Integrated processing of brewer's spent grain into value-added protein feedstuff and cellulose adsorbent

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ABSTRACT

Brewer's spent grain (BSG) is the major byproduct generated by the brewing industry, which contains 14–30% protein and 50–70% of fiber. Currently, BSG is predominantly used as low-value cattle feed or buried in landfills, which is a considerable loss of valuable resources, leading to economic loss and environmental problems. Although research has been done on BSG valorization, the studies are limited to producing a single product (e.g., polyphenols, ethanol, or active carbon) and without further utilization of the produced products. Besides, the economic information available about the production of value-added products from BSG is insufficient. The overall goal of this research is to develop an integrated process to convert BSG into value-added protein-rich feedstuff and cellulose absorbent. The objectives of the research detailed here were to 1) develop a process to simultaneously produce protein-rich (PP) and fiber-rich products (FP) from BSG, 2) assess the replacement of fishmeal with PP in shrimp feed, 3) evaluate the economics of the overall process of PP production at a commercial scale, and 4) explore the potential use of cellulose adsorbent obtained from the FP in removing heavy metals from contaminated water.

To attain these objectives, BSG was first subjected to a wet fractionation process to produce PP and FP using different chemical/biological treatments, where the effects of sodium hydroxide, sodium bisulfite, and a protease (Alcalase) at different concentrations were investigated. Under the optimized conditions, the produced PP contained 46% protein and less than 1% fiber. The effectiveness of using PP to replace fishmeal at increasing levels (10–70%) was then evaluated by performing shrimp feeding trials. The results showed that up to 50% of fishmeal in shrimp feed can be replaced by PP without affecting shrimp growth and feed utilization. Moving forward, a techno-economic analysis was conducted to evaluate the economic feasibility of the production of PP. The experimental conditions and results were input into the process simulation model for determining the

mass and energy flows. For a processing plant with a capacity of 590 t wet BSG per day, the minimum selling price of PP to achieve a 5% return was determined to be $1044/t, lower than the price of fishmeal, indicating that the use of PP to replace fishmeal in shrimp feed could potentially reduce shrimp farming cost. The utilization of FP will further improve the economic feasibility of the fractionation process. FP was sequentially treated by dilute acid, alkali, and bleach to produce purified cellulose fibers, which were then modified by 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) oxidation to produce a cellulose adsorbent. The feasibility of the adsorbent in removing heavy metals (especially lead and manganese) from contaminated water was then investigated. Based on the results, the produced cellulose adsorbent showed high adsorption capacities for lead (272.5 mg/g) and manganese (52.9 mg/g). Overall, this study demonstrated that BSG can be upcycled into multiple value-added products via an integrated process. The outcomes of this study not only provide a low-cost and sustainable protein source to the aquaculture industry, and provide a novel adsorbent for the water treatment industry, but also offer alternative ways for the brewing industry to manage BSG.

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GENERAL AUDIENCE ABSTRACT

Brewer's spent grain (BSG) is the major byproduct generated by the brewing industry. Currently, BSG is predominantly used as low-value cattle feed or buried in landfills due to its high fiber and low protein contents, which is a considerable loss of valuable resources. Besides, raw BSG contains other nutrients and high water content, the inappropriate management of BSG may introduce environmental concerns. Though technologies have been investigated to valorize BSG by extracting protein from it, the process scaled-up is limited by the high drying costs of wet BSG, heavy chemical consumptions, and a large amount of fiber residue. The overall goal of this research is to develop an integrated process to convert BSG into value-added protein-rich feedstuff and cellulose absorbent.

In this study, we developed and optimized a process to produce protein and fiber products from wet BSG. The protein content of the produced protein product was doubled and the fiber content was reduced significantly compared with the raw BSG, which lighted the use of the protein product as an alternative to fishmeal. Fishmeal is an expensive aquafeed ingredient, the aquaculture industry is looking for alternatives to replace it. Herein, we investigated the effectiveness of the protein product as an alternative to fishmeal by conducting shrimp trials. A further economic analysis was conducted to evaluate the economic feasibility of the proposed process for protein and fiber production from BSG. In addition, the fiber product was used for producing a cellulose adsorbent to remove heavy metals from contaminated water.

Overall, this study demonstrated that BSG can be upcycled into multiple value-added products via an integrated process. The outcomes of this study not only provide a low-cost and sustainable protein source to the aquaculture industry, and provide a novel adsorbent for the water treatment industry, but also provide alternative ways for the brewing industry to manage BSG.

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BSG, brewer’s spent grain; AC, activated carbon; TEA, techno-economic analysis; PP, protein-rich product; FP, fiber-rich product; ADF, acid detergent fiber; NDF, neutral detergent fiber; PWG, percent weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; MSPP, minimum selling price of protein-rich product; NDF, neutral detergent fiber; ANOVA, analysis of variance; TCI, total capital investment; FCI, fixed capital investment; CP, crude protein; t, metric ton; Tukey’s HSD, Tukey’s honestly significant difference; DDGS, distiller's spent grain with solubles; CF, cellulose fiber; TOCF, TEMPO-oxidized fiber; UCF, BSG cellulose fibers with ultrasonic fibrillation; UTOCF, BSG cellulose fibers with both TEMPO-mediated oxidation and ultrasonic fibrillation; TOC, total organic carbon; ICP-MS, inductively coupled plasma mass spectrometry; HPLC, high-performance liquid chromatography; SEM, scanning electron microscopy; XRD, X-ray diffraction; FTIR, Fourier transform infrared spectroscopy.

Attributions

Several colleagues contributed to chapters 3–6 of this dissertation. A brief description of their contributions is listed below.

Chapter 3: Wet fractionation process to produce high protein and high fiber products from brewer's spent grain

David D. Kuhn, Ph.D., a current professor and extension specialist in the Department of Food Science and Technology at Virginia Tech contributed to the compilation and completion of the manuscript.

Jactone A. Ogejo, Ph.D., a current professor and extension specialist in the Department of Biological Systems Engineering at Virginia Tech contributed to the compilation and completion of the manuscript.

Sean F. O'Keefe, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech contributed to the compilation, and completion of the manuscript.

Cristina F. Fraguas, Ph.D., a former professor in the Department of Food Science and Technology at Virginia Tech contributed to the compilation, and completion of the manuscript.

Brian D. Wiersema, M.S., the current pilot plant manager in the Department of Food Science and Technology at Virginia Tech assisted with product separation.

Qing Jin, Ph.D., a current postdoctoral associate in the Department of Food Science and Technology at Virginia Tech assisted with experiment design and data collection.

Dajun Yu, a current doctoral student in the Department of Food Science and Technology at Virginia Tech contributed to the data collection.

Haibo Huang, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation, and completion of the manuscript.

Chapter 4: Protein-rich product recovered from brewer's spent grain can partially replace fishmeal in diets of Pacific white shrimp, *Litopenaeus vannamei*

Oscar A. Galagarza, Ph.D., a former postdoctoral associate in the Department of Food Science and Technology at Virginia Tech contributed to the production of shrimp feed pellets, the making of formulation and shrimp feeding trial.

Hengjian Wang, Ph.D., a current laboratory specialist in the Department of Food Science and Technology at Virginia Tech contributed to the production of shrimp feed, shrimp feeding trials, and protein and fat analysis.

Zachary W. Taylor, M.S., a graduate student in the Department of Food Science and Technology at Virginia Tech contributed to the shrimp feeding trial.

Clay S. Ferguson, a current doctoral student in the Department of Food Science and Technology at Virginia Tech contributed to the shrimp feeding trial and compilation of the manuscript.

Jactone A. Ogejo, Ph.D., a current professor and extension specialist in the Department of Biological Systems Engineering at Virginia Tech contributed to the compilation and completion of the manuscript.

Sean F. O'Keefe, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech contributed to the fatty acids analysis, the compilation, and completion of the manuscript.

Cristina F. Fraguas, Ph.D., a former professor in the Department of Food Science and Technology at Virginia Tech contributed to the compilation, and completion of the manuscript.

Dajun Yu, a current doctoral student in the Department of Food Science and Technology at Virginia Tech contributed to the amino acid analysis.

Nicholas E. Poe, M.S., a graduate student in the Department of Food Science and Technology at Virginia Tech contributed to the collection and pretreatment of brewer's spent grain.

Brian D. Wiersema, M.S., the pilot plant manager in the Department of Food Science and Technology at Virginia Tech assisted with products separation.

David D. Kuhn, Ph.D., a current professor and extension specialist in the Department of Food Science and Technology at Virginia Tech contributed to the study design, compilation, and completion of the manuscript.

Haibo Huang, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation, and completion of the manuscript.

Chapter 5 Protein production from brewer's spent grain via wet fractionation: process optimization and techno-economic analysis

David D. Kuhn, Ph.D., a current professor and extension specialist in the Department of Food Science and Technology at Virginia Tech contributed to the compilation and completion of the manuscript.

Sean F. O'Keefe, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech contributed to the compilation, and completion of the manuscript.

Jactone A. Ogejo, Ph.D., a current professor and extension specialist in the Department of Biological Systems Engineering at Virginia Tech contributed to the compilation and completion of the manuscript.

Cristina F. Fraguas, Ph.D., a former professor in the Department of Food Science and Technology at Virginia Tech contributed to the compilation, and completion of the manuscript.

Hengjian Wang, Ph.D., a current laboratory specialist in the Department of Food Science and Technology at Virginia Tech contributed to the product separation and protein analysis.

Haibo Huang, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the techno-economic analysis model design, the compilation, and completion of the manuscript.

Chapter 6 Cellulosic adsorbent produced from brewer's spent grain fiber and its application in the removal of Mn and Pb from water

Andrea M. Dietrich, Ph.D., a current professor in the Department of Civil and Environmental Engineering at Virginia Tech assisted with study design, the compilation of the manuscript.

Qing Jin, Ph.D., a current postdoctoral associate in the Department of Food Science and Technology at Virginia Tech assisted with FTIR and SEM data collection.

Tiantian Lin, Ph.D., a current postdoctoral associate in the Department of Food Science at Cornell University contributed to fiber size analysis.

Dajun Yu, a current doctoral student in the Department of Food Science and Technology at Virginia Tech contributed to the XRD data collection.

Haibo Huang, Ph.D., a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the compilation of the manuscript.

Chapter 1. Introduction and objectives

Increasing demands for food and natural resources are derived from the continuous growth of the world's population. The global population will exceed 9 billion by 2050 (UN, 2019). Vigorous industrialization and inefficient resource management in the food industry usually lead to negative environmental impacts. In recent years, there is growing political and social pressure on the reduction of pollutants coming from industrial activities. Meanwhile, the idea of sustainability has been recognized by more people. To achieve a more sustainable future for all mankind, the United Nations in 2015 adopted 17 Sustainable Development Goals as the blueprint to address the global challenges we face, including those related to affordable and clean energy, industry innovation, responsible consumption, and production (UN, 2015). In addition, food processing wastes usually contain abundant carbon and nitrogen sources, including protein, fiber, starch, and lipid, which are the potential feedstock for the production of value-added products. Consequently, large food processing companies no longer consider residues/by-products as wastes, but as raw materials to produce other useful products.

Beer is one of the most widely consumed alcoholic beverages in the world. The brewing industry generates two major solid wastes: brewer's spent grain (BSG) and brewer's yeast. The Environmental Performance of the European Brewing Sector defines the BSG as the materials which remain after the starch has been solubilized from grains, and brewer's yeast is a kind of yeast and used for fermenting beer (Donoghue et al., 2012). BSG represents 85% of total byproducts generated during beer production (Tang et al., 2009). In 2017, 1.95 billion hectoliters of beer were produced globally (Statista, 2017). For every 100 L of brewed beer, approximately 20 kg of wet BSG is produced (Mussatto et al., 2006), which indicates that 45.8 million tons of wet BSG were co-generated with the production of 1.95 billion hectoliters of beer in 2017. The United States is the second leading country in beer production and produced an average of 4.4 million tons of wet BSG annually between 2010 and 2020 (TTB, 2021). Although the majority of BSG is repurposed, for example, diverting to local farms for animal feed or composting, a portion of BSG is ultimately disposed of in landfills (EPA, 2012). BSG is a lignocellulosic material with high fiber and low protein contents. Its high moisture content (around 80%) and other nutrients make it is not stable and prone to microbiological spoilage during storage (Ivanova et al., 2017). The spoilage of BSG will lead to increased mold, decreased moisture, and reduced palatability.

Furthermore, BSG spoils quickly owing to its high nitrogen and phosphorus contents, which, if not handled properly, may impose an environmental burden and cause microbial contamination. Therefore, there is an acute need to appropriately manage BSG from both resources recycling and environmental protection perspectives.

The underutilized BSG is considered a potential source of protein and fiber if these two can be successfully separated and recovered at relatively high purity. The separated protein-rich product could be used as a cost-effective protein source for animal feed and human food; and the separated fiber-rich product are also valuable feedstock for producing biofuels, dietary fibers, and adsorbent materials.

The rising demand for seafood, together with the over-exploited marine resources, has made aquaculture the fastest-growing segment in global food production. One of the challenges the aquaculture industry faces is finding out sustainable and economically viable alternatives to fishmeal (Gatlin et al., 2007). Fishmeal is a solid product obtained by removing most of the water and some/all of the oil from fish or fish waste. Fishmeal is generally sold as a powder and is used mostly in compound foods for poultry, pigs, and farmed fish. Fishmeal has been widely used as the primary protein source in aquafeed because of its balanced amino acid composition, high digestibility, palatability, and general lack of antinutrients (Huang et al., 2018). Around 30% of the global shrimp production is from aquaculture, and 75–80% of farmed shrimp are grown on commercial feed (Delgado et al., 2021). In commercial shrimp farming, feed cost accounts for between 50% and 80% of a producer's operational expenses (Cummins et al., 2017). Fishmeal is the most expensive ingredient and typically accounts for 20% to 50% of the total feed weight. While the harvest of species used to produce fishmeal has been on a steady decline due to the overexploitation and the finite nature of global marine resources, leading to an increase in fishmeal price from around $500 to $2,000 in the past two decades (Indexmundi, 2021). The price of fishmeal is likely to increase due to the increasing demand and unstable global supply. Consequently, there is a need to seek alternatives to fishmeal to secure the sustainable development of the shrimp farming industry or the aquaculture industry.

Water is essential to life. However, the rapidly growing world population, accelerated industrialization, and climate change have resulted in a decrease in water supply and an increase in water contamination (UN-Water, 2020). The release of substances produced by human activities into the water not only makes water unsafe to drink but also disrupts aquatic ecosystems. It is

estimated that 785 million people lack basic drinking water services, and contaminated drinking water has been causing 485,000 diarrheal deaths each year; by 2025, half of the world's population will be living in water-stressed areas (WHO, 2019). Among the water pollutants, heavy metal contamination is posing a threat to human society. Adsorption is commonly regarded as an effective and economical method for the removal of heavy metals from water (Zhou et al., 2020). Biomass materials, such as cellulose, is a promising adsorbent for heavy metal removal since it is cheap, abundant, easily modified, efficient, and eco-friendly (Hokkanen et al., 2016).

The overall goal of this research is to develop an integrated process to convert BSG into value-added protein-rich feedstuff and cellulose adsorbent. The central hypothesis is that through a rationally designed process, the low-protein and high-fiber BSG can be upgraded to an economic protein-rich feedstuff used as an alternative to fishmeal in shrimp feed, and the coproduct fiber-rich product could be used to produce an efficient cellulose adsorbent for heavy metals removal to improve water quality. To meet the overall goal and test the central hypothesis, the following objectives were proposed:

Objective 1: Develop and optimize a fractionation process to produce protein-rich and fiber-rich products from BSG. It is hypothesized that the produced protein-rich product from the optimized process will contain over 40% protein and less than 6% fiber.

Objective 2: Evaluate the performance of partial replacement of fishmeal with the protein-rich product obtained from BSG in shrimp diets. It is hypothesized that the BSG protein product can effectively replace fishmeal in shrimp diets at a high level (> 50%) without influencing the growth and health of shrimps.

Objective 3: Conduct the techno-economic analysis to evaluate the economic feasibility of the production of protein-rich and fiber-rich products from BSG. It is hypothesized that the minimum selling price of the protein-rich product will be lower than the price of fishmeal.

Objective 4: Evaluate the feasibility of using the fiber-rich product to generate cellulose adsorbent for heavy metals removal. It is hypothesized that the generated cellulose adsorbent has a high adsorption capacity for heavy metals.

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Chapter 2 Literature review

2.1 Generation and characteristics of brewer's spent grain

Beer is not only a pleasant beverage, but also a source of water that contains calories and nutrients (O'Keefe, 2004). Barley is the fourth most important cereal crop in the world after corn, wheat, and rice, and is the primary cereal used in brewing. Two-row and six-row are the most popular types of barley used for beer production. The process of beer production varies depending on the beer style. **Figure 2.1** shows an overview of the basic process of the production of beer and brewer's spent grain (BSG). Malted barley is milled to allow for rapid hydration and enhanced enzymatic reaction in the followed mashing step. In the mashing step, the milled barley is mixed with water, and starch is hydrolyzed by amylases into simple sugars, which can be used for beer fermentation by yeast. After the starch hydrolysis in the mashing step, the wort (liquid part) containing sugars, proteins, and other soluble components is separated from the solid residues by filtration in the lautering step. The sweet wort is heated, added with hops and yeast, then fermented into beer. The solid residues produced in the lautering step are known as BSG. It is estimated that 20 kg of BSG is generated from every 100 L of beer production (Pinheiro et al., 2019).

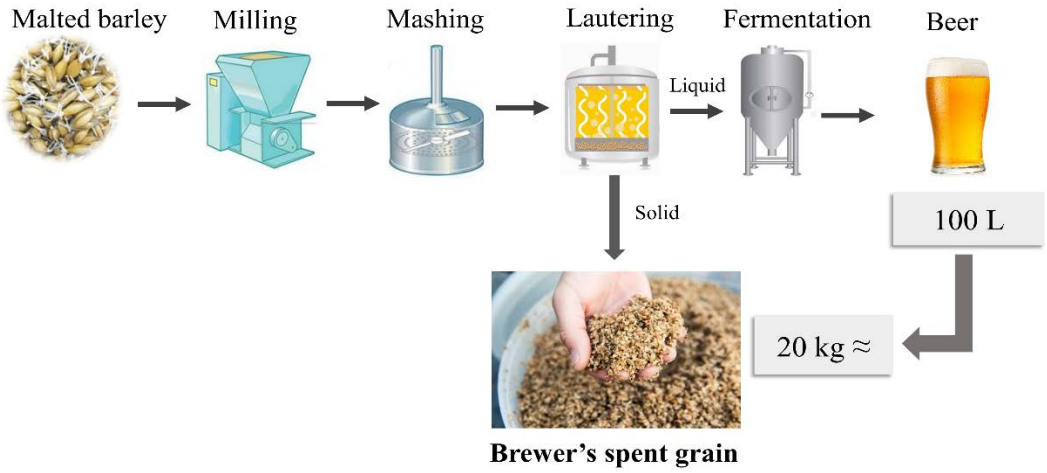

Figure 2.1 Schematic representation of the production of brewer's spent grain

BSG consists of insoluble components including the seed coat-pericarp-husk layers covering the original barley grain and other additional ingredients, such as insoluble protein and ash (Verni et al., 2020). Wet BSG contains 70–80% of water (He et al., 2021). Dry BSG contains around 70% fiber (19–42% hemicellulose, 12–26% cellulose, 10–28% lignin), 14–30% protein,

3–13% lipid, and 1–5% ash (Lynch et al., 2016). The high fiber concentration of BSG is because starch and free sugars are removed from barley grain during the mashing process. It is considered that most starch of raw feedstock is converted into fermentable sugars in the mashing step. However, depending on the procedures, incomplete hydrolysis may lead to part of starch goes to the BSG stream, resulting in starch content in BSG ranges from 1.6% to 13% (Lynch et al., 2016; Robertson et al., 2010; Rojas-Chamorro et al., 2020). BSG also contains minerals (copper, cobalt, iron, potassium, selenium, sulphur, sodium, selenium, magnesium, phosphorus, manganese), vitamins (folic acid, niacin, biotin, choline, thiamine, pantothenic acid, pyridoxine and riboflavin), and both essential and nonessential amino acids (Cooray et al., 2017). The composition of BSG depends on the raw materials and selected brewing techniques including barley variety, malting and mashing conditions, and the type and quality of other cereals added to the brewing process.

Wet BSG is not stable due to its high moisture content and nutrients such as sugars, proteins, and fats. It will rapidly spoil if not processed and stored properly. Drying is the most effective method used to prolong the shelf life of BSG, while freezing is inappropriate as a large storage volume is required (Chanie and FieVez, 2017; Mussatto et al., 2006a). Freeze-drying does not affect BSG composition, but it is not economical for large-scale applications. Consequently, oven-drying is considered an ideal technique. Considering the high moisture of wet BSG, oven drying is energy-intensive, and drying temperatures higher than 60 ℃ will result in discoloration, unpleased flavor, or even odor pollution (Ikram et al., 2017). Therefore, most BSG is used as wet material.

2.2 Current utilization of brewer's spent grain

Most breweries brew several times a week, if not daily, throughout the year; therefore, BSG is generated continuously. Currently, BSG used as livestock feed is a relatively common practice. However, its low-energy content, high content of structural carbohydrates (hemicellulose and cellulose), and insufficient essential amino acids such as lysine, threonine, and tryptophan limit the inclusion rate of BSG in both ruminants and nonruminants feed (Westendorf and Wohlt, 2002). Because of its easy spoilage and costive transportation, wet BSG is usually fed to livestock on local farms. If local farmers are willing to pick up this wet material, breweries may charge it at a low price. However, if breweries cannot find a way to get rid of this "waste", they have to pay for the disposal (Jackowski et al., 2020).

2.3 Valorization of brewer's spent grain

2.3.1 Dietary fiber

BSG can be incorporated into food diets due to its food-grade nature and potential nutritional value. Especially, BSG is a source of dietary fiber, which is defined as a group of edible components that resistant to digestion and absorption in the human small intestine but can be totally or partially degraded by the gut microbiota consortium in the large bowel. Dietary fiber includes non-starch polysaccharides (e.g., pectin, cellulose), oligosaccharides such as galactooligosaccharides, and non-carbohydrate-based polymers (e.g., lignan) (Armstrong et al., 2021). Dietary fiber intake provides some health benefits, including reduced risk of diabetes, coronary heart disease, cardiovascular disease, and improvement of immune function (Anderson et al., 2009).

BSG used as a functional baking ingredient has been reported. The addition of BSG at levels between 15% and 35% significantly increased the dietary fiber content of baked snacks (breadsticks) and had a negligible effect on their shelf-life; while the structure of breadsticks was changed. Therefore, further research on quality improvement was needed (Ktenioudaki et al., 2012). In another study, it was found that the white flour bread with 10% of BSG had good organoleptic attributes, acceptability, and enhanced nutritional value (improved contents of fiber, protein, fat, and minerals) (Fărcaș et al., 2014). Adding BSG to bread at a 30% inclusion rate increased the dietary fiber content in bread up to fivefold, and using suitable enzymes or forming sourdough could improve loaf volume, texture, and shelf of the bread added with BSG (Stojceska, 2011). Fifteen percent of wheat flour replaced with BSG flours could increase 23% of dietary fiber in muffins and without affected consumer acceptance (Shih et al., 2020).

BSG can also be incorporated into non-bakery foods. In one study, BSG was ground into different particle sizes (< 212, 212–425, and 425–850 μm) and added into Frankfurters at varied levels (1%, 3%, and 5%) (Özvural et al., 2009). The results showed that with the increasing BSG content, the sensory scores of the prepared Frankfurters decreased, but overall, the sensory parameters were not deleteriously affected. BSG was also blended into smoked sausage at different levels (1.5%, 3%, and 6%) (Nagy et al., 2017). The results revealed that there were no significant differences among the hedonic scores for sensory attributes (appearance, color, texture, odor, taste, and overall acceptability) of the smoked sausages had or had no BSG. Choi et al. (2014) suggested

BSG could be used to replace 20–25% of pork back fat to make low-fat chicken sausages. BSG (5%) enriched pasta was reported to have comparable nutritional and sensorial characteristics with traditional durum wheat pasta (Nocente et al., 2019).

2.3.2 Protein

Protein is vital in the creation and maintenance of our bodies. With the development of society, the public's awareness of protein as a macronutrient in the diet is higher than ever. Eating high-protein and low-carbohydrate diets is becoming a popular way to lose weight and improve health. Protein intake at the expense of carbohydrates may beneficially influence blood pressure (Tielemans et al., 2013). Plant protein is an inevitable topic for anyone whose dietary choices emphasize plant foods. Plant protein replaces animal proteins contributing to the modest improvements in glycemic control in individuals with diabetes (Viguiliouk et al., 2015) and prevention of the onset of risk factors associated with cardiovascular disease (Chalvon-Demersay et al., 2017). Although proteins obtained from cereal and legume crops are especially insufficient in lysine and methionine comparing with animal protein (Galili and Amir, 2013), the insufficient levels of essential amino acids can be complemented by a combination of different plant proteins. Therefore, diets contained diverse plant proteins can provide adequate essential amino acids to healthy adults (Mariotti, 2017). Moreover, when consuming plant proteins, people also receive other beneficial compounds such as polyphenols, vitamins, fibers, and bioactive peptides (Pihlanto et al., 2017). Besides health benefits, environmental protection is another factor affecting the consumption of animal protein. Animal-based foods are associated with higher greenhouse gas emissions than plant-based foods are (González et al., 2011). The production of animal protein shares the largest greenhouse gas emissions of any food industry (Boehm et al., 2018). Therefore, the plant protein market is expanding for both public health and environmental reasons.

Protein is the second-largest component following fiber in BSG. BSG has a higher protein content than most agricultural byproducts, such as grape pomace (7–15%), wheat straw (2–6%), corn stover (4–9%) (Jin et al., 2018); however, it has a lower protein content than some plant-based protein sources, such as canola meal (35–45%), pea protein concentrate (35–50%), soybean meal (40–50%), and corn gluten meal (60–70%) (Choi and Han, 2001; Gatlin et al., 2007; Overland et al., 2009). The quantity of protein with a high proportion of essential amino acids

(around 30% of total protein content, and lysine accounts for 14%) make BSG a potential feed/food ingredient (Waters et al., 2012).

The separation and recovery of protein from BSG have received significant attention in the past years. The richest protein in BSG is hordeins (43% of total protein content), also called prolamins due to their high contents of proline and glutamine, which are soluble in alcohols in the presence of a reducing agent (Ikram et al., 2017). Glutelin is the second richest protein in BSG, accounting for around 22% of total BSG proteins, and it can be extracted with dilute acid, alkali, or detergents (Celus et al., 2006). It is challenging to extract BSG protein as BSG is a lignocellulosic material, its complex fiber network traps protein inside. Hence, different extraction methods including alkali-soluble and acid precipitation, diluted acid extraction, hydrothermal extraction, mechanical extraction, ultrasound/steam explosion/carbohydrate hydrolysis-assisted extraction, subcritical water extraction, and a combination of the above-mentioned methods have been extensively investigated in extracting protein from BSG (**Table 2.1**). These methods can be classified into physical, chemical, biochemical, and physical-chemical approaches based on the main driving-force agents.

Table 2.1 Summary of the methods used for protein recovery from BSG.

Methods	**Conditions**	**Protein recovery (%)**	**References**
Sodium dodecyl sulphate (SDS)	3% SDS-0.5% Na_2HPO_4 (pH 7.0), 100 ℃, 1 h	49%	(Ervin et al., 1989)
Alkali	BSG: alkaline 17% w/v, 0.1 M NaOH, 60 ℃, 60 min. Protein precipitation: pH 4.0, 2.0 M citric acid	41%	(Celus et al., 2007a)
Alkali	BSG: alkaline 5% w/v, 0.11 M NaOH, 60 ℃, two 1-h extractions. Protein precipitation: pH 3.8	Pale BSG: 88.2%; Black BSG: 64.3%	(Connolly et al., 2013)
Alkali (sequential extraction)	BSG: alkaline 20% w/v. S1: 0.1 M KOH, 24 h (room temperature); S2: 0.5 M KOH; S3: 4 M KOH. Protein precipitation: pH 3	83%	(Vieira et al., 2014)
1. water-alkaline-acid sequential extraction **2.** Alkaline-acid sequential extraction	**1.** Defatted BSG: water 5% w/v, 25℃,1.5 h; then substrate: alkaline 5% w/v, 0.11M NaOH, 50 ℃, 1 h; 16.15 g H_2SO_4/g substrate, 25℃, 1 h then 121℃, 1 h.	**1.** 94% **2.** 95% **3.** condition 1: 85%, condition 2 90% **4.** 64–66% (60℃, 1–24 h) solid to liquid ratios	(Qin et al., 2018)

3. One-step dilute acid extraction **4.** Hydrothermal **5.** Alkaline with ammonium carbonate **6.** Enzyme	**2.** The same conditions with method 1 except for the absence of water extraction. **3.** Condition 1: BSG (defatted and raw): H_2SO_4 (12% w/w) 10% w/w, 120℃, 27 min; condition 2: BSG (defatted and raw): H_2SO_4 (4% w/w) 0.35% w/w, 121℃, 1 h. **4.** Raw BSG: water 2.5–6.67% w/v, temperature 30–135℃, 1–4 h (defatted BSG: water 2.5% w/v, 60℃, 1–24 h). **5.** Raw BSG: ammonium carbonate solution 2.5–6.67% w/v, 60℃, 1–24 h. **6.** Raw BSG: water (pH 6.25) or 10 mM ammonium carbonate solution (pH 8.0) 2.5–6.67% w/v, 100 µL of alcalase® 2.4L/g raw BSG, 60℃, 2–24 h.	and whether the BSG is defatted or not didn't significantly affect the protein separation. Increasing the reaction temperature to 90 ℃ did not promote the protein separation. When the temperature over 100 ℃, protein recovery decreased. **5.** 38–40%, solid to liquid ratios and reaction time increased from 2 h to 24 h had a minor effect on protein recovery. **6.** 43-52%	
Ultrasound-assisted extraction	BSG: sodium carbonate buffer (pH 10) 10% w/v, 1 h	N/A	(Tang et al., 2009)
Ultrasonic-assisted extraction	BSG powder (80-mesh sieve)	N/A	(Tang et al., 2010)
Enzyme with ultrasound pretreatment	20 µL Alcalase /g BSG (ultrasound pretreated), pH 8, 60 ℃, 3.1 h	69.8%	(Yu et al., 2020)
Enzyme	**1.** Acidic enzymes: cod stomach pepsin and hog pepsin (20 µL or 20 mg/g BSG), cheese rennet (200 µL/g BSG); solid loading 3.33% w/v, 30 ℃ for 18 h. **2.** Neutral or basic enzymes: Alcalase, Neutrase, Protamex, Papain, and Bromelain. pH control: 0.1 M Na_2HPO_4/NaH_2PO_4, 50 and 60 ℃	77% (pH 8, 4 h, 10–20 µL Alcalase / g BSG; 64% (pH 8, 4 h, 1.2 µL Alcalase / g BSG	(Treimo et al., 2008)
Enzyme	1. BSG was pretreated with Depol 749L (100 U/g BSG), 5 h, 50 ℃; 2. Pretreated BSG hydrolyzed by enzymes including Alcalse 2.4L (pH 9.5), Promod 144GL (pH 6.5), and Acid Protease A (pH	76% (Alcalase)	(Niemi et al., 2013)

	3.5). Solid content 10% w/v, 40 ℃, 4 h.		
Enzyme	BSG was pretreated with a steam explosion (200 ℃, 10 min) and further treated with carbohydrate hydrolysis; alkaline protease, pH 10, 50 ℃, 5 h	65%	(Rommi et al., 2018)
Hydro-mechanical processing	A colloid mill was used to pretreat BSG, then the protein-rich product was separated from the coarse husk by centrifugation.	38%	(Ibbett et al., 2019)
Deep eutectic solvent	Extraction: 90% w/w, NaAcO: urea (molar ratio 1:2), 80 ℃, 2 h.	79%	(Wahlstrom et al., 2017)
1. Subcritical water **2.** Enzyme **3.** Alkali	**1.** Water flow rate 4 mL/min, operating pressure 5 MPa, 125–185 ℃, 4h **2.** Enzyme loading 6% w/w, protease, xylanase, and cellulose, 50 ℃, 4 h. **3.** NaOH (0.01, 0.1, 1M), 50 ℃, 4 h.	1. 78% (185 ℃, 4 h) 2. 47% (protease) 3. 51% (0.1 M NaOH)	(Alonso-Riaño et al., 2021)

Among these methods, alkaline extraction is the most used approach due to its high yield and ease of operation (Contreras et al., 2019). However, the severe alkaline treatment causes protein denaturation, racemization, and lysinoalanine formation, which negatively affect protein functionality and nutritional value (Sari et al., 2015). Moreover, other components such as lignin can also be solubilized in alkaline solution, making the purification of protein more complex. Enzymatic extraction is getting popular because it is a mild treatment and environmentally friendly. Besides, enzyme-assisted extraction induces fewer salts and non-desirable compounds comparing with alkaline extraction does. Carbohydrases and protease are two types of enzymes used for protein extraction. Among different enzymes, Alcalase 2.4L has been reported as the most efficient enzyme for protein separation from BSG (Niemi et al., 2013; Treimo et al., 2009). What is more, enzymatic hydrolysis of proteins involves the break of peptide bonds. Different enzyme varieties and hydrolysis durations applied to extract protein from BSG may lead to proteins with different functional properties, including solubility, viscosity, emulsifying, foaming, and sensory properties (Celus et al., 2007b). Excessive hydrolysis may produce more small peptides inducing bitterness and odor (Day, 2013). The high cost of the enzyme is a major obstacle to its use in protein extraction.

Extracted proteins from BSG have been used in different products. In a previous study, BSG protein was utilized for making packaging films. To be specific, BSG protein was extracted by using 0.1 N NaOH and precipitated with 70% ammonium sulfate. BSG protein composite film was produced by mixing 3% BSG protein and 2% chitosan solutions at a ratio of 1:1. The incorporation of chitosan was to improve the physical and mechanical properties of BSG protein film. The prepared film showed both antimicrobial and antioxidant properties (Lee et al., 2015). A film made from BSG protein adding with 0.1 g polyethylene glycol (a plasticizer) showed promising mechanical, water-barrier, and antioxidant properties, which could be used as active food packaging material (Proano et al., 2020). Additionally, BSG protein could be a source of functional food ingredients. Incorporating BSG protein hydrolysate obtained using Alcalase hydrolysis into low-fat milk can confer anti-inflammatory effects in Jurkat T cells (Crowley et al., 2015). BSG peptides prepared by enzymatic hydrolysis showed significant hypotensive effects in rats experiment (Cermeño et al., 2019). What is more, BSG protein hydrolysate has higher digestibility compared with non-hydrolysis protein. Therefore, protein hydrolysate obtained from BSG hydrolysis with enzyme (Protamex® and Celluclast®) was successfully used in the feed of gilt-head sea bream at a 20% level (San Martin et al., 2020).

2.3.3 Polyphenols

Polyphenols are plant-based secondary metabolites, they are essential in a wide range of plant functions and capable of bioactive functionality in humans (Petti and Scully, 2009). Consumption of polyphenol-rich diets has been associated with human health, the potential benefits include decreasing the incidence of colon cancer, cardiovascular diseases, diabetes, obesity, liver disorders, and neurodegenerative diseases (Rasouli et al., 2017). Besides antioxidant and anti-inflammatory properties, polyphenols also contribute to color and sensory properties such as bitterness and astringency (Pandey and Rizvi, 2009; Silva and Pogačnik, 2020). The above-mentioned properties attracted researchers to study polyphenols. The most of phenolic compounds of barley grain present in the husk, and husk is the major component of BSG, which makes BSG a promising raw material for polyphenols production. BSG has higher concentrations of polyphenols than brans of rice, corn, and wheat (Stefanello et al., 2018). Ferulic acid, *p*-coumaric acid, sinapic acid, and syringic acid are the major abundant polyphenols in BSG (Birsan et al., 2019). Total phenolic content in dry BSG ranged from 1.3 to 9.9 mg gallic acid (GA)/g (Birsan et

al., 2019; McCarthy et al., 2013; Meneses et al., 2013). Phenolic components extracted from BSG can increase ferric reducing the antioxidant power of cranberry juice (McCarthy et al., 2013). The following methods have been used for the extraction of phenolic compounds from BSG:

1) Solvent extraction

   Solid-liquid extraction is a common method used for antioxidants extraction from plant materials. The extracted content and characteristics of phenolic compounds highly depend on the solvent (Socaci et al., 2018a). Phenolic compounds such as ferulic and *p*-coumaric acids in BSG can be extracted with organic solvents including methanol, hexane, ethanol, acetone, water, ethyl acetate, or aqueous phase of solvent mixtures. In a previous study, Meneses et al. (2013) evaluated the efficiency of different solvents for extracting antioxidant phenolic compounds from BSG. It was found that an acetone: water mixture (60% v/v) had the highest efficacy in total phenols extraction (9.9 GA/g BSG) and the greatest antioxidant potential of the extracted product among solvents. Nevertheless, this method has some drawbacks, for example, , extraction time is long and the product is impure (Guido and Moreira, 2017).

