Valorisation of brewers' spent grains for food applications

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Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

23 May 2024

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Date

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Chin Yi Ling

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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This thesis contains material from 4 papers published in the following peer-reviewed journals in which I am listed as an author.

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- Chin, Y.L. conducted the experiments and prepared the manuscript draft
- Keppler, J.K. helped with conceptualization and reviewed the manuscript draft
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Contents

Chapter 1

Introduction

Globally, about 13% of the world's food is lost before reaching the retail level (FAO, 2021). Food losses and waste put unnecessary pressure on the environment and the natural resources that were used to produce these foods in the first place. With the world population projected to reach almost 10 billion in 2050, the demand for agricultural products is forecast to increase by 35-50% between 2012 and 2050 (FAO, 2019). In order to ensure our food security, there is an urgent need to minimise food losses and waste and make the most out of our limited resources. Furthermore, reducing food losses and waste will help to mitigate environmental impacts in terms of lowering greenhouse gas emissions and alleviating pressure on water and land resources (Nyhan, Sahin, Schmitz, Siegel, & Arendt, 2023). Besides the environmental benefits, interventions to reduce food loss and waste can lead to increased productivity and economic gains, for example, by decreasing the need for disposal and its associated costs (Ishangulyyev, Kim, & Lee, 2019). In this thesis, we focus on food losses generated from the brewing industry, in particular, the leftover barley grains from beer production, known as brewers' spent grains (BSG).

1.1. Generation of brewers' spent grains (BSG)

Barley is the main raw material used in beer production. It comprises of the germ, endosperm and grain coverings. The grain coverings are further subdivided into the aleurone layer, seed coat, pericarp and husk (Figure 1.1).

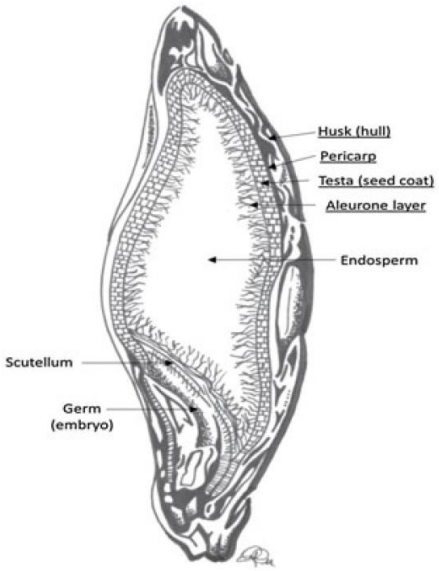


Figure 1.1. Cross-section of a barley kernel showing the grain coverings (underlined) that constitute BSG (taken from Lynch, Steffen, and Arendt (2016))

Prior to brewing, barley grains undergo a controlled germination process called malting, to promote the synthesis and activation of enzymes within the grains. More than 70% of the proteins in barley are broken down to smaller peptides by endoproteases (Osman et al., 2002).

In the brewery, malted barley is milled and mixed with water in the mash tun. The temperature is increased from 37 to 78°C to allow gelatinisation and hydrolysis of starch to fermentable sugars while proteases are deactivated. This enzymatic conversion stage, known as mashing, produces wort, which is a sugar-rich liquid containing degraded and solubilised proteins (Jaeger, Sahin, Nyhan, Zannini, & Arendt, 2023).

In the next step called lautering, the insoluble parts of the malted barley grain sediment towards the bottom of the mash tun and the wort is filtered. The filtered wort is subsequently used as the fermentation medium to produce beer, leaving behind a residual solid fraction known as brewers' spent grains (BSG) (Mussatto, Dragone, & Roberto, 2006). A simplified diagram of the brewing process is shown in Figure 1.2.

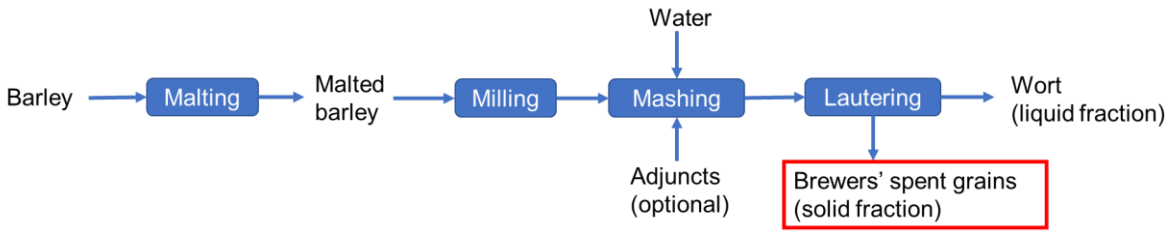


Figure 1.2. Simplified diagram of brewing process up till the generation of brewers' spent grains

BSG, which is primarily composed of the insoluble barley grain coverings, accounts for 85% of the total byproducts from the brewing industry (Nyhan et al., 2023). For every 100 litres of beer brewed, 20 kg of wet BSG is produced, and this amounts to 39 million tonnes of BSG generated worldwide on a yearly basis (Lynch et al., 2016). Despite the staggering amount of BSG produced, the majority of BSG is currently used as animal feed or disposed of, as the high moisture content and the presence of proteins and sugars makes it a very unstable material that is liable to rapid deterioration by microbial activity (Mussatto, 2014). It has been reported that every ton of BSG disposed to the environment releases 513 kg carbon dioxide equivalent of greenhouse gases (Kavalopoulos et al., 2021), therefore more sustainable practices to valorise BSG is necessary.

Recent studies have started to recognise that BSG is a valuable byproduct as it contains high nutritional value and health-promoting bioactive components including arabinoxylans, phenolic compounds and protein hydrolysates (Lynch et al., 2016; Nyhan et al., 2023). As such, there has been ongoing research to incorporate BSG directly into food products like bread, pasta, yogurt and sausages (Naibaho et al., 2022; Nocente, Taddei, Galassi, & Gazza, 2019; Talens et al., 2022; Waters, Jacob, Titze, Arendt, & Zannini, 2012; Yitayew, Moges, & Satheesh, 2022). Thanks to the high content of fibre, protein and minerals present, the incorporation of BSG in the human diet was demonstrated to increase prebiotic activity,

improve glycemic control, increase faecal weight and fat excretion, decrease gallstone incidence and reduce plasma cholesterol levels (Brennan & Cleary, 2005; Fastnaught, 2001; Nyhan et al., 2023). However, the implementation of BSG into foods was shown to negatively impact the technofunctional and sensorial characteristics of the food matrices and reduces the acceptability of the final product due to changes in physical properties, particularly with respect to the (brown) colour and texture that is largely attributed to the presence of fibres (Combest & Warren, 2018; Naibaho et al., 2022). Most studies have only been able to partially replace wheat flour in bread and pasta up to about 10% for an acceptable sensory profile (Nocente et al., 2019; Waters et al., 2012; Yitayew et al., 2022). With the supply of BSG far exceeding its demand, further processing strategies to increase the functionality and applications of BSG in food should be explored.

1.2. Composition of BSG

BSG is mainly composed of fibres and proteins, with small amounts of lipids, phenolics, vitamins and minerals. The starch content is negligible, as most of it is degraded during mashing. Table 1.1 shows the composition of BSG compared across various literature. The composition of BSG varies across studies due to differences in barley variety, harvest time, malting and mashing processes and the presence of adjuncts during the brewing process (Lynch et al., 2016). Despite these differences, majority of BSG is made up of about 60-70% fibres and 20-30% proteins, with minor amounts of lipid and ash.

Table 1.1. Composition of BSG compared across various literature. n.d. means not determined.

Composition (% dry weight)	Niemi, Faulds, et al. (2012)	Lynch et al. (2021)	Zeko-Pivač et al. (2022)	Sganzerla, Ampese, Mussatto, and Forster-Carneiro (2021)
Cellulose	20.9	43.0*	21.42	17.9
Arabinoxylan	22.9	21.9	30.6	35.7
Lignin	19.4	n.d.	11.41	17.8
Protein	23.3	31.0	20.93	19.2
Lipid	7.8	9.7	8.52	n.d.
Ash	4.9	3.6	3.68	3.9

* reported as insoluble high molecular weight fibre which can include lignin

1.2.1. Fibres

As the largest component in BSG, fibres make up about 60-70% of the whole material. Of these fibres, there is about 17% cellulose, 28% hemicellulose and 28% lignin, which collectively make up the lignocellulosic matrix of BSG (Mussatto et al., 2006). The chemical

structures of cellulose, hemicellulose (in particular, arabinoxylan) and lignin are presented in Figure 1.3.

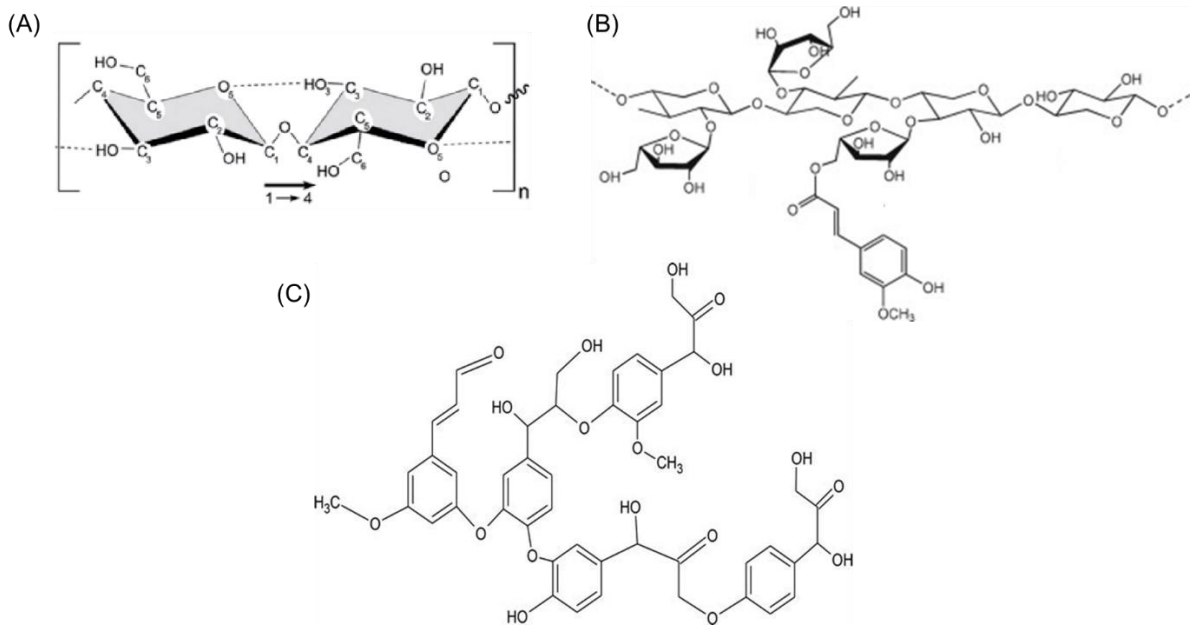


Figure 1.3. Chemical structure of (A) cellulose, (B) arabinoxylan and (C) lignin. Images were taken from Ilyas, Sapuan, Ishak, Zainudin, and Atikah (2018), Buanafina (2009) and Zahed et al. (2018) respectively.

Cellulose is a highly stable, semi-crystalline homogenous polymer consisting of glucose units joined by glycosidic linkages, while hemicellulose is a heterogeneous polymer made up of five-carbon and six-carbon monomeric residues (Anwar, Gulfraz, & Irshad, 2014). In cereals like BSG, arabinoxylan is the main non-cellulose polysaccharide with a linear backbone of xylose units linked by glycosidic bonds, which can be substituted with arabinose residues, where ferulic acid can be esterified to arabinose. BSG arabinoxylans were also found to have arabinose residues at the terminals that may allow for additional substituted groups to be present (Coelho, Rocha, Moreira, Domingues, & Coimbra, 2016). Arabinoxylan chains are cross-linked to one another via diferulic acid bridges and are attached to cellulose fibrils by hydrogen bonding (Mandalari et al., 2005). Lignin, a polyphenolic macromolecule of complex structure, is formed by phenylpropane units linked by ether bonds and is covalently linked to the polysaccharides through ether and/or ester bonds (Lv et al., 2013).

With all the fibre components together, cellulose and hemicellulose contribute to the structure of plant cell walls by providing mechanical strength while lignin forms a protective seal around cellulose and hemicellulose to shield them from physical and biological disturbances (Bilal et al., 2020). The difficulty in separating lignin from the non-starch polysaccharides accounts for the recalcitrant nature of the lignocellulosic biomass.

1.2.2. Proteins

Besides fibres, there is about 20-30% protein in BSG (Mussatto et al., 2006). Kemppainen, Rommi, Holopainen, and Kruus (2016) found that a substantial proportion of protein is found in aleuronic cells which appear to remain relatively intact after mashing. The low solubility of proteins in BSG may be due to association between cellulosic material and proteins (Crowe, Alli, & Baker, 1985) as well as disulfide bond formation leading to protein aggregation during brewing (Celus, Brijs, & Delcour, 2006).

In barley, proteins are classified based on their sequential extractabilities (Osborne, 1924), comprising of albumins, globulins, prolamins (hordeins) and glutelins. Albumins and globulins, which are water-soluble and salt-soluble respectively, are solubilised in the wort during mashing and only a small amount remains in BSG, and the majority of the BSG proteins are the hordeins and glutelins. Hordein, which is the main barley storage protein that is soluble in alcohol and alkalis especially in the presence of a reducing agent (Shewry, Field, Kirkman, Faulks, & Miflin, 1980), make up 43% of the total barley grain proteins (Celus et al., 2006) and is divided into 5 groups (A, B, C, D and γ hordeins) based on their electrophoretic mobilities and amino acid compositions. In BSG, B and C hordeins represent 70-80% and 10-20% of the hordein fraction respectively, while A, D and γ-hordeins make up less than 5% of the total hordein fraction (Shewry, 1993; Tatham & Shewry, 1995). C and some B hordeins appear as monomers, while most low molecular weight B hordeins and high molecular weight D hordeins are linked by interchain disulfide bridges. Glutelins, the second most abundant fraction in barley storage proteins after hordein (Lâsztity, 2017), are soluble in dilute alkali, detergents, and/or chaotropic agents usually in the presence of a reducing agent. Little is known about glutelin proteins, as it is difficult to prepare an undenatured glutelin fraction free of hordein (Celus et al., 2006).

During malting, disulfide bonds are reduced and some B and D hordeins are proteolytically degraded by enzymes secreted in the endosperm into albumins and globulins (Baxter, 1981). In the mashing process, an extended disulfide bonded network of gel protein composed of B hordeins in which C hordeins are entrapped (Celus et al., 2006). An earlier study by (Moonen, Graveland, & Muts, 1987) also highlighted that an impenetrable complex is formed between the residual high molecular weight aggregates composed of proteins and carbohydrates (gelprotein) in the malt and the glutelins.

In terms of amino acid profile, around 40% of the amino acids in barley hordeins and glutelins are hydrophobic, corresponding to the presence of proline, leucine, valine and alanine next to a large amount of hydrophilic amino acid glutamine (Wang et al., 2010). This balanced ratio of polar and non-polar amino acid endows barley proteins with good emulsifying properties

(Zhao, Tian, & Chen, 2010; Zhao, Tian, & Chen, 2011). In a study by Boostani et al. (2019), hordein-based particles were shown to have a significantly higher physical stability as a triphasic Pickering emulsion compared to zein-based particles.

1.2.3. Other valuable fractions

Apart from fibres and proteins, the lipids in BSG (typically less than 10%) were shown to be a source of valuable phytochemicals, with fatty acids, triglycerides and phytosterols being the predominant compounds with antioxidant potential (del Río, Prinsen, & Gutiérrez, 2013; Niemi, Tamminen, et al., 2012; Parekh, Khanvilkar, & Naik, 2017). In addition, there is an abundance of phenolic compounds in BSG, namely, ferulic and p-coumaric acid, with concentrations ranging from 1860 to 1948 mg/g and from 565 to 794 mg/g, respectively (McCarthy, O'Callaghan, Piggott, FitzGerald, & O'Brien, 2013). A previous study demonstrated that the phenolic compounds in barley exist mostly in an insoluble form, bounded to lignans, arabinoxylans and proteins (Madhujith & Shahidi, 2009). Phenolic extracts from BSG have shown bioactive properties such as antioxidant, DNA-protective and antimutagenic activities, antimicrobial properties and immunomodulatory effects (McCarthy et al., 2012). In addition, other studies have also shown that BSG can be an important source of vitamins and minerals (Huige, 2006; Pomeranz & Dikeman, 1976).

1.3. BSG valorisation approaches

Being available throughout the year at low or no cost at all, BSG presents itself as a widely available raw material that can be considered in many types of applications. As introduced in the earlier section, BSG still contains a substantial amount of valuable nutrients that are unexploited and should be recovered. Therefore, more efforts are needed to divert this resource away from landfills. In this section, various approaches to valorise BSG are briefly summarised.

1.3.1. Separation of useful components

The wide variety of useful fractions in BSG such as proteins, fibres and phenolic compounds has invited researchers to develop methods to valorise BSG. In particular, the latest shift towards alternative sources of proteins has encouraged research in the recovery of proteins from sustainable sources like agricultural residues or byproducts from food processing (Kavalopoulos et al., 2021). However, the resistance of the lignocellulosic BSG to deconstruction poses a challenge for nutrient recovery of BSG (Pabbathi et al., 2022), therefore pretreatment is often necessary. Physical, chemical, biological methods or a combination of these are applied to initiate structural and compositional changes that improve the accessibility of nutrients.

1.3.1.1. Pretreatment of lignocellulosic material

Physical pretreatment can be referred to as mechanical comminution such as chipping, milling and grinding, which leads to a reduction of particle size and disruption of the cell wall structure. This often results in an increased surface area, a depolymerised structure, reduced cellulose crystallinity and an improved enzymatic accessibility and digestibility. An earlier study by Jay et al. (2008) suggested that dried and milled BSG which passed through 106 and 55 µm sieves was fine, crumb-like and enriched in protein and starch with a decrease in cell wall polymers while the material retained on sieves of 500, 250 and 150 µm was rich in arabinoxylans. This suggested that the physical separation of BSG by particle sizes after comminution could be used for fractionating various components. Later, a study by Niemi, Faulds, et al. (2012) compared dry and wet milling of BSG and found that wet milled BSG produced more uniform particle size distributions than dry milling with barely any visible cellular structures when viewed under a light microscope, suggesting that a greater level of disruption was achieved with wet than dry milling. More recent studies have also proven that it is possible to obtain separate fractions that are protein-rich and fibre-rich by wet milling (He et al., 2019; Ibbett, White, Tucker, & Foster, 2019). Nonetheless, regardless of the type of milling used, a significant proportion of the material remained insoluble, indicating the recalcitrance of the complex cell wall structure (Niemi, Faulds, et al., 2012).

Chemical methods to pretreat BSG may involve acids, alkalis or organic solvents. Acid pre-treatment works on the basis of hydrolysing the hemicellulosic fraction and converting them to fermentable sugars. An example of a dilute acid hydrolysis was carried out by Mussatto and Roberto (2005). They used diluted sulfuric acid to hydrolyse BSG and produced a liquor rich in xylose. More commonly, alkaline treatment is used instead due to its effectiveness and lower production of degradation products (Fernández-Delgado et al., 2019; Kan, Zhang, Tong, & Wang, 2018; Mosier et al., 2005; Singhvi & Gokhale, 2019). Alkalis delignify biomass by saponifying the intermolecular ester bonds between lignin and hemicelluloses and solubilize lignin (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010; Balan, 2014; Sun & Cheng, 2002). However, alkalis are not totally selective for lignin as proteins, phenolics and hemicellulose are also better soluble in alkalis (Avgerinos & Wang, 1983; Balan, 2014; Brodeur et al., 2011; Kan et al., 2018). This leads to complex purification steps if these latter fractions are of interest.

Biological pretreatment involves the direct use of biological agents like enzymes or microorganisms that contain suitable enzymes to break down the lignocellulosic material. Enzymes are highly specific and only require mild conditions to be activated to break down lignocellulosic material, but the use of proprietary enzymes can be costly, especially in large-scale processing. A more cost-effective 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, Bountas, Banat, & Kanellakl, 2007; Canedo, de Paula, da Silva, & Vendruscolo, 2016; Cooray & Chen, 2018; Marcus & Fox, 2021). The added benefit of solid-state fermentation with fungi is the accumulation of protein in the form of fungal biomass.

1.3.1.2. Extraction of proteins

Following pretreatment of BSG, the fraction of interest can be extracted by water, enzymes or other chemicals. As proteins were the main focus of this thesis, therefore only the extraction of proteins from BSG will be discussed in this section.

Although most of BSG is derived from barley grains, there are some differences in terms of protein extractability, as previously discussed in section 1.2.2. Therefore, BSG protein extraction is likely more difficult than from barley due to its processing history. Harsher extraction methods may have to be employed to separate the proteins from its matrix. The most effective method for protein isolation is by alkaline extraction followed by isoelectric precipitation (Celus, Brijs, & Delcour, 2007; Connolly, Piggott, & FitzGerald, 2013; Vieira et al., 2014), but this fractionation is challenged by the co-extraction of hemicellulose, phenolics and lignin (Rommi, Niemi, Kemppainen, & Kruus, 2018). Proteins and these alkali-soluble components could interact under alkaline conditions, for example, via the formation of covalent protein-phenol interactions, and lead to structural changes in the protein which impacts its functionality.

A more environmentally friendly method is to employ an organic solvent like ethanol for protein extraction. Previous work by Celus et al. (2006) and Wang et al. (2010) employed an aqueous alcohol mixture to isolate hordein from BSG and barley. Addition of a reducing agent helps to overcome the disulfide bonds present in the aggregates, resulting in a higher protein yield. However, although ethanol can be recovered by distillation and is a food-grade solvent, recovery can be energy-intensive and losses of ethanol can be significant (Jankowiak, Jonkman, Rossier-Miranda, van der Goot, & Boom, 2014).

Another approach is to solubilise the protein fractions by using commercial peptidases and obtain protein hydrolysates which may have improved techno-functional properties, as demonstrated by previous studies (Celus et al., 2007; Connolly et al., 2019; Treimo, Aspmo, Eijsink, & Horn, 2008b). However, hydrolysates may not necessarily always have better properties than intact proteins. The functionality of hydrolysed proteins depend on the cleavage specificity of the enzymes used and the degree of hydrolysis. It was concluded by Smulders (2000) that peptides need a certain minimum molar mass to be able to stabilise an emulsion effectively. More specifically, a large effective molar mass may lead to poor emulsion formation while a low molar mass implies lesser emulsion stability against coalescence.

Evidently, many factors have to be considered when useful components are selectively fractionated, including the temperature, time, and solid-liquid ratios. In a practical context, enrichment alone may be sufficient to achieve a desired functionality without maximising protein purity. Previous studies have shown that mildly purified mixtures containing minor amounts of proteins may be sufficient to exhibit desired properties such as emulsification (Karefyllakis, Octaviana, van der Goot, & Nikiforidis, 2019; Sridharan, Meinders, Bitter, & Nikiforidis, 2020b), and extraction may not always be necessary. In fact, the influence of other components and their interactions in mixtures could be interesting to explore when extraction of purified ingredients is avoided.

1.3.2. Full utilisation of BSG for food

As a nutrient-rich byproduct, BSG has been used as a low-cost substrate for the cultivation of microorganisms. Several studies successfully attempted to grow edible mushrooms such as *Aspergillus*, *Pleurotus*, *Lentinus*, *Trametes*, *Neurospora* and *Agrocybe* on BSG. Not only did the elevated moisture content, presence of proteins and sugars provide favourable conditions for fungal growth, but some physical properties of the material such as particle size, porosity and water holding capacities also influenced the growth of these microorganisms (Szponar, Pawlik, Gamian, & Dey, 2003; Wang, Sakoda, & Suzuki, 2001). If microorganisms that are Generally Recognised As Safe (GRAS) are deployed, the entire fermented product can be consumed as a functional food ingredient (Cooray & Chen, 2018; Costa et al., 2021; Tan, Mok, & Chen, 2020), or in other cases, the microorganisms are capable of secreting valuable molecules like enzymes or biosurfactants after cultivation on BSG (Bogar, Szakacs, Tengerdy, Linden, & Pandey, 2002; Moshtagh, Hawboldt, & Zhang, 2018; Tišma, Jurić, Bucić-Kojić, Panjičko, & Planinić, 2018). Moreover, microorganisms can also digest BSG fibres like arabinoxylans to arabino-xylo-oligosaccharide (AXOS) which is a prebiotic (Sajib et al., 2018).

1.3.3. Non-food uses of BSG

Due to the high insoluble fibre content in BSG, various non-food uses of BSG have been explored. Besides that, BSG has been incorporated in bricks, as fillers in wood polymer composites, as a raw material in paper manufacturing or as a biodegradable packaging material (Ferreira, Martins, Carvalho, & Magalhães, 2019; Hejna et al., 2021; Ishiwaki, Murayama, Awayama, Kanauchi, & Sato, 2000; Russ, Mörtel, & Meyer-Pittroff, 2005). As a lignocellulosic material, studies by Kezerle, Velic, Hasenay, and Kovacevic (2018) and Pedro Silva et al. (2004) considered the use of BSG as adsorbent for removing synthetic dyes from wastewaters, with removal percentages reaching over 90%. Application as a biosorbent to remove metallic ions such as iron and manganese in groundwater and surface water have been successful as well (Fontana, Peterson, & Cechinel, 2018). BSG is also used for energy production through direct combustion or biogas and bioethanol production (Arauzo et al., 2019;

Čater, Fanedl, Malovrh, & Marinšek Logar, 2015; Liguori, Soccol, Porto de Souza Vandenberghe, Woiciechowski, & Faraco, 2015), although this is hampered by the high water content just after brewing.

1.4. Aim and outline of this thesis

As a versatile and food-safe raw material, BSG has the potential to be reused as a food ingredient. From an economic and environmental point of view, upcycling BSG can help to address the ongoing global food crisis and decrease pressure on our natural resources (Chetrariu & Dabija, 2020). Therefore, the overall aim of this work is to explore the possibilities of processing BSG to attain desirable functionalities that are relevant in the food industry, with a main focus on the proteins in BSG. A key aspect is to understand how processing changes the composition, structure and function of the resulting ingredients. An underlying hypothesis is that a high degree of refining may not always be necessary for functionality, especially since food exists as a heterogeneous mixture. In this thesis, chemical, physical and biological methods are adopted to process BSG as described in Figure 1.4. The sub-objective of each chapter is stated in the following paragraphs.

Existing literature has shown that BSG proteins can be successfully extracted with various methods including the use of alkali, aqueous ethanol and enzymes such as proteases and carbohydrases. However, each of these methods come with their disadvantages: alkaline conditions can co-extract proteins with arabinoxylans and phenolics, aqueous ethanol should be used with a reducing agent due to the disulfide linkages in the protein, and enzymatic methods can hydrolyse proteins entirely into short-chain peptides that have no technofunctionality. Therefore, in **Chapter 2** we compare the effect of these extraction methods on the composition, structure and function of the extracted protein. For the enzymatic extraction, a commercial BSG protein isolate extracted by proprietary enzymes is used. Proximate analysis is performed, and structural differences are analysed by spectroscopy and gel electrophoresis. The physicochemical and functional properties including surface hydrophobicity, solubility, water and oil holding capacity, emulsifying properties, gelling capacity and antioxidant activity are evaluated. Potential applications based on the changes in composition, structure and function are proposed.

Given that BSG is generated in the brewery in a wet state, it is interesting to explore the possibility of using wet milling to process BSG and explore the functionality of milled BSG. This helps to avoid the need for energy-intensive drying and large amounts of BSG can be processed at once. Therefore, **Chapter 3** investigates the use of colloid milling to physically treat BSG and evaluates the emulsifying abilities of the milled BSG and its insoluble fraction. The dispersions are first characterised based on their composition, physical behaviour,

particle size, morphology and interfacial properties. Subsequently, emulsions are prepared and assessed by their droplet sizes, charges, protein surface load, microstructure and creaming stability over a period of 10 days.

Current studies have shown that enzymatic hydrolysis is effective in breaking down the recalcitrant structure of BSG, but a cheaper alternative is via fermentation. **Chapter 4** examines the biological pretreatment of BSG via microbial fermentation and its effects on the resulting protein and hydrolysates. A food-grade fungus is cultivated on BSG to break down the lignocellulosic matrix by secreting proteases and carbohydrases that enable the release of nutrients from the cell wall material. BSG proteins are extracted using an ethanolic-alkali mixture with subsequent isoelectric precipitation and evaporative removal of ethanol. The protein extracts are characterised based on amino acid composition, degree of hydrolysis and molecular weight. Functional properties including protein solubility, emulsifying and foaming properties as well as water and oil binding capacities are evaluated. The bioactivity and cytotoxicity of the protein extracts are also determined by antioxidant and viability assays respectively. Finally, the extracts are used to prepare mayonnaise as a proof-of-concept regarding its potential as a plant-based emulsifier.

Chapter 5 concludes with a general discussion of all results presented in the thesis. It starts with a summary of the main findings, after which they are put into a wider perspective and important considerations of valorisation are discussed. The chapter ends with an outlook on future scientific research on fractionation strategies and other possible applications of BSG in food.

