Evaluation of chemical and biological refinement strategies for transforming brewer's spent grain into a high-quality protein source for fish feed

Karlsen, Freja

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

DTU Aqua
National Institute of Aquatic Resources

Evaluation of chemical and biological refinement strategies for transforming brewer's spent grain into a high-quality protein source for fish feed

Freja Manø Busk Karlsen

PhD Thesis

DTU Aqua

National Institute of Aquatic Resources

Evaluation of chemical and biological refinement strategies for transforming brewer's spent grain into a high-quality protein source for fish feed

PhD Thesis by

Freja Manø Busk Karlsen

Main supervisor:

Peter Vilhelm Skov

Co-supervisor:

Ivar Lund

Technical University of Denmark

National Institute of Aquatic Resources

Section for Aquaculture, Hirtshals Denmark

Submitted on 20 September 2022

Table of Contents

Manuscript 3: Evaluation of brewer’s spent grain as a feedstock for amino acid production by submerged fermentation

Manuscript 4: Evaluating brewer’s spent grain and Ligno biomass as potential substrates for single cell protein production through submerged fermentation

Preface

This PhD thesis is submitted in partial fulfilment for obtaining a Doctor of Philosophy (Ph.D.) degree. The work was conducted during my PhD enrolment at The Technical University of Denmark (DTU), National Institute of Aquatic Resources (Aqua) in Hirtshals (Denmark). Furthermore, a great part of the work was performed in collaboration with the Industrial Biotechnology group at the Norwegian Research Centre (NORCE) in Stavanger (Norway).

The work was undertaken under supervision by principal supervisor Associate Professor Peter Vilhelm Skov and co-supervisor Senior Researcher Ivar Lund. In addition, Senior Researcher Sushil Gaykawad and Chief Scientist Catherine Boccadoro were responsible for the work performed at NORCE.

During my PhD enrolment, several people have played an indispensable role to whom I want to express my gratitude. First, I would like to thank my main supervisor Peter Vilhelm Skov for giving me the opportunity to pursue my dreams of a research career and for always believing in my skills. Many thanks to Laboratory Technician Ulla Sproegel for assisting with the practical work, especially the sample analysis. Huge thanks to my colleague and friend Carlos Octavio Letelier-Gordo for offering his point of view on my work and for being supportive in times of doubt. A great thanks to Sushil Gaykawad and the rest of the people from the Industrial Biotechnology group at NORCE for integrating me in the group and for assisting with the planning and conduction of experiments. It has been an unforgettable experience to work with you!

Last but not least, I want to thank my family for their endless support and patience.

Abstract

Aquaculture is the major supplier of fish for human consumption. An expanding human population will lead to intensification of fish farming as well as production of aquafeeds in the coming years. Fishmeal and soybean meal constitute the most common protein sources in aquafeeds due to their high nutritional value. Yet, increasing demands and competition for resources have driven up the prices of these protein sources which have heightened a need to implement sustainable alternatives. Brewer's spent grain (BSG) is the most abundant by-product from beer production. Due to its low market value, immense availability throughout the year and relatively high protein content (20-30% DM), BSG represents a potential protein source for aquafeeds. Inclusion of unrefined BSG in aquafeeds is hindered due to its high levels of lignin, cellulose and hemicellulose which are antinutritional factors (ANFs) for fish. Furthermore, the protein content of unrefined BSG is too low to meet the protein requirement of most fish species (25-50%). The main objective of this PhD project was to evaluate different refinement strategies for BSG that may allow for its incorporation in aquafeeds. The PhD project consisted of three parts: 1) protein extraction, 2) delignification by solid-state fermentation (SSF) and 3) valorization of cellulose and hemicellulose using a combination of hydrolysis and submerged fermentation.

Part I intended to optimize an alkaline process for extracting proteins from BSG. For this purpose, several parameters were evaluated including temperature (30, 45, 60°C), duration (30, 60, 120, 180 min), isolation mode (precipitation vs freeze-drying), and repeated extraction (single vs triple). The following conditions led to the highest protein content (55% DM) but, unfortunately, the lowest recovery (~10%): 60°C, 30 min, precipitation and single extraction. Conversely, freeze-drying combined with triple extraction maximized the protein recovery (~45%) but did not enrich the protein content of isolates (~25% DM).

Part II explored the potential of utilizing SSF to delignify BSG using the fungus *Phanerochaete chrysosporium*. In an attempt to enhance delignification, *P. chrysosporium* was cultured on solid BSG substrates supplemented with veratryl alcohol, MnO_2 and excess trace elements. The obtained results indicated that delignification occurred at a slow pace (0.75%/day) and that chitin accumulated during

cultivation. Surprisingly, no differences were found between the supplemented substrates and the control (no supplementation).

Part III had a dual purpose. First, it aimed to hydrolyse cellulose and hemicellulose into fermentable sugars, thereby forming a liquid BSG hydrolysate for fermentation. Second, the sugar-containing BSG hydrolysate was utilized as a substrate for production of free amino acids and single cell protein (SCP) by submerged fermentation. Initially, different microorganisms were screened with respect to their ability to grow and produce amino acids in BSG-based media. This screening resulted in selection of two microorganisms: *Saccharomyces cerevisiae* and *Corynebacterium glutamicum*. *C. glutamicum* was the most promising as it produced several amino acids in shake flasks and bioreactor including alanine (Ala), proline (Pro), valine (Val) and glycine (Gly). The amino acid composition and protein content were determined for *S. cerevisiae* biomass to assess its potential as a protein source. This analysis showed a relatively high protein content (~38% DM) and an amino acid profile resembling that of fishmeal except for deficiencies in methionine (Met) and histidine (His).

In conclusion, this PhD project provided vital insight into various refinement strategies of BSG. Low efficiencies were a general limitation of all evaluated processes and therefore further process optimization should be the focus of future research.

Resumé

Globalt set udgør akvakultur den primære producent af marine fødevarer. Som følge af den globale befolkningstilvækst forventes det, at produktionen af opdrættede fisk vil intensiveres i de kommende år. Protein er den vigtigste ingrediens i fiskefoder, idet fisk har et højt proteinbehov (25-50%). De mest udbredte proteinkilder inden for akvakultur er fiskemel og sojabønnemel, som har et højt indhold af protein og en favorabel aminosyresammensætning. En stigende efterspørgsel på fødevarer kombineret med en øget konkurrence om naturlige ressourcer har dog afstedkommet en enorm prisstigning på fiskemel og sojabønnemel. Disse omstændigheder har øget behovet for at finde og implementere bæredygtige alternativer. Mask, som er det primære restprodukt fra ølbrygning, udgør en potential ny proteinkilde til fiskefoder, da det produceres billigt, er tilgængeligt i store mængder hele året og har et relativt højt proteinindhold (20-30% af tørstof). Inkludering af ubehandlet mask i fiskefoder begrænses dog af et højt indhold af lignin, cellulose og hemicellulose, som er antinæringsstoffer for fisk. Derudover er proteinindholdet i ubehandlet mask for lavt til, at det kan anvendes som hovedingrediens i fiskefoder. Hovedformålet med dette PhD projekt var at evaluere forskellige valoriseringsstrategier for mask som muligvis vil kunne facilitere en implementering i fiskefoder. Projektet bestod af tre overordnede dele: 1) protein ekstrahering, 2) ligninfjernelse ved faststoffermentering (”solid-state fermentation”) og 3) udnyttelse af cellulose og hemicellulose via hydrolyse og fermentering i flydende medium (”submerged fermentation”).

Del I havde til formål at optimere en basisk proces til ekstrahering af protein fra mask. I denne optimering blev betydningen af forskellige parametre undersøgt, herunder temperatur (30, 45, 60°C), varighed (30, 60, 120, 180 min), isoleringsmetode (udfældning vs frysetørring) og multipel ekstrahering (single vs triple). Følgende betingelser gav det højeste proteinindhold i koncentrat fra mask (~55% af tørstof) men samtidig også det laveste proteinudbytte (~10%): 60°C, 30 min, udfældning og single ekstrahering. Omvendt blev proteinudbyttet (~45%) maksimeret ved triple ekstrahering og frysetørring, imens proteinindholdet (~25% af tørstof) ikke blev signifikant forbedret under disse betingelser.

Del II undersøgte muligheden for at anvende faststoffermentering til fjernelse af lignin fra mask. Den ligninnedbrydende svamp *Phanerochaete chrysosporium* blev dyrket

på masksubstrater tilsat veratryl alkohol, MnO_2 eller overskud af sporstoffer. Resultaterne fra dette vækstforsøg indikerede en langsom ligninnedbrydning af masken (0.75%/dag) samt en ophobning af kitin. Det mest overraskende resultat var dog, at der ikke var signifikant forskel på ligninnedbrydningen i de forskellige masksubstrater, hvilket indikerede at de tilsatte additiver ingen betydning havde for svampens evne til at nedbryde lignin.

Del III var todelt. I den første del blev cellulose og hemicellulose hydrolyseret enzymatisk, hvor der blev dannet et flydende maskhydrolysat bestående af simple sukre. I den anden del blev dette hydrolysat anvendt som substrat til at producere frie aminosyrer og mikrobielt protein (”single cell protein”) ved en klassisk fermenteringsprocess i flydende medium. Indledningsvist blev en række mikroorganismer testet for deres evne til at gro og producere aminosyrer i et mask-baseret medium, hvilket resulterede i selektering af to aminosyre-producerende mikroorganismer, nemlig bakterien *Corynebacterium glutamicum* og gærsvampen *Saccharomyces cerevisiae*. Af disse to var *C. glutamicum* den mest lovende da den producerede forskellige aminosyrer både i rystekolber og i bioreaktor, herunder alanin (Ala), prolin (Pro), valin (Val) og glycin (Gly). Aminosyresammensætningen og proteinindholdet i biomasse fra *S. cerevisiae* blev desuden bestemt og brugt til at vurdere hvorvidt mask-dyrket *S. cerevisiae* kan anvendes som en anden potentiel proteinkilde i fiskefoder. Denne analyse viste et relativt højt proteinindhold på ~38% af tørstoffet samt en aminosyresammensætning, der ligner den i fiskemel med undtagelse af methionin (Met) og histidin (His) som begge var mangelfulde i biomasse fra *S. cerevisiae*.

Overordnet set kan det konkluderes, at dette PhD projekt har bidraget med vigtig viden om potentielle valoriseringsstrategier for mask. En lav effektivitet syntes dog at være en begrænsning for alle de testede processer. Derfor bør fremtidige studier fokusere på at optimere processerne, som er rapporteret i denne afhandling før en egentlig implementering i fiskefoder kan realiseres.

Synopsis

1. Introduction

1.1 Current challenges in aquafeed production

Fish is an important source of nutrients for humans, particularly protein, fatty acids, vitamins and minerals (Mishra, 2020). Capture fisheries and aquaculture constitute the major producers of fish for human consumption. Traditionally, capture fisheries have been the main supplier of fish; however, the growing demand for marine protein combined with declining stocks of wild fish have accelerated a transition from capture fisheries to aquaculture (Boyd, 2015). From 1985 to 2018, aquaculture production increased from ~15 to ~82 million tonnes while capture fisheries remained stable around 90 million tonnes with minor annual fluctuations (FAO, 2020). Over the next decade, aquaculture will continue to increase and has been projected to reach 105 million tonnes by 2029, thereby surpassing the production of fish from capture fisheries (OECD/FAO, 2020). To meet the forecasted food demand of the world's population in 2050, aquaculture production must increase from ~82 (2018) to 129 million tonnes (Boyd et al., 2022). This will inevitably lead to intensification of aquafeed production, hence increasing the exploitation of the resources used to produce the main feed ingredients. Fishmeal and soybean meal are staple ingredients in aquafeeds due to their high protein content and well-balanced amino acid profile. Still, the intensified use of these ingredients is considered economically unsustainable owing to their high prices caused by limited availability of resources and increased competition (Jannathulla et al., 2019). Therefore, the growing demand for fish, along with increasing consumption of fishmeal and soybean meal, have prompted endeavors to identify alternative ingredients for aquafeeds. Proteinaceous waste from industrial production could potentially be utilized as novel ingredients in aquafeed although implementation of these by-products often requires extensive valorization. In this context, the PhD project sought to explore different strategies to valorize industrial residues for aquafeed production focusing on lignocellulosic by-products. Throughout the thesis, the terms aquafeed and fish feed are used interchangeably.

1.2 An introduction to fish nutrition

1.2.1 General principles

Formulating feeds that meet the nutritional requirements of fish is crucial to sustain a profitable aquaculture production and minimize loss to the environment. Like other animals, fish require nutrients to grow and reproduce optimally. The nutritional constituents of a fish feed can be divided into two major classes: the macronutrients, also known as the energy-yielding nutrients, and the micronutrients, which include vitamins and minerals (Craig and Kuhn, 2017; Gatlin, 2010). The functions of micronutrients lie beyond the scope of this PhD project and will therefore not be discussed further here.

The macronutrients comprise three distinct groups including carbohydrates, lipids and proteins which generate different amounts of energy by oxidation. These nutrients provide building blocks for growth and an energy source for ATP production (Lall and Dumas, 2015). The chemical energy of carbohydrates, lipids and proteins make up the total amount of feed energy that can be ingested by fish. The energy of the ingested feed is either lost to the surroundings or assimilated in biomass, mainly as proteins and lipids. Energy losses result from faecal excretion of undigested nutrients or production of ammonia which are the main nitrogenous waste from protein digestion in fish (Lovell, 1998). For diets of high nutritional quality, energy loss is minimal due to high assimilation of nutrients in fish tissue.

The requirement and utilization of macronutrients differ between fish and endothermic animals in a number of aspects. One significant difference is that fish, unlike other animals, do not have a dietary carbohydrate requirement; yet, most fish species are able to oxidize carbohydrates in the feed to yield energy. Even though dietary carbohydrates are not essential for fish, they commonly provided in the feed to minimize oxidation of proteins and lipids (Wilson, 1994). Another important difference is the utilization of dietary protein and lipids. In warm-blooded animals, protein and lipids are mainly deposited in tissue while they constitute the principal energy sources in fish. In addition, protein represents the most important macronutrient in fish feed as fish are known to have a higher protein requirement than endothermic animals. For fish, the dietary protein requirement is generally found in the range of 25%-50% and varies greatly depending on the natural feeding behaviour, life-stage and abiotic

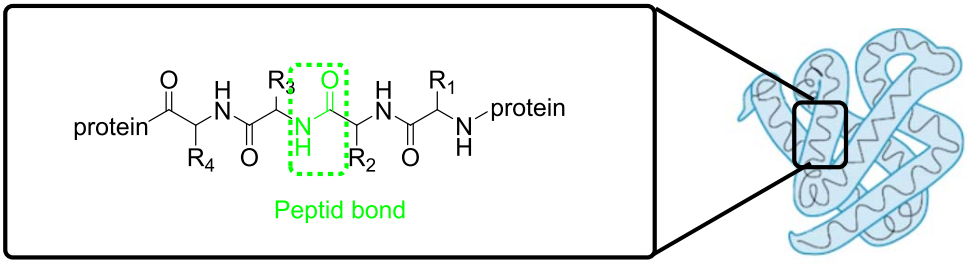
factors (Teles et al., 2020). The next section will explore the most significant facets of protein nutrition in fish including basic properties, digestion and nutritional aspects.