2) Alkaline hydrolysis

   BSG fiber mainly includes hemicellulose and lignin. Ferulic acid is in etherified linkages with both arabinoses in hemicellulose and lignin (Fengel and Wegener, 1983). It was reported that alkaline hydrolysis using NaOH (2.0%) with a solid/liquid ratio of 1:20 w/w at 120 ℃ for 90 min could extract 9.65 mg ferulic acid and 9.22 mg *p*-coumaric acids from per gram of solubilized lignin in BSG; this method is more effective in ferulic acid extraction than enzymatic extraction is, which releases not more than 70% of ferulic acid (Mussatto et al., 2007).

3) Microwave-assisted extraction

   Microwaves are electromagnetic radiations. The dipole rotation of solvent and solid sample molecules and ionic conduction lead to very fast heating of solvents and samples, contributing to a shorter extraction time, less consumption of solvents, and improved extraction efficiency compared to traditional extraction methods (Kaufmann and Christen, 2002). Recently, studies have been conducted to use microwave-assisted extraction techniques to recover bioactive phenolic compounds from BSG. Moreira et al (2012) compared the microwave and non-microwave assisted methods for the extraction of

polyphenols from BSG. They found that the microwave-assisted alkaline hydrolysis method achieved around fivefold higher ferulic acid yield from BSG comparing to the non-microwave assistance method, and the extraction time for the microwave-assisted alkaline hydrolysis method was only 15 min. In another study, it was reported that microwave-assisted choline-chloride-glycerol extraction recovered more phenolic compounds (2.3 mg GA/g BSG) than traditional extraction did (1.2 mg GA/g BSG) (Lopez-Linares et al., 2021).

4) Ultrasound-assisted extraction

The principle of ultrasound-assisted extraction is that the mechanic vibration caused by high-frequency sound waves produces bubbles and bubble collapse, and the implosion of bubbles hits the surface of solid samples, resulting in the release of desired compounds (Guido and Moreira, 2017). Ultrasound-assisted extraction was previously used to extract polyphenols from barley, and under the optimized conditions (60 mL of ethanol per gram of barley grain, extraction time of 18 min, and temperature of 50 ℃), the phenolic acid yield was 19.86 mg GA/g barley grain (Wang et al., 2013). Ultrasound-assisted extraction was also reported to be more efficient for the extraction of phenolic compounds from BSG comparing with conventional water extraction, and water was proved as a good solvent (Alonso-Riano et al., 2020).

5) Supercritical fluid extraction

Supercritical fluid extraction is a green technology. A supercritical fluid is a fluid at a temperature and pressure above its critical point, where the fluid has properties that are similar to both liquid and gas. It diffuses quickly like gas and acts as a solvent similar to liquid (Ahmad et al., 2019). Carbon dioxide (CO_2) is the most commonly used as a supercritical solvent as it is cheap, environmentally friendly and it allows extraction at a low temperature and pressure (Da Porto and Natolino, 2017). Supercritical-CO_2 fluid extraction is rapid and suitable for the extraction of thermally liable substances (Ye et al., 2019). Though CO_2 is preferred for the extraction of non-polar compounds, its low polarity induces disadvantages in polar compounds extraction. Nevertheless, the polarity of supercritical-CO_2 can be improved by adding modifiers (co-solvents) such as ethanol. Spinelli et al (2016) reported that a proper supercritical fluid composed of CO_2 + 60% ethanol (v/v) for polyphenols extraction from BSG. Under the best conditions (35 MPa of

pressure, 40 ℃ of temperature, and 240 min of extraction time), 0.35 mg phenolic compounds can be extracted per gram of BSG.

6) Subcritical water extraction

Increased temperature and pressure would break the strong hydrogen bonds in the water molecules. Subcritical water is the water that sustains a liquid state when it is heated from 100 ℃ to 374 ℃ under a pressure of 1–22.1 MPa (Zhang et al., 2020). Subcritical water has a lower dielectric constant than normal water, indicating it can solubilize hydrophobic organic compounds (Toor et al., 2011). Alonso-Riaño et al (2021) reported subcritical water for polyphenols extraction from BSG. Under operating pressure of 5 MPa, the temperature of 185 ℃, and reaction time of 240 min, the yield was 33.0 mg GEA/g dry BSG, which was higher than the yields got from ultrasound-assisted extraction and acid/alkaline hydrolysis.

2.3.4 Hemicellulose, cellulose, and lignin

Hemicellulose is one of the most abundant carbohydrates in BSG, accounting for 19–42% of the total weight of dry BSG. This hemicellulose content in BSG is similar or superior to that in some other crop by-products, such as rice husk (17.3%), wheat straw (16.1%), barley straw (22.2%), rice straw (22.9%), and oat straw (23.4%), corn stover (26.6%) and sugarcane bagasse (25.2%) (Mussatto, 2014). BSG hemicellulose is mainly composed of pentose (mostly xylose and arabinose), hexose sugars, as well as some other small components, for example, phenolic and ferulic acids. Hydrolysis of hemicellulose can separate these components, which can be used for the production of wide value-added products, such as fuels, emulsifiers, prebiotics, xylo-oligosaccharides, and furfuryl (Qaseem et al., 2021). Xylitol, a sweetener with multiple health benefits, can be produced by fermentation of hemicellulosic sugars by yeast or bacteria strains. Dilute acid pretreatment is widely used for hemicellulosic sugar recovery (85–95%) from lignocellulosic material, leaving a residue riches in cellulose and is more accessible to enzymes (Zheng et al., 2014). BSG pretreated with dilute acid at optimized conditions (liquid/solid ratio of 8 g/g, 100 mg H_2SO_4/g dry BSG 120 ℃, 17 min) could extract 92.7% of hemicellulosic sugars, and the hydrolysis efficiency of xylan and arabinan was 88.7% and 100%, respectively. The hydrolyzed sugars (mainly xylose) were fermented by *Candida guilliermondii* to produce xylitol at a yield of 0.7 g xylitol per gram of xylose (Mussatto and Roberto, 2005). Changes in acid

hydrolysis conditions of BSG can affect the production of hemicellulosic sugars. For instance, when BSG was treated with 120 mg H_2SO_4/g dry BSG, liquid/solid ratio of 10 g/g, at 120 ℃ for 27 min, 67% of xylose and 97.8% of arabinose sugars were recovered from BSG (Mussatto et al., 2006b). BSG is also a promising feedstock for producing prebiotics due to its high xylose content. For example, Gómez et al (2015) produced a mixture of arabino-xylo-oligosaccharide from BSG using a series of treatments including hydrothermal treatment, membrane filtration, ion exchange, and enzymatic hydrolysis.

Cellulose is another important carbohydrate in BSG, accounting for 12–26% of dry BSG weight. Cellulose is a major structural component of plant cell walls, and it is a homo-polysaccharide composed entirely of D-glucose subunits linked by β-(1,4)-glycosidic bonds. Cellulose is surrounded by hemicellulose and lignin. Lignin is a complex aromatic polymer, and it accounts for 10–28% of dry BSG weight. Hemicellulose is less rigid than cellulose. The removal or structure break of hemicelluloses and lignin helps expose cellulose and making it more enzymatically accessible (Alvira et al., 2010). Biomass conversion technologies normally involve a pretreatment step to break the rigid and complex structure of lignocellulosic material. Typical pretreatment methods include: 1) physical pretreatments such as wet disc milling and extrusion, 2) biological pretreatments, 3) chemical pretreatments, and 4) physicochemical pretreatments.

Renewable energy plays a significant role in developing a sustainable society, and bioenergy as an important part of renewable energy has gained more and more attention in the global energy system. BSG as lignocellulosic biomass has great potential for bioenergy production due to its abundance, availability throughout the year, and low cost. BSG used as a feedstock for bioenergy (biofuel and biogas) production usually involves chemical or physicochemical pretreatment followed by enzymatic hydrolysis to release edible compounds for microorganisms. In a study that used BSG to produce bioethanol, BSG was milled with a hammer mill, pretreated with acid, and hydrolyzed with enzyme mixtures (cellulase, β-glucosidase, hemicellulase, and xylanase) to produce sugar hydrolysate containing 27.0 g/L glucose, 16.7 g/L xylose, and 11.9 g/L arabinoses. The sugar hydrolysate was then fermented by *Pichia stipitis* to produce ethanol with a yield of 0.32 g ethanol/g sugar (White et al., 2008). In another study, BSG was first pretreated with sulfuric acid for converting hemicellulose into monomer sugars and then sodium hydroxide for solubilizing lignin. The remained solid with 86% of cellulose was saccharified with enzymes to produce glucose. The sugars from the pretreatment and enzymatic hydrolysis were mixed and

fermented by S. *cerevisiae NRRL YB2293* to produce ethanol. The yield was 0.28 g ethanol/g of glucose (Liguori et al., 2015). Besides ethanol production, butanol can also be produced from BSG using a similar process for bioethanol production: pretreatment, enzymatic hydrolysis, and fermentation. However, instead of using yeast (for ethanol production), Clostridial strains, such as *Clostridium beijerinckii* and *Clostridium acetotutilycum*, are usually used for butanol production via restrictive anaerobic fermentation (Giacobbe et al., 2019; Plaza et al., 2020; Plaza et al., 2017). Additionally, BSG has also been used as a feedstock for the production of biohydrogen (Zhang and Zang, 2016) and biogas (Dudek et al., 2019; Panjičko et al., 2017).

The abovementioned studies were focused on the production of value-added products from hemicellulose and cellulose of BSG. Besides hemicellulose and cellulose, lignin in BSG is also a potential component for producing high-value products such as activated carbon, carbon fibers, aromatic rich pyrolysis oil, and mixed phenols (Wang et al., 2019). The detailed composition and structure of BSG were thoroughly characterized by Rencoret et al. (2015), which provides references for future utilization of BSG lignin. In the study of activated carbon production from BSG lignin (Mussatto et al., 2010), BSG was pretreated with dilute sulfuric acid and then alkaline solution to produce a black liquor contained lignin. The black liquor was acidified to pH 2.15 then filtered to get lignin, which was activated with H_3PO_4 and carbonized at 600 ℃ to produce activated carbon. The activated carbon was able to purify the sugar hydrolysate from the acid pretreatment of BSG. In another study, lignin as a polyphenolic macromolecule was used to produce a lignin-rich insoluble residue, which showed a hypocholesterolemic effect and beneficial systemic changes in mice via gut microbiota and bile acids (Raza et al., 2018).

Other utilization of hemicellulose, cellulose, and lignin components in BSG include the productions of wood-based particleboard manufacture (Klímek et al., 2017) and combustible material to increase the porosity of brick (Russ et al., 2005).

2.4 Potential alternatives to aquafeed proteins and their limitations

The continued growth of aquaculture is unsustainable if fishmeal remains the primary protein source (Hardy, 2010). Therefore, it is important to identify alternative protein sources to feed fish. During the past few decades, studies have been conducted to use different sources of plant- and animal-based proteins to replace 20% to 70% of fishmeal in aquafeed formulation (Bulbul et al., 2016; Nyina-wamwiza et al., 2010; Panserat et al., 2009; Xie et al., 2016). Rendered

products have traditionally been used in animal feeding because the protein level and amino acid profile in animal by-products are close to fish meal. It was reported that porcine meat meal can replace up to 35% of fishmeal without adverse effects on shrimp growth (Hernandez et al., 2008). Poultry by-product was used to replace 10% of fishmeal in the diet of Florida pompano *Trachinotus carolinus L.* and no negative effects were observed; however, a complete replacement of fishmeal with poultry by-product resulted in a depression of fish growth performance (Rossi Jr and Davis, 2012). In another study, Ye et al. (2011) found that different levels (35% or 51%) of fishmeal can be replaced by a protein alternate which was a combination of meat and bone meal, poultry by-product meal, blood meal, and corn gluten meal in the diets of Pacific white shrimp without affecting the feed utilization efficiency or shrimp growth performance. Although animal by-products have been a long-time ingredient to provide a rich source of amino acids and minerals to livestock and fish worldwide, the use of rendered animal products in fish feeds is constrained by the lack of consumer acceptance and the regulatory environment (Naylor et al., 2009).

Plant proteins have become a fishmeal substitution in aquaculture feed. Compared with fishmeal, plant-based proteins have a relatively low price and consistent supply. The most promising alternatives to fishmeal are high-protein concentrates produced from soybean, wheat, and other grains or oilseeds. Protein from soybean meal could replace up to 75% of fishmeal protein without influencing the growth of tilapia (Lin and Luo, 2011). Studies also reported that 80% of soybean meal and 5% of brewer's grain with yeast can replace all the fishmeal in the diet of juvenile (0.2–3.1 g) red claw crayfish (Muzinic et al., 2004). However, the low concentrations of methionine and threonine, together with the high concentrations of carbohydrates could be a limitation for soybean product inclusion in aquaculture (Cummins et al., 2017; Gatlin et al., 2007). Moreover, a rapid increase in the price of soybean meal may preclude its extensive use in aquaculture. Canola meal is another popular alternative protein to fishmeal and it was reported that canola meal can replace up to 20% of fishmeal without influencing the growth of Japanese seabass (Cheng et al., 2010). Pea protein concentrate was used to replace 20% of fishmeal protein without any adverse effect on Atlantic salmon growth performance (Overland et al., 2009).

Plant-based proteins for partially or replacing fishmeal protein in shrimp diets were evaluated. A high level (54%–58%) of soybean meal combined with poultry by-product meal, distiller's dried grains, and pea meal showed the same shrimp growth performance compared with a diet containing the same level of soybean meal and partial fishmeal (Sookying and Davis, 2011).

The result indicates that fishmeal in the shrimp diet may be completely replaced by a mixture of plant-and animal-based proteins. In another study, it was reported that fishmeal can be completely replaced with soy protein concentrate and microbial floc meal which was obtained from super-intensive shrimp farm effluent without adverse effects on shrimp growth (Bauer et al., 2012). Rice protein can also be used to substitute fishmeal. A study suggested that rice protein concentrate had the potential to replace 50% of fishmeal protein in the Pacific White shrimp diet (Oujifard et al., 2012).

On the other hand, though the world production of grains and oilseeds has increased over the past two decades, their prices also increased as a result of growing demand inform the animal feed, food, and biofuel industries (Hardy, 2010). Thus, non-food-based proteins, such as microbial floc meal, were studied for fishmeal replacement. Kuhn and his coworkers have done a series of studies of using microbial floc meal to replace fishmeal and soybean protein in shrimp feed (Kuhn et al., 2009; Kuhn et al., 2010; Kuhn et al., 2016), and they found: 1) for shrimp control diet contained 15% fishmeal and 7.9% soybean meal, microbial floc meal obtained from biological treatment of fish effluent replaced 67% of fishmeal or 100% of soybean not only did not affect the shrimp survival rate but also enhanced shrimp growth performance; 2) for shrimp control diet contained 13% fishmeal and 45.7% soybean meal, microbial bioflocs can successfully replace 20% of fishmeal or 30% of soybean meal without affecting shrimp growth performance. Taking into account that BSG is a food processing by-product and its large quantities, it can be an alternative protein source to food-based and fishmeal proteins. BSG used as an ingredient in the diet of some fish species has been reported. The diet of gilthead seabream (*Sparus aurata*) contained 15% of BSG and 30% of brewer's spent yeast gave similar results in fish growth performance to the control diet with fishmeal as the main protein source (Estévez et al., 2021). A higher BSG inclusion level (20%) in the feeds of rainbow trout (*Oncorhyncus mykiss*) and gilthead seabream (*Sparus aurata*) was reported had similar results to the feed with fishmeal as the main protein source and good digestibility in protein, lipid, and amino acid (Nazzaro et al., 2021). In another fish feeding study, BSG replaced up to 50% of soybean meal (75.4% in the control diet) in the feed of striped catfish (*Pangasianodon hypophthalmus*) without any negative effect on the growth, nutrient utilization, and feed conversion of the fish (Jayant et al., 2018). Despite this, BSG used in the other aquafeeds such as shrimp feeds has not been studied.

BSG was tested as an ingredient in aquafeed with limited success, mainly due to its excess fiber and inadequate protein content (Cheng and Hardy, 2004; Stone et al., 2005). Fiber is not digestible by most fish, thus BSG is associated with decreased digestibility and increased fecal losses, and negative ecosystem impacts (Cheng et al., 2004). Besides, fiber might be an anti-nutritional parameter for fish (Francis et al., 2001). Therefore, processes to increase protein content and reduce fiber content in BSG are necessary before it can be used as an alternative feedstuff to fishmeal. Biological processes involving solid-state fungal fermentations have been developed to produce protein-enriched fishmeal replacement from processing byproducts, such as BSG (Canedo et al., 2016), canola meal (Croat et al., 2016), soybean cotyledon fiber and distiller's dried grains with solubles (Lio and Wang, 2012). These processes take advantage of the metabolic diversity of microorganisms to convert fiber and carbohydrates into protein-rich biomass. After fermentation, protein concentration is increased and fiber concentration is decreased, but the increase in protein concentration is limited to about 10%. For example, the protein concentration of spent grain is increased from 23% to 32% after fungal fermentation (Canedo et al., 2016), which remains insufficient to replace fishmeal. Potential contamination problems and the large reactor volume required with the long fermentation time (usually several weeks) are also of concern.

2.5 BSG-based adsorbents for the removal of heavy metals from water

Water is an essential substance for life. However, the rapidly growing world population, accelerated industrialization, and climate change have resulted in an increase in water contamination. 785 million people lack basic drinking water services, and contaminated drinking water is estimated to cause 485,000 diarrheal deaths each year; by 2025, half of the world's population will be living in water-stressed areas (WHO, 2019). Among the water pollutants, heavy metal contamination is posing a threat to human society. Heavy metals are those naturally occurring elements that have high atomic weight and a density at least five times higher than water (Tchounwou et al., 2012). The common heavy metals that have been recognized in polluted water, and their related standard concentration limits regulated by the United States Environmental Protection Agency include lead (Pb, 0.015 mg/L), copper (Cu, 1.3 mg/L), arsenic (As, 0.01 mg/L), zinc (Zn, 0.8 mg/L), cadmium (Cd, 0.005 mg/L), chromium (Cr, 0.1 mg/L), nickel (Ni, 0.2 mg/L) and mercury (Hg, 0.00003 mg/L) (Azimi et al., 2017). Normally, humans are exposed to these metals through ingestions (drinking or eating), inhalation (breathing), and osmotic effect (Joseph

et al., 2019). Heavy metals are difficult to degrade, their accumulation in organisms with high concentrations produces health risks to life and ecological disturbances (Akpor and Muchie, 2010). Current methods for removing heavy metals include: 1) chemical precipitation, the most common and economic method; however, it is inefficient in treating contaminated water within high acid content, and the produced toxic sludge needs further treatment; 2) chemical coagulation, it shares similar properties with chemical precipitation; 3) ion exchange, the adsorbed metals have high reusability, however, this method is expensive, high acid and metals levels in water limit its use; 4) membrane filtration, membrane-based separation is an attractive method because of its environment-friendly and zero sludge production; 5) adsorption. Among these, adsorption is commonly regarded as an effective and economical method for wastewater treatment (ManufacturingNet, 2016; Pei et al., 2021).

Lignocellulosic biomass-based adsorbents used for heavy metals removal from the contaminated water have gained a lot of attention due to their renewable, low cost, biodegradable, and highly efficient (Zhong et al., 2012). BSG as a food processing by-product and lignocellulosic biomass has a great potential for adsorbent production. Raw BSG used as an adsorbent showed an increased adsorption capacity on heavy metals including $Mn^{2+} \approx Zn^{2+} < Ni^{2+} < Cd^{2+} < Cu^{2+} < Pb^{2+}$ (Wierzba and Kłos, 2019). However, the applicability of BSG in its original state might be constrained by its relatively small surface area and limited functional groups. Raw BSG was reported to have lower sorption efficiency for Cu^{2+} compared to conditioning BSG in a hydrochloric acid solution (Wierzba et al., 2019). Besides, when biomass is exposed to water, some organic components may leach out leading to the increase of chemical oxygen demand or total organic carbon (Czikkely et al., 2018). Therefore, producing carbonaceous adsorbents such as activated carbon, biochar, and cellulose adsorbents, which have a relatively higher adsorption capacity, are the potential valorization direction of BSG.

2.5.1 Activated carbon

Activated carbon (AC) is a highly porous solid that has a large internal surface area and pore volume for holding kinds of organics and non-organics. It has been widely recognized as one of the most popular adsorbents for contaminated water treatment (Bhatnagar et al., 2013). Carbonaceous rich materials such as wood, nutshells, coconut shell, and lignite are traditional feedstock for AC production. Nowadays, efforts have been spent on the exploit of lignocellulosic

biomass including rice husks, corn cobs, and other food processing wastes as raw materials for AC production. Carbonization and activation are two typical steps in producing AC. The properties of AC depend on the raw material as well as conditions of production. In the carbonization step, raw materials are pyrolyzed in the absence of oxygen at 400–600 ℃, maybe higher, but less than 1000 ℃ (Bansal and Goyal, 2005). Noncarbon impurities such as oxygen, nitrogen, hydrogen, etc. are removed by converting into volatile gaseous. The resulted char requires activation to improve its adsorption capacity by developing a highly porous structure. Two methods are usually used to achieve this goal, physical activation, and chemical activation. In the physical activation process, the carbonized material reacts with steam, carbon dioxide, etc. in an oxidizing atmosphere with temperatures ranging from 350 to 900 ℃ depending on the gas used (Bedia et al., 2018). While for the chemical activation, the carbonized material char is then impregnated with KOH or NaOH followed by pyrolysis at 800–1000 ℃. The other method of chemical activation is that raw material is firstly impregnated with strongly dehydrating and oxidizing chemicals such as H_3PO_4, $ZnCl_2$, and $FeCl_3$, then thermally treated at 400–800 ℃ (Bedia et al., 2018). Comparing with physical activation, chemical activation contributes to the higher porous and specific surface area, as well as high product yield with a relatively lower temperature (Bergna et al., 2018; Yang et al., 2021).

Research on AC production from raw BSG or BSG lignin has been conducted. AC obtained from BSG by chemical activation with H_3PO_4 and KOH could remove 77% of lead (initial lead concentration was 100 mg/L) from water in one hour (Osman et al., 2020). AC production from raw BSG with pyrolysis followed by steam activation was studied in the adsorption of toxic chromium (VI) (Vanderheyden et al., 2018). The AC was able to remove 99% of Cr (VI) with an initial concentration of 10 mg/L at pH 2 with optimal dosage (0.75 g AC/L). In another study, AC obtained with BSG lignin (carbon yield was 8.3%) using chemical activation (3 g H_3PO_4/g lignin, 600 ℃) showed a similar or even better adsorption capacity for nickel, iron, chromium, and silicon than commercial AC (Mussatto et al., 2010).

2.5.2 Biochar

Biochar is a new potential adsorbent within comparable or better adsorption properties with AC. It is black carbon made from thermal degradation of carbon-rich biomass with limited oxygen at relatively low temperatures (< 700℃) (Tan et al., 2017). Moreover, biochar has a lower environmental impact than activated carbon, and the average cost of biochar is $5/kg while it is

$5.6/kg for activated carbon (Alhashimi and Aktas, 2017). Methods used in converting biomass into biochar include pyrolysis, gasification, torrefaction, and hydrothermal carbonization (Meyer et al., 2011). Raw biochar may have limited adsorption capacity especially for high concentrations of polluted water. Hence, methods such as steam activation, heat treatment, acid modification, alkaline modification, and impregnation have been studied to improve the adsorption capacity of biochar (Ahmed et al., 2016). Heavy metal removal mechanisms of biochar include physical sorption, ion exchange, electrostatic interactions, complexation, and precipitation (Inyang et al., 2016). In general, raw materials, activation methods, and target heavy metals are factors affecting the adsorption capacity of biochar.

There are some studies related to the sorption of heavy metal ions using biochar obtained from agricultural waste. Biochar produced from corn straw using pyrolysis at 600 ℃ for 2 h showed maximum Cu and Zn adsorption capacities of 12.5 and 11.0 mg/g, respectively (Chen et al., 2011). Sesame straw was converted into biochar at 700 ℃ for 4 h demonstrating varied maximum adsorption capacities for Pb (102 mg/g), Cd (86 mg/g), Cr (65 mg/g), Cu (55 mg/g), and Zn (34 mg/g) (Park et al., 2016). Grape pomace lignin pyrolyzed at 700 ℃ for 2 h was reported to have a maximum adsorption capacity (134 mg/g) for Pb at pH 6.5 (Jin et al., 2020). Biochar production from BSG has been reported. However, BSG biochar used in the adsorption of the heavy metals hasn't been found. BSG mixed with sewage sludge (80:20, wt%) pyrolyzed at 600 ℃ for 2 h showed more than 60% of removal efficiency on ammonia-nitrogen (Zhang and Wang, 2016). Other studies regarding BSG biochar utilization include soil amendment (Amoriello et al., 2020; Yoo et al., 2021), adsorbent for the removal of pesticide pymetrozine from aqueous solution (Xi et al., 2014), feedstock to crease biogas production from the anaerobic digestion of BSG (Dudek et al., 2019), material for the production of screen-printed electrodes (Cancelliere et al., 2019).

2.5.3 Cellulose adsorbent

Cellulose is the most abundant natural polymer in the world. It is cheap, biodegradable, renewable, and harmless to the environment. Cellulose can be extracted from different agricultural wastes such as corn stalk, palm shell, and cotton waste by applying alkaline-acid, enzymatic, and ionic liquid treatments (Farooq et al., 2020). Cellulose separation from BSG with dilute-acid hydrolysis followed by soda pulping was reported (Mussatto et al., 2008), in which BSG was first

treated with H_2SO_4 (100 g/g BSG) at solid/liquid ratio of 12.5% w/w at 120 ℃ for 17 min; the acid pretreated BSG was then submitted to pulping reactions. Under the best pulping conditions (2% soda concentration, solid/liquid ratio of 5% w/w, 120 ℃, 90 min), the obtained pulp contained 90.4% of cellulose. A similar pulp with a cellulose content of 90.1% was produced from BSG using an alkaline treatment, the same conditions as the previous best pulping. The treated solid residue was then bleached with sodium chlorite (2% w/w $NaClO_2$ in water) with a solid/liquid ratio of 2% w/v at 80 ℃ for 4 h (dos Santos et al., 2015). In another study, the deproteinized BSG obtained from alkaline treatment (solid/liquid ratio of 17% w/v, 0.1 M NaOH at 60 ℃ for 60 min) was treated by boiled sodium chlorite (0.7% w/v) at pH 4 for 2 h, followed by sodium bisulfite (5% w/v) treatment at room temperature for 1 h. The washed solid residue was treated with NaOH (17.5% w/v) at room temperature for 8 h to produce a solid with 100% of cellulose (Mishra et al., 2017).

One potential utilization of cellulose is as an adsorbent in various forms including raw cellulose, modified cellulose, or cellulose-based activated carbon, to remove heavy metals from contaminated water (Gupta et al., 2016). Compared to activated carbon and biochar, the generation of cellulose adsorbent does not need a high temperature to carbonize raw material. Most of the modified cellulose adsorbents are re-usable after several adsorption/desorption cycles, which makes it possible to recover heavy metals from aqueous solutions (O'Connell et al., 2008).

Raw cellulose usually has a low metal adsorption capacity because of its abundant hydroxyl groups and hydrogen bonds between the molecular chains (Li et al., 2017). To improve adsorption ability and efficiency, many researchers have attempted to modify cellulose. Reducing cellulosic dimension to nanometric levels or functionalizing cellulose can improve its adsorption ability and heavy metals removal efficiency (Varghese et al., 2019). Cellulosic materials can be converted into nanocellulose by mechanical/chemical treatment or electrospinning. Grinding/crushing, high-pressure homogenization, microfluidization, and ultrasonication are typical mechanical treatments (Wang, 2019). Chemical treatment includes acid hydrolysis, 2,2,6,6-tetramethylpiperidinyl-1-oxyl radical (TEMPO)-mediated oxidation, and ammonium persulfate treatment (Farooq et al., 2020). BSG treated with 0.5 M NaOH at room temperature for 4 h had a higher adsorption capacity for Cd (17.3 mg/g) and Pb (35.5 mg/g) than non-treated BSG or acid-treated BSG (Low et al., 2000).

Functional groups attach to the hydroxyl group in cellulose through techniques such as carboxylation, etherification, esterification, oxidation, and phosphorylation can make cellulose

adsorbent selectively uptake metal ions (Wang, 2019). The thiol-functionalized BSG had a higher adsorption capacity for Zn^{2+} (227.4 mg/g) than raw BSG (125.8 mg/g) (Chai et al., 2010). TEMPO-mediated oxidation, which involves the oxidation of C6 of the D-glucose unite of cellulose by substituting the –OH group by a COOH or COONa group, is one of the excellent chemical modification methods, and it has been widely used in lab-scale production in recent years (Fiol et al., 2019; Liu et al., 2020). TEMPO-oxidized softwood cellulose was reported to have 1.7 mmol carboxylate content per gram of cellulose(Fukuzumi et al., 2010)The modified cellulose has been used for heavy metal ions removal from aqueous solution due to the carboxylate groups of modified cellulose can electrostatically interact with metal ions. TEMPO-oxidized cellulose produced from corn stalk with a nanofunctionalized surface and abundant carboxyl groups showed maximum adsorption capacity (5.8 mg/g) to Cd^{2+} (Yu et al., 2021). In another converting agricultural wastes into efficient adsorbents, Liu et al. (2019) modified the cellulose obtain from lotus seedpods by TEMPO oxidation. The modified cellulose exhibited an excellent Pb^{2+} removal, 111.1 mg/g. The TEMPO oxidation was also reported can increase the adsorption capacity of the cellulose hydrogel that was produced from ashless filter paper pulp to Cu^{2+}. The maximum adsorption capacity was 268.2 mg/g. The TEMPO-oxidized cellulose hydrogel showed high adsorption capacity for other metal ions including Zn^{3+}, Fe^{3+}, Cd^{2+}, and Cs^{+} as well (Isobe et al., 2013). Abou-Zeid et al. (2018) reported that the TEMPO-oxidized bagasse cellulose nanofiber had 0.7 mmol/g of carboxyl content, and this content can reach 3 mmol/g by further oxidizing C-2 and C-2 of cellulose with periodate-chlorite oxidation, resulting in the adsorption of Cu^{2+} and Pb^{2+} increased from 92.2 mg/g to 97.3 mg/g. Zhang et al. (2016) modified the TEMPO-oxidized cotton cellulose by using polyethyleneimine grafting to further introduce functional amino groups. The modified cellulose showed the maximum adsorption capacity, 52.3 mg/g, to Cu^{2+}. However, BSG cellulose with TEMPO-oxidation used in heavy metals removal from water hasn't been studied.

2.6 Techno-economic analysis

Techno-economic analysis (TEA) is a method that can be used to evaluate the economic performance of a proposed process for the production of new products. It combines process modeling and engineering design with economic evaluation to provide information about capital cost, operation cost, anticipated selling price of new products, future cash flows, and the likely return on investment. TEA allows researchers to identify bottlenecks of the proposed process and

technology. The more defined the project is, the more accurate the TEA will be. Lab or pilot scale data is usually used to simulate commercial-scale production, which will be achieved by using TEA software tools such as SuperPro Designer (Intelligen, Inc. USA), Aspen Process Economic Analyzer (Aspen Technology, Inc., USA), and Biosolve Process (Biopharm Servies Ltd. UK).

TEA used to analyze technologies in bioenergy production has been widely reported. The National Renewable Energy Laboratory described the use of TEA on the evaluation of converting lignocellulosic biomass to ethanol in detail (Humbird et al., 2011). TEA used for transportation fuel production from microalgae (Ou et al., 2015), cellulosic butanol production from corn stover (Baral and Shah, 2016), biodiesel and ethanol production from lipid-producing sugarcane (Huang et al., 2016), anthocyanin and ethanol production from corn (Somavat et al., 2018), ethanol production from sugarcane bagasse (Cheng et al., 2019) were reported. For an existing product, an updated selling price could also be obtained by using information regarding improved process technology, materials prices, and other relevant data. For instance, the outdated cost of algae biodiesel was $0.53–$0.85/L, while the updated cost was $0.42–$0.97/L (Nagarajan et al., 2013).

Similarly, TEA has been used to evaluate technologies on the production of value-added products from BSG. TEA was used in evaluating the production of xylitol, lactic acid, activated carbon, and phenolic acids from BSG based on a biorefinery, indicating that production costs of products changed with process designs, and the scenario that combined energy and mass integration had the greatest potential (Mussatto et al., 2013). In another biorefinery approach for the production of xylitol, ethanol, and polyhydroxybutyrate from BSG, the TEA results showed that it was not feasible to produce ethanol or polyhydroxybutyrate from BSG alone without heat integration, while it was feasible to produce xylitol with or without heart integration (Davila et al., 2016). Recently, TEA of biodiesel production from BSG (Sae-ngae et al., 2020), biomethane, electricity, thermal energy, and fertilizer production from BSG in a biorefinery concept, and xylitol and xylo-oligosaccharides production from BSG were reported (Sganzerla et al., 2021; Swart et al., 2021). However, there has been little information about TEA of protein production from BSG.

2.7 Conclusions

From the above literature review, we know that BSG is a valuable “waste”, it could be used as a feedstock for the production of varied value-added products. Protein and fiber as the two major components of BSG have gained a lot of attention. The enzyme, especially protease, has been

recognized as an efficient and environmentally friendly reagent for BSG protein recovery. However, the current methods are focused on pure protein separation, and the processes used are usually limited to lab scales. In addition, most of the studies focus on the production of a single product from BSG, for example, production protein from BSG while neglecting the use of the abundant fiber residue. Therefore, an integrated and scaled-up process for multiple products production from BSG needs to be developed. In addition, it is interesting and important to investigate wide applications of these produced products. Since simultaneously produce protein feedstuff and cellulose adsorbent from BSG, as well as the use of BSG protein to replace fishmeal in shrimp feed, use BSG cellulose adsorbent for removing heavy metals from contaminated water, TEA of BSG protein production based on the proposed process haven't been studied. Therefore, the purpose of the work in the following chapters was to design a process to produce protein-rich and fiber-rich products from BSG. Then evaluate the feasibility of using the protein-rich product to replace fishmeal in shrimp diets through shrimp feeding trials. After getting promising results from the shrimp feeding trial, TEA was used to assess the overall process economics of protein-rich product production from BSG at a commercial scale. The fiber-rich product got from BSG was used for the production of cellulose adsorbent. The adsorption capacity of BSG cellulose to heavy metals was subsequently evaluated.

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Chapter 3. Wet fractionation process to produce protein-rich and fiber-rich products from brewer's spent grain

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ABSTRACT

Brewer's spent grain (BSG) is the major waste generated by breweries, which contains 20–30% (w/w) proteins and 40–60% (w/w) of fibers. Technologies have been investigated to valorize BSG by extracting proteins from BSG; however, none of them has been implemented on an industrial scale due to the challenges including high drying cost of wet BSG, heavy chemical consumptions, and a large volume of secondary waste after protein extraction. Herein, a wet fractionation process was proposed to fully utilize BSG by simultaneously producing protein-rich product (PP) and fiber-rich product (FP) through the separation of proteins and fibers from BSG. The effect of different concentrations (1, 3, and 5%, w/w) of chemicals (sodium hydroxide and sodium bisulfite) and enzyme (Alcalase, 5, 20 and 35 μL/g dry BSG) treatments on product yield and composition was investigated to maximize the process separation efficiency and potentially reduce the chemical and enzyme consumptions. The sodium hydroxide and Alcalase treatments improved the protein and fiber separation compared to the sodium bisulfite treatment. Under the optimal condition (20 μL/g dry BSG) using Alcalase, the protein separation efficiency was 84%, and the protein concentration in PP was 43% (w/w), almost double the protein concentration (23%,

w/w) in original BSG. The fiber content of the FP was more than 80% (w/w), of which hemicellulose is the dominant fiber component.