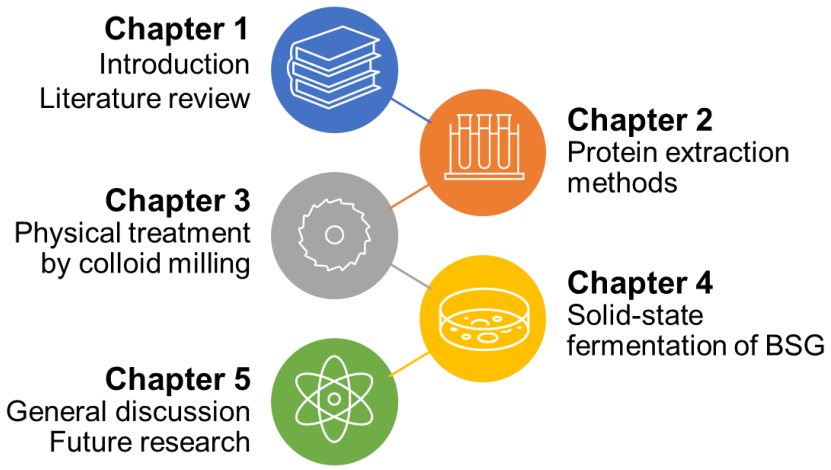


Figure 1.4. Schematic overview of the thesis outline

Chapter 2

Brewers’ spent grain proteins: the extraction method determines the functional properties

This chapter is modified from: Chin, Y. L., Keppler, J. K., Dinani, S. T., Chen, W. N., & Boom, R. (2024). Brewers' spent grain proteins: The extraction method determines the functional properties. *Innovative Food Science & Emerging Technologies, 94,* 103666. doi:https://doi.org/10.1016/j.ifset.2024.103666

Abstract

Brewers' spent grains (BSG) are a major byproduct from the brewing industry that are currently discarded or used as animal feed. This study systematically explored the effects of BSG protein extraction methods using alkali, ethanol or enzymes on the protein's structure, composition and function. Proteins extracted at pH 12 were partially unfolded and glutelin-rich while those extracted by 55% ethanol containing 2-mercaptoethanol were highly aggregated and hordein-rich. Enzymatic-assisted extraction resulted in peptides below 10 kDa that had no distinct structural elements. Being completely water-soluble, these peptides gave good emulsifying properties (activity: 83 m^2/g protein; stability: 35 min) and a high antioxidant activity. On the other hand, alkali-extracted and ethanol-extracted proteins were mostly insoluble but exhibited high water holding capacities (2.5-4.0 g/g) that enabled gelation. The results highlighted that selection of the extraction method is critical, as the composition, structure and function of proteins are modified, which affects its potential applications.

Industrial relevance

As a major side stream from the production of beer, brewers' spent grains are present in huge amounts. These residues are a promising source of alternative proteins, but before separating them, it is important to first consider an extraction method that does not compromise on its functionality. This study offered insights into the influence of extraction methods on the structure and function of the resulting protein concentrates as well as its future applications.

2.1. Introduction

Fuelled by the need to reduce environmental waste, the recovery of valuable components from agricultural residues and byproducts from food processing has become increasingly relevant. Coupled with the shift towards alternative sources of proteins in recent years, it would be desirable to recover proteins from food waste residues. Brewers' spent grains (BSG), a byproduct of the beer brewing industry, make up 85% of brewing waste and largely consist of insoluble proteins (20%), fibres (70%) and an abundance of phenolic compounds (Mussatto et al., 2006). As the mashed barley residues that are generated after starch is converted to sugars, BSG is a major side stream that that can be further exploited for other applications. Annually, an estimated 39 million tonnes of BSG is generated worldwide (Macias-Garbett, Serna-Hernández, Sosa-Hernández, & Parra-Saldívar, 2021), yet most of this waste is currently discarded or used as animal feed due to microbial instability caused by its high moisture content (Mussatto et al., 2006).

Utilisation of the BSG protein fraction is not straightforward as the proteins that remain in BSG are by definition insoluble (Shewry et al., 1980). During lautering in beer brewing, soluble proteins are removed as the wort, leaving behind insoluble proteins that remain in BSG (see Figure 1.1). These proteins are mostly storage proteins known as hordeins and glutelins. Hordeins, which in their native form are alcohol and alkali-soluble, make up about 35-55% of the total barley grain proteins and can be further subdivided into A, B, C, D and γ hordeins based on their electrophoretic mobility and amino acid compositions (Celus et al., 2006). Glutelins, which are in native form also alkali- but not alcohol-soluble, constitute 23% of the total barley proteins. Although BSG typically originates from barley, there are differences in terms of protein extractability as a result of the brewing process. During the mashing process of beer brewing, a complex is formed between the residual high molecular weight aggregates composed of proteins and carbohydrates (gelprotein) in the malt and the glutelins that form an impenetrable layer on BSG (Moonen et al., 1987). Celus et al. (2006) showed that mashing likely induces the formation of disulfide bonds, resulting in strong protein aggregation. Therefore, protein extraction from a byproduct like BSG is more challenging than from unprocessed barley due to the physical and thermal processing history. More extreme extraction strategies, for example, a longer extraction time or harsher chemicals, may thus be necessary to separate the proteins from its matrix.

Alkaline extraction is one of the most common ways to fractionate BSG proteins (Connolly et al., 2013). At an alkaline pH, the lignocellulosic BSG matrix is partially broken up and proteins in the system attain a net negative charge. The increased repulsion within and between polypeptide chains lead to enhanced protein solubilisation. Adjustment of the pH of the system to the isoelectric point of the proteins, causes the proteins to become insoluble and precipitate.

The preference for alkali extraction of protein stems from its high extraction yield of about 82-85% (Vieira et al., 2014). However, this method comes at the expense of changes in the protein's structure, composition and function (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015; Yang, Zamani, Liang, & Chen, 2021), which were not extensively investigated in previous studies but are critical when deciding on suitable applications for these proteins. Furthermore, alkalis are capable of solubilising other components in a lignocellulosic matrix including hemicellulose and lignin (Vieira et al., 2014), resulting in a compositional change of the fractions which may influence its functionality.

Owing to the limited solubility of BSG proteins, another existing method is via enzymatic action, which involves the use of proteases and carbohydrases directly on BSG to open its matrix and release peptides, or to extract proteins by alkali first followed by enzymatic hydrolysis (Celus et al., 2007; Connolly et al., 2019). The resulting protein hydrolysates, containing 66-77% protein and a yield as high as 92% (Celus et al., 2007), showed increased solubility and improved emulsifying and foaming properties when compared to the alkali-extracted protein concentrate. However, the use of enzymes could be costly and difficult to scale industrially. A third option, as demonstrated by Celus et al. (2006), is to use an aqueous alcohol mixture containing a reducing agent to reduce the disulfide bridges between hordeins and solubilise these proteins. A resulting extraction yield of about 12% was obtained but the functional properties of the resulting BSG protein concentrate were not examined. In addition, food-grade reagents were not employed in the abovementioned literature, thereby restricting its applications in food.

Several studies showed that the choice of extraction method significantly affects the protein's composition, structure and function. Yang et al. (2021) demonstrated that alkaline extraction of pea protein had more profound impact on the protein conformation than salt extraction and altered its gelling properties. Similar results were found by Hadnađev et al. (2018) with hempseed meal protein isolates, in which the structural differences in the protein secondary structure impacted the water retention capacity. From this aspect, it is important to select a suitable protein extraction method based on its desired functionality and intended application before optimising the extraction process to attain desirable yields.

To the best of our knowledge, few studies adopted aqueous alcohol protein extraction for BSG and no work has yet been performed to compare BSG proteins obtained by different extraction techniques. While there are other recent technologies such as ultrasound, microwave or pulsed electric field that aim to improve extraction yields or reduce extraction time, the focus of this work is to examine how solvents modify the protein's structure and composition. Given the existing information, we hypothesise that different ways of extraction will produce protein

concentrates that vary in composition, structure and functional properties. Therefore, the objective of this work is to systematically explore how the extraction method influences the composition, structure and function of protein from BSG. The proteins were extracted separately using alkaline extraction and aqueous ethanol. A commercial BSG protein hydrolysate was compared. Proximate analysis of the various samples was performed, and their structures were analysed by spectroscopy and electrophoresis. The physicochemical and technofunctional properties were also evaluated. Finally, potential food applications pertaining to the composition, structure and function of BSG-derived protein fractions were discussed.

2.2. Materials and methods

2.2.1. Materials

BSG was kindly supplied by Asia Pacific Breweries Pte Ltd (Singapore) and stored in polyethylene bags at -20 ^{o}C before freeze-drying (FreeZone 2.5, Labconco, Kansas, US) at -50 ^{o}C, 0.01 mbar for 5 days. The freeze-dried BSG was milled in a rotor mill (Pulverisette 14, Fritsch, Idar-Oberstein, Germany) and passed through a 400 µm air-jet sieve (e200LS, Hosokawa Alpine, Augsburg, Germany). Barley protein isolate (BPI), obtained by pH-adjusted enzymatic extraction (confidential information), was kindly supplied by EverGrain LLC (St. Louis, USA). Soybean oil containing 92 g fat per 100 ml was purchased from a local supermarket (The Netherlands). Acetone and hexane (100%) were purchased from Actu-All Chemicals B.V. (Oss, The Netherlands). Ethanol (≥ 96%) was purchased from VWR Chemicals (Amsterdam, The Netherlands). Folin-Ciocalteu reagent and Pierce™ BCA Protein Assay Kit were purchased from Thermo Fisher Scientific (Waltham, USA). 2x Laemmli sample buffer, running buffer, Precision Plus Protein Dual Color Standards and Bio-safe Coomassie Stain were purchased from Bio-Rad Laboratories (Hercules, USA). 8-anilinonapthalene-1-sulfonic acid ammonium salt (ANSA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Alfa Aesar (Ward Hill, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, USA). For all experiments, Milli-Q water was used.

2.2.2. Preparation of defatted and dephenolised BSG (dBSG)

Milled BSG was defatted with hexane in a SOXTHERM® unit (SOX416, Gerhardt, Germany) and left to evaporate overnight in a fume hood. Phenolics were removed according to the protocol described by Meneses, Martins, Teixeira, and Mussatto (2013). Briefly, an acetone-water mixture (60:40, v/v) was added to defatted BSG at a solid-liquid ratio of 1:20 (w/v) and incubated at 60 ^{o}C for 30 min with agitation. The mixture was centrifuged at 4500g for 20 min at 20 ^{o}C followed by vacuum filtration. The residue was dried in a vacuum oven (VD53, Binder, Tuttlingen, Germany) to a dry matter content of about 90% and termed as dBSG.

2.2.2.1. Alkali-extracted BSG protein concentrate (A-BPC)

To prepare A-BPC, dBSG was dispersed in Milli-Q water at a solid-liquid ratio of 1:20 and pH was adjusted to 12 using 6 M NaOH. The extraction was carried out for 24 h at 20 ºC and 40 rpm (SB3 rotator, Stuart, UK). The solid-liquid mixture was separated by centrifugation at 10,000g for 20 min at 20 ºC. The supernatant was collected and pH was adjusted to 4 using 6 M HCl for isoelectric precipitation of proteins. A second centrifugation step at 10,000g for 20 min at 4 ºC was applied to collect the precipitates before neutralising the pH and freeze-drying the precipitate (Epsilon 2-10D LSCplus, Martin Christ, Germany) at -20 ºC, 0.01 mbar for 3 days.

2.2.2.2. Preparation of ethanol-extracted BSG protein concentrate (E-BPC)

To prepare E-BPC, dBSG was added to 55% ethanol containing 0.5% 2-mercaptoethanol at a solid-liquid ratio of 1:10. The mixture was incubated at 60 ºC for 1 h in a shaking water bath at 200 rpm (SW22, Julabo, The Netherlands) and centrifuged at 10,000g for 20 min at 20 ºC. The supernatant was collected and rotary evaporated (RC600, KNF, USA) at 40 ºC. Precipitates were collected by centrifugation at 10,000g for 20 min at 4 ºC, washed several times with water and freeze-dried.

2.2.3. Compositional analysis

The nitrogen content was determined with the Dumas combustion method (FlashSmart™ Elemental Analyzer, Thermo Scientific, US), with a protein conversion factor of 5.83 (Jones, 1931). The lipid content was determined using hexane in a SOXTHERM® unit. Ash content was determined by gravimetric method in a furnace at 550 ºC for 24 h (AAF1100, Carbolite, UK). The fibre content was determined by difference. The samples were dispersed at pH 12 (protein concentration of 10 mg/ml) and centrifuged at 10,000g for 10 min. The supernatant was used to determine the total phenolic content (TPC) by the Folin-Ciocalteu method as described previously (Chin, Chai, & Chen, 2022), and was expressed as gallic acid equivalent (GAE).

2.2.4. Molecular weight estimation of protein by gel electrophoresis

The molecular weight ranges of the 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. Sample buffer (65.8 mM Tris-HCl pH 6.8, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) was mixed with 2-mercaptoethanol at a ratio of 19:1 (v/v). All samples were dispersed in the reducing sample buffer, ensuring that the final protein concentration was 2 mg/ml. After a centrifugation step at 10,000g for 10 min to separate insoluble parts, the supernatant was heated at 95ºC for 10 min and centrifuged at 10,000g for 5 min. Then, 15 µl of the supernatant and 5 µl of Precision Plus Protein Dual Color

Standards were loaded on a 12% Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Hercules, USA) in different lanes. The electrophoresis was carried out at 200 V for approximately 40 min. The gel was washed three times with MilliQ water and stained with the Bio-safe Coomassie Stain. Excess stain was removed with MilliQ water. The GS-900 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, USA) was used for gel imaging.

2.2.5. Protein secondary structure by Fourier-Transform Infrared (FTIR) Spectroscopy

The secondary structure of the proteins was investigated using an ATR-FTIR (ALPHA II-Platinum ATR, Bruker, USA). About 20 mg of sample was placed on the crystal cell of the spectrometer at room temperature before being pressed. The FTIR spectrum for each sample, measured in at least triplicates, was recorded at a resolution of 4 cm^{-1} and averaged over 32 scans across the spectral range of 400-4000 cm^{-1}. For interpretation of the results, baseline correction and vector normalisation were applied on the OPUS 8.1 software (Bruker, USA). The secondary structure in the range of 1600-1700 cm^{-1} was processed using the derivative option on the software with 9 smoothing points. The peaks in the second derivative spectra were assigned as follows: β-turns (1662-1684 cm^{-1}), α-helix (1655-1658 cm^{-1}), random coils (1640-1650 cm^{-1}), intramolecular β-sheets (1629-1632 cm^{-1}) and intermolecular β-sheets (1619-1621 cm^{-1}), respectively (Keppler, Heyn, Meissner, Schrader, & Schwarz, 2019).

2.2.6. Surface hydrophobicity

The surface hydrophobicity was measured using ANSA as a fluorescence probe. Samples were dispersed in 10 mM phosphate buffer (pH 7) and incubated at 60 ^{o}C for 1 h at 500 rpm (ThermoMixer C, Eppendorf, Germany) before being centrifuged at 4000g for 30 min. The protein concentration in the supernatant was determined by the BCA assay and diluted with phosphate buffer to a range between 0.005 mg/ml to 0.3 mg/ml. To 4 ml of diluted samples, 20 µl of 8 mM ANSA in 10 mM phosphate buffer (pH 7) was added and left in the dark for 15 min. The fluorescence intensity of the samples was measured at the excitation wavelength of 390 nm and the emission wavelength of 470 nm (RF-6000, Shimadzu, Japan). The net fluorescence intensity was calculated by subtracting the fluorescence of proteins without ANSA from the protein samples with ANSA at each protein concentration.

2.2.7. Water and oil holding capacities (WHC) and nitrogen solubility index (NSI)

Dried samples with known masses (M_0) were added to water or oil respectively at a solid-liquid ratio of 1:50 (w/v) in pre-weighed tubes and left to mix at 40 rpm for 20 h (SB3 rotator, Stuart, UK). Subsequently, the samples were centrifuged at 4800g for 30 min at 20 ^{o}C. The supernatant was carefully removed with a pipette and the mass of the wet pellet was recorded (M_1). The WHC was calculated according to Equation 2.1. The wet pellet was then freeze-

dried to remove the water and its protein content was determined using Dumas. The amount of solubilised protein was calculated by difference (Equation 2.2).

WHC (g water/g dry sample) = $\frac{M_1 - M_0}{M_0}$ (Equation 2.1)

NSI (%) = $\frac{Protein\ content\ _{initial\ sample} - Protein\ content\ _{dry\ pellet}}{Protein\ content\ _{initial\ sample}} \times 100\%$ (Equation 2.2)

2.2.8. Emulsifying properties

Samples were dispersed in 10 mM phosphate buffer (pH 7) at a protein concentration of 0.1% (w/v). Then, 5 ml of soybean oil was added to 15 ml of each sample and homogenised at 20,000 rpm for 1 min (Ultra Turrax T18 Digital, IKA, Germany). In the next step, 50 µl of each emulsion was pipetted from the bottom of the container at 0 and 10 min after homogenisation, then diluted with 5 ml of 0.1% SDS solution. The absorbances of the diluted emulsions (A_0 and A_{10}, for 0 and 10 min respectively) were read at 500 nm (DR 6000, Hach, US), and used to calculate the emulsifying activity index (EAI) and emulsion stability index (ESI) according to Equations 2.3 and 2.4.

EAI (m^2/g protein) = $\frac{2 \times 2.303 \times A_0 \times dilution\ factor}{C \times \emptyset \times 10{,}000}$, (Equation 2.3)

where dilution factor is 100, C = weight of protein per unit volume (g/ml), Φ = oil fraction of emulsion

ESI (min) = $\frac{A_0 \text{ x } \Delta t}{A_0 - A_{10}}$, where Δt = 10 min (Equation 2.4)

2.2.9. Gelling capacity

The thermal gelation behaviour of BSG proteins was determined by the lowest gelling concentration according to Yang et al. (2021) with some modifications. Protein-rich samples (A-BPC, E-BPC and BPI) between the protein concentrations of 2% to 20% (w/v) were dispersed in 10 mM phosphate buffer (pH 7) and allowed to hydrate for at least 3 h with vigorous agitation. The samples were subsequently heated at 95 ^{o}C for 1 h and cooled immediately by ice bath and stored at 4 ^{o}C overnight. The lowest gelling concentration was defined as the concentration at which the sample did not fall or slip when the tube was inverted.

2.2.10. Antioxidant activity

In view of the possible differences in solubility of antioxidants, two antioxidant assays were selected to eliminate possible influences of solvent choice on measured antioxidant activity.

2.2.10.1. 2,2'-9-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

An ABTS assay was performed by solubilising 10 mg of ABTS in 2.6 ml of 2.45 mM potassium persulfate solution to yield a final ABTS concentration of 7 mM. The concentrated ABTS

solution was stored in the dark at room temperature for 16 h and then diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Samples were diluted to a protein concentration of 0.1 mg/ml using 55% ethanol (adjusted to pH 12). Then, 300 µl of the supernatant was mixed with 900 µl of the diluted ABTS and stored in the dark at room temperature for 1 h. The absorbance was read at 734 nm. Gallic acid was used as a standard.

2.2.10.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

A DPPH assay was performed according to Xu, Wei, Xue, and Huang (2022) with slight modifications. DPPH solution (1.75×10^{-4} M) was freshly prepared in methanol and samples were diluted to a protein concentration of 0.3 mg/ml using 55% ethanol (adjusted to pH 12). Subsequently, 500 µl of the supernatant was mixed with 500 µl of DPPH solution and the absorbance was measured at 517 nm after 1 h. Gallic acid was used as a standard.

2.2.11. Statistical analysis

All experimental data were analysed using SPSS Statistical Software (Version 28, IBM, US). A descriptive Duncan's test was used to evaluate the statistical significance between samples at a significance level of 95% ($P \leq 0.05$). All reported results are shown as mean ± standard deviation and all tests were done at least in duplicate.

2.3. Results and discussion

2.3.1. Composition

The composition of BSG, dBSG, A-BPC, E-BPC and BPI are shown in Table 2.1. Our results agree well with values reported previously in literature (Connolly et al., 2013; Mussatto et al., 2006), in which the majority of BSG was comprised of proteins and fibres with a small amount of lipids. After defatting and removal of the phenolics, the lipid content of dBSG was significantly reduced ($P \leq 0.05$) and there was a slight reduction in phenolic content ($P > 0.05$) as compared to the initial BSG. The protein content of dBSG was not changed significantly ($P > 0.05$) and the overall distribution of components in dBSG was roughly similar to the initial BSG, in which proteins and fibres form the bulk of the material. This also suggests that the effects due to the high fibre content in BSG and dBSG should not be neglected.

Table 2.1. Compositions of BSG, dBSG, A-BPC, E-BPC and BPI on a dry basis. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Fibre was calculated from the other components by mass balance. Different small upper letters in the same row indicate significant difference at P ≤ 0.05.

Composition (%)	**BSG**	**dBSG**	**A-BPC**	**E-BPC**	**BPI**
Protein	22.7 ± 0.5^{c}	24.2 ± 0.5^{c}	73.1 ± 2.4^{b}	74.1 ± 0.7^{b}	78.6 ± 0.04^{a}
Lipid	9.0 ± 0.1^{a}	1.4 ± 0.3^{c}	4.8 ± 1.4^{b}	5.2 ± 0.3^{b}	1.3 ± 0.1^{c}
Phenolics*	$0.09 \pm 0.000^{c,d}$	0.03 ± 0.002^{d}	2.5 ± 0.07^{b}	0.2 ± 0.009^{c}	2.7 ± 0.06^{a}
Ash	3.9 ± 0.05^{b}	3.5 ± 0.02^{c}	0.6 ± 0.1^{d}	0.2 ± 0.001^{e}	4.7 ± 0.02^{a}
Fibre	64.3	70.9	19	20.3	12.7

*Phenolics were first quantified as mg GAE/ml and converted to mg GAE/g BSG. This is then converted into a percentage (1 mg/g = 0.1%)

In A-BPC and E-BPC, protein content was significantly increased to more than 70% ($P \leq 0.05$), which indicates that the extraction methods were effective for enrichment in proteins. We observed an increase in lipid content relative to dBSG, which may be related to the comparatively lower amount of fibre present. BPI was commercially supplied and did not originate from dBSG, but it also showed a protein content higher than 70%, with a low amount of lipids and fibres.

The Folin-Ciocalteu assay, performed with only the soluble fraction of each material, showed that A-BPC and BPI had a significantly higher phenolic content than the other samples ($P \leq 0.05$). In both cases, alkaline pH and enzymatic hydrolysis (possibly with xylanases) could have released the insoluble-bound phenolics from cell wall materials such as hemicellulose (Madhujith & Shahidi, 2009; Yu, Vasanthan, & Temelli, 2001), resulting in this higher phenolic

content. This agrees well with previously published work, in which alkali-extracted and enzymatically hydrolysed BSG were relatively high in phenolics (Connolly et al., 2013; Verni et al., 2020). The presence of free phenolics can subsequently lead to covalent or non-covalent interactions between proteins and phenolics. It is worth mentioning that proteins are reactive to Folin-Ciocalteu reagent (Everette et al., 2010) and may contribute to the perceived phenolic content.

2.3.2. Molecular weight estimation by SDS-PAGE

Figure 2.1 shows the SDS-PAGE gel profile of samples under reducing conditions. The objective of determining the molecular weights of the proteins is to verify their identities with existing literature, which may provide some insight into their structure. In BSG, about six bands at 100 kDa, ~60 kDa, 50 kDa, ~30 kDa, ~22 kDa and 10-15 kDa were evident. These bands in the 10-100 kDa range could represent a small amount of residual water- and salt-soluble proteins, namely the albumins and globulins, left in BSG after the malting and mashing process (Celus et al., 2006). Most of the albumins and globulins are likely to have solubilised in the wort during the brewing process. It was reported previously that barley albumins and globulins are present in the range of 21-58 kDa and 16.5-53 kDa respectively (Linko, Lapvetelainen, Laakso, & Kallio, 1989). A small amount of hordeins is also expected due to the breakdown of storage proteins during malting, which explains the presence of a faint band at 100 kDa.

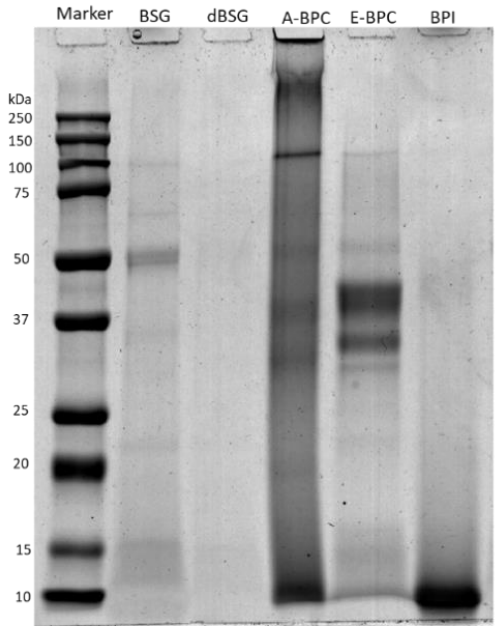


Figure 2.1. SDS-PAGE of different samples under reducing conditions. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-

extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively.

After defatting and dephenolisation, the bands initially present in BSG were no longer seen in dBSG. This is because the residual proteins were washed away and therefore the majority of the proteins present in dBSG were insoluble and trapped within the matrix. Acetone, which is typically used for protein precipitation, could also have caused aggregation of proteins such that their solubility is reduced. Without prior extraction or pretreatment, these proteins would not solubilise in the buffer and therefore no bands were present for dBSG. The A-BPC and E-BPC protein extracts revealed proteins that were slightly more soluble in the buffer (see section 2.3.5), so bands were present again in the gel.

In A-BPC, a distinct band at 100 kDa, plus some faint bands in the range of 30-50 kDa and around 20 kDa could be observed against a smeared background throughout the lane. These bands could represent D-hordeins or glutelins, B-hordeins and A hordeins (Celus et al., 2006). The band smearing suggests that prolonged exposure of the protein aggregates at high pH may have led to protein fragmentation with varying degrees of hydrolysis (Wang, Li, Liu, Ren, & Qu, 2016). Another possibility is that some proteins were associated with other alkali-soluble components such as phenolics or cell wall polysaccharides like hemicellulose. The formation of cross-linked, high molecular weight complexes is confirmed by the presence of a band at the top of the lane. It is known that protein-phenol or protein-polysaccharide interactions can be induced under the applied alkaline conditions (Mohamed, Hojilla-Evangelista, Peterson, & Biresaw, 2007). Sęczyk, Świeca, Kapusta, and Gawlik-Dziki (2019) observed that the addition of phenolics to white bean proteins not only produced a band at the bottom of the well but also resulted in smears below the interface between the stacking and resolving gels.

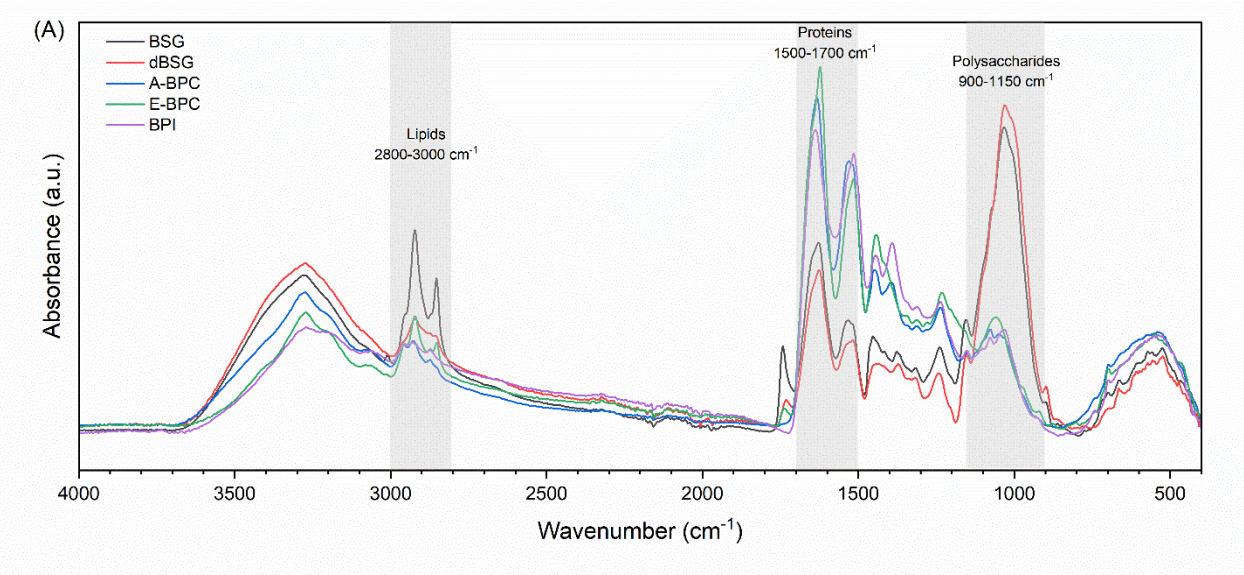
For E-BPC, distinctive bands could be seen that are representative of typical hordein subunits. The bands at 100 kDa, 55-80 kDa, 35-50 kDa and below 15 kDa likely represent D-hordeins, C-hordeins, B-hordeins and A-hordeins respectively (Wang et al., 2010). These bands were also found in A-BPC, albeit at different intensities. In particular, the 100 kDa band was more prominent and the 35-50 kDa band was less intense in A-BPC than in E-BPC. This likely indicates that A-BPC is composed of hordeins and glutelins, while E-BPC is enriched in hordeins. Both hordeins and glutelins can be solubilised under alkaline conditions, and previous studies have also shown that hordeins cannot be completely separated from glutelin (Celus et al., 2006), therefore the presence of hordeins in A-BPC is expected.

Lastly, the bands for BPI were diffuse and in the lower molecular weight range, indicating that the enzymatic hydrolysis produced short-chain peptides and amino acids. This is expected from using the proprietary enzymatic formulation in its production and is aligned with the

results of previous studies showing a high proportion of peptides after hydrolysis (Celus et al., 2007; Connolly et al., 2019). The results show that BPI has a different composition and a much smaller size than A-BPC and E-BPC, which may subsequently affect its functionality.

2.3.3. Composition and protein secondary structure by FTIR spectroscopy

FTIR analysis was used to compare the structural differences resulting from the different extraction methods. Typically, absorption bands at 900-1150 cm^{-1}, 1500-1700 cm^{-1} and 2800-3000 cm^{-1} are assigned to the respective functional groups of C-O stretching, C=O stretching and N-H bending, as well as C-H stretching. Each wavenumber region is indicative of carbohydrates, proteins or lipids, although not necessarily limited to those. In the full spectra (Figure 2.2A), the absorbance was higher for A-BPC, E-BPC and BPI in the amide I and II region (1500-1700 cm^{-1}) as compared to the lipid and polysaccharide regions (2800-3000 cm^{-1} and 900-1150 cm^{-1}, respectively). Conversely, the absorbance was higher in the polysaccharide region than the lipid and protein regions for BSG and dBSG. This is in line with the composition analysis in Table 2.1: the protein is the major component in A-BPC, E-BPC and BPI while the fibre content is highest in BSG and dBSG.