1.2.2 Protein

1.2.2.1 Structure, function and digestion

Proteins are polymers composed of 20 different amino acids that are linked together via peptide bonds (Figure 1A). Based on their biological functions, amino acids are classified into essential (EAA) and non-essential amino acids (NEAA). The EAAs cannot by synthesized by the organism and are therefore obtained from the diet while the NEAAs are produced de novo from intermediates of the central C-metabolism. Yet, the cellular synthesis of NEAAs is often too slow to meet the requirements for maximal performance and thus adequate quantities of NEAAs must be supplied in the feed (Oliva-Teles, 2012; Teles et al., 2020). Ten amino acids are essential for fish growth and health which include: threonine (Thr), methionine (Met), isoleucine (Ile), leucine (Leu), valine (Val), tryptophan (Trp), phenylalanine (Phe), histidine (His), lysine (Lys) and arginine (Arg). Likewise, there are ten amino acids, which are not essential for fish but still need to be present in the feed to achieve maximum performance. These are: serine (Ser), glycine (Gly), alanine (Ala), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), proline (Pro), cysteine (Cys) and tyrosine (Tyr) (Figure 1B). There is a tendency to report the nitrogen requirement of fish in terms of dietary protein content despite the fact that fish do not have a true protein requirement. Instead, fish require an adequate mixture of EAAs and NEAAs which are obtained from dietary proteins (Wilson and Halver, 1986).

A) Structural characteristics of proteins



B) Classification and structure of amino acids

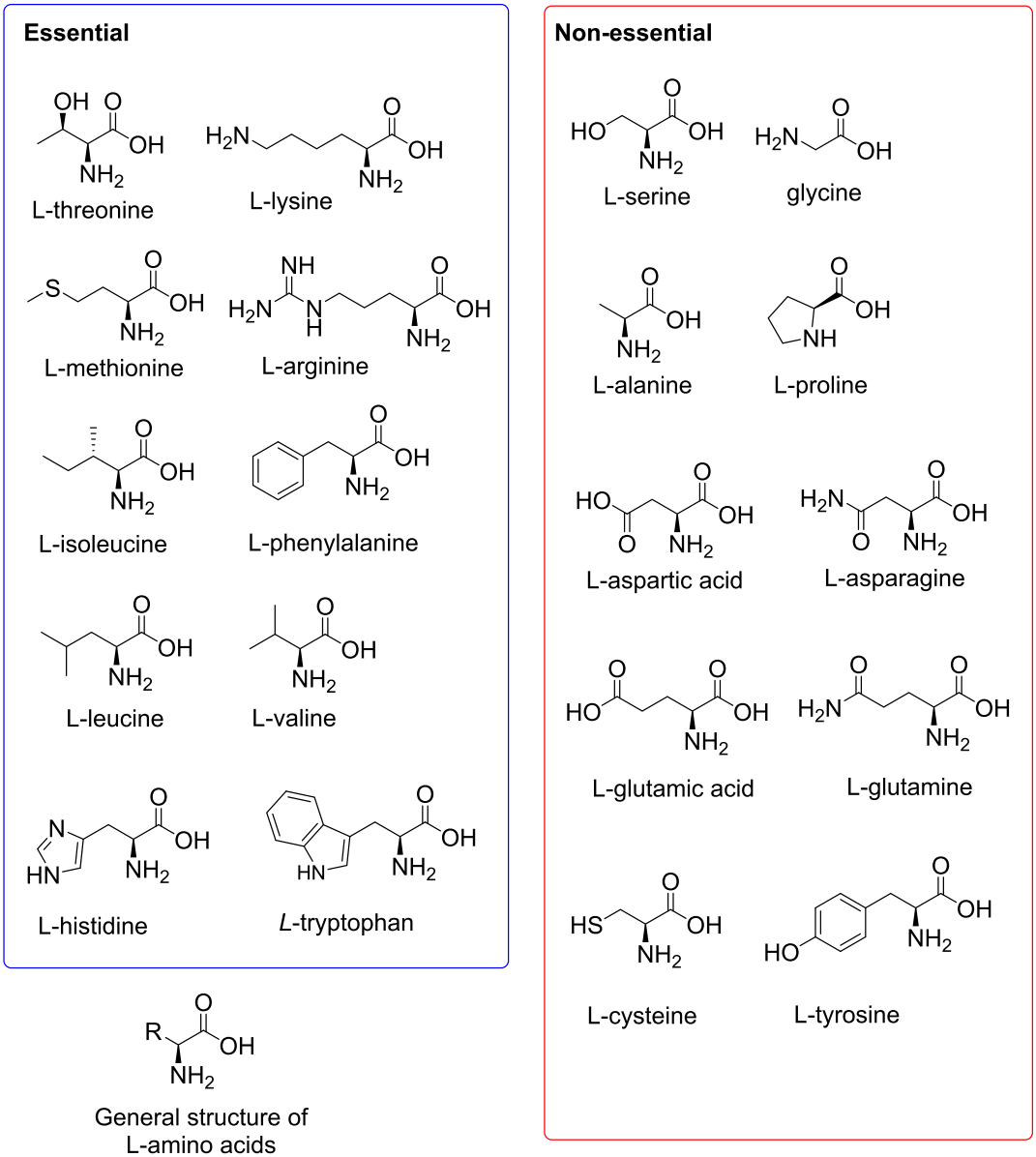


Figure 1. Structure of proteins (A) and amino acids (B).

During digestion, proteins are hydrolysed by digestive enzymes (proteases) to produce free amino acids which are utilized in two main metabolic processes. First, amino acids can be used as building blocks for the synthesis of cellular proteins which are deposited in tissue. Second, amino acids are the major metabolic fuels for energy production in fish which occurs via oxidative deamination, a process that yields CO_2 and ammonia as the primary wastes (Falco et al., 2020). Besides protein synthesis and energy generation, it has been established that amino acids serve as substrates for the synthesis of several metabolites such as nitric oxide, creatine and purines which are involved in essential physiological processes (Li et al., 2021). The digestion of proteins, as well as other macronutrients, occurs in the gastrointestinal (GI) tract and is similar to that of other monogastric animals. It starts in the stomach where proteins are partially degraded by pepsin under acidic conditions. In the intestine, the acidic mixture from the stomach is neutralized and acted on by proteases to completely degrade proteins to amino acids. These are absorbed into the blood stream and directed to different organs where they are utilized in protein synthesis or for energy production (Li et al., 2021; Wright, 1995).

Protein digestion is an integral part of the nitrogen balance in fish which encompasses several key processes as delineated in Figure 2. Ideally, all the feed protein (P_{feed}) should be ingested during feeding; however, a minor portion of it will unavoidably be lost as rejected feed items ($P_{uneated}$). Depending on the nutritional quality of the feed, some of the ingested protein will pass undigested through the GI tract and be excreted in the feces (P_{feces}). The remaining fraction of protein, defined as the digestible protein ($P_{digestible}$), is degraded to form free amino acids that are utilized for synthesis new proteins or respired to produce ATP. In all animals, respiration of amino acids generates different nitrogenous wastes, the most predominant being uric acid, urea and ammonia. Uric acid and urea are the major wastes in mammals and birds as these animals have low tolerance towards ammonia. By contrast, fish produce mainly ammonia but, like terrestrial animals, most fish can also produce smaller amounts of uric acid and urea (Wright, 1995). Furthermore, mobilization of stored protein may occur during prolonged starvation leading to an increase in the intracellular pool of amino acids. The mobilized amino acids are typically respired to produce energy when lipid and glycogen reserves have been depleted. In addition, certain amino acids (e.g. Ala, Ser, Glu, Asp and Gln) may be used as substrates in gluconeogenesis to produce

glucose that is utilized to restore the glycogen reserve in the liver (Falco et al., 2020; Pérez-Jiménez et al., 2011).

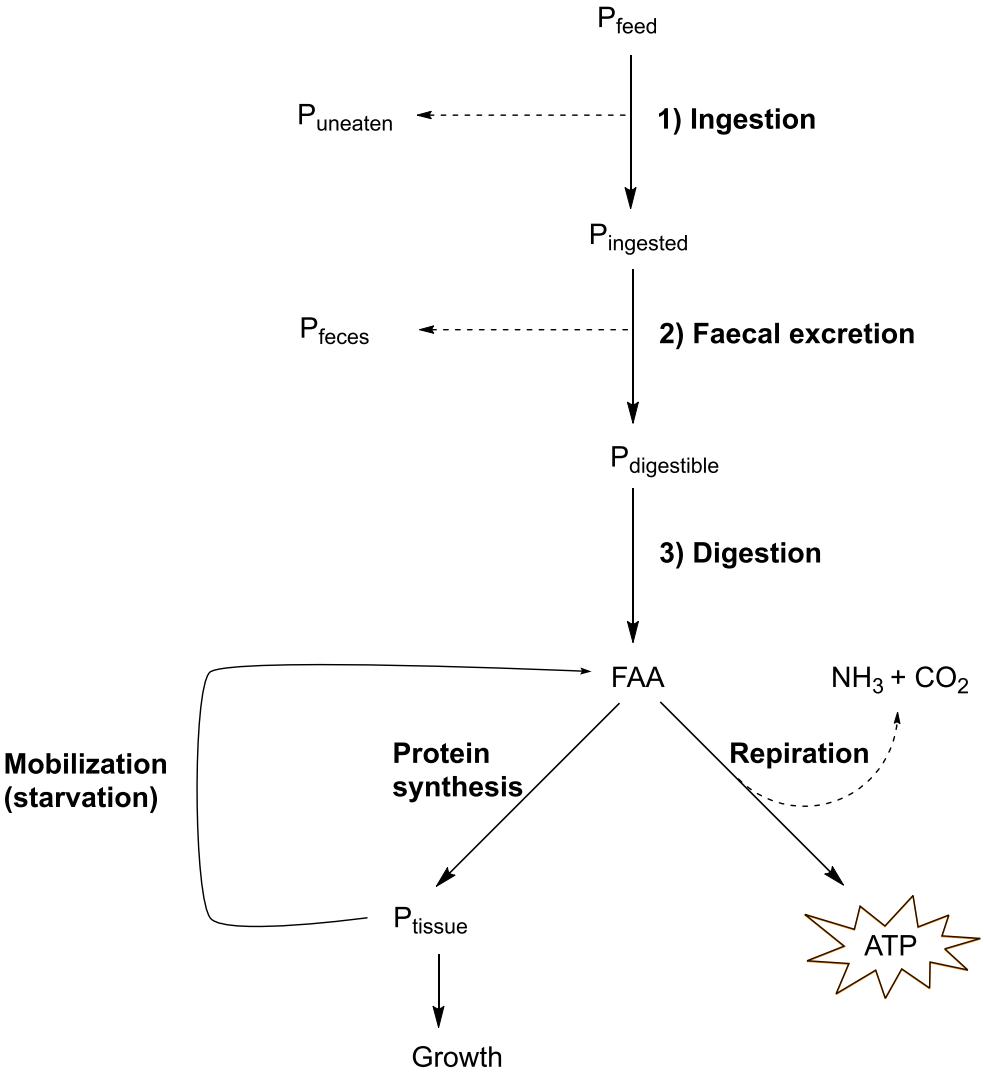


Figure 2. Simplified illustration of the nitrogen balance in fish demonstrating essential processes for dietary protein (P_{feed}). Abbreviations: P = protein and FAA = free amino acids liberated during protein digestion. For simplification, excretion of urea and uric acid has been omitted since these wastes are of minor importance for fish.

Protein retention refers to the amount of feed protein that is assimilated as cellular protein in fish tissue. Mathematically, protein retention can be expressed using equation (1):

Retained protein = $P_{tissue} = P_{feed} - P_{total\ loss}$ (1)

Feed rejection, faecal excretion and production of nitrogenous waste, mainly ammonia, contribute to the total loss of feed protein. For an optimized feed, the total loss must be kept at a minimum which can be assured by selecting protein sources that fulfil multiple criteria. The following section outlines the considerations that should be taken into account when assessing the suitability of a specific protein source for fish feed.

1.2.2.2 Nutritional aspects

Several factors must to be considered when evaluating the nutritional potential of a protein source. These include: 1) dietary protein content, 2) amino acid profile, 3) palatability, 4) content and types of antinutritional factors (ANFs) and 5) the protein to energy ratio.

It is crucial that the protein content of the diet meets the protein requirement of the cultured fish species since unbalanced protein levels may have adverse effects on the nutritional status of the fish as well as the environment. It is well documented that protein deficit impairs growth while excess protein is associated with increased excretion of nitrogenous compounds (Bibiano Melo et al., 2006; Carvalho et al., 2017; De Carvalho et al., 2010). Dietary protein requirement is not an absolute value as it is influenced by various factors including trophic level of the fish, abiotic factors, fish size and the nutritional value of the feed or feed ingredients (Teles et al., 2020). A positive correlation between trophic level and dietary protein requirement has been established which can explain the interspecific variations in protein requirement. As a result, carnivorous fish have higher protein requirements (40-50%) than herbivorous and omnivorous fish (25-35%) (Gatlin, 2010; Teles et al., 2020). Conversely, intraspecific variations in protein requirement may result from differences in abiotic factors such as water temperature, fish size and feed quality. In some fish species, the protein requirement decreases with increasing water temperatures, which is probably a consequence of improved protein digestibility and better carbohydrate utilization at higher temperatures (Amin et al., 2014; Glencross and Bermudes, 2010). Similarly, an inverse relationship between fish size and protein requirement has been shown for some species indicating that larger fish require less protein than smaller ones (Page and Andrews, 1973; Yoshimatsu et al., 1992). Finally, the protein requirement is typically lower for high-quality protein sources due to high bioavailability and digestibility of proteins (Oliva-Teles, 2012).

The amino profile and the EAA/NEAA ratio are known to be influencing the protein retention and ammonia excretion as well as the feed intake. Generally, maximum protein retention is obtained when the amino acid composition of the feed meets the dietary amino acid requirement of the cultured species. On the other hand, increased ammonia excretion occurs if dietary levels of amino acids surpass the amino acid requirement because excess amino acids might be used for energy production by oxidative deamination (Green et al., 2002a). A number of studies have evaluated the effect of EAA/NEAA ratio on protein retention and ammonia excretion in several fish species (Green et al., 2002b; Mambrini and Kaushik, 1994; Peres and Oliva-Teles, 2006). These studies demonstrated that protein retention increased and ammonia excretion decreased with increasing EAA/NEAA values. Furthermore, an EAA/NEAA ratio of 50:50 was reported to be preferable for all examined fish species as this ratio promoted maximum protein retention (P_{tissue}) and feed intake while minimizing discharge of ammonia.

In broad terms, antinutritional factors (ANFs) can be defined as biologically active molecules that reduce the bioavailability of nutrients resulting in inhibited digestion. In nature, ANFs constitute an essential part of the chemical defence system utilized by plants and therefore ANFs are most prevalent in plant-derived fish feeds (Samtiya et al., 2020). Antinutritional factors (ANFs) are commonly grouped into six major classes: alkaloids, saponins, phytate, protease inhibitors, tannins and structural cell wall constituents including cellulose, hemicellulose and lignin. While some ANFs directly impact the digestion of nutrients (phytate, protease inhibitors, tannins and cell wall components), others act as deterrents due to their bitter taste, thus reducing feed intake (alkaloids and saponins)(Glencross et al., 2020; Sinha et al., 2011). Concentrations of ANFs in fish feeds should be kept at an absolute minimum to enhance feed intake and reduce nutrient losses in feces.