3.1 Introduction

Brewer's spent grain (BSG) is the main byproduct in the brewing industry, representing 85% (w/w) of the total byproducts generated in beer production (Reis and Abu-Ghannam, 2014). The estimated global annual production of BSG is around 38,600,000 metric tons (wet basis) (Mussatto, 2014). BSG is the insoluble residual fraction obtained after mashing and filtration during beer making process. It is mainly composed of husks, bran, endosperm residue of barley and other grains used for beer production (Mussatto et al., 2006). Chemically, 100 g dry BSG typically contains about 15–25 g protein, 50–70 g fiber (hemicellulose, cellulose, and lignin), 5–10 g fat, and 2–5 gash (Aliyu and Bala, 2011; Mussatto, 2014). Currently, because of its inherent high fiber content, the use of BSG has been limited to low-value cattle feed or disposed in landfills. Furthermore, BSG spoils quickly owing to its high nitrogen and phosphorus content, which, if not handled properly, may impose the environmental burden and cause microbial contamination. Therefore, there is an acute need to explore new uses of BSG.

One way to valorize BSG is through deconstruction, separation, and recovery of valuable components it contains, such as proteins and fibers. Separation and recovery of proteins from BSG has received significant attention in the past years, due to the increasing protein demand as food and animal feed ingredients (Qin et al., 2018). Numerous studies have been conducted to recover proteins from BSG using traditional alkaline extraction of proteins, followed by acid precipitation (Connolly et al., 2017, 2013; Vieira et al., 2014). Recently, enzymatic methods using proteases have been investigated to produce protein hydrolysates from BSG (Bi et al., 2018; Treimo et al., 2008). Nevertheless, none of the developed separation technologies has been implemented on an industrial scale up till now due to the known limitations including high drying cost to remove moisture, high chemical (e.g., alkali and acid) consumptions and the related environmental concerns, high costs of enzymes, and low protein separation efficiency (Mussatto, 2014; Connolly et al., 2013; Treimo et al.,2008). These limitations hinder the commercialization of the value-added processing of BSG. Furthermore, most of previous studies only focused on the extraction of protein from BSG but with little attention on the recovery of fiber, which is another valuable and more abundant component in BSG. The separated fiber could be used as dietary fiber, as substrate for

biofuel production, or as feedstock for making packaging materials, whereas the separated high protein product could be a high-value protein source for aquaculture or poultry farming, thereby greatly contributing to the economic feasibility of the BSG processing. More importantly, the simultaneous production of protein and fibers allows the full utilization of BSG and minimize the waste disposal after BSG processing.

The aim of study was to develop a novel wet milling fractionation process to simultaneously produce protein-rich product (PP) and fiber-rich product (FP) through the effective recovery of proteins and fibers from BSG. The following fractionation process that combines wet milling, chemical and enzymatic incubation, and size-controlled sieving for BSG fractionation has not been reported in the scientific literature. After receiving the BSG from the brewery, it was wet milled to small particle sizes using a disk mill. The milled sample was incubated with reagents of sodium hydroxide, sodium bisulfite and Alcalase, respectively, to improve the protein and fiber separation efficiency. Finally, the incubated BSG sample was sieved to separate small solubilized proteins and large insoluble fibers to produce PP and FP. The key variables were studied to understand the performance of the process, including the BSG particle size, reagent type, and reagent loading. The outcomes from this study will provide new renewable sources of proteins and fibers from BSG, concurrently mitigating the environmental concerns caused by BSG disposal.

3.2 Materials and methods

3.2.1 Materials

BSG was obtained from the beer fermentation of bar-ley in a commercial brewery (Lexington, VA) in January, 2017, frozen and stored in the dark (−20 ℃) until use. The enzyme Alcalase 2.4L FG was provided by Novozymes Inc. (Franklinton, NC). Alcalase 2.4L FG is a nonspecific proteolytic enzyme produced by controlled fermentation of *Bacillus licheniformis*. The enzymatic activity was determined as 2.2 ± 0.0 (mean ± standard deviation) AU/g using the Sigma's Universal Protease Activity Assay with casein as a substrate (Sigma-Aldrich, 1999). All chemicals (analytical grade) were purchased from Fisher Scientific Co. (Hampton, NH).

3.2.2 Wet milling process to produce PP and FP from BSG

The wet milling process of BSG to produce PP and FP is illustrated in **Figure 3.1**. After thawing at 4 ℃ overnight, wet BSG was milled to reduce particle size using a disk mill (Model 4-

E, Quaker City Grinding Mills, Phoenixville, PA) (Huang et al.,2012 a,b). The milled BSG (2 g in dry basis) was mixed with deionized water to make a slurry with 5% (w/w) total solids. The BSG slurry was then added to the designated amount of reagent (**Table 3.1**), followed by incubation at 60 ℃ for 4 h in a shaking water bath to solubilize proteins. The selection of the reagents and their loadings were based on their distinct reaction mechanisms with proteins (**Table 3.1**). The incubated samples were transferred to a sieve shaker (RX-29, W.S. tyler, OH) and shaken for 15 min to separate small solubilized proteins and large insoluble fibers. The sieving method was adapted from Eckhoff et al. (1996) developed to separate proteins, fibers and starch from corn. Briefly, the method entailed using a sieve with pore size of 75 µm operated at a speed of 278 ± 10 oscillations per minute (Eckhoff et al., 1996). During sieving, samples were continuously dispersed by spatula and washed using 120 mL of deionized water. The material that passed through the sieve was collected and designated as PP, while the retentate (the material retained on the sieve) was collected and named as FP. Both PP and FP were dried in a convection oven at 60 ℃ for up to 24 h. The dried sample was stored at −20 ℃ for further analysis.

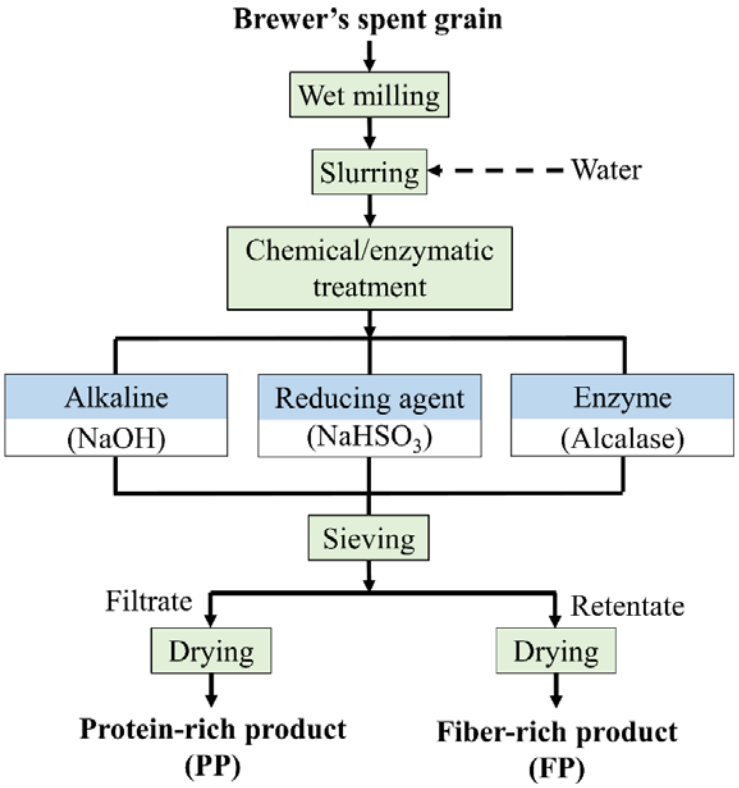

Figure 3.1 Schematic of the wet milling process to produce protein-rich product (PP) and fiber-rich product (FP) from BSG. Reagent (Alkaline, reducing agent or Enzyme) was used respectively during the incubation process to produce PP and FP.

Table 3.1 Experimental design of brewer's spent grain incubation with different reagents and loadings.

Reagent	Reagent Loading	Incubation pH	Principles of breaking protein matrix
Sodium hydroxide	0,1, 3, 5[a]	Different with loadings	Break hydrogen bonds, unfold protein matrix, and increase protein solubility in aqueous solution.
Sodium bisulfite	0,1, 3, 5[a]	pH 5.0	Break disulfide bonds in protein to s-sulfonate derivatives, increase protein solubility.
Alcalase	0, 5, 20, 35[b]	pH 8.0	Hydrolyze peptide bonds in protein matrix, reduce protein size, and increase protein solubility.

[a] % w/w based on dry weight BSG.
[b] μL/g of dry BSG.

3.2.3 Analytical methods

3.2.3.1 Determination of particle size distribution of the original and milled BSG

BSG particle size plays a significant role in the separation process because it affects the available surface area of BSG particles reacting with reagents and the amount of matter passing through the sieve. In this study, the BSG samples were milled once, twice or four times using a disk mill in order to obtain different particle size distributions. The particle size (Sauter mean diameter) distributions of the original and milled BSG samples were determined by a Laser Diffraction Particle Size Analyzer (LA-950, HORIBA, Ltd., TX). The principle of the method is measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample. Large particles scatter light at smaller angles than small particles do. And the particle size distribution is calculated using Mie theory of light scattering, with all particles being assumed spherically shaped. The particle size is reported as the diameter of an equivalent sphere having the same volume as the measured particle. Briefly, 1.5 g of the milled BSG sample was introduced into the dispersion unit device of the analyzer; and the introduced sample was diluted by the auto-dilution system of the particle size analyzer to reach the appropriate concentration for laser scanning.

3.2.3.2 Moisture content and chemical composition determination

Moisture content was determined using the gravimetric method by drying in the oven at 135 ℃ for 2 h (AOAC,2005). Ash was determined by weight difference before and after incineration of samples in a muffle furnace at 550 ℃ for 12 h (Niemi et al., 2012). Crude protein was determined by following the Kjeldahl procedure (AOAC 2001.11), multiplying the total nitrogen by 6.25. The Foss TecatorTM Digestor and Kjeltec TM 8100 Distillation Unit (FOSS North America, Eden Prairie, MN) were used to determine the total nitrogen content. Crude fat was determined by Ran-dall/Soxtec/Hexanes Extraction–Submersion Method (AOAC2003.05) using petroleum ether as an extraction solvent. FOSS2055 Soxtec Avanti Manual System (FOSS North America, Eden Prairie, MN) was used. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) of samples were quantified by ANKOM Filter Bag System (ANKOM 2000 automated fiber analyzer, ANKOM Technology, Macedon, NY). Amino acid profiles were determined at the University of Missouri-Columbia experiment station chemical labs by following the AOAC Official Method (982.30 E (a, b)).

Hemicellulose, cellulose, lignin contents of FP were deter-mined following the National Renewable Energy Laboratory (NREL) standard procedures: “Determination of structural carbohydrates and lignin in biomass (TP-510-42618)” (Sluiteret al., 2008). Briefly, non-structural materials were first removed by utilizing sequential water and ethanol extraction with Dionex ASE 350 Accelerated Solvent Extractor (Thermo Fisher Scientific Inc., MA). The extractive free samples were then incubated with 72% (w/w) sulfuric acid at 30 ℃ for 1 h, diluted to 4% (w/w) sulfuric acid using deionized water, then autoclaved for 1 h at 121℃. The acid hydrolyzed samples were then filtered through crucibles. The solid residues in crucibles were dried in a convection oven at 105 ℃ for 12 h, and the ash contents were determined by burning samples at 575 ℃ for4 h. The solid residue after the acid hydrolysis was determined gravimetrically to determine the acid insoluble lignin content. The filtrate containing hydrolyzed sugars were analyzed using Agilent 1200 HPLC system (Agilent Technologies, CA) equipped with a 1260 refractive index detector. Bio-Rad Aminex HPX-87P column (Bio-Rad, CA) was used with deionized water as a mobile phase at flow rate of 0.6 mL/min at 80 ℃. The total running time was 30 min, and the injection volume of sample was 10 µL. The content of cellulose was calculated from glucose while hemicellulose was calculated from the sum of xylose, arabinose and galactose. The composition analysis for each sample was conducted in duplicate.

3.2.4 Evaluation of the process performance

The performance of the separation process was assessed based on the PP and FP recovery rates from BSG, protein separation efficiency, and protein concentrations in PP and FP, which were calculated based on the following equations.

$$\text{PP or FP recovery (\%)} = \frac{\text{PP or FP (g)}}{\text{Total weight of PP and FP (g)}} \times 100 \quad \text{3-1}$$

$$\text{Protein Concentration (\%)} = \frac{\text{Protein in the PP or FP (g)}}{\text{PP or FP (g)}} \times 100 \quad \text{3-2}$$

$$\text{Protein separation efficiency (\%)} = \frac{\text{Protein in the PP (g)}}{\text{Total protein in the PP and FP (g)}} \times 100 \quad \text{3-3}$$

3.2.5 Statistical analysis

Each treatment of the separation process was conducted in triplicate. The chemical composition of samples (PP and FP) produced from each treatment was analyzed in duplicate. Analytical replicates were averaged together to create a composite value for each process replicate. One-way analysis of variance (ANOVA) was conducted to determine any treatment effect and then the Tukey' HSD (honestly significant difference) test for multiple comparisons was used to discern differences in treatment means. Statistical significance level was set at $p < 0.05$. The statistical analysis was performed using JMP Pro13® desktop software (SAS, Cary, NC).

3.3 Results and discussion

3.3.1 Chemical composition of BSG

The chemical composition of BSG is presented in **Table 3.2**. The most abundant component in dry BSG is NDF, which accounts for 44.7% (w/w) of total dry matter. NDF represents the structural fiber components in plant cells, i.e. cellulose, hemicellulose, and lignin. The reason for the high concentration of NDF is that starch is hydrolyzed and removed from malted barley during the mashing and filtration process, making NDF concentrated in BSG. Because the feed digestibility and the feed intake of animal are greatly influenced by the high levels of NDF (Mertens, 1987; Harper and McNeill, 2015), BSG is currently being used as low-value animal feed.

Protein is the second largest component in BSG, accounting for 22.9% (w/w) of total dry matter. The protein concentration in BSG in this study is within the typical range (15–25%, w/w) of protein concentrations in BSG reported by other studies (Meneses et al., 2013; Mussatto and Roberto, 2006; Xiros and Christakopoulos, 2012). The protein concentration in BSG is higher than some agricultural feed stocks, like corn (6–12%, w/w), wheat straw, and corn stover (2–7%, w/w) (Dhillon et al.,2016; Adapa et al., 2009). Meanwhile, this value is much lower than in other popular protein sources used to make animal feed, such as canola meals (35–45%, w/w), soybean meal (40–50%, w/w), and corn gluten meal (60–70%, w/w) (Johnson and May, 2003; Toda et al., 2016; Wu et al., 2016; Broderick et al., 2016). Therefore, there is a justification to develop a separation process to concentrate proteins in BSG to expand the utilizations of BSG. Moreover, BSG contains 19.8% (w/w) of acid detergent fiber (ADF), which represents least-digestible of fibers in BSG.

Furthermore, BSG is considered as a lignocellulosic material with relatively low lignin content (11.3%, w/w) compared to wheat straw (19.8%, w/w), corncob (21.63%, w/w), sugar-cane bagasse (28.4%, w/w) and rice straw (20.2%, w/w) (Akpinaret al., 2009; Su et al., 2015; Sakdaronnarong et al., 2017). The lower lignin could be an advantage for BSG fiber processing and utilization. Lignin is a highly complex and amorphous polymer, and it protects cellulose and hemicellulose from hydrolytic attacks (dos Santos et al., 2019). Therefore, the lignocellulosic material with lower lignin content is usually more accessible to enzymatic hydrolysis. Moreover, BSG contains higher crude fat (8.5%, w/w) than other lignocellulosic feed-stock, such as corn stover (1.0%, w/w), barley straws, and wheat straws (0.5–0.8%, w/w) (Falls et al., 2017; Pronyk and Mazza, 2012), making BSG a good lipid source to feed animals.

Table 3.2 Proximate composition of original brewer's spent grain in dry matter basis.

Components	Concentration (%) [1]
Crude protein	22.9 ± 1.6
Neutral detergent fiber (NDF)	44.7 ± 0.2
Acid detergent fiber (ADF)	19.8 ± 0.3
Lignin	11.3 ± 1.4
Crude fat	8.5 ± 0.2
Ash	4.3 ± 0.0

[1] Values are means ± standard deviation. % w/w in dry basis.

3.3.2 Particle size distribution of milled BSG

The geometric mean sizes of the original BSG and BSG milled once, twice and four times are 354.1 ± 4.2, 161.7 ± 3.9, 111.1 ± 3.7 and 100.0 ± 3.3 μm, respectively. **Figure 3.2** shows the detailed particle size distributions of the original and milled BSG. The milled BSG had larger fractions (> 68%, w/w) of particles with smaller diameters (i.e. 0–200 and 201–400 μm) and smaller percentages of large-size particles (i.e. > 600 μm) compared with the original BSG. Milling BSG twice produced particles with smaller sizes compared to milling once. Biomass particle size could largely affect the chemical and enzymatic treatment for protein and fiber separation. Le Gall et al. (2005) reported that the decrease in particle size of peas increased protein separation efficiency from peas due to an improved access of solvents and enzymes to proteins. Khullar et al. (2013) found that there was an increasing trend in enzymatic hydrolysis efficiency of fibers with decreasing mean particle size of Miscanthus (an energy crop). Both of these findings pointed out that decreased particle size by milling could benefit the hydrolysis and separation efficiencies of protein and fiber.

The particle sizes resulting from milling BSG twice were not significantly different from those milled for four times ($p > 0.05$). Based on this result and the high energy demand typically associated with disk milling process (Kim et al., 2016; Ortega-Rivas, 2012), BSG was milled twice in the subsequent experiments.

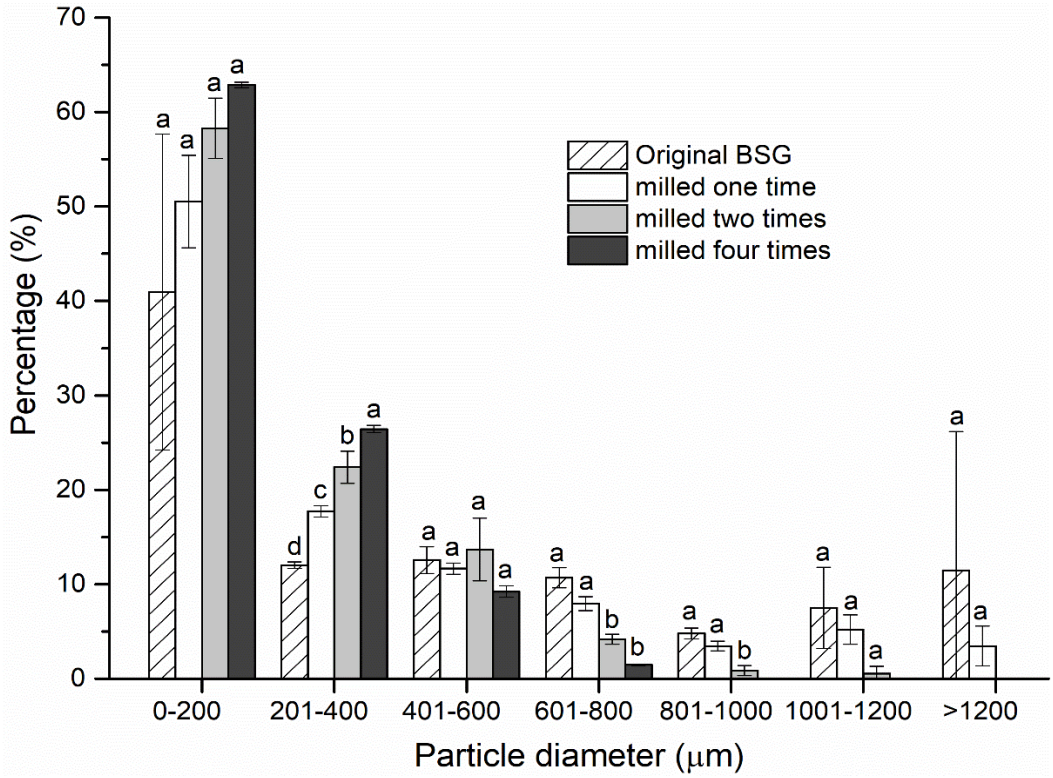

Figure 3.2 Particle size (Sauter mean diameter) distribution of the original and milled brewer's spent grain (BSG). Values are presented as means ± standard deviation (n = 2). Same letter within the same particle diameter range indicates not significantly different (p < 0.05)

3.3.3 Effects of incubation reagents and their loadings on PP and FP production

PP and FP recoveries, protein concentrations in PP and FP, and protein separation efficiencies are summarized in **Table 3.3**. BSG was wet milled, incubated with sodium hydroxide, sodium bisulfite, or Alcalase, and then sieved to separate PP and FP from BSG. The incubation of BSG was conducted in shaking flasks without mechanical agitators; therefore, 5% (w/w) of solid content was chosen to ensure sufficient mix of the slurry. However, it is worth to note that 5% solid content in slurry is relatively low com-pared to what would typically be used for an industrial process. In previous studies, a 10% (w/w) solid content was used for biomass hydrolysis to design a commercial-scale lignocellulosic-ethanol process with mechanical agitation (Humbird et al., 2011; Tao et al., 2012).

3.3.3.1 Sodium hydroxide treatment

With sodium hydroxide treatment, about 34.8–54.8% (w/w) of each BSG sample passed through the sieve and was recovered as PP. Higher amounts of PP were recovered from BSG when

the sodium hydroxide loading increased from 0 (control) to 5% (w/w) based on the dry matter of BSG. This could be attributed to higher concentration of sodium hydroxide enabling more solubilization of protein and carbohydrates. Accordingly, the amount of materials retained on sieve, collected as FP, reduced significantly from 65.3% to 45.2% (w/w) when the sodium hydroxide loading increased. The protein separation efficiency increased significantly from 60.7% to 81.8% (w/w) when the sodium hydroxide loading increased from 0 to 5%.

A high protein concentration is desirable when PP is used as a protein ingredient for food or feed. Our results showed that the protein concentration in PP decreased from 44.1% to 36.5% (w/w) as the sodium hydroxide loading increased (**Table 3.3**). This result is understandable because sodium hydroxide not only solubilize proteins in BSG, but also partially solubilize fibers, especially hemicellulose and lignin, by breaking the linkages between lignin and hemicellulose (Jin et al., 2018; Qing et al., 2017). At higher sodium hydroxide loadings, a larger amount of fibers in BSG solubilized during incubation, passed through the sieve, and became a part of PP (Hendriks and Zeeman, 2009). Accordingly, the protein concentration in PP became proportionally smaller. The market values of protein feeds (or food) are usually positively correlated with their protein concentration. With the increasing of sodium hydroxide, the protein separation efficiency and PP recovery from BSG increased; however, this happened with the decreased protein concentration (thus decreased market value) of PP. Therefore, there might be a trade-off point for the sodium hydroxide loading to produce PP from the quality and economic aspect.

3.3.3.2 Alcalase treatment

When the Alcalase loading increased from 0 (control) to 20 μL/g, the protein separation efficiency increased from 70.8% to 83.7%, and the PP recovery increased from 39.3% to 43.7% significantly (**Table 3.3**). This result clearly indicated that the Alcalase treatment could improve the protein separation and PP recovery from BSG. This result aligns with other studies, which have reported that enzymatic treatment improved protein separation from BSG and other agricultural products (Kim et al., 2016; Treimo et al., 2008). The significant effect of Alcalase treatment was also revealed on the low amount of protein left in FP. After the sodium hydroxide treatment, FP still contained no less than 10% (w/w) protein; while after the Alcalase treatment, FP contained less than 7% (w/w) protein. Unlike sodium hydroxide that solubilizes both protein and fiber, Alcalase is more specifically to solubilize protein by cleaving the peptide bonds in protein macromolecules. Therefore, the protein concentration in PP increased from 39.4% to 42.8% (w/w)

when the Alcalase loading increased from 0 to 20 μL/g. It is worth noting that no improvement in protein separation was observed when the Alcalase loading was increased above 20 μL/g.

3.3.3.3 Sodium bisulfite treatment

The treatment with sodium bisulfite for the protein and fiber separation from BSG was overall not effective (**Table 3.3**). When the sodium bisulfite loading increased from 0 to 5% (w/w), the protein separation efficiency remained at a similar level of 68%. As a reducing agent, sodium bisulfite has the capability to cleave disulfide bonds in proteins to increase protein solubility and improve protein and fiber separation efficiency (Abtahi and Aminlari, 1997). Previous studies have showed that sodium bisulfite effectively increased the solubility of soy-bean and corn proteins (Abtahi and Aminlari, 1997; Parris and Dickey, 2001); however, sodium bisulfite did not work well in our study. One possible reason for the low solubility of protein in BSG may be due to the link between cellulosic material and protein (Crowe et al., 1985; Celus et al., 2006), making the BSG proteins recalcitrant to sodium bisulfite. Studies had showed the combination of the reducing agent and detergent [e.g. sodium dodecyl sulfate (SDS)] to improve the protein solubility in BSG to extract proteins (Celus et al., 2006). Another possible reason for the low protein solubility was the pH of the slurry. In this study, the pH value of the sodium bisulfite treatment was set at 5.0 based on the steeping pH of the corn wet milling industry (Eckhoff et al., 1996). The pH 5.0 is close to the isoelectric point (5–6) of most proteins (Mejri et al., 2005). It is a well-known fact that protein solubility is lowest at the isoelectric point. In the future, it would be interesting to investigate the effect of sodium bisulfite treatment under different pH conditions.

Table 3.3 A summary of wet fractionation of brewer's spent grain to produce PP and FP.

Reagent	Reagent loading	PP Recovery (%) [1]	FP Recovery (%) [2]	Protein conc. in PP (%)	Protein conc. in FP (%)	Protein Separation Efficiency (%)
	0 [3]	34.8 ± 1.5[d, 5]	65.3 ± 1.5 [a]	44.1 ± 0.8 [a]	15.2± 0.5 [a]	60.7 ± 0.9 [c]
Sodium	1	40.3 ± 2.1 [c]	59.7 ± 2.1 [b]	43.0 ± 1.4 [a]	15.5 ± 0.5 [a]	65.2 ± 3.3 [c]
Hydroxide	3	46.3 ± 0.6 [b]	53.7 ± 0.6 [c]	38.7 ± 0.5 [b]	13.7 ± 0.6 [b]	71.0 ± 1.1 [b]
	5	54.8 ± 0.6 [a]	45.2 ± 0.6 [d]	36.5 ± 0.7 [b]	9.8 ± 0.0 [c]	81.8 ± 0.7 [a]
	0 [3]	37.9 ± 0.8 [b]	62.1 ± 0.8 [a]	42.3 ± 1.3 [a]	12.2 ± 1.1 [a]	68.0 ± 0.6 [a]
Sodium	1	39.6 ± 0.5 [a]	60.4 ± 0.5 [b]	41.8 ± 1.6 [a]	12.6 ± 1.2 [a]	68.5 ± 1.5 [a]
bisulfite	3	40.6 ± 0.5 [a]	59.4 ± 0.5 [b]	39.2 ± 0.6 [a]	12.8 ± 0.9 [a]	67.8 ± 2.3 [a]
	5	41.0 ± 0.6 [a]	59.0 ± 0.6[b]	38.7 ± 1.5 [a]	12.5 ± 0.7 [a]	68.3 ± 2.5 [a]
	0[4]	39.3 ± 0.3 [b]	60.7 ± 0.3 [a]	39.4 ± 1.7 [a]	10.6 ± 0.1 [a]	70.8 ± 1.4[b]
Alcalase	5	39.5 ± 1.1 [b]	60.5 ± 1.1 [a]	35.6 ± 4.1 [a]	6.7 ± 0.2 [b]	76.9 ± 2.6 [b]
(Enzyme)	20	43.7 ± 0.3 [a]	56.4 ± 0.3 [b]	42.8 ± 0.1 [a]	6.5 ± 1.1[bc]	83.7 ± 2.2 [a]
	35	41.4 ± 0.9 [ab]	58.6 ± 0.9 [ab]	38.3 ± 4.6 [a]	5.4 ± 0.5 [c]	83.4 ± 1.6 [a]

[1] Percentage of dry mass recovered as PP as shown in equation 3-1.

[2] Percentage of dry mass recovered as FP as shown in equation 3-1.

[3] With the unit of % w/w based on dry weight BSG.

[4] With the unit of μL/g of dry BSG.

[5] Statistical analyses were carried out with one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Different letters within the same column of the same reagent are significantly different ($p < 0.05$). Values are means ± standard deviation.

3.3.4 Chemical composition of PP and FP from representative treatments

3.3.4.1 Major components in PP and FP

The chemical compositions were analyzed for the representative PP and FP produced from the following three treatments: 1) sodium hydroxide at 5% (w/w), 2) sodium bisulfite at 5% (w/w), and 3) Alcalase at 20 μL/g dry BSG. These three treatments were chosen because they had the best PP recovery and protein separation efficiency at each of the three reagents. The results indicated that the sodium hydroxide, sodium bisulfite and Alcalase treatments affected the composition of the resulting PP and FP (**Table 3.4**). Among the PP produced from the three treatments, the PP produced from the Alcalase treatment had the highest protein (42.8%, w/w) concentration and lowest NDF (10.2%, w/w) and ADF (6.6%, w/w) concentrations. This is particularly desired if PP is used as an animal feed because the feeding value is positively correlated with protein contents and negatively correlated with NDF and ADF contents. The increased protein concentration in PP allows it to be used for animals that require high protein diets. For example, in the aquaculture industry, the shrimp diet usually requires 35–50% (w/w) of protein concentration (Van Wyk et al., 1999), and the protein concentration in PP aligns well with this requirement. The high protein concentration and low NDF and ADF concentrations in PP produced by the Alcalase treatment are probably attributed to the high specificity of enzyme to solubilize protein in BSG, but remain fibers untouched. The PP produced from the sodium hydroxide treatment had the highest NDF and ADF contents among the three, which again reveals that the sodium hydroxide treatment non-specifically solubilizes fibers.

FP is another valuable product from the processing of BSG. Generally, high NDF and low protein concentrations in FP are desired as a fiber product. The NDF concentrations in FP from the three treatments ranged from 75% to 85% (w/w). The FP got from the sodium hydroxide treatment had the highest NDF concentration (84.6%, w/w), and the FP from the sodium bisulfite had the lowest NDF concentration (74.8%, w/w). In terms of protein, the FP from the Alcalase treatment had the lowest protein concentrations (6.5%, w/w) among the three, which corresponded well with the high protein concentration in PP from the Alcalase treatment.

The lipid concentrations were also quantified in both PP and FP (**Table 3.4**). The results showed the lipid concentrations in PP were higher than those in FP, indicating that majority of lipids entered into the PP fraction during the fractionation process. For instance, when the Alcalase

pretreatment was performed, more than 63% (w/w) of lipid was distributed in PP product based on the mass balance and the rest of 36% was distributed in FP. Lipid is an important nutrient in PP for feeding animal; therefore, it is favorable to have more lipid entering to the PP fraction. It is interesting to note that both PP and FP produced from the sodium hydroxide treatment had the lowest lipid concentrations among the three treatments. This is probably because of saponification, the alkaline hydrolysis of fatty acid esters. During the incubation process, triglycerides, the predominant lipids in BSG (Niemiet al., 2012), react with sodium hydroxide and produce glycerol and fatty acid salts. This reaction results in the decreased lipid contents in PP and FP after the sodium hydroxide treatment. Ash is composed of various types of minerals. High amounts of calcium, magnesium, silica and phosphorus were reported in BSG (Khidzir et al., 2010). The ash in PP obtained from the three treatments was less than 3% (w/w), lower than the value (6%, w/w) in soybean meal (Sharawy et al., 2016).

Table 3.4 Chemical composition of representative PP and FP produced from three reagents.

		Protein	Neutral detergent fiber (NDF, %[2])	Acid detergent fiber (ADF, %)	Lipids (%)	Ash (%)
PP	Sodium Hydroxide	36.5 ± 0.7^{b,1}	16.1 ± 1.1^{a}	13.6 ± 1.4^{a}	9.7 ± 0.4^{b}	2.5 ± 0.1^{a}
	Sodium bisulfite	38.7 ± 1.5^{b}	15.8 ± 0.4^{a}	10.3 ± 0.3^{ab}	12.2 ± 0.1^{a}	2.7 ± 0.2^{a}
	Alcalase	42.8 ± 0.1^{a}	10.2 ± 0.6^{b}	6.6 ± 0.9^{b}	13.4 ± 0.3^{a}	2.6 ± 0.0^{a}
FP	Sodium Hydroxide	9.8 ± 0.0^{b}	84.6 ± 1.6^{a}	37.7 ± 0.8^{a}	4.4 ± 0.3^{b}	12.8 ± 0.2^{a}
	Sodium bisulfite	12.5 ± 0.7^{a}	74.8 ± 1.6^{b}	34.0 ± 1.7^{a}	6.3 ± 0.2^{a}	11.6 ± 0.0^{b}
	Alcalase	6.5 ± 1.1^{c}	80.4 ± 2.4^{ab}	38.6 ± 0.7^{a}	5.9 ± 0.1^{a}	7.5 ± 0.1^{c}

[1] Statistical analyses were carried out with one-way ANOVA followed by Tukey's HSD test for multiple comparisons.

[2] Different letters in the same column indicate significant differences ($p < 0.05$). Values are means ± standard deviation. % w/w in dry basis.

3.3.4.2 Amino acid profile in PP and fiber composition in FP

Protein is the most important component in PP; and fiber is the most important component in FP. Therefore, we further investigated the amino acid profiles of protein in PP and the cellulose, hemicellulose and lignin contents in FP. This information is critical to identify the potential uses of PP and FP.

The amino acid profiles of representative PP are shown in **Figure 3.3**. The predominant amino acids in PP were glutamine and proline, which constituted more than 30% (w/w) of the total amino acids. The result agreed well with the amino acid composition reported by Treimo et al. (2008). Glutamine/glutamic acid and proline are the main components of hordeins, which are the main storage proteins in barley (Robertson et al., 2010). Besides glutamic acid and proline, PP also contained significant amounts of essential amino acids, such as leucine, phenylalanine and valine. Lysine is another important essential amino acid; however, most cereal proteins, such as barley, corn and wheat, contain a low content of lysine (less than 4%, w/w) (Gatlin et al., 2007). In our study, the PP produced from the sodium hydroxide treatment had the lowest lysine content (3.1%, w/w) and the PP from the Alcalase treatment had the highest lysine content (4.1%, w/w). Papadopoulos (1985) found the elevated NaOH concentrations increased losses of lysine, and reduced the digestibility of essential amino acids, such as lysine, methionine and histidine. Severe treatment of isolated soy protein with alkali of pH 12.2 at 60 ℃ also caused a decreased protein digestibility (De Groot and Slump, 1969). This result indicates that the Alcalase treatment release lysine more effectively than the sodium hydroxide and sodium bisulfite treatments do, resulting in a higher amount of lysine ended in PP.