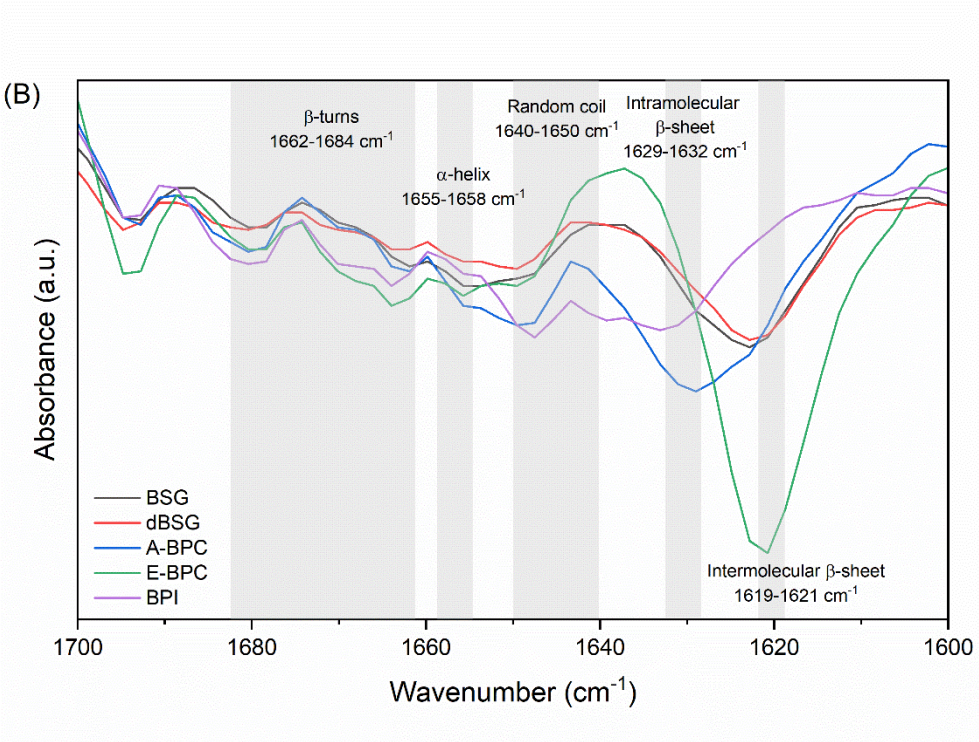


Figure 2.2. (A) Full FTIR spectra from 400 to 4000 cm^{-1} of dried samples, (B) FTIR spectra of the second derivative of the amide I region. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively.

With the second derivative of the amide I region (Figure 2.2B), the secondary structure of the proteins can be determined. The samples possessed a mixture of β-turns (1662-1684 cm^{-1}), α-helix (1655-1658 cm^{-1}), random coils (1640-1650 cm^{-1}) and intra- and intermolecular β-sheets (1629-1632 cm^{-1} and 1619-1621 cm^{-1}, respectively). The proteins that were initially present in BSG and dBSG contained intermolecular β-sheets as shown at 1621 cm^{-1}, and these likely arose from the thermal processes of beer brewing that unfolded the protein and reduced native barley protein structural elements such as intramolecular β-sheets and α-helices (Hadnađev et al., 2018). Consequently, hydrophobic interactions were increased and the partially unfolded proteins associated to form aggregates, which are presented as intermolecular β-sheets (Shivu et al., 2013). The mashing process was also reported by Celus et al. (2006) to encourage the formation of disulfide bonds, leading to protein aggregation.

Between A-BPC, E-BPC and BPI, significant differences can be seen. BPI did not show distinct structural elements since these proteins were enzymatically hydrolysed into peptides. E-BPC had the most intermolecular aggregates out of all samples, indicating severe protein unfolding in the presence of the reducing agent followed by subsequent aggregation during precipitation. In A-BPC, these aggregates were absent and a distinct signal at 1630 cm^{-1} was observed, indicative of intramolecular β-sheets. A similar observation was also made by Jia,

Sethi, van der Goot, and Keppler (2022), who found a high signal intensity corresponding to intramolecular β-sheets in covalently and non-covalently modified sunflower proteins. Since alkaline extraction is likely to induce partial protein unfolding (Jiang, Chen, & Xiong, 2009), more phenol-binding sites are exposed. Under alkaline conditions, bound phenolics are released from cell wall polysaccharides and can bind to these sites, resulting in a change in protein secondary structure. However, one should exercise caution in interpreting results from heterogeneous samples because also non-protein material could induce a signal at this wavenumber.

2.3.4. Surface hydrophobicity of the soluble fraction by ANSA

The protein surface hydrophobicity is a measure of the number of hydrophobic groups on a protein's surface in contact with a polar aqueous environment. In a native protein, these hydrophobic groups are mostly buried inside the core of the folded protein. From Figure 2.3, A-BPC had the highest surface hydrophobicity in its soluble fraction, which could arise due to partial protein unfolding and exposure of hydrophobic groups. This is probably a result of conjugation or complexation of proteins to phenolic compounds, as supported by previous studies which indicate that protein unfolding occurs when proteins interact with phenolics (Cao & Xiong, 2017; Jiang, Zhang, Zhao, & Liu, 2018; Xu, Han, Huang, & Xu, 2021). The phenolics attached to the protein could also have a higher number of aromatic rings with a low number of hydroxyl groups, contributing to an increase in surface hydrophobicity. The increased surface hydrophobicity can also be caused by a different protein composition in A-BPC as compared to the other samples. From SDS-PAGE (section 2.3.2), we can deduce that A-BPC contains both hordeins and glutelins, both of which are proteins that have large numbers of non-polar amino acids, whereas E-BPC is composed of only hordeins. This difference could result in A-BPC having larger surface hydrophobicity. It is important to mention that all fractions except BPI displayed low overall solubility (see section 2.3.5), therefore only the soluble fraction was used for determination of surface hydrophobicity and is not representative of the whole sample.

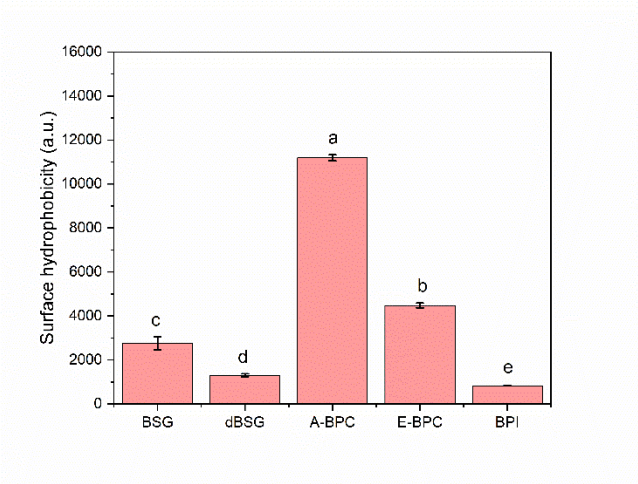


Figure 2.3. Surface hydrophobicity of soluble fraction by ANSA. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at P ≤ 0.05.

In the case of BPI, hydrophobic groups are also exposed due to the enzymatic hydrolysis, but the smaller peptide size means that there are fewer hydrophobic binding sites, resulting in BPI having the lowest surface hydrophobicity (Bamdad, Wu, & Chen, 2011; Celus et al., 2007). For E-BPC, BSG and dBSG, the FTIR signal gave evidence that these proteins form aggregates in a hydrophilic, aqueous environment (Figure 2.2B, at 1621 cm^{-1}), and therefore the hydrophobic groups were hidden and interacted minimally with the ANSA fluorescent probe under aqueous conditions. Generally, it can be concluded that different extraction methods resulted in proteins of varied compositions and structures, which will impact their functional properties.

2.3.5. Nitrogen solubility index

The protein solubility is a critical factor that impacts most functional properties of a protein. According to Figure 2.4, only about 10% of BSG proteins are soluble in water. These are likely residual proteins that remained in the interstitial pores of the spent grains after lautering, since BSG proteins should be insoluble as it is the insoluble residue from mashing. In line with the results from SDS-PAGE, defatting and dephenolisation removed most of these water-soluble proteins, resulting in an even lower NSI of dBSG.

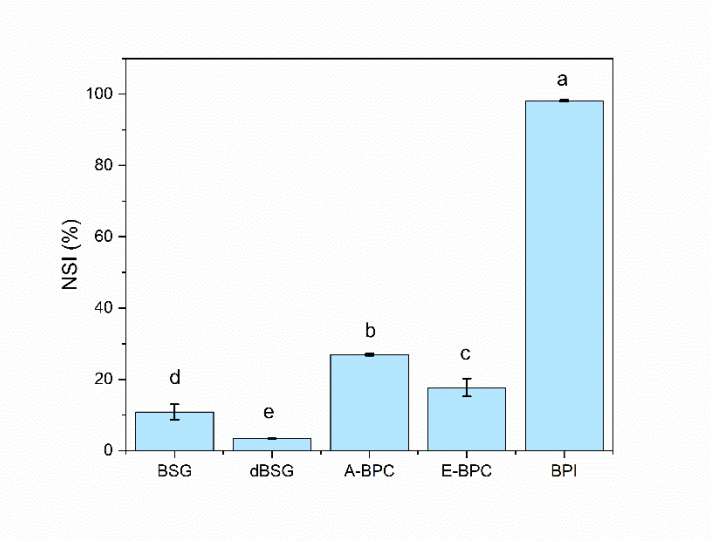


Figure 2.4. Nitrogen solubility index (NSI) of all samples dispersed in water. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at $P \leq 0.05$.

Protein extraction by alkaline and ethanolic methods doubled the NSI for both A-BPC and E-BPC relative to BSG, although the protein solubility in water remained relatively poor (27% and 18%, in A-BPC and E-BPC respectively) as compared to pea protein isolates with solubilities ranging from 46 to 91% (Stone et al., 2015). The NSI is limited due to the high incidence of hydrophobic amino acids in hordeins and glutelins (Wang et al., 2010). A-BPC had a higher NSI than E-BPC despite having a higher surface hydrophobicity of the soluble fraction in A-BPC (Figure 2.3). This is likely because E-BPC was highly aggregated (Figure 2.2B) and therefore does not solubilise well anymore. The relatively higher protein solubility could also be due to protein-phenol interactions that result in partial unfolding of the protein to expose or block hydrophobic and hydrophilic groups on its surface, or the obstruction of charged amino groups on protein by phenolics that lead to a shift in its isoelectric point, thus improving the protein solubility (Rawel, Rohn, Kruse, & Kroll, 2002; Sęczyk et al., 2019; Xu et al., 2022). In addition, other studies have shown that in the absence of phenolics, the protein solubility can already be enhanced through a pH shift from alkaline to neutral as a result of conformational changes at extreme pH conditions (Jia et al., 2022).

Of all the protein-rich fractions, BPI had the highest NSI ($P \leq 0.05$), and in fact was completely soluble in water. This can be ascribed to the reduction in molecular weight into short-chain peptides after enzymatic hydrolysis. Most of their tertiary structure is probably lost, such that the residual chains can reorient to expose the more hydrophilic amino acids, enabling solubility to be enhanced strongly. Polar amino acids that are exposed can interact with water molecules through hydrogen bonding and electrostatic interactions, leading to increased protein solubility.

2.3.6. Water and oil holding capacity

The ability to bind water and oil depends on the polar and nonpolar side chains of the protein respectively, as well as the protein or aggregate conformation and surface hydrophobicity. Figure 2.5 shows the WHC and OHC of all five samples. BPI was completely water soluble after hydrolysis (Figure 2.5) and thus was not capable of holding water as a solid, but the exposure of some hydrophobic groups after hydrolysis enabled BPI to hold some oil. A-BPC and E-BPC have a WHC of about 4 g/g and 2 g/g respectively, which is relatively similar to the values obtained by Wang et al. (2010) using alkaline and alcohol methods to isolate barley proteins. The WHC for A-BPC is also higher than an alkali-extracted pea protein isolate reported previously (Stone et al., 2015).

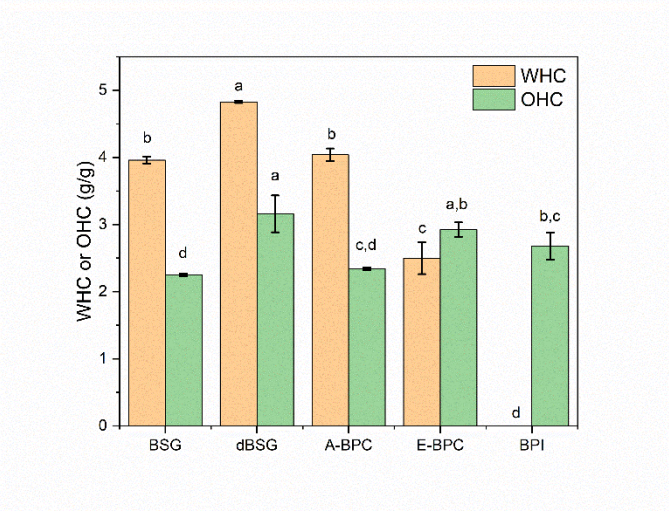


Figure 2.5. Water holding capacity (WHC) and oil holding capacity (OHC) of all samples. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at $P \leq 0.05$.

As a partially unfolded protein, A-BPC has exposed polar amino acid side chains allowing it to hold water better than E-BPC. Furthermore, A-BPC contains high molecular weight glutelins (Figure 2.1) which can form a network that can hold more water than E-BPC which only contains hordeins. Moreover, interactions between proteins and phenolics result in conformational changes that expose hydrophilic groups and increase their accessibility for interacting with water molecules, resulting in an increase in WHC.

Conversely, according to Figure 2.2B, the proteins in E-BPC are strongly aggregated, implying that the hydrophobic regions are exposed, which can help to create pores and networks in oil and increase the OHC (de Vries, Wesseling, van der Linden, & Scholten, 2017), which supports the higher OHC in E-BPC than A-BPC ($P \leq 0.05$). Since E-BPC is rich in hordeins which contains many hydrophobic amino acids such as proline, it is also expected to have a

higher oil uptake. It is noteworthy that the results from the surface hydrophobicity experiment (section 2.3.4) cannot be directly correlated with OHC as the latter is based on the soluble aqueous fraction whereas the OHC also comprises of insoluble components. For example, BSG and dBSG are shown to have a high WHC and OHC despite having a low protein content and poor surface hydrophobicity. This can be attributed to the presence of fibres to hold water and oil (Wang, Suo, de Wit, Boom, & Schutyser, 2016). We conclude that, of all protein-rich fractions, A-BPC has the highest WHC and OHC ($P \leq 0.05$), which is a useful property in applications involving food structuring such as meat analogues, as they influence the mouthfeel and texture of the product.

2.3.7. Emulsifying properties

When proteins are able to rapidly diffuse to, adsorb onto and unfold at an oil-water interface and form a viscoelastic film around the oil droplet, they can help to stabilise emulsions (Wang et al., 2010). Emulsification requires an interplay between protein amphiphilicity, solubility, and water and oil holding capacities (Wang et al., 2010). Figure 2.6 illustrates the emulsifying properties of all five samples. In general, all fractions showed an EAI ranging from 24 to 83 m^2/g and ESI ranging from 13 to 35 min. These values compare relatively well to soy protein isolates (26-41 m^2/g) and egg albumin (49 m^2/g), but are still poorer than for sodium caseinate and β-lactoglobulin (166 and 153 m^2/g respectively) (Pearce & Kinsella, 1978). It should be pointed out that due to differences in the composition, structure and size of proteins as well as the presence of non-protein components in all samples, their mechanisms for emulsification are expected to be different.

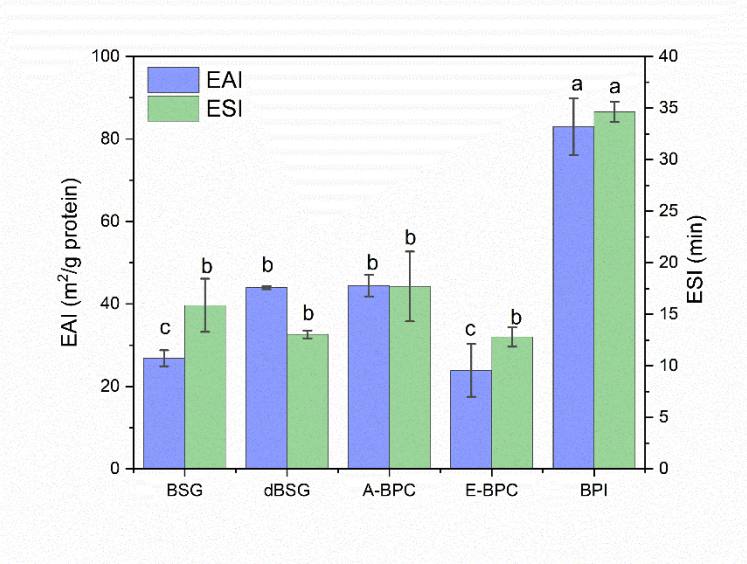


Figure 2.6. Emulsifying activity index (EAI) and emulsifying stability index (ESI) of all samples. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at $P \leq 0.05$.

BPI exhibited a significantly higher emulsifying activity and stability than the other samples ($P \leq 0.05$). This can be attributed to BPI having a lower molecular weight and a significantly higher protein solubility ($P \leq 0.05$), that enables it to migrate and adsorb easily from the bulk phase to the oil-water interface and provide better emulsification. Similar findings were made by Celus et al. (2007) and Chin et al. (2022) regarding the improved emulsion-forming abilities of hydrolysed proteins.

With partial protein unfolding in A-BPC, hydrophobic and hydrophilic groups were exposed, resulting in high WHC and OHC and thus an amphiphilic property was expected. However, an overall poor protein solubility of 27% likely prevented the migration of proteins to the oil-water interface (Malik & Saini, 2017). Soluble proteins in A-BPC could also form crosslinks with phenolics, which may hinder the ability for proteins to rearrange at the interface and form an interfacial film (Salgado, Molina Ortiz, Petruccelli, & Mauri, 2012). It is also worth mentioning that the surface hydrophobicity measurement in section 2.3.4 was only applied on the soluble fraction and is not representative of the whole sample, which was used for emulsification.

Between A-BPC and E-BPC, E-BPC showed poorer emulsification. The reason is two-fold: firstly, A-BPC has a higher protein solubility than E-BPC, which enables more proteins to diffuse to the oil-water interface. Additionally, the soluble portion of A-BPC showed higher surface hydrophobicity, in which the exposed hydrophobic groups can orient more readily towards the oil phase while the polar groups are directed towards the water phase to lower surface free energy, resulting in an increase in emulsifying activity. Secondly, A-BPC showed

a stronger ability to attract water and oil phases, evident from its higher WHC and OHC than E-BPC (Figure 2.5). Conjugation of proteins with phenolics lead to protein unfolding which exposes hydrophobic and hydrophilic groups, which in turn improves surface activity (Cao & Xiong, 2017; Jiang et al., 2018; Xu et al., 2021). These results also corroborate with Stone et al. (2015), who showed that alcohol-soluble wheat proteins, analogous to E-BPC, has poorer emulsifying properties than alkali-extracted pea protein isolates.

Besides emulsification, foaming is also an important functionality for proteins. However, good solubility is a pre-requisite for good foaming behaviour, and the presence of insoluble fibres in A-BPC and E-BPC will likely destabilise the lamellae between foam bubbles, making it difficult for foams to form. Particle-stabilised foams were previously reported to be possible (Dickinson, 2010), but this is only for particles in the size range of a few nanometres to tens of micrometres, which is not the case in this study. Therefore, we did not evaluate foaming properties in detail even though some interfacial activity might be possible.

2.3.8. Gelation

With the exception of BPI, most fractions had low amounts of soluble protein (Figure 2.4). Therefore, it was interesting to explore other functionalities such as gelation that do not depend on high soluble protein content. According to Figure 2.7, the LGC for A-BPC and E-BPC was 6% and 20% respectively, whereas BPI was not able to gelate across the entire concentration range selected. Barley proteins were previously reported to form a gel layer (‘oberteig’) on top of the spent grains after the malting and mashing steps in beer brewing. This gel layer was said to be composed of high molecular weight proteins and carbohydrates (Moonen et al., 1987). Our SDS-PAGE results similarly show that high molecular weight fractions were present in A-BPC and E-BPC but were absent in BPI (Figure 2.1). When heated, these fractions in A-BPC and E-BPC may unfold and aggregate to form a three-dimensional network that immobilises water. For BPI, however, there are no distinct structural elements (Figure 2.2B) that will enable protein aggregation. A previous study by Kotlar, Ponce, and Roura (2013) also showed that no gelation occurred for protein hydrolysates up to a concentration of 16%, which agrees well with our results. This is likely due to the break-up of hydrophobic regions by hydrolysis and therefore protein-protein interactions cannot occur to form gels.

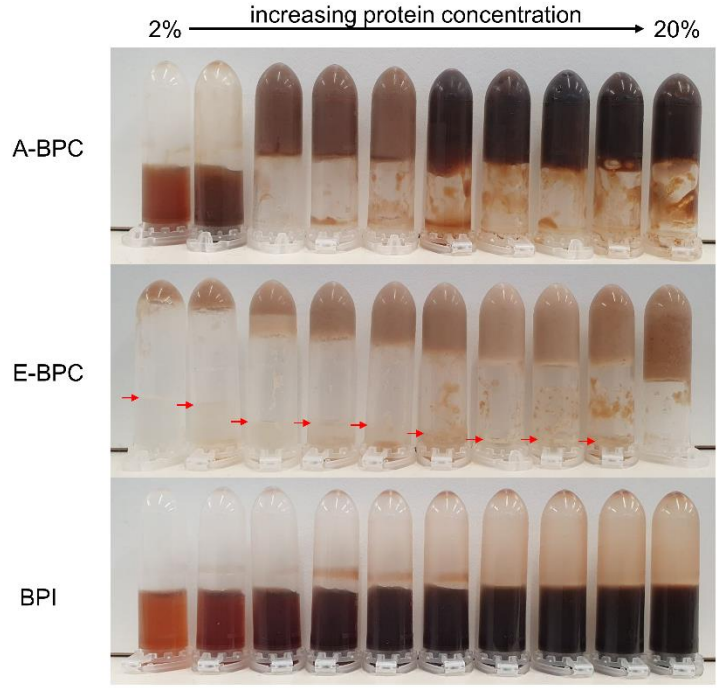


Figure 2.7. Gelation of BSG proteins obtained by different extraction methods. A-BPC, E-BPC and BPI refer to alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. The protein concentration increases from left (2%, w/v) to right (20%, w/v), with an increment of 2%. The red arrows in E-BPC indicate the excess liquid at the bottom of the tube when inverted.

Without protein aggregation, BPI was unable to bind water (Figure 2.5), which is a pre-requisite for forming a gel (Banerjee & Bhattacharya, 2012). On the other hand, the limited protein solubilities of A-BPC and E-BPC imply that these fractions result in dispersions of protein particles with only a small amount of proteins being solubilised. When subjected to thermal treatment without agitation, the particles sedimented over time in a swollen state, leading to particle jamming (Berghout, Boom, & van der Goot, 2015). A-BPC showed a better gelling capacity than E-BPC, as illustrated by a lower LGC. This could be a result of the alkaline extraction, in which hemicellulose (arabinoxylan) could be co-extracted and contributed to improved gelation as these biopolymers retain water very easily (Carvajal-Millan et al., 2007). Furthermore, the presence of phenolics in A-BPC increased the WHC of the proteins which in turn influenced its gelation, as previously demonstrated in sunflower protein isolate by Malik and Saini (2017). Proteins that are crosslinked with phenolics have more exposed hydrophobic regions as seen by an increase in surface hydrophobicity in Figure 2.3. These protein-phenolic conjugates can also lead to additional interactions through hydrogen bonding and hydrophobic interactions. As they interact, they can form swollen flocs with an open structure, therefore enabling gel formation at a low protein concentration of A-BPC. In addition, as hypothesised earlier in this study, A-BPC likely contains more glutelins than E-BPC, and previous work concluded that glutelins are involved in the formation of gel-like

aggregates (Moonen et al., 1987). For E-BPC, a self-supporting gel could be formed at protein concentrations as low as 2%, but due to the poor WHC and likely poor swelling capacity, syneresis resulted in an excess layer of liquid on top of the gel. When the protein concentration was increased to 20%, the layer of particle extended throughout the total sample, resulting in a single gel layer. Syneresis was observed in E-BPC due to gravitation and density differences between the particles and water, causing the particles to rearrange and result in an endogenous pressure large enough for water to flow outside of the gel (Mizrahi, 2010). When there are sufficient particles to fill up the whole volume, more water can be trapped within the gel structure and syneresis does not occur. Further experiments on rheology and texture analysis of the gels are required to determine the gel strength and the extent of these interactions. Given that thermal gelation is necessary for meat analogue formation, A-BPC proves to be a relatively suitable candidate due to its low LGC in addition to its high WHC and OHC as previously discussed.

2.3.9. Antioxidant activity of the soluble fraction

Figure 2.8 depicts the antioxidant activity based on DPPH and ABTS assays. In these assays, the source or type of antioxidant cannot be distinguished, for example, between bound phenolics and free phenolics. Both assays test the ability of compounds to act as free radical scavengers or hydrogen donors. Since DPPH is only soluble in alcoholic media and ABTS is soluble in both water and ethanol, there is a consistently poorer antioxidant activity of all fractions measured with the DPPH assay. The choice of solvent may affect the measured antioxidant activity, but the results of both antioxidant assays correlate strongly, which suggests that the assays are appropriate. Similar trends in antioxidant activity across all samples were observed in both assays, with dBSG having the least antioxidant activity and BPI and A-BPC having the most ($P \leq 0.05$). Due to the presence of a small amount of free phenolics, BSG showed minor antioxidant activity. After dephenolisation, free phenolics were removed and the antioxidant activity in dBSG was lowered.

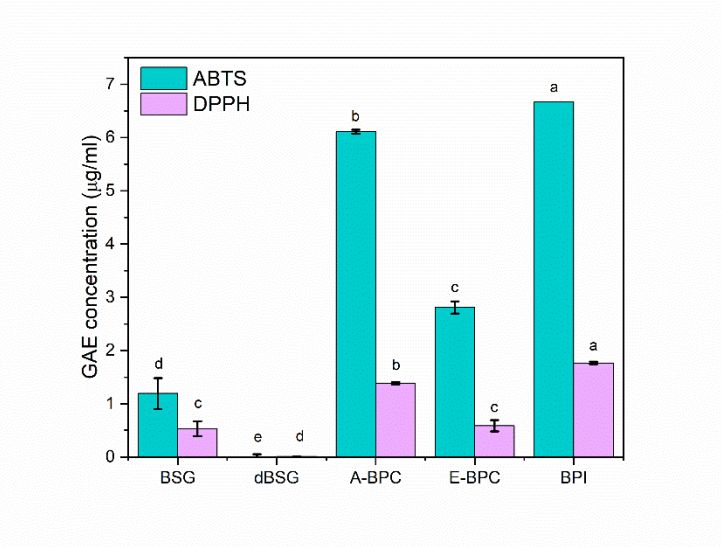


Figure 2.8. Antioxidant activity based on ABTS and DPPH assay. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at P ≤ 0.05.

As previously mentioned in section 2.3.1, insoluble-bound phenolics that were present in dBSG were released under alkaline conditions and enzymatic hydrolysis (possibly by xylanases), resulting in a higher total phenolic content present in A-BPC and BPI. For A-BPC, the phenolics were bounded to proteins and were less reactive to the radicals. On the other hand, the phenolics in BPI were bounded to short-chain peptides that have less steric hindrance towards radicals and therefore show higher antioxidant activity. Additionally, in BPI, the possible presence of bioactive peptides contributes to more radical scavenging activity (Connolly et al., 2019). Based on the typical amino acid composition found in barley, antioxidant amino acids such as tyrosine, methionine, histidine, lysine and tryptophan are present and available for redox reactions (Chin et al., 2022; Xu, Chen, & Liu, 2017). Since BPI is completely soluble in water, coupled with the small peptide size after hydrolysis (Figure 2.1), these protein hydrolysates have more exposed antioxidant groups than non-hydrolysed fractions which are hidden away from solution by folding and aggregation (Bamdad et al., 2011). Therefore, BPI has greater accessibility and diffusivity to scavenge free radicals than A-BPC. Furthermore, antioxidative peptides may exert a strong synergistic effect with other antioxidants such as phenolics (Saito et al., 2003), which may explain for the significantly higher antioxidant activity in BPI as compared to A-BPC.

Despite the low phenolic content in E-BPC, there was still some antioxidant activity. This observation was also made previously by Chanput, Theerakulkait, and Nakai (2009) regarding barley hordeins, and can be attributed to the presence of some hydrophobic amino acids which have antioxidant activity (Bamdad et al., 2011).

2.4. Conclusion

We examined the influence of different protein extraction methods on the composition, conformation and functional properties of BSG extracted proteins. In all the extraction methods applied, protein content was enriched to more than 70%. With alkaline and enzymatic extraction (A-BPC and BPI respectively), the proteins also contained about 3% phenolics. Alkaline conditions likely released bound phenolics on cell wall material which covalently interacted with proteins. Consequently, A-BPC was in a partially unfolded state with exposed hydrophobic and hydrophilic groups. Similarly, with enzymatic hydrolysis, possibly by a combination of xylanases and proteases, low-molecular weight peptides without distinct structural elements were obtained. Ethanolic extraction resulted in proteins that were characteristic of barley hordeins and were highly aggregated.