Utilization of ingredients that improve the overall feed palatability and attractability may promote feed intake and diminish waste production stemming from uningested feeds (Al-Souti et al., 2019). While the term palatability is related to taste and smell, attractability is mainly controlled by the visibility of the feed. The physical properties of a feed are determined by a number of chemical factors including the quantities of macronutrients, ANFs, toxins and stimulants/attractants which indirectly affect feed palatability and attractability (Eriegha and Ekokotu, 2017).

Finally, feed intake and protein utilization are influenced by the digestible protein to digestible energy (DP/DE) ratio which varies depending on the feed composition of lipids, carbohydrates and protein. In bioenergetics, digestible energy (DE) is defined as the difference between gross energy of the feed (GE) and the energy lost in feces (FE) (Kaushik et al., 2002). The effect of varying DP/DE ratios has been investigated for several fish species including rainbow trout (*Oncorhynchus mykiss*) (Lee and Putnam, 1973), Nile tilapia (*Oreochromis niloticus*) (Fernandes et al., 2016) and channel catfish (*Ictalurus punctatus*) (Garling and Wilson, 1976). As a whole, these studies established that the DP/DE ratios required for maximum protein retention vary among different fish species. Generally, low DP/DE ratios, which are characteristic of high-energy diets with low protein content, promote the utilization of lipids and carbohydrates as energy sources instead of protein, a phenomenon known as protein sparing. Although protein sparing reduces ammonia excretion, it may lead to insufficient protein intake since fish eat to meet their energy requirements. In contrast, high DP/DE ratios (low energy, high protein) are often associated with excess consumption of protein and consequently increased ammonia excretion. This can be explained by the fact that fish need larger amounts of feed to meet their energy demands when fed low-energy diets (Kaushik et al., 2002; Oliva-Teles, 2012; Wilson and Halver, 1986). Having considered the key aspects of fish nutrition with focus on protein, we will now move on to discuss the basic principles of aquafeed production.

1.2.3 Fundamentals of aquafeed production

Insight into the nutritional requirements of fish and the nutritive value of the feed ingredients is of prime importance for producing nutritionally adequate fish feeds. Overall, the manufacturing of fish feed entails three major operations: 1) ingredient selection, 2) feed formulation and 3) feed production. In the feed manufacturing industry, it is commonplace to express nutrient values as percentage of the dry matter content (DM). Accordingly, all values presented in this thesis will be reported on a dry matter basis also known as “as-fed” basis.

Fish feeds are composed of six main ingredients derived from different sources including: 1) protein supplements (>20% protein), 2) energy supplements, mostly fat and oils (<20% protein), 3) soluble carbohydrates, 4) mineral supplements, 5) vitamin supplements and 6) additives. Additives do not add any nutritional value to the feed but they are still of high value. These compounds include filling agents, such as

cellulose, palatability enhancers, medication, antimicrobial agents and enzymes (Gatlin, 2010; Hardy and Barrows, 2003). As previously stated, dietary carbohydrates are not essential for fish; nevertheless, most formulated diets contain appreciable levels of soluble carbohydrates such as starch (15-20%) which are cheap energy sources and possess a high binding capacity (Craig and Kuhn, 2017). Protein is the most important ingredient in fish diets because of the high dietary demand for this nutrient. Many feeds consist of multiple protein sources mixed in adequate quantities to meet the protein requirements of fish. Selection of protein sources for feed formulation is based on different key considerations. First, a protein source should be rich in high-quality protein (50-70%) and palatable. Second, stable annual availabilities and low costs are essential prerequisites for ensuring an economically sustainable production of fish feeds.

The ultimate goal of feed formulation is to compute quantities of selected ingredients required for a nutritionally balanced diet that meets the minimum requirements of fish (Hardy and Barrows, 2003). Formulation of fish feeds that consist of multiple ingredients are performed in formulation softwares with inbuilt calculation spreadsheets. During feed production, calculated amounts of selected ingredients are mixed with water to form a homogeneous mixture that is further processed by pelletization and drying to form compact feeds of desired shape, texture and density (Sayooj and Ebeneezar, 2021). In the manufacturing of feed pellets two major technologies prevail, namely extrusion or steam compression. The type of processing mode determines the physical characteristics of a feed which may affect the performance of fish. Fish differ in the way they respond to and utilize different types of pellets. In general, extruded diets have several advantages over steam-compressed diets. These include a high utilization efficiency and hence low wastage, increased starch gelatinization, high durability and excellent water absorption capacity (Hilton et al., 1981). On the contrary, extrusion may have a negative impact on feed quality due to heat-induced degradation of vitamins and amino acids in extruded pellets (Misra et al., 2002). In addition, the hardness of extruded pellets may reduce feed intake and disturb the digestive processes in some fish species (Jacobsen et al., 2018).

Aside from the nutritional and economical considerations of feed formulation, it is important to evaluate the practical aspects of the manufacturing process including type of pelletization mode, which indirectly affect feed quality and thus fish performance.

1.3 The protein source in fish feed

1.3.1 Conventional protein sources

Currently, two major types of protein supplements are applied in formulated aquafeeds: animal-based and plant-based meals (Tacon et al., 2011). The animal-based meals are derived from marine or terrestrial sources. They include fishmeal and meals produced from bone, blood, feathers, shrimp and poultry by-products (Hardy and Barrows, 2003). Plant-based protein is mainly sourced from oil seeds such as soybean, rapeseed, sunflower, groundnut, cottonseed and palm kernel (Salin et al., 2018). To date, fishmeal and soybean meal represent the most widely used protein sources in aquafeed production.

Fishmeal is obtained from different sources, mostly wild-caught fish but also fish processing waste such as frames, offal and trimmings (Ghaly et al., 2013; Péron et al., 2010). Whole fish or fish by-products are reduced to fishmeal in a three-step process which involves cooking, drying and grinding. Apart from fishmeal, this process also generates fish oil commonly used as energy supplements in fish feeds (Ghaly et al., 2013). Fishmeal constitutes the most attractive ingredient in formulated fish feeds as it is associated with a high feed intake, good fish performance and minimal nitrogen excretion (Oliva-Teles, 2012). These benefits result from its excellent nutritional properties including high palatability, high protein content (50-70%), well-balanced amino acid profile and no ANFs. In addition, fishmeal is rich in other essential nutrients such as fatty acids and vitamins (Cho and Kim, 2011). As such, fishmeal is distinguished as the most complete feed ingredient reported to date. Dependence of fishmeal as the main protein source in fish feeds is a continuing concern within the aquaculture industry as an increasing demand for fish protein may lead to shortage of the small pelagic fish species exploited for fishmeal production (Olsen and Hasan, 2012; Salin et al., 2018). In recent years, fishmeal prices have escalated due to reduced availability of wild fish in the oceans combined with an increasing aquaculture production. From 2000 to 2018, the global fishmeal prices underwent a steep increase from 452 USD/tonnes to 1596 USD/tonnes (Jannathulla et al., 2019). Concurrently,

fishmeal production from non-targeted captures declined from ~7.3 million tonnes in 1994 to ~5.0 million tonnes in 2020 (FAO, 2020). These challenges have bolstered efforts to implement alternative protein sources in aquafeed production, thereby reducing the reliance on fishmeal.

The last decades have seen a growing trend in the use of soy protein owing to its wide availability and high nutritional quality. Soy protein is available in the form of soybean meal and soy protein concentrate which differ with respect to protein content, ANFs levels and amino acid profiles. Soy protein concentrates are characterized by a high protein content (>75%), insignificant levels of ANFs and a favourable amino acid profile similar to that of fishmeal (Dersjant-li, 2002). In comparison, soybean meal has a lower protein content (40-50%) and contains considerable amounts of different ANFs, primarily trypsin inhibitors and phytate. Furthermore, soybean meals are deficient in certain amino acids such as lysine and methionine (Hardy, 2010). Consequently, soybean meals have lower nutritional value than soy protein concentrates, and therefore high inclusion levels soybean meals adversely affect nutrient utilization in most fish species (Kaushik et al., 1995). Although soybean meals have lower nutritional quality as compared to soy protein concentrates, they are still the most common type of soy protein in aquafeed. Besides nutritional limitations, the usage of soybean meals in aquafeed poses an environmental challenge because soy production is associated with intensified land use and high CO_2 emission caused by increased deforestation (Pelletier et al., 2018). Furthermore, a high content of phytate in soybean meal is problematic in two respects. Firstly, phytate increases faecal excretion of phosphorus because fish lack the enzyme phytase and are therefore unable to utilize phytate-bound phosphorus. Secondly, phytate inhibits the absorption of minerals and protein via complexation which prevents utilization of these nutrients in fish (Dalsgaard et al., 2009; Kumar et al., 2012).

The challenges imposed by conventional protein sources, have spurred a need to identify and implement sustainable alternatives in aquafeeds. Potential alternative protein sources will be explored in the next section focusing on agroindustrial by-products, which are abundant and rich in recoverable protein.

1.3.2 Agroindustrial by-products as alternative protein sources

Agroindustrial by-products are a diverse group of residues with high protein content and as such, they represent potential sources of protein for aquafeed. Despite this, agroindustrial by-products are mostly disposed of through landfilling and incineration.

Depending on their origin, agroindustrial by-products can be classified into animal-and plant-derived by-products (Reguengo et al., 2022). The animal-derived by-products comprise a small group, consisting of meat waste from fish and livestock as well as dairy by-products. On the other hand, the plant-derived by-products form a large group of multiple residues emanating from the processing of different plant materials.

Three of the most abundant plant-derived by-products are: 1) grape pomace from wineries, 2) dried distiller's grain (DDG) from ethanol production) and 3) brewer's spent grain (BSG) from beer brewing (Petruccioli et al., 2011). Recently, these plant-derived by-products have attracted increasing attention as potential protein sources for fish feed because of their wide distribution, exorbitant quantities and low prices (Diógenes et al., 2019; Jayant et al., 2018; Peña et al., 2020). Table 1 compares the proximate compositions and global production volumes reported for BSG, DDG and grape pomace.

Table 1. Composition and worldwide production of three abundant plant-derived agroindustrial by-products.*

	Protein (%DM)	Lignin[a] (%DM)	Cellulose[b] (%DM)	Hemicellulose[c] (%DM)	Global production in 2020 (mio. tonnes)[#]	References
Brewer's spent grain (BSG)	25.8	5.4	16.5	34.4	36.4	(Heuzé et al, 2017; Karlsen and Skov, 2022)
Dried distiller's grain (DDG)	29.5	4.3	9.3	20.6	24.0	(Heuzé et al, 2015; USDA, 2021; Shahbandeh, 2022)
Grape pomace	13.6	33.7	18.9	8.2	8.50	(Castellanos-Gallo et al., 2022; Heuzé and Tran, 2020)

*All nutritional data is average values acquired from the open access information system Feedipedia. The reported compositions of the three by-products are based on: 1) dehydrated, unground BSG, 2) maize distiller's dried grain with solubles and 3) dehydrated grape pomace containing stems, seeds and skins. #Note on data sources: production volumes of dried distiller's grain (DDG) were estimated as the sum of values reported for United States (~24 mio. tonnes) (USDA, 2021) and Europe (~4.2 mio. tonnes) (Shahbandeh, 2022) as no single source was available. Contributions from UK and other DDG suppliers are not included in this estimation. [a]Lignin content is determined as acid detergent lignin (ADL) and excludes the acid-soluble lignin fraction. [b]Cellulose content is calculated as the difference between acid detergent fibre (ADF) and ADL: cellulose = ADF – ADL. [c]Hemicellulose content is calculated by subtracting ADF from the neutral detergent fibre (NDF): hemicellulose = NDF – ADF

Currently, the use of these by-products in aquaculture has been restricted by two major drawbacks. Firstly, their protein content is too low to meet the high protein requirement of most fish species. Secondly, these residues contain considerable amounts of lignin, cellulose and hemicellulose which are antinutritional factors (ANFs) for fish (Kokou and Fountoulaki, 2018). To realize the potential of these residues as aquafeed ingredients, they must undergo a refinement in which the protein content is increased and the lignocellulosic material is removed. In a broader perspective, the

valorization of these residues as aquafeed ingredients may contribute to the development of a circular economy because value is added to waste that would otherwise be dumped in landfills or incinerated.

This PhD project addresses the possibility of implementing BSG as an alternative protein source in fish feed with focus on different valorization strategies. Yet, all refinement methods considered for BSG could be extended to other plant-derived by-products due to the compositional similarity between these residues.

1.3.3 Brewer's spent grain (BSG) as an alternative protein source for fish feed

1.3.3.1 Production of BSG: the beer brewing process

Brewing is an industrial process that transforms cereal grains into beer. In addition, brewing generates huge quantities of by-products including BSG and brewer's yeast (Rachwał et al., 2020). Brewer's spent grain is the most abundant by-product representing ~85% of the total waste streams generated during production of beer. Barley (*Hordeum vulgare*) is the main ingredient in the beer brewing process. Structurally, a barley grain is compartmentalized into three sections: the embryo (germ), the endosperm and the grain covering. The endosperm comprises a matrix of starch and protein encapsulated in a layer of aleurone cells that separates it from the grain covering. The grain covering surrounds the inner parts of the grain (embryo and endosperm) and consists of three distinct layers which include the teste (inner seed coat), the pericarp (middle layer) and the husk (outer layer). The latter is a protective layer consisting mainly of dead cells rich in lignocellulose and silica (Mosher and Trantham, 2017; Willows et al., 2017). During the initial stages of beer brewing, the starchy endosperm undergoes substantial alterations resulting in the production of fermentable sugars. Transformation of the endosperm takes place in two separate processes known as malting and mashing. Malting is the germination of barley grains in which the endosperm is chemically modified by synthesis and activation of hydrolytic enzymes. After malting, the germinated barley grains, referred to as the malt, are milled to separate the grain covering from the endosperm and increase the exposure of starch and proteins to hydrolytic enzymes. Subsequently, the milled malt undergoes mashing in which a sweet wort is produced under action of the hydrolytic enzymes produced during malting. This liquid forms the primary fermentation medium for beer

production as it contains significant amounts of fermentable sugars (glucose) generated by enzymatic hydrolysis of endosperm starch. After mashing, the wort is enriched by hops and sugars to form a so-called hopped wort. Finally, the hopped wort is isolated by filtration through a bed of insoluble and undegraded malt, which accumulates and forms a filter in the bottom of the mash tun. Brewer's spent grain constitutes the wet, grainy material obtained from wort filtration and is mainly composed of grain coverings as well as endosperm fragments (Lewis and Young, 2001; Mussatto et al., 2006).

1.3.3.2 Chemical composition of BSG

Brewer's spent grain is heterogeneous biomass majorly composed of lignin (~12-28% DM), cellulose (12-25% DM), hemicellulose (20-25% DM) and protein (20-30% DM). Furthermore, BSG contains several minor constituents including lipids (7-10% DM), ash (2-5% DM), minerals (~1%), mono-and disaccharides such as glucose, fructose and sucrose, vitamins in traceable amounts and endosperm-derived β-glucans and starch (~1%) (Steiner et al., 2015; Waters et al., 2012).