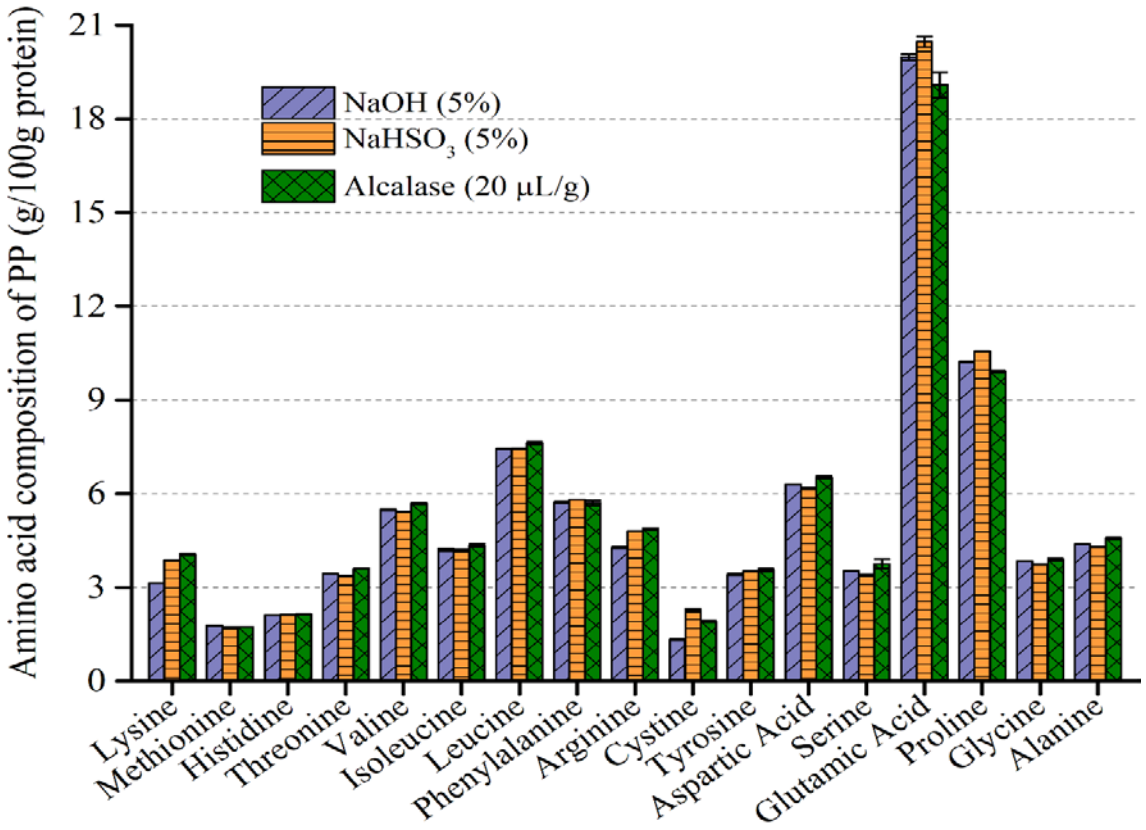

Figure 3.3. Amino acid profiles of selected PP produced from the sodium hydroxide, sodium bisulfite and Alcalase treatments. Values are presented as means ± standard deviation (n = 2).

The predominant fiber components in FP are cellulose, hemicellulose, and lignin. Cellulose is a linear polymer com-posed of glucose linked by beta 1,4 glyosidic bonds (Pérezet al., 2002). Hemicellulose is a complex carbohydrate polymer consisting of xylose, arabinose, galactose, mannose and other minor galacturonic and glucuronic acids (Pérez et al.,2002). Lignin is a cross-linked phenolic polymer consisting of three common monolignols: paracoumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These three components possess distinct physical and chemical properties and the ratio of these largely determine the end uses of FP as a fiber material (Collard and Blin, 2014). The concentrations of cellulose, hemi-cellulose, and lignin in representative FP are summarized in **Table 3.5**. The cellulose concentrations in FP were between 24% and 27% (w/w). The lignin concentration in FP was between 8% and 11% (w/w). The hemicellulose concentrations in FP were between 41% and 43% (w/w), much higher than the cellulose and lignin concentrations. The high concentration of hemicellulose makes FP a unique fiber source. For example, arabinoxylans, a major component of hemicellulose in BSG, has been

used as dietary fiber and proved to have high prebiotic potential for the modulation of gut microbiota (Reisand Abu-Ghannam, 2014). Mussatto and Roberto also applied acid hydrolysis and fermentation processes of BSG to pro-duce xylitol by taking advantages of the high concentration of hemicellulose in BSG fiber (Mussatto and Roberto, 2005).

Table 3.5 Cellulose, hemicellulose and lignin concentrations of selected FP.

Incubation agent	Cellulose (%[2])	Hemicellulose (%)	Lignin (%)
Sodium Hydroxide	26.8 ± 0.9 [a,1]	41.9 ± 0.5 [ab]	11.2 ± 0.2 [a]
Sodium bisulfite	23.5 ± 0.2 [a]	41.1 ± 0.0 [b]	8.2 ± 0.1[b]
Enzyme (Alcalase)	24.2 ± 1.1 [a]	42.7 ± 0.2 [a]	10.4 ± 1.0 [ab]

[1] Statistical analyses were carried out with one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Different letters in the same column indicate significant differences ($p < 0.05$). Values are means ± standard deviation.

[2] % w/w in dry basis.

3.4 Conclusions

This study designed a wet fractionation process to produce value-added PP and FP through separating and recovering proteins and fibers from BSG. Three different reagents (i.e. sodium hydroxide, sodium bisulfite, and Alcalase) with distinct reaction mechanisms with BSG were investigated to improve the production of PP and FP. Among the three reagents, Alcalase showed great promise to produce PP and FP from BSG, followed by sodium hydroxide. Under the optimal condition with Alcalase treatment, the protein separation efficiency was as high as 83.7% with the PP recovery rate of 43.7%, and the protein concentration in PP was 42.8% (w/w). Produced FP contains more than 80% (w/w) of fiber, of which hemicellulose is a dominant fiber component. The amino acid analysis showed that the glutamine, proline, and leucine are the predominant amino acids in produced PP; and the hemicellulose amounted for more than 40% (w/w) of the dry matter in produced FP. This study could potentially increase the value of BSG and support the sustainability of agriculture and food industry. In the future, detailed techno-economic analysis will be addressed to evaluate the economic feasibility of the process before scaling it up to a commercial level.

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Chapter 4. Protein-rich product recovered from brewer's spent grain can partially replace fishmeal in diets of Pacific white shrimp, *Litopenaeus vannamei*

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ABSTRACT

A protein-rich product (PP) with 46% protein and less than 1% fiber was recovered from brewery's spent grain. This study aimed to investigate the effects of replacing dietary fishmeal with PP on the growth, feed utilization efficiency, and nutritional composition of Pacific white shrimp, *Litopenaeus vannamei*. The control diet (PP0, containing 35% fishmeal) was compared with four isonitrogenous (44% crude protein), isolipidic (10% crude fat), and isocaloric (20 kJ/g) test diets PP10, PP30, PP50 and PP70, which were formulated using PP protein to replace 10%, 30%, 50% and 70% of fishmeal protein. Sextuplicate groups of shrimp (averaging 1.10 g) were fed each of the five diets for 8 weeks. The results showed that up to 50% of fishmeal replaced with PP did not negatively affect the shrimp survival, growth performance, feed utilization efficiency, or the protein content and amino acid profile of shrimp. However, replacing 70% of fishmeal protein with PP protein negatively affected the percent weight gain and specific growth rate of shrimp, although the shrimp survival rate and feed conversion ratio were not affected.

4.1 Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is a tropical marine species that is widely farmed globally, accounting for more than 75% of annual shrimp production in the world (FAO, 2016). Although wild-caught shrimp continue to contribute a large fraction of the total supply, 55% of the shrimp produced globally is currently farmed (FAO, 2018; Gaille, 2018). Fishmeal has historically been the primary protein source in shrimp feeds due to its balanced amino acid profile, high digestibility, palatability and general lack of antinutrients (Shao et al., 2019). Commercial shrimp feeds usually include 25 to 50% fishmeal (Amaya et al., 2007; Xie et al., 2016; Yun et al., 2017). During the past twenty years, the fishmeal price has increased from $350 to $1,600 per metric ton (Fishmeal-Monthly-Price, 2019), and further increases are expected due to growing demands and an unstable global supply of fishmeal (FAO, 2018). Since 40 to 60% of shrimp production expenses are from feed cost, of which fishmeal is the most expensive component (Hu et al., 2019). It is important to seek alternatives to fishmeal to secure the sustainable development of the shrimp farming industry.

In this context, plant-based proteins could be potential alternatives to fishmeal for the aquaculture industry. Compared with fishmeal, plant proteins have a lower price and more consistent supply (Bulbul et al., 2016; Chakraborty et al., 2019). A potential plant protein source is brewer's spent grain (BSG), the main by-product of brewing industries. BSG is one of the most underutilized plant protein sources (Mussatto et al., 2006; Tang et al., 2009). Typically, raw BSG contains 77–81% moisture. Dry BSG contains 14–30% protein, 50–70% fiber, 5–10% fat, and 2–5% ash (Aliyu and Bala, 2011; Ivanova et al., 2017; Lynch et al., 2016; Mussatto, 2014). Approximately 20 kg of wet BSG is generated from each 100 L of beer produced (Reinold, 1997). Since beer is the third most popular drink in the world, the global annual production of wet BSG is as high as 38,600,000 metric tons (Mussatto, 2014). Currently, BSG is predominantly used as cattle feed or disposed of in landfills. Due to its high moisture content, the easily spoiled BSG is also a potential threat to the environment (Jayant et al., 2018). On the other hand, besides protein, BSG also contains minerals (copper, cobalt, iron, potassium, selenium, sulfur, sodium, selenium, magnesium, phosphorus, manganese), vitamins (folic acid, niacin, biotin, choline, thiamine, pantothenic acid, pyridoxine, and riboflavin), both essential and nonessential amino acids, and polyphenols (Cooray et al., 2017; Mussatto, 2009), making BSG a potential valuable ingredient in aquafeed (Ivanova et al., 2017).

Some pioneering studies have been conducted to explore using BSG in aquaculture. Oduro-Boateng and Bart-Plange (1988) investigated the replacement of fishmeal with Pito brewery waste in the diet of Tilapia *busumana fry*. Zerai et al. (2008) conducted a 10-week feeding trial to evaluate the use of raw BSG (32% protein and 10% fiber) to replace fishmeal in Nile Tilapia (*Orechromis niloticus*) diets. Their results showed that replacing 50% of fishmeal with BSG did not negatively impact fish growth. Cheng et al. (2004) evaluated the incorporation of raw BSG (21% protein and 15% fiber) in the diet of rainbow trout, which is a carnivorous species and has a higher protein requirement than Tilapia (an omnivorous species) does. They found that incorporation of 30% of BSG in the diets reduced the apparent digestibility coefficients of formulated diets because of the high-fiber content of BSG. Previous literatures have pointed out that the ideal fishmeal alternative should possess a high protein content (> 40%), low fiber content (< 6%), and minimal antinutrients to be successfully used as a protein ingredient (Gatlin et al., 2007; Naylor et al., 2009).

In a previous study, we developed an enzymatic-based wet fractionation process that removes fibers and concentrate proteins from BSG to produce a protein-rich product (PP) (He et al., 2019). This PP contains more than 40% protein and less than 1% fiber, which makes it more in line with ideal fishmeal replacement compared with raw BSG. Furthermore, PP is produced by the enzymatic hydrolysis of BSG protein. Thus, the molecular size of protein in PP is smaller than that in raw BSG (Niemi et al., 2013), making PP potentially more digestible in shrimp digestion system. These changes might make PP more competitive than raw BSG in fishmeal replacement in aquafeed. However, the effectiveness of incorporation of PP in aquaculture diet on the shrimp (or any fish) growth performance and survival rate has not been evaluated before. Moreover, it is still unknown regarding the effect of PP incorporation in shrimp diet on the nutritional composition of raised shrimp.

Consequently, the objectives of this study were as follows: (a) to evaluate the growth performance of shrimp fed diets where fishmeal was gradually replaced by PP, and (b) to evaluate the effects of PP incorporation on the nutritional composition of harvested shrimp. The new knowledge gained from this study not only will enable the development of novel strategies to upgrade low-value BSG to a highly nutritious protein ingredient for the aquaculture industry, but may also be used to upgrade other food byproducts that have high-fiber and low-protein contents, such as rice bran, distiller's spent grain, and wheat bran.

4.2 Materials and methods

4.2.1 PP production

The raw BSG was obtained from Parkway Brewing Company (Salem, VA, USA). Raw BSG contained 79% moisture (w/w, wet basis), 45% fiber, 22% protein, and 8% fat (w/w, dry basis) was milled directly to reduce particle size. The PP production from milled BSG followed a modified method adopted from our previous study (He et al., 2019). Briefly, the raw BSG was wet milled and then enzyme hydrolyzed by Alcalase 2.4L FG (Novozymes Inc. Franklinton, NC, USA) with a loading of 5 μL Alcalase/g dry BSG, solid content 9% w/w, pH 8.0, and under temperature 60 ℃ for 1 h. The hydrolysate rich in protein was then separated with a cold press (X-1, Goodnature Products, Inc. NY, USA) followed by a sieve shaker (RX-29, W.S. tyler, OH, USA). The material that passed through the sieve was concentrated using an evaporator and dried to less than 10% of moisture content. This dried product was named PP and was subjected to the proximate composition analysis based on the methods described in section 4.2.4. The PP had 45.8% crude protein, 9.5% crude fat, 0.8% neutral detergent fiber, and 6.7% ash, with other detailed composition shown in **Table 4.1**. The total carbohydrates in PP was calculated by the weight difference between the total dry matter and the sum of crude protein, total fat, and ash according to the reference (Merrill and Watt, 1973). The carbohydrates are mainly composed of arabinoxylan, glucans, starch and mixed linked β-glucan (Niemi et al., 2012). PP was stored at -80 ℃ until use.

4.2.2 Diet formulation and preparation

The proximate compositions of major diet ingredients and feed formulation are shown in **Tables 4.1** and **4.2**, respectively. The control diet (PP0) containing 35% (w/w) of fishmeal was formulated based on previous studies (Bulbul et al., 2015; Hulefeld et al., 2018; Liu et al., 2012). The other four test diets were formulated by replacing fishmeal with PP at increasing levels of 10%, 30%, 50%, and 70%, and designated as PP10, PP30, PP50, and PP70, respectively. All ingredients were mixed thoroughly in a Kitchen Aid^{TM} (Pro 600, Whirlpool Corp., Benton Harbor, MI, USA) for 30 min. Distilled water was then added slowly with mixing for an additional 20 min. The final mixture was extruded using a grinder attached to the Kitchen Aid^{TM} stand mixer. Extruded feeds were subsequently cut into pellets of approximately 10 mm long and 2 mm diameter, and were air dried in 5-mm layers for around 48 h to < 10% moisture content at room

temperature. The feeds were stored at -20 ℃ until the shrimp feeding trial was completed (within three months). The proximate compositions of diets were analyzed in duplicate based on methods listed in part 4.2.4.

Table 4.1 Composition of experimental ingredients (dry-weight basis).

Parameter	PP	Fish meal	Soybean meal	Krill meal
Proximate and mineral (g/100 g dry matter)				
Crude protein	45.80	70.90	51.43	63.47
Crude fat	9.50	9.31	1.04	22.35
Carbohydrate[1]	38.01	0.00	40.76	4.81
Ash	6.69	20.65	6.77	9.37
NDF[2]	0.81	5.88	6.53	-
Calcium	0.43	5.15	0.53	1.56
Sodium	1.81	0.94	0.01	1.40
Phosphorus	1.00	3.29	0.78	1.43
Potassium	0.16	1.27	2.52	0.56
Magnesium	0.38	0.21	0.32	0.41
Essential amino acid (g/100 g dry matter)				
Leucine	3.53	4.69	4.09	4.84
Valine	2.60	3.38	2.65	3.61
Threonine	1.62	2.64	2.03	2.56
Isoleucine	2.05	2.85	2.56	3.40
Arginine	2.09	4.11	3.85	3.64
Phenylalanine	2.78	2.72	2.78	2.89
Lysine	1.85	5.17	3.39	4.69
Methionine	0.89	1.76	0.73	1.66
Histidine	0.96	1.88	1.37	1.23
Tryptophan	0.60	0.70	0.75	0.78
Non-essential amino acid (g/100 g dry matter)				
Glutamic acid	9.81	8.29	9.52	7.31
Aspartic acid	3.18	5.87	5.94	6.10
Alanine	2.15	4.39	2.31	3.22
Glycine	1.74	5.25	2.23	2.62
Serine	1.71	2.32	2.30	2.14
Proline	4.55	3.25	2.70	2.40
Tyrosine	1.69	2.06	1.97	3.08
Cysteine	0.91	0.59	0.80	0.47
Trace element levels (mg/1000 g dry matter)				
Manganese	59.22	29.49	52.76	2.10
Zinc	90.83	103.86	66.75	55.42
Copper	19.83	6.28	17.79	90.05

[1] Calculated by difference (Merrill and Watt, 1973): total dry matter- (crude protein + total fat + ash). [2] NDF: Neutral detergent fiber

Table 4.2 Formulation of experimental diets (% dry matter). Diets with graded levels of PP as a replacement for menhaden fish meal.

Ingredients	Diet groups				
	PP0	PP10	PP30	PP50	PP70
PP[1]	0.00	5.42	16.25	27.09	37.93
Menhaden Fish meal[2]	35.00	31.50	24.50	17.50	10.50
Soybean meal	25.00	25.00	25.00	25.00	25.00
Corn starch	24.49	21.82	17.37	12.77	8.07
Beef gelatin	2.00	2.00	2.00	2.00	2.00
Menhaden fish oil	2.70	2.50	2.10	1.90	1.70
Krill meal	6.00	6.00	6.00	6.00	6.10
Soybean lecithin	2.20	2.20	2.20	2.20	2.20
Potassium chloride	1.75	1.82	1.95	2.08	2.23
Calcium carbonate	0.00	0.87	1.73	2.53	3.32
Vitamin mix[3]	0.50	0.50	0.50	0.50	0.50
Mineral mix[4]	0.10	0.10	0.10	0.10	0.10
Vitamin C	0.02	0.02	0.02	0.02	0.02
Cholesterol[5]	0.14	0.14	0.14	0.14	0.14
Methionine	0.10	0.11	0.14	0.17	0.19

[1] The protein-rich product that was separated from brewer's spent grain with enzyme.

[2] Omega Protein, Houston, TX, USA.

[3] Pentair Aquatic Eco-Systems, Cary, NC, USA. Composition of vitamin mix (per 454 g): vitamin A, 325,000 USP units; vitamin E, 32,500 IU; Vitamin B12, 10.08 μmol; vitamin D3, 65,000 USP units; vitamin K, 793.65 mg; ρ-pantothenic acid, 16,500 mg; choline, 2,600 mg; pyridoxine, 2,600 mg; ascorbic acid, 87,100 mg; BHT, 200 mg; riboflavin, 3,250 mg; niacin, 19,500 mg; thiamine, 2,600 mg; folic acid, 780 mg; biotin, 40 mg; inositol, 13,000 mg.

[4] Florida Aqua Farms Inc., Dade, FL, USA. Composition of mineral mix: manganese, 60 ppm; zinc, 50 ppm; iron, 40 ppm; copper, 4.0 ppm; cobalt, 0.5 ppm; iodine, 40 ppm; selenium, 0.4 ppm.

[5] MP Biomedicals, Solon, OH, USA.

4.2.3 Experimental shrimp and feeding trial

The shrimp feeding trial does not need an ethical approval based on the Virginia Tech Institutional Animal Care and Use Committee (IACUC). Post-larvae 12- to 15-day-old (PL-10 to PL-15) Pacific white shrimp were obtained from Miami Aqua-Culture, Inc. (Boynton Beach, FL, USA). Shrimp were initially cultured in a 480-L recirculated seawater system containing 260 L of 25 parts per thousand (ppt) synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA), equipped with mechanical and biological filtration. The shrimp were fed a starter commercial diet followed by a larger feed (2.4 mm diameter, with a minimum crude protein content of 35% and maximum crude fiber of 4%) twice per day until they grew close to 1 g.

The shrimp feeding trial was carried out in six aquatic habitat systems (A-habb, Pentair, Minneapolis, MN, USA), each equipped with five 10-L tanks. Two hundred and forty shrimp were randomly selected from the 480 L tank and distributed evenly to the new systems with 30 tanks. Eight shrimp were allocated to each tank which provided one extra shrimp per tank in case of mortalities occurring due to transportation or acclimation stress. Shrimp were acclimated to the new system for 3 days and fed the same commercial feed mentioned above. The commercial diet was then replaced with the control diet at a gradually increasing ratio of 20%, 40%, 60%, 80% and 100% (w/w) in the following 5 days. During this eight-day acclimation period, shrimp were fed twice daily for a total feed rate of 8% (w/w).

After the acclimation period, shrimp numbers were adjusted to seven in each tank to start the feeding trial within a density of 107 shrimp/m^2. The initial weight of each shrimp for the feeding trial was 1.10 ± 0.06 g (mean ± standard deviation). Each dietary treatment consisted of six replicates (six tanks) in the six aquatic habitat systems. During the feeding trial, shrimp were fed manually three times a day at 08:30, 15:30 and 22:00. Shrimp were weighted on a tank basis every early Thursday morning to track growth and feed efficiency. The weight gain data was used to estimate the daily weight gain for the following week. The feeding rate was adjusted weekly based on shrimp weight and remained consistent across all treatment groups on the same day within the range of 5.5–8.5% (g dry feed per g wet body) based on the cumulative tank shrimp weight. Mortality was checked several times a day. Any moribund or dead shrimp were removed

from the tanks, and the feed weight was immediately adjusted based on the weight of shrimp remaining in the tank.

The water quality was monitored daily for dissolved oxygen and temperature, using the ProDO meter (Yellow Springs Instruments, OH, USA). The salinity of the systems was measured daily with a refractometer (WL0020-ATC, Agriculture Solutions LLC, ME, USA). Total ammonia-N, alkalinity as calcium carbonate, nitrite-N, nitrate-N, and pH were monitored three times per week (APHA, 2012). Sea salt and sodium bicarbonate were used to adjust salinity and alkalinity, respectively, during the feeding trial. During the shrimp feeding trial, the values of the water quality parameters were within desired ranges for Pacific white shrimp (Kuhn et al., 2010; Schuler et al., 2010; Van Wyk et al., 1999) (mean ± standard error): temperature (29.6 ± 0.0 °C), dissolved oxygen (6.2 ± 0.0 mg/L), salinity (25.2 ± 0.0 ppt), alkalinity (107.8 ± 1.3 mg $CaCO_3$/L), pH (7.9 ± 0.0), total ammonia-N (0.3 ± 0.0 mg/L), nitrite-N (0.1 ± 0.0 mg/L), nitrate-N (13.3 ± 0.5 mg/L) (**Table 4.3**).

Table 4.3 Water quality results for the six systems used during the feeding trial. Number of sampling events denoted by n [1].

System	Temperature	Dissolved oxygen (mg/L)	Salinity (ppt)	pH	Total ammonia-N (mg/L)	Nitrite-N (mg/L)	Nitrate-N (mg/L)	Alkalinity (mg/L, $CaCO_3$)
	n = 56	n = 56	n = 56	n = 24	n = 24	n = 24	n = 24	n = 24
1	29.6 ± 0.1	6.19 ± 0.02^{a}	25.3 ± 0.1	7.81 ± 0.02	0.22 ± 0.02^{b}	0.04 ± 0.01	13.84 ± 1.28	105 ± 3^{ab}
2	29.5 ± 0.1	6.17 ± 0.02^{ab}	25.1 ± 0.1	7.89 ± 0.02	0.23 ± 0.02^{b}	0.04 ± 0.01	13.01 ± 1.28	111 ± 3^{ab}
3	29.7 ± 0.1	6.18 ± 0.02^{ab}	24.9 ± 0.1	7.90 ± 0.02	0.26 ± 0.02^{ab}	0.04 ± 0.01	12.56 ± 1.28	105 ± 3^{ab}
4	29.5 ± 0.1	6.10 ± 0.02^{b}	25.2 ± 0.1	7.88 ± 0.02	0.25 ± 0.02^{ab}	0.07 ± 0.01	13.92 ± 1.28	113 ± 3^{a}
5	29.5 ± 0.1	6.20 ± 0.02^{a}	25.2 ± 0.1	7.88 ± 0.02	0.28 ± 0.02^{ab}	0.05 ± 0.01	12.40 ± 1.28	104 ± 3^{ab}
6	29.5± 0.1	6.22 ± 0.02^{a}	25.1 ± 0.1	7.85 ± 0.02	0.31 ± 0.02^{a}	0.04 ± 0.01	14.18 ± 1.28	100 ± 3^{b}

[1] Means ± standard error of six replicates groups of shrimp where values in the same column with different superscripts are significantly different ($p < 0.05$). Absence of superscripts indicates no significant difference between treatments.

4.2.4 Sample collection and biochemical analysis

At the end of the feeding trial, all shrimp were fasted for 24 h after the last feeding. Final survival rates and total weight of shrimp were recorded at the tank level. Shrimp from each tank were subsequently euthanized in an ice bath (< 4 ℃), then vacuum packaged separately and stored at -80 ℃. The frozen shrimp were freeze-dried and milled into powder. These 30 powder samples that, respectively, obtained from the surviving shrimp in 30 tanks were then submitted to composition analysis. Hence, six replicates of growth performance and composition analysis data were received for each diet treatment.

Proximate composition analysis of the diets and shrimp whole body was determined following the standard AOAC methods (AOAC, 2016). The moisture content was determined gravimetrically by oven-drying at 105 ℃ to constant weight. The freeze-dried shrimp were also dried in oven to determine moistures due to the incomplete moisture evaporation during freeze drying. Ash was determined by weight difference before and after the incineration of samples in a muffle furnace at 550 ℃ for 12 h. Crude protein was determined by following the Kjeldahl procedure, multiplying the total nitrogen by 6.25. The Foss TecatorTM Digestor and KjeltecTM 8100 Distillation Unit (FOSS North America, Eden Prairie, MN, USA) were used to determine the total nitrogen content. Crude fat was determined by Randall/Soxtec/Hexanes extraction-submersion method using petroleum ether as an extraction solvent, and FOSS 2055 Soxtec Avanti Manual System (FOSS North America, Eden Prairie, MN, USA) was used. Neutral detergent fiber (NDF) contents were quantified by using the ANKOM Filter Bag System (ANKOM 2000 automated fiber analyzer, ANKOM Technology, Macedon, NY, USA). High performance liquid chromatography (HPLC, Agilent Technologies, CA, USA) combined with modified performic acid oxidation was used to analyze amino acids. Minerals were determined using a modified method adopted from a previous study (Farzad et al., 2019). Briefly, 0.5 g dry diet or shrimp body sample was transferred to a polytetrafluoroethylene tube. Four milliliters of nitric acid (69% w/v), four milliliters of distilled water, and two milliliters of hydrogen peroxide (30% w/v) were added. The samples were then heated to a temperature of 180 ℃ and held at this temperature for 15 min using a microwave power of 800 W. After digestion, samples were diluted and sent to inductively coupled plasma mass spectrometry for mineral analysis (Agilent 7900 ICP-MS, Santa Clara, CA, USA). For the fatty acid determination, the lipids in diets were extracted using

chloroform/methanol (2:1, v/v) following the method of Folch et al. (1957). The extracted lipids were transformed to fatty acid methyl esters (AOCS method Ce 1b-89, 1989), which were analyzed using gas chromatograph mass spectrometer (QP2010, Shimadzu, Kyoto, Japan), equipped with column ZB_WAXplus (60m × 0.25 mm, thickness 0.25 mm), a FID detector, and an auto injector AOC-20i. The injection mode was split. The carrier gas was helium. The temperature program included a gradient from 175 to 225 ℃ with an increase rate of 2.0 ℃/min.

4.2.5 Shrimp performance indicators

The shrimp growth performance was analyzed using the following equations:

$$\text{Percent weight gain (PWG\%)} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} \times 100 \quad \text{4-1}$$

$$\text{Specific growth rate (SGR, g/day)} = \frac{(\ln(final\ weight) - \ln(initial\ weight))}{feeding\ trial\ \ period\ (56\ days)} \times 100 \quad \text{4-2}$$

$$\text{Feed conversion ratio (FCR)} = \frac{feed\ consumed\ (g, dry\ weight)}{live\ weight\ gain\ (g, wet\ weight)} \quad \text{4-3}$$

$$\text{Survival (\%)} = \frac{final\ number\ of\ shrimp}{initial\ number\ of\ shrimp} \times 100 \quad \text{4-4}$$

$$\text{Protein efficiency ratio (PER)} = \frac{weight\ gain\ (g, wet\ weight)}{protein\ intake\ (g, dry\ weight)} \quad \text{4-5}$$

4.2.6 Analysis of data

All data were presented as means ± standard error unless specifically pointed out. The effects of dietary treatment on shrimp growth performance and shrimp whole-body composition were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey's honestly significant difference (HSD) to identify differences among experimental groups. ANOVA was also applied to test the differences in water quality in the six aquatic systems. The significance level was chosen at 0.05. The Statistics software JMP Pro14® (SAS, Cary, NC, USA) was used for the analyses in this study.

4.3 Results and discussion

4.3.1 The formulation of shrimp diets

Shrimp growth can be affected by many factors, such as water quality, feeding management, feed formulation, and manufacture. In this study, shrimp diets were formulated with an emphasis on maintaining similar levels of protein, crude fat, and calories. Additionally, a selected number of amino acids and minerals were also adjusted for equivalence. The proximate compositions of diets are shown in **Tables 4.4**. Gross energy was estimated based on the feed composition according to previous studies (Bulbul et al., 2015; Kuhn et al., 2016).

Table 4.4 Proximate nutrients, essential amino acids, trace element levels and determined energy of five practical diets (dry-weight basis)[1]

Parameters	PP0	PP10	PP30	PP50	PP70
Proximate nutrients (g/100 g dry diet)					
Crude protein	43.36 ± 0.24	44.06 ± 0.24	43.74 ± 0.24	43.55 ± 0.24	44.01± 0.24
Crude fat	9.55 ± 0.07^{b}	9.58 ± 0.07^{b}	9.58 ± 0.07^{b}	9.59 ± 0.07^{ab}	9.98 ± 0.07^{a}
Total ash	12.13 ± 0.10^{b}	12.92 ± 0.10^{a}	12.65 ± 0.10^{ab}	12.50 ± 0.10^{ab}	12.26 ± 0.10^{b}
NDF[2]	4.84 ± 0.14^{a}	4.19 ± 0.14^{ab}	3.92 ± 0.14^{b}	3.95 ± 0.14^{b}	3.41 ± 0.14^{b}
Carbohydrate[3]	34.97	33.45	34.03	34.37	33.76
Calcium	3.02 ± 0.38	3.19 ± 0.38	3.11 ± 0.38	2.56 ± 0.38	2.39 ± 0.38
Phosphorus	2.08 ± 0.21	1.88 ± 0.21	1.71 ± 0.21	1.27 ± 0.21	1.08 ± 0.21
Potassium	2.80 ± 0.25	2.30 ± 0.25	2.68 ± 0.25	2.15 ± 0.25	2.19 ± 0.25
Magnesium	0.25 ± 0.03	0.24 ± 0.03	0.29 ± 0.03	0.26 ± 0.03	0.29 ± 0.03
Sodium	0.59 ± 0.07	0.56 ± 0.07	0.81 ± 0.07	0.76 ± 0.07	0.93 ± 0.07
Essential amino acid levels (g/100g dry diet)					
Leucine	2.91 ± 0.03^{c}	3.02 ± 0.03^{bc}	3.06 ± 0.03^{abc}	3.10 ± 0.03^{ab}	3.18 ± 0.03^{a}
Valine	2.02 ± 0.02^{c}	2.11 ± 0.02^{bc}	2.16 ± 0.02^{ab}	2.21 ± 0.02^{ab}	2.27 ± 0.02^{a}
Threonine	1.57 ± 0.01	1.60 ± 0.01	1.58 ± 0.01	1.56 ± 0.01	1.58 ± 0.01
Isoleucine	1.78 ± 0.02^{b}	1.86 ± 0.02^{ab}	1.88 ± 0.02^{ab}	1.90 ± 0.02^{ab}	1.93 ± 0.02^{a}
Arginine	2.70 ± 0.02^{ab}	2.74 ± 0.02^{a}	2.69 ± 0.02^{ab}	2.63 ± 0.02^{ab}	2.60 ± 0.02^{b}
Phenylalanine	1.81 ± 0.02^{e}	1.91 ± 0.02^{d}	2.02 ± 0.02^{c}	2.12 ± 0.02^{b}	2.24 ± 0.02^{a}
Lysine	2.94 ± 0.04^{a}	2.95 ± 0.04^{a}	2.78 ± 0.04^{ab}	2.61 ± 0.04^{bc}	2.46 ± 0.04^{c}
Methionine	0.98 ± 0.03	1.04 ± 0.03	0.98 ± 0.03	0.96 ± 0.03	1.01 ± 0.03
Histidine	1.02 ± 0.01^{ab}	1.05 ± 0.01^{a}	1.02 ± 0.01^{ab}	1.00 ± 0.01^{b}	0.98 ± 0.01^{b}
Tryptophan	0.47 ± 0.01	0.50 ± 0.01	0.51 ± 0.01	0.50 ± 0.01	0.52 ± 0.01
Trace element levels (mg/kg dry diet)					
Copper	45.00 ± 6.51	41.50 ± 6.51	55.50 ± 6.51	39.00 ± 6.51	43.50 ± 6.51
Zinc	111.00 ± 9.74	98.50 ± 9.74	111.50 ± 9.74	93.50 ± 9.74	104.00 ± 9.74
Manganese	65.50 ± 6.80	62.50 ± 6.80	71.50 ± 6.80	59.50 ± 6.80	70.00 ± 6.80
Selenium	2.76 ± 0.25	2.05 ± 0.25	2.00 ± 0.25	1.65 ± 0.25	1.59 ± 0.25
The ratio of fishmeal protein to feed protein (%)					

	57	51	40	28	17
Gross energy (kJ/g dry diet)[4]					
	20.02	19.93	19.96	19.98	20.13

[1] All data are presented based on dry weight of diets. Values are means ± standard error from duplicate. Values in the same row with different superscripts are significantly different ($p < 0.05$). Absence of superscripts indicates no significant different between treatments.

[2] NDF: Neutral detergent fiber

[3] Calculated by difference (Merrill and Watt, 1973): total dry matter- (crude protein + total fat+ ash). The values of crude protein, total fat and ash were mean value.

[4] Calculation based on combustion values of protein, lipid and carbohydrate with 23.6, 39.5 and 17.2 kJ/g, respectively (Blaxter, 1989).

The levels of the essential amino acids including lysine, arginine, methionine and histidine in PP were 20% lower than those in fishmeal. Thus, it is challenging to formulate cost-effective feeds that meet all essential amino acid requirements. Three approaches are commonly used to deal with the deficiency in amino acids: (a) increasing the total protein of diet, (b) addition of crystalline amino acids, and (c) combined use of protein sources with innately different amino acid profiles (NRC, 2011). Through the combined use of other ingredients including soybean meal, corn starch, krill meal, and addition of a small amount of DL-methionine (0.1–0.19 g per 100 g dry diet), the contents of the four aforementioned essential amino acids in our formulated diets were in a safe range for shrimp growth. For shrimp diets containing 35–45% crude protein, the recommended contents of lysine, methionine, histidine and arginine in dry diets are 1.6–2.4%, 0.9 –1.4%, 0.8–1.0% and 1.9–2.6%, respectively (Akiyama, 1992; Fox et al., 1995; Millamena et al., 1998; Millamena et al., 1999; Nunes et al., 2014; Van Wyk et al., 1999; Xie et al., 2012; Zhou et al., 2012). Besides amino acids, minerals such as potassium and calcium were supplemented during the feed formulation because of their vital role in shrimp growth and metabolism, and the differences in their levels between PP and fishmeal were significant ($p < 0.05$). Since this study focused on the fishmeal protein replacement, the difference in fatty acids profile between PP and fishmeal was not considered. However, this could be a target in future studies.

In addition to nutritional requirements on shrimp diets, sensorial attraction is another important factor to consider. Shrimp rely on chemosensory systems to identify, locate, and ingest food (Derby and Sorensen, 2008; Lee and Meyers, 1996). “Unattractive” diets would affect feed intake and shrimp growth performance (Alvarez et al., 2007; Bulbul et al., 2013). In this study,

shrimp did not refuse test diets based on observation. It might be due to the addition of krill meal, which can help increase the attraction of diets. Krill meal is easy to be recognized by shrimp due to its chemical compounds including free amino acids, nucleotides, amines and nucleosides, thus it could improve the attractiveness of diets (Derby et al., 2016; Suresh et al., 2011).

The lipid levels in the five formulated diets were less 10%, which were within the recommended level in shrimp feed (Gonzalez-Felix et al., 2002). However, the fatty acids of the diets were not balanced in this study (**Table 4.5**). The increasing level of PP in the diets resulted in decreased amount of some fatty acids, such as C16:1n7, C18:0, C18:1n7, C18:4n3, C20:5n4, and C20:6n3. The results are in correspondence with those reported on fishmeal replacement with rice protein in white shrimp diets (Oujifard et al., 2012).

Table 4.5 Fatty acids composition of five practical diets (dry-weight basis)[1].