The highest protein solubility was found in BPI, which allowed better emulsification and may be relevant in applications where emulsions are concerned. The proteins in BSG and its non-hydrolysed protein extracts are primarily insoluble, and therefore perform better in those applications where solubility is not a pre-requisite, such as in the formation of protein gels. In particular, A-BPC showed good gelation combined with a high WHC and antioxidant activity, of which the former two qualities are important for meat analogue applications. Despite the poorer WHC caused by aggregation, E-BPC demonstrated its ability to form gels readily at low concentrations. In the brewing industry, BSG is typically discarded, but we showed that the protein concentrates could be promising for a range of food structuring applications. Further studies into the gelling properties of BSG proteins such as gel strength and morphology should be examined in the future when exploring opportunities to valorise BSG.

2.5. Supplementary Information

The intrinsic fluorescence of the soluble fraction was determined by fluorescence spectroscopy (RF-6000, Shimadzu, Japan), in accordance to Liu, Ma, McClements, and Gao (2017). Samples were dispersed in 55% ethanol (adjusted to pH 12) at a protein concentration of 0.2 mg/ml. After centrifuging at 10,000g for 10 min, the supernatant of each sample was collected and the fluorescence emission was read. The excitation wavelength was at 280 nm and the emission spectrum was scanned from 290-450 nm using the widths of 5 nm for the excitation and emission slit. Each emission spectrum was the average of two runs.

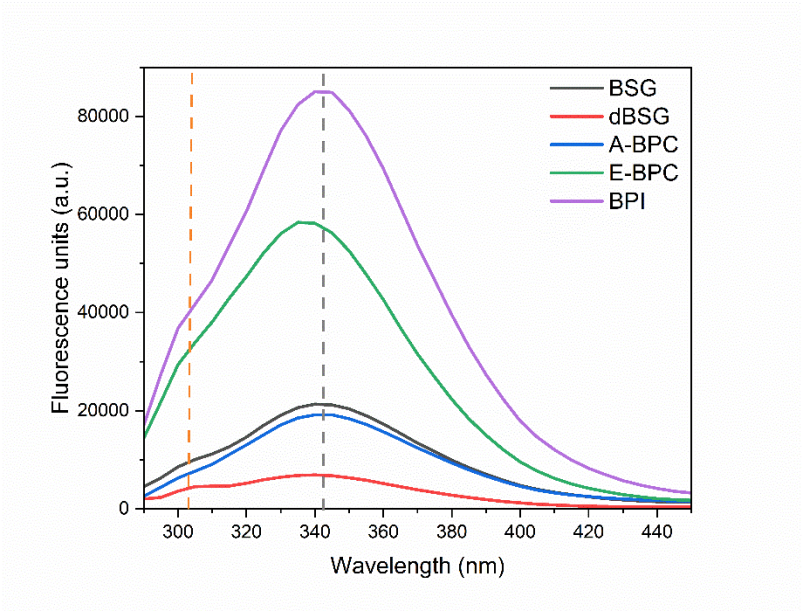


Figure 2.9. Intrinsic fluorescence of soluble proteins after excitation at 280 nm. The maximum emission wavelength for tryptophan and tyrosine is indicated in the grey and orange vertical dashed lines respectively. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively.

In the presence of an excitation source, soluble conjugated molecules such as the aromatic amino acids in proteins will fluoresce, allowing us to determine changes in the tertiary structure of proteins. When excited at 280 nm, aromatic amino acids like tryptophan and tyrosine absorb the incident light and emit fluorescence near their typical characteristic wavelengths of 350 nm and 304 nm, respectively (Kronman & Holmes, 1971). From Supplementary Figure 2.9, the maximum emission wavelengths for intrinsic fluorescence of tryptophan amino acids in all samples occurred around 342 nm except for E-BPC, which was blue-shifted to a lower wavelength, indicating an increase in hydrophobicity of the protein. It is probable that tryptophan residues were occluded in an aqueous environment, which is consistent with the presence of protein aggregation of E-BPC depicted in the FTIR second-derivative spectrum (Figure 2.2B). It is worth mentioning that due to differences in protein solubility, the

fluorescence intensities of the samples cannot be directly compared, but in theory the presence of phenolics can quench the fluorescence of proteins (Czubinski & Dwiecki, 2017). It should also be noted that a small emission maximum at 304 nm, representative of the intrinsic fluorescence from tyrosine amino acids, can be seen in the spectra, but the signal is relatively weak.

Chapter 3

The emulsifying performance of brewers' spent grains treated by colloid milling

This chapter is modified from: Chin, Y.L., Dinani, S.T., Chen, W.N., & Boom, R. (2024). The emulsifying performance of brewers' spent grains treated by colloid milling. *Innovative Food Science & Emerging Technologies, 91,* 103541. doi:https://doi.org/10.1016/j.ifset.2023.103541

Abstract

The sustainable production of food ingredients can be achieved by utilising waste streams, but it is difficult to isolate pure components from a heterogeneous matrix. In this work, brewers' spent grains (BSG), which is a byproduct from the brewing industry, was mildly treated with a colloid mill and its emulsifying ability was evaluated. The milled samples contained 26-32% protein and 52-62% fibre and were generally smaller than 10 µm. In its soluble fraction, a small amount of surface-active proteins was present and reduced oil-water interfacial tension by 35%. These soluble proteins impacted the stabilisation of the emulsion, resulting in incomplete surface coverage and droplet sizes up to 40 µm. Nonetheless, emulsions prepared with the milled BSG and its insoluble particles were stable against coalescence for 10 days, which is attributed to steric stabilisation from fibres. The fibres in the continuous phase were bounded to proteins that adsorbed on the interface, which enabled Pickering stabilisation. This created a fibre network in the emulsion but resulted in bridging of oil droplets and subsequently creaming. Nonetheless, our results suggest that obtaining pure components may not be necessary for emulsification purposes, especially for the case of particle-stabilised emulsions.

Industrial relevance

Industrial byproducts from food processing are generated in large quantities and typically discarded due to their recalcitrant nature. Yet, there is more that can be exploited from this waste stream including proteins and fibres. Separation of these components in their purified form would require harsh methods that are not environmentally sustainable. This study provided insights into the use of a mild and scalable process that can produce functionally active dispersions that are of interest in the preparation of emulsions.

3.1. Introduction

Every year, 39 million tonnes of brewers' spent grains (BSG) are generated around the world as a side stream of the brewing industry (Macias-Garbett et al., 2021). BSG makes up 85% of the total byproducts from the brewing industry and is produced after the malting and mashing process, during which starch in barley grains is converted into soluble sugars. This leaves behind the original barley grain coverings, such as the pericarp, husk and aleurone layers which are rich in fibres and proteins. As a largely water-insoluble material with a high moisture content, BSG is highly susceptible to microbial contamination and most BSG is currently discarded or used as animal feed. BSG fibres comprise of 17% cellulose, 28% hemicellulose and 28% lignin, while the proteins, which make up 20% of the material, are mostly alcohol-soluble prolamins and alkali-soluble glutelins that remain in the barley grains (Mussatto et al., 2006). These proteins have a good balance of hydrophobic and hydrophilic amino acids, which should allow them to exhibit good emulsifying properties (Wang et al., 2010).

Various methods to put BSG to valuable use have been explored, including the extraction of BSG fibres and proteins under alkaline conditions (Connolly et al., 2013; Vieira et al., 2014). In particular, the enzymatic hydrolysis of BSG has been widely investigated to generate protein hydrolysates that have better technofunctional and bioactive properties (Celus et al., 2007; Connolly et al., 2019; Niemi, Martins, Buchert, & Faulds, 2013). However, these fractionation processes often involve intensive physicochemical and thermal steps, the use of harsh solvents or end in large mass losses. In addition, previous studies have demonstrated that mildly purified mixtures can also be functional, depending on their intended application (Karefyllakis et al., 2019; Möller, van der Padt, & van der Goot, 2022; Sridharan et al., 2020b), thus complex purification steps may in fact not be necessary. A straightforward and more sustainable approach is to utilise BSG as such. One example is the direct incorporation of BSG 'as is' in food products such as baked goods, pasta or yogurt, with the purpose of improving the dietary fibre content in the final application (Mussatto, 2014). Being a nutrient-rich material, BSG was also used as a substrate for growing microorganisms to produce industrially relevant compounds (Lynch et al., 2016), or by employing microbes to break down the lignocellulosic structure and produce other useful components (Chin et al., 2022; Cooray & Chen, 2018; Tan et al., 2020).

However, until now, little attention has been paid towards physical modifications that could impart new or better functional properties to the whole BSG material. A recent study by Ibbett et al. (2019) showed that colloid milling of BSG produced a protein-rich, fine dispersion with the ability to stabilise oil-in-water emulsions, but the interfacial properties and emulsifying behaviour were not investigated in further detail. In addition, only the proteins in BSG were considered for its potential to stabilise emulsions, neglecting the possibility that fibres could

also have an effect on emulsification and emulsion stability. Previous studies have shown that insoluble fibres that are pretreated via a homogenisation step can possess emulsifying properties and stabilise emulsions through a combination of Pickering stabilisation and the formation of a fibre-based network (Bao et al., 2021; Wallecan, McCrae, Debon, Dong, & Mazoyer, 2015; Yang, Liu, Li, & Tang, 2019). The homogenisation process disrupts the plant cell wall matrix and can be carried out by high pressure homogenisation, ultrasonication or wet media milling. Emulsions stabilised by these fibres have demonstrated high stability against pH and ionic strength variations, with long-term storage stability. Similarly, water-insoluble proteins from corn and pea have also demonstrated to be effective particle stabilisers of oil-in-water emulsions (de Folter, van Ruijven, & Velikov, 2012; Hinderink, Schröder, Sagis, Schroën, & Berton-Carabin, 2021).

In recent years, substantial interest in clean-label and sustainable products has led to extensive research on particle-stabilised emulsions, i.e. Pickering emulsions, mainly due to their high physical stability (Schröder, Laguerre, Tenon, Schroën, & Berton-Carabin, 2021). For these reasons, it is relevant to consider exploiting the insolubility of BSG for Pickering stabilisation. We hypothesise that as a mixture containing amphiphilic proteins and a large amount of fibres, BSG can be used to stabilise emulsions without isolation of pure components. Therefore, the aim of this study is to characterise colloid mill-treated BSG and investigate the emulsifying ability of BSG as a whole and more specifically of its insoluble fraction. In this work, BSG was first treated with a colloid mill and separated by centrifugation to obtain an insoluble pellet fraction (insoluble BSG). The composition, physical behaviour, particle size and morphology of full and insoluble BSG dispersions were evaluated while the interfacial properties of the soluble component from centrifugation was determined. Emulsions were prepared with full and insoluble BSG and assessed based on their droplet sizes, charges, protein surface load, microstructure and creaming stability.

3.2. Materials and methods

3.2.1. Materials

BSG was kindly supplied by Heineken Nederland B.V. (The Netherlands). Hexane was purchased from Actu-All Chemicals B.V. (The Netherlands). Soybean oil was purchased from a local supermarket (The Netherlands). Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (USA). Calcofluor White and Fast Green FCF were purchased from Sigma-Aldrich (USA). BODIPY™ 493/503 was purchased from Thermo Fisher Scientific (USA). All other reagents were purchased from Sigma-Aldrich (USA) unless otherwise stated. For all experiments, Milli-Q water was used.

3.2.2. Preparation of BSG for colloid milling

Wet BSG was dried in a fluidised bed dryer (TG200, Retsch GmbH, Germany) at 55^{o}C for 3 h in batches using an airflow of 70 m^{3}/h. The dried BSG (moisture content <4%) was impact-milled (Multimill, Hosokawa Alpine, Germany) with the ZPS configuration at 7000 rpm and a classifier wheel speed of 2500 rpm. Air flow was fixed at 52 m3/h and feed rate was 3 kg/h. Milled BSG was then passed through a 100 µm air-jet sieve (e200LS, Hosokawa Alpine, Germany).

3.2.3. Preparation of full BSG and insoluble BSG dispersions

BSG (< 100 µm) was dispersed in water (2 wt%) and magnetically stirred at 600 rpm for 30 min at room temperature. The dispersion was colloid milled (magic LAB, IKA, Germany) at 20,000 rpm for 60 min using the MK module set at a gap size of 0.159 mm. To prepare the insoluble BSG, full BSG was centrifuged at 10,000 g for 10 min at 20^{o}C. The supernatant was collected and stored at 4^{o}C for further analysis, while the pellet was collected and freeze-dried (Epsilon 2-10D LSCplus, Martin Christ, Germany).

3.2.4. Compositional analysis

The nitrogen content was determined by the Dumas combustion method (FlashSmart™ Elemental Analyzer, Thermo Scientific, US), with a protein conversion factor of 5.83 (Jones, 1931). The lipid content was determined using hexane in a SOXTHERM® unit. The ash content was determined by gravimetric method in a furnace at 550^{o}C for 24 h (AAF1100, Carbolite, UK). The fibre content was determined by difference.

3.2.5. Microscopy, particle size and morphology

The aggregation behaviour of particles in aqueous media and the microstructure of emulsions were observed by light microscopy using a Carl Zeiss Axioscope A1 microscopy equipped with a camera (Axiocam 305 colour). Particle sizes of the BSG before and after colloid milling, and its supernatant was first measured by laser light diffraction (Mastersizer 3000, Malvern Instruments, UK) using the Hydro MV module. A refractive index of 1.468 for the dispersed phase with an absorption index of 0.01 were used. Accounting for the particle morphology, the particle sizes and elongation of colloid mill-treated dispersions were then determined by automated imaging (Morphologi 4, Malvern Instruments, UK) with at least 100,000 particles and estimated as a circle-equivalent diameter. Dried particles were also visualised using a scanning electron microscope (JCM-7000 NeoScope™ Benchtop SEM, JEOL, Japan). Emulsions were observed by confocal laser scanning microscopy using a ReScan Confocal Microscope (Confocal.nl, The Netherlands) fitted with a 60x water objective and a camera. Samples were simultaneously stained with Calcofluor White, Fast Green FCF and BODIPY™ for the fibres, proteins and oil phase, respectively. BODIPY™ was dissolved in ethanol, while

the other two stains were dissolved in water. The emission wavelengths used were 405, 488 and 640 nm for Calcofluor White, BODIPY™ and Fast Green FCF respectively.

3.2.6. Interfacial tension and dilatational rheology

The interfacial tension between stripped soybean oil and the soluble filtered fraction (< 0.2 µm) of the supernatant was measured using an automated drop tensiometer (Tracker, Teclis Scientific, France) used in the rising drop configuration. An oil droplet with a surface area of 30 mm^2 was formed at the tip of a stainless steel needle immersed in the dispersion at 20°C. The shape of the oil droplet was continuously monitored using a camera and transformed into interfacial tension by Wdrop® software (Teclis Scientific, France). The interfacial tension reached a steady value after 3 h and the dilatational viscoelasticity was measured using an amplitude sweep test. The droplet was subjected to sinusoidal amplitude deformations that varied its projection area by 5%, 10%, 15%, 20%, 25%, 30%. The interfacial tension was recorded as a function of time together with the amplitude and phase shift of the deformation. These measurements were used to estimate the interfacial dilatational modulus (E_d), which is a measure of the resistance to surface disturbances and can be defined as $E_d = E_d' + E_d''$, with E_d' and E_d'' referring to the storage and loss modulus respectively, obtained from the amplitude sweep tests. Additional experiments pertaining to the identification and quantitation of proteins in the soluble filtered fraction were also performed using Fourier-Transform Infrared Spectroscopy (FTIR) and a bicinchoninic acid assay (Thermo Fisher Scientific, USA).

3.2.7. Emulsion preparation

Full BSG dispersions were diluted with Milli-Q water and lyophilised insoluble BSG was re-dispersed in water. Soybean oil (10 wt%) was added to an aqueous phase (90 wt%) containing 0.5, 1 or 2 wt% of particles. A coarse emulsion was prepared using a rotor-stator homogeniser (Ultra Turrax T18 Digital, IKA, Germany) at 11,000 rpm for 2 min. Subsequently, the emulsion was processed with an ultrasonic probe (Branson Digital Sonifier SFX 550, Emerson, USA) using a 5 mm probe at 70% amplitude for 5 min (3 s on/3 s off). Emulsions were immersed in an ice bath to prevent excessive heating. At least two emulsions were prepared independently for each measurement.

3.2.8. Emulsion droplet size

Droplet size measurements were performed by laser light diffraction (Mastersizer 3000, Malvern Instruments, UK) using the Hydro MV module. A refractive index of 1.465 for the dispersed phase with an absorption index of 0.01 were used. For measurements with SDS, 1% SDS was diluted with the sample in a 1:1 ratio. Each measurement was performed in triplicate on days 0, 5 and 10.

3.2.9. Zeta potential of emulsions

The surface charge of the emulsion droplets was measured by electrophoretic light scattering (Zetasizer Ultra, Malvern Instruments, UK) using a Malvern folded cuvette (DTS1070) at 25°C. Before measurement, the emulsion samples were diluted 500 times in Milli-Q water to prevent multiple scattering. Each measurement was performed in triplicate.

3.2.10. Protein surface load of oil droplets

The protein surface load of oil droplets was determined in accordance to Sridharan et al. (2020b) with slight modifications. The prepared emulsions were centrifuged at 10,000 g for 30 min at 4°C and subsequently stored at -20°C for overnight. The cream layer was carefully separated from the frozen serum and dried in the oven at 60°C for 48 h. The protein content in the dried cream layer was determined by Dumas as mentioned in section 3.2.4. Protein surface load (Γ_S, in mg/m^2) was computed by Equations 3.1a and 3.1b.

$$\Gamma_S = \frac{\Gamma_T}{S_T} \quad \text{(Equation 3.1a)}$$

$$S_T = \frac{6}{d_{32}} V_{oil} \quad \text{(Equation 3.1b)}$$

Where Γ_T is the measured amount of protein at the interface, S_T is the total surface area, d_{32} is the surface mean diameter from laser diffraction experiments and V_{oil} is the volume of oil in the emulsion.

3.2.11. Creaming index

As the emulsions prepared with 2 wt% dispersions had the smallest droplet sizes, the storage stability of these emulsions against creaming was evaluated. Emulsions were stored at 4°C and creaming was determined by recording the total height of the emulsion (Ht) and the height of the transparent or turbid layer at the bottom of the container (Hs) on days 0, 3, 5, 8 and 10. The creaming index was calculated using Equation 3.2.

$$\text{Creaming index (\%)} = \frac{Hs}{Ht} \times 100\% \quad \text{(Equation 3.2)}$$

3.2.12. Statistical analysis

All experimental data were analysed using SPSS Statistical Software (Version 28, IBM, US). Significant differences were analysed with one-way ANOVA using a descriptive Duncan's test at a significant level of 95% ($P \leq 0.05$). All experiments were done at least in duplicate and reported results are shown as mean ± standard deviation.

3.3. Results and discussion

3.3.1. Characterisation of particle dispersions

3.3.1.1. Composition of BSG dispersions

The effects of colloid milling and subsequent centrifugation on the composition of BSG were compared as shown in Table 3.1.

Table 3.1. Composition of BSG before and after colloid milling and the remaining pellet after centrifugation. Fibre was calculated from the other components by mass balance Different small upper letters in the same row indicate significant difference at $P \leqslant 0.05$.

Composition (%)	**Initial BSG before colloid milling**	**Colloid-milled BSG (Full BSG)**	**Pellet after centrifugation (Insoluble BSG)**
Protein	31.4 ± 0.5 [a]	31.6 ± 0.9 [a]	25.8 ± 2.1 [b]
Lipids	12.8 ± 0.5 [b]	14.6 ± 0.4 [a]	8.8 ± 0.4 [c]
Ash	3.7 ± 0.09 [a]	2.4 ± 0.004 [c]	3.1 ± 0.09 [b]
Fibre	52.1	51.5	62.3

The main components in the initial BSG comprised of proteins and fibres, which is in line with those previously reported (Lynch et al., 2016; Mussatto et al., 2006). Colloid milling was used as a physical treatment to reduce particle size as large particles will sediment quickly and are less likely to exert any stabilising effects on an oil-water interface. After colloid milling, the protein content in full BSG remained the same as the initial BSG while there was a slight increase in the lipid content ($P \leq 0.05$), probably because colloid milling released some oil bodies from the endosperm, embryo and aleurone layer of the barley grain (Neuberger et al., 2008; Niemi, Tamminen, et al., 2012). Following centrifugation, the protein and lipid content decreased in the insoluble BSG while the fibre content increased. It was previously reported by Ibbett et al. (2019) that after colloid milling and centrifugation, an upper fine fraction that is richer in proteins and lipids relative to the initial BSG was generated. As a result of colloid milling, protein-containing tissues are broken into smaller fragments and the smaller protein-containing particles sediment more slowly than the larger husk or pericarp particles upon centrifugation. Since lipids and proteins are also components of protein bodies in barley (Becker, 2007), it is therefore likely that the supernatant which is more enriched in proteins would also have an improvement in lipid content, thus the remaining insoluble BSG pellet would have lesser proteins and lipids. Overall, despite the minor differences in the composition of full BSG and insoluble BSG, the distribution of components remained relatively similar, in which proteins and fibres make up the bulk of the material. It is also worth mentioning that in

this study, BSG was dried before colloid milling to ensure stability of the material against microbial degradation. However, in reality, BSG is already in a wet state after brewing, and it would be more practical to directly process it in the colloid mill immediately after brewing. This would help to save costs involved in drying.

3.3.1.2. Physical behaviour, particle size and morphology

Following the compositional analysis, the dispersibility and aggregation behaviour of BSG before and after colloid milling was investigated. Initially, BSG fibres form a dense network in the suspension (Figure 3.1A). After colloid milling, the particles appear more fibrillated with a less dense network but they remained in clusters comprising of different shapes and sizes (Figure 3.1B). This moderate aggregation of particles in an aqueous phase could be associated to flocculation caused by fibres as they collide and entangle after disintegration, and interact through electrostatic interactions and hydrogen bonding (Saarikoski, Saarinen, Salmela, & Seppälä, 2012; Ullah et al., 2018). It may also suggest that the particles are somewhat hydrophobic, which may indicate their suitability for forming stable Pickering oil-in-water emulsions (Aveyard, Binks, & Clint, 2003; Binks, 2002).

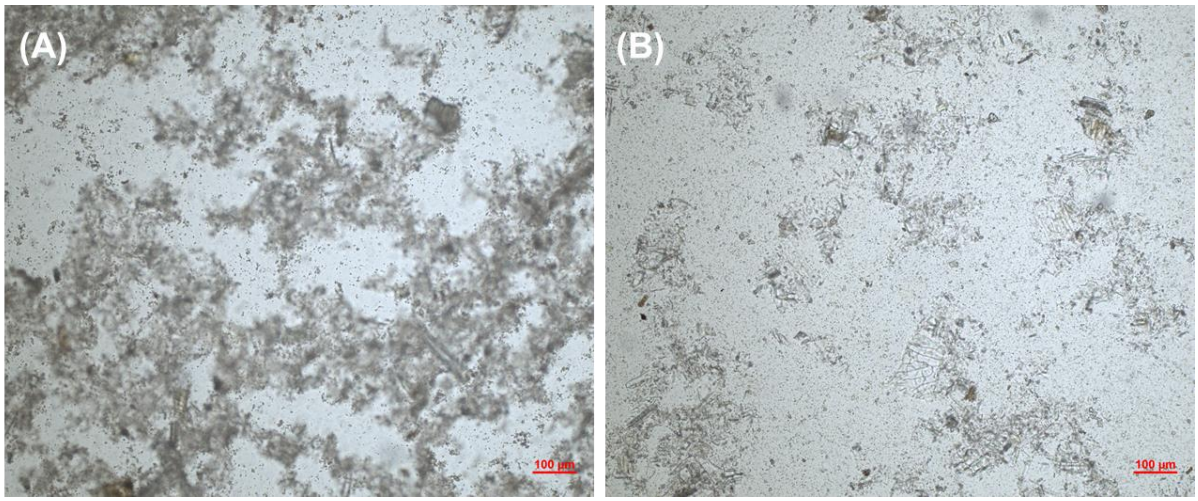


Figure 3.1. Particle dispersion of (A) BSG before colloid miling and (B) BSG after colloid milling under the light microscope. Scale bar represents 100 µm.

As the shape of the particles will influence their interfacial behaviour and their ability to stabilise interfaces (Berton-Carabin & Schroen, 2015), the morphology of the particles was examined in their dried form using SEM. The SEM images in Figure 3.2 show that BSG particles generally have an irregular shape with an uneven surface. Colloid milling seemed to loosen up the structure and result in gaps between the particles that were initially more intact. Using automated imaging analysis, the elongation for the colloid mill-treated BSG, defined as $\left(1 - \frac{width\ of\ particle}{length\ of\ particle}\right)$, was 0.33 ± 0.03. This implies that the particles had an anisotropic shape which could be related to the presence of the fibres. With a greater particle aspect ratio, the

particles can cover a larger interfacial area, resulting in a higher interfacial packing and likely shape-induced attractive capillary interactions, leading to a more viscoelastic behaviour. Additionally, the adsorption of an anisotropic particle is stronger than for a spherical particle as the interfacial area-to-particle size ratio is larger, which helps to prevent coalescence (Madivala, Vandebril, Fransaer, & Vermant, 2009). Furthermore, an anistropic shape also induces the formation of a network-like structure at the emulsion droplet surface through jamming. Overall, emulsion stability is improved with anisotropic particles (Dugyala, Daware, & Basavaraj, 2013).

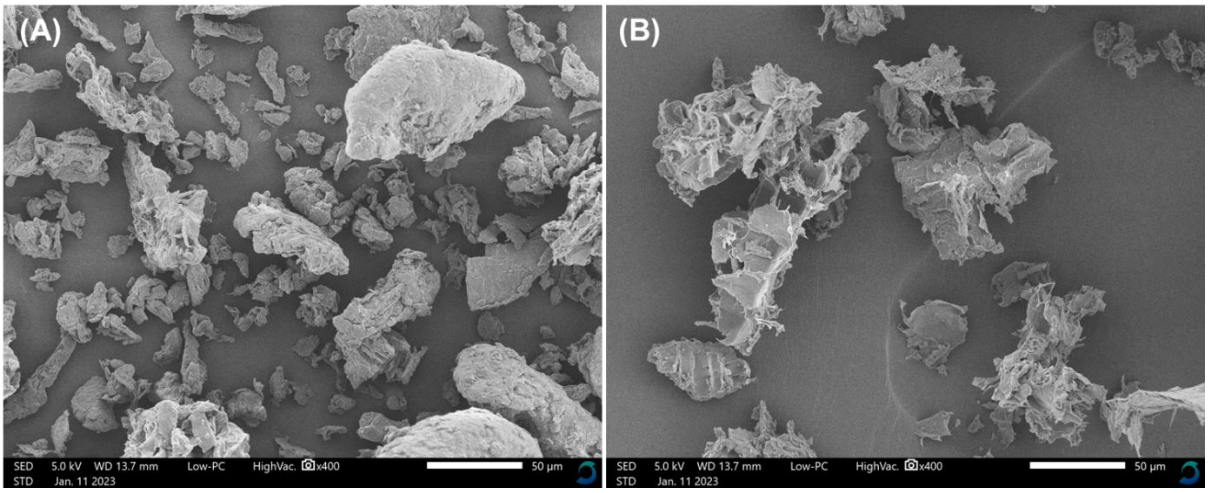


Figure 3.2. SEM images of the dispersion of (A) BSG before colloid miling and (B) BSG after colloid milling. Scale bar represents 50 µm.

Figure 3.3A depicts the particle size distributions of the homogenized dispersions of BSG before and after colloid milling determined by laser diffraction. The particle sizes were reduced from the initial BSG after colloid milling, and some particles were smaller than 1 µm. After centrifugation to remove large insoluble particles from full BSG, the supernatant showed a significant portion of particles centered around 0.4 µm, indicating the presence of fines in full BSG, which was also reported earlier by Ibbett et al. (2019). In that study, they obtained fine BSG particles in the supernatant around 1-10 µm in size after colloid milling and centrifugation and found that the fine particles were rich in proteins with the ability to stabilise oil-in-water emulsions.

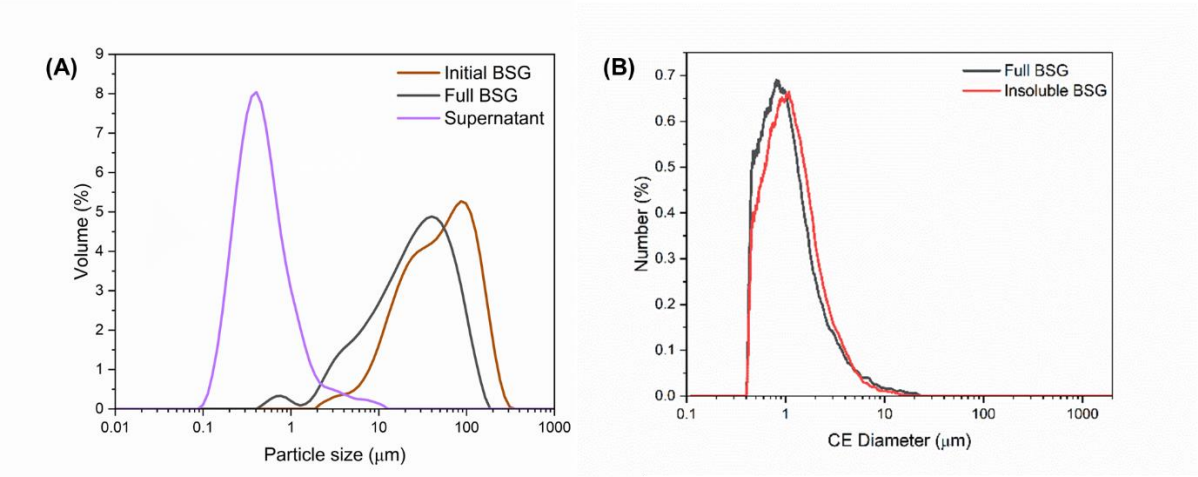


Figure 3.3. (A) Particle size distribution of dispersions from initial BSG before colloid milling, full BSG after colloid milling and the supernatant after centrifugation of full BSG (by laser diffraction); (B) Particle size distribution of dispersions from full BSG and insoluble BSG after homogenization (by automated particle imaging)

Assuming the particles were spherical, an asymmetric particle size distribution based on a circle-equivalent (CE) diameter was obtained through the image analysis software for full and insoluble BSG dispersions (Figure 3.3B). Majority of the particles in both dispersions had a circle-equivalent diameter below 10 μm. In this particle size distribution, full BSG has slightly more particles below 1 μm than insoluble BSG, which can be attributed to the generation of fine particles after colloid milling. These fines are removed from insoluble BSG after centrifugation.