Lignin is a phenolic polymer composed of three alcohol monomers: p-coumaryl, coniferyl and sinapyl. To synthesize lignin, these monomeric precursors are coupled randomly via a radical-induced polymerization reaction resulting in the formation of a complex aromatic network consisting of several C-C and ether linkages. Due to its structural complexity and high content a stabile bonds (C-C and C-O), lignin is highly recalcitrant towards degradation (Yinghuai et al., 2013). Hemicellulose and cellulose are collectively referred to as non-starch polysaccharides (NSP). Structurally, cellulose is a linear polysaccharide of glucose monomers linked together by β-1,4-glycosidic bonds. Because of its high content, cellulose represents the primary source of glucose in BSG while starch and β-glucans account for a minor fraction of glucose. Arabinoxylan (AX) constitutes the most abundant hemicellulose in BSG and is an amorphous polysaccharide composed of a xylose backbone with a complex substitution pattern. The xylose backbone is mainly substituted with arabinose moieties at O-2 and/or O-3 but it can also be linked to other substituents such as uronic acids, methylated uronic acid and acetyl groups (Coelho et al., 2016). Furthermore, xylose-linked arabinose can be esterified to ferulic acids which may result in formation of diferrulic acid cross-linkages between adjoining AX strands under oxidative conditions (Lynch et al., 2016). Lignin, hemicellulose and cellulose are arranged in a

compact structure known as lignocellulose. In this arrangement, cellulose and hemicellulose are held together via hydrogen bonds while hemicellulose forms ether and ester linkages with lignin (Mandalari et al., 2005).

After lignocellulose, protein is the second most abundant component in BSG representing up to 30% of DM. The proteins of BSG belong to the class of cereal protein which are divided into four major types based on their extractability differences in different solvents. These include: 1) water-soluble albumins, 2) salt-soluble globulins, 3) alcohol-soluble prolamins and 4) acid/alkaline soluble glutelins (Byers et al., 1983). Hordeins (barley prolamins) are the most abundant protein type in BSG (>50% of total protein) followed by glutelins while albumins and globulins represent the smallest fraction of the total BSG protein (~2%) (Celus et al., 2006). From a nutritional perspective, the proteins of BSG can be defined as high-quality proteins due to a high content of amino acids that are essential for fish (~65% of total protein). Among these, Lys, Leu, Phe and Ile are the most abundant with concentrations estimated at ~14%, ~6%, ~5% and ~3% of the total protein, respectively (Waters et al., 2012).

1.3.3.3 Potential of utilizing BSG as an alternative fish feed ingredient

Since the advent of the brewing industry, BSG has mainly found use as a low-cost feed supplement for livestock. However, the current oversupply of BSG combined with its poor storability pose challenges for the transportation from breweries to farmers (Steiner et al., 2015). As a result, surplus BSG is disposed of by other means, mainly landfilling or incineration which represent environmental nuisances (Johnson et al., 2010). Hence, incorporation of BSG as a second-generation feedstuff in aquafeed could be a way to diminish the environmental impact caused by current waste management practices.

To date, a number of studies have addressed the use of raw or semi-refined BSG as a feed ingredient for different fish species (Fernandes et al., 2022; He et al., 2020; Jayant et al., 2018; Nazzaro et al., 2021). These studies have tended to focus more on the nutritional aspects of utilizing BSG in aquafeeds rather than the socio-economic implications. One recent study of He et al. (2021) considered the economic aspects related to production of a BSG-derived protein concentrate intended for fish feed. They concluded that the production costs of this BSG product (1043 USD/t) were

considerably lower compared with the current fishmeal price (1449 USD/t). This finding suggests that BSG valorization is economically viable provided that cost-effective methods are employed. Studies on the nutritional impact of utilizing BSG in aquafeed found that the maximum allowed inclusion levels varied depending on fish species. Expectedly, carnivorous fish species displayed a lower tolerance towards high inclusion levels of BSG as compared to omnivorous and herbivorous fish. This was demonstrated by Nazzaro et al. (2021) who found that low inclusion of 20-30% raw BSG was appropriate for maintaining a high protein digestibility in rainbow trout and gilthead seabream (*Sparus aurata*) which are both carnivorous fish species. Similarly, Jayant et al. (2018) established that replacing soybean meal with up to 50% of raw BSG did not negatively affect growth or protein digestion in striped catfish (*Pangasianodon hypophthalmus*) which is an omnivorous fish species. Furthermore, the benefits of BSG valorization were substantiated by He et al. (2020) who found that growth and feed utilization of Pacific white shrimp (*Litopenaeus vannamei*) were unaffected when fishmeal was replaced by up to 50% of a BSG-derived product with high protein content (46% DM). Likewise, Fernandes et al. (2022) showed that the growth performance and protein digestion of European seabass (*Dicentrarchus labrax*) increased with increasing levels of an enzyme extract produced by solid-state fermentation of BSG.

Previous studies have not been able to incorporate BSG at inclusion levels higher than 50% which can be attributed to the nutritional constraints of raw and semi-refined BSG. To enable higher inclusion in aquafeeds, BSG must therefore be extensively refined. The prospects of developing of an economic refinement method for BSG, thereby allowing for higher inclusion levels (60-90%) in aquafeeds, has been the primary motivation for this PhD project.

1.4 PhD study: major objectives and structure

The overarching aim of the PhD project was to evaluate the suitability of different valorization methods for converting BSG into a high-quality protein source for fish feed. Initially, a systematic review was performed to attain a complete overview of existing research into BSG valorization. The knowledge gained from this literature study was compiled in a review article (**Paper 1**) which served as a reference work in elaborating the experimental studies. To address the main objective of the PhD project, two general refinement strategies were considered as outlined in **Paper 1**:

1) *Chemical fractionation*, in which protein is separated from lignocellulose by extraction.
2) *Conversion of lignocellulose*, in which the lignocellulosic components are transformed into compounds that can be digested by fish or utilized as starting materials in production of various value-added compounds.

Based on these strategies, the experimental work was divided into three parts:

- **Part I** intended to optimize the protein extraction of BSG by testing the effect of various parameters on protein yield and protein content of BSG-derived isolates. All results from this part are presented in **Paper 2**.
- **Part II** examined the possibility of eliminating lignin by a fungal delignification strategy using solid-state fermentation (SSF) under different cultivation conditions.
- **Part III** had two major objectives. First, the cellulose and hemicellulose of BSG were hydrolysed to yield a liquid hydrolysate containing NSP-derived monosaccharides. Second, this BSG hydrolysate was utilized as a substrate for production of two types of value-added products by submerged fermentation: 1) free amino acids and 2) single cell protein (SCP). Results from the fermentation experiments are compiled in **Manuscript 3** and **Manuscript 4**.

The following chapters provide an overview of the major findings of this PhD project along with a short theoretical background of the applied valorization methods. For a detailed overview of the various refinement methods reported to date, the reader is referred to **Paper 1**.

2. Storage and initial processing of BSG

2.1 Storage of BSG

The high moisture content of fresh BSG (70-80%) coupled with a favourable chemical composition makes it an excellent substrate for microbial growth (Robertson et al., 2010a). Consequently, wet BSG is highly susceptible to spoilage under improper storage conditions which is problematic in two respects. First, microbial colonization will lead to chemical deterioration of BSG eventually impairing its nutritional quality (Robertson et al., 2010b). Second, poor storage may increase the production of aflatoxins by inherent aflatoxin-producing fungi growing on BSG, mainly species belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* (Gerbaldo et al., 2011; Sodhi et al., 1985). Inappropriate storage facilitates the proliferation of these microbes leading to production and accumulation of aflatoxins (Asurmendi et al., 2013; Sodhi et al., 1985). These compounds are harmful for fish although tolerance differs greatly among different fish species. Hence, employment of adequate storage conditions is of paramount importance to prevent chemical alterations and aflatoxin contamination of BSG. A detailed description of the toxicogenic effects of aflatoxins and different detoxification strategies can be found in **Paper 1** (Section 4.2.3.1 Aflatoxins).

The most common methods for BSG storage encompass drying and freezing. While drying renders BSG microbiologically stable and more manageable by removing water, freezing is used for long-term preservation of wet BSG. The major disadvantages of freezing are the demand for bulk storage area and the fact that the arabinoxylan (AX) content is significantly reduced during thawing of BSG (Bartolomeé et al., 2002). For aquafeed applications, however, a reduction in the AX content might be beneficial due to the antinutritional effect of AX on most fish species (Sinha et al., 2011). Overall, there are three main types of drying: freeze-drying, oven-drying and superheat steam (SS) drying. Freeze-drying and oven-drying are energy-intensive techniques and as a result less suitable for large-scale applications. Furthermore, these drying methods apply harsh conditions which might induce compositional changes in BSG, mainly decreases in protein and lipid contents stemming from partial degradation of these components (Santos et al., 2003). On the other hand, SS drying is a mild technique known to have negligible impact on the composition of BSG; yet,

the main weakness of this method is a requirement for technically advanced and costly equipment (Tang et al., 2005).

The storage and initial processing of the BSG batches employed in this PhD project involved the following consecutive processes:

I. Storage of wet BSG by freezing (-20°C)
II. Slow thawing of frozen BSG followed by autoclaving (121°C for 15 min)
III. Water removal by drying at 60°C
IV. Mechanical pretreatment by micronization

Autoclaving was performed to remove any indigenous microbial activity from BSG. As established by Robertson et al. (2010a), autoclaving may lead to heat-induced decomposition of certain constituents in BSG such as starch and phenolic compounds. To ascertain whether proteins and amino acids were degraded during autoclaving, the contents of these compounds were determined in autoclaved and non-autoclaved BSG. Results from this analysis clearly showed that autoclaving did not affect the composition of protein and amino acids (data not shown). Furthermore, autoclaving may lead to structural alterations of proteins which could affect the physico-chemical properties, and hence the extractability, of proteins from BSG. Even so, the effect of autoclaving on protein extractability was not examined in this PhD project.

Micronization was performed at the Danish Technological Institute to reduce the particle size of BSG, thereby increasing the surface area and reducing the cellulose crystallinity. This process is believed to enhance the efficacy of BSG refinement due to increased exposure of macromolecules to enzymes and/or extraction solvent. Furthermore, micronization might lead to a partial fragmentation of the lignocellulosic structure which may increase the accessibility of cellulose and hemicellulose to enzymatic attack (Kumar et al., 2017). In the section that follows, the principles of the micronization method applied for BSG will be outlined followed by an evaluation of the proximate composition of micronized BSG fractions.

2.2 Micronization of BSG

The micronization method used in this PhD project can be divided into two basic processes: milling and sifting. Whilst milling reduces the overall particle size of a

biomass resulting in a mixture of different size fractions, sifting is used to segregate the milled material into separate fractions of uniform size distribution.

Traditionally, the milling process has been classified into two main types based on their mode of grinding: knife milling and hammer milling. During knife milling, biomass is chopped into smaller fragments while hammer milling squeezes the biomass to form long fibrous chains (Montgomery and Bochmann, 2014). For the purpose of this PhD project, BSG was milled employing a knife milling system. Specifically, dried BSG entered a milling chamber via a feed throat (hopper) wherein it was shredded by the rotation of a blade assembly anchored to a central rotor. This process generates a fierce centrifugal force which causes extrusion of milled material through a sieve that surrounds the milling chamber. Finally, the milled BSG was collected in a receptacle connected to the milling chamber. Prior to milling, BSG was dried at 60°C to obtain a dry matter content higher than 90% as a high moisture content is associated with increased operation costs owing to strengthened shearing forces of the wet material (Barakat et al., 2015).

Sifting is used to separate the milled biomass into different fractions based on particle size. This process is commonly carried out using a horizontal vibratory sifter which contains a stack of sieves with decreasing mesh size (Liu, 2009). The idea of this technique is that smaller particles will pass through the sieve apertures while oversize particles are rejected and conveyed to the opposite end of the sifter where they are collected. Thus, the largest fraction is always obtained from the upper sieve while the smallest fraction passes through all sieves and is collected under the lowest sieve. Previous studies have established that particle size influences the chemical composition of the sieved fractions, thus indirectly affecting their nutritional quality. Often, larger size fractions have lower nutritional value due to a lower protein content. Conversely, smaller size fractions tend to have higher protein content and thus a higher nutritional value (Hanif et al., 2014; Neto et al., 2016). Consequently, sifting may offer a powerful tool for eliminating fractions of lower quality from the initial biomass.

Micronization of BSG resulted in obtainment of three size fractions, referred to as small (<125 µm), medium (125-250 µm) and large (>250 µm). The medium fraction represented the most abundant fraction totalling ~66% of the micronized BSG followed

by the small fraction (~25%) and the large fraction (~9%). Following micronization, the proximate composition of all size fractions were determined (Table 2).

Table 2. Proximate composition of size fractions obtained from micronization of brewer's spent grain (BSG). All fractions were analysed in duplicates and results reported as mean±SD.

BSG fractions	Dry matter (%DM)	Ash (%DM)	Lipid (%DM)	Protein (%DM)*
Small (<125 µm)	94.6 ± 0.00	3.50 ± 0.03	14.2 ± 0.1	36.8 ± 0.10
Medium (125-250 µm)	95.0 ± 0.00	4.21 ± 0.03	9.90 ± 0.04	20.2 ± 0.10
Large (>250 µm)	95.5 ± 0.00	4.27 ± 0.00	5.60 ± 0.03	14.1 ± 0.10

*Protein content was determined with the Kjeldahl method and includes non-protein nitrogen (NPN) as well as protein nitrogen. To calculate the protein content, Kjeldahl N was multiplied by a conversion factor of 6.25.

The most compelling result was that the protein content was ~2.6 fold higher in the small fraction (~37%) as compared to the large fraction (~14%). This finding was encouraging because it suggests that the protein content of BSG could be increased by mechanical treatment. Although interesting, the potential of valorizing BSG by mechanical processing was not explored further as this PhD project focused exclusively on chemical and biological valorization methods. Even though the small size fraction had the highest protein content, it was considered unsuitable for chemical/biological treatments due to its dusty nature which may complicate its handling and pose a health risk. On the other hand, the medium size fraction presents a reasonable trade-off between high protein content and low dustiness. Therefore, this size fraction was employed in the experimental work of this PhD project.

3. Part I: fractionation of BSG focusing on protein extraction

3.1 Fractionation methods for BSG

Chemical fractionation is the process of separating a biomass into its macromolecular constituents. In overall terms, a fractionation process consists of two individual steps: extraction and isolation. During extraction, the biomass is mixed with a solvent, known as the extractant, resulting in the production of an extract that contains the target compound as well co-extracted substances. By isolation, the target compound is separated from the extract with a suitable method, the most common being precipitation and filtration (Galanakis, 2015). Characteristics of a successful fractionation process include a high recovery of the target compound and minimal content of co-extracted compounds in the final product. This may be achieved by selecting extraction and isolation conditions that maximize solubilization and separation of the target compound.

In the context of this PhD project, the overarching goal of utilizing fractionation was to produce a protein-rich product from BSG. Drawing on existing literature, fractionation of BSG can be categorized into two different approaches:

1) Fractionation-based removal of lignocellulosic components
2) Fractionation-based isolation of proteins

Both fractionation methods are believed to have their advantages and drawbacks. One major benefit of the first method may be that it allows for a better exploitation of all components in BSG by generating a protein-rich product applicable for feeds and lignocellulosic fractions useful for a variety of biotechnological applications. By contrast, the second method may be more efficient in obtaining a protein-rich product from BSG as it involves fewer extractions; however, it does not ensure full utilization of all constituents present in BSG.