Parameters	Diet groups				
	PP0	PP10	PP30	PP50	PP70
C14:0	7.86 ± 1.32	7.97 ± 1.32	7.31 ± 1.32	7.48 ± 1.32	6.49 ± 1.32
C16:0	12.02 ± 0.81	9.71 ± 0.81	8.75 ± 0.81	9.29 ± 0.81	10.91 ± 0.81
C16:1n7	11.65 ± 0.18^{a}	11.00 ± 0.18^{a}	9.43 ± 0.18^{b}	8.00 ± 0.18^{c}	6.72 ± 0.18^{d}
C18:0	5.40 ± 0.09^{a}	5.29 ± 0.09^{a}	4.95 ± 0.09^{ab}	4.55 ± 0.09^{bc}	4.17 ± 0.09^{c}
C18:1n9	12.36 ± 0.20^{c}	12.71 ± 0.20^{bc}	13.23 ± 0.20^{abc}	13.55 ± 0.20^{ab}	13.91 ± 0.20^{a}
C18:1n7	5.31 ± 0.11^{a}	5.13 ± 0.11^{ab}	4.69 ± 0.11^{bc}	4.20 ± 0.11^{cd}	3.73 ± 0.11^{d}
C18:2n6	15.62 ± 0.25^{e}	19.18 ± 0.25^{d}	25.41 ± 0.25^{c}	30.13 ± 0.25^{b}	34.69 ± 0.25^{a}
C18:3n3	2.69 ± 0.09^{c}	3.15 ± 0.09^{c}	4.02 ± 0.09^{b}	4.69 ± 0.09^{a}	5.16 ± 0.09^{a}
C18:4n3	2.43 ± 0.10^{a}	2.38 ± 0.10^{a}	2.10 ± 0.10^{ab}	1.75 ± 0.10^{bc}	1.41 ± 0.10^{c}
C20:5n3 (EPA)[2]	13.79 ± 0.33^{a}	13.18 ± 0.33^{ab}	11.48 ± 0.33^{bc}	9.62 ± 0.33^{cd}	7.84 ± 0.33^{d}
C22:6n3 (DHA)[3]	10.87 ± 0.35^{a}	10.30 ± 0.35^{ab}	8.63 ± 0.35^{bc}	6.74 ± 0.35^{cd}	4.95 ± 0.35^{d}

[1] Fatty acid values (percentages of total fatty acid methyl esters) were adjusted to express a percent of total area identified in the chromatograms, and unidentified peaks were not considered in the computations; Values are least squares means ± standard error from duplicate groups. Values in the same row with different superscripts are significantly different ($p < 0.05$). Absence of superscripts indicates no significant different between treatments.

[2] EPA: eicosapentaenoic acid.

[3] DHA: docosahexaenoic acid

4.3.2 Shrimp growth performance and feed utilization efficiency

Table 4.6 shows the final weight, weight gain, SGR, FCR, survival, and PER of shrimp fed diets with different levels of fishmeal protein replaced with PP protein after the eight-week feeding trial. There were no significant differences in survival (76.2–94.3%), PER (1.2–1.3) or FCR (1.8–1.9) among dietary treatments (PP0, PP10, PP30, PP50 and PP70). However, when 70% of fishmeal protein was replaced by PP protein in shrimp diet (PP70), the PWG and SGR of shrimp were significantly lower than that in the control group ($p < 0.05$). To better monitor the effect of increased PP in shrimp diet on feed utilization, the FCR changes within and between dietary groups in each week were compared (**Figure 4.1**). In the initial 2 weeks, the FCR increased and fluctuated among the test dietary groups comparing with the control group. There were no significant differences in FCR among dietary groups in week 3 to 8, except for week 7.

Table 4.6 Growth performance and feed utilization of shrimp fed different diets after an 8-week feeding trial [1].

Parameters	Diet groups				
	PP0	PP10	PP30	PP50	PP70
Initial weight (g)	1.10 ± 0.03	1.10 ± 0.03	1.08 ± 0.03	1.08 ± 0.03	1.13 ± 0.03
Final weight (g)	10.80 ± 0.37^{a}	10.21 ± 0.41^{ab}	8.87 ± 0.37^{bc}	9.12 ± 0.37^{bc}	8.57 ± 0.37^{c}
PWG (%)[2]	892.08 ± 37.27^{a}	829.35 ± 40.83^{ab}	721.91 ± 37.27^{bc}	741.76 ± 37.27^{abc}	655.42 ± 37.27^{c}
SGR[3]	4.08 ± 0.08^{a}	3.98 ± 0.09^{ab}	3.76 ± 0.08^{bc}	3.79 ± 0.08^{abc}	3.60 ± 0.08^{c}
FCR[4]	1.81 ± 0.06	1.76 ± 0.06	1.84 ± 0.06	1.83 ± 0.06	1.94 ± 0.06
Survival	76.19 ± 5.22	94.28 ± 5.71	85.71 ± 5.22	76.19 ± 5.22	80.95 ± 5.22
PER[5]	1.28 ± 0.04	1.29 ± 0.04	1.24 ± 0.04	1.26 ± 0.04	1.17 ± 0.04

[1] Values are means ± standard error. Means in each row with different letters are significantly different ($p < 0.05$). Absence of letters indicates no significant different between treatments.
[2] PWG: percent weight gain.
[3] SGR: specific growth rate, g day^{-1}.
[4] FCR: feed conversion ratio.
[5] PER: protein efficiency ratio.

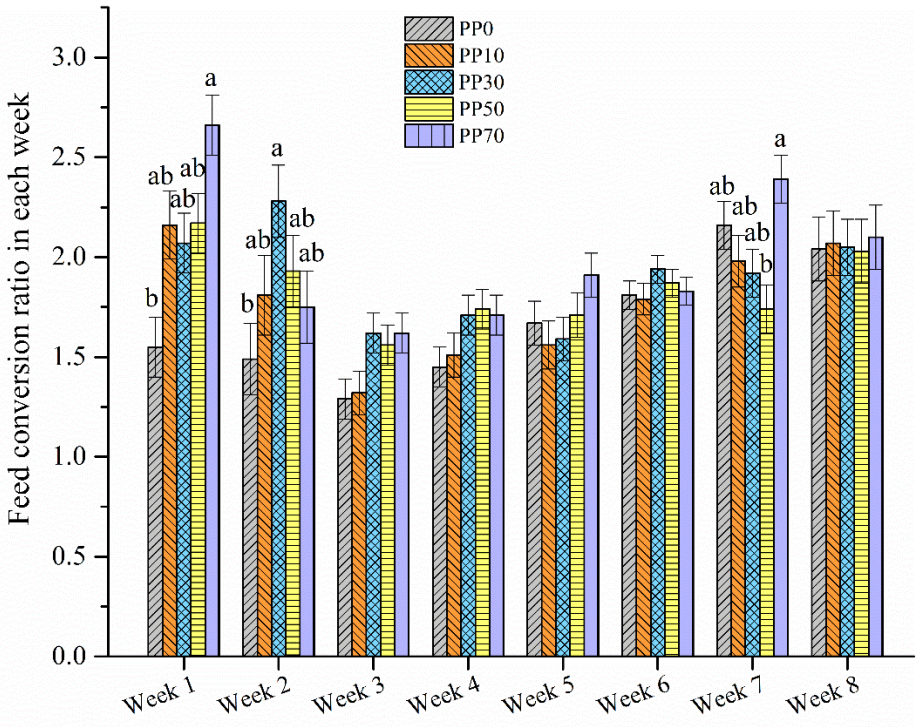

Figure 4.1 Feed conversion ratios of shrimp fed different PP inclusion rates during the 8-week feeding trial. Values are presented as means ± standard error (n=6). Same letter within the same week indicates no significance (p < 0.05). Absence of letter indicates no significant difference exist.

The shrimp survival and feed utilization indicators, including FCR, survival, and PER, were not affected when as much as 70% of fishmeal was replaced with PP in shrimp diets (**Table 4.6**). One reason might be the low fiber content and the high protein content of PP (**Table 4.1**). Because fiber is not digestible by fish or shrimp, adding high-fiber ingredient to aquaculture diets increases fecal losses, thus negatively impacting feed utilization efficiency (Naylor et al., 2009). Unlike raw BSG which has 50–70% of fiber, the upgraded PP (from BSG) has less than 1% fiber (NDF) on a dry basis; therefore, the negative effect of the fiber in PP on feed digestibility is minimal. The high level of fishmeal replacement with PP could also be attributed to the balanced amino acid profiles obtained by supplementing lysine and methionine as well as the low antinutritional compounds in PP. The lysine in PP protein (4.04%, w/w) was 45% lower (relative basis) than in fishmeal protein (7.29%, w/w); however, this deficit was eliminated by mixing the soybean meal and krill meal in diets, which adjusted the final lysine content in the diets to a range of 2.4–3.0% (w/w). A direct addition of synthetic methionine (0.1–0.2%, w/w) into diets offset lower levels of methionine in PP. Moreover, unlike some plant-based proteins such as canola meal and soymeal, which contain certain levels of antinutrients (Gatlin et al., 2007), the PP produced

from BSG (originally from barley), contains insignificant levels of antinutrients to fish and shrimp. Moreover, the small molecular size of PP protein could also be a contributor to the high-level replacement of fishmeal.

Although the FCR, PER, and survival were not affected, we observed the differences in shrimp growth performance among different groups. The shrimp fed PP50 did not show significantly different SGR compared to the control group (PP0); however, the shrimp fed PP70 showed a significantly lower SGR compared to the control group. The lower SGR was likely due to the delayed adaptation to PP diets at the beginning of the trial, especially during the first week. The high levels of plant protein could cause a change in the texture of diets (Alceste, 2000). The peptides and free amino acids in PP may also affect the palatability (Song et al., 2014). Thus, the shrimp had to adjust to the new diets with the different smell and taste, and change their metabolism functions to more efficiently digest the new diet (Aksnes et al., 2006). As mentioned in the methodology section (subsection 4.2.3), before starting the test diets, shrimp were fed increasing levels of the control diet (PP0) to replace the commercial diet. Therefore, shrimp that received the control diet (PP0) experienced a likely advantageous acclimation prior to the trial initiation and hence grew faster than shrimps in the other four dietary groups during the week one. In this study, the amount of feed assigned to each group was calculated based on the previous week's shrimp weight in that group, thus the treatment group that grew faster in week one received a higher amount of feed than the other groups. This exaggerated the weight gain difference among different treatments. Therefore, although FCR was similar among groups (**Figure 4.1**), the SGR of the shrimp fed PP70 was lower. Besides, the shrimp fed PP70 also had the lowest final weight and PWG among all groups (**Figure 4.2** and **Table 4.6**). In the future, it might be better to feed all shrimp groups with the same commercial feed prior to trial initiation, in order to eliminate the growth difference caused by this acclimation issue. Penaeid shrimp have limited ability to synthesize highly unsaturated fatty acids such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA). The decreased weight gains in shrimp fed PP70 might be also due to the imbalanced fatty acids and lower content of EPA and DHA in the diet compared with PP0. It was reported that dietary fatty acids affected the growth and immune system of white shrimp (Zhang et al., 2014).

In a summary, our results showed that 70% replacement of fishmeal protein with PP protein was not appropriate in shrimp feed, as well as 50% of replacement of fishmeal protein with PP

protein was able to maintain the growth (SGR), feed utilization efficiency (FCR and PER), and survival of shrimp.

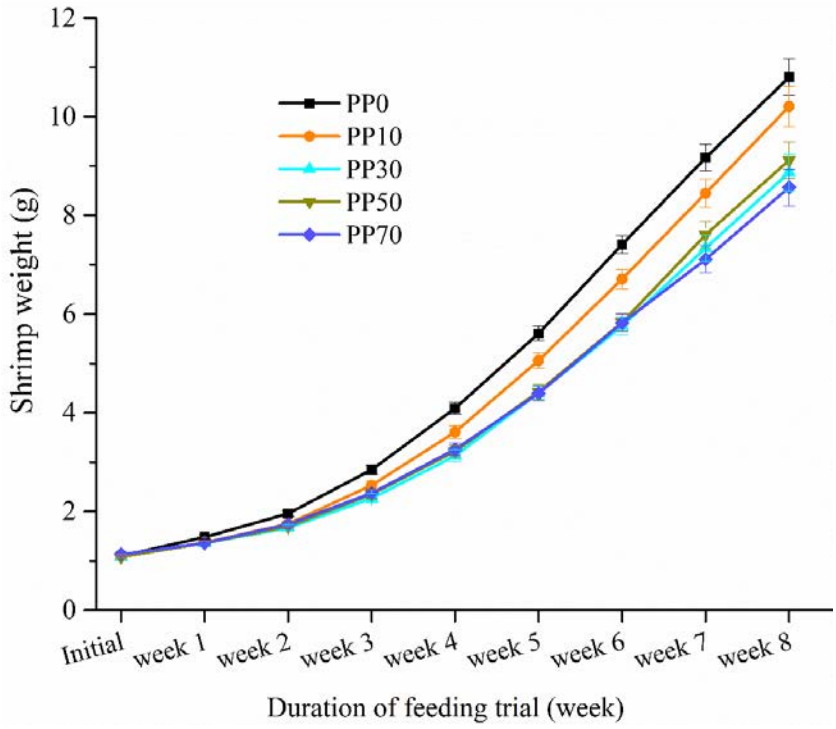

Figure 4.2 Shrimp weight per week during the 8-week feeding trial. Values are presented as means ± standard error (n=6).

4.3.3 Whole-body proximate composition of shrimp

There was no significant difference in the dry matter content of the shrimp whole body (23.85 ± 0.16%) among dietary groups. The whole-body proximate composition of shrimp at the end of the feeding trial is summarized in **Table 4.7**. The protein contents of the shrimp whole body increased with incremental additions of PP in diets. The shrimp in diet group PP70 had a higher crude protein content compared with the shrimp fed control diet (PP0), but the difference is < 2%. PP was produced by the wet fractionation process, where BSG was subjected to the enzymatic hydrolysis to break the peptide bonds of proteins (He et al., 2019; Niemi et al., 2013; Yu et al., 2019). It was reported that the ingestion of protein hydrolysates with small molecular size led to the increase of skeletal muscle protein synthesis (Koopman et al., 2009). On the other hand, the fat content of shrimp decreased successively with the incremental addition of PP in diets, the crude fat content of the shrimp body decreased from 6.42% to 5.46%. This result is in agreement with previous studies on fishmeal replacement with plant protein (Kissil et al., 2000; Robaina et al.,

1998). Robaina et al. (1998) found that the total lipid content in sea bream decreased when 30% of soy protein was incorporated in diets. Kissil et al. (2000) also reported that a decrease in fat content in whole-body fish when fishmeal in diet was replaced by soy and rapeseed protein concentrates in an 8-week feeding trial. Our results together with previous studies indicate that the inclusion of some types of plant proteins in diets could affect lipid digestion and accumulation in fish/shrimp. This could be due to several factors, such as the presence of embedded compounds (e.g. fibers) that reduce lipid digestion and the physicochemical properties of lipids themselves in plant protein ingredients. Some lipids are easily digested by the lipases in shrimp digestive systems, while some are not (Gunasekera et al., 2002).

Table 4.7 Whole-body nutrition and mineral contents of shrimp fed different diets after an 8-week feeding trial (dry weight basis)[1].

Parameters	Diet groups				
	PP0	PP10	PP30	PP50	PP70
Proximate nutrients (g/100 g)					
Crude protein	73.61 ± 0.33^{b}	74.45 ± 0.36^{ab}	74.41 ± 0.33^{ab}	74.94 ± 0.33^{ab}	75.06 ± 0.33^{a}
Crude fat	6.42 ± 0.11^{a}	6.07 ± 0.12^{ab}	5.61 ± 0.11^{bc}	5.59 ± 0.11^{c}	5.46 ± 0.11^{c}
Total ash	11.94 ± 0.31^{b}	12.21 ± 0.34^{b}	12.51 ± 0.31^{ab}	13.60 ± 0.31^{a}	13.04 ± 0.31^{ab}
Macro mineral contents (g/kg)					
Sodium	8.91 ± 0.20^{b}	9.46 ± 0.21^{ab}	9.70 ± 0.20^{ab}	10.15 ± 0.20^{a}	10.06 ± 0.20^{a}
Calcium	26.88 ± 1.36	29.09 ± 1.49	27.13 ± 1.36	31.16 ± 1.36	32.18 ± 1.36
Phosphorus	9.47 ± 0.09^{ab}	9.63 ± 0.10^{a}	9.61 ± 0.09^{a}	9.47 ± 0.09^{ab}	9.13 ± 0.09^{b}
Potassium	12.17 ± 0.16^{b}	12.66 ± 0.18^{ab}	12.77 ± 0.16^{ab}	12.88 ± 0.16^{a}	12.80 ± 0.16^{ab}
Magnesium	2.40 ± 0.08^{b}	2.61 ± 0.08^{ab}	2.50 ± 0.07^{ab}	2.73 ± 0.07^{a}	2.77 ± 0.07^{a}
Micro mineral contents (mg/kg)					
Iron	11.75 ± 0.59	12.35 ± 0.64	13.23 ± 0.59	14.10 ± 0.64	14.20 ± 0.59
Zinc	62.41 ± 0.76^{c}	63.50 ± 0.83^{bc}	64.62 ± 0.76^{abc}	67.57 ± 0.76^{a}	66.62 ± 0.76^{ab}
Copper	64.86 ± 1.92^{b}	67.34 ± 2.10^{b}	68.89 ± 1.20^{b}	79.50 ± 1.92^{a}	82.55 ± 1.92^{a}
Manganese	1.14 ± 0.04^{c}	1.26 ± 0.04^{bc}	1.36 ± 0.04^{ab}	1.50 ± 0.04^{a}	1.45 ± 0.04^{a}

[1] Values are least squares means ± standard error from six replicates groups. Values in the same row with different superscripts are significantly different ($p < 0.05$). Absence of superscripts indicates no significant different between treatments.

Regarding the mineral contents, high levels of PP in diets resulted in sodium accumulation in shrimp bodies. The shrimp fed PP50 and PP70 diets had significantly higher contents of sodium, magnesium, zinc, copper, and manganese compared with the shrimp fed the control diet. However, no difference in the contents of calcium and iron was observed among the dietary groups. Based on previous studies (Ikram et al., 2017; Mussatto et al, 2006), phosphorus, calcium and magnesium are the most abundant minerals in raw BSG, whereas, in PP, sodium was the predominant mineral, probably due to the addition of sodium hydroxide for adjusting pH to 8.0 for enzymatic hydrolysis during the PP production from BSG. The sodium in PP was almost double that of fishmeal (**Table 4.1**). Thus, the sodium content in the shrimp body increased in response to the increasing level of PP in diets. However, *Litopenaeus vannamei* are euryhaline, tolerating salinities ranging from 1 g/L to over 40 g/L (Bray et al., 1994; Van Wyk et al., 1999). Shrimp cultured in lower salinity water will benefit physiologically from receiving higher dietary sodium.

One concern about the fishmeal replacement with plant proteins is a reduction in trace minerals. Some minerals, such as magnesium, copper, zinc and manganese, are vital for shrimp growth, metabolism, and health (Bharadwaj et al., 2014; Katya et al., 2016), but that too high of a concentration can be detrimental to shrimp health (Aruna and Felix, 2017; Kuhn et al., 2017; Muralisankar et al., 2014; Zhou et al., 2017). PP had higher contents of magnesium, copper and manganese than fishmeal and the reverse was true for Zinc. However, there were no differences in these minerals in the diets as shown in **Table 4.4**. This was probably because the other ingredients, such as corn starch, contributed to the balance of these minerals in the formulated feeds. Yet, the higher contents of magnesium, copper, zinc and manganese in shrimp fed higher levels of PP were observed (**Table 4.7**), indicating that incorporation of PP in diets could enhance mineral absorption. It should be noted that the minerals contents in all shrimp from this study are lower than the values observed in intensively cultured shrimp (Wu & Yang, 2011).

The effect of the different diets on the amino acid profiles of shrimp whole body is shown in **Table 4.8**. There were no significant differences in the threonine, isoleucine or methionine levels between the shrimp fed the PP diets and the shrimp fed the control diet. Furthermore, the amounts of these three essential amino acids in the shrimp fed PP50 were slightly lower than those in the shrimp fed other diets. However, we did not observe any difference in the remaining essential amino acids or non-essential amino acids between any of the dietary groups.

Table 4.8 Whole-body amino acids contents of shrimp fed different diets after an 8-week feeding trial (g/100g dry weight basis)[1].

Parameters	Diet groups				
	PP0	PP10	PP30	PP50	PP70
Essential amino acid					
Leucine	7.86 ± 0.30	7.97 ± 0.32	7.43 ± 0.30	7.05 ± 0.30	7.63 ± 0.30
Valine	0.41 ± 0.05	0.57 ± 0.06	0.41 ± 0.05	0.43 ± 0.05	0.45 ± 0.05
Threonine	2.54 ± 0.10^{ab}	2.62 ± 0.11^{ab}	2.35 ± 0.10^{ab}	2.20 ± 0.10^{b}	2.66 ± 0.10^{a}
Isoleucine	2.45 ± 0.11^{ab}	2.83 ± 0.12^{a}	2.65 ± 0.11^{ab}	2.26 ± 0.11^{b}	2.86 ± 0.11^{a}
Arginine	8.24 ± 0.35	8.59 ± 0.38	7.80 ± 0.35	7.26 ± 0.35	8.28 ± 0.35
Phenylalanine	3.45 ± 0.14	3.67 ± 0.15	3.33 ± 0.14	3.16 ± 0.14	3.67 ± 0.14
Lysine	4.38 ± 0.38	4.56 ± 0.42	4.14 ± 0.38	3.46 ± 0.38	3.96 ± 0.38
Methionine	4.13 ± 0.20^{ab}	4.54 ± 0.21^{ab}	4.26 ± 0.20^{ab}	3.74 ± 0.20^{b}	4.72 ± 0.20^{a}
Histidine	1.49 ± 0.06	1.60 ± 0.06	1.46 ± 0.06	1.37 ± 0.06	1.59 ± 0.06
Non-essential amino acid					
Glutamic acid	10.11 ± 0.43	10.81 ± 0.47	9.69 ± 0.43	9.42 ± 0.43	10.61 ± 0.43
Aspartic acid	9.21 ± 0.38	9.80 ± 0.41	8.82 ± 0.38	8.41 ± 0.38	9.44 ± 0.38
Alanine	4.86 ± 0.20	5.23 ± 0.21	4.68 ± 0.20	4.45 ± 0.20	4.92 ± 0.20
Glycine	5.45 ± 0.26	6.09 ± 0.28	5.70 ± 0.26	5.28 ± 0.26	6.17 ± 0.26
Serine	2.78 ± 0.12	2.86 ± 0.13	2.53 ± 0.12	2.42 ± 0.12	2.77 ± 0.12
Cysteine	1.73 ± 0.11	1.61 ± 0.12	1.68 ± 0.11	1.57 ± 0.11	1.82 ± 0.11

[1] Values are least squares means ± standard error from six replicates groups. Values in the same row with different superscripts are significantly different ($p < 0.05$). Absence of superscripts indicates no significant different between treatments.

There are many dietary factors affecting the shrimp growth, such as digestibility, enzymatic activity, antinutritional factors, low palatability and indigestible carbohydrates, imbalanced fatty acid profiles or amino acid profiles. In this study, we mainly focused on the effect of protein and amino acid profiles on shrimp growth performance and survivals. It is the first study to use the protein-rich product recovered from BSG to replace fishmeal in shrimp diet, with 50% replacement without affecting shrimp growth and feed utilization efficiency. However, this study is not without

limitations. First, as mentioned before, the shrimp of group diet PP70 showed a significant lower specific growth rate and weight gain, which could be due to the acclimation issue. Therefore, if the shrimp was fed the same commercial feed (instead of control feed with high fishmeal) before trial initiation to eliminate the acclimation issue, 70% replacement of fishmeal protein with PP might be achievable. Second, due to the small tank volume (10 L), only seven shrimps were raised in each tank to have a reasonable shrimp density during the feeding trial. The small number of shrimps in each tank might be the reason for the high standard deviation on shrimp survival even with six replicates for each treatment. In the future, larger tanks could be used to have more shrimps in each tank to reduce the deviations on shrimp survival and other parameters. Third, with the increasing amount of PP in diets, unbalanced fatty acids profiles among diets were observed. Although the effect of unbalanced fatty acids in diets on shrimp growth was not the focus of our research, it would be interesting to investigate this effect by measuring the fatty acid composition of feeding diets and harvested shrimps. Last but not least, the negative impacts of replacement of fishmeal with PP might not have been shown during the 8-week feeding trial. In the future, it will be beneficial to extend the feeding trial duration until shrimps grow to commercial size, in order to have more comprehensive results.

4.4 Conclusions

In summary, up to 50% of fishmeal was replaced by PP in shrimp diets without compromising shrimp survival, growth performance, and feed utilization during an 8-week feeding trial. In the whole-body shrimp composition analysis, the shrimp fed with the highest PP diet (PP70) had higher contents of crude protein, magnesium, zinc, copper, and manganese compared with the shrimp fed with the control diet. The amino acid composition of shrimp protein was not affected by the replacement of fishmeal with PP in diets. To our knowledge, this is the first study to evaluate protein-rich products recovered from BSG with wet fractionation process as an alternative to fishmeal in shrimp diets. The outcomes of this study could provide a low-cost and sustainable protein source to the aquaculture industry, meanwhile, it could create a new way for the brewing industry to manage the waste materials. In the future, detailed techno-economic analysis and enterprise budgets will be addressed to comprehensively evaluate the economic feasibility of PP production from BSG for fishmeal replacement in the shrimp industry.

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Chapter 5. Protein production from brewer's spent grain via wet fractionation: process optimization and techno-economic analysis

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ABSTRACT

Brewer's spent grain (BSG) is the major byproduct generated by the brewing industry. It has 50–70% fiber and 14–30% protein contents. This study investigates the technical and economic performances for producing protein-rich product (PP) from BSG using enzyme-assisted fractionation process. This was done through process optimization, scale-up verification, and techno-economic analysis (TEA). The experiment was conducted with varying enzyme (Alcalase) loadings and enzymatic hydrolysis times. The results showed that the optimal condition was using Alcalase loading of 5 μL/g with hydrolysis time of 1 h for achieving a high protein concentration (46%) in PP and protein separation efficiency (80%). Using the optimal condition, the scaled-up process resulted in a consistent PP composition and protein separation efficiency. The experimental conditions and results were input into process simulation model for determining the mass and energy flows, from which TEA is derived. For a processing plant with a capacity of 590 t wet BSG per day, the minimum selling price of PP (MSPP) to achieve a 5% return was determined to be 1044 USD/t. Sensitivity analysis revealed that Alcalase and BSG costs have the

most effect on the MSPP. Besides, protein separation efficiency is an important processing parameter in determining the MSPP.

5.1 Introduction

In beer making, brewer's spent grain (BSG) is generated as a solid residue after wort production. Approximately 20 kg of wet BSG is generated for every 100 L of beer produced (Mussatto et al., 2006). BSG is the major byproduct of the beer industry, representing about 85% of the total byproducts generated (Mussatto, 2014). The USA is the second-largest beer producer globally, with an annual BSG production of 4.5 Mt in the past decade (TTB, 2019). The top fifteen breweries in the USA produce an average of 0.2 Mt wet BSG per brewery per year (TTB, 2019). BSG comprised primarily of husks, bran, and endosperm residue of barley and other grains used in making beer. It is a lignocellulosic material, with an abundant fiber component containing cellulose (12–25%), hemicellulose (20–35%) and lignin (7–28%) (Aliyu and Bala, 2011; Wen et al., 2019). The second-largest component of BSG is proteins that account for 14–30% of dry weight. Besides fiber and protein, fat, minerals (especially silicon, phosphorus, and calcium) and vitamins are also found in BSG (Mussatto et al., 2006).

Traditionally, breweries sell or donate wet BSG to local farmers as feed for animals, which not only benefits agriculture but also keeps BSG out of the waste stream. However, BSG is easily spoiled due to its nutrients and high moisture content (70–80%), leading to a short storage time in warm weather, potent smell, and microbial contamination. Attempts have been conducted to exploit the alternative use of BSG in producing functional foods and nutritional supplements, such as oligosaccharides (Carvalheiro et al., 2004b), xylitol (Carvalheiro et al., 2005), lactic acid (Mussatto et al., 2007), functional ingredient for breadsticks (Ktenioudaki et al., 2012), polyphenols and flavonoids (Spinelli et al., 2016). Moreover, BSG is used as feedstock for bioenergy production, including biogas (Cater et al., 2015), ethanol (Liguori et al., 2015), biohydrogen (Zhang and Zang, 2016) and biobutanol (Plaza et al., 2017), or used as biosorbent material (Lu and Gibb, 2008), active carbon (Mussatto et al., 2010), enzyme carrier (Pospiskova and Safarik, 2013). The use of extracted protein from BSG as an antimicrobial and antioxidative packaging material for foods has also been reported (Lee et al., 2015).

Recently, a wet fractionation process to separate proteins and fibers in BSG to produce protein-rich and fiber-rich products at a lab-scale was developed (He et al., 2019). The

fractionation of proteins and fibers from BSG with sodium hydroxide, sodium bisulfide, and enzyme (Alcalase) was investigated. The results showed that Alcalase had the best performance in terms of protein separation efficiency and protein concentration in the protein-rich product (PP) among all three agents. Moving forward, because of the need of sustainable development of the aquaculture industry, the produced PP was used as an alternative to fishmeal protein, the main and most expensive protein source in aquafeeds (He et al., 2020). The results showed that PP was able to replace up to 50% of fishmeal in the Pacific white shrimp diet without affecting shrimp growth performance and feed utilization efficiency. On the other hand, the fiber-rich product (FP) that contains around 80% neutral detergent fiber (NDF) could potentially be used as fiber-rich animal feed or as feedstock to produce dietary fibers, packaging materials, and lignocellulosic biofuels. The wet fractionation technology shows a great technical potential to valorize BSG; however, further process optimization and techno-economic analysis (TEA) are warranted to estimate the PP production cost from BSG and evaluate the overall process economics before the adoption of this technology at a commercial scale.

Therefore, the goal of this study is to conduct TEA of PP production from BSG, to understand better the economic feasibility of the wet fractionation technology to produce PP, and to identify the most significant opportunities for reducing the PP production cost in the future. First, the most influential variables, i.e., enzyme loading and hydrolysis time, were investigated to obtain a low level of enzyme loading and hydrolysis time for PP production from BSG in lab-scale experiments. Second, the lab-scale process (60 mL per batch) with optimized parameters was scaled up to 10 L per batch to verify its scalability on protein separation efficiency and PP composition. The obtained results from the scaled-up process were then used as a basis for the TEA to estimate the total capital costs, operating costs, and minimum selling price of PP (MSPP), and for identifying the items that have the most positive effect on the reduction of production cost. It is expected that the information gained from this study will provide a reference to improve and commercialize the production process of PP from BSG.

5.2 Materials and methods

5.2.1 Materials

The raw BSG, with 79% of moisture content (wet basis), was obtained from a commercial brewery (Salem, VA, USA). The BSG was vacuum packed and stored frozen in the dark (- 20 ℃)

until use. The enzyme Alcalase 2.4L FG was provided by Novozymes Inc. (Franklin, NC, USA). The enzymatic activity was determined as 2.2 ± 0.0 (mean ± standard deviation) AU/g using the Sigma's Universal Protease Activity Assay with casein as a substrate (Sigma-Aldrich, 1999). Sodium hydroxide (analytical grade) with a purity of 98.9% was purchased from Fisher Scientific Co. (Hampton, NH, USA).

5.2.2 Bench-scale investigations of enzyme loading and hydrolysis time for optimized PP production

The wet fractionation process (**Figure 5.1**) was adopted from a previous study (He et al., 2019), with a focus on identifying the optimal enzyme loading and hydrolysis time for PP production. The BSG was thawed and then wet milled with a disc mill (Model 4 E, Quaker City Grinding Mills, PA, USA) to reduce the particle size to < 1 mm. After milling, 14.1 g (containing 3 g of dry matter) of BSG was mixed with 46 mL of deionized water to obtain a ~60 mL slurry with a solids content of 5% (w/w). The resulting slurry was adjusted to pH 8.0 with 5 N NaOH and then hydrolyzed with Alcalase at a loading of 0, 5, 10, 15, or 20 µL per g of dry BSG to solubilize protein. According to the product sheet, the optimal temperature for the enzyme Alcalase was 60 ℃; thus, the enzymatic hydrolysis was conducted at 60 ℃ for 4 hours in a shaking water bath (GYROMAX 939 XL, Amerex Instruments, Inc. CA, USA). The hydrolyzed BSG slurry was then sent to a sieve shaker with a mesh size of 75 µm (RX-29, W.S. Tyler, OH, USA) to separate the hydrolyzed proteins from the large insoluble fibers. The sieving process lasted 3 min. During the 3-minute sieving, 100 mL deionized water was poured on the retentate (the material retained on the sieve) evenly to wash out the attached proteins. The filtrate, rich in protein hydrolysates, was collected and dried to make the PP, while the retentate, rich in fibers, was collected and dried to make FP. The optimal enzyme loading that contributed to a high protein concentration in PP (Eq. 5-1) and protein separation efficiency (Eq. 5-2) was chosen to investigate the best hydrolysis time. The hydrolysis time of 1, 2, 3 or 4 h was investigated with the same procedures (**Figure 5.1**). Each treatment was conducted in duplicate.

$$\text{Protein Concentration (\%)} = \frac{\text{Mass of protein in PP (g)}}{\text{Total mass of PP (g)}} \times 100 \qquad 5\text{-}1$$

$$\text{Protein separation efficiency (\%)} = \frac{\text{Mass of protein in PP (g)}}{\text{Total protein in the PP and FP (g)}} \times 100 \qquad 5\text{-}2$$

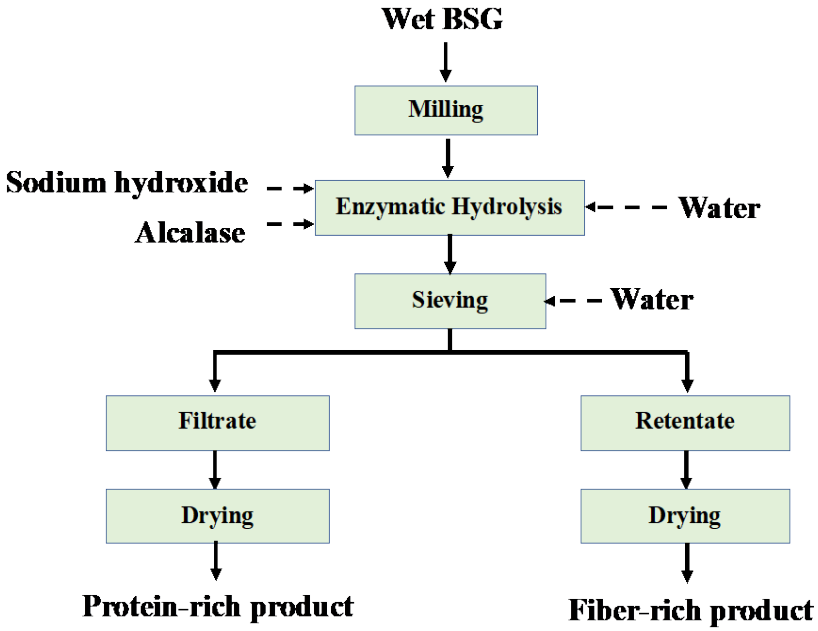

Figure 5.1 The wet milling process to produce protein-rich product and fiber-rich product from brewer's spent grain (BSG).