Particles that are at least one order of magnitude smaller than the targeted emulsion droplet size generally provide good surface coverage and can give effective Pickering stabilisation (Schulman & Leja, 1954). However, it is also possible to make stable emulsions comprising of oil droplets that are similar in size as the particles in dispersion, as demonstrated by Gould, Vieira, and Wolf (2013) and Kurukji, Pichot, Spyropoulos, and Norton (2013) using cocoa particles and sodium stearoyllactylate aggregates respectively. This can be explained by the emulsification process itself being capable of concurrently deaggregating the particles and reducing their size further during oil droplet breakup. Furthermore, only the finest particles will act as stabilisers while the larger fractions remain unadsorbed. With majority of the particles centering around 1 μm, we can expect to create stable emulsion droplets that are larger than 10 μm.

3.3.1.3. Interfacial tension and dilatational rheology

In previous studies, colloid milling was found to disintegrate the physical structure of fibres and result in an increase in soluble components (Ullah et al., 2018). These soluble components may be surface-active and have an effect on the interfacial behaviour. Therefore,

full BSG was centrifuged and filtered to measure interfacial activity over time. On the other hand, insoluble BSG is free from soluble components and the adsorption of insoluble particles at the interface does not influence the interfacial tension (Berton-Carabin & Schroen, 2015; Vignati, Piazza, & Lockhart, 2003). The results from the interfacial tension measurement of the soluble fraction are illustrated in Figure 3.4A.

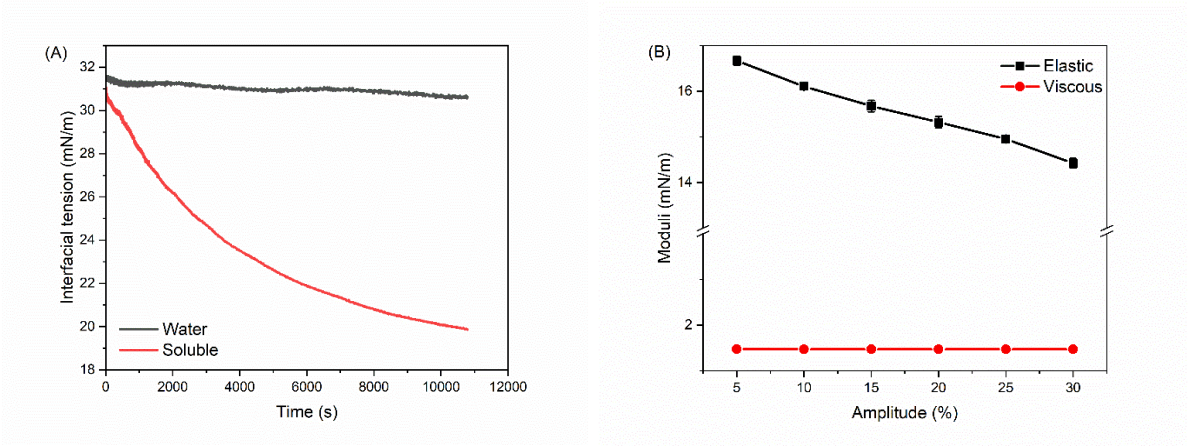


Figure 3.4. (A) Interfacial tension of oil-water interface stabilised by the soluble component in the supernatant as a function of time; (B) Dilatational elastic and viscous modulus of soluble component in the supernatant as a function of amplitude of droplet deformation

The interfacial tension between stripped soybean oil and water without any BSG addition was around 31 mN/m and remained stable over the timescale of the experiment. The soluble components in the supernatant gradually decreased the interfacial tension and reached about 20 mN/m after 3 h. To verify whether soluble proteins could be the cause for the reduction of the interfacial tension, FTIR was used to identify bands in the amide I and II region (1500-1700 cm^{-1}) that are typical of proteins (Supplementary Information, Figure 3.12). A bicinchoninic acid assay was also performed to estimate the protein content present. The results suggest that proteins with a concentration of 0.2 ± 0.02 mg/ml were indeed present in the soluble fraction, which could arise from residual wort proteins remaining in the BSG after lautering (Celus et al., 2006) or from the release of soluble proteins as a result of colloid milling (Anderson & Guraya, 2001). Therefore, the reduction in interfacial tension suggests that the soluble proteins are surface-active and diffused towards the oil-water interface. In the case of full BSG where soluble proteins exist together with insoluble particles, soluble proteins can adsorb on the particles or compete with particles to adsorb at the interface (Berton-Carabin & Schroen, 2015; Dickinson, 2012). It is also possible that the soluble proteins reduce the interfacial tension to an extent that the high desorption energy for particles is lowered and adsorbed particles on the interface are displaced, as observed by Vashisth, Whitby, Fornasiero, and Ralston (2010) between silica nanoparticles and SDS.

Apart from the ability to reduce the interfacial tension, the viscoelasticity of the interface formed by the soluble fraction of the supernatant was examined by dilatational rheology (Figure 3.4B). The elastic modulus (14-17 mN/m) was much higher than viscous modulus (~1.5 mN/m), indicating predominantly elastic behaviour, which is characteristic of protein adsorption at the interface (Evans, Ratcliffe, & Williams, 2013).

3.3.2. Characterisation of emulsion and its physical stability over time

3.3.2.1. Droplet size

Oil-in-water emulsions (10 wt%) were prepared with full BSG and insoluble BSG at concentrations of 0.5, 1 and 2 wt% in the aqueous phase. The emulsion droplet size was determined to investigate the emulsifying ability of these multicomponent dispersions. Both light microscopy and laser diffraction were used to estimate the actual droplet size as non-adsorbed particles could also contribute to the scattered light signal. Based on light microscopy images without SDS added (Figure 3.5), the individual emulsion droplet sizes of fresh emulsions prepared with full BSG and insoluble BSG were estimated to be 40 µm and 25 µm respectively. As expected, the large particle sizes present in the dispersions contributed to the large droplet sizes of both emulsions. Both emulsions also showed network formation in the continuous phase, which could be due to flocculation or particle bridging. To determine whether flocculation occurred, SDS was used as an anionic detergent to disrupt hydrogen bonds that developed among proteins and other dispersed components. Full BSG emulsions diluted with SDS showed more well-dispersed droplets in the continuous phase, indeed indicating reversible flocculation. On the other hand, clusters of droplets were still seen in the insoluble BSG emulsion, which may suggest that particle bridging was more dominant here, since particle adsorption to the oil-water interface is not likely to be affected by SDS.

The differences in droplet sizes and aggregated states of emulsions prepared with full BSG and insoluble BSG could be attributed to the presence of surface-active proteins in the full BSG. Studies have shown that multicomponent systems, as in the case of full BSG containing surface-active molecules and particles, these free molecules may adsorb onto particles and modify their wettability through hydrophobic or electrostatic interactions, inducing particle flocculation (Binks, Rodrigues, & Frith, 2007; Dickinson, 2012). Above the isoelectric point, soluble proteins are typically positively charged and the particles could be negatively charged, leading to electrostatic adsorption of the soluble proteins onto the particle surface. Consequently, the particles become hydrophobic and uncharged, resulting in particle flocculation (Binks et al., 2007). Addition of SDS makes the particles negatively charged again and overcomes flocculation. Alternatively, these soluble proteins can also compete with particles to adsorb on the oil-water interface (Berton-Carabin & Schroen, 2015). In both cases, they can result in incomplete surface coverage, leading to larger emulsion droplets. Particles

which are also present in full BSG later adsorb on the interface and prevent the droplets from becoming larger and coalescing.

On the other hand, insoluble BSG consisted of mainly insoluble fibres and proteins. It was previously reported that cell wall polysaccharides in BSG are associated with proteins (Crowe et al., 1985; Ibbett et al., 2019; Niemi et al., 2013). Colloid milling partially disintegrated the cell wall structure, resulting in fibrils that collide and entangle through electrostatic interactions and hydrogen bonding (Saarikoski et al., 2012; Ullah et al., 2018). These fibres contain protein particles that are amphiphilic and can anchor on the oil-water interface (Dickinson, 2009). Therefore, by means of the proteins residing on the interface, the fibres can act as Pickering particles and form an interconnected network which bridges oil droplets.

Despite instability through flocculation and particle bridging in the emulsions, the droplet sizes remained relatively constant over time based on microscopic observations, indicating that minimal coalescence of droplets occurred during storage. This can be attributed to steric stabilisation from the particles, giving rise to an interfacial film with a thickness is at least equal to the size of the particles. Further on in section 3.3.2.2, it is also observed that there is sufficiently strong electrostatic repulsion between droplets that help to prevent coalescence.

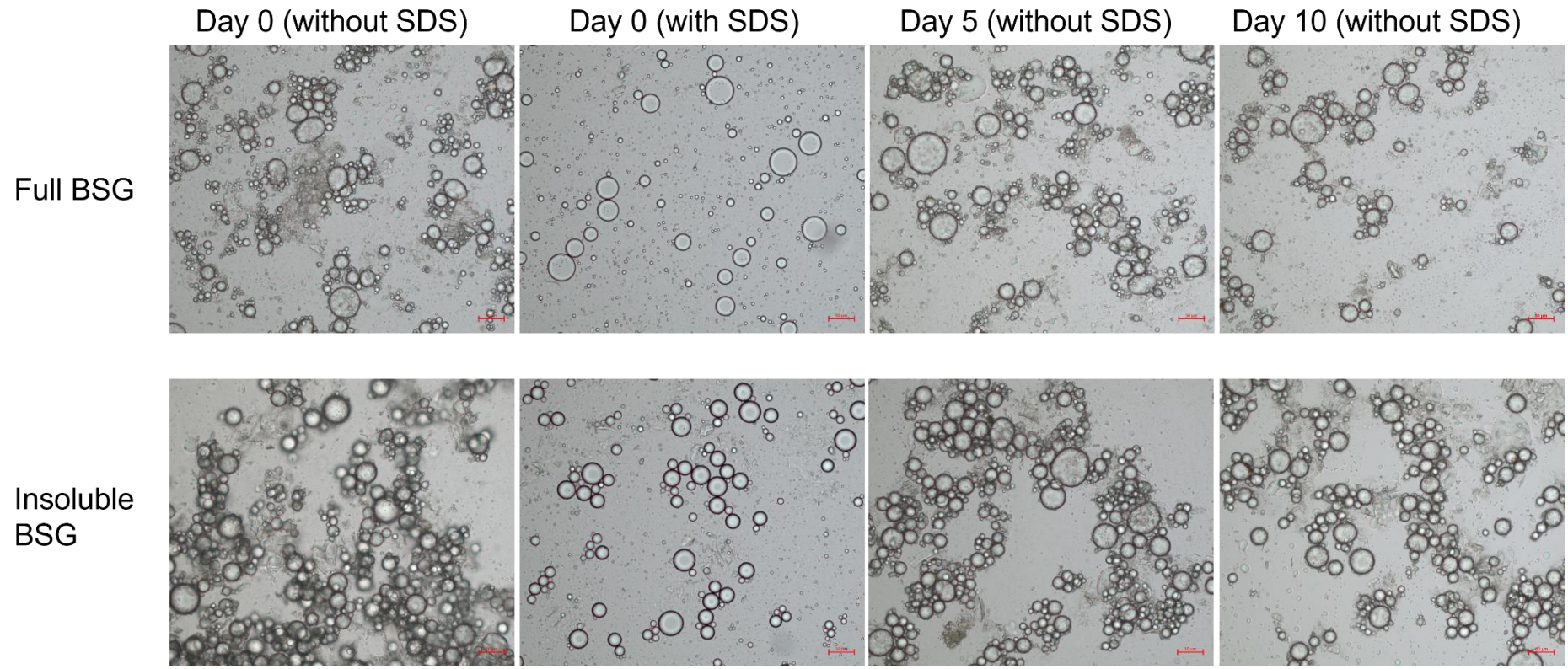


Figure 3.5. Light micrographs of emulsions prepared with full BSG and insoluble BSG prepared at 2 wt% at day 0, 5, 10. Scale bar represents 50 µm.

The droplet size distribution of both emulsions was also investigated with laser light diffraction. From Figure 3.6, both emulsions displayed a similar polydisperse distribution, except for a large proportion of droplets above 100 µm in full BSG, which could suggest flocculation or coalescence. When SDS was added to the emulsions, there was a reduction in droplet size above 100 µm in full BSG, indicative of flocs that were broken up by SDS, which was not observed with insoluble BSG. This aligns with what was observed and explained earlier with the light microscope images, in which the adsorption of soluble proteins result in particle flocculation. Over time, the droplet sizes (d_{32}) of both emulsions did not change significantly over time ($P \leq 0.05$), which is in line with our results using light microscopy.

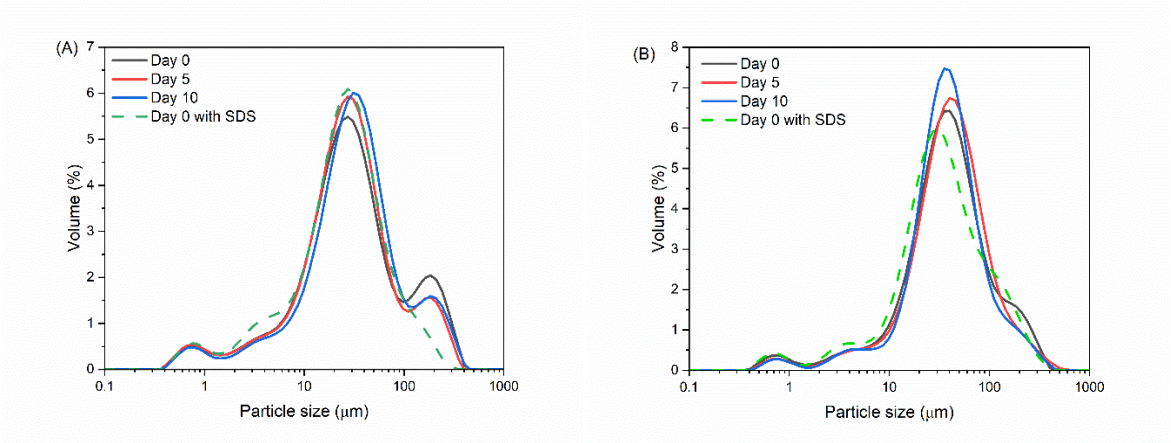


Figure 3.6. Emulsion droplet size distribution of 2 wt% (A) full BSG and (B) insoluble BSG over time

The concentration of particles also influenced the size of the emulsion droplets. Irrespective of the dispersion used, increasing the particle concentration shifted the size distribution to the left (Figure 3.7), since more particles were present to stabilise the extra oil-water interface required for smaller droplets. However, we can observe that at 2 wt% particle concentration, the distribution becomes trimodal. The peak at the smaller size range could refer to the emulsion droplets in the system while the peak at the larger size range could be related to the higher degree of flocculation in the system. This can be related to the aggregation of fibres at higher concentration due to the formation of a fibre-based network among oil droplets (Nomena et al., 2018; Qi, Song, Zeng, & Liao, 2021; Winuprasith & Suphantharika, 2013). It could also be a case of depletion flocculation, in which an excess of non-adsorbed particles result in an attractive force between oil droplets as their depletion zones overlap.

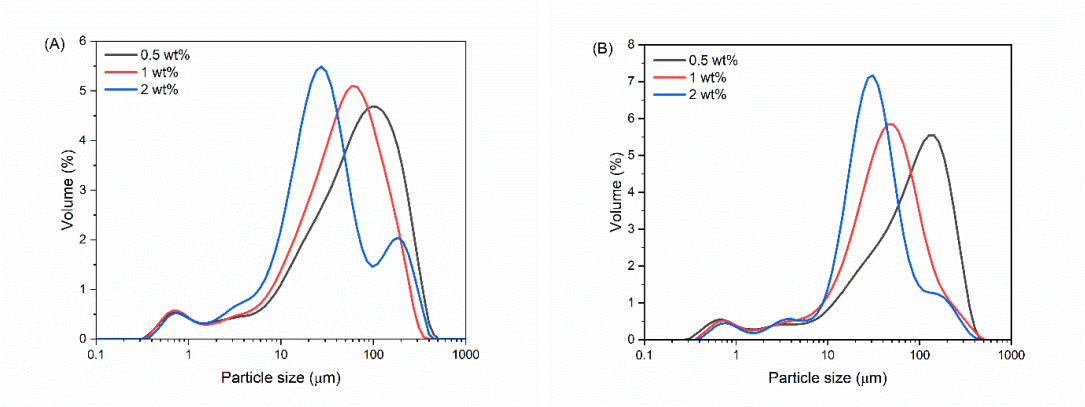


Figure 3.7. Emulsion droplet size distributions of (A) full BSG and (B) insoluble BSG with solid content of 0.5, 1, 2 wt% at day 0

3.3.2.2. Zeta-potential

The zeta potential is a measure of the surface charges present on the oil droplets, which is an indicator of colloidal stability. All emulsions had a negative charge, with an initial zeta potential of about -28 mV and -26 mV for full BSG and insoluble BSG emulsion respectively (Figure 3.8). The adsorption of proteins would lead to a net negative surface potential and hence negative zeta potential. Over time, there was a slight increase in the net zeta potential (more negatively charged) which could be due to conformational rearrangement of the proteins at the interface (Wiącek & Chibowski, 2002). Across all days and both samples, there was no significant difference ($P > 0.05$) in the surface charge.

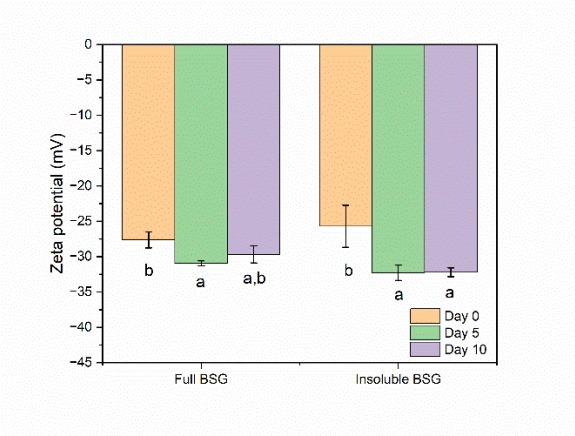


Figure 3.8. Zeta potential of emulsions prepared with full BSG and insoluble BSG at 0.5 wt% in aqueous phase over time. Different small upper letters in the same type of sample indicate significant difference at $P \leqslant 0.05$. Error bars refer to standard deviations.

With this surface charge, emulsion droplets are likely to experience strong electrostatic repulsion (Freitas & Müller, 1998), which suggests good emulsion stability as droplets are not likely to coalesce. These net zeta potentials are higher (absolute value) than those of emulsions stabilised with full and insoluble pea proteins with values of -16 mV and -18 mV

respectively (Hinderink et al., 2021), but relatively similar to emulsions stabilised by microfibrillated cellulose particles (Winuprasith & Suphantharika, 2013). This could indicate the presence of fibres, namely cellulose, which contribute negative charges by the presence of carboxyl and hydroxyl groups (Sjostrom, 1989; Stana-Kleinschek & Ribitsch, 1998). As mentioned earlier, the emulsion droplets are stabilised by protein particles which are connected to fibres to form a network, therefore the overall electrical charge of droplets measured was based on fibres.

3.3.2.3. Protein surface load

The protein surface load of the emulsions was determined to examine whether the amount of proteins adsorbed at the interface was influenced by the choice of dispersion (Figure 3.9).

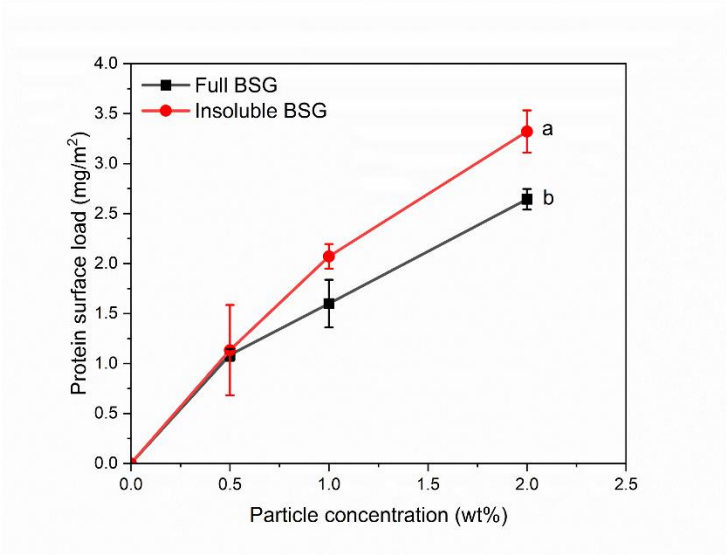


Figure 3.9. Protein surface load as a function of particle concentration for full BSG and insoluble BSG. Different small letters indicate significant difference at $P \leqslant 0.05$. Error bars refer to standard deviations.

In both emulsions, a higher particle (protein) concentration led to more protein adsorption at the interface. At low particle concentrations, there was no significant difference in protein surface load between full BSG and insoluble BSG, presumably due to less soluble proteins present in full BSG, thereby the effect of soluble proteins on particles is less pronounced and particles have sufficient time to adsorb on the interface. However, at 2 wt% particle concentration, full BSG had a significantly lower protein surface load ($P \leq 0.05$) than insoluble BSG despite the higher protein content in the former (Table 3.1). This is because soluble proteins in full BSG have greater binding affinities and surface activities than particles, and generally a lower amount of soluble proteins is required to stabilise the emulsion (Dickinson, 2009). Therefore, full BSG emulsion, which comprises of a combination of soluble proteins and particles, does not require as much proteins than insoluble BSG to stabilise the interface.

However, it is worth noting that the difference in protein surface load, although statistically lower in full BSG, is not as drastic as expected, perhaps due to low soluble protein concentration. Particle-stabilised interfaces like insoluble BSG have an interfacial thickness that is at least equal to the particle size for a monolayer, which is much larger than conventional emulsifiers, therefore a higher surface load is expected (Berton-Carabin & Schroen, 2015). In fact, the protein surface load for an emulsion prepared using conventional emulsifiers is typically around 1-2 mg/m^2 (Dickinson, 2009), whereas an emulsion made with zein particles was 109 mg/m^2 (Feng & Lee, 2016). However, our results show a low protein surface load of only 3.3 mg/m^2 was obtained, which clearly suggests that there are insufficient protein particles adsorbed at the interface to stabilise the emulsions. Yet, the emulsions remained stable against coalescence, which suggests that non-protein particles, i.e. fibres, contributed to emulsion stability by forming a steric barrier that efficiently prevented droplet coalescence. This was previously reported by Wallecan et al. (2015) who proved that functionalised orange pulp fibres can be used as Pickering particles that are anchored at the oil surface by means of proteins. The study also showed that opening up the microstructure of the fibres made them more accessible, which could help to reduce the amount of particles required to stabilise the interface.

3.3.2.4. Microstructure of emulsions

To visualise the spatial arrangement of particles at the oil-water interface, confocal laser scanning microscopy was carried out.

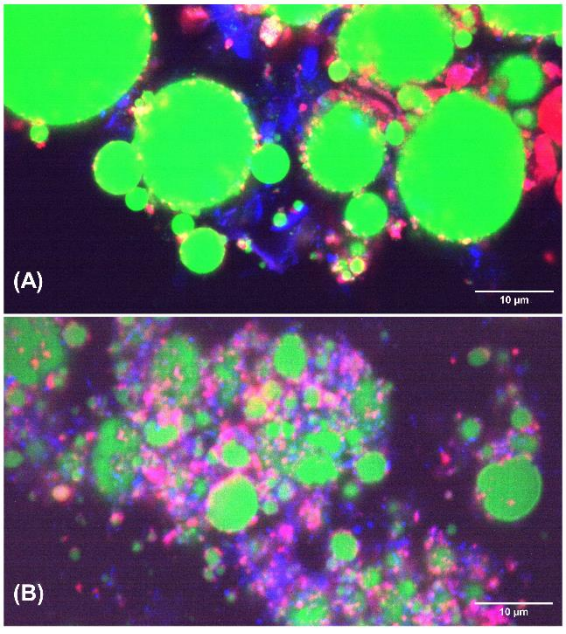


Figure 3.10. CLSM micrograph of emulsions of (A) full BSG and (B) insoluble BSG. Lipids, fibres and proteins are coloured green, blue and red respectively. Scale bar represents 10 µm.

From the confocal micrograph in Figure 3.10, both emulsions have protein particles (red) surrounding oil droplets (green), evident from the brighter yellow spots produced from the combination of the oil droplets and protein on the interface. This confirms that in both emulsions, protein particles are adsorbed at the oil-water interface, but only in sparse amounts, which is in line with the results from the protein surface load (section 3.3.2.3). Besides soluble proteins, protein particles are also involved in stabilising the full BSG emulsion, which could be due to a low concentration of soluble proteins present. With incomplete coverage from the soluble proteins, the particles have sufficient time and area to adsorb on the interface.

Fibre particles (blue colour) were found in the vicinity of the protein particles and oil droplets, suggesting that these particles might play a secondary role in stabilising (but also bridging) the oil droplets. This is consistent with our previous explanation and past studies that the separated fibre strands contain protein regions that act as the surface active component and reside in the oil-water interface (Dickinson, 2003; Nomena et al., 2018; Wallecan et al., 2015). Primarily, the protein particles are the emulsifiers and the fibres that are bound to these proteins form a network and create steric stabilisation. The interconnected structure, however, induces bridging between the oil droplets, which is later observed as creaming in the emulsions (Figure 3.11).

3.3.2.5. Macroscopic behaviour of emulsions

The macroscopic behaviour of the emulsions was assessed by visual appearance and creaming stability measurements. Due to the large droplet sizes and the bridging of oil droplets in both emulsions, creaming of the emulsions was expected and evaluated by monitoring the creaming index.

From Figure 3.11A, the creaming index significantly increased over time ($P \leq 0.05$) and remained constant after day 8 in both emulsions. Between both samples, creaming was initially similar in both emulsions but after day 5, faster creaming was seen in the full BSG emulsion than the insoluble BSG emulsion ($P \leq 0.05$). This is associated to the larger emulsion droplets in full BSG as previously seen in section 3.3.2.1, which indicates poorer emulsion stability. Smaller droplet sizes provide larger interfacial surface area which contributes to emulsion stability. After 10 days of storage, no free oil separation was observed for the emulsions, suggesting that no extensive coalescence occurred.

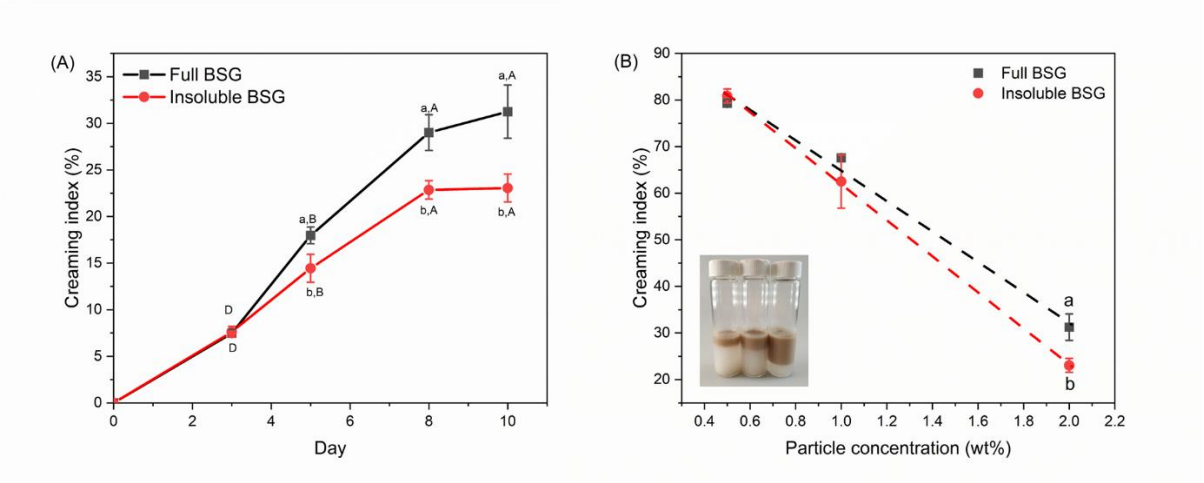


Figure 3.11. (A) Creaming index of 2 wt% full BSG and insoluble BSG stored at 4°C for 10 days; (B) Creaming index as a function of increasing particle concentration at day 10 (photo: an emulsion prepared with full BSG after 10 days, with increasing particle concentration from left to right). Different small letters indicate significant difference ($P \leqslant 0.05$) between full and insoluble BSG, and different big letters indicate significant difference ($P \leqslant 0.05$) between days. Error bars refer to standard deviations.

As presented in the photo of Figure 3.11B, the emulsion was separated into two phases during storage, and this was observed in both emulsions. The top emulsion phase was a cream (brown) layer likely formed by particle aggregates or droplet flocculation. The bottom phase, a white turbid layer, possibly contained small oil droplets that remained stable and were able to scatter light. According to Figure 3.11B, as particle concentration increased, creaming was significantly reduced at 2 wt% ($P \leq 0.05$). We hypothesise that the particles need a certain minimum concentration to form a network around the oil droplets. When more particles are present in the continuous phase, network formation is promoted (Binks, 2002; Wallecan et al., 2015), therefore the upper layer is proportionally thicker. When there are sufficient BSG particles present, no creaming would occur. A correlation between particle concentration and the creaming index was determined to find out the minimum particle concentration that prevents creaming. Based on our calculations, emulsions prepared using full BSG or insoluble BSG as dispersions should contain a particle concentration of at least 3 wt% to ensure that creaming does not occur. This agrees with our results that there is insufficient protein particles on the emulsion droplets as visualised by CLSM and also discussed in section 3.3.2.3. An increase in particle concentration will help to increase the emulsion stability, enabling more protein particles to adsorb on the oil-water interface through the fibre network.