3.1.1 Method 1: fractionation-based removal of lignocellulosic components

A growing body of literature has studied the fractionation of lignocellulosic components and proteins in BSG. While some studies have focused on the extraction of single

constituents, mainly lignin and AX, others have evaluated sequential processes for complete fractionation of lignocellulose. Extraction of AX from BSG has been the primary focus of most studies due to the well-documented health benefits of this polysaccharide (Mendis and Simsek, 2014). One study by Mandalari et al. (2005) demonstrated a highly selective procedure in which AX was sequentially extracted from BSG using alkaline solutions of increasing strength (0M to 4M KOH). Similarly, Laine et al. (2015) found that AX could be obtained from a single alkaline extraction; however, this method was associated with poor selectivity resulting in simultaneous extraction of proteins, lignin and lipids. In another study, Reis et al. (2015) reported the fractionation of AX from BSG by ultrasound-sound assisted extraction (UAE) and concluded that UAE and alkaline extraction were equally efficient in extracting AX. As previously reported by Mussatto et al. (2007), lignin can be removed from BSG by acidic treatment with sulfuric acid where the highest removal was obtained at pH ~2 (~80%). More interestingly, sequential fractionation of BSG into lignin, cellulose, AX and protein fractions was demonstrated for the first time by D'Heysselaer et al. (2022). In this seminal study, BSG was subjected to acidic treatment followed by alkaline extraction. These processes led to AX isolation (acidic treatment) and production of two products (alkaline treatment): a solid cellulosic fraction and a liquid fraction consisting mainly of lignin and proteins. Lignin and protein from the liquid fraction were separated into two fractions by acidic precipitation of lignin (D'Heysselaer et al., 2022).

3.1.2 Method 2: fractionation-based isolation of proteins

To date, protein extraction presents the most well-studied fractionation method for BSG. Previous studies have evaluated the usefulness of numerous extraction procedures. These include sequential extractions in different solvents (Osborne extraction), alkaline and acidic extractions, hydrothermal extraction, detergent extractions, protease-assisted extraction and ultrasonic-assisted extraction (Celus et al., 2006; Crowe et al., 1985; He et al., 2021; Qin et al., 2018; Tang et al., 2010; Vieira et al., 2014). Additionally, a few studies have investigated different procedures for isolation of solubilized proteins including isoelectric precipitation, which occurs at the isoelectric point of proteins, ethanol precipitation with and without refrigeration (4°C) and ultrafiltration (Ervin et al., 1989; Tang et al., 2009). The most intriguing result from the study by Ervin et al. (1989) was that ethanol precipitation appeared to be more efficient in recovering proteins from BSG than isoelectric precipitation. Furthermore,

ethanol precipitation with refrigeration increased the protein recovery nearly two-fold as compared to ethanol precipitation without refrigeration which suggests that cooling promotes precipitation of proteins from the extract. The study by Tang et al. (2009) demonstrated the use ultrafiltration for protein isolation, a process that retains proteins on membranes while eliminating salts and other low-molecular weight compounds by filtration. Even though several methods have been successfully employed in the literature, alkaline extraction coupled with isoelectric precipitation continues to be the most preferred procedure for extracting and isolating proteins from BSG (Wen et al., 2019). Therefore, this section focused entirely on alkaline extraction combined with isoelectric precipitation.

3.2 Alkaline protein extraction of BSG

Proteins were obtained from BSG by alkaline extraction (NaOH) followed by isoelectric precipitation as schematized in Figure 3.

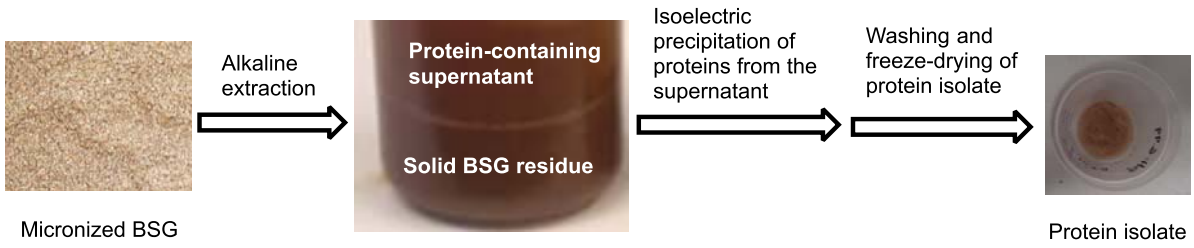


Figure 3. Protein fractionation of brewer's spent grain (BSG) using a combination of alkaline extraction and isoelectric precipitation. First, proteins are extracted with an alkaline solution. After extraction, the protein-rich supernatant is separated from the solid BSG residue by centrifugation. Then, proteins are isolated from the supernatant by isoelectric precipitation resulting in production of a protein isolate which is washed with water, neutralized and freeze-dried.

During alkaline extraction, proteins are separated from the biomass and solubilized in alkaline solution (NaOH or KOH). Protein solubilization occurs via unfolding and partial hydrolysis of proteins into shorter peptides which are more soluble in alkaline solution as compared to the folded proteins. Opon extraction, the solid residue is separated from the liquid fraction by centrifugation. Under ideal extraction conditions, most of the protein from the original biomass will be recovered in the supernatant. During isoelectric precipitation, proteins are isolated from the supernatant by adjusting pH to the isoelectric point (pI) of proteins after which the precipitated proteins are washed with water, neutralized and freeze-dried. Typical pI values of plant proteins are found

within the range of 4-5 (Lam et al., 2018). Protein precipitation occurs because proteins are less soluble near their pI. In contrast, proteins display higher solubility at pH values above or below their pI. This trend can be explained by the fact that proteins are positively charged at pH lower than pI, negatively charged at pH higher than pI and neutral at pH equal to pI. Positively and negatively charged proteins will not precipitate due to repulsive forces that prevent the formation of insoluble protein aggregates (Pace et al., 2004; Türker et al., 2021). Several factors have been found to influence extraction and precipitation efficacies, thus affecting the overall protein recovery and the protein content of the produced isolates. Major determinants of protein extractability include: 1) interactions between proteins and other macromolecules in the vegetal matrix, 2) extraction pH and salinity, 3) solid to liquid (S:L) ratio, 4) temperature and 5) extraction time (Rodrigues et al., 2012; Sari et al., 2015b, 2015a). Precipitation efficiency is largely dependent on temperature and precipitation pH since even small pH deviations from pI may cause significant reductions in the amount of precipitated protein (Ervin et al., 1989; Veide Vilg and Undeland, 2017).

3.2.1 Optimization of alkaline protein extraction of BSG

Part I investigated the impact of variuous parameters on protein recovery and protein content of isolates obtained from alkaline extraction/isoelectric precipitation of BSG. The results obtained from **Part I** are compiled in **Paper 2**. Therefore, the aim of this section is not to provide a detailed exposition of all results, but merely to summarize and explain the most significant findings.

Protein content of isolates and protein yield were used for assessing the efficiency of protein extraction and isolation under different conditions. Protein content provides insight into the purity of the isolated product, and it was determined as crude protein (C_p) or true protein (AA_p). The C_p was measured with the Kjeldahl method and includes both non-protein-and protein nitrogen. By contrast, AA_p includes only protein nitrogen and was calculated as the sum of all amino acids. Protein yield (Y), also known as protein recovery, expresses the percentage of proteins recovered from the original biomass and can be calculated using equation (2):

$$Y = \frac{protein\ in\ isolate\ (g)}{protein\ in\ biomass\ (g)} * 100\% \quad (2)$$

Protein yield reflects the extractability of proteins—that is how easily proteins are separated from other biomass constituents—but also the overall efficiency of the fractionation process.

Temperature and time: initially, the effect of temperature and time on protein content and yield were evaluated by a two-factorial experiment including three temperature levels (30, 45 and 60°C) and four time levels (30, 60, 120 and 180 min). Overall, the results indicated that the protein content decreased with temperature while the protein yield increased with temperature until a certain point beyond which it started to decrease. In contrast, protein yield and content appeared to be inversely correlated with extraction time. The inverse correlation between time and yield may be ascribed to complete protein hydrolysis yielding free amino acids that would remain in the liquid fraction during precipitation. The decreasing protein content, however, might be a result of enhanced solubilization of lignin and lignin-AX adducts at longer extraction times as lignin is partially soluble in alkaline solutions (Melro et al., 2020; Sari et al., 2015b). All protein isolates had a relatively high protein content ranging from 40% to 55%, which might be acceptable for aquafeed applications depending on the target fish species. Despite that, all combinations resulted in low yields (6-10%) which could be related to low extractability or loss of protein. As shown in Figure 4, protein losses might occur during precipitation or washing with water.

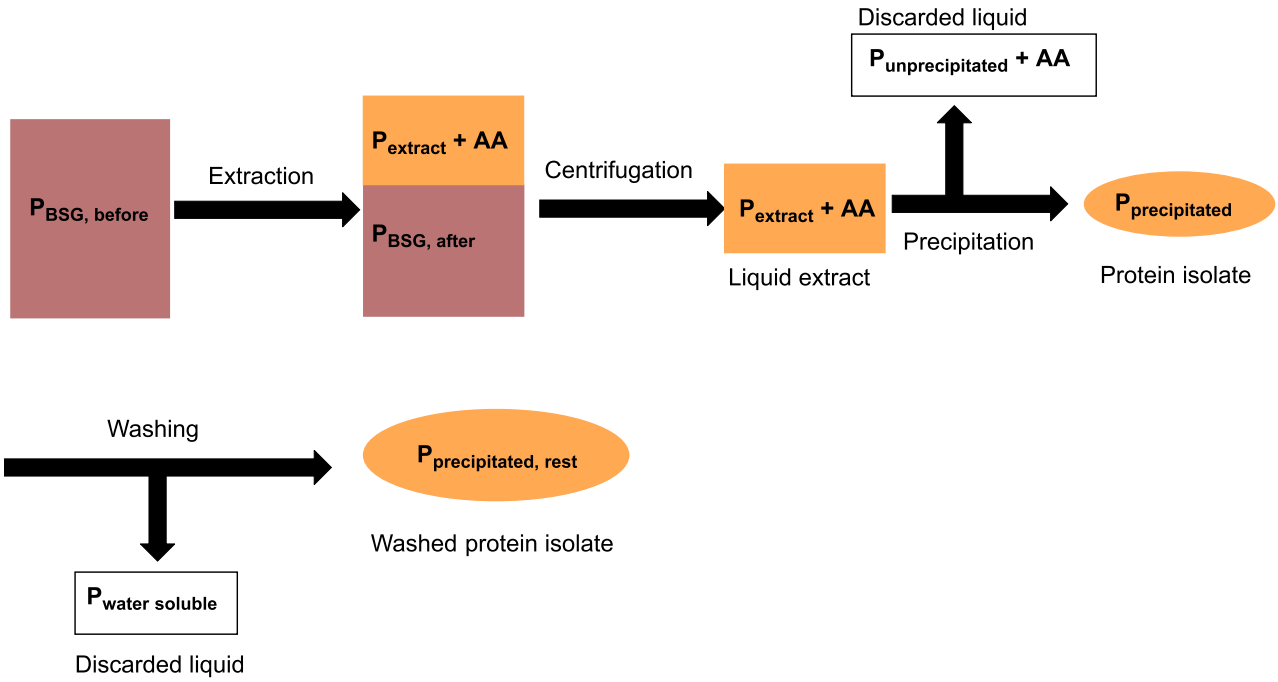


Figure 4. Partitioning of protein during alkaline extraction and isoelectric precipitation of brewer's spent grain (BSG). Potential losses may occur during precipitation and washing. Abbreviations: P = protein, AA = amino acids.

It is possible that precipitation losses stem from protein-derived amino acids produced by complete hydrolysis during alkaline extraction. Alternatively, protein losses could be due to incomplete precipitation resulting from indefinite pI values of solubilized proteins or inadequate precipitation pH (Ervin et al., 1989). Protein losses occurring during the washing step, however, may originate from solubilization of water-soluble proteins such as albumins. Furthermore, reduced protein extractability might be due to improper agitation during extraction and/or interactions with other biomass constituents. To elucidate whether the low yield emerged from poor extractability, protein losses or both, five optimization experiments were conducted.

Precipitation salinity: based on the principles of the salting-out effect, it was hypothesized that increased salinity would enhance protein precipitation. This hypothesis was tested by comparing results obtained from precipitation of a saline extract (0.5 M NaCl) and a control (0.0 M NaCl). Contrary to our expectations, increased salinity substantially reduced the protein yield and content compared with the control. This rather contradictory result may be explained by the fact that solubility is affected by a multitude of factors including salinity, pH, protein structure and amino

acid profile (Sousa et al., 2007). These factors, particularly pH, may contribute to an increased protein solubility, and hence reduced precipitation, near the isoelectric point (4.5) as reported for pea proteins by (Chavan et al., 2001).

Agitation mode: Two different agitation modes were evaluated: orbital shaking and magnetic stirring, of which the latter was expected to be more efficient in extracting proteins from BSG. Surprisingly, these agitation modes did not differ with respect to protein content and protein yield which was supported by previous studies (Crowe et al., 1985; Surasani et al., 2017).

Chemical pretreatment: three chemical pretreatments were examined to determine whether the low yield was due to interactions of proteins with lipids, lignin or both. These pretreatments included 1) defatting, 2) delignification and 3) a combination of defatting and delignification. Delignification had a positive effect on the protein content whereas it did not affect the protein yield. These findings suggest that protein and lignin do not form associations and that lignin is simultaneously extracted with proteins. Lipid-protein interactions have previously been demonstrated to hamper protein extraction; a problem that might be overcome by defatting (Sari et al., 2015b). Surprisingly, defatting negatively affected the protein yield, which is probably related to the methanol-chloroform system used for defatting. A comparison between the protein content of defatted and untreated BSG revealed a lower protein content of defatted BSG which suggests that proteins are lost during defatting, probably because of moderate methanol solubility. The combined pretreatment was associated with increased protein content and reduced protein yield which are almost certainly due to the single effects of delignification and defatting, respectively.

Isolation mode and triple extraction: to rule out the possibility that the low yield was due to precipitation losses, isolates produced by freeze-drying and isoelectric precipitation of extracts were compared. This comparison revealed that the protein yield was almost 3-fold higher for isolates generated by freeze-drying (~30%) compared to isolates obtained from isoelectric precipitation (~10%). Evidently, protein loss occurred during precipitation which may partly explain the poor protein yield. To optimize the protein yield further, isolates were generated using a combination of freeze-drying and triple extraction. Results obtained under these conditions showed that the protein yield increased from 10% to 45% when freeze-drying and triple

extractions were applied instead of precipitation and a single extraction. Although this approach may appear promising, it suffers from two serious limitations. First, the protein yield (45%) was low in comparison with previous research which reported yields higher than 70% (Qin et al., 2018). Second, the protein content of isolates was comparable to that of unprocessed BSG (20-25% DM) and as a result, these isolates may have low nutritional value.

The results accumulated from **Part I** suggest that the low protein yield is caused by a combination of low extraction efficiency and precipitation losses. Obviously, further optimization is needed to develop a method that allows for maximization of the protein yield without compromising the nutritional quality of isolates.