5.2.3 Process scale up to produce PP from BSG

Scaled-up wet fractionation process was conducted in a 13.5 L bioreactor equipped with a temperature controller and a mechanical agitator. The solid content of the slurry was increased from 5% in the bench-scale experiments to 9% in the scaled-up experiments because the mechanical agitator allowed handling a more viscous slurry compared to shaking flasks in the bench-scale experiments. In the scaled-up process, 4.23 kg of BSG containing 900 g of dry matter was mixed with 5.8 L of water to make around 10 L of BSG slurry with a 9% (w/w) solid content. The slurry was adjusted to pH 8.0 with 10 N NaOH. The optimal enzyme loading and hydrolysis time identified from the lab-scale experiments were applied to the scaled-up process. After the enzymatic hydrolysis, the BSG slurry was subjected to a vertical filter press with a pore size of 250 µm (X-1, Goodnature Products, Inc. NY, USA) to separate the filtrate rich in proteins and the retentate rich in fibers. The separated retentate was washed with water then filtered again to remove any attached proteins. The filtrate from the two filtrations was combined and transferred to a sieve shaker to remove fine-size fibers. The filtrate from the sieve shaker was sent to a customized evaporator (height = 1.57 m, diameter = 0.45 m) at a recirculating flow rate of 6 kg/min under

room temperature to concentrate the solids content to 10% solids content. The concentrated slurry was frozen in a freezer at -20 ℃. After being completely frozen, the samples were freeze-dried in a freeze-dryer (FreeZone 18 L, Labconco Corp., MO, USA), where the condenser temperature is -50 ℃ and vacuum pressure is 28 Pa. The sublimation was conducted at 0 ℃ for 24 h, 15 ℃ for 24 h and 24 ℃ for 24 h. The freeze-dried samples were ground by a knife mill (Arthur H. Thomas CO., PA, USA) to produce the final product – PP. PP was then stored at -20 ℃. The experiment was conducted ten times.

5.2.4 Composition and statistical analysis

Moisture content of the samples were determined by drying samples in the oven at 105 ℃ for constant weight according to the AOAC method of 925.10 (AOAC, 2000). Crude protein content was determined following the Kjeldahl procedures according to the AOAC method of 990.03 (AOAC, 2000). Ash was determined by burning samples in a muffle furnace at 550 ℃ for 12 h (Niemi et al., 2012). Crude fat content was determined following Randall/Soxtec/Submersion Method (Thiex et al., 2003). NDF was determined using the ANKOM 2000 Fiber Analyzer (ANKOM Technology, Macedon, NY) (ANKOM, 2017). The chemical composition of samples was analyzed in duplicate, and the average ± standard deviation reported. Comparisons among treatments were analyzed with one-way ANOVA followed by Tukey's HSD test using JMP Pro14® desktop software (SAS, Cary, NC, USA). Differences were considered significant when $p < 0.05$.

5.2.5 Techno-economic analysis of wet fractionation process for PP and FP production

The TEA model for PP and FP production from BSG included a process flow diagram, rigorous process modeling based on the mass and energy balance, and economics of materials, utilities and equipment using SuperPro Designer 11.0 (Intelligen, Inc., NJ, USA). Given the USA has a high number of large breweries to provide a centralized BSG resource, and the BSG itself is highly perishable and costly to ship, it is assumed that the BSG processing plant is co-located at a large brewery. The co-location strategy has certain advantages, including avoiding the shipping cost of BSG and preventing BSG from spoiling. The wet fractionation process was designed to handle 590 t of wet BSG per day, which is the average BSG generation rate by large breweries in the USA (TTB, 2019). The process runs 330 days (7,920 hours) per year with three shifts per day; the remained 35 days are scheduled for equipment maintenance and cleaning (Davis et al., 2011).

The cost of maintenance is included in the category of fixed operating costs. PP was assigned as the main product, and FP was a coproduct to be sold for credits.

5.2.5.1 Process model description

The flow diagram of PP and FP production from BSG is shown in **Figure 5.2**. The first step of the process is grinding wet raw BSG using a disc mill. The ground BSG is then conveyed to a slurry mixing tank equipped with a mechanical agitator and mixed with process water to make a slurry. Sodium hydroxide (NaOH) solution is added to adjust the pH of the slurry to 8.0, after which Alcalase is added at a loading of 5 μL per g of dry BSG. The resulting slurry with a solids content of approximately 9% (w/w) is then heated to 60 ℃ by steam in a heat exchanger and pumped to a hydrolysis tank, where the enzymatic hydrolysis reaction lasts for 1 hour. After the enzymatic hydrolysis, the hydrolysate is mixed with recycled liquid from the fiber pressing and fresh water using a mixer to create a uniform slurry, which is then passed through a sieve shaker to separate proteins and fibers. The retentate is then dewatered with a filter press to a solids content of 40% and then dried to 90% solids (10% moisture content) using a rotary dryer at 250 ℃ to produce FP. The liquid stream from the filter press is recycled to mix with the hydrolysate before sieving. Since a large portion of soluble compounds in the recycled liquid pass through the sieve shaker and enter the filtrate (rich in protein hydrolysis), the cycling of liquid will not cause the accumulation of soluble compounds. The filtrate is concentrated to a solids content of 35% using a multiple-effect evaporator and further dried to 90% solids using a spray dryer to produce PP powder. The condensed vapor from the evaporator is recycled as process water for enzymatic hydrolysis and fiber wash, a strategy for reducing freshwater consumption.

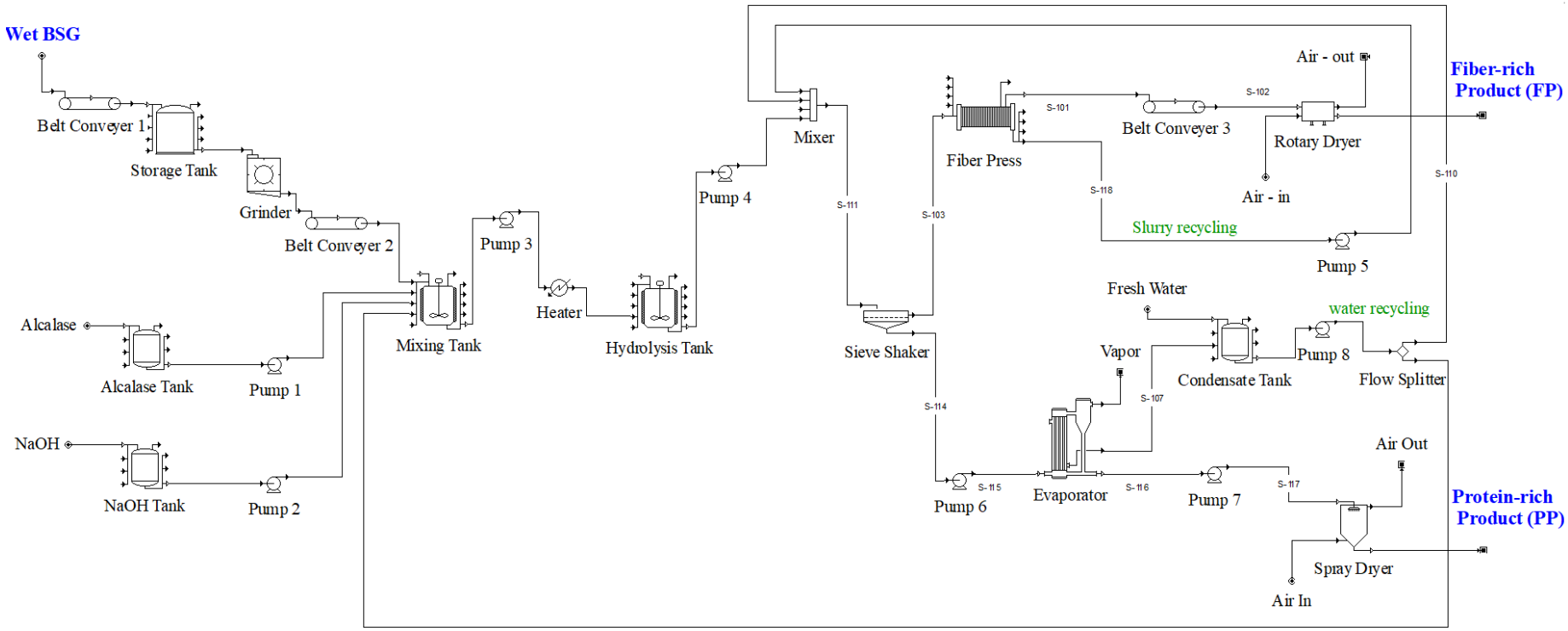

Figure 5.2 The process model flow for PP and FP production from brewer's spent grain (BSG) using SuperPro Designer.

5.2.5.2 Total capital investment, operating costs, and unit production cost of PP

The equipment purchase costs were obtained from previous studies (Juneja et al., 2019; Ramirez et al., 2008), equipment suppliers, or the build-in equipment cost in SuperPro Designer software. Grinding equipment is used to reduce particle size. Screen and filter are used to separate components. Evaporator and dryers remove water. Conveyers and pumps, and tanks are used transport or store streams. All economic calculations were performed for the year of 2019. The equipment cost obtained in earlier years was inflated to the year 2019 using the Chemical Engineering Plant Cost Index. The cost of the equipment with a different size was adjusted based on the scaling equation (5-3) (Humbird et al., 2011):

$$\text{New cost} = (\text{Base cost})\left(\frac{New\ size}{Base\ size}\right)^{n} \quad \text{5-3}$$

where n is the scaling factor, usually in the range of 0.5 to 0.8 (Humbird et al., 2011). Total capital investment (TCI) includes the purchase of equipment and their installations, services building, and other related costs that needed to bring a project to a commercially operable status. TCI is the sum of total fixed capital investment (FCI) and working capital. FCI cost consists of direct costs (e.g. equipment installation, warehouse, site development and piping) and indirect costs (e.g. portable expense, field expense, home office and construction fee, project contingency, project permits), which are both calculated based on equipment cost (Humbird et al., 2011). Working capital was set as 5% of FCI (Somavat et al., 2018).

The details of the variable and fixed operating costs were listed in **Table 5.1**. The variable operating cost includes raw material costs, utility costs, and by-product credits. The unit prices of materials and utilities were determined by their market prices in the year 2019 based on the industrial quotes or the data from literature. It is not straightforward to obtain the exact market price of wet BSG. Buffington (2014) reported that the market price of wet BSG with 30% dry solids is around 44.2 USD/t in the USA. Given the BSG used in the study had a lower dry solids content (21%), a corrected wet BSG price of 31.4 USD/t was used based on the same dry solids content. The sensitivity analysis of the BSG price on PP production cost was also conducted in the section 5.3.4.3. The fixed operating cost includes labor salaries, labor burden, maintenance materials, and property insurance as described by Humbird et al. (2011).

Table 5.1 Variable and fixed operating costs in this study.

Item	Cost (USD)	Unit	Reference
Variable operating cost items			
Raw materials utilities			
Brewer's spent grain	31.4[1]	t	Buffington (2014)
Alcalase	34.0	kg	Industrial quote [2]
Sodium hydroxide	410.0	t	Huang et al. (2016)
Water	0.353	t	Huang et al. (2016)
Utilities			
Steam	17.0	t	Huang et al. (2016)
Natural gas	188	t	EIA (2019a,b)
Electricity	0.0642	KWh	EIA (2019a,b)
Co-product credits			
Fiber-rich product	120[3]	t	Anon (2020)
Fixed operating cost items			
Total salaries	350,000[4]		
Labor burden (90% of total salaries)	315,000		
Maintenance	3.0% of inside-battery-limits equipment costs (Humbird et al., 2011)		
Property insurance	0.7% of fixed capital investment (Humbird et al., 2011)		
Depreciation	The MACRS[5] 7-year depreciation schedule		

[1] The cost of wet BSG (with 79% moisture content) is calculated to be 31.4 USD/t based on the 44.2 USD/t market price of wet BSG with 70% moisture content. (Buffington, 2014).
[2] Bulk price quotes from Novozymes Inc.
[3] The selling price of fiber-rich product (FP) is assumed based on the selling price of soy hulls because of the similar chemical composition and applications (animal feed) between FP and soy hulls.
[4] Assuming 7 employees (1 manager, 6 shift operators) with an average annual salary of 50,000 USD per employee.
[5] MACRS: modified accelerated cost recovery system

5.2.5.3 Minimum selling price of PP and sensitivity analysis

Once the TCI, variable operating costs, and fixed operating costs are calculated, the minimum selling price of PP (MSPP) was determined by the net present value becoming zero for the entire plant's lifetime using the parameters listed in **Table 5.2**. After identifying the MSPP, a single-point sensitivity analysis was performed to test variables that were uncertain or significantly affected MSPP. Seven variables were chosen for the sensitivity analysis, including feedstock and utility costs (BSG, enzyme and steam), processing parameters (solid loading of hydrolysis and protein separation efficiency), and daily input of BSG. The variables were analyzed by changing their values at ± 20% (Zang et al., 2020).

Table 5.2 Main parameters used for determining the minimum selling price of PP.

Parameter	Value
Project lifetime	30 years
Salvage value of equipment	0
General plant depreciation	MACRS 7-year depreciation schedule
Income Tax	35%
Working Capital	5% of fixed capital costs
Construction period	1 year
Start-up time	3 months
Revenues during start-up	50%
Variable costs incurred during start-up	75%
Fixed costs incurred start-up	100%

5.3 Results and discussion

5.3.1 Chemical composition of raw BSG

The raw BSG had a moisture content of 79%. The approximate chemical composition of the BSG on a dry basis is presented in **Table 5.3**. NDF is the most abundant component in BSG, accounting for 46% of the total dry matter of BSG. The NDF content is similar to those reported in previous studies (Shen et al., 2019), though the BSG was obtained from different sources. Crude protein (CP) is the second abundant component in BSG, accounting for 30% of the total dry matter, a typical value similar to those reported by other researches (Nascimento et al., 2017). Overall,

BSG has a lower CP and higher NDF content compared to some popular plant protein sources, such as soybean meal (CP 42–51%, NDF 9–11% (Paula et al., 2020; Sanchez-Duarte et al., 2019)), canola meal (CP around 40%, NDF 28% (Zhang et al., 2017)), sunflower meal (CP 32– 36%, NDF 44–49% (Li et al., 2018; Vanegas et al., 2017)), cottonseed meal (CP around 40%, NDF 32% (Imaizumi et al., 2016), peanut meal (CP 45–53%, NDF 12–19% (Dias et al., 2018; Li et al., 2018)). The low CP and high NDF in BSG have limited its application to ruminant feed. Therefore, the fractionation process for removing NDF and concentrating CP could potentially expand BSG applications to other end uses, such as being used as protein ingredients in aquaculture feed and human food products. Crude fat in BSG was 8%, which is slightly lower than the reported values of 9–11% in literature (del Río et al., 2013; Niemi et al., 2012). Ash content was the same to that reported by Mussatto and Robert (Mussatto and Robert, 2006).

Table 5.3 Proximate composition of brewer's spent grain (BSG) in dry basis.

Components	Concentration (g/100g dry weight)[1]
Crude protein	21.9 ± 0.0
Neutral detergent fiber (NDF)	45.5 ± 0.8
Crude fat	8.3 ± 0.1
Ash	4.6 ± 0.2

[1] Values are means ± standard deviation.

5.3.2 Identifying the optimal hydrolysis condition in the lab-scale process

Figures 5.3A and **5.3B** show the protein concentration in PP and protein separation efficiency when BSG was hydrolyzed with different concentrations of Alcalase for four hours. When no enzyme was added (control), the protein concentration in PP was 45%, and the protein separation efficiency was 68%. Enzymatic hydrolysis significantly ($p < 0.05$) improved both protein concentration of PP and protein separation efficiency. When the Alcalase loading was at 5 µl/g, the protein concentration in PP and protein separation efficiency increased to 48% and 80%, respectively. However, no significant improvements in protein concentrations or protein separation efficiency were observed at enzyme loading above 5 µL/g. The result was consistent with a previous study, which reported that increased Alcalase loading only slightly increased BSG protein solubilization (Treimo et al., 2008). Therefore, 5 µL/g of enzyme loading was selected for the subsequent studies.

The effect of hydrolysis time on PP production were conducted at the same Alcalase loading (5 µL/g). The protein concentration in PP decreased from 49% to 46% as the hydrolysis time decreased from four hours to one hour; however, statistical analysis showed no significant differences ($p > 0.05$) among all treatments with different hydrolysis times (**Figure 5.3C**). The protein separation efficiencies from all treatments stabilized at around 80% with no significant differences (**Figure 5.3D**). This result indicates that extending the enzymatic hydrolysis time from one hour to four hours does not improve PP recovery from BSG. Therefore, Alcalase loading at 5 µL/g and hydrolysis time of one hour were selected as the optimal condition for the following scaled-up process for PP production.

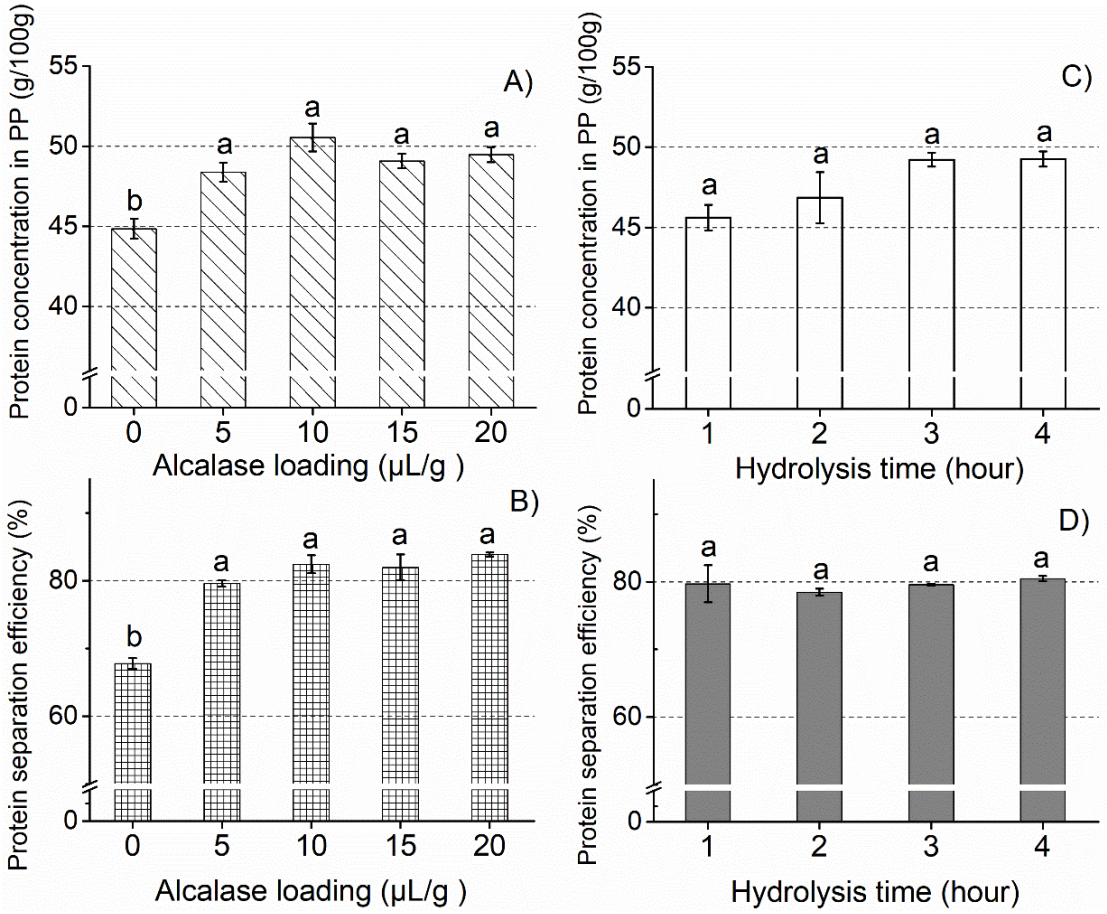

Figure 5.3 Protein-rich product (PP) separated from spent grain with varied Alcalase loadings at 60 °C for 4 h (A and B); PP separated from spent grain with Alcalase loading of 5 µl/g at 60 °C for different hydrolysis time (C and D). PP separation was evaluated with protein concentration in PP (A and C) and protein separation efficiency (B and D). Vertical bars show standard deviation. Same letter indicates no significant different (p > 0.05).

5.3.3 Scaled-up process for PP production from BSG

The chemical compositions of PP and FP obtained from the scaled-up process are shown in **Table 5.4**. PP had 47% protein, double that in raw BSG (22%). The result was consistent with the lab-scale experiments, which resulted in PP with protein content of 46% at the same hydrolysis condition (**Figure 5.3A, C**). The PP protein content is similar to commercial soybean meal, which is between 45% and 50%. Contrarily, the content of NDF (1%) in PP was much lower than that in raw BSG (45% NDF) because most fibers were removed from PP during the filtration and sieving process. The FP had a low protein content of 11% and a high fiber content of 64%, which is understandable because of most of BSG fibers were accumulated in FP. The composition of the FP is similar to that of soy hulls, a major byproduct in soybean processing. Soy hulls have been used to replace forage or hay in animal diets to balance protein and energy (Fustini et al., 2017). Therefore, use of FP like the soy hulls as a viable fiber source in animal feeds was referred in this study. Based on the mass balance analysis (**Figure 5.4**), 312.2 g PP and 586.3 g FP were produced from 900 g dry BSG, corresponding to a PP yield of 35% and a FP yield of 65%. Meanwhile, the scaled-up process consumed around 8.8 L of water and 4.5 mL of Alcalase, plus the associated energy for evaporation and drying. Overall, the results from the scaled-up process indicate that the developed process can be scalable with a consistent product (PP and FP) composition and yield.

Table 5.4 Chemical composition of protein-rich product and fiber-rich product (g/100g, dry weight) from the scaled-up tests.[1]

Parameter	PP	FP
Crude protein	47.2 ± 0.6	11.1 ± 0.5
Crude fat	8.1 ± 0.3	7.8 ± 0.3
Neutral detergent fiber (NDF)	1.0 ± 0.3	63.5 ± 2.3
Total ash	6.8 ± 0.6	4.4 ± 0.4

[1] Values are means ± standard deviation.

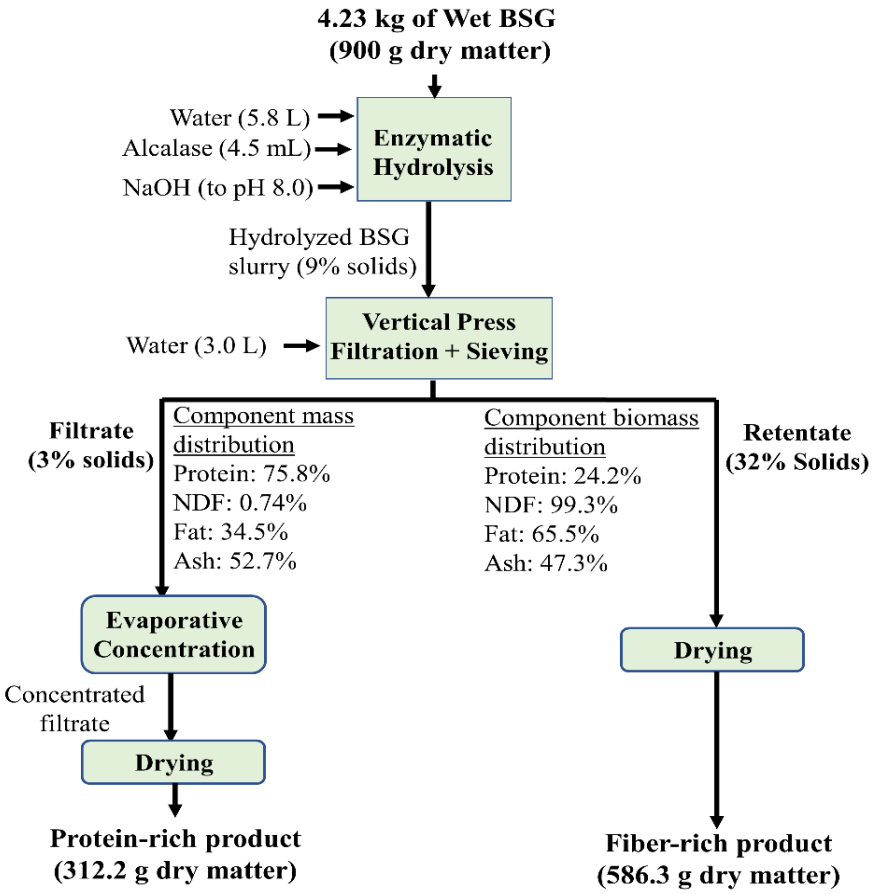

Figure 5.4 Process and mass balance of protein-rich and fiber-rich products (PP and FP) production from brewer's spent grain (BSG).

5.3.4 Techno-economic analysis of PP production from BSG

5.3.4.1 Total capital investment of the processing plant

The TCI (**Table 5.5**) for the plant with an annual processing capacity of 194,700 t wet BSG per year is estimated to be 11.2 million USD, of which, the total installed equipment costs 6.1 million USD, accounting for the largest portion (55%) of the TCI. The dewatering equipment, including filter press, rotary dryer, multi-effector evaporator, and spray dryer, share the largest total installed equipment costs. In the wet fractionation process, a large amount of water is added during enzymatic incubation and sieving, and this water has to be removed to produce dried PP and FP. The flow rate of the PP slurry from sieving is 65 t/h, which contains as high as 97% water. Moreover, some nutrients dissolved in the water and cannot be separated out easily. Hence, the PP slurry is first concentrated using a multiple-effect evaporator and then further dried using a spray dryer to produce PP powders. Both of the two equipment are capitally expensive. For the FP stream,

because fiber particles are large and insoluble in water, a filter press and a rotary dryer are used to produce dried FP. Other equipment, such as storage tanks, disc mills, conveyors and pumps, also contribute significantly to the total capital investment.

Table 5.5 Capital investment for the processing plant with an annual capacity of 194,700 t wet BSG and equipment specification.

Item	Size/capacity	Construction of materials	Cost (USD, thousands)
Process equipment			
BSG storage tank	641.8 m^3	304 s.s.	127.5
BSG disc mill	24.6 t/h	304 s.s.	114.9
Alcalase storage tank	5.8 m^3	304 s.s.	35.6
NaOH storage tank	19.0 m^3	304s.s.	72.4
Slurry mixing tank	16.5 m^3	304s.s.	49.4
Slurry heater	5.3 m^2	304 s.s.	19.5
Hydrolysis tank	66.2 m^3	304s.s.	113.7
Vibrating screen	76.2 t/h	316 s.s.	36.8
Fiber-rich product filter press	13.7 m^2	316 s.s.	228.6
Fiber-rich product rotary dryer	150. m^2	c.s.	734.1
Protein-rich product evaporator	135.3 m^2	316 s.s.	409.5
Process condensate tank	330.8 m^3	304 s.s.	85.0
Spray dryer	3.3 t/h	316 s.s.	1,031.7
Belt conveyer (3)	various	c.s.	164.2
Pump (8)	various	316 s.s.	180.8
Total equipment cost (TEC)			3,403.7
Total installed equipment cost[1]			6,137.8
Warehouse, 4% TEC			136.1
Site development, 9% TEC			306.3
Additional piping, 4.5% TEC			91.5
Total direct cost (TDC)			6,671.8
Prorateable expense, 10% TDC			667.2
Field expense, 10% TDC			667.2

Home office & construction expense, 20% TDC	1,334.4
Project contingency, 10% TDC	667.2
Other costs (start up, permits), 10% TDC	667.2
Total indirect cost (TIC)	4,003.1
Fixed Capital Investment (FCI), TDC+TIC	10,674.9
Working Capital (WC), 5% FCI	533.7
Total Capital Investment, FCI + WC	11,208.6

[1] Total installed equipment cost = cost of equipment purchased × multiplier. The multiplier varies from 1.5 to 2.3 depending on different equipment (Humbird et al., 2011).

5.3.4.2 PP production and unit cost

The annual operating costs of PP production facility are shown in **Table 5.6**. Based on the annual operating costs, the minimum selling price of PP (MSPP) was calculated at 1,043.5 USD/t. The MSPP of the designed process includes a variable cost (raw materials, utility cost, and co-product credit), fixed cost (labor and overheads), capital depreciation, average income tax, and an average return on investment. Among these costs, raw material cost contributed most to the MSPP; within the materials cost, the enzyme and raw BSG costs accounted for the greatest portion. Specially, the enzyme and raw BSG costs contributed to 492.4 USD/t and 362.9 USD/t of the MSPP, respectively. Although a low enzyme dosage (5 μL/g BSG) was used based on the lab experiments, the unit price of the enzyme (food-grade Alcalase, 34 USD/kg, a bulk price quoted from Novozymes Inc.) still makes it the highest cost for PP production. This high contribution of the enzyme to MSPP highlights the importance of exploring a low-cost but effective enzyme. On-site crude enzyme production using fungi could also be a possible option as several studies have reported that on-site enzyme production could greatly reduce the enzyme cost in the biofuel industry (Olofsson et al., 2017). After the cost of enzyme, the cost of wet BSG was the second-largest contributor to the overall operating cost. In this study, a cost of 31.4 USD/t wet BSG was used based on a market survey (Buffingt, 2014). To be noted, in reality, the cost could be zero as some breweries are willing to give away their BSG as a means to get rid of the waste materials (McHugh et al., 2020). Thus, the PP production cost could potentially be reduced if a low-cost BSG source can be secured. Besides the cost of the raw materials, utility costs, including the costs of electricity, natural gas, and steam, accounted for 224.9 USD/t (22%) of the MSPP. Among the

utility costs, the steam cost constitutes the highest portion (64%), which was attributed to the need to remove a large amount of water from PP slurry during evaporation and spray drying to produce dried PP, and to the fact that the heating source for both evaporation and drying is steam. The FP annual production was 29,825 t, and the selling price of FP was assumed at 120 USD/t, which was based on its nutritional composition identical to soybean hulls (**Table 5.4**). Income from the sale of FP was claimed as a coproduct credit and contributed to around 20% reduction in PP production cost.

A previous study showed that PP could replace 50% of fishmeal in shrimp diet without affecting shrimp growth and survival rates (He et al., 2020). The PP production cost of 1043.5 USD/t estimated in this study was lower than the average fishmeal price of 1,449 USD/t in the past five years (YCharts, 2019), indicating that the process of PP production from BSG might be economically feasible. The PP made by this process can also potentially be used as a plant-based protein ingredient for food products. Considering that soybean protein concentrate, a popular plant-based protein source, has a current market price over 2,000 USD to 3,000 USD/t (Soto-Sierra et al., 2018), PP from BSG could be competitively priced. However, further product development research is warranted to incorporate PP into food products, such as determination of the functional properties and flavor profiles of PP.

Table 5.6 Annual production cost of 16,847 t of PP from BSG

Description	Annual use (thousands)	Annual cost (USD, thousands)	Unit cost (USD/t)
Raw materials (t)			
BSG	194.7	6,113.6	
Enzyme	0.24	8,296.0	
Sodium hydroxide	0.53	216.5	
Water	6.93	2.6	
Subtotal raw materials		*14,628.7*	*868.3*
Utilities			
Electricity (KWh)	8,297.8	532.7	
Natural gas (t)	4.5	837.9	
Steam (t)	142.3	2,419.0	
Subtotal utilities		*3,789.6*	*224.9*
Labor			
Total salaries		350.0	
Labor burden		315.0	
Subtotal labor		*665.0*	*39.5*
Other overhead			
Maintenance		286.2	
Property insurance		74.7	
Subtotal overhead		*361.0*	*21.4*
Co-product (FP)credit		*-3,579.0*	*- 212.5*
Capital depreciation		355.8	21.1
Average Income Tax		279.3	16.6
Average Return on Investment		1,079.0	64.1
Net operating costs			1,043.5

5.3.4.3 Sensitivity analysis

The sensitivity analysis (**Figure 5.5**) showed that Alcalase price is the most influential to the MSPP. When Alcalase price changed in the range of 34 ± 6.8 USD/kg, MSPP ranged from 944.5 to 1142.5 USD/t. BSG price also highly influences the PP production cost. When BSG price changed in the range of 31.4 ± 6.3 USD/t, MSPP increased or decreased by 7%, varying between 970.5 and 1,116.5 USD/t. Compared to the raw material cost, steam price, FP selling price, and BSG processing capacity have less influences on MSPP. This result indicates that using cheaper BSG and Alcalase can significantly reduce the PP production cost.

Between the two processing parameters (protein separation efficiency and solid loading of hydrolysis), the MSPP is more sensitive to the change of protein separation efficiency. When protein separation efficiency increased from 76% to 91%, the MSPP decreased by 7% to 968.1 USD/t; on the other hand, when the protein separation efficiency decreased from 76% to 61%, the MSPP increased by 9% to 1132.5 USD/t. The solid loading of hydrolysis has a lower impact compared to protein separation efficiency. These findings suggest that increasing protein separation efficiency is more efficient to improve the economics of PP production.

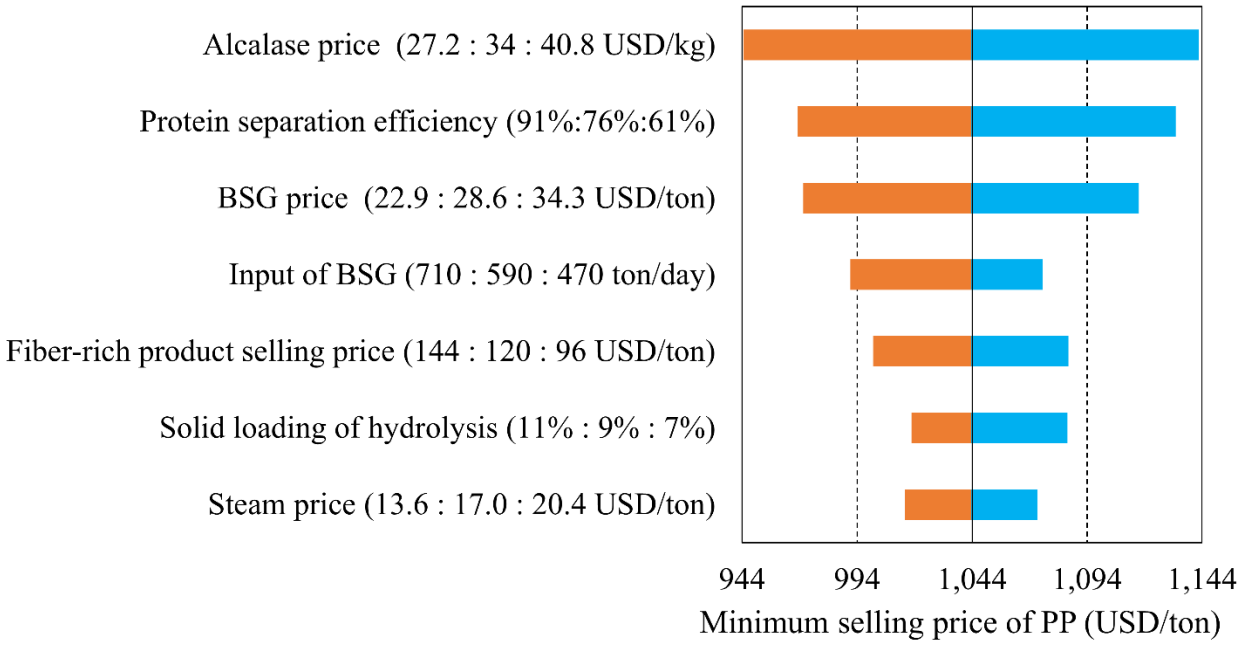

Figure 5.5 Sensitivity of PP production cost to different parameters. The numbers in brackets in Y-axis are the potential low, base and high values of each parameter.

Based on the single-point sensitivity analysis, the Alcalase price is the most influential parameters to MSPP. With the development of an enzyme production technology, Alcalase price is expected to decrease. Therefore, there is a great potential to reduce the PP production cost in the future if a lower-cost enzyme can be used to hydrolyze proteins. The detailed effects of enzyme price on the PP production cost were further investigated (**Figure 5.6a**). In corn ethanol industry, protease was used to treat corn to produce ethanol and DDGS (distiller's spent grain with solubles) as animal feed, and the price of the protease is about 0.5 USD/kg. When the enzyme price reduced from 34.0 USD/kg to 0.5 USD/kg, PP production cost decreased by 47% from 1043.5 USD/t to 555.7 USD/t. BSG price is another uncertainty but very important variable to estimate MSPP. In the base scenario, the BSG price is assumed to be 31.4 USD/t. However, as mentioned before, some breweries are providing BSG for free to local farmers. When the BSG price decreased from 31.4 USD/t to 0 USD/t, the MSPP decreased by 35% to 678.6 USD/t (**Figure 6b**). This analysis result indicates that the PP production cost has the potential for significant improvement in the future if a lower-cost enzyme and BSG can be secured for the process.