3.4. Conclusion

We investigated the emulsifying performance of brewers' spent grains after colloid milling. After physical treatment, full and insoluble BSG were still largely composed of proteins and fibres which moderately aggregate in an aqueous dispersion due to interactions between the fibres. A small amount of soluble surface-active proteins were detected in the full BSG dispersion by interfacial tension measurements and its presence was confirmed by FTIR. These soluble proteins have an effect on emulsion stability. During homogenisation, the soluble proteins diffuse rapidly towards the interface, but they can also adsorb on the particles or compete with the particles for adsorption on the interface. As the soluble protein concentration is relatively low, there is incomplete coverage on the interface, allowing particles to have sufficient time and area to also adsorb on the interface. This was confirmed through microscopy images that showed protein particles adsorbed on the droplet, indicating that both soluble proteins and protein particles are likely responsible for emulsion stability in full BSG. Larger droplet sizes of full BSG emulsion can also be explained by the incomplete coverage by the soluble proteins that initially adsorbed, followed by the later adsorption of protein particles, which prevented the droplets from increasing in size and coalescing. On the other hand, insoluble BSG emulsions were likely stabilised by a true Pickering mechanism comprising of protein particles bounded to fibres in the continuous phase. Polysaccharides are associated with amphiphilic protein regions which can act as an anchor on an oil-water interface. Colloid milling broke down these fibres and created an interconnected network of fibres that enabled steric stabilisation of the interface. Microscopy images showed that the fibres were in the vicinity of the droplets and likely formed a bridge between them. These fibres were also critical in ensuring sufficient electrostatic repulsion and allowed the emulsions to remain physically stable over a 10-day period despite the large droplet sizes. Our findings suggest that mild processing of BSG that is rich in proteins and fibres can lead to emulsions with promising performance and comply with sustainable and clean-label strategies. Without further purification, colloid mill-treated BSG can be used as a plant-based emulsifier. The presence of fibres also improve the nutritional quality of the emulsified product as it can serve as a prebiotic (Abdi & Joye, 2021).

3.5. Supplementary Information

The identity of the soluble filtered component in the supernatant was determined using ATR-Fourier Transform Infrared Spectroscopy (FTIR) with a thermally controlled Bio ATR2 unit at 25^{o}C and MCT detector (Confocheck^{TM} system, Bruker Optics, Germany). The FTIR spectrum was recorded at a resolution of 4 cm^{-1} and averaged over 64 scans across the spectral range of 400-4000 cm^{-1}. For each measurement (performed at least thrice), 20 µl of sample was placed on the cell. For interpretation of results, baseline correction and vector normalisation were applied on the OPUS 8.1 software (Bruker, USA). BCA assay (Thermo Fisher Scientific, USA) was performed in accordance to the supplier's instructions.

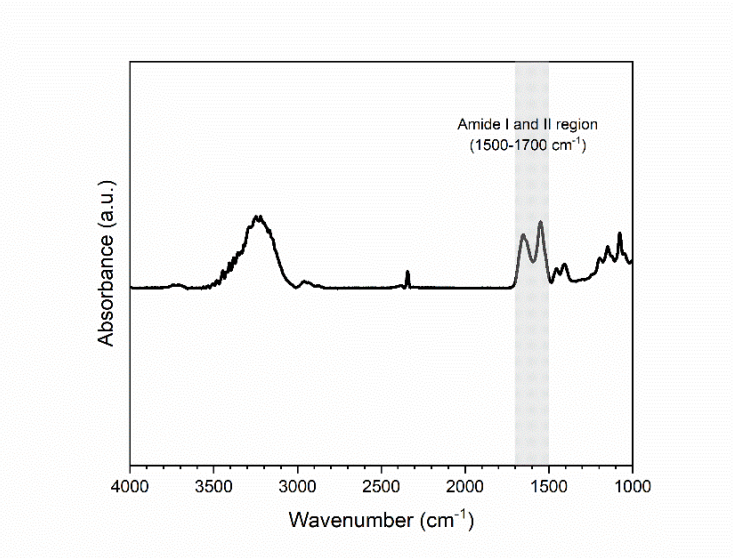


Figure 3.12. FTIR identification of the soluble filtered component in the supernatant suggests the possibility of soluble proteins based on the bands present; grey shaded area is the amide I and II region (1500-1700 cm^{-1}) that are typical for proteins

Chapter 4

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

This chapter is modified from: Chin, Y.L., Chai, K.F., & Chen, W.N. (2022). Upcycling of brewers' spent grains via solid-state fermentation for the production of protein hydrolysates with antioxidant and techno-functional properties. *Food Chemistry: X, 13*, 100184. doi:https://doi.org/10.1016/j.fochx.2021.100184

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 were more soluble than unfermented ones, especially at alkaline pH. Fermented proteins also presented an oil binding capacity that was almost twice that of unfermented extracts. The emulsifying activity and stability after fermentation doubled that of the unfermented extracts, but there was limited improvement in foaming properties. The improved functionalities were generally attributed to improved solubility and exposure of hydrophobic groups due to protein unfolding. Greater antioxidant activities were found with the fermented extract, as indicated by a higher ABTS inhibition and stronger reducing power. No cytotoxic effect was detected in the range of 2-10 mg/mL. When applied in a mayonnaise formulation, fermented hydrolysates had larger oil droplets and lower viscosity than egg-based mayonnaise, but could be used as a partial substitute for eggs. Our results suggest that fermented BSG protein is a potential plant-based emulsifier for food applications.

4.1. 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, Niedźwiecki, Jagiełło, Uchańska, & Trusek, 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; Niemi et al., 2013) 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 a mixture of proteins and polysaccharides would be obtained, making it challenging to isolate pure BSG protein if that is of interest. 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; Treimo, Aspmo, Eijsink, & Horn, 2008a). Even though enzymes are highly specific and only require mild conditions for protein solubilization, the 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.

4.2. Materials and methods

4.2.1. 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 ^{o}C. The BSG was thawed at room temperature and autoclaved (Hirayama HG-80, Japan) prior to fermentation.

4.2.2. Fermentation of BSG

The fermentation of BSG was adopted from the study described by Cooray and Chen (2018). BSG was fermented with *Rhizopus oligosporus* ATCC 64063 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 ^{o}C for three days. Both fermented and unfermented BSG were freeze-dried, grinded into a powder and passed through a sieve size of 400 microns.

4.2.3. 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 ^{o}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 (Jones, 1931).

4.2.4. Characterisation of protein extracts

4.2.4.1. Amino acid profile

The amino acids present in the extract were determined according to Perez-Palacios, Barroso, Ruiz, and Antequera (2015) with some modifications. Protein extracts were hydrolysed using 6 M HCl at 105 ^{o}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, USA) coupled to a mass spectrometer (5975C inert MSD with Triple Axis Detector, Agilent Technologies, USA) equipped with a HP-5MS capillary column. Amino acid standard (AAS18) from Sigma-Aldrich was used for quantitation.

4.2.4.2. Degree of protein hydrolysis

The degree of hydrolysis (DH) was quantified using the trinitrobenzenesulphonic acid (TNBS) method described by Spellman, McEvoy, O'Cuinn, and FitzGerald (2003). Briefly, 5% (w/v) TNBS (Thermo Scientific, USA) 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 ^{o}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, USA). A calibration curve using L-leucine (0-2.0 mM) in 1% SDS was constructed for quantitation. DH was calculated using: DH% = $100AN_2 – AN_1Npb$, where AN_1 and 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, Piggott, & FitzGerald, 2014).

4.2.4.3. 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 ^{o}C for 5 min. After a short centrifugation and cooling to room temperature, 10 µL 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.

4.2.5. Functional properties

4.2.5.1. Protein solubility

The extracts solubility at different pH was determined by following the method of Intarasirisawat, Benjakul, Visessanguan, and Wu (2012). 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:

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

4.2.5.2. 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, Ahmedna, and Goktepe (2007).

4.2.5.3. Emulsifying activity and stability

The emulsifying properties of the extracts were investigated following the method of Klompong, Benjakul, Kantachote, and Shahidi (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 µL 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:

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

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

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

4.2.5.4. Foaming properties

The foaming capacity and stability of the extracts were investigated based on the method described by Klompong et al. (2007) and Intarasirisawat 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:

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

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

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

4.2.6. Antioxidative assays

4.2.6.1. 2,2'-9-azinobis-(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, Switzerland) stock solution with a known concentration of potassium persulfate (Sigma-Aldrich, USA) 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 solution with an absorbance of 0.70 ± 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 µL of ABTS and mixed thoroughly in a microplate well before being read at 734 nm (Varioskan LUX, Thermo Fisher Scientific, USA). The antioxidant capacity was calculated as: $\%\,\mathrm{Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$, where $A_{control}$ is the absorbance of the working ABTS solution and A_{sample} is the absorbance of the samples with ABTS reagent. Gallic acid was used as a standard.

4.2.6.2. Ferric Ion Reducing Antioxidant Power (FRAP)

The FRAP assay was conducted as described previously by Benzie and 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 ^{o}C for 10 min before 950 µL was added to 50 µL of protein extract or standard. The samples were incubated at 37 ^{o}C for 30 min and absorbance was read at 593 nm (Varioskan LUX, Thermo Fisher Scientific, USA). Gallic acid was used as a reference standard, with concentrations ranging from 0.1 mg/ml to 0.5 mg/mL.

4.2.6.3. Total phenolic content (TPC)

The Folin-Ciocalteu (F-C) assay was used to determine the phenolic content of the protein extracts. To 50 µL of sample solution (10 mg/mL), 20 µL of F-C reagent (Sigma-Aldrich, USA) followed by 930 µL 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.

4.2.7. 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 x 10^4 cells/well and incubated overnight at 37 ºC in a 5% CO_2 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 ºC. 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 ºC. Next, MTT solution was removed by pipetting and 100 µL 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, USA) and compared to the control (protein extract absent). The relative cell viability (%) was calculated according to: $\%\ \text{Relative viability} = \frac{Abs_{sample\ @\ 540\ nm}}{Abs_{control\ @\ 540\ nm}} \times 100\%$

4.2.8. Application as a plant-based emulsifier

4.2.8.1. 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, Mazaheri Tehrani, and Daneshvar (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% BSGP or FBSGP + 60% whole egg (BSGP40 or FBSGP40), 60% BSGP or FBSGP + 40% whole egg (BSGP60 or FBSGP60), 100% BSGP or FBSGP (BSGP100 or FBSGP100). Prepared mayonnaise emulsions were stored at 4 ºC for further characterization.

4.2.8.2. Optical microscope observation

Samples were observed under a light microscope (Olympus IX71, Japan) equipped with a digital camera (DP70, Olympus, Japan) to compare the microstructure of the emulsions. The size of the emulsion droplets were estimated with a sample size of 50 droplets using ImageJ software.

4.2.8.3. 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 ºC at a shear rate of 50 s^{-1} as described by Rahmati et al. (2014).

4.2.8.4. 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 ^{o}C water bath for 20 min prior to centrifugation.

4.2.9. Statistical analysis

All experimental data were analysed using Minitab v. 19 Statistical Software (Minitab Inc., Coventry, UK). Experimental data were analysed using student's t-test or one-way ANOVA with Tukey's post hoc test (for more than 2 groups). The results were then expressed as mean value ± standard deviation at 95% confidence intervals.

4.3. Results and Discussion

4.3.1. 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 FBSG 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 minimising 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 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.

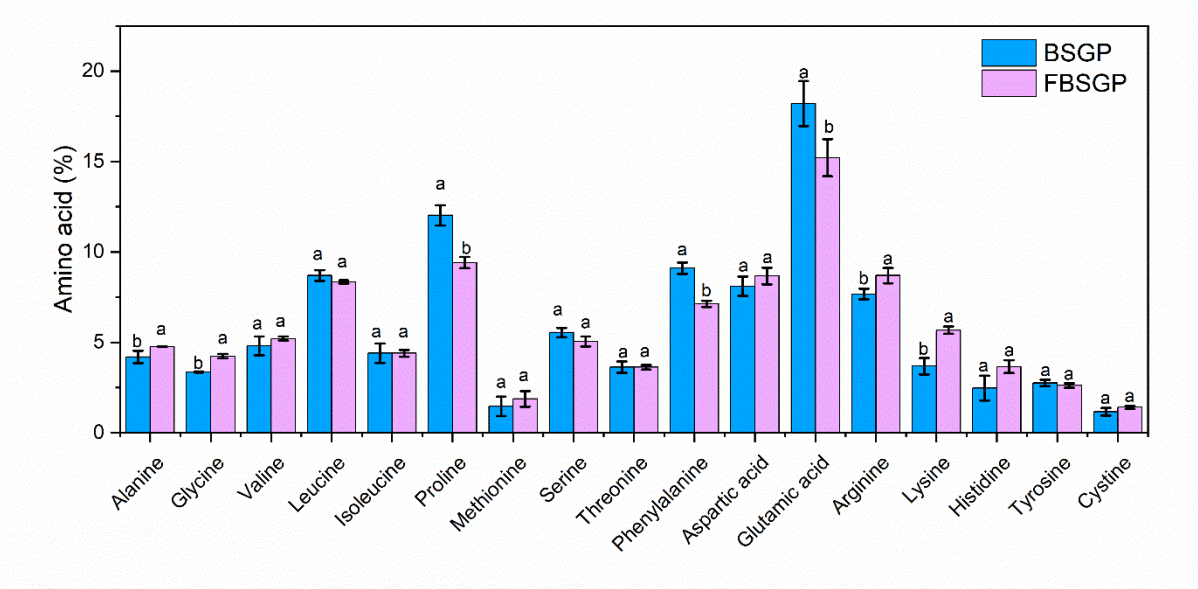


Figure 4.1. Changes in amino acid composition for unfermented and fermented BSG after three days. Aspartic acid includes asparagine. Glutamic acid includes glutamine. Results are means of triplicate determinations. Values with different letters denote significant differences ($P \leq 0.05$).

Changes in amino acid composition were also observed between BSGP and FBSGP as shown in Figure 4.1. 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. (2008a) during enzymatic hydrolysis of BSG proteins. This indicates an improvement in nutritional status of the fermented product and hence its extracted proteins.

Previous studies have reported that fermentation changes the amino acid profile of the initial BSG (Cooray & Chen, 2018; Tan, Mok, Lee, Kim, & Chen, 2019). The ratio of hydrophobic to hydrophilic amino acids was therefore examined to determine if the extracted proteins have a preference towards an organic or aqueous phase. Due to the complexity of defining amino acid hydrophobicity in literature, a few papers were studied (Biswas, DeVido, & Dorsey, 2003;

Hopp & Woods, 1981; Kyte & Doolittle, 1982) and the hydrophobic amino acids in this study were specified as: alanine, glycine, valine, leucine, isoleucine, proline, methionine, phenylalanine and cystine. Based on our results, fermentation did not drastically change the ratio of hydrophobic to hydrophilic amino acids. About 47% of amino acids in both BSGP and FBSGP were considered hydrophobic, implying that the proteins are likely amphiphilic and may have a possible emulsification property.

BSGP and FBSGP were subjected to SDS-PAGE as shown in Figure 4.2. The bands shown are representative of hordeins and glutelins in barley (Wang et al., 2010). Barley hordeins, which are extracted by alcohols in the presence of a reducing agent, are classified into 5 groups (A, B, C, D and γ-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). Barley glutelins, which can be extracted by alkali, show bands around 35-55 kDa, 25 kDa and below 20 kDa. Our 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 glutelins (Connolly et al., 2013; Wang et al., 2010).

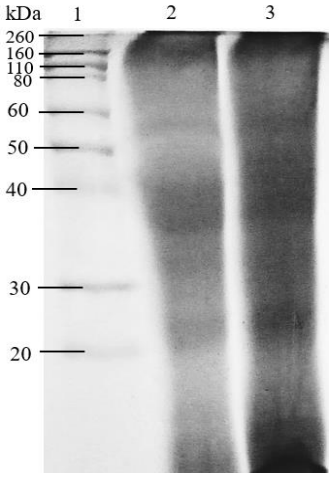


Figure 4.2. SDS-PAGE patterns of BSGP and FBSGP where lanes 1, 2 and 3 are molecular weight marker, BSGP and FBSGP, respectively

In this study, both protein extracts presented similar bands ranging from about 20-55 kDa and some bands below 20 kDa, suggesting that there was limited hydrolysis of the proteins therefore both extracts contained polypeptides of similar molecular weights. However, the bands were more diffused in FBSGP as compared to BSGP and below 20 kDa there were more intense bands, indicating some protein degradation as a result of enzymatic hydrolysis. Celus, Brijs, and Delcour (2009) reported that BSG protein hydrolysates with good emulsifying activity have a high surface hydrophobicity and contained less than 40% of protein fragments

with molecular weight exceeding 14.5 kDa. On the other hand, BSG protein hydrolysates with good foaming properties generally have a low surface hydrophobicity and contained less than 10% of material with molecular weight lower than 1.7 kDa. This was attributed to the need for retaining a minimum three-dimensional structure so that stable protein films can be formed at the interfaces. Extensive hydrolysis may instead lead to the loss of functionality.

4.3.2. Functional properties of BSGP and FBSGP

4.3.2.1. 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 (Małecki, Muszyński, & Sołowiej, 2021).

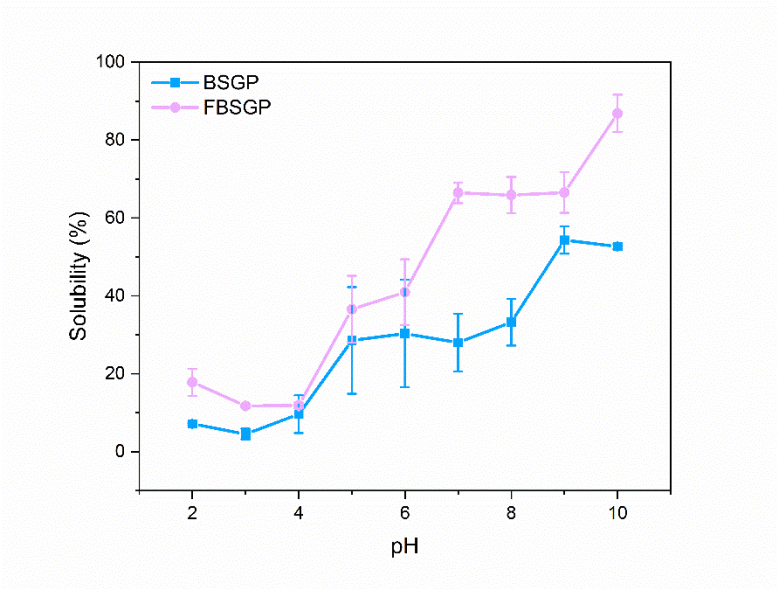


Figure 4.3. Solubility of BSGP and FBSGP as influenced by pH

Figure 4.3 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 4. 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, Raasmika, & Sangeetha, 2021). The fungal fermentation process adopted in this study increased the solubility of the proteins by hydrolysing some of the complex structure of proteins to peptides through the proteases secreted by *R. oligosporus*. This facilitates the

proteins to solubilise in the extracting solution. When peptide bonds are broken, the native protein conformation is lost, resulting in protein unfolding. Nonpolar and some polar amino acid groups that were previously buried inside the protein could be exposed to the surface. In particular, since BSG proteins largely comprise of hordeins and glutelins that are rich in hydrophobic amino acids, there is an increase in hydrophobicity of the proteins. Besides hydrophobic groups, exposed polar amino acids may interact with water molecules through hydrogen bonds and charged residues can repel each other through electrostatic interactions and subsequently increase the protein solubility (Celus et al., 2007).

The low solubility of these proteins at pH 4 could possibly be due to the lowest repulsion of proteins at their isoelectric point, which is around pH 4-5 based on previous studies (Kotlar et al., 2013; Wang et al., 2010; Yalçın & Çelik, 2007). At neutral pH, BSGP is poorly soluble in water due to protein aggregation caused by the formation of disulfide bonds induced during mashing (Celus et al., 2006). Fermentation released peptides and amino acids which gives FBSGP a much higher solubility at neutral pH. At alkaline pH, the proteins and peptides experience increased electrostatic repulsion, leading to improved solubility. FBSGP with high solubility over a wide pH range could therefore be applied widely in formulated food systems.

4.3.2.2. Water and oil holding capacities

WHC is a measure of the water-binding of the proteins by polar side chains while OHC is a measure of the fat-binding capacity of proteins via nonpolar side chains. Figure 4.4 shows the water and oil holding capacities of both BSGP and FBSGP.

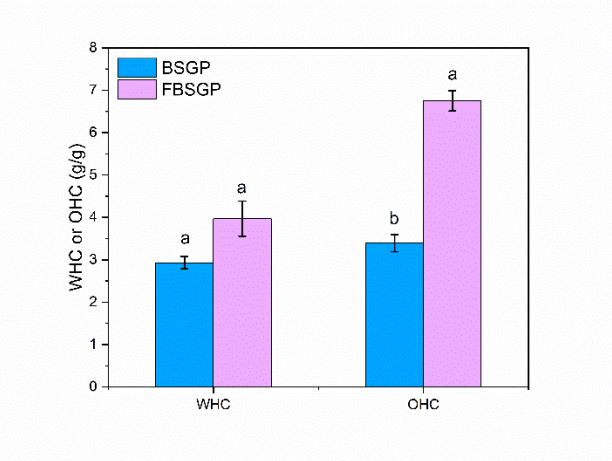


Figure 4.4. Water holding capacity (WHC) and oil holding capacity (OHC) of BSGP and FBSGP. Values with different letters denote significant differences ($P \leq 0.05$) between BSGP and FBSGP.

BSGP exhibited WHC and OHC of 2.9 g/g and 3.4 g/g respectively, which is relatively similar to that of barley hordeins and glutelins reported by Wang et al. (2010). On the other hand, FBSGP had a WHC and OHC of about 4.0 g/g and 6.7 g/g respectively. There was no

significant difference in WHC between BSGP and FBSGP ($P > 0.05$), but the OHC of FBSGP was significantly higher ($P \leq 0.05$) compared to that of BSGP. In a native protein, hydrophobic groups are buried inside the folded protein structure. During fermentation, enzymes that are secreted hydrolyse the proteins, which resulted in protein unfolding and exposed hydrophobic groups. This increased surface hydrophobicity enabled the proteins to bind more oil. Generally, a balanced water and oil holding capacity is useful for applications involving the stabilisation of oil-water interfaces.

4.3.2.3. Emulsifying properties

Since proteins are amphoteric polyelectrolytes, their emulsifying and foaming behaviours are expected to vary with different pH 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 pH are depicted in Figure 4.5A and Figure 4.5B respectively.

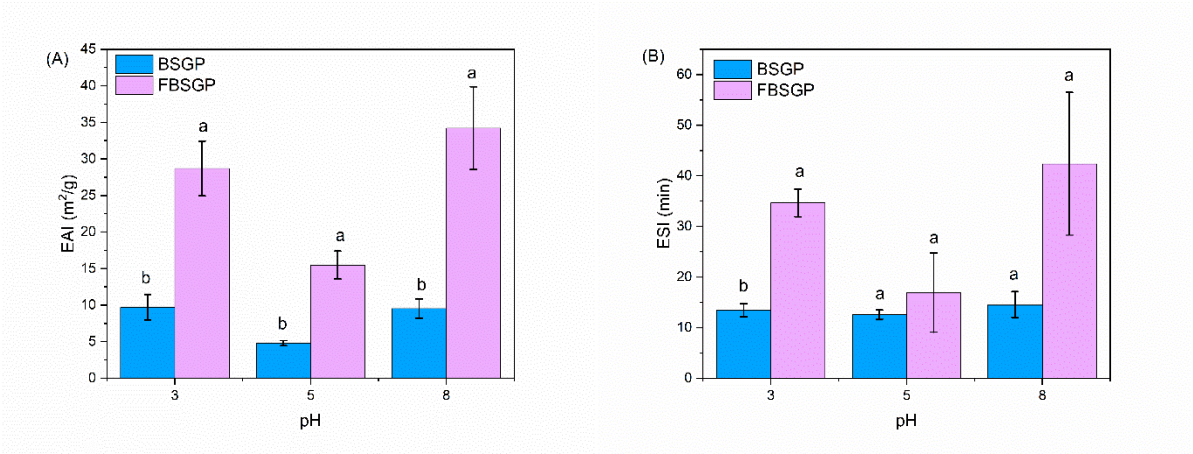


Figure 4.5. (A) Emulsifying activity index (EAI) and (B) emulsifying stability index of BSGP and FBSGP as influenced by pH. Values with different letters denote significant differences ($P \leq 0.05$) between BSGP and FBSGP.

FBSGP had a significantly higher EAI than BSGP ($P \leq 0.05$), but there was no significant difference ($P > 0.05$) in ESI between the two extracts except at pH 3. Better emulsifying properties was shown by FBSGP largely due to the structural modification after fermentation in which some peptides were liberated from their inert parent proteins upon microbial fermentation, leading to protein unfolding (Chai, Voo, & Chen, 2020; Görgüç, Gençdağ, & Yılmaz, 2020; Saadi, Saari, Anwar, Abdul Hamid, & Ghazali, 2015). Hydrophobic groups that were previously buried inside the proteins are exposed, and the small and soluble peptides in FBSGP rapidly diffuse and adsorb at the oil-water interface to form a viscoelastic film, resulting in higher emulsion activity and stability. This agrees well with the results on WHC and OHC in the previous section, showing that fermentation increased the oil uptake of the proteins. Also

our results corroborate with Park, Chang, and Cho (2019) who also demonstrated that the protein hydrolysates obtained from soybean meal after fermentation contained more short chain peptides and amino acids and thus showed better emulsifying properties. The high EAI values suggest that hydrolysates may be able to act as emulsifiers in formulated food products such as mayonnaise, soups and sauces.

At pH 5, both types of BSG proteins exhibited the lowest EAI and ESI. When the pH was decreased to pH 3 or increased to pH 8, the EAI and ESI of both proteins increased. These results show the same trends as the work by Celus et al. (2007) and Connolly et al. (2014), in which enzymatically hydrolysed BSG protein concentrates and isolates have a lower EAI and ESI near the isoelectric point and improved emulsifying properties away from the isoelectric point. Previous studies have shown that a positive correlation exists between solubility and emulsifying properties (Bilgi & Çelik, 2004; Connolly et al., 2014), and adjusting pH affects protein solubility (Figure 4.3). Near the isoelectric point, there is increased protein-protein interactions, leading to low surface hydrophobicity and reduced protein solubility. As such, oil droplets experience lesser electrostatic repulsion, leading to the aggregation of droplets and a reduction in emulsion stability. Away from the isoelectric point, proteins and peptides are more soluble and there is an increase in electrostatic interactions between droplets, therefore emulsion stability is improved.

4.3.2.4. Foaming properties

The pH dependence of foaming capacity (FC) and foaming stability (FS) of BSGP and FBSGP are shown in Figure 4.6A and Figure 4.6B, respectively.

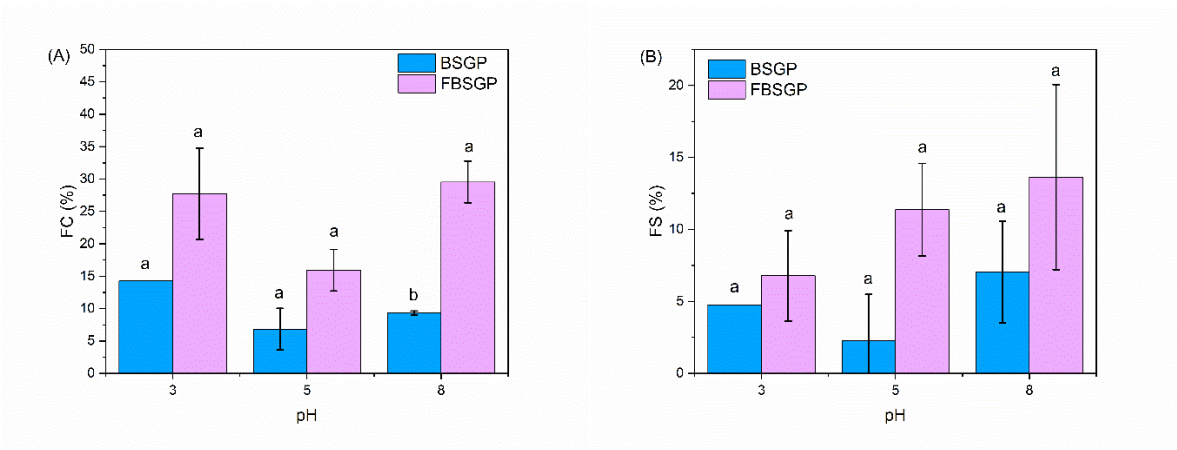


Figure 4.6. (A) Foaming capacity (FC) and (B) foaming stability (FS) of BSGP and FBSGP as influenced by pH. Values with different letters denote significant differences ($P \leq 0.05$) between BSGP and FBSGP.

The trends in foaming capacity and foaming stability were largely similar to that of emulsification, since these functionalities depend on the protein's interfacial behaviour, which

are influenced by solubility, size and surface hydrophobicity. However, there was no significant difference between BSGP and FBSGP for most FC and FS values ($P > 0.05$) apart from a significantly higher FC in FBSGP at pH 8 ($P \leq 0.05$). The similarity in FC and FS between BSGP and FBSGP can be attributed to the presence of only a small amount of short chain peptides present in FBSGP despite fermentation, as evident from SDS-PAGE in Figure 4.2. A previous study by Yalçın, Çelik, and İbanoğlu (2008) showed that an increase in degree of hydrolysis led to higher solubility of barley protein hydrolysates, resulting in better foamability. This suggests that foamability is largely influenced by low molecular weight components and fermentation of BSG probably did not liberate sufficient short chain peptides to substantially improve foaming properties. Also, the exposed hydrophobic groups in FBSGP that was discussed previously, did not appear to influence the foaming behaviour, which could indicate that protein hydrophobicity may not be an important factor when stabilising an air-water interface as compared to an oil-water interface. This is supported by Molina-Ortiz and Wagner (2002) who reported that protein solubility has a greater impact than surface hydrophobicity on the ability of hydrolysates to form and stabilise foams.

