ reagent.

*2.5.3.3. Ferric ion reducing antioxidant power (FRAP).* The FRAP assay was carried out according to Chin et al. (2022). The FRAP working solution was prepared by mixing 300 mM acetate buffer (pH 3.6), 30 mM ferric chloride, and 40 mM 2,4,6-Tripyridyl-s-Triazine (dissolved in 40 mM HCl) at a ratio of 10:1:1. The resulting mixture was warmed at 37 °C for 10 min. Subsequently, 950 μL of the prepared FRAP solution was combined with 50 μL of either the FBPs sample or a standard compound (gallic acid). After incubating the mixture at 37 °C for 30 min, the absorbance of the samples was measured at 593 nm using a Varioskan LUX spectrophotometer from Thermo Fisher Scientific.

*2.5.4. Protein solubility*

The solubility of FBPs at various pHs was determined using the method described by Intarasirisawat, Benjakul, Visessanguan, and Wu (2012). The pH of FBPs in water was modified using either 6 N HCl or 6 N NaOH, covering a pH range of 2.0 to 10.0. The final concentration of FBPs was maintained at 10 mg/mL. The mixture was stirred for 90 min and subsequently subjected to centrifugation at 1800 ×*g* for 30 min. The protein content in the supernatant was determined using the Bradford protein assay. The solubility of FBPs was then calculated using the formula below.

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

*2.5.5. Emulsifying and foaming properties*

The methods described by Chin et al. (2022) were adopted to determine the emulsifying activity index (EAI), emulsion stability index (ESI), foaming capacity (FC) and foaming stability (FS) of FBPs. The following formulae were used to obtain the EAI, ESI, FC and FS values:

$$EAI\ \left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A_{0min}}{0.25 \times protein\ weight\ (g)}$$

$$ESI\ (min) = A_{0min} \times \frac{\Delta t}{\Delta A}$$

$$FC\ (\%) = \frac{(B - C)}{C} \times 100\%$$

$$FS\ (\%) = \frac{B_{30min} - C}{B_{0min} - C} \times 100\%$$

where A = absorbance value at 500 nm, Δt = 10 min, $\Delta A = A_{0min} - A_{10min}$, B = volume after whipping (mL) and C = volume before whipping (mL).

*2.5.6. Water and oil binding capacities*

The water and oil binding capacities of FBPs were evaluated by adding distilled water and soybean oil, respectively, to FBPs at a solid-liquid ratio of 1:40 (*w/v*). The samples were vortexed for 30 s and left undisturbed at room temperature for 30 min. Subsequently, centrifugation was performed at 4000 ×*g* for a duration of 25 min. The mass of the bound water or oil was determined by calculating the difference in mass between the samples before and after decanting the supernatant (Yu, Ahmedna, & Goktepe, 2007).

### 2.6. Statistical analysis

The analyses were conducted in triplicate, and all experimental data were analyzed using Minitab v.19 Statistical Software (Minitab Inc., Coventry, UK). One-way ANOVA followed by Tukey's test was performed for data analysis. The results were presented as mean values ± standard deviation at a 95% confidence interval.

## 3. Results and discussion

### 3.1. Protein content

BSG and fermented BSG were reported to contain 19–30% and 34% of crude protein, respectively (Chin et al., 2022; Macias-Garbett et al., 2021). Fig. 1 shows that there was a significant difference ($p < 0.05$) between the protein content in the extracts obtained via TPP and MATPP. The extract obtained from MATPP recovered 82.2% of FBPs and was about two times greater than that of the extract obtained via TPP (41.8%) alone. Our previous study showed that by using an ethanolic-alkali mixture, 66.2% of protein was recovered from FBPs (Chin et al., 2022). The MATPP extraction method used in this study successfully recovered a higher protein content from fermented BSG compared to that extracted using TPP and ethanolic-alkali mixture. Similar results were reported by Bedin, Netto, Bragagnolo, and Taranto (2019) and Phongthai, Lim, and Rawdkuen (2016) who managed to obtain a higher protein content from rice bran by 1.02–1.07-fold and 1.54-fold, respectively, using microwave-assisted extraction compared to conventional alkaline extraction. Varghese and Pare (2019) also found that microwave-assisted extraction improved the protein content of soymilk by 44.4% compared to conventional extraction. The protein content in the extract obtained through MATPP was enhanced by the application of microwaves, which are non-ionizing electromagnetic radiation. These microwaves induce the rotation of polar molecules within the fermented BSG at a consistent frequency, leading to molecular friction, the generation of heat, and an increase in temperature. This phenomenon ultimately contributes to the release and extraction of proteins, resulting