3.2.2 Recommendations for future research

Extraction efficiency may potentially be improved through optimization of extraction pH and the solid to liquid (S:L) ratio which have been shown to influence protein extraction in earlier studies (Gao et al., 2020; Ruiz et al., 2016; Surasani et al., 2017; Vareltzis and Undeland, 2012). Furthermore, the protein loss emerging from precipitation could be minimized by optimizing the precipitation pH. In this work, an extraction pH of 12.40 was necessary to maintain alkaline conditions during extraction of BSG; therefore, future research should evaluate extraction pH values above 12.40. A S:L ratio of 1:15 was utilized in the present work; yet, increasing the extraction volume might improve protein extraction due to increased exposure of protein to extractant. Thus, it is recommended that future studies investigate the impact of S:L ratio, extraction pH and precipitation pH on protein yield and content. Simultaneous assessment of these factors might be accomplished by using a three-factorial design which can be constructed as exemplified in Figure 5.

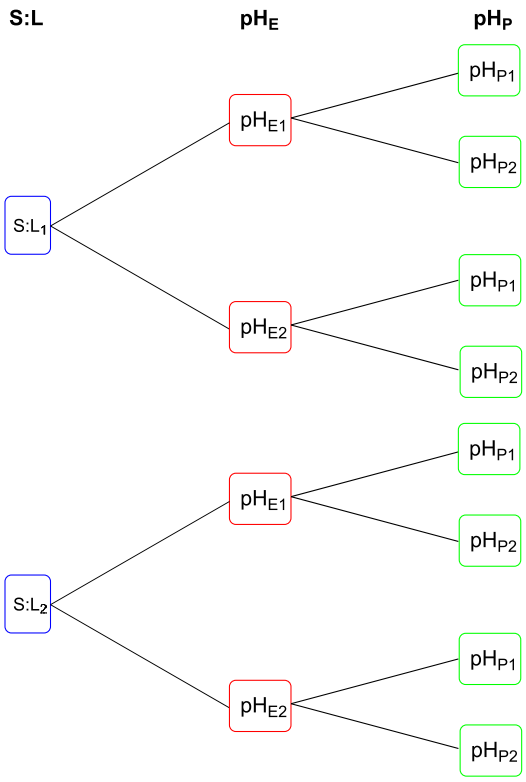


Figure 5. Proposal of a nested design for optimization of solid to liquid (S:L) ratio, extraction pH (pH_E) and precipitation pH (pH_P). For simplification, two levels of each parameter were shown in this figure.

It is well-established that alkaline conditions combined with high temperatures might decrease the nutritional value of protein isolates because of an altered tertiary protein structure and enhanced production of Maillard reaction products (Deleu et al., 2019). Structural alterations of proteins are often associated with reduced solubility in aqueous environment. Maillard reactions are known to lower the bioavailability of amino acids, especially lysine, which are consumed to form Maillard reaction products (Van Rooijen et al., 2013). In consequence, future studies should also consider these aspects when evaluating the nutritional quality of protein isolates produced by alkaline extraction of BSG.

4. Part II: delignification of BSG with focus on biological methods

4.1 Delignification strategies

Historically, the term delignification has been used to describe the natural process of wood decay in which lignin is slowly degraded by microorganisms. In modern terms, this definition has been broadened to include any process that results in degradation or removal of lignin from a biomass (Wool, 2005). In a biotechnological context, delignification serves a dual purpose. Firstly, it may improve the nutritional quality of lignocellulosic residues by eliminating the antinutritional effect of lignin, thereby enabling their use in feed applications. Secondly, delignification favors downstream processing of lignocellulosic biomasses as it increases the access of hydrolytic enzymes to cellulose and hemicellulose. Delignification methods can be grouped into two broad categories: chemical and biological methods. Chemical delignification relies on the extraction of lignin using different solvent systems including alkaline solutions, organic solvents (ethanol or acetone) and ionic liquids while biological methods employ lignin-degrading microorganisms to delignify a biomass (Christopher et al., 2014; Prado et al., 2013). The major advantage of biological methods is a low by-product formation due to the mild operation conditions. In contrast, chemical methods often involve harsh conditions which may cause biomass deterioration. On an industrial scale, however, biological methods are currently of limited use because they require long incubation times (up to 50 days) and may lead to consumption of other essential biomass components such as protein and carbohydrates (Tocco et al., 2021).

This PhD project aimed to develop a better understanding of the biological delignification of BSG. Before proceeding to examine the main findings of **Part II**, it is important to understand the key aspects of biological delignification which will therefore be presented in the following pages.

4.1.1 Biological delignification systems

Biological delignification is principally carried out using solid-state fermentation (SSF); a process that involves the cultivation of microorganisms on a solid substrate in the absence and near-absence of free water. Water is an essential element for all

microbial processes; therefore, minimum moisture levels are required to sustain growth during SSF (Lizardi-Jiménez and Hernández-Martínez, 2017).

Lignin-degrading microorganisms comprise a diverse group of bacteria and fungi (Atiwesh et al., 2022). Generally, fungi are more efficient in lignin degradation as compared to bacteria. Lignin-degrading fungi are classified into three main groups depending on their ecology and substrate preference: 1) white-rot fungi, 2) brown-rot fungi and 3) soft-rot fungi (Janusz et al., 2017). The brown-rot fungi are able to depolymerize cellulose and hemicellulose while their lignin degradation is inefficient. Soft-rot fungi are non-selective degraders of lignin, cellulose and hemicellulose whereas most white-rot fungi selectively degrade lignin, thus leaving cellulose and hemicellulose untouched (Blanchette, 1995). In addition, white-rot fungi are the most efficient lignin degraders because they are capable of oxidizing lignin completely to CO_2 and H_2O under aerobic conditions. The possibility of utilizing white-rot fungi in biological delignification has drawn special attention due to their high substrate specificity and effective lignin degradation (Abdel-Hamid et al., 2013). *Phanerochaete chrysosporium* represents the most well-characterized white-rot fungus, and it has been extensively used to study different aspects of fungal delignification (Schoemaker and Leisola, 1990).

Lignin-degrading enzymes are secreted extracellularly by fungi and comprise three major types: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. The LiP and MnP share several structural and functional similarities as they are both H_2O_2-dependent enzymes with a heme group embedded in the active site. Production and activity of these enzymes depend largely on the culture conditions (Singh and Chen, 2008). To establish conditions that enhance enzyme production and activity, it is important to be familiar with the degradative mechanisms which differ among laccase, MnP and LiP (Wong, 2009). MnP, LiP and laccase are collectively known as lignin modifying enzymes (LME) because they are directly involved in the degradation of lignin. In addition to LMEs, white-rot fungi produce various accessory enzymes which play a crucial role in delignification but cannot degrade lignin on their own. These enzymes are often responsible for the production of extracellular H_2O_2 consumed by LiP and MnP; therefore, medium supplementation with H_2O_2 is not necessary for maintaining high activities of LiP and MnP (Janusz et al., 2017).

Lignin peroxidase: unlike MnP and laccase, oxidation of phenolic and non-phenolic lignin structures by LiP does not require redox mediators, which are defined as small compounds or ions that function as electron carriers between enzyme and substrate (Christopher et al., 2014; Vandana et al., 2019). This may be explained by the high redox potential of LiP (E^o ~1.2 V) which makes it an excellent electron acceptor (oxidant) as compared to MnP and laccase (E^o ~0.5-0.8 V) (Wong, 2009). Due to the lower redox potential of MnP and laccase, these enzymes are incapable of oxidizing the non-phenolic structures of lignin in the absence of a mediator. As displayed in Figure 6, the catalytic cycle of LiP encompasses three main steps:

1. Oxidation of the native LiP enzyme to Compound I by a two-electron transfer process in which H_2O_2 serves as the electron acceptor.
2. Reduction of Compound I to Compound II coupled with the oxidation of phenolic or non-phenolic lignin substrates (S_{red}).
3. Reduction of Compound II to the native state of LiP and simultaneous oxidation of another lignin substrate (S_{red}).

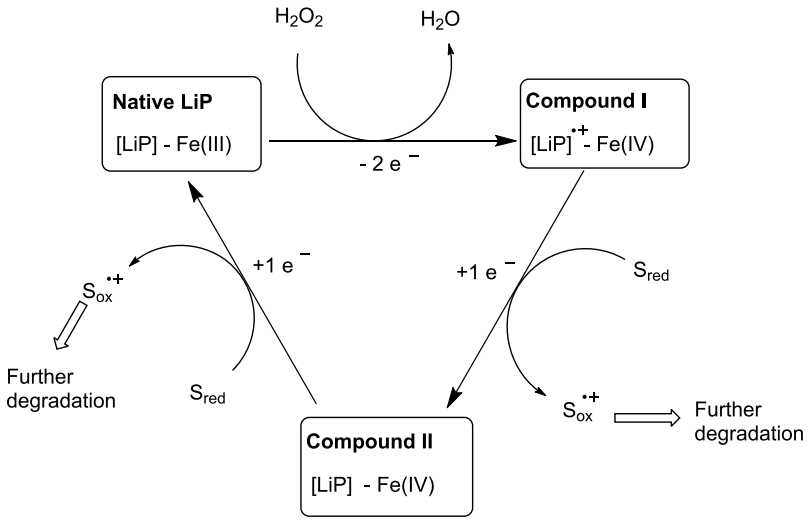


Figure 6. Catalytic cycle of lignin peroxidase (LiP) involved in lignin degradation. S_{red} is a phenolic or non-phenolic substrate of the lignin polymer.

The redox processes in step 2-3 proceed via transfer of a single electron resulting in the oxidation of lignin substrates (S_{red}) to cation radicals ($S_{ox}^{\cdot+}$). Subsequently, these reactive intermediates undergo various degradative reactions such as C-C and ether cleavages, aromatic ring opening, demethoxylation and decarboxylation (Schoemaker and Leisola, 1990).

Manganese peroxidase: mechanistically, the catalytic cycle of MnP resembles that of LiP; however, the main difference is that MnP-bound Mn^{2+} ions, located near the active site, are utilized as redox mediators (Figure 7).

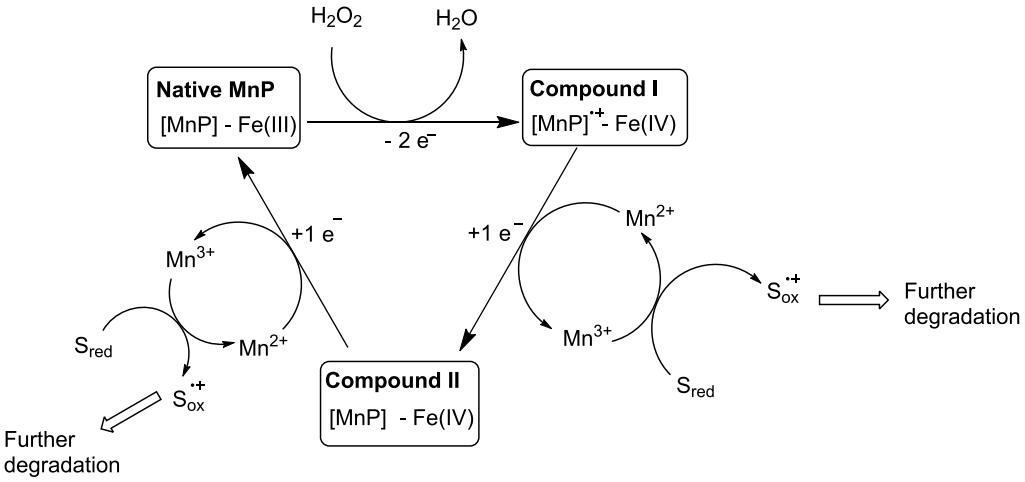


Figure 7. Catalytic cycle of manganese peroxidase (MnP) involved in lignin degradation. S_{red} is a phenolic or non-phenolic substrate of lignin.

Reduction of the oxidized MnP intermediates (Compound I and II) is accompanied by the oxidation of Mn^{2+} to Mn^{3+}. The formed Mn^{3+} ions are detached from MnP and stabilized by chelation with organic acids. Oxidation of lignin substrates is facilitated by reduction of the chelated Mn^{3+} ions to Mn^{2+} ions which may reassociate with MnP and be utilized in the oxidation of a new lignin substrate (Wong, 2009).

Laccase: laccases are multicopper-containing phenol oxidases that catalyze the oxidation of phenolic and non-phenolic substrates with simultaneous reduction of molecular oxygen to water (Thurston, 1994). Oxidation proceeds via laccase-mediator systems in which small aromatic compounds act as mediators. The mediators help overcome steric hindrance between substrates and laccase and facilitate the oxidation of non-phenolic lignin substrates which are highly recalcitrant and therefore require strong oxidants (Li et al., 1999). The mechanistic aspects of substrate oxidation by laccase-mediator systems are delineated in Figure 8. Initially, the mediator is oxidized by laccase to generate an intermediate of high redox potential ($mediator_{ox}$) which dissociates from laccase and oxidizes the substrate (S_{red}). During this reaction, the mediator is converted to its reduced form that reenters the catalytic cycle of the laccase-mediator system (Christopher et al., 2014; Shraddha et al., 2011).

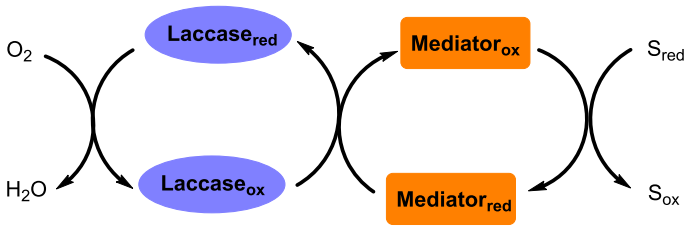


Figure 8. Catalytic cycle of a laccase-mediator system. The mediator acts as an electron shuttle between laccase and lignin substrates (S_{red}). The terminal electron acceptor is O_2 which is reduced H_2O during the catalytic cycle.

Previous research has indicated that phenolic structures of lignin are preferentially oxidised before the non-phenolic lignin structures. During the initial oxidation of phenolic structures, small phenolic compounds are formed which serve as mediators to oxidize the non-phenolic parts of lignin (Camarero et al., 1994). Examples of naturally-occurring phenolic compounds, which have proven to be effective mediators, include syringaldehyde, p-coumaric acid and vanillin (Camarero et al., 2005).

Because *P. chrysosporium* formed the central focus of **Part II**, the remaining part of this chapter will concentrate on describing key features of this white-rot fungus. It has been established that *P. chrysosporium* produces multiple extracellular isoenzymes of LiP and MnP which act in a synergistic manner to degrade lignin (Singh and Chen, 2008). There is a general belief that *P. chrysosporium* does not produce any laccase. Yet, high laccase activities have been reported for cultures of *P. chrysosporium* supplemented with MnO_2 indicating that MnO_2 may act as a stabilizer of laccase (Couto et al., 1998; Rodríguez Couto et al., 2000). Another unique characteristic of *P. chrysosporium* is its ability to grow optimally at 40°C. A large volume of literature has investigated the factors involved in regulating the activity of lignin-degrading peroxidases (LDPs), including LiP and MnP, produced by *P. chrysosporium* (Cancel et al., 1993; Couto et al., 1998; Faison and Kirk, 1985; Hammel et al., 1985; Kirk et al., 1986; Schoemaker and Leisola, 1990; Venkatadri and Irvine, 1990). Based on these studies, it can be concluded that the following conditions have a stimulatory effect on LDP activity, particularly LiP, in *P. chrysosporium*:

1. Nutrient limitation, mainly nitrogen and carbon limited conditions.
2. Presence of veratryl alcohol (VA); a natural secondary metabolite of *P. chrysosporium*.
3. Addition of MnO_2

4. Excess of trace elements
5. High oxygen tension

The enhanced LDP activity observed under nutrient scarcity suggests that production of MnP and LiP is part of the secondary metabolism of *P. chrysosporium.* By contrast, excess levels of nutrients appeared to have a suppressive effect on LDP activities (Faison and Kirk, 1985). Moreover, some studies have considered the effect of agitation under submerged conditions and found that LDP activity was strongly inhibited by agitation, probably because of mechanical inactivation (Faison and Kirk, 1985; Venkatadri and Irvine, 1990).