The low solubility at pH 5 (Figure 4.3) limited the amount of peptides that could adsorb at the air-water interface. At lower or higher pH, the peptides have an increased net charge and higher solubility. Consequently, there is a decrease in attractive hydrophobic forces which increases protein flexibility and enables the protein to diffuse more rapidly to the air-water interface to stabilise air molecules (Connolly et al., 2014; Wang et al., 2010), which explains the higher FC in FBSGP at pH 3 and 8. At pH 3, the peptides that stabilised the foam probably formed a thin and weak interfacial film due to the lack of interaction with other peptides (Yalçın et al., 2008), resulting in a low FS of FBSGP. When pH is increased to 5, the neutral net charge promotes protein-protein interactions and creates a stronger and thicker interfacial film that is more stable (Wang et al., 2010). At pH 8, due to higher solubility, there are more peptides present to strengthen the interfacial film and further increase the stability of the interface. Connolly et al. (2014) also reported similar results for their BSG hydrolysates where the FS of their hydrolysates increased with pH.

4.3.3. 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 4.1), which can be explained by the contribution from phenolic compounds and amino acids present in the extract.

Table 4.1. Antioxidant activity and total phenolic content of BSGP and FBSGP. Results are means of triplicate determinations. Values with different letters denote significant differences ($P \leq 0.05$)

	ABTS (µg GAE/mg protein DW)	**FRAP (µg GAE/mg protein DW)**	**Total phenolic content (µg GAE/mg protein DW)**
BSGP	42.0 ± 17.7^{b}	129.40 ± 2.98^{b}	52.34 ± 0.45^{b}
FBSGP	84.5 ± 29.4^{a}	140.31 ± 3.86^{a}	53.75 ± 0.29^{a}

Antioxidants are often associated with the presence of phenolic compounds as they possess the ability to scavenge free radicals and chelate metal ions (Pereira, Valentão, Pereira, & Andrade, 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 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 & Medina-Meza, 2020; Cooray & Chen, 2018).

In addition to phenolic compounds, the peptides and amino acids produced after hydrolysis, can play a part in contributing to the antioxidant activity (Chai et al., 2020; Connolly et al., 2019; Vieira, da Silva, Carmo, & Ferreira, 2017). In the ABTS assay, antioxidants present in solution would donate a hydrogen atom to the synthesised ABTS radical, 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). After fermentation, FBSGP has smaller peptides which can diffuse quickly to scavenge free radicals. Furthermore, as discussed previously, fermentation can help to expose electron-dense amino acid side chain groups, such as polar or charged moieties or increase free amino acids that act as an additional source of protons and electrons (Bamdad et al., 2011), both of which can help to increase reducing power and thus antioxidant activity.

Generally, amino acids such as tyrosine, methionine, histidine, lysine and tryptophan are considered to possess antioxidant activity and demonstrate higher antioxidative activity as part of a peptide (Saito et al., 2003; Sanjukta et al., 2021). Of these amino acids, there was only a significant increase in lysine content in FBSGP ($P \leq 0.05$) which may not sufficiently

explain the significant improvement in antioxidant activity. It is important to note that the antioxidant activity in these peptides are not only influenced by the composition of amino acids but also the sequence and length of the peptide. Peptidomics studies may be necessary to isolate the peptides after fermentation to completely elucidate the core of the antioxidant activity.

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, increased diffusivity of peptides and enhanced amount of antioxidative peptides present.

4.3.4. 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, Niazi, Jahan, Ahmad, & Hussain, 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.

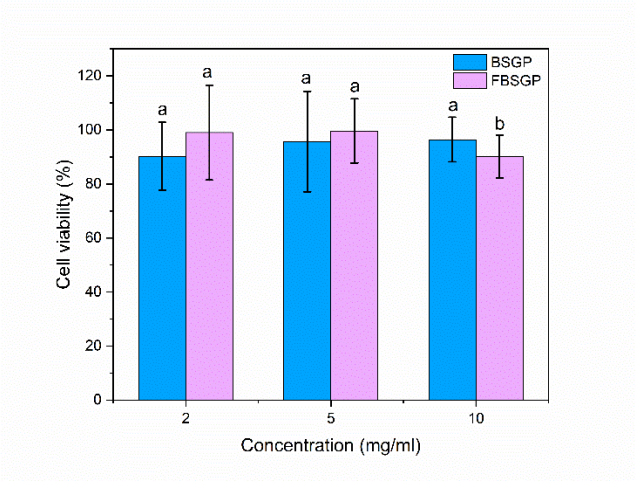


Figure 4.7. Cytotoxic effect of BSGP and FBSGP from MTT assay. Concentrations of 2, 5 and 10 mg/ml were tested for each sample. Results are means of triplicate determinations. Values with different letters denote significant differences (P ≤ 0.05).

Based on our results (Figure 4.7), 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.

4.3.5. 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.

4.3.5.1. 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 Figure 4.8. In general, all samples had tightly packed microstructures, except for the BSGP100. With a high oil volume fraction, it is expected that the droplets interact more with each other and are compressed to polyhedral shapes. The estimated droplet sizes of the emulsions were generally in the range of 7-60 µm with a wide size distribution for each emulsion, which is expected from homogenizing using a commercial immersion blender for a short time as compared to homogenisers in the industry that yield more uniform and smaller droplet sizes (Aganovic, Bindrich, & Heinz, 2018). It was not possible to estimate the droplet size of BSGP100 emulsion from the microscope image, as the mayonnaise broke (Figure 4.10d).

Eggs and in particular, the egg yolk, contains small surface-active molecules in the nanometre range like lecithin and low-density lipoproteins that confer good emulsifying abilities (Bengoechea, Lopez, Cordobes, & Guerrero, 2009). In contrast, BSGP and FBSGP, are made of comparatively larger proteins and peptides. Therefore, the emulsion droplet sizes of mayonnaise made with egg yolk and whole egg are expected to be the smallest among all samples, which aligns with our results.

Emulsions of BSGP40, BSGP60, FBSGP40 and FBSGP60 had larger droplet sizes than both controls. This is due to the low concentration of soluble proteins in BSGP and FBSGP. With a higher soluble protein concentration, a larger interfacial area can be covered by the proteins, resulting in in smaller droplets. The emulsion droplet sizes of BSGP and FBSGP were relatively similar. Certainly the surface-active egg (yolk) components played a significant role in the emulsion stability, but there was also contribution from FBSGP as we observed a slightly

smaller droplet size in the emulsions with FBSGP. Due to the addition of vinegar, mayonnaise is made at an acidic pH of ~3.6-4.0 (Xiong, Xie, & Edmondson, 2000). As seen previously in Figure 4.5, at pH 3, the EAI and ESI of FBSGP was significantly higher than BSGP, which is attributed to the improved protein solubility and exposed hydrophobic groups after fermentation. There was also significantly higher OHC in FBSGP (Figure 4.4). These soluble proteins and peptides in FBSGP can diffuse rapidly towards the oil droplets and stabilise them. In addition, previous studies have suggested that soy and lentil proteins can stabilise smaller oil droplets in an acidic environment as the proteins dissociate from their multimeric form into subunits that have an increased exposed hydrophobicity (Chang, Tu, Ghosh, & Nickerson, 2015; Jarpa-Parra et al., 2015). As a result, this encourages hydrophobic adsorption of proteins to the droplet interface. Sridharan, Meinders, Bitter, and Nikiforidis (2020a) also proved that at pH 3, which is below the isoelectric point of pea proteins, pea protein molecules instead of particles, were the primary stabilisers of oil droplets. Thus, it is reasonable to expect a slightly smaller droplet size of FBSGP in mayonnaise.

For FBSGP100 emulsion, droplet sizes are substantially larger than that of 40% and 60% formulations as well as whole egg and yolk controls. In the absence of egg (yolk), there was insufficient proteins or peptides to cover the entire oil droplet initially, therefore the droplets coalesced into larger droplets that remained stable when there was sufficient coverage. With BSGP100, it was believed that phase inversion occurred. Due to a low amount of soluble proteins and low surface hydrophobicity of BSGP, the oil fraction was too high and the oil-in-water emulsion inverted to a water-in-oil emulsion.

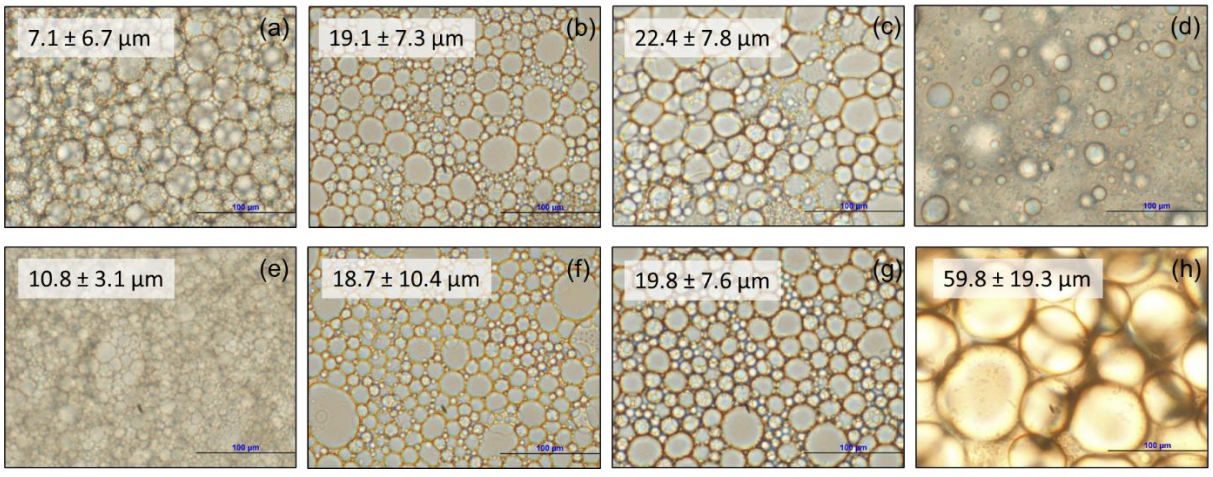


Figure 4.8. Optical microscope images of mayonnaise samples, magnification was ×60, scale bar represents 100 µm. The estimated droplet sizes are indicated on the top left corner of the

images. (a) Yolk control, (b) BSGP40, (c) BSGP60, (d) BSGP100, (e) whole egg control, (f) FBSGP40, (g) FBSGP60 and (h) FBSGP100 as the emulsifier of the emulsions

4.3.5.2. Viscosity of emulsions

The viscosity of the emulsions prepared using different types of emulsifiers was tested and the results are presented in Figure 4.9.

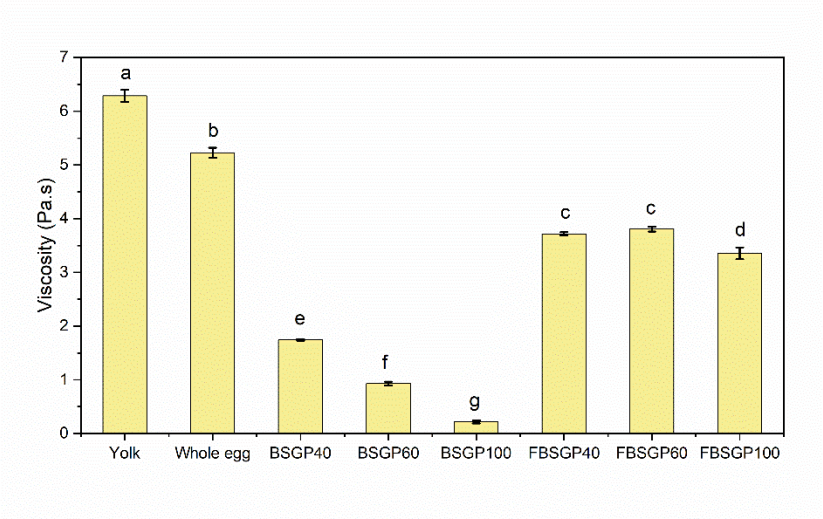


Figure 4.9. Viscosity of different samples with yolk, whole egg, different ratios of whole egg to BSGP or whole egg to FBSGP as the emulsifier in the mayonnaise. Values with different letters denote significant differences ($P \leq 0.05$). BSGP40, BSGP60, BSGP100, FBSGP40, FBSGP60 and FBSGP100 denote the ratios of BSGP-to-whole egg 40:60, BSGP-to-whole egg 60:40, BSGP-to-whole egg 100:0, FBSGP-to-whole egg 40:60, FBSGP-to-whole egg 60:40 and FBSGP-to-whole egg 100:0 respectively

There was a significant difference ($P \leq 0.05$) in viscosity among the samples. Emulsion prepared from egg yolk was the most viscous, followed by those prepared from whole egg, FBSGP and BSGP. In highly concentrated emulsions like mayonnaise, droplets come into close contact with each other to form a network structure of thin liquid films of continuous phase. Generally, smaller droplet sizes decrease the mean distance of separation between droplets and increases hydrodynamic interaction, which leads to an increase in viscosity of the emulsion (Pal, 1996). This agrees with the estimated droplet sizes in Figure 4.8, in which egg yolk and whole egg emulsions correspond to the smallest droplets and have the highest viscosities. However, despite estimating to have similar droplet sizes, FBSGP had a significantly higher viscosity than BSGP emulsions. This is perhaps due to a stronger protein network and attractive interactions with the junction points surrounding the deformed oil droplets of FBSGP because of higher solubility and hydrophobicity of the proteins. When subjected to shearing, the thin interfacial film was ruptured less easily in FBSGP leading to higher viscosity of the emulsion. This was especially evident in BSGP emulsions in which the viscosity decreased with decreasing amount of whole egg incorporated, implying a weakening of the protein network formed by egg components.

Our results for both controls and samples, showed comparable viscosities to that of soy milk formulated mayonnaise by Rahmati et al. (2014). In his study, the viscosity of mayonnaise was unaffected up to 75% replacement of egg with soy milk. From a textural attribute point of view, there was no significant difference between mayonnaise prepared with whole egg and soy milk, indicating that consumer perception to viscosity may not be as sensitive.

4.3.5.3. Creaming and thermal creaming of mayonnaise emulsions

Figure 4.10 shows the mayonnaise produced using various concentrations of BSGP and FSGP with yolk and whole egg as the controls. All samples, except for BSGP100, had a thick visual appearance that is typical of commercial mayonnaise. The egg yolk and whole egg control was pale yellow and white in colour respectively, while the rest of the emulsions had a light brown colour. Emulsions that contained FBSGP had some brown bits, which was due to larger particles that were not sufficiently dispersed in water. In Figure 4.10d, it was clear that the BSGP100 emulsion broke as it had a darker yellow colour and behaved more like a liquid than a weak gel like mayonnaise. The viscosity, as measured in Figure 4.9, was very low and close to the viscosity of oil, which points to phase inversion of the emulsion.

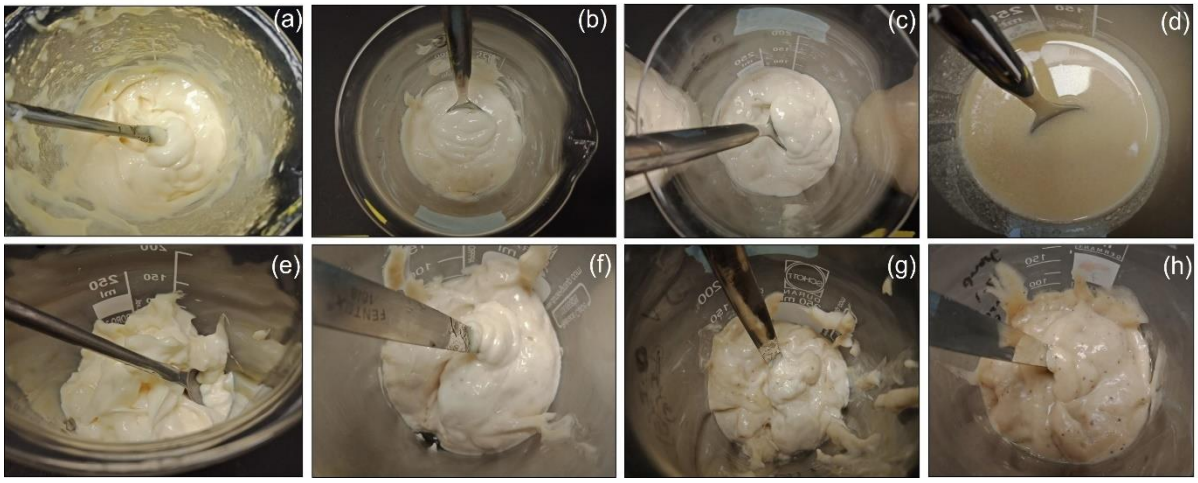


Figure 4.10. Mayonnaise emulsions produced using (a) egg yolk, (b) 40% BSGP/whole egg, (c) 60% BSGP/whole egg, (d) 100% BSGP, (e) whole egg, (f) 40% FBSGP/whole egg, (g) 60% FBSGP/whole egg and (h) 100% FBSGP as the emulsifier

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 Figure 4.11.

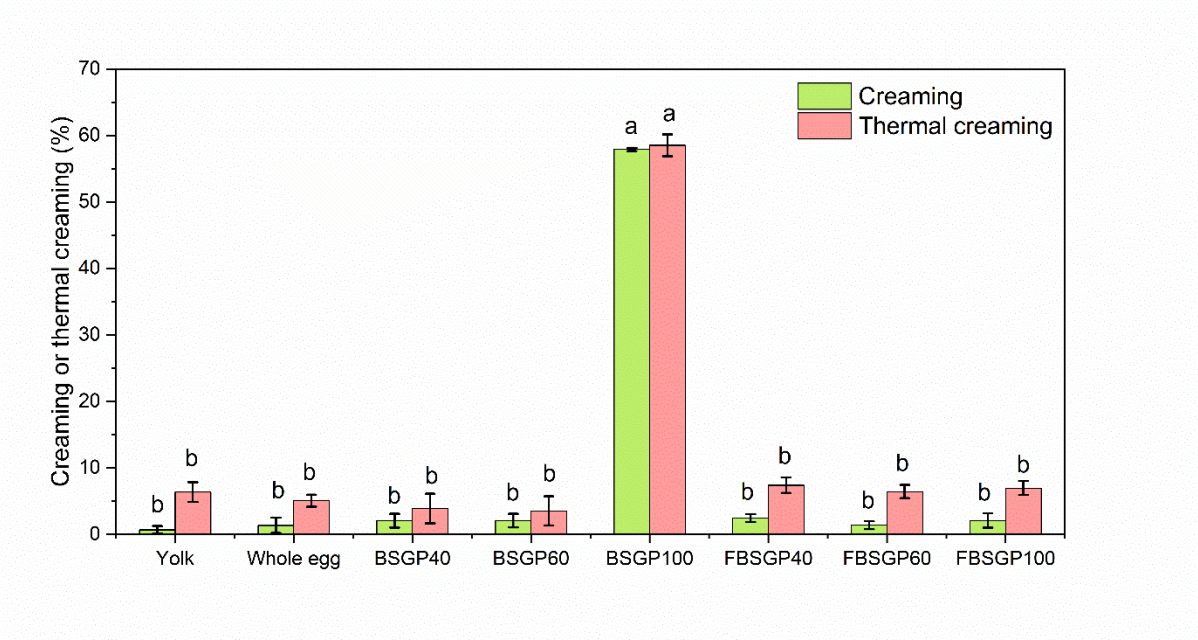


Figure 4.11. Creaming or thermal creaming of different samples with yolk, whole egg, different ratios of whole egg to BSGP or whole egg to FBSGP as the emulsifier in the mayonnaise. Values with different letters denote significant differences ($P \leq 0.05$). BSGP40, BSGP60, BSGP100, FBSGP40, FBSGP60 and FBSGP100 denote the ratios of BSGP-to-whole egg 40:60, BSGP-to-whole egg 60:40, BSGP-to-whole egg 100:0, FBSGP-to-whole egg 40:60, FBSGP-to-whole egg 60:40 and FBSGP-to-whole egg 100:0

Using different concentrations of BSGP and FBSGP as the emulsifier in producing mayonnaise had no significant difference ($P > 0.05$) in both creaming and thermal creaming tests when comparing to the controls, except for BSGP100 in which an oil layer was separated from the emulsion after centrifugation. This again confirms that phase inversion to a water-in-oil emulsion occurred and BSGP alone cannot be used to substitute egg yolk or whole egg in the production of mayonnaise. With a concentrated emulsion like mayonnaise, the tightly packed structure caused by the high oil fraction prevents droplets from moving during centrifugation (McClements, 2015), therefore creaming is unlikely to happen in mayonnaise which is shown as a low level of creaming in our results. At elevated temperatures, Brownian motion of the droplets is promoted and the viscosity of the continuous phase is decreased, which may contribute to a partial breakdown of the emulsion. This is seen as an increase in thermal creaming values in our results, although differences were not significant. Further experiments are required to determine the storage stability of these emulsions.

4.4. 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 solubility, oil binding capacities and emulsifying properties 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 lower viscosities than egg-based mayonnaise but could potentially be used as a partial substitute for eggs. However, BSGP did not perform well in the absence of egg and the emulsion was phase inverted. 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.

Chapter 5

General discussion

A section in this chapter was published as: Teng, T.S., Chin, Y.L**.**, Chai, K.F., & Chen, W.N. (2021). Fermentation for future food systems: Precision fermentation can complement the scope and applications of traditional fermentation. *EMBO Rep, 22*(5), e52680. doi:https://doi.org/10.15252/embr.202152680

5.1. Outline

Brewers' spent grains (BSG) have shown potential as a source of protein and other valuable components. After harnessing the starch-rich endosperm to produce sugars for beer brewing, BSG is currently discarded or used as animal feed (Nyhan et al., 2023; Zeko-Pivač et al., 2022). However, with the large amounts of BSG produced from brewing, more efficient uses should be sought to fully utilise our available resources. Therefore, the motivation of the research described in this thesis was to explore the possibilities of processing spent grains in a way that imparts desirable properties that have potential applications in food. We aim to obtain an understanding of how the choice of fractionation affects composition, structure and function, with a specific emphasis on the proteins in BSG. We hypothesised that a highly refined ingredient may not always be necessary for function and subsequent food application. This chapter summarises the main findings of the preceding chapters and from these compiles a processing approach aiming at functional fractions with minimal waste produced. The chapter concludes with some points to consider about BSG valorisation and an outlook for future research on valorisation methods.

5.2. Main findings and conclusions

Unlike unprocessed barley, BSG has a physical and thermal processing history which makes extracting proteins more complex. The treatment of the barley grains during brewing results in a matrix that is difficult to penetrate, and promotes the formation of disulfide bonds that cause strong protein aggregation (Celus et al., 2006; Moonen et al., 1987). Therefore, protein extraction from a byproduct like BSG is more challenging and more extensive extraction strategies are necessary to separate the proteins from the matrix. In Chapter 2, we systematically explored the effect of various protein extraction methods involving alkali, ethanol or enzymes, on the composition, structure and function of the extracted protein. We concluded that the protein concentrates exhibited distinct differences dependent on the isolation technique employed. Alkaline extracted proteins mainly comprising of glutelins, were partially unfolded and had a fraction of high molecular weight complexes while those extracted by ethanol were highly aggregated and enriched in hordeins. Enzymatic assisted extraction led to low molecular weight peptides with no prominent structural elements. These structural and compositional differences consequently resulted in differences in functional properties, mostly ascribed to solubility differences. Protein hydrolysates from enzymatically assisted extraction were the most soluble in water and showed good emulsifying properties and high antioxidant activity. Alkali-extracted and ethanol-extracted proteins were poorly water-soluble but displayed high water and oil holding capacities that enabled them to form gels. The results from this study showed that the choice of extraction method should be driven by the desired

functionality as the extraction method clearly affects the potential of the protein concentrates for different applications.

The goal of protein extraction is usually to obtain a highly purified fraction with certain desirable functionalities. However, the yield-purity dilemma, which refers to the difficulty in extracting high purity proteins with high yield, as described by Tamayo Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, and van der Goot (2018), is something to consider for large-scale extraction of functional proteins. Besides, previous studies suggested that mildly purified mixtures can also be functional (Karefyllakis et al., 2019; Möller et al., 2022; Sridharan et al., 2020b), thus extensive and complex purification procedures may not be necessary. As such, in Chapter 3, we considered the use of non-purified proteins and fibres in BSG for the stabilisation of oil-in-water emulsions. Colloid milling was used to reduce particle sizes and centrifugation was used to obtain protein-fibre mixtures with and without soluble components (termed as full BSG and insoluble BSG, respectively). Emulsions prepared with full BSG showed reversible flocculation of droplets while those with insoluble BSG formed particle bridges. Despite the large droplet sizes, the emulsions were physically stable against coalescence after 10 days, partly due to the adsorption of soluble proteins at the interface and Pickering stabilisation. These results therefore suggest that pure protein components are indeed not necessary for emulsification purposes, especially in the case of particle-stabilised emulsions.

Since BSG proteins are entrapped within a complex carbohydrate structure (Connolly et al., 2019; Niemi et al., 2013), in Chapter 4, we studied another approach in which microbial fermentation was employed to open the BSG matrix and produce protein hydrolysates. Fermentation is a more cost-effective method than enzymatic extraction since the microorganisms directly secrete the required enzymes without incurring the need for enzyme purification and its associated high costs; however it may yield different functionality. Solid-state fermentation of BSG with a food-grade fungus *Rhizopus oligosporus* successfully produced proteins and hydrolysates that had better solubility, oil binding capacity and emulsifying properties than unfermented BSG. In addition, a higher antioxidant activity was found in fermented BSG protein extracts, which could be due to the release of phenolic compounds after fermentation as well as the presence of a larger amount of exposed antioxidative peptides and amino acids. Since the protein extracts did not show any cytotoxic effects against liver cells and food-safe solvents were used throughout the production, a mayonnaise emulsion was prepared. With the fermented BSG protein extracts, droplet sizes were larger and the viscosity was lower than an egg-based mayonnaise, but a complete substitution of egg was possible, whereas the emulsion broke for the unfermented extract.

This study showed that there is potential for the fermented extract to be used as a plant-based emulsifier, and fermentation is a promising approach to process BSG for future applications.

5.3. Evaluation of processing methods

In Chapters 2 and 4, proteins were extracted from BSG and the functionalities and antioxidant activities of these extracts were determined. As similar methodologies were used, it is possible to compare some of these results and gain new insights into how the processing methods influenced protein yield, functionality and antioxidant capacity.

5.3.1. Protein extraction yield

Figure 5.1 depicts a plot of the protein yield and purity from the respective extractions. As discussed earlier, the yield-purity dilemma, in which a higher protein purity leads to decreased yields, is clearly shown here. A recent study by Karlsen, Lund, and Skov (2022) highlighted the difficulty in obtaining high protein yield and purity when extracting BSG proteins using alkaline conditions. In his study, a maximum protein content of 40-55% could be achieved but with poor protein yields of 5-10%. On the other hand, when the extraction was repeated three times, the protein yield increased to 45-50% but the protein content was reduced to 20-25%.

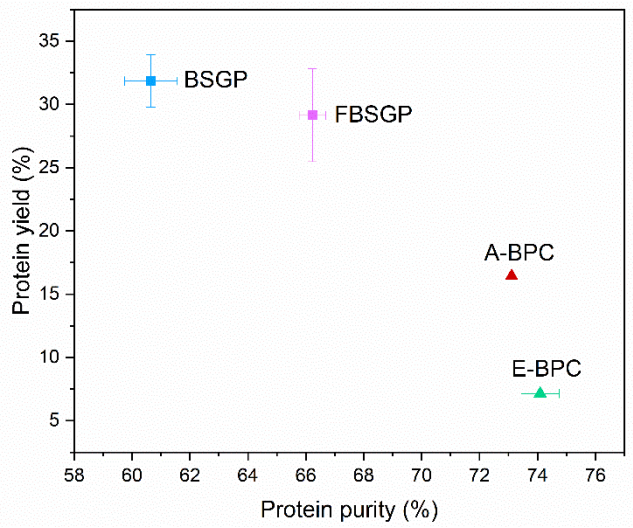


Figure 5.1. Plot of protein yield and purity from the extraction processes in Chapters 2 and 4

In our study, the protein extraction yields were also relatively poor, especially when alkali or aqueous ethanol alone was applied (Chapter 2), referring to A-BPC and E-BPC respectively. When an alkali-ethanol mixture was used (Chapter 4), an increase in protein yield is observed in BSGP and FBSGP. This is related to the respective isolation of glutelins with alkali and the isolation of hordeins with aqueous ethanol, whereas a combination of these was able to double the protein yield. However, the protein purity was reduced. This could be related to the use of a different BSG source between the two studies (Figure 5.5). For instance, the higher fibre content in the initial BSG may lead to more arabinoxylans that were co-extracted in BSGP and

FBSGP, resulting in a lower protein purity. Overall, an increase in protein yield was achieved when a combination of alkali and ethanol is used, and an optimisation of the extraction process can probably help to increase the yield further.

5.3.2. Protein functionality

Protein functionality in terms of solubility, water and oil holding capacities as well emulsifying properties were compared between Chapters 2 and 4 in Figure 5.2.