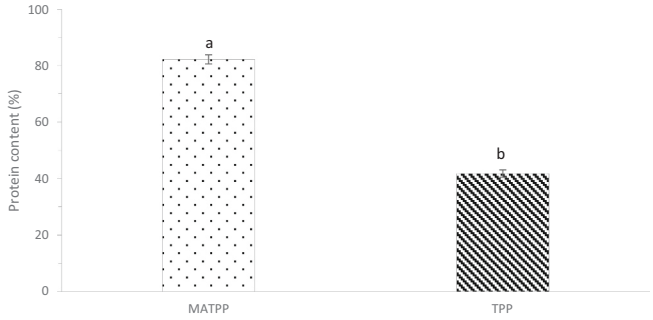


**Fig. 1.** Protein content of FBPs obtained via MATPP and TPP. Graph bars marked with different letters denote significantly difference ($p < 0.05$).

in an increased protein content in the extract (Chew, Chia, Lee, Zhu, & Show, 2019). The application of heat in the MATPP process affects the fermented BSG through two simultaneous mechanisms i.e., dipole rotation and ionic conduction. This combined action leads to the disruption of hydrogen bonds that are present in the cell wall structure. As a result, the porosity of the cell wall increases, enabling the solvent to penetrate more effectively into the cells. This enhanced penetration facilitates the efficient release of proteins into the solvent system, thereby facilitating the extraction process (Kumar et al., 2021). Since there was no alteration on the cell structure or membrane on the fermented BSG without microwave treatment, lesser proteins were released into the extracting system and hence, contributing to the lesser amount of protein in the extract.

### 3.2. Amino acid composition

The amino acid composition of FBPs obtained from TPP and MATPP are shown in Table 1. It can be noticed that microwave treatment did not significantly affect ($P > 0.05$) the amino acid profile of FBPs with glutamic acid (14.51–14.88%) and proline (9.28–9.33%) being the major amino acids of the hydrolysates. Similar results were observed in the studies reported by Chin et al. (2022) and Yu et al. (2020). It has been reported that BSG is abundant in hordeins which are also known as prolamins due to the presence of high proline and glutamine contents that make up 43% of the total BSG proteins, indicating that hordeins are the main storage proteins in barley (Ikram, Huang, Zhang, Wang, & Yin, 2017). It can also be seen in Table 1 that FBPs is a good source of both essential and nonessential amino acids. Chin et al. (2022) mentioned that hydrolysis of BSG proteins efficiently utilizes glutamine and proline, causing an increase in certain essential amino acids and consequently, improving the nutritional status of FBP. Besides, both types of FBPs analyzed in this study exhibited a relatively balanced ratio of hydrophobic to hydrophilic amino acids, suggesting the presence of an optimal equilibrium between surface hydrophilicity and hydrophobicity. Given that no significant differences were observed in the amino acid composition of the FBPs extracted using both TPP and MATPP techniques, the microwave application employed in the study could be deemed useful.