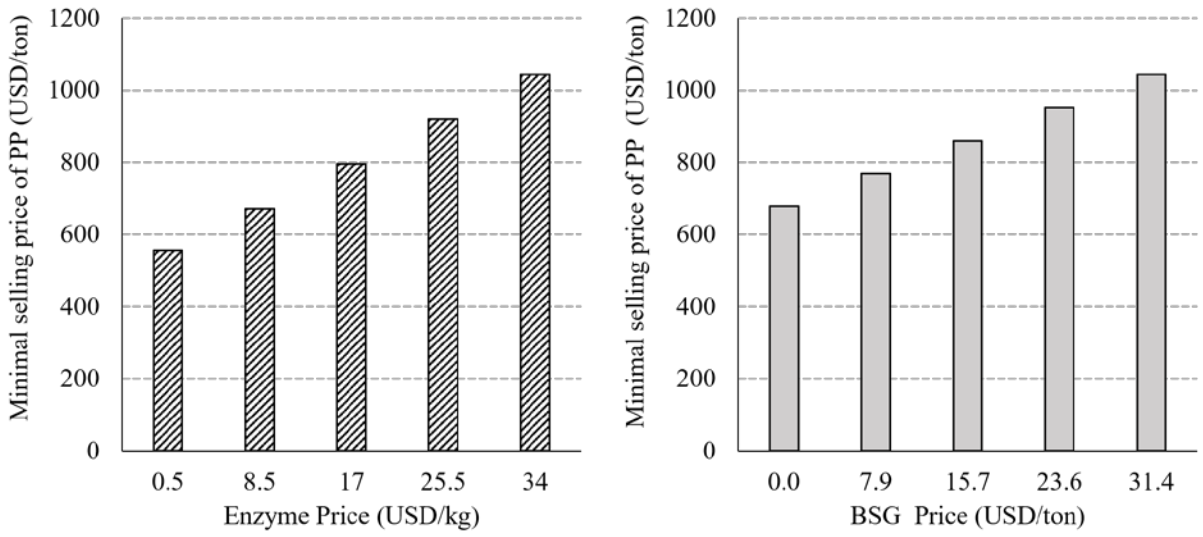

Figure 5.6 Effect of enzyme price (a) and BSG price (b) on MSPP of PP.

5.4 Conclusions

Lab-scale experiments were conducted to identify the optimal hydrolysis conditions of the wet fractionation process. The enzyme loading of 5 μL/g and hydrolysis time of 1 h were identified as the optimal condition for achieving a high protein concentration (46%) in PP and protein

separation efficiency (80%). The identified processing conditions were further verified by the scaled-up process. The techno-economic analysis showed that the total capital investment of the processing plant with an annual processing capacity of 194,700 t wet BSG was 11.2 million USD. The MSPP to achieve 5% return was determined to be 1043.5 USD/t. The costs of Alcalase and BSG contributed significantly to MSPP, accounting for 43% and 31%, respectively. Overall, PP production from BSG is promising, and it is not only providing a potential route for breweries to deal with this brewing by-product, but also for generating protein-rich feedstuff to the animal feeding industry. Developing a low-cost and effective protein separation enzyme, accessing low cost BSG, and improving the protein separation efficiency of the process would have a great potential to reduce the PP production cost for ensuring its competitiveness in the market.

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Chapter 6. Cellulosic adsorbent produced from brewer's spent grain fiber and its application in the removal of Mn and Pb from water

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ABSTRACT

Removal of heavy metals from drinking water is of high importance to human health. This study reports the fabrication of a novel cellulose adsorbent from a secondary waste material, i.e. a fiber-rich product (FP) from brewer's spent grain processing, for Mn and Pb removal from water. FP was sequentially treated by dilute acid, alkali, and bleach to produce purified cellulose fibers, which were then modified by 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) oxidation. The adsorption data fitted the Langmuir isotherm and the maximum adsorption capacity of the adsorbent was determined to be 272.5 mg/g for Pb (II) and 52.9 mg/g for Mn (II) at pH 6.5. Kinetic studies showed that the adsorption process followed the pseudo-second-order rate model. The performance of the adsorbent on the removal of Pb and Mn from real contaminated tap water was further evaluated. The removal efficiency for Pb and Mn was 87.4% and 83.9%, respectively. This study provides a novel insight into the fabrication of low-cost and high-capacity cellulose adsorbents from a waste material for water purification. Meanwhile, it allows the integrated utilization of brewer's spent grain, thus supporting the sustainable resource management and circular economy development.

6.1 Introduction

The demand for freshwater parallels the increase in the world population. However, the rapid industrialization, urbanization, agricultural practices, human activities, and improper

discharge of wastewater have led to the rising water pollution problem. Water pollutants are usually complex and majorly consist of organic, inorganic (salts and metals), and biological (pathogens) contaminants (Ghangrekar & Chatterjee, 2018). Particularly, heavy metals are toxic at a low level of exposure, non-biodegradable, and threatening human health via the food chain (Bolisetty et al., 2019). Exposure to Pb-containing drinking water causes toxicity in adults and children (Yao et al., 2021), and the United States Environmental Protection Agency (EPA) has set a regulatory action level at 0.015 mg/L (EPA, 2009). The presence of Mn in water can cause severe discoloration of drinking water (Cerrato et al., 2006; Sain et al., 2014) and concentrations > 0.2 mg/L may lead to neurotoxicity in humans and animals (Therdkiattikul et al., 2020), and especially in children (Iyare, 2019). The EPA has set the secondary maximum contaminant limit of manganese in drinking water at 0.05 mg/L (EPA, 2009). Adsorption is recognized as one of the most suitable and promising methods used for heavy metal removal from water (Zhao et al., 2016).

The cellulose-based adsorbent has been reported as an efficient adsorbent for heavy metal removal from water such as gold and platinum (Biswas et al., 2021), lead (Li et al., 2020), chromium (Park et al., 2020), as well as cadmium, zinc and copper (Fakhre et al., 2018). Cellulose is the most abundant biomass in the world, and it is one of the major components of lignocellulosic biomass. Using lignocellulosic biomass obtained from food processing waste to produce cellulose adsorbent is not only contributing to the waste recycle but also reducing the environmental burden, which is in line with circular bioeconomy. However, raw cellulose has limited adsorption capacity due to its hydroxyl group. Therefore, surface modifications are usually needed to transform the hydroxyl groups into carboxyl, amino and sulfo groups to improve the adsorption performance (Wang et al., 2017). Besides, the size reduction by using methods such as high-pressure homogenization, and ultrasonication is expected to increase the specific area of adsorbent to enhance the adsorption capacity (Dilamian & Noroozi, 2019).

Brewer's spent grain (BSG) is the major byproduct after the mashing and lautering process in the beer production. The United States as the second leading country in beer production annually produces 190.2 million barrels of beer in the past decade (TTB, 2021). Since 20 kg of BSG is cogenerated with every 100 L of beer production (Mussatto et al., 2006a), it is estimated that annual BSG production was around 4.4 million tons. BSG usually contains 14–30% of protein, 50–70% of fiber (on a dry basis), and other minor components including lipids, minerals and phenolic compounds (Lynch et al., 2016). Because of its high fiber content, the use of BSG has

been limited to low-value cattle feed with the surplus being disposed of in landfills, leading to substantial resource losses. Moreover, BSG's high moisture content (77–81%) and fair amounts of nutrients (e.g., proteins, fats, vitamins) make it susceptible to microbial growth and spoilage. Spoiled BSG would cause significant environmental concerns such as soil acidification and groundwater contamination.

Effective valorization of BSG into value-added products will greatly support sustainable resource management and circular economy development. Recently, numerous studies have been investigated to valorize BSG through recovering proteins from BSG using alkaline extraction or enzymatic hydrolysis of proteins (Connolly et al., 2019; He et al., 2019, He et al., 2021a; Yu et al., 2020). These studies have received significant attention because the recovered proteins from BSG could be used as a plant-based protein in human diets and a fishmeal replacement in aquaculture feed (He et al., 2020). However, all of these studies only focused on the recovery and utilization of protein from BSG but with little attention to the utilization of fiber, which is the most abundant component in BSG. Furthermore, valorization of BSG without considering the fiber utilization would leave a large amount of secondary waste (i.e., the fiber-rich materials) to dispose, which has caused economic and environmental concerns and hindered the commercialization of the value-added processing BSG. This study was to directly address this knowledge gap by converting the fiber-rich product (FP) from BSG processing (for protein recovery) into cellulose-based adsorbents for heavy metals removal.

The overall goal of this work is to fabricate a cellulosic adsorbent using the FP obtained from BSG processing and investigate its feasibility to remove Pb and Mn from water. The specific objectives are: 1) extract and characterize cellulose from FP through a sequential acid, alkali, and blench treatment; 2) apply TEMPO-mediated oxidation to introduce carboxyl groups onto the cellulose fiber; 3) determine the effect of ultrasonication on the adsorption capacity of the prepared cellulosic adsorbent; 4) investigate the ability of the prepared cellulosic fiber to capture metal ions including Pb (II) and Mn (II) via static adsorption and to determine the kinetics and possible adsorption mechanisms; and 5) characterize the particle size and surface properties of the TEMPO-mediated oxidation cellulosic fiber (TOCF). To the best of our knowledge, the current study was the first report on Pb and Mn removal from contaminated water with BSG cellulose-based adsorbent. The promising fabrication of cellulosic adsorbent by using the fiber residues from BSG

processing provides a potentially cost-effective material for drinking water purification and paves a novel way for the integrated utilization of BSG.

6.2 Materials and methods

6.2.1 Preparation of cellulosic adsorbent

6.2.1.1 Cellulose fiber extraction from FP

FP obtained from our previous study that focused on the protein recovery from BSG (He et al., 2020) was oven-dried at 60 ℃. The particles that passed through a No. 18 sieve (particle size ⩽ 1 mm) were collected and used for the production of cellulose adsorbent. The FP was first pretreated with dilute acid for removing hemicellulose under the optimized conditions identified in our previous study (He et al., 2021b). Briefly, three hundred grams of FP was mixed with 0.5% (v/v) H_2SO_4 solution in a 1:10 w/v solid: liquid ratio and heated at 121℃ for 60 min. After the treatment, the resulting solid residue was separated using a sieve with a pore size of 75 μm, and washed with deionized water until neutral pH, and dried in an oven at 65 ± 2 ℃ to attain 10% moisture content. The acid pretreated FP were then treated with 2% (w/v) sodium hydroxide solution in a solid: liquid ratio of 1:20 w/w at 121℃ for 90 min to remove lignin (Mussatto et al., 2006b). The obtained black slurry was filtered and the solid residue was washed with deionized water to neutral pH and dried at 50 ± 2 ℃ in an air-circulating oven to attain 10% moisture content. The dried fibers with yellow/brownish color were then submitted to a bleaching process, where the fibers were treated with a solution made up of equal parts (v/v) of acetate buffer (pH 4.8) and aqueous sodium chlorite (2% w/w, $NaClO_2$ in water) in a solid: liquid ratio of 1:50 (w/v) at 80 ℃ for 4 h (dos Santos et al., 2015). The mixture was then allowed to cool and the solid was separated using a sieve with a pore size of 75 μm followed by deionized water wash to neutral pH. The white color-bleached fibers were dried at 50 ± 2 ℃ in an oven to attain 10% moisture content.

6.2.1.2 TEMPO-mediated oxidation of cellulose fibers and ultrasonic fibrillation

TEMPO-mediated oxidation was applied to the cellulose fibers with NaClO, TEMPO, and NaBr to introduce carboxyl groups according to the previously reported method (Fiol et al., 2019). Specifically, 5 g cellulose fibers were dispersed in 500 ml deionized water containing TEMPO (0.016 g per g of fiber) and NaBr (0.1 g per g of fiber). The mixture was stirred for 15 min to

assure good dispersion of all the substances. The TEMPO oxidation of the fiber slurry was started by adding 50 ml (10 ml/g fiber) of sodium hypochlorite solution (NaClO, 10%). The NaClO was added drop-wise to the pulp slurry to maintain the pH at 10. Once all the NaClO was added, the pH was further maintained at 10 by adding 0.5 M NaOH. When the pH was stable at 10 and no more NaOH was consumed, the reaction was quenched by adding ethanol (100%). The TEMPO-oxidized fiber mixture was submitted to sieving which the pore size of the sieve was 75 µm. The fiber that was left on the sieve was purified by repeatedly adding water, dispersion (30 min), and sieving three times, then freeze-dried to avoid aggregation. The content of carboxylate groups of oxidized fiber was determined to be 3.3 mmol/g using conductometric titration (Li et al., 2020). To be specific, 0.05 g fiber was suspended in 50 mL of water, and 0.1 mol/L of HCl solution was used to adjust the pH value to 2.5–3.0. Sodium hydroxide solution (0.025 mol/L) was then used to nutrilize the fiber mixture. The content of carboxylate groups was calculated based on Eq. 6-1.

$$C_{-COO^-} = C_{NaOH} \times V_{NaOH}/m \quad \text{6-1}$$

where C_{-COO^-} is the content of carboxylate groups (mmol/g), C_{NaOH} is the concentration of NaOH (mol/L), V_{NaOH} is the volume of consumed NaOH (mL), and m is the mass of fiber (g).

Both oxidized and non-oxidized fibers were then fibrillated by ultrasonication. Briefly, the fiber suspension prepared by soaking fibers (1% w/v) in deionized water was sonicated by an ultrasound processor (Model 505, 500 W, 20 kHz, Fisher Scientific Inc., Waltham, MA, USA) equipped with a 6.0-mm-diameter ultrasound probe. Power output (amplitude 60%) and treatment time (30 min) was applied with a pulse cycle of 10 sec on and 2 sec off. The ultrasonic treatment was carried out in an ice bath throughout the entire ultrasonication.

6.2.2 Adsorption experiments to screen cellulose adsorbents

To investigate the effects of TEMPO-mediated oxidation and ultrasonic fibrillation on heavy metals adsorption of the BSG cellulosic adsorbents, a screening test was conducted to identify the cellulosic adsorbent with the highest Pb and Mn adsorption capacity for the subsequent kinetic and isotherm studies. Four adsorbents, FP cellulose fibers (CF), CF treated with ultrasonic fibrillation (UCF), CF treated with TEMPO-mediated oxidation (TOCF), and TOCF treated with ultrasonic fibrillation (UTOCF) were evaluated by exposing to 0.3 mM of Pb and Mn solution separately with the same dose (0.03 g/30 mL) (Li et al., 2020). The pH of initial solutions was 6.5 (adjusted with 0.1N NaOH or HCl), which was within the pH range (6.5–8.5) of drinking water

and the solubility of Pb and Mn (Huang et al., 2020). The test tubes were placed into a water bath (GYROMAX 939, Amerex Instruments, Inc., Concord, CA, USA) at 25 ℃ with constant shaking (150 rpm) for 48 h to reach the equilibrium state. Water samples (2 mL) collected at 1, 6, 12, 24, and 48 h were filtered through a 0.45 μm filter. The filtrates were collected, and the metal concentrations in the filtrates were quantified using a Thermo Electron iCAP-RQ inductively coupled plasma mass spectrometer (ICP-MS, Thermo Electron North America LLC, West Palm Beach, FL, USA) with a method reporting limits of 0.1 μg/L for both Mn and Pb. The adsorption capacity (Q_t, mg/g) at time t was calculated using the following Eq. 6-2:

$$Q_t = (C_0 - C_t) \times V/m \quad \text{6-2}$$

where, C_0 and C_t correspond to initial and metal ion concentrations at time t (mg/L), and V is the volume (L) of a solution, m is the mass (g) of adsorbent.

6.2.3 Studies on the adsorption behaviors

6.2.3.1 Adsorption kinetics

To study adsorption kinetics, 15 mg of the selected adsorbent was added into 30 mL Mn solution with an initial concentration of 30 mg/L or Pb solution with an initial concentration of 140 mg/L under the same conditions as described previously. Liquid was sampled at 6-time points in 1 h, followed by filtration and analysis with ICP-MS. The pseudo-first-order (Eq. 6-3) and pseudo-second-order (Eq. 6-4) models were used to evaluate the possible mechanisms controlling the adsorption rate (Inyinbor et al., 2016).

$$\ln(Q_e - Q_t) = lnQ_e - k_1 t \quad \text{6-3}$$

where Q_t is the quantity absorbed at time t (mg/g), Q_e is adsorption capacity at equilibrium status (mg/g), k_1 (1/min) is the rate constant for the first-order sorption.

$$t/Q_t = 1/(k_2 Q_e^2) + t/Q_e \quad \text{6-4}$$

where k_2 is the rate constant of the second-order kinetic sorption.

6.2.3.2 Adsorption isotherms

In the adsorption isotherms study, 15 mg of the selected cellulose adsorbent was added to 30 mL of Mn or Pb water solutions with concentrations in the range of 1–160 mg/L and 4–603 mg/L, respectively. The experimental temperature and pH were the same as the adsorption tests

described earlier. Three adsorption isotherms: Langmuir (Eq. 6-5 and 6-6), Freundlich (Eq. 6-7), and Temkin (Eq. 6-8 and 6-9) models were used to analyze the adsorption data (Jin et al., 2020).

$$Q_e = \mathrm{Q}_m K_L C_e/(1 + K_L C_e) \quad 6\text{-}5$$

$$R_L = 1/(1 + K_L\, C_0) \quad 6\text{-}6$$

where Q_m (mg/g) is the maximum adsorption capacity, K_L (L/mg) is the equilibrium constant, C_e is the concentration of adsorbate in solution at equilibrium (mg/L).

$$Q_e = \mathrm{K}_F C_e^{1/n} \quad 6\text{-}7$$

where K_F (mg/g) is the Freundlich capacity constant and n is the adsorption intensity.

$$Q_e = \mathrm{BlnA} + \mathrm{Bln}C_e \quad 6\text{-}8$$

$$B = RT/b \quad 6\text{-}9$$

where B is a constant related to the heat of adsorption, A is the Temkin isotherm constant (L/g), R is the gas constant (8.314 J/mol K), T is the absolute temperature (K), and b is the Temkin constant (J/mol).

6.2.4 Adsorption of Mn and Pb from tap water

To study the adsorption of Mn and Pb onto the selected cellulose fiber in real water, adsorption experiments were conducted in intentionally contaminated tap water (Blacksburg, VA, USA). One tap water was adjusted the pH value to 6.5, containing 2.5 mg/L of Mn and 0.30 mg/L of Pb. The other tap water was adjusted the pH value to 7.5 while containing 2.5 mg/L Mn and 0.03 mg/L Pb (due to the lower solubility of Pb at higher pH). Another two plain tap water samples were only with pH adjustment and no addition of Mn or Pb. For each experiment, 15 mg of the selected cellulose adsorbent was added into 30 mL of the tap water sample, and the cellulose adsorbent added water sample was shaken at 150 rpm for 1 h at 25 ℃. Concentrations of metals in the water samples were analyzed using ICP-MS as previously mentioned.

6.2.5 Characterization and statistical analysis

Hemicellulose, cellulose, and lignin contents of FP (before and after each treatment) were determined by following the National Renewable Energy Laboratory standard procedures (Sluiter et al., 2008). The particle size profile of the four adsorbents was measured by using a laser diffraction particle size analyzer (LS13320XR, Beckman Coulter, Inc., Brea, CA, USA). Before the particle size measurement, fibers were dispersed in distilled water and diluted until the solution

looks transparent. The crystal structure of the selected fiber was investigated by X-ray diffraction (XRD) analysis, which was conducted using a MiniFlex II diffractometer (Rigaku Corp., Tokyo, Japan) with a Cu Kα radiation (λ = 1.54 Å). Diffractograms were collected in the 2θ range from 5 ^{o} to 50 ^{o} at a scanning rate of 0.5 ^{o} min^{-1}, and the step size was 0.02 ^{o}. The morphology of the selected adsorbent before and after adsorption was examined by a JEOL IT-500HR scanning electron microscopy (SEM), samples were coated with a thin layer of Au to make the surfaces conductive. Fourier transform infrared (FTIR) spectroscopy was performed with an 8700 Nicolet Thermo Electron to determine the functional groups in the corresponding cellulose fibers before and after Mn and Pb adsorption. To be specific, about 2–4 mg of sample was mixed with 100 mg of pre-dried spectroscopic-grade KBr (Thermo Fisher Scientific) to form pellets for FTIR analysis. Spectra in the range of 500–4000 cm^{-1} with a resolution of 4 cm^{-1} were recorded. The leaching of organics indicated by total organic carbon (TOC) was analyzed using a Shimadzu Total Organic Carbon Analyzer (TOC)-VCSN.

Experiments including adsorbent production, adsorption, kinetics, and isotherms were conducted in duplicate and the results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to test the significant differences among treatments, followed by a Tukey's HSD (honestly significant difference) for a pair-wise comparison of means using JMP Pro15® (SAS, Cary, NC, USA). The confidence level was 95% ($p < 0.05$).

6.3 Results and Discussion

6.3.1 Chemical composition of FP at different treatment stages

The chemical composition of FP before and after sequential acid-, alkali-, and bleaching-treatment are summarized in **Table 6.1**. The untreated FP had higher contents of hemicellulose and lignin, and a lower content of cellulose than treated fiber. The original FP contained 81.8% of fiber, including 48.1% hemicellulose, 25.5% cellulose, and 8.2% lignin. The FP cannot be used as a cellulose adsorbent directly due to its low cellulose content. To extract cellulose from FP, treatment to break the lignin and degrade surrounding hemicellulose is necessary. Dilute sulfuric acid can effectively degrade BSG hemicellulose into xylose while leaving cellulose and lignin fractions almost unaltered (Mussatto & Roberto, 2005). After the acid treatment, a marked decrease in the hemicellulose content of FP was observed, decreasing from 48.1% to 11.1%.

Further alkali treatment was able to completely remove the remaining hemicellulose and diminish the lignin content from 9.5% to 1.8%, and increased the cellulose content in the treated fiber from 25.5% to 95.2%. The resulting yellow/brownish fiber was hypochlorite bleached, thereby producing white color fiber, which contained 98.7% cellulose. A cellulose pulp obtained from our study had a higher purity than those obtained from previous studies. A cellulose pulp produced from BSG using the similar acid and alkali treatment contained 90.4% cellulose (Mussatto et al., 2008), and bleached cellulose extracted from BSG contained 90.1% cellulose (dos Santos et al., 2015). The main differences between our study and the previous studies were the starting materials. Mussato et al. (2008) and dos Santos et al. (2005) used raw BSG to produce cellulose pulp, whereas our study used FP (the remained fiber after protein removal from BSG). The results indicate using FP may be more suitable for cellulose adsorbent production than raw BSG.

Table 6.1 Cellulose, hemicellulose, and lignin concentrations of FP at different stages of treatment (dry mass basis, mean ± SD).

Fibers	**Contents (%, dry mass basis)**			
	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Total fiber
FP [1]	25.5 ± 2.8	48.1± 7.2	8.2 ± 0.2	81.8 ± 9.7
Acid treated FP	49.2 ± 1.0	11.1 ± 1.5	9.5 ± 0.8	69.9 ± 1.6
Acid-alkali treated FP	95.2 ± 0.8	0.0	1.8 ± 0.0	97.0 ± 0.8
Cellulose fiber	98.7 ± 0.3	0.0	0.0	98.7 ± 0.3

[1] Fiber-rich product (FP) obtained from brewer's spent grain.

6.3.2 Screening of the four BSG cellulose fibers for Mn and Pb adsorption

6.3.2.1. Particle size of cellulose fibers

Fiber with a small particle size provides a high specific surface area and short intraparticle diffusion distance, contributing to high metal adsorption and fast kinetics (Liu et al., 2016). Therefore, both oxidized and non-oxidized cellulose fibers were submitted to ultrasonication treatment to reduce particle size. The differential volume versus the particle size distribution of fiber solutions and the photos of the fibers dispersed in water (1% w/v) are shown in **Figure 6.1**. Among the four fibers, CF without TEMPO oxidation or ultrasonic treatment (**Figure 6.1**A) had

the widest particle size distribution, with particle diameters from 4 µm to 3780 µm. When the CF was treated with ultrasound to get UCF, the particle diameters of UCF were in the same range as those of CF, but the differential volume of smaller particles of UCF increased (**Figure 6.1**B). This was also observed in the decreased values of the area-based mean diameter D (3,2) and the volume-based diameter D (4,3) shown in **Table 6.2**. It indicates that ultrasonication did help to reduce the particle size of CF, but the fiber size was not at the nanoscale (~100 nm) (Nanodic, 2021), which might be due to the low power (500 W) used for ultrasonic treatment. Nano-scale cellulose fiber was reported when the power was increased to 1,000 W while the ultrasonic processing time was the same with the current study (30 min) and ultrasonication frequency was 20–25 kHz (Chen et al., 2011). The UCF did not sediment but remained suspended in water because of its increased swelling capacity and higher surface charge density (Huerta & Saldaña, 2019) compared to CF. The CF after TEMPO oxidation (TOCF) showed a narrower particle diameter distribution, which was in the range of 6 µm to 1738 µm, and an increased differential volume of small particles (**Figure 6.1**C), which might be due to the fact that TEMPO oxidation introduced negative charges leading to electrostatic repulsion forces to disperse cellulose microfibrils (Serra et al., 2017). Further ultrasonication contributed to a sharp decrease in particle diameters (15 nm–360 µm) (**Figure 6.1**D) and the lowest values in D (3,2) and D (4,3) values (**Table 6.2**), which was due to the formation of carboxyl groups during the TEMPO oxidation, and a mild mechanical treatment is enough for the fibrillation of cellulose fibers (Levanič et al., 2020). Moreover, the sonication power destroyed the intermolecular hydrogen bonds in cellulose, resulting in the production of nano-scale fibers (Nasri-Nasrabadi et al., 2014). The transparency of the suspension of UTOCF as shown in **Figure 6.1**D is due to the small fiber particles of UTOCF that cannot reflect light as the other three fibers.

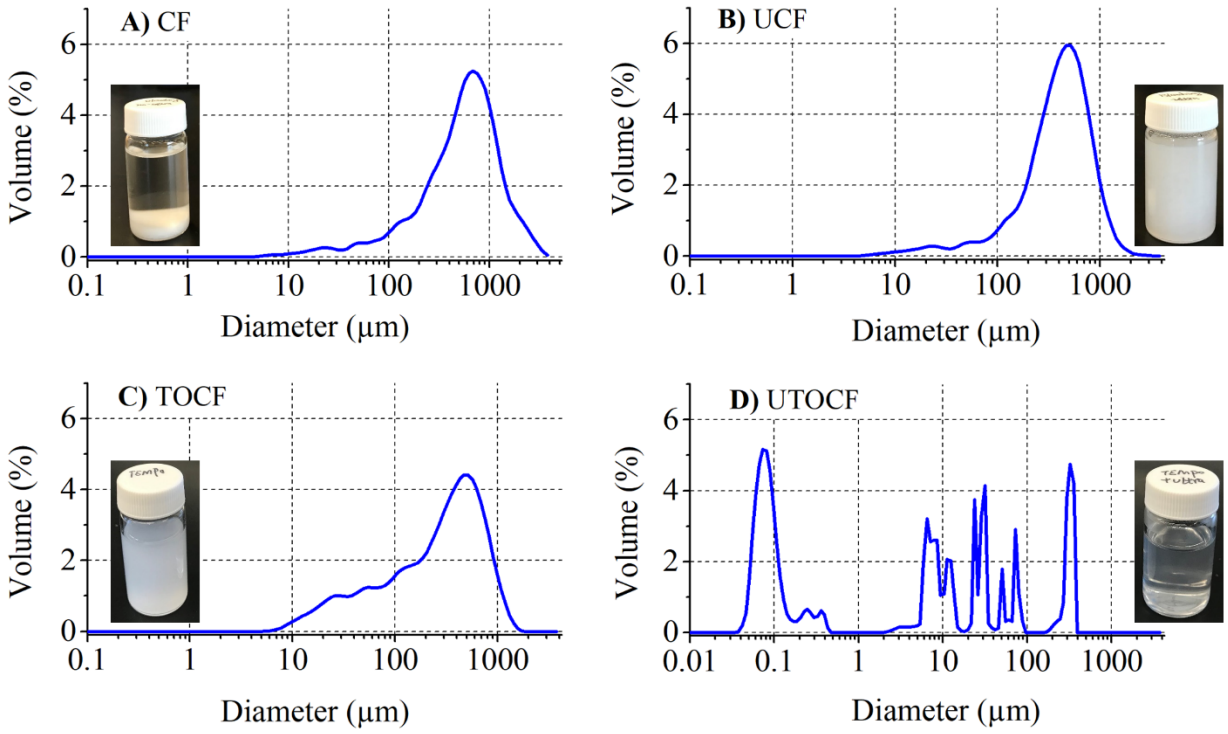

Figure 6.1 The differential volume versus the particle size distribution of four cellulose fibers produced from the fiber-rich product grained from brewer's spent grain. CF: cellulose fibers; UCF: CF treated with ultrasound; TOCF: CF treated with TEMPO-mediated oxidation; and UTOCF: TOCF treated with ultrasound.

Table 6.2 Detailed particle size information of the four fibers.

Parameter (μm)	Four cellulose fibers produced from fiber-rich product obtained from brewer's spent grain[1]			
	CF	UCF	TOCF	UTOCF
D (3,2)	208.6 ± 5.6^{a,2}	180.3 ± 5.8^{b}	96.3 ± 2.6^{c}	0.2 ± 0.0^{d}
D (4,3)	681.4 ± 34.0^{a}	469.2 ± 19.5^{b}	357.2 ± 10.1^{c}	92.8 ± 14.4^{d}
D10	128.6 ± 3.9^{a}	123.8 ± 5.2^{a}	34.1 ± 1.1^{b}	0.1 ± 0.0^{c}
D50	576.1 ± 14.2^{a}	419.0 ± 16.8^{b}	302.3 ± 10.7^{c}	7.7 ± 1.0^{d}
D90	1343.7 ± 81.7^{a}	861.0 ± 47.4^{b}	767.5 ± 19.5^{b}	335.2 ± 18.7^{c}

[1] CF: cellulose fibers; UCF: CF treated with ultrasound; TOCF: CF treated with TEMPO-mediated oxidation; and UTOCF: TOCF treated with ultrasound.

[2] Values in the same row with different superscripts are significantly different ($p < 0.05$).

6.3.2.2 Metal ions adsorption of cellulose fibers

All four cellulose fibers were tested for the adsorption of Pb (II) and Mn (II) that with an initial concentration of 0.3 mM. The adsorption results are shown in **Figure 6.2**. The TEMPO oxidation of cellulose fiber can significantly increase the adsorption of Pb and Mn due to the induced carboxylic groups. CF and UCF, which were not undergone TEMPO oxidation had limited ability for the removal of Pb and Mn. TOCF showed the highest Pb and Mn adsorption at 1 h, 56.7 mg/g and 16.6 mg/g, respectively. Longer adsorption time did not improve its adsorption capacity. The results agree with a previous study in which commercial cellulose fabrics oxidized with TEMPO sorbed Pb at the initial Pb concentration of 58.3 mg/L (0.28 mM), the adsorption reached equilibrium status in 1 h and the adsorption capacity was ~50 mg/g at pH 5 (Li et al., 2020). It should be point out that although the pH value (5) was different with the current study (pH 6.5), the adsorption capacity was similar, which agreed with a previous report that the adsorption capacity of TEMPO-oxidized cellulose fibers to heavy metal ions is not affected at pH $\geqslant$ 4.0 (Isobe et al., 2013). Compared to TOCF, UTOCF had lower Pb and Mn adsorption at 1 h, which may have resulted from the small UTOCF with particle size $\leqslant$ 0.45 μm that were not separated by the filtration, the fibers with adsorbed metals went to the filtrate leading to the high values of measured metals concentrations.

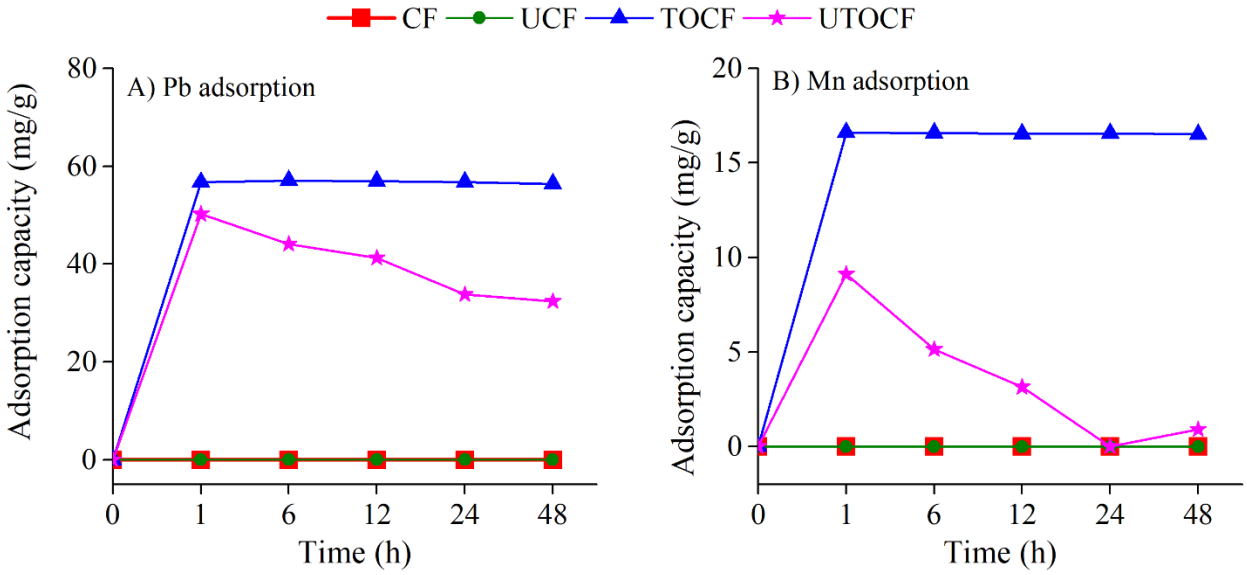

Figure 6.2 Pb and Mn adsorption with four cellulose fibers that were produced from the fiber-rich product (FP) obtained from brewer's spent grain. CF: cellulose fibers; UCF: CF treated with ultrasound; TOCF: CF treated with TEMPO-mediated oxidation; and UTOCF: TOCF treated with ultrasound. Adsorption capacity indicates mg of metals ions is adsorbed by per g of cellulose fiber.

Moreover, using small-particle-size cellulose adsorbent may release organic compounds into the water if the followed filtration treatment is not efficient enough, leading to the total organic carbon (TOC) exceeding the guidance level (⩽ 2 mg/L) in tap water (EPA, 2016). The TOC concentrations in the supernatant obtained after the fiber adsorption at 24 h were: CF 1.4 ± 0.2 mg/L, UCF 1.7 ± 0.2 mg/L, TOCF 14.7 ± 0.7 mg/L, and UTOCF 46.2 ± 0.8 mg/L, respectively. However, when the TOCF dosage decreased from 1.0 g/L to 0.5 g/L, TOC was measured at 2.0 ± 0.5 mg/L, which was around the guidance level. Therefore, TOCF was chosen in the following adsorption study, and the fixed dosage of 0.5 g/L TOCF was used.

6.3.3 Adsorption kinetics and isotherms

6.3.3.1 Adsorption kinetics

The time-dependent adsorption experiments were performed with the initial concentrations of Pb and Mn were at 140 mg/L and 30 mg/L, respectively. The pH values of the solutions were 6.5. The adsorption capacity of TOCF increased rapidly at the beginning 5 min followed by a subsequent slow increase from 5 to 15 min, and then approached the equilibrium status for both Mn and Pb adsorption (**Figure 6. 3** A and B), indicating that there was a strong interaction between TOCF and metal ions (Mn and Pb). Specifically, the adsorption capacity of TOCF for Mn was 44.6 ± 0.7 mg/g at 1 min, it increased to 47.3 ± 0.5 mg/g at 60 min. However, there were no significant differences ($p > 0.05$) among the adsorption capacities got from experiments with adsorption time changing from 3 min to 60 min. The Mn removal was changed from 79.0% to 83.9% when the adsorption time increased from 1 min to 60 min. The adsorption capacity of TOCF for Pb increased from 222.1 ± 5.2 mg/g to 239.4 ± 3.2 mg/g, and removal changed from 78.0% to 84.0% when the adsorption time changed from 1 min to 60 min, while there was no significant increase in adsorption capacity and removal when the adsorption time longer than 3 min. The corresponding fitting results with pseudo-first-order and pseudo-second-order kinetic models were used to describe the kinetics of the adsorption of metal ions onto TOCF (**Figure 6.3** C and D). The kinetic parameters obtained from both models were listed in **Table 6.3**. It was found that the time-dependent adsorption behavior was better described by the pseudo-second-order kinetic model (R^2 = 1.00) than the pseudo-first-order kinetic model (R^2 = 0.74). Moreover, the experimental adsorption capacity ($Q_{e,\,exp}$, 47.7 mg/g for Mn and 241.7 mg/g for Pb) and the calculated adsorption

capacity ($Q_{e, cal}$, 47.4 mg/g for Mn, 239.8 mg/g for Pb) based on the pseudo-second-order kinetic model agreed well with each other. Therefore, the pseudo-second-order kinetic model was considered to provide the most accurate and comprehensive reflection of the adsorption mechanism of TOCF, indicating that the adsorption behavior of Pb and Mn was controlled by the chemical adsorption by electron sharing and exchange between the metals ions and carboxylate groups on TOCF (Li et al., 2020; Wang et al., 2019).