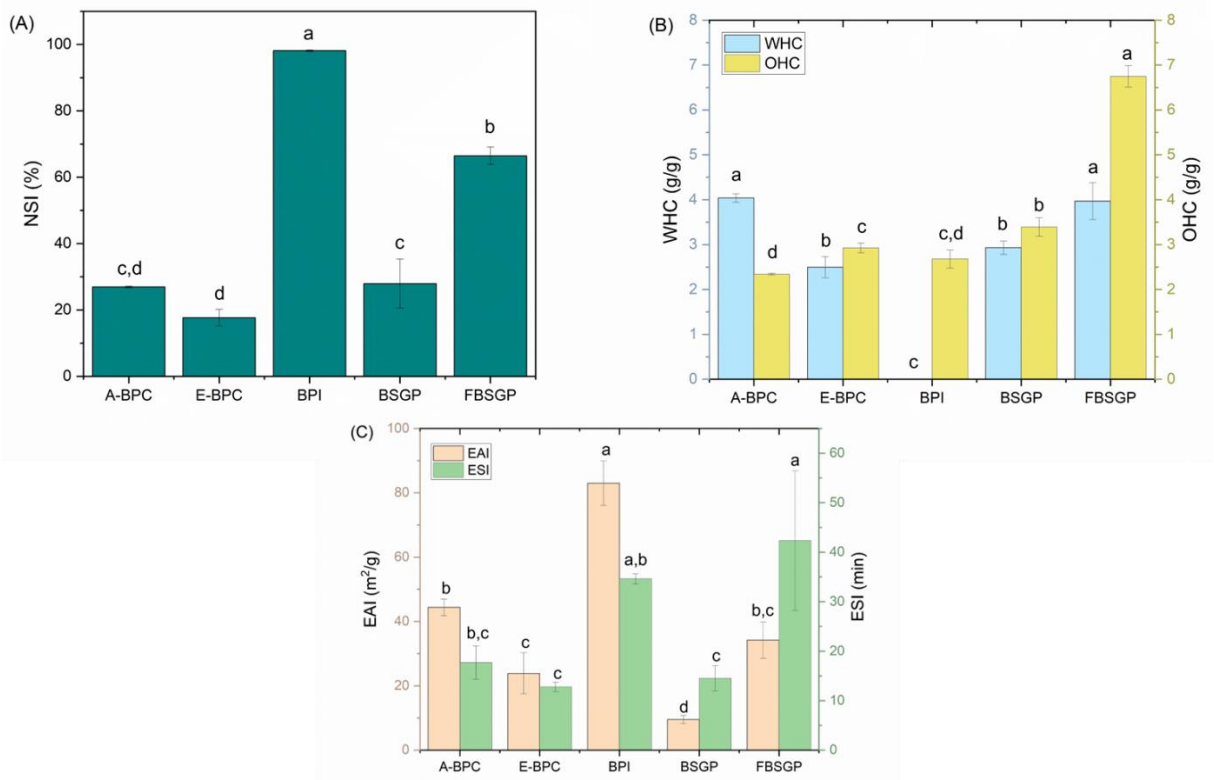


Figure 5.2. (A) Nitrogen solubility index, (B) water and oil holding capacity and (C) emulsifying activity index and emulsion stability index for the extracts in Chapters 2 and 4. Values with different letters denote significant differences ($P \leq 0.05$) for the same parameter.

Figure 5.2A shows that the highest protein solubility was from BPI followed by FBSGP. Based on the estimated molecular weights using SDS-PAGE (Figures 2.1 and 4.2), BPI was presumably hydrolysed to a larger extent than FBSGP, resulting in shorter peptides and amino acids and consequently, a significantly higher solubility than FBSGP ($P \leq 0.05$). These results indicate that hydrolysis induced by either enzymes or microbial fermentation, is most effective in improving protein solubility from BSG than extractions from alkali and aqueous ethanol.

However, with extensive hydrolysis, BPI was completely degraded to peptides, leaving it incapable of holding water unlike FBSGP (Figure 5.2B). Both FBSGP and A-BPC showed the highest WHC ($P \leq 0.05$), which can be explained by protein unfolding that occurred during fermentation and alkaline extraction, as discussed in the earlier chapters. The extent of

unfolding was perhaps significant enough in FBSGP to result in the highest OHC ($P \leq 0.05$), indicative of a large amount of exposed hydrophobic groups.

Regarding emulsification, the highest EAI came from BPI followed by FBSGP and A-BPC (Figure 5.2C). This is likely related to the highest protein solubility in BPI, which allowed the peptides to diffuse rapidly and adsorb at the oil-water interface. However, for FBSGP and A-BPC, a higher EAI could be associated with a higher WHC as previous studies have shown significantly positive correlations between emulsification capacity and WHC (Qiao, Fletcher, Smith, & Northcutt, 2001). Therefore, despite poorer protein solubility, a higher WHC was able to compensate for the difference and enable FBSGP and A-BPC to have relatively higher EAI. On the other hand, ESI is largely influenced by the protein solubility since the ESI was highest for BPI and FBSGP, corresponding to the extracts with the highest solubility.

The results from protein solubility, water and oil holding capacities as well emulsifying properties highlighted the importance of solubility on functionality, but also how structural changes like protein unfolding or extensive hydrolysis induced by processing can impact functionality.

5.3.3 Antioxidant capacity and total phenolic content

The antioxidant activity by ABTS assay and the total phenolic content of the extracts from Chapters 2 and 4 are compared in Figure 5.3.

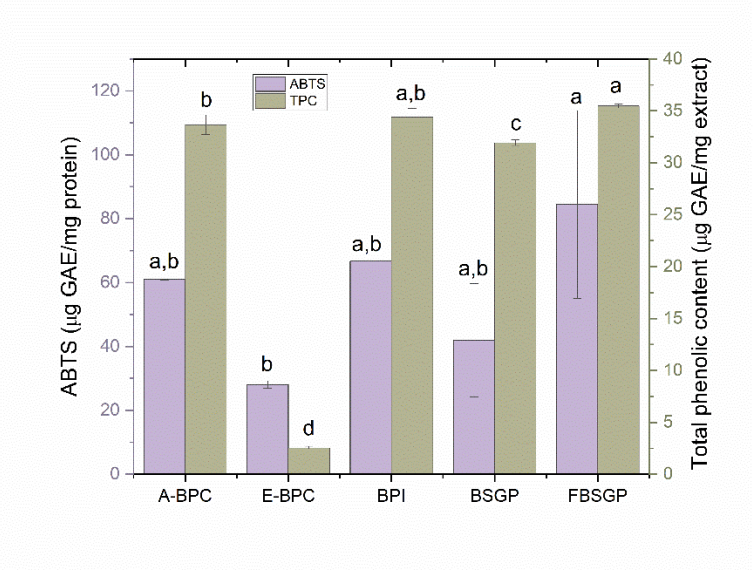


Figure 5.3. Antioxidant activity by ABTS assay and total phenolic content for the extracts in Chapters 2 and 4. Values with different letters denote significant differences ($P \leq 0.05$) for the same parameter.

From Figure 5.3, the antioxidant capacity measured by ABTS assay and the total phenolic content was generally similar for all extracts with the exception of E-BPC. As discussed

previously, antioxidant activity can be influenced by the presence of phenolics and antioxidative peptides (Saito et al., 2003). Majority of the free phenolics were removed prior to the extraction of E-BPC and the use of aqueous ethanol did not release unbound phenolics, therefore the phenolic content remained low. Furthermore, antioxidative peptides were absent from E-BPC since hydrolysis was not performed. More importantly, E-BPC was the least soluble of all (Figure 5.2A), therefore the ability to scavenge radicals is limited and it is reasonable to expect the lowest antioxidant activity. The minor amount of antioxidant activity in E-BPC is attributed to the presence of hydrophobic amino acids in the small amount of soluble proteins of E-BPC (Bamdad et al., 2011).

For hydrolysed extracts, namely BPI and FBSGP, it was not surprising to observe a high antioxidant capacity due to the release of phenolics and antioxidative peptides or amino acids released. Clearly, the use of alkali was also beneficial in improving phenolic content in A-BPC and BSGP which led to higher antioxidant activities. Our results suggest that hydrolysis and alkaline conditions are useful in increasing antioxidant activity, but the basis of this improvement is again related to solubility. It is probable that E-BPC has a relatively high amount of hydrophobic amino acids which could have led to a high antioxidant activity but the protein was strongly aggregated which resulted in poor solubility.

5.4. Processing for real-world applications

Food losses occur along the supply chain, during transportation, storage and processing. In many traditional food production processes, the aim is to convert one or more raw materials to one final ingredient or product, but this often results in side streams which might be utilised as byproducts or discarded as waste. Cereals and pulses, which are usually cultivated for their starches, have been identified as the largest contributor to water scarcity and the biggest contributor to greenhouse gas emissions associated with food loss and waste (FAO, 2019). Owing to the significant impact on our environment and resources, there is a need to devise solutions that can allow more efficient use of side streams from processing cereals and pulses.

5.4.1. Towards functional fractions

BSG is a heterogenous material, largely comprising of proteins and fibres. In conventional fractionation processes, one generally aims to obtain highly refined ingredients such as protein isolates, but this can be challenging to achieve due to the inherent properties of the material. In the case of BSG, the recalcitrant nature of the lignocellulosic matrix prevents the direct utilisation of fibre components since they are intricately connected, and the proteins are denatured and highly aggregated as a result of the brewing process. Several means are available to obtain purified components, as demonstrated in Chapter 2, but they come at the expense of considerable amounts of water, chemicals and energy (Loveday, 2020), which is

not only bad for the environment but also incurs high costs of production. This brings to mind, what the purpose is of obtaining pure components. From Chapter 2, we saw that despite the low protein content of BSG and defatted and dephenolised BSG, they still could hold water and oil to degrees that were comparable to those of protein-rich fractions and in Chapter 3, we showed that unpurified protein fractions can be used directly to stabilise emulsions. These findings reveal that pure components may not be necessary if the desired functionality can be attained with unpurified fractions. The presence of “impurities” could impart certain properties that are not even attainable with pure components. For instance, in Chapter 2, we showed that the interaction of proteins and phenolics could result in improved solubility due to the hydroxyl groups of the phenolics, and that the co-extraction of arabinoxylans and proteins can improve the water holding capacity.

Real food systems are almost always mixtures, so it is only practical that we shift our focus from obtaining high purity ingredients to functional fractions when dealing with complex materials. With this in mind, it is relevant to determine up to what concentration “impurities” can be tolerated before the functionality is altered or lost. A functionality-driven processing concept rather than one aiming for high purity is further motivated by the current trend for clean-label and organic products that put restrictions on the chemicals that can be used. Of course this is not to imply that pure fractions are no longer important, but they should only be pursued in the construction of model systems or for the mechanistic understanding of certain phenomena. In situations where it is necessary to obtain enriched protein fractions, the extraction method should be carefully selected and guided by the intended application, since the composition and structure will be affected and eventually influence its functionality.

5.4.2. Towards a circular economy

In a linear economy, environmental resources are used to manufacture useful products and side streams from processing are simply disposed of. This is far from sustainable as a large amount of waste is generated and often leaves behind residues that could be put to better use. From a circular economy perspective, it is desirable to minimise side streams from food processing and fully utilise the raw materials. This means that for better sustainability, cycles need to be closed, in which the products and residues from one industry serve as the raw material for others (Vardanega, Prado, & Meireles, 2015). In this way, maximum use is extracted at each stage and all materials are recovered or regenerated when the product life ends. The selective isolation of just useful components should be avoided, and instead we should aim to utilise the whole material as much as possible, which also aligns with our previous discussion on the use of unpurified fractions.

In Chapter 4, fermentation of BSG was carried out but only protein hydrolysates were extracted, leaving behind a wasted biomass after the process. With a biorefinery approach, a single raw material can be processed into several products in an integrative, cost-effective manner. The insoluble fibrous structure that has been partially broken down by microbial enzymes can be used as an input for colloid milling as in Chapter 3. A possible route for complete valorisation of BSG via fermentation and colloid milling is suggested in Figure 6.1.

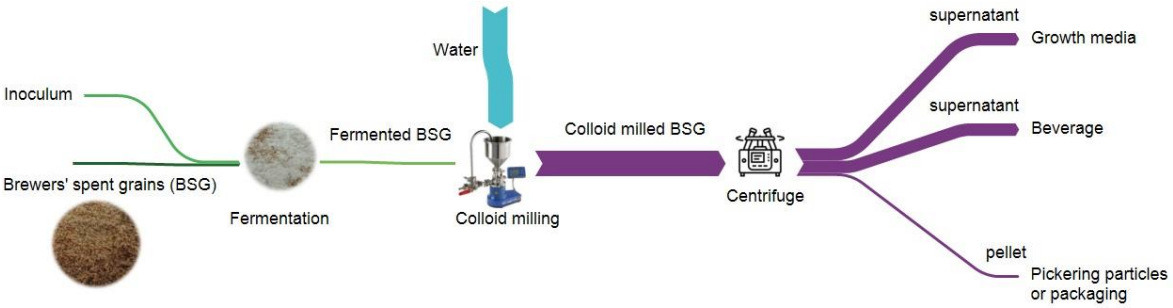


Figure 5.4. Complete valorisation of BSG through fermentation and colloid milling. Mass flows are not drawn to scale.

Previous studies highlighted the importance of using enzymatic methods to unlock the nutrients that are trapped within the matrix (Celus et al., 2007; Niemi et al., 2013), and fermentation helps to achieve that through a low-cost and sustainable approach. BSG, which contains more than 70% moisture just after being separated from the wort, has all necessary nutrients to support microbial growth. Microbes such as fungi, yeast and lactic acid bacteria can be directly inoculated onto BSG without the need for drying BSG as shown in Chapter 4, which helps to reduce energy consumption. Subsequently, using fermented BSG as an input for colloid milling again avoids the need for drying BSG and saves energy. Furthermore, fermentation enhances the accessibility for soluble fractions, which will be useful in the colloid milling step as more soluble components can be released. Our results in Chapter 3 suggest that the emulsion produced with insoluble BSG was Pickering stabilised, and these emulsions are more stable against coalescence. The colloid mill-treated BSG can be centrifuged and separated by its soluble and insoluble parts. The insoluble pellet could be used as Pickering particles for the purpose of stabilising emulsions as performed in Chapter 3, or as a composite material in packaging (Ferreira et al., 2019). On the other hand, the supernatant is a nutrient-rich liquid comprising of protein hydrolysates and oligosaccharides, which can be considered for several applications such as a growth media for yeasts that are used in producing beer (Cooray & Chen, 2018), or as a novel non-alcoholic nutritional beverage (Tan et al., 2020). In doing so, all parts of the BSG are utilised and waste streams can be eliminated to a large extent. Fermentation is an environmental-friendly method of treating byproducts (Nyhan et al., 2023) and colloid milling can process large amounts of BSG without energy-intensive drying

(Niemi, Faulds, et al., 2012), therefore an ideal scenario like this helps to minimise the environmental impact of BSG while at the same time create novel ingredients for food applications.

5.5. Important considerations for the valorisation of BSG

5.5.1. Challenges at an industrial scale

For a suitable valorisation approach, the challenges that may appear at an industrial scale should be considered in terms of a sustainability assessment. Due to the high moisture content and fermentable sugars present in BSG, it is highly susceptible to microbial spoilage (Mussatto, 2014). To ensure the long-term storage stability of BSG, several preservation methods have been investigated in previous studies, including oven drying, freeze-drying and freezing. Freezing was deemed unrealistic as large volumes have to be stored and leads to changes in arabinose content, while both drying methods decrease the volume of material without compositional changes (Bartolomé, Santos, Jiménez, del Nozal, & Gómez-Cordovés, 2002). However, freeze-drying is not economically viable and oven-drying consumes an excessive amount of energy and risks the toasting or burning of grains. Drying is also not practical if water were to be added to the dried BSG in subsequent processing. As such, a more feasible approach is to immediately use the BSG as soon as it leaves the lauter tun. This can only be possible if there is an integration of the brewery to other processing sites, for example, to a bioreactor for fermentation. Transportation costs are also reduced in this situation.

Another challenge to consider is the upscaling of the fermentation. In this work, we considered using solid-state fermentation (SSF) as a means of pretreating BSG. SSF is advantageous in terms of simplicity, higher fermentation productivity, lower energy requirement and smaller reactor volume, translating to a reduction in capital cost. However, there are several engineering problems associated with operating SSF on a large scale, mainly due to the accumulation of heat, uneven distribution of nutrients, moisture content and aeration, amongst others (Manan & Webb, 2017). Schutyser (2003) used a discrete particle modelling approach in a mixed SSF and proposed several methods that could help to overcome some of these issues, including the spraying of water over a short period with mixing followed by a period of only intensive mixing to minimise temperature gradients in the substrate bed. Alternatively, submerged fermentation, which is more common in the fermentation industry such as brewing, allows better control over environmental parameters and requires less space. However, this type of fermentation is less suitable for filamentous fungi which can grow better on a solid substrate. Furthermore, lower product yields and longer fermentation times can be expected. Excessive foaming could also occur after prolonged periods of time and prevent sufficient

oxygen transfer. Therefore, innovative engineering solutions are needed for fermentation of BSG on a larger scale.

5.5.2. Origin and processing history of BSG

In this study, we assumed that BSG was made up primarily of barley residue, but it is essential to note that breweries can include other cereal adjuncts such as wheat, rice or maize. Furthermore, variations in barley variety, time of harvest and brewing conditions or technology influence the composition of BSG (Mussatto, 2014). Other factors that may affect the consistency of BSG include the processing of barley grains prior to its use in brewing, such as the choice of malted or unmalted barley, the degree of pearling and roasting of the grains. For instance, protein isolates of black BSG that was roasted prior to brewing, were shown to have degraded to molecular weights below 5 kDa even though it had a higher phenolic content and antioxidant activity than pale BSG (Connolly et al., 2013). Figure 6.2 illustrates the compositional differences that are already evident in the spent grains supplied by different breweries for this study.

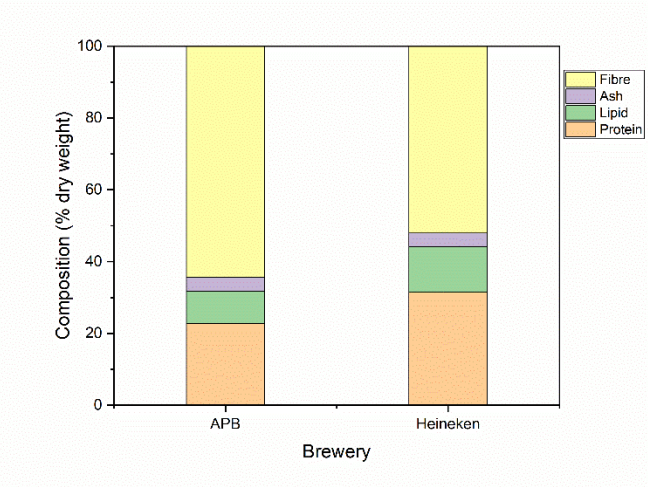


Figure 5.5. Comparison of BSG composition between Asia Pacific Breweries (APB, Singapore) and Heineken Zoeterwoude (The Netherlands), the two breweries that supplied BSG in this thesis

Variability in the chemical composition of BSG deters the standardisation of processes and exploitation of these byproducts on a large scale (de Crane d'Heysselaer, Bockstal, Jacquet, Schmetz, & Richel, 2021), as the final ingredient or product quality will be inconsistent between batches and consumer acceptability would be poor. Therefore, batch variability could be one of the biggest hurdles for upcycled BSG to be more well accepted by the industry and consumers.

5.6. Outlook

5.6.1. Alternative fractionation strategies

Several strategies to utilise BSG were explored in this work, with physical treatment and biological fermentation (Chapters 3 and 4) being the most appropriate from economic and environmental perspectives. Previous studies demonstrated good separation of protein and fibre-rich fractions of barley flours by dry fractionation routes including milling and air classification (Andersson, Andersson, & Åman, 2000; Silventoinen, Sipponen, Holopainen-Mantila, Poutanen, & Sozer, 2018). However, keeping in mind that BSG is produced in a wet state, dry fractionation routes are not recommended unless the separation efficiency and yield is high enough to justify the cost of drying BSG. Conversely, a wet fractionation route such as wet milling followed by sieving was shown to adequately separate proteins and fibres based on particle size (Kanauchi & Agata, 1997), but a large amount of water is required to prevent clogging of the mill and sieve and the process has to be repeated several times for effective separation. Therefore, alternative approaches should be considered. In recent years, novel technologies involving ultrasound, microwave and pulsed electric fields have been introduced as potential extraction techniques that generate minimal waste. Other solutions include the use of natural deep eutectic solvents (NADES) or supercritical fluids which are non-toxic and recyclable solvents (Fernández, Rodriguez, García, de Lucas, & Gracia, 2008; López-Linares, Campillo, Coca, Lucas, & García-Cubero, 2021; Spinelli, Conte, Lecce, Padalino, & Del Nobile, 2016; Wahlström et al., 2017). A preliminary investigation (not reported in this thesis) to combine ultrasound-assisted extraction with NADES suggested that strong interactions exist between proteins and phenolics, and there are difficulties in separating NADES from the extract. Depending on the intended application, complex downstream processing may be required, and further evaluation on its extraction efficiency is necessary.

5.6.2. Further applications in food

The research described in this thesis reveals the potential of using BSG for making emulsions that are either stabilised by particles or soluble proteins/peptides (Chapters 3 and 4), but its applications can be far wider. In light of the current trend to reduce meat consumption for social, environmental and health concerns, BSG may contribute as a plant-based ingredient that is rich in fibres and proteins. In Chapter 2, we saw that BSG contains a large amount of fibres and proteins, and possess good water and oil holding capacities. In addition, unhydrolysed BSG proteins were able to form heat-induced gels. These results point at the potential for incorporating BSG in shear-induced structure formation, such as in the application of meat analogues, since it naturally has both protein and polysaccharide phases (Grabowska et al., 2016), which may enable the formation of a fibrous structure.

Regardless of the final food application, consumer acceptance will remain an important factor that determines whether a novel food innovation will eventually be successful. For a satisfactory product, the sensory attributes, digestibility, food safety and nutritional qualities are some of the elements that should be evaluated. Nutritionally, one study found that essential amino acids constitute 30% of the total protein content in BSG, with lysine being most abundant (Waters et al., 2012). This is of importance since lysine is often deficient in cereal foods. Furthermore, the high dietary fibre content helps to decrease the glycemic index while also serving as a prebiotic (Naibaho et al., 2022). However, it is essential to note that the consumption of barley (and hence BSG) may not be suitable for those who are sensitive to gluten, as they are derived from the same cereal family. Further studies to assess the quality and allergenic risk of BSG-fortified food products are required for incorporating BSG proteins into human nutrition.

5.6.3. Potential of fermentation in upcycling food loss streams

Beyond BSG, many other food loss streams, ranging from fruit peels to vegetable pomace, are generated during food production and processing. Valorising these byproducts through fermentation can help to maximise the outputs from food and agriculture. In a broader sense, fermentation can be defined as the cultivation of microorganisms such as bacteria, yeasts and fungi to break down complex molecules into simpler ones, notably organic acids, alcohols or esters. As a cost-efficient, versatile and proven technology, fermentation was traditionally used to extend the shelf life of food products. In recent years, studies have shown that fermentation also enhances the nutritional content, texture and flavour of foods, alongside a myriad of health benefits including probiotic effects and antihypertensive properties (Teng, Chin, Chai, & Chen, 2021). Fermentation enables us to exploit new substrates from waste streams to create higher-value products, such as an increase in nutritional value from the fermentation of soybean residues (okara) by probiotic bacteria *Bacillus subtilis* (Mok, Tan, Lee, Kim, & Chen, 2019). The CRUST Group, a company in Singapore, collects unwanted bread from bakeries and restaurants as substitute for malted barley: the sugars are extracted as wort and fermented to ‘bread ale’. Efforts have also been made to valorise the seed of an exotic fruit, rambutan by fermenting, drying and roasting the fruit pulp and seed in a manner similar to cocoa bean processing to produce a cocoa powder-like product (Chai et al, 2019). These and other studies showed that fermentation can become an important solution for sustainable food production by converting byproducts into useful end products. Despite the advantages of fermentation, there are still inherent risks involved, such as the presence of pathogenic microorganisms or harmful metabolites, which will need to be carefully examined through food safety analysis and genomic assessments.

5.7. Conclusion

BSG is a byproduct from the brewing industry that is mainly discarded or used as animal feed. Valuable components like proteins, fibres and phenolics are abundant in this byproduct, which therefore has potential to be used as a food ingredient. In this thesis, several approaches to upcycle BSG were proposed, initially focusing on protein concentrates as a starting point but later extending towards the utilisation of BSG as a whole material through physical or biological means. This research has proven that mild processing methods through colloid milling and fermentation can impart functionality to BSG, and fractions that are less refined can already be sufficiently useful for several food applications. Complex purification steps towards highly refined ingredients result in a change in the composition, structure and function, which limits the applications that are possible for the ingredient. The alternative processing strategies of BSG proposed in this thesis provide new insights into the opportunities and challenges that await the valorisation of BSG in the future.

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Summary

As the world population continues to grow and food demand is projected to increase, our global food security is facing an impending threat. At the same time, food losses and waste are increasing, which not only brings about negative repercussions to the environment, and limits the efficiency in which we use our resources, but also signifies economic loss. As such, it is pertinent to critically examine stages of the production chain where food losses can be cut and convert these wastes from a problem into a resource. Side streams from food processing usually contain underutilised fractions that have potential in other applications but they are often disposed in landfills or used as animal feed. Brewers' spent grains (BSG), which is a major byproduct of the brewing industry, represent an inexpensive and valuable resource owing to its high protein and fibre content, alongside other nutrients such as phenolic compounds and lipids. With rising consumer interest in alternative sources of proteins, BSG fits well into the scene as a plant-based product derived from sustainable sources.

The objective of this thesis is to obtain an understanding of the processing and fractionation methods that are possible for BSG to possess functional properties which are industrially relevant. This thesis starts with the conventional approach of extracting proteins from BSG that aims at comparing the influence of extraction methods on the characteristics of proteins and their properties. Given the heterogeneous composition of BSG, it is however more realistic to consider the use of functional fractions or mixtures rather than purified components. Being rich in nutrients and high in moisture content, BSG is prone to microbial degradation, but this also illustrates the possibility of using fermentation to upgrade its properties.

Chapter 2 reports on a study of the effect of BSG protein extraction methods using alkali, ethanol and enzymes, on the composition, structure and function of the proteins. Fractions extracted with alkali, ethanol and enzymes contain more than 70% protein, with some carbohydrates and lipids present. Alkali-extracted proteins were more enriched in glutelins and were partially unfolded while the ethanol-extracted proteins comprised mostly of hordeins that were strongly aggregated. Enzymatic hydrolysis resulted in peptides smaller than 10 kDa with no distinct structural elements. The changes in composition and structure led to different functional properties, mainly related to solubility differences. Protein hydrolysates from enzymatic extraction were completely soluble in water and exhibited good emulsifying properties and high antioxidant activity. Conversely, alkali-extracted and ethanol-extracted proteins were poorly soluble in water but demonstrated good water and oil holding capacities that allowed heat-induced gelation from as low as 6% protein. These results indicate that the extraction method impacts the protein composition and structure which in turn affects

functionality. Therefore the intended application of the protein concentrate should drive the choice of the procedure for protein extraction.

As different protein extraction methods give clear differences in functionality, the possibility of using non-purified BSG containing proteins and fibres to stabilise oil-in-water emulsions was investigated in Chapter 3. First, BSG was mildly treated by a colloid mill to reduce particle size. Colloid mill-treated BSG yielded samples with about 26-32% protein and 52-62% fibre, with sizes generally smaller than 10 µm. The soluble fraction in full BSG without centrifugation yielded a 35% reduction in the interfacial tension of oil-water interfaces with an interfacial elasticity that is characteristic of soluble proteins. Emulsions prepared with full BSG were reversibly flocculated whereas those with insoluble BSG showed particle bridging. Despite sufficient electrostatic repulsion between the emulsion droplets, flocculation still occurred due to the presence of large amounts of fibres. Even though the emulsion droplet sizes ranged between 25 to 40 µm, both emulsions remained stable against coalescence after 10 days. This was attributed to Pickering stabilisation by insoluble proteins bounded to fibres. The fibres created a network in the continuous phase that sterically hindered droplets from coalescence. These findings indicate that mild processing methods like colloid milling can be sufficient to functionalise BSG for emulsification purposes, and chemicals and harsh treatments to obtain pure components are not always necessary, especially for particle-stabilised emulsions.

Considering that BSG is a nutrient-rich material, Chapter 4 approached the treatment of BSG from a different perspective by employing fungal fermentation to secrete enzymes that degrade the lignocellulosic BSG and enable the release of valuable compounds. In particular, protein extracts obtained from fermented BSG showed that up to 15% of the original protein was hydrolysed, and the amino acid profile revealed that proline and glutamic acid were prominently present, due to the extraction of prolamins in BSG. There was a good balance of hydrophobic and hydrophilic amino acids, which indicated the possibility of emulsification. The fermentation improved the protein solubility, alongside other functionalities such as emulsifying properties as well as oil holding capacities. Protein extract from fermented BSG also presented a higher antioxidant activity and non-cytotoxic effects. To utilise these promising characteristics, the fermented BSG protein extract was applied in a mayonnaise-type product to prove its ability as a plant-based emulsifier. Fermented hydrolysates had larger oil droplets and lower viscosity than egg-based mayonnaise, but could be used as a partial substitute for eggs.

Chapter 5 concludes this thesis with a general discussion of the main findings. Processing methods that were applied in Chapters 2 and 4 were compared in terms of yield, functionality and the antioxidant activities of the extracts. The findings were put into a wider perspective

that examines how BSG can be processed for real-world applications, emphasising the need to shift our focus from high purity ingredients to functional fractions instead, especially for natural complex materials that are composed of more than one component. The concept of a circular economy was discussed, and an integrated approach of the total use of BSG in a low-cost and sustainable manner was proposed. The chapter ends with a few considerations concerning BSG valorisation and recommendations for future research.

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Chin Yi Ling was born in Singapore on 18th September 1995. She attended Nanyang Technological University (NTU) in Singapore, where she obtained her Bachelor's degree with Honours in Chemical and Biomolecular Engineering, with a second major in Food Science and Technology in partnership with the Wageningen University & Research (WUR), The Netherlands. In 2019, she started her joint NTU-WUR PhD programme under the supervision of Professor Chen Wei Ning William and Professor Remko Boom, of which the findings are discussed in this thesis.

Publications

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