### 3.3. Molecular weight of FBPs

SDS-PAGE was used to determine the molecular weight of FBPs and the protein patterns are presented in Fig. 2. It can be noticed that the protein bands of MATPP-extracted FBPs (Fig. 2, lane b) were slightly deepened compared to those of TPP-extracted FBPs (Fig. 2, lane c), suggesting that more proteins were dissolved during extraction. Regardless of the extraction method used, FBPs exhibited protein subunits of molecular weight between 20 and 50 kDa with main bands of 40–50 kDa. Some very light bands of <20, 50–60 and 160–260 kDa were also observed in both FBPs extracted using TPP and MATPP. Similar findings were reported by Chin et al. (2022), Li, Yang, Coldea, and Zhao (2021) and Yu et al. (2020). As aforementioned, hordein (43%) is the major protein found in BSG. It is made up of A, B, C and D hordeins with molecular weights of about 15, 30–50, 55–80 and 95 kDa, respectively (Ikram et al., 2017; Li et al., 2021). Therefore, the bands in the range of 40–50 and 50–60 kDa could be attributed to the presence of B and C hordeins, respectively (Fig. 2). These results are in parallel to those reported in the previous studies which mentioned that B and C hordeins represent 70–80% and 10–20% of the BSG hordein fraction, respectively, while the remaining minor portions are composed of other hordein groups (Celus, Brijs, & Delcour, 2006; Chin et al., 2022; Li et al., 2021). The subunits with molecular weight of <20 kDa could be due to the presence of A hordein. In addition, the 40–50 kDA subunits might be ascribed to protein Z, a barley protein that survived the malting process with a molecular weight of about 43 kDa (Li et al., 2021). It is worth noting that there was no visual difference between the protein patterns exhibited by FBPs obtained from TPP and MATPP, indicating that microwave pretreatment did not alter the molecular weight of the extracted protein hydrolysates.

**Table 1**
Amino acid composition of FBPs obtained via MATPP and TPP.

| Amino acid | MATPP (%) | TPP (%) |
|---|---|---|
| Alanine | 4.49 ± 0.45[a] | 4.87 ± 0.14[a] |
| Glycine | 4.16 ± 0.13[a] | 4.20 ± 0.15[a] |
| Valine | 5.23 ± 0.08[a] | 5.32 ± 0.09[a] |
| Leucine | 8.33 ± 0.06[a] | 8.15 ± 0.09[a] |
| Isoleucine | 4.35 ± 0.08[a] | 4.11 ± 0.12[a] |
| Proline | 9.28 ± 0.14[a] | 9.33 ± 0.10[a] |
| Methionine | 1.85 ± 0.06[a] | 1.92 ± 0.05[a] |
| Serine | 5.14 ± 0.11[a] | 5.21 ± 0.09[a] |
| Threonine | 3.56 ± 0.07[a] | 3.52 ± 0.16[a] |
| Phenylalanine | 7.14 ± 0.10[a] | 7.23 ± 0.09[a] |
| Aspartic acid | 8.82 ± 0.12[a] | 8.72 ± 0.07[a] |
| Glutamic acid | 14.88 ± 0.67[a] | 14.51 ± 0.66[a] |
| Arginine | 8.62 ± 0.20[a] | 8.58 ± 0.13[a] |
| Lysine | 5.51 ± 0.20[a] | 5.58 ± 0.20[a] |
| Histidine | 3.57 ± 0.08[a] | 3.68 ± 0.09[a] |
| Tyrosine | 3.65 ± 0.10[a] | 3.62 ± 0.10[a] |
| Cystine | 1.43 ± 0.07[a] | 1.45 ± 10.07[a] |

Mean ± standard deviation values with similar letters within the same row are not significantly different (p > 0.05). MATPP and TPP denote microwave-assisted three phase partitioning and three phase partitioning, respectively.