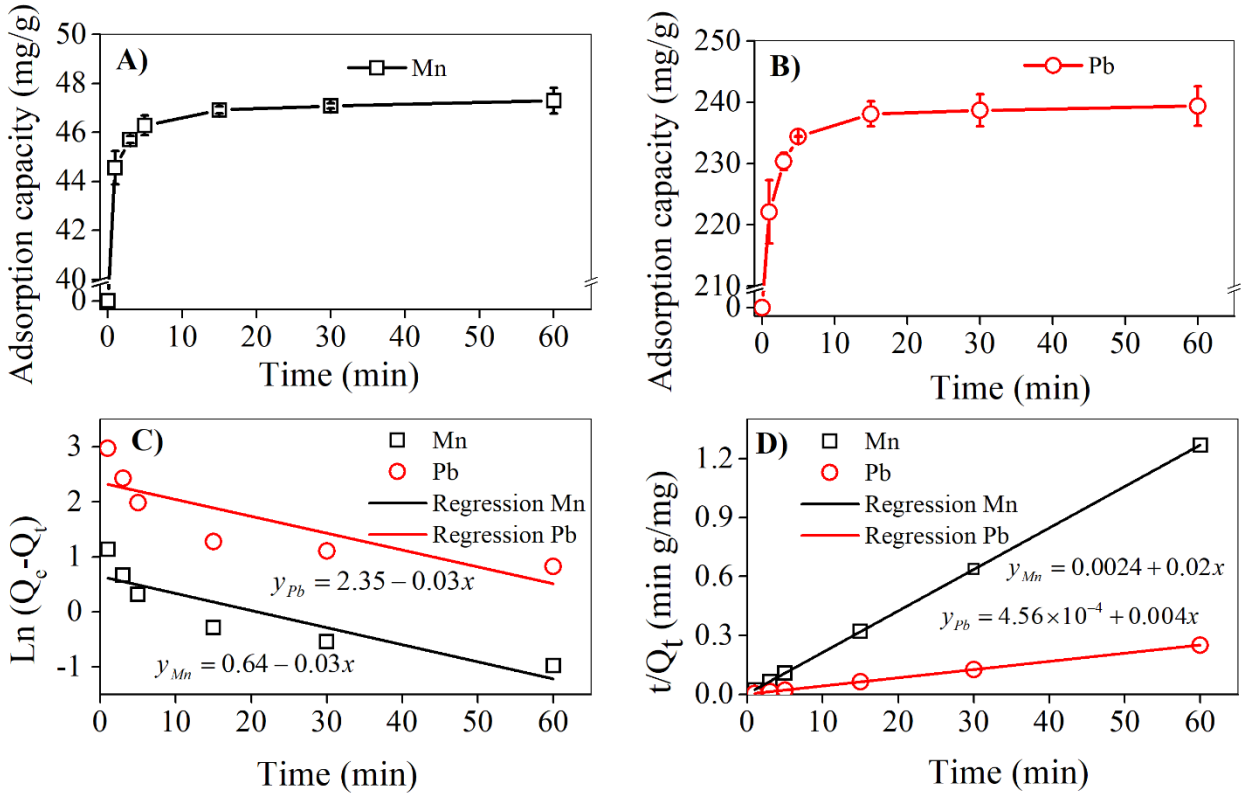

Figure 6.3 Adsorption capacities of TOCF for Mn (A) and Pb (B) as a function of time, and fitting results with the pseudo-first-order kinetic model (C) and pseudo-second-order kinetic model (D)

6.3.3.2 Adsorption isotherms

An adsorption isotherm is useful to describe the relationship between the metal adsorbate concentration in solution and the amount adsorbed at the TOCF interface at a constant temperature. In this study, three adsorption isotherms, Langmuir, Freundlich, and Temkin, were used to analyze the data. **Figure 6.4** shows the adsorption capacity (Q_e, mg/g) for Mn and Pb ions as a function of the initial ion concentrations, as well as the fitting results with the three models. All the resulting parameters are shown in **Table 6.3**. A comparison of the three models revealed that R^2 values by a fit with the Langmuir model for both Mn and Pb were 0.998, whereas those of the Freundlich

and Temkin models were in the range 0.808–0.917 and 0.837–0.954, respectively, suggesting that the Langmuir model well-describes the adsorption behavior of Mn and Pb onto the TOCF. The Langmuir model assumes that monolayer adsorption with homogeneous binding sites and no interactions among adsorbed species (Ma et al., 2016). The maximum adsorption capacity (Q_m) of the TOCF for Pb (272.5 mg/g) was much higher than Mn (52.9 mg/g) on a mass basis, and more similar on a molar basis, Pb (1.3 mM/g) compared to Mn (0.96 mM/g).

The Pb adsorption capacity of TOCF, 272.5 mg/g produced in the current study was very similar to TEMPO-oxidized cellulose hydrogel modified by polyethyleneimine (279.3 mg/g) (Xing et al., 2021), but higher than the cellulose-based adsorbent prepared with other methods, such as cellulose chemically modified with succinic anhydride (205.9 mg/g) (Gurgel et al., 2008) and guanyl-modified cellulose (52.0 mg/g) (Kenawy et al., 2018). This was the first study using the modified absorbent made from BSG cellulose to remove Mn in contaminated water. The adsorption capacity of TOCF (52.9 mg/g) for Mn was lower than amino-functionalized graphene oxide (161.3 mg/g) (Suddai et al., 2018), while higher than other adsorbents, such as granular activated carbon (2.6–7.6 mg/g) (Goher et al., 2015), graphene oxide (41.7 mg/g) (Suddai et al., 2018), manure derived biochar (2.8–6.7 mg/g) (Idrees et al., 2018). Overall, TOCF produced in our study using BSG fiber had a great adsorption capacity for both Pb and Mn.

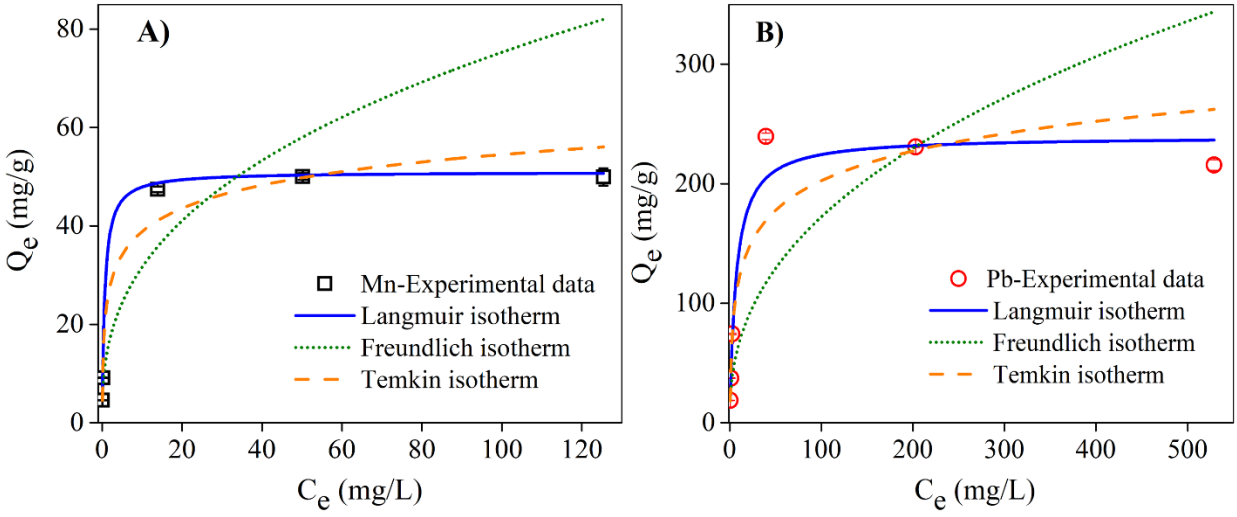

Figure 6.4 Isotherms showing the adsorption of Mn (A) and Pb (B) ions onto TOCF and fitting results with Langmuir, Freundlich, and Temkin isotherm models.

Table 6.3 Kinetic and isotherm model parameters for Mn and Pb adsorption onto TOCF.

Type of models	Parameters	TOCF	
		Mn	Pb
Kinetic models			
	$Q_{e,\,exp}$ (mg/g)	47.67	241.69
Pseudo-first order	$Q_{e,\,cal}$ (mg/g)	1.90	10.45
	K_1 (min^{-1})	-5.18×10^{-4}	-5.10×10^{-4}
	R^2	0.7416	0.6146
Pseudo-second order	$Q_{e,\,cal}$ (mg/g)	47.35	239.81
	K_2 (min^{-1})	0.19	0.04
	R^2	1.000	1.000
Isotherm models			
Langmuir	Q_m (mg/g)	52.94	272.48
	K_L (L/mg)	1.5100	0.1275
	R^2	0.9978	0.9979
	R_L	0.0044-1.000	0.0122-0.4408
Freundlich	K_F (mg/g)	14.4115	36.4083
	1/n	0.31567	0.35162
	R^2	0.9167	0.8083
Temkin	A (L/mg)	36.9442	3.9050
	B (J/mol)	6.54473	33.9418
	R^2	0.95408	0.8366

6.3.4 Adsorption in tap water

The adsorption of metal ions can be affected by factors such as initial metal concentration, adsorbent dosage, pH, temperature, and the presence of other metals (Da'na & Sayari, 2012). Therefore, the TOCF removal of low concentrations of Mn and Pb together with interfering ions, typically present at mg/L concentrations in tap water, as well as the effect of pH, were investigated. The solubility of Pb was limited by pH-dependent precipitation of lead carbonate in tap water, so the initial concentration of Pb was varied depending on the pH: 0.32 mg/L (pH 6.5) and 0.03 mg/L (pH 7.5), meanwhile the measured initial concentration of Mn was 2.35 mg/L (pH 6.5) and 2.42 mg/L (pH 7.5). Plain tap water without adding Mn and Pb was also used for the comparison. The results are shown in **Table 6.4**. TOCF adsorbed divalent metal ions in plain tap water, especially Ca, Mg, Zn, and Cu. The initial concentrations of these competing divalent metal ions in the tap water were: 11.2 mg/L Ca, 4.7 mg/L Mg, 0.2 mg/L Zn, and 0.1 mg/L Cu. The total cation adsorption capacity (~ 830 µM cations/g TOCF) was not significantly affected by the pH of the water. When the water pH was 6.5, though the presence of Mn and Pb did not change the total

adsorption capacity dramatically, the adsorption of Mg, Cu, and Zn decreased. Interestedly, though the tap water (pH 7.5) with a lower initial Pb concentration (0.03 mg/L) than the tap water with pH 6.5 (0.32 mg/L), the total adsorption capacity on TOCF was the highest (870 μM divalent cations/g) due to the increased adsorption of Mn. To be noted, there was 1.8–1.9 mM of monovalent Na/g TOCF leached into the solution during the adsorption; the Na leached is approximately two times of the adsorption of the charges for the sum of divalent cations, ~ 0.9 mM/g (**Table 6.4**). For the contaminated tap water of pH 6.5, the concentration of Mn decreased from 2.35 mg/L to 0.38 mg/L, and Pb changed from 0.32 mg/L to 0.07 mg/L after adsorption. Meanwhile, the concentration of Mn decreased from 2.42 mg/L to 0.33 mg/L, and Pb changed from 0.32 mg/L to 0.00 mg/L after adsorption at pH 7.5. The Pb was removed below its USEPA regulatory level, although the Mn remaining in solution was higher and its target 0.05 mg/L. The above results indicated that the presence of interfering ions in tap water competed with and negatively affected the adsorption of Mn of Pb, for the active binding sites like carboxylic groups on the surface of TOCF. Similar results were reported that the adsorption of Cd and Pb on cellulose-based adsorbent decreased due to the presence of various other metals in an aqueous solution (Yakout et al., 2016).

Table 6.4 The adsorption capacity of metals in tap water onto TOCF (mean or mean ± SD)

Tap water[1]	Adsorption capacity (µM/g)[1]						
	Mn	Pb	Mg	Ca	Cu	Zn	**Sum[3]**
pH 6.5[2]							
Plain	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}	327.3 ± 2.0^{a}	496.3 ± 3.5^{a}	2.9 ± 0.0^{a}	4.9 ± 0.0^{a}	831.5 ± 5.6^{c}
+ Mn & Pb	72.1 ± 1.1^{b}	2.4 ± 0.2^{a}	296.5 ± 1.8^{c}	472.4 ± 3.3^{b}	2.8 ± 0.1^{a}	4.6 ± 0.1^{b}	850.9 ± 6.5^{b}
pH 7.5[2]							
Plain	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}	328.3 ± 0.5^{a}	497.0 ± 2.9^{a}	2.7 ± 0.0^{a}	4.9 ± 0.0^{a}	832.9 ± 3.4^{bc}
+ Mn & Pb	75.9 ± 0.3^{a}	0.3 ± 0.0^{b}	306.8 ± 2.1^{b}	479.8 ± 1.8^{b}	2.7 ± 0.0^{a}	4.8 ± 0.0^{ab}	870.3 ± 0.1^{a}

[1] Plain tap water and contaminated tap water with Mn and Pb (+ Mn & Pb).

[2] Values in the same row with different superscripts are significantly different ($p < 0.05$).

[3] Summation of all individual metals as µM cations/g TOCF.

6.3.5 Surface characterization of TOCF before and after adsorption of Mn or Pb

6.3.5.1 Scanning electron microscopy (SEM)

The effects of TEMPO oxidation and metal ions adsorption on the morphology of cellulose fiber were revealed by SEM images. SEM images of CF and TOCF before and after the adsorptions of Mn and Pb are shown in **Figure 6.5**. A comparison of CF to TOCF revealed clear morphological differences. TEMPO oxidation unfolded the structure of CF and reduced the thickness of the fiber. The surface of TOCF looks smoother than that of CF due to its smaller particle size. These changes were in line with a previous morphology study which showed that TEMPO-oxidized cellulose fiber had a smoother surface than untreated cellulose fiber had (Levanič et al., 2020). However, the adsorption of Mn and Pb onto TOCF decreased the smoothness of TOCF. This suggests that Mn or Pb was adsorbed on the surface of TOCF and formed a layer of Mn or Pb that covered the TOCF. The adsorption of Cu onto TEMPO-oxidized cellulose nanofibers also showed a reduction of the smoothness of the fibers (Zhu et al., 2017).

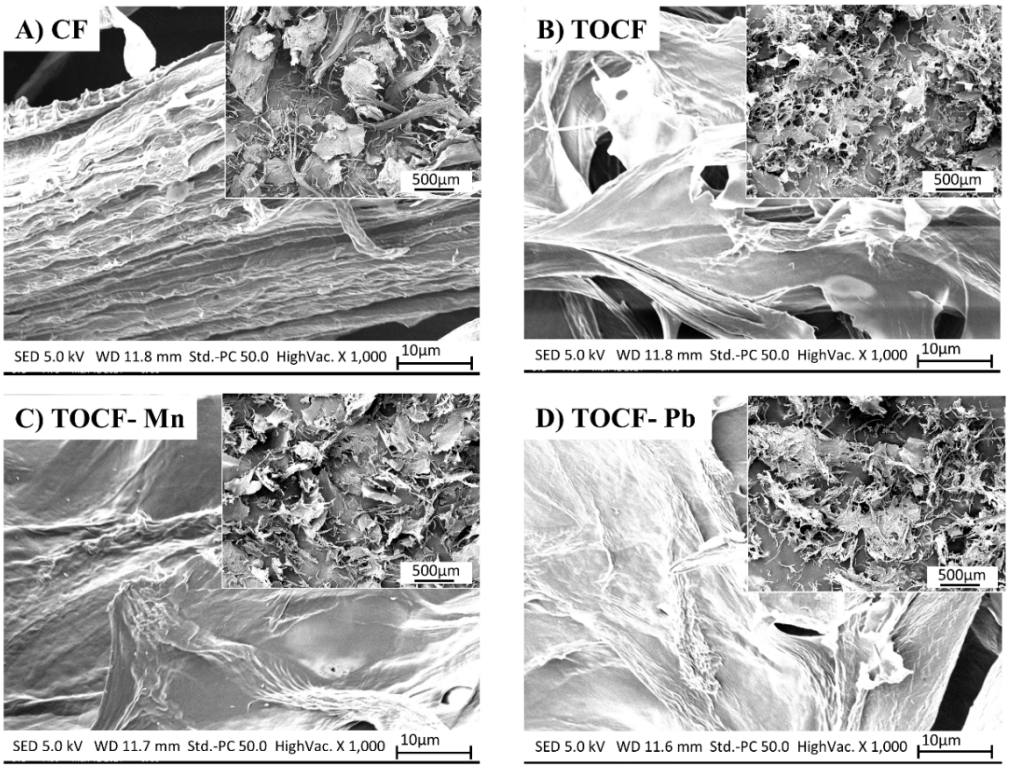

Figure 6.5 Scanning electron microscopy images (1,000 ×) of A) brewer's spent grain cellulose fiber (CF), B) TEMPO-oxidized fiber (TOCF), C) TOCF after adsorption of Mn, D) TOCF after adsorption of Pb.

6.3.5.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of pristine CF, pristine TOCF, and TOCF after adsorption of Mn and Pb are shown in **Figure 6.6**. The dominant spectral bands of CF (cellulose before oxidation) at 3390 cm^{-1}, 2899 cm^{-1}, 1060 cm^{-1} are ascribed to the stretching vibration of O—H, C—H, and C—O—C, respectively (Wang et al., 2019). These spectral bands were not affected by TEMPO-mediated oxidation and metal ions adsorption. Comparing the spectrum of TOCF (cellulose after TEMPO-mediated oxidation) with that of CF, a significant peak appears at 1610^{-1} corresponding to the antisymmetric stretching of COO— in carboxylate salts, indicating that the carboxyl groups were introduced on the cellulose after the TEMPO-mediated oxidation (Li et al., 2015). The peak at 1610 cm^{-1} shifted after Pb and Mn adsorption due to the combination of the carboxylate group with metal ions, indicating that metal adsorption on the TOCF affected the chemical bonds change. The results suggested that the ion exchange affected the adsorption behavior of TOCF, and the chemical adsorption was predominated (Wang et al., 2017). The results were consistent with that the pseudo-second-order kinetic model was suitable for the adsorption of Mn and Pb onto TOCF.

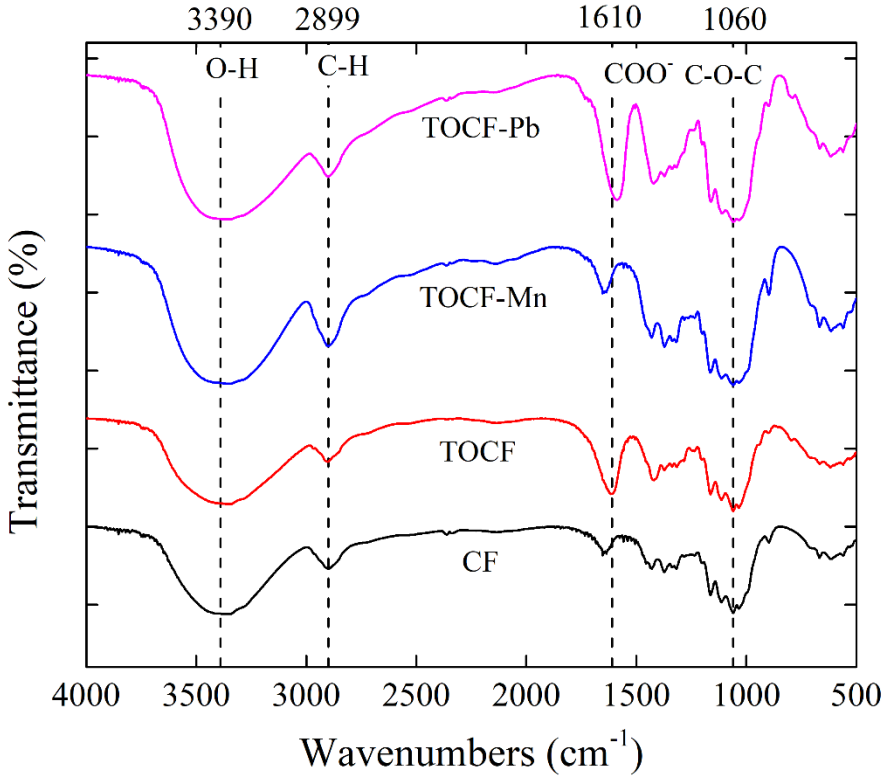

Figure 6.6 FTIR spectra of pristine cellulose fiber (CF), pristine TEMPO-oxidized CF (TOCF), and TOCF after adsorption of Mn and Pb.

6.3.5.3 X-ray diffraction (XRD)

The XRD patterns of CF and TOCF before and after Mn and Pb adsorptions are shown in **Figure 6.7**. The diffraction peaks at 2θ = 15.12º, 22.22º, 34.24º, corresponding to the ($1\bar{1}0$), (200), and (040) crystallographic planes, are typical cellulose I crystalline forms (Tang et al., 2017). The main 2θ diffraction peaks are identical for both CF and TOCF, indicating cellulose I crystals are resistant to TEMPO oxidation, and carboxyl groups introduced during oxidation were selectively formed on the crystal surface and amorphous regions not the inside of crystallites (El Bakkari et al., 2019). The occurrence of oxidation reaction resulted in a decrease in the peak intensity (Rohaizu & Wanrosli, 2017). TOCF peak positions remained unaltered after the Mn/Pb adsorption, demonstrating that the metal ions did not react or affect the crystalline structure (Hernández-Francisco et al., 2020).

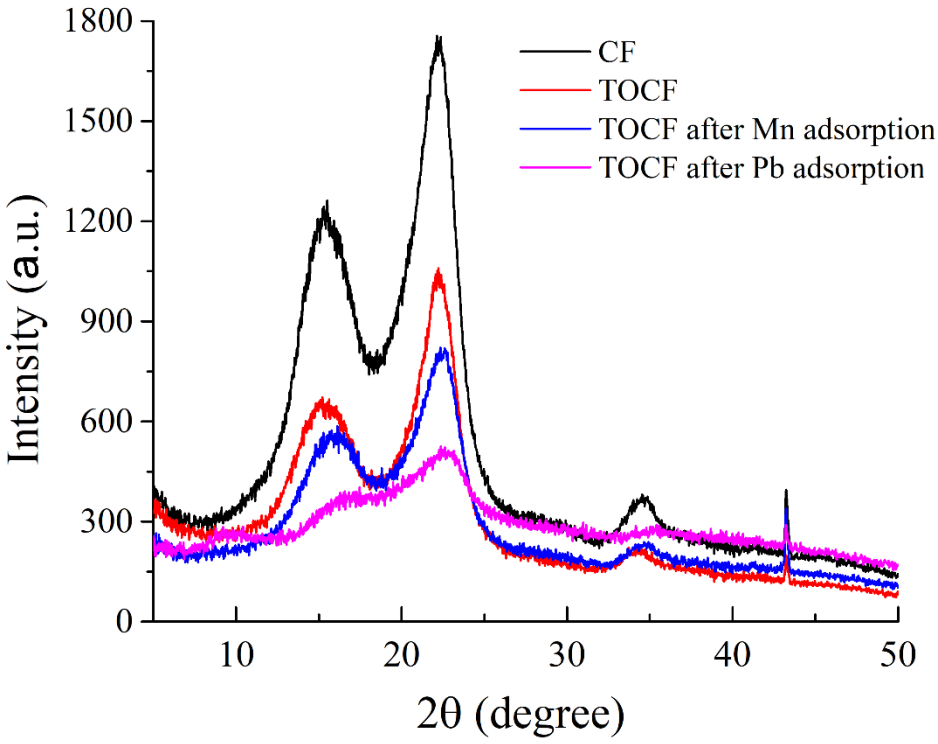

Figure 6.7 XRD patterns of TOCF before and after metal ions adsorption. CF: cellulose fibers; TOCF: CF treated with TEMPO-mediated oxidation.

6.3.6 Mass balance for cellulosic fiber production

The dry weights of biomass after each step (acid treatment, base treatment, bleaching, and oxidation) were used to develop a mass balance of the production of TOCF from FP, which was

originally from BSG (**Figure 6.8**). For each 1 kg of FP, 467.0 g of the biomass was recovered after the dilute acid treatment. Further alkali treatment reduced this recovered biomass to 161.6 g due to the removal of hemicellulose and most of lignin. The yield of CF in the followed bleaching step was up to 94.9% (153.3 g CF/161.6 g acid-alkali treated FP). The high yield was due to the non-cellulose components were mostly removed in the previous acid, the alkali treatment and bleaching only removed the low content of residual lignin and components that contribute to the yellow/brownish color of the fiber. The yield of TOCF during the TEMPO oxidation step was 83.1% (127.4 g/153.3 g). The relatively low yield was attributed to the sieving method (section 6.2.1) used to separate the high hydrophilicity of TOCF from liquid after the TEMPO oxidation. As mentioned, the particle size distribution of TOCF was 6 µm to 1738 µm (section 6.3.2.1), while the pore size of the sieve used in sieving was 75 µm that led to those particles with size less than 75 µm went to the waste stream. Overall, 127.4 g TOCF can be produced from 1 kg dry FP, which represents a 12.7% yield.

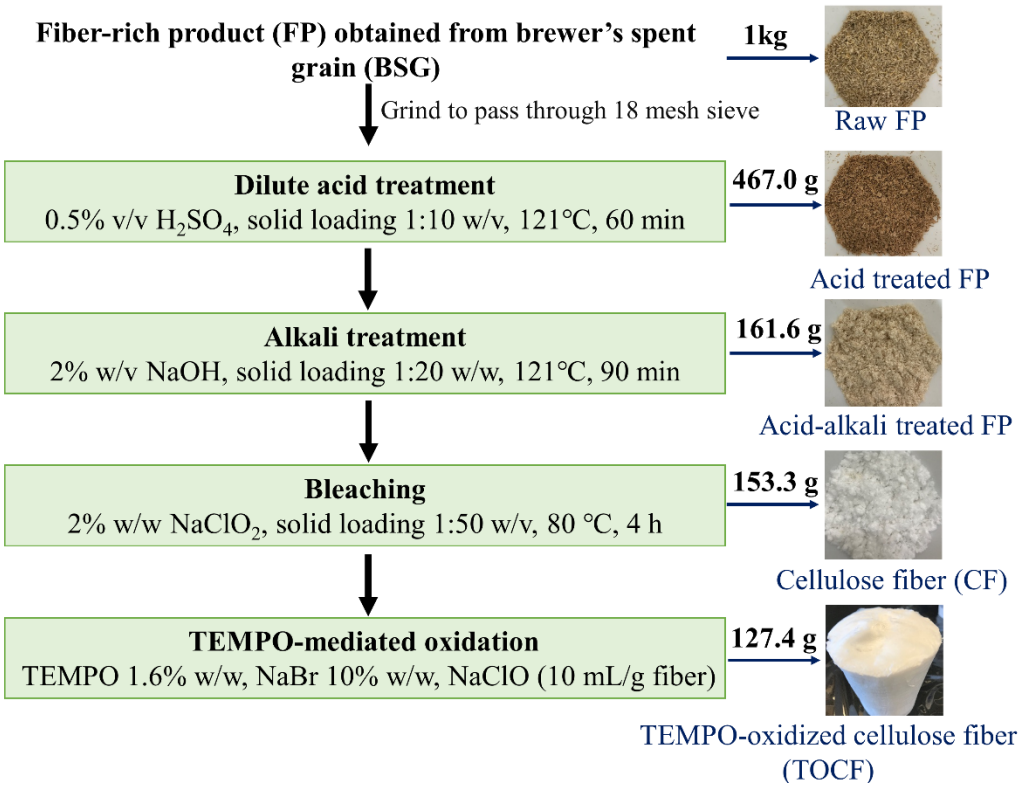

Figure 6.8 Mass balance for producing TEMPO-oxidized cellulose fiber (TOCF) adsorbent from 1 kg of fiber-rich product produced from brewer's spent grain.

6.4 Conclusions

FP obtained from BSG processing was successfully modified as an adsorbent (TOCF) for efficient removal of Mn (II) and Pb (II) from contaminated water. The kinetic study showed that the adsorption of metal ions on TOCF fitted the pseudo-second-order kinetic model with removal nearly complete within 15 minutes. The single metal adsorption isotherms were better described by the Langmuir model, and TOCF showed a high maximum adsorption capacity up to 272.5 mg/g for Pb and 52.9 mg/g for Mn. The TOCF produced from FP is expected to be a good candidate for an effective adsorbent of heavy metal ions in contaminated water.

Acknowledgements

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Chapter 7. Conclusions and Future Work

The overall goal of this work was to develop an integrated process to make full use of brewer's spent grain (BSG) and minimize waste generation during processing by producing value-added protein feedstuff and cellulose adsorbent. To achieve this goal, a wet fractionation process was developed to separate protein-rich and fiber-rich products from BSG (**Chapter 3**). Among the three reagents (sodium hydroxide, sodium bisulfite, and enzyme) that were used for assisting BSG protein separation, the enzyme (Alcalase) showed the best performance. Under the optimal conditions (Alcalase loading was 20 μL/g dry BSG, solid loading was 5% w/w, 4 h incubation time, 60 ℃ reaction time, and pH 8.0), 84% of the total protein in products was separated into the protein-rich product (PP), and the protein concentration in PP was 43%, almost double the protein concentration (23%) in original BSG. Further process optimization focused on the decrease of enzyme loading and incubation time, as well as the increase of the solid loading for enhancing the quality of PP and improving the economic feasibility of the process. Under the optimal conditions, the produced PP contains 46% protein. The PP was evaluated as an alternative to fishmeal in shrimp feed by conducting shrimp feeding trials (**Chapter 4**). The results indicated that up to 50% of fishmeal can be replaced by PP in the shrimp diet without compromising shrimp growth performance or feed utilization efficiency. After getting the promising results from the shrimp feeding trials, a techno-economic analysis was conducted to evaluate the economic feasibility of PP production from BSG (**Chapter 5**). The results showed that the minimum selling price of PP was lower than the current market price of fishmeal, indicating that using PP to replace fishmeal can potentially reduce feed costs for aquaculture farmers. The fiber-rich product, a co-product of the wet fractionation process and riches in cellulose and hemicellulose, was used to produce a cellulose adsorbent, which had a great potential to adsorb heavy metals including Pb and Mn from contaminated water. Overall, this work took advantage of the fractionation technique to upgrade a low-value waste material (BSG) to 1) PP as an alternative to the expensive fishmeal in aquafeed, and 2) cellulose adsorbent used for water treatment. The outcomes of this study could provide a low-cost and sustainable protein source to the aquaculture industry and an adsorbent for the water treatment industry, meanwhile, it created new ways for the brewing industry to manage the waste materials.

The work described in this dissertation provided possible valorization ways of food processing byproducts, especially upgrading those with high-fiber and low-protein contents. As mentioned, enzymatic hydrolysis is an effective and environmentally friendly technology for protein separation. However, its wide utilization might be limited by the high cost of the enzyme. Cooperation with people who have expertise in enzymes to develop low-cost and efficient enzymes might be a way to enhance the valorization of food processing byproducts. In addition, this work demonstrated the protein-rich product obtained from BSG could be used as a feed ingredient in shrimp feed. Future studies could focus more on its effect on the gut microbiota of animals, and the gained knowledge might be helpful for the process optimization of production to further increase the inclusion ratio of the BSG protein products in animal feed. What is more, studies on using adsorbents including biochar, active carbon, and cellulosic adsorbents obtained from agriculture waste to treat intentionally contaminated water have been widely conducted, future research focusing on real contaminated water may provide more useful references to the practical application. Last but not least, the size of the above-mentioned adsorbents is usually in micro- or nano-scale level, which poses a high process requirement for the followed adsorbent separation after adsorption. Hence, future efforts could be on the improvement of adsorbents separation after the adsorption.

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Chapter 3. Wet fractionation process to produce protein-rich and fiber-rich products from brewer's spent grain

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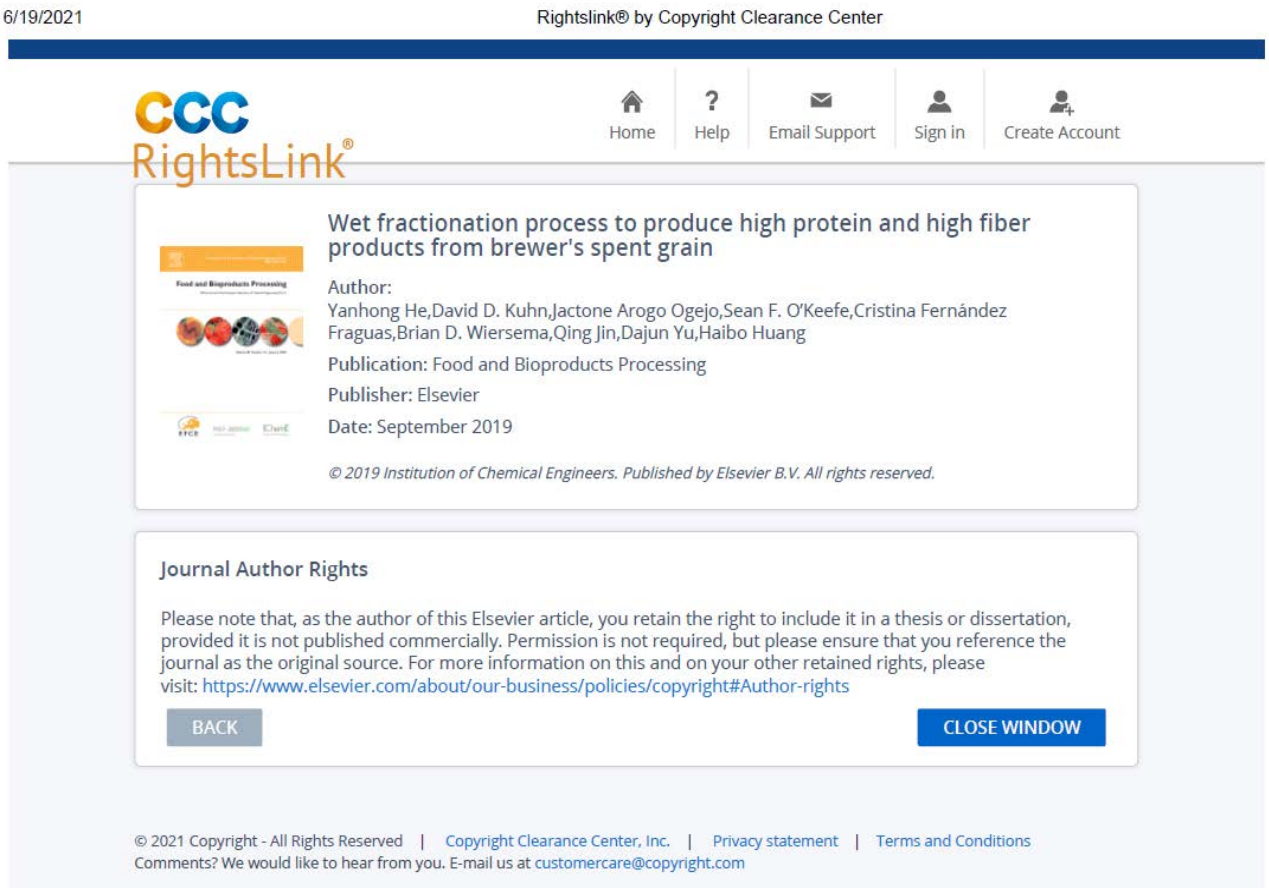

Chapter 4. Protein-rich product recovered from brewer's spent grain can partially replace fishmeal in diets of Pacific white shrimp, *Litopenaeus vannamei*

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