The positive effect of VA on LiP activity is well-documented; though, the exact role of VA has been subject to considerable debate. Until recently, VA was believed to be an inducer of the ligninolytic system of *P. chrysosporium*, thereby increasing protein synthesis of LiP (Leisola et al., 1984). Nonetheless, this theory was refuted by Cancel et al. (1993) who found strong evidence that VA protects LiP against H_2O_2–induced inactivation and decay rather than activating the synthesis of LiP. This conclusion was based on the findings that external addition of VA caused a stable LiP activity under prolonged H_2O_2 exposure while it did not increase the transcription of LiP encoding genes (Cancel et al., 1993).

In **Part II**, SSF using *P. chrysosporium* was explored with the aim of optimizing delignification of BSG. To date, a number of studies have demonstrated the use of BSG as a SSF substrate for production of protein, amino acids and enzymes by different fungi (Ibarruri et al., 2019; Sousa et al., 2018; Tan et al., 2019). To the best of the author's knowledge, no previous studies have considered the possibility of utilizing *P. chrysosporium* to delignify BSG by SSF. The following section outlines the experimental design and presents major results obtained from this work; furthermore, it provides a technical description of the method applied for quantification of lignin in fermented and unfermented BSG.

4.2 Employing solid-state fermentation for delignification of BSG

4.2.1 Quantification of lignin and insoluble polysaccharides by the Van Soest method

The Van Soest method is based on the fractionation of lignocellulosic biomass into three insoluble fractions known as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL). As depicted in Figure 9, these fractions can be utilized for estimating the contents of cellulose and insoluble hemicellulose in a biomass. In addition to lignocellulose and soluble components (proteins, lipids and sugars), biomasses contain ash. As a result, the NDF, ADF and ADL fractions must be corrected for the ash content of the biomass. Each fraction is determined gravimetrically as the solid residues retained on porous crucibles after extraction and removal of solubilized material by filtration. The contents of NDF, ADF and ADL are generally expressed as percentages of the dry matter content (Mongeau and Brooks, 2015).

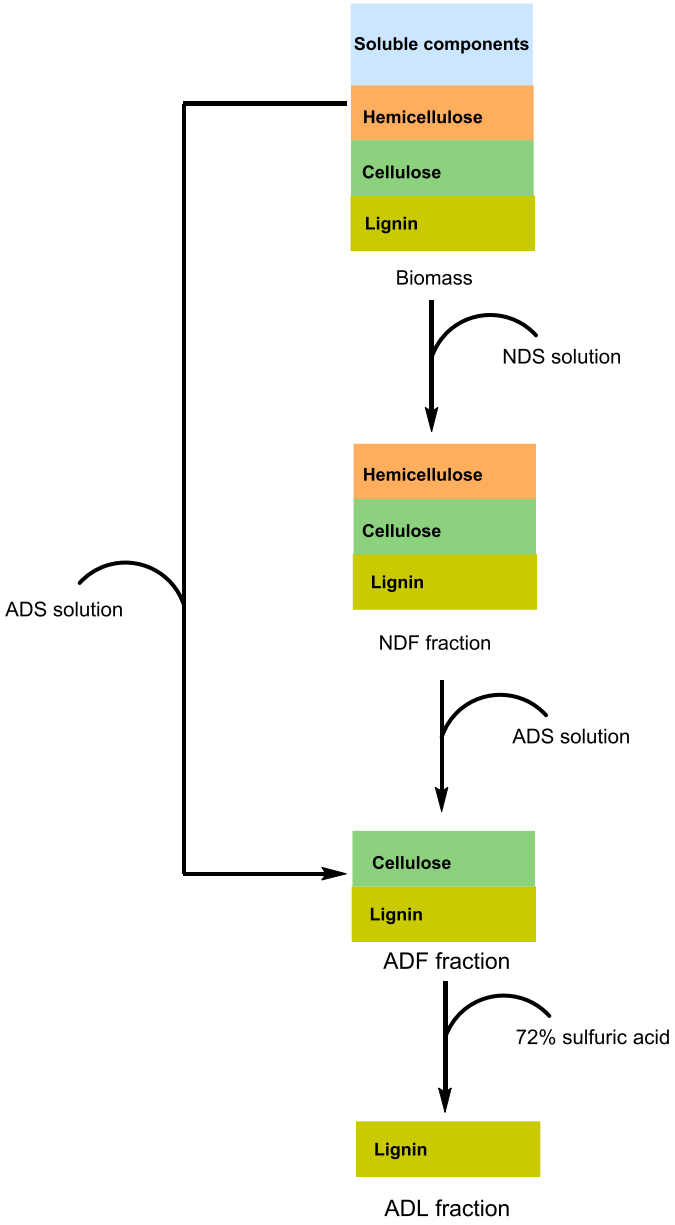


Figure 9. Principles of the Van Soest method. For the sake of simplicity, the ash content is omitted from the figure. Soluble components are comprised of proteins, lipids and various sugars. Abbreviations: NDS = neutral detergent solution, NDF = neutral detergent fibre, ADS = acid detergent solution, ADF = acid detergent fibre and ADL = acid detergent lignin.

NDF is the solid fraction remaining after treatment with neutral detergent solution (NDS) and α-amylase which dissolve the soluble fraction of the biomass including protein, lipids, starch and pectin. Further, enzymatic treatment with amylase eliminates interferences with starch, consequently increasing the accuracy of NDF determination

(Van Soest et al., 1991). The NDF fraction, which typically comprises cellulose, hemicellulose and lignin, is calculated according to equation (3):

$$\mathrm{NDF}(\%) = \frac{\mathrm{m_{dry\ NDF}\ (g) - m_{ash}(g)}}{\mathrm{m_{sample}(g)}} * 100\% \quad (3)$$

where $\mathrm{m_{sample}}$ is the sample weight before analysis, $\mathrm{m_{dry\ NDF}}$ is the sample weight after extraction with NDS, filtration and drying and $\mathrm{m_{ash}}$ is the weight of ashes generated by incineration of the dried NDF residue.

ADF is defined as the insoluble fraction that remains after extraction with acid detergent solution (ADS); it is chiefly composed of cellulose and lignin. The ADF may be determined either by extracting the intact biomass or the NDF fraction with ADS, and it is calculated using equation (4):

$$\mathrm{ADF}(\%) = \frac{\mathrm{m_{dry\ ADF}(g) - m_{ash}(g)}}{\mathrm{m_{sample}(g)}} * 100\% \quad (4)$$

Definitions of all variables in equation (4) are equivalent to those in equation (3), the only difference being that equation (4) is based on the ADF fraction instead of the NDF fraction.

Acid detergent lignin (ADL) refers to the solid residue obtained from extraction of the ADF fraction with 72% sulfuric acid. Lignin is known to be composed of an acid-soluble and an acid-insoluble fraction, with the latter representing the most abundant fraction in most lignocellulosic biomasses. The ADL fraction excludes acid-soluble lignin and therefore it slightly underestimates the true lignin content.

Cellulose and hemicellulose can be easily deduced from the NDF, ADF and ADL contents by using equation (5) and (6):

$$\mathrm{Hemicellulose}(\%) = \mathrm{NDF}(\%) - \mathrm{ADF}(\%) \quad (5)$$

$$\mathrm{Cellulose}(\%) = \mathrm{ADF}(\%) - \mathrm{ADL}(\%) \quad (6)$$

The Van Soest method is a facile approach for determining the contents of structural polysaccharides and lignin in biomasses; yet, it has a number of flaws. First, it does not provide insight into the structural characteristics of the isolated fractions. Second, it may be inaccurate for complex biomasses containing interfering compounds, such as chitin which are insoluble in ADS and NDS. When present in the biomass, such

components may impede the fractionation process, eventually resulting in overestimation of the NDF and ADF contents (Mould and Robbins, 1981).

4.2.2 Solid-state fermentation of BSG using Phanerochaete chrysosporium

Solid-state fermentation (SSF) was conducted by adopting the procedure described by Couto et al. (1998). The principal goal of this work was to evaluate the suitability of different BSG media as potential SSF substrates for delignification by *P. chrysosporium*. Specifically, this work aimed to test the hypothesis that fungal delignification of BSG can be increased by supplementation with veratryl alcohol (VA), MnO_2 and excess levels of trace elements (6X). In addition, it was hypothesized that excess levels of trace elements combined with VA or MnO_2 may have an additive positive effect on delignification. All experimental details are illustrated graphically in Figure 10.

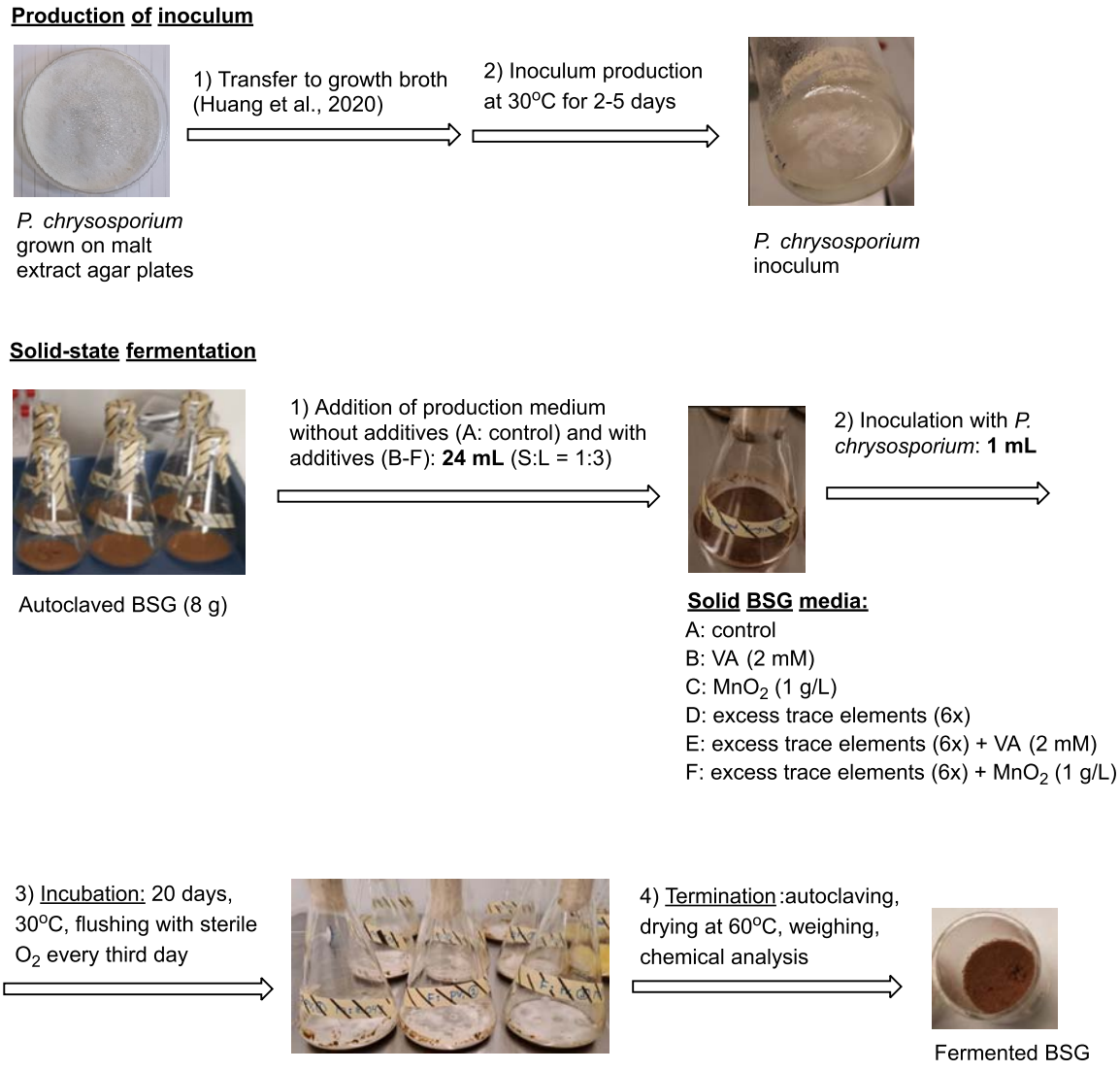


Figure 10. Design of solid-state fermentation (SSF) of brewer's spent grain (BSG) using the fungus Phanerochaete chrysosporium. First, an inoculum of P. chrysosporium was produced in liquid medium and used for inoculation of six solid BSG media (A-F). P. chrysosporium was grown on the BSG media for 20 days and sterile O_2 was added every third day to enhance enzymatic lignin degradation. VA: veratryl alcohol, S:L: solid to liquid ratio.

The growth broth used for inoculum production was prepared according to Huang et al. (2020) and had a glucose concentration of 10 g/L. The production medium, which was added to the autoclaved BSG, had the same composition as the growth broth expect for a glucose concentration of 2 g/L (Couto et al., 1998). Prior to inoculation of the solid BSG media, the spore concentration of the inoculum was measured as described by Tien and Kirk (1988). In summary, the inoculum was homogenized by manual agitation and a small volume (~10 mL) was drawn out and filtered through a

cell strainer (40 µm) to produce a mycelium-free spore suspension. The spore density was determined spectrophotometrically by measuring the absorbance at 650 nm, and the measured absorbance was converted into spore concentration assuming that an absorbance of one unit corresponds to $5 \cdot 10^6$ spores/mL. In the present work, all BSG media were inoculated with ~100.000 spores which was considered a sufficient quantity for establishing new cultures of *P. chrysosporium* on solid substrates. On completion of the SSF experiment, fermented BSG samples were autoclaved to quench microbial activity, dried at 60°C and analysed for ADF, ADL and protein. Compositional differences between fermented and unfermented BSG are summarized in Table 4.

Table 4. Comparison of unfermented and fermented brewer's spent grain (BSG) obtained from solid-state fermentation (SSF) with *Phanerochaete chrysosporium* on different BSG media (A-F). For unfermented BSG, the protein content was determined in duplicates while acid detergent fibre (ADF) and acid detergent lignin (ADL) were quantified in triplicates. The SSF treatments (media A-F) were carried out in triplicates and a single analysis was performed for each triplicate. All data is presented as mean±SD*.