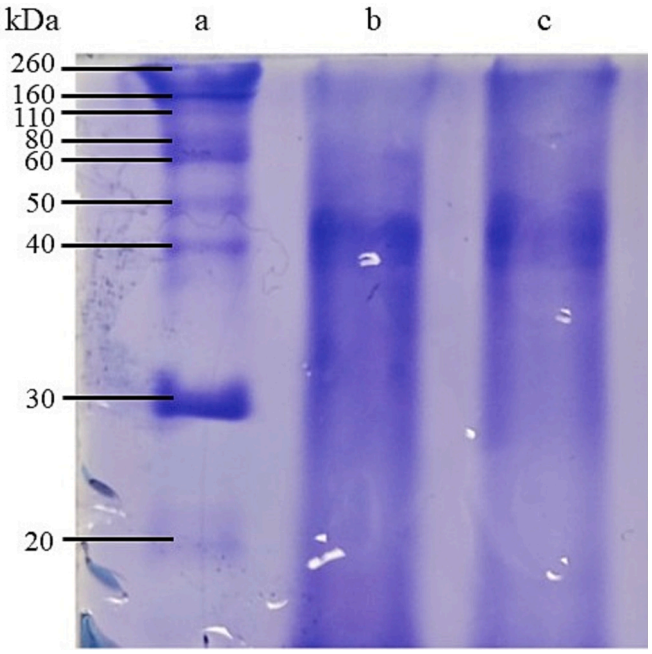


**Fig. 2.** SDS-PAGE of molecular weight marker (lane a) and FBPs obtained via MATPP (lane b) and TPP (lane c).

### 3.4. Antioxidant properties

Barley grain has been shown to be an excellent source of phenolic compounds such as phenolic acids, proanthocyannidins, tannins, flavonoids, and amino phenolic compounds, which contribute to its high antioxidant capacity (Meneses, Martins, Teixeira, & Mussatto, 2013). Hence, the total phenolic content (TPC) of TPP- and MATPP-extracted FBPs were investigated, and their antioxidant properties were evaluated based on FRAP and ABTS assays. The TPC of both the FBPs obtained via TPP and MATPP is shown in Fig. 3a. There was a significant difference ($p < 0.05$) between the FBPs obtained using different extraction method on the TPC, with MATPP-extracted FBPs showed a higher TPC

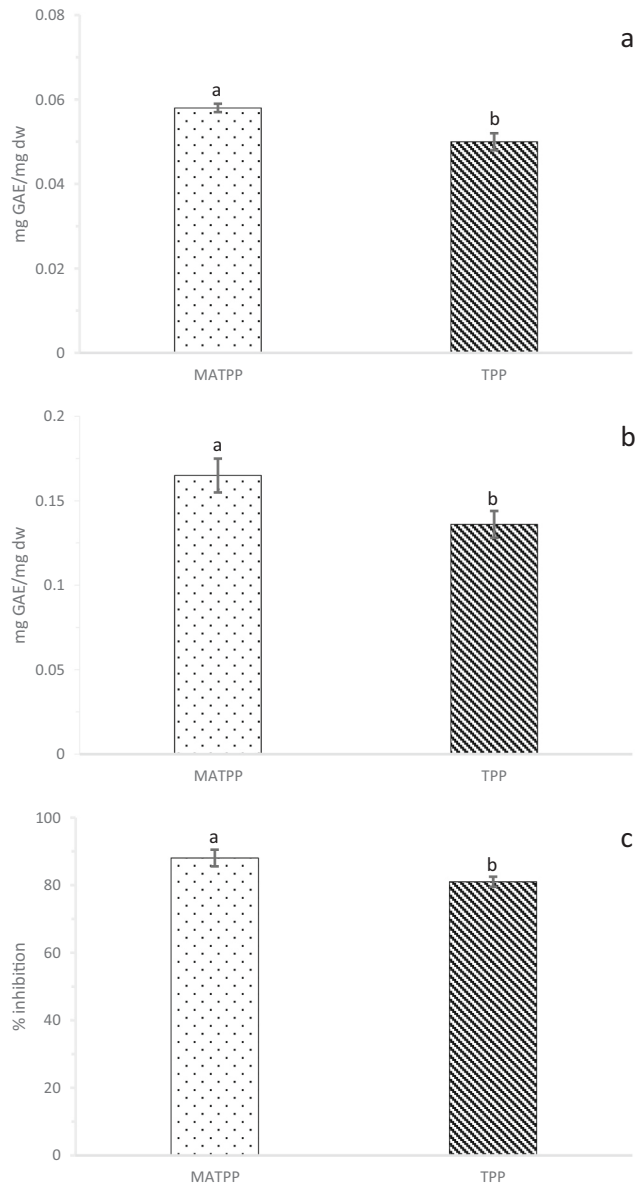


**Fig. 3.** Total phenolic content (TPC) (a) and antioxidant activities of FBPs obtained via MATPP and TPP based on FRAP (b) and ABTS (c) assays. Graph bars marked with different letters denote significantly different ($p < 0.05$).

(by 14%) compared to that of TPP-extracted FBPs. Similar trend was reported by Zago et al. (2022) who also found that the TPC of BSG increased significantly after it was treated with 600 W microwave for 30 min. Studies have shown that the content of gallic acid increased with microwave treatment which could be due to the heat produced in the process has the tendency to enhance the solubility of the compound and accelerate the extraction rate and subsequently, increasing the TPC of the extract (Patrignani, Brantsen, Awika, & Conforti, 2021). Similar effects were reported for caffeic acid, p-coumaric and ferulic acid with microwave treatment (Patrignani et al., 2021). The elevated TPC observed in FBPs extracted through MATPP can also be attributed to the thermal degradation of cell walls. This is because ferulic acid, which is commonly esterified to hemicellulose, has the potential to be released when subjected to heat (Patrignani et al., 2021). Additionally, the disruption of cell walls during the extraction process enhances the interaction between the extracting solvent and the plant material. This increased interaction promotes better penetration of the extracting solvent, resulting in a higher extraction yield (López-Linares, Campillo, Coca, Lucas, & García-Cubero, 2021). The greater antioxidant activity could also be explained by the formation of chlorogenic acids and melanoidins, the Maillard reaction products, formed by polymerization of furanose rings (Patrignani et al., 2021).

The FRAP assay utilized in this study measures the capacity of a compound to reduce ferric ions ($Fe^{3+}$) to ferrous ions ($Fe^{2+}$). On the other hand, the ABTS assay relies on the decolorization reaction that takes place when the radical cation $ABTS^{\bullet+}$ is reduced to its non-radical form, known as ABTS' (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)). The results from both FRAP and ABTS assays depicted in Fig. 3b and c, respectively, showed that MATPP-extracted FBPs had a significantly ($p < 0.05$) greater antioxidant activity compared to TPP-extracted FBPs, largely contributing to the higher TPC in MATPP-extracted FBPs. Similar findings were reported by Patrignani et al. (2021) who showed that microwave-treated BSG exhibited higher antioxidant activity. In addition to the aforementioned important antioxidant compounds formed after microwave treatment, the proteins, peptides, and amino acids synthesized during the microbial fermentation also contribute to the high antioxidant activity of FBPs (Chai et al., 2020).

### 3.5. Functional properties of FBPs

#### 3.5.1. Solubility

The effect of pH on the solubility of FBPs obtained from TPP and MATPP is shown in Fig. 4a. It can be noticed that, regardless of extraction process, FBPs were soluble over a wide range of pH. The solubility of FBPs was highly dependent on pH, and a higher solubility was attained at alkaline pH conditions (pH 8-pH 10). In general, the solubility of both FBPs decreased from pH 2 and attained their lowest solubility at pH 4 which is known to be the isoelectric point of barley proteins (Kotlar, Ponce, & Roura, 2013), and finally increased with an increase in pH. Similar pH dependent solubility trend was also reported in the works studied by Celus et al. (2007), Chin et al. (2022) and Kotlar et al. (2013). BSG proteins are known to have poor solubility (~8%) (Celus et al., 2007) and are contrast with the FBPs under study. The higher solubility of FBPs is largely due to the fungal fermentation process adopted in the study. Proteases secreted by *R. oligosporus* during fermentation hydrolyze globular BSG proteins into smaller peptides and amino acids with lower molecular weight, thereby facilitating the proteins to solubilize (Chai et al., 2020). Furthermore, during the fermentation process, the complex structure of BSG proteins undergoes unfolding, leading to the exposure of previously buried polar and nonpolar amino acid groups. These exposed groups have the ability to interact with water molecules through hydrogen bonds and electrostatic interactions. As a result, the solubility of the proteins increases due to the improved ability to interact with the solvent (Chin et al., 2022). The observed low solubility of FBPs at pH 4, which corresponds to their isoelectric point, could be attributed to minimal repulsion between the proteins. At the isoelectric point, the net charge of the proteins is zero, leading to reduced electrostatic repulsion among protein molecules. Consequently, this decreased repulsion can contribute to lower solubility (Intarasirisawat et al., 2012).

#### 3.5.2. Emulsifying properties

Fig. 4b and c show the emulsifying activity index (EAI) and emulsion stability index (ESI) of FBPs obtained from TPP and MATPP at three different pHs, respectively. Both TPP- and MATPP-extracted FBPs showed similar trends in both EAI and ESI where these hydrolysates exhibited the lowest values of EAI and ESI at pH 5 and higher values at pH 3 and pH 8. Higher values of EAI and ESI were also observed in MATPP-extracted FBPs compared to that of TPP-extracted FBPs. The findings of this study are in parallel to those reported by Chin et al. (2022) and Connolly, Piggott, and FitzGerald (2014) who mentioned that BSG hydrolysates exhibited a significantly higher values of EAI and ESI at alkaline pH conditions and is possibly due to the association between increased solubility and higher emulsifying properties. The

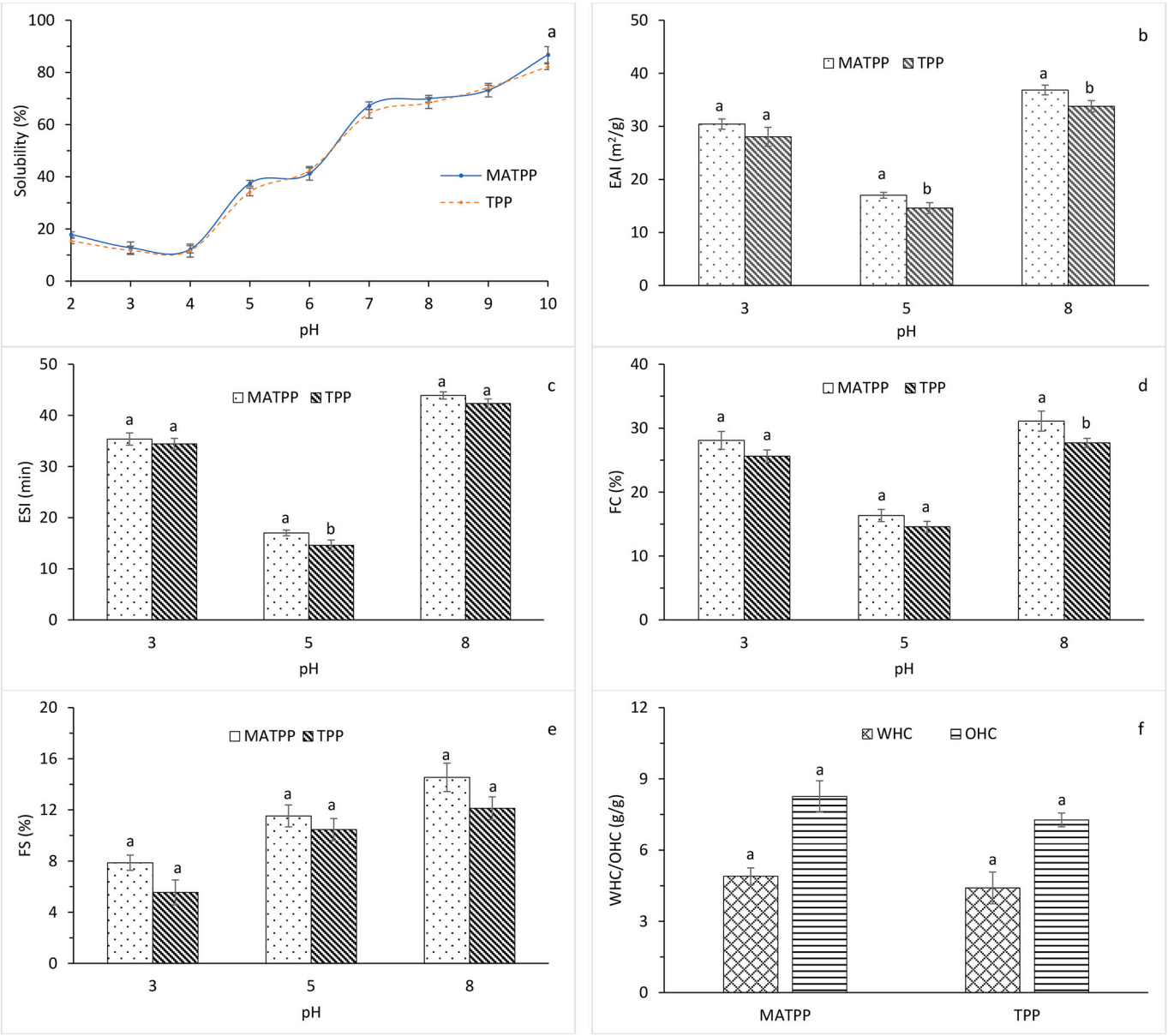


**Fig. 4.** Solubility (a), emulsifying activity index (EAI) (b), emulsion stability index (ESI) (c), foaming capacity (FC) (d), foaming stability (FS) (e), and water holding capacity (WHC) and oil holding capacity (OHC) (f) of FBPs obtained via MATPP and TPP. Graph bars marked with different letters denote significantly different ($p < 0.05$).

structural modification of BSG proteins during fermentation, which involves the liberation of low-molecular-weight peptides from their originally inert, globular parent proteins together with their higher solubility properties facilitate the diffusion and spread at oil-water interfaces. The exposed hydrophobic groups enhanced the interaction between proteins and lipids, thereby elevating the emulsifying properties of the resulting FBPs (Chai et al., 2020; Wu, Hettiarachchy, & Qi, 1998). Besides, the greater emulsifying properties exhibited by MATPP-extracted FBPs could be explained by the higher levels of protein found in the extract treated with microwave compared to that of the control (Fig. 1), indicating that MATPP is an efficient extraction technique.

### 3.5.3. Foaming properties

Fig. 4d and e depict the foaming capacity (FC) and foaming stability (FS) of FBPs extracted from TPP and MATPP. As can be seen, the foaming properties of FBPs are similar to other functional properties that are highly pH dependent. Both types of FBPs exhibited the lowest FC and FS at pH 5 and higher FC and FS at pH 3 and pH 8. The results corroborate with those reported by Chin et al. (2022), Connolly et al. (2014) and Wang et al. (2010). Regardless of extraction method, FBPs showed higher FC and FS at alkaline conditions. At higher pH values, the proteins undergo an increase in net charge, leading to a reduction in attractive hydrophobic forces. This change in charge can enhance protein flexibility, facilitating their diffusion to the air-water interface and enabling the encapsulation of air molecules (Connolly et al., 2014). Besides, the presence of hordeins and protein Z in BSG has been demonstrated to be foam-active proteins, thus, contributing to the strong foaming properties of FBPs (Li et al., 2021). It could also be observed that MATPP-extracted FBPs had better foaming properties than TPP-extracted FBPs, mainly again due to the higher content of protein found in the MATPP-extracted FBPs (Fig. 1).

### 3.5.4. Water and oil holding capacities

The water and oil holding capacities of FBPs were assessed to

determine its ability to bind these substances through its polar and nonpolar side chains and the results are shown in Fig. 4f. There was no significant difference ($p > 0.05$) on the water and oil holding capacities between FBPs extracted from TPP and MATPP. Both types of FBPs had relatively higher water (4.4–4.9 g/g) and oil (7.3–8.3 g/g) holding capacities compared to BSG proteins which have a water and oil holding capacities of <4 g/g (Chin et al., 2022). In addition, the FBPs under study demonstrated a notably higher oil holding capacity when compared to oil seed protein isolates, which are recognized for their typically high oil holding capacities with values below 4 g/g (Wang et al., 2010). The enhanced water and oil holding capacities of FBPs can be attributed to the increased presence of polar and nonpolar groups in the BSG proteins after microbial hydrolysis. This hydrolysis process leads to the generation of smaller peptides and amino acids, which in turn results in a larger number of polar and nonpolar groups and subsequently, increasing the exposure of the hydrophilic and hydrophobic groups to the water and oil interfaces (Chin et al., 2022; Wasswa, Tang, Gu, & Yuan, 2007).

## 4. Conclusion

In this study, TPP and MATPP were used for the first time to recover proteins from fermented BSG. The results showed that MATPP provided a significantly greater protein yield ($p < 0.05$) compared to TPP. Additionally, the chemical and functional analyses showed that MATPP did not significantly alter ($p > 0.05$) the amino acid composition, protein pattern, and some functional properties of FBPs. Moreover, MATPP was shown to enhance the antioxidant activities of FBPs. Based on these findings, it can be concluded that MATPP is a useful and efficient bioseparation technique for recovering proteins from fermented BSG due to its effectiveness, simplicity and speed. The results of this study can serve as a basis for future research aimed at increasing the success of adopting the MATPP bioseparation technique in protein recovery at the industrial level.

## Declaration of generative AI in scientific writing

The authors declare that no generative AI and AI-assisted technologies were used in the writing process.

## Author statement

We declare that no AI and no AI-assisted technologies were used in the writing process of this manuscript.

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

## Data availability

Data will be made available on request.

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