	ADF (% DM)	ADL (% DM)	Protein (% DM) **
Unfermented BSG	22.8 ± 1.15^a	3.00 ± 0.48^a	20.2 ± 0.10^a
Fermented BSG			
Medium A	31.5 ± 0.07^b	2.55 ± 0.11^b	23.7 ± 0.13^b
Medium B	33.1 ± 0.64^b	2.67 ± 0.26^b	24.0 ± 0.58^b
Medium C	31.4 ± 0.10^b	2.71 ± 0.07^b	23.5 ± 0.16^b
Medium D	31.4 ± 0.17^b	2.68 ± 0.16^b	23.4 ± 0.12^b
Medium E	31.6 ± 0.61^b	2.82 ± 0.06^b	23.5 ± 0.27^b
Medium F	30.5 ± 1.68^b	2.92 ± 0.26^b	23.0 ± 0.28^b

*To evaluate the effect of media type (A-F) on the composition of fermented BSG, data was subjected to a one-way analysis of variance (ANOVA) while a t-test was employed for pairwise comparison of unfermented BSG and fermented BSG from different SSF treatments (medium A-F). Lowercase letters designate significant differences between fermented BSG from different SSF treatments as well as fermented and unfermented BSG. **Protein content was measured with the Kjeldahl method using a conversion factor of 6.25. For unfermented BSG, protein data was retrieved from Table 2.

Lignin (ADL): a statistically significant reduction in the lignin content was observed for all SSF treatments (media A-F) which indicated that *P. chrysosporium* has the capability to delignify BSG. In contrast, no significant differences were found between the different BSG media which suggest that addition of VA, MnO_2 and excess levels of trace elements did not promote the delignification of BSG. From a nutritional and

economic point of view, the observed lignin reduction was not meaningful as only 10-15% of lignin was removed after 20 days. There might be several plausible explanations for the low delignification rates. Firstly, it could be due to matrix interactions which may restrict the access of enzymes to lignin. Secondly, it might be caused by high levels of secreted polysaccharides which are known to inhibit lignin degradation in *P. chrysosporium* (Huang et al., 2020). Thirdly, the results might be explained by insufficient enzyme production. This theory finds support in the work of Gassara et al. (2010) who investigated the production of LiP, MnP and laccase by solid-state cultures of *P. chrysosporium* grown on BSG with and without VA. Interestingly, there was no production of LiP and laccase while high MnP production was observed in the presence and absence of VA. It is well-known that fungal delignification is controlled by the concerted action of different lignin-degrading enzymes. Thus, the slow delignification observed in the present work could be due to the absence of one or more enzyme activities. To establish this, however, activities of laccase, LiP and MnP should be assayed for each SSF treatment.

Protein: a significant increase (15-20%) was observed in the protein content of BSG for all SSF treatments; however, the protein content of fermented BSG (23-24%) was still too low to be considered suitable for aquafeed.

ADF: SSF led to a noticeable increase in the ADF content of BSG (~40%). This finding is probably due to accumulation of chitin, which is insoluble in ADS. Production of chitin may be used as an indirect measure of a fungus ability to proliferate on a certain substrate since chitin is an important structural polysaccharide of fungal cell walls (Blumenthal and Roseman, 1957). Hence, the high ADF levels imply that the conditions were favourable for growth but did not facilitate efficient lignin degradation. High chitin levels in fermented BSG may hinder its use in aquafeeds as chitin is an antinutritional factor (ANF) for some fish species (Karlsen et al., 2017).

As a whole, the findings of **Part II** were less promising because the nutritional quality of BSG did not change considerably during SSF. Undoubtedly, further work is needed to develop a more effective method for delignification of BSG.

4.2.3 Suggestions for future research

Solid-state fermentation can be considered a less feasible approach to delignify BSG as it is time-consuming, ineffective and leads to accumulation of chitin. These

challenges could conceivably be addressed by employing an enzymatic strategy. In their pioneering work, Fan et al. (2019) established an enzymatic method in which corn stover was treated with a LiP isolated from submerged cultures of *Aspergillus oryzae*. Furthermore, they identified ideal conditions for delignification of corn stover by systematic screening of multiple parameters including temperature, time, buffer pH, enzyme load, H_2O_2 concentrations and S:L ratio. In view of their findings, it is suggested that future research adopt a similar approach to investigate the enzymatic delignification of BSG.

5. Part III: valorization of non-starch polysaccharides in BSG using a combination of hydrolysis and submerged fermentation

The third part of the PhD project is concerned with valorization of the NSP (cellulose and hemicellulose) in BSG using a combination of hydrolysis and submerged fermentation. **Part III** was undertaking with two major objectives. First, it pursued to increase the nutritional value of BSG by hydrolysing hard-to-degrade cellulose and hemicellulose to monosaccharides including glucose, xylose and arabinose. Second, **Part III** investigated the possibility of utilizing the BSG-derived monosaccharides as substrates for production of free amino acids (**Manuscript 3**) and single cell protein (SCP) (**Manuscript 4**) by submerged fermentation. For convenience, the work of **Part III** will be divided into two sections. The first section lays out the theoretical dimensions of NSP hydrolysis and presents the primary results from the optimization experiments that were performed before the fermentations. The second section deals with submerged fermentation of BSG-derived sugars and is composed of four consecutive parts including: 1) short background, 2) outline of main findings from the experimental work and 3) recommendations for future research. In addition, the second section considers how the fermentation products may contribute to the development of sustainable aquafeeds. The different segments of **Part III** are summarized in Figure 11.

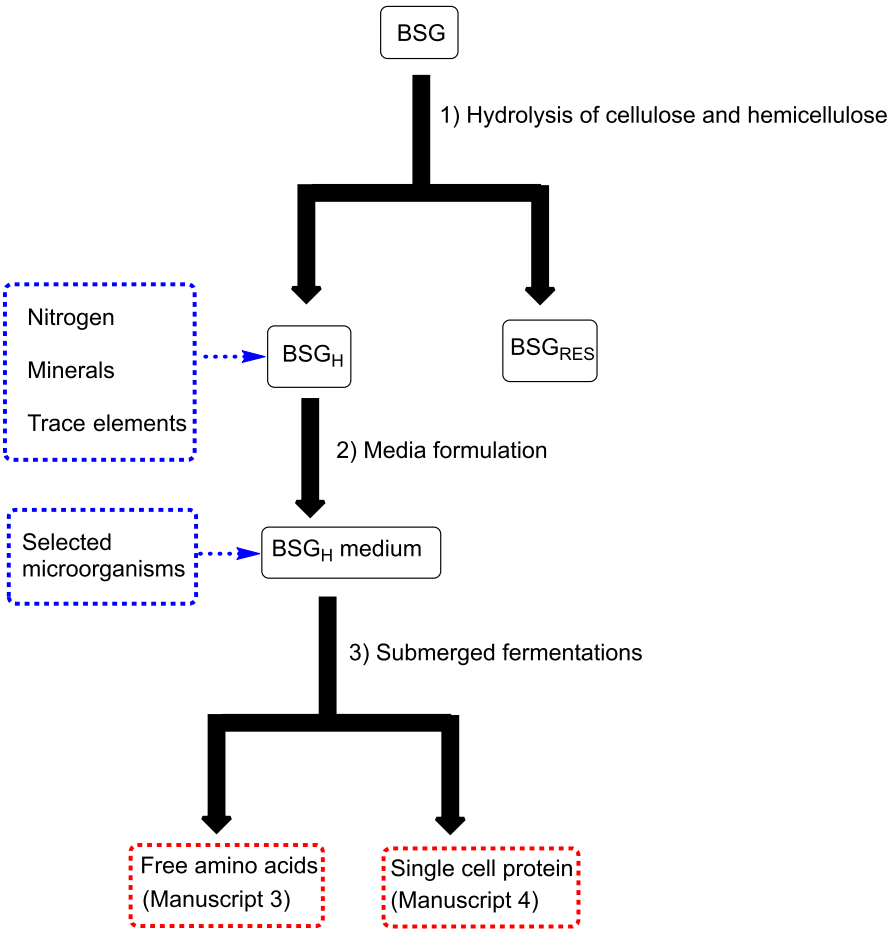


*Figure 11. Schematic overview of the experimental work of **Part III**. Abbreviations: BSG = brewer's spent grain, BSG_H = hydrolysate of brewer's spent grain and BSG_{RES} = hydrolysed residue of brewer's spent grain.*

5.1 Hydrolysis of cellulose and hemicellulose

5.1.1 Hydrolysis methods

During hydrolysis of cellulose and hemicellulose, the polymeric structures are broken down into their monomeric building blocks comprising glucose, xylose and arabinose (Laca et al., 2019). These monosaccharides are liberated from the BSG matrix resulting in production of a solid BSG residue (BSG_{RES}) and a sugar-rich hydrolysate (BSG_H) that may serve as a C-source for fermentative processes (Figure 11). Under optimal conditions, protein will be retained in solid BSG_{RES} fraction while cellulose and hemicellulose are recovered as fermentable sugars in the BSG_H fraction (Mussatto and Teixeira, 2010). Generally, hydrolysis of cellulose and hemicellulose can be achieved by means of chemical and enzymatic methods (Galbe and Zacchi, 2002).

The chemical method relies on acidic hydrolysis in which an acid catalyses the cleavage of polymeric bonds in cellulose and hemicellulose. The main advantage of acidic hydrolysis is that it does not require a preliminary delignification step because acid molecules easily penetrate the lignin barrier that protects cellulose and hemicellulose (Verardi et al., 2012). Acidic hydrolysis is performed using either concentrated (30-70%) or diluted acid (2-5%), with the most widely used acids being hydrochloric acid (HCl) and sulfuric acid (H_2SO_4). One advantage of using concentrated acid solutions is that high hydrolysis yields can be obtained at low temperatures (Galbe and Zacchi, 2002). On the other hand, this approach involves a risk of equipment corrosion due to the high acid concentrations. To overcome this challenge, diluted acid may be employed to hydrolyse cellulose and hemicellulose. Yet, the main limitation of dilute acid processes is that high temperatures (200-240°C) are required for obtaining acceptable hydrolysis yields which may increase the production of various sugar decomposition products such as furfural, 5-hydroxymethylfurfural (5-HMF) and acetic acid (Niju et al., 2019). Cellulose hydrolysis leads to production of glucose as the sole product while hydrolysis of hemicellulose generates assorted products including arabinose, xylose and acetic acid. Harsh conditions, such as high temperatures combined with acidic media, favour the degradation of glucose to 5-HMF and acetic acid whereas xylose and arabinose are degraded to furfural (Fiorella et al., 2015). Excessive concentrations of these by-products are undesirable for fermentative processes because they are toxic to many types of fermenting microorganisms. As documented in previous studies, these compounds inhibit growth as well as product formation during fermentation; therefore, their production should be avoided (Baek et al., 2008; Casey et al., 2010; Dessie et al., 2019; Martín and Jönsson, 2003). Recently, enzymatic hydrolysis has become an attractive alternative because it is associated with minimal formation of fermentation inhibitors due to the mild reaction conditions. Despite this, enzymatic hydrolysis of NSP is yet to be implemented on industrial scale owing to the high costs of enzymes and a relatively low enzyme activity compared to other commercial enzymes (Laca et al., 2019; Verardi et al., 2012).

Enzymatic hydrolysis of cellulose and hemicellulose takes place by two distinct systems which rely on the synergism of multiple enzymes. The conversion of crystalline cellulose into glucose is carried out by the concerted action of cellulases, a

class of enzymes that catalyse the hydrolysis of β-(1,4) glycosidic bonds. The cellulases involved in the depolymerization of cellulose comprise three types with different substrate specificity and mechanism of action (Figure 12). These are: 1) β-1,4-endoglucanase, 2) β-1,4-exoglucanase and 3) β-glucosidase (Singhania, 2011). The β-1,4-endoglucanase hydrolyses internal bonds located in the amorphous regions of crystalline cellulose which results in the formation of cellulose chains of varying length. The β-1,4-exoglucanase, also known as cellobiohydrolase, catalyses the cleavage of cellobiose units from the non-reducing ends of the cellulose chains. Finally, the liberated cellobiose dimers are hydrolysed to glucose monomers by β-glucosidase (Béguin and Aubert, 1994; Gomez Del Pulgar and Saadeddin, 2014).

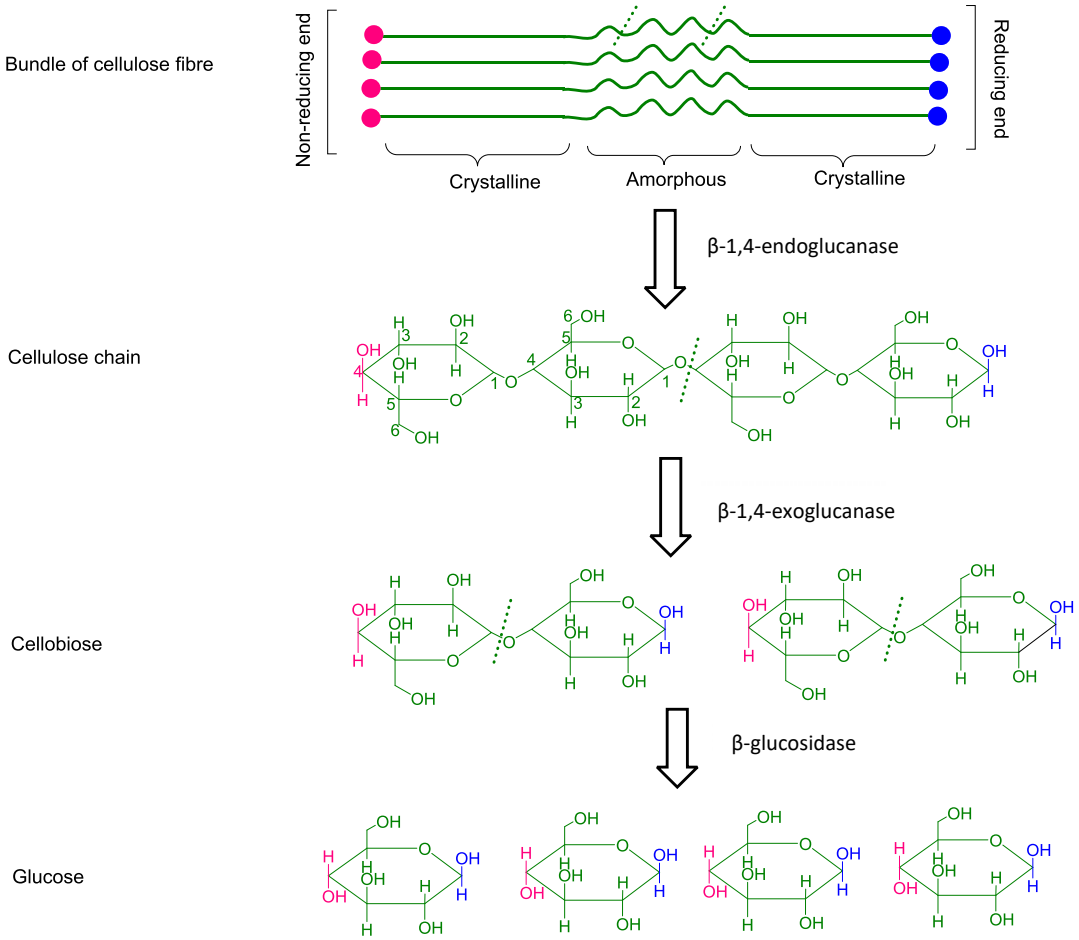


Figure 12. Enzymatic conversion of cellulose. Reducing and non-reducing ends are highlighted in blue and pink, respectively. Dashed lines indicate the site of cleavage during enzymatic hydrolysis.

Owing to the structural complexity of hemicellulose, total hydrolysis of this NSP depends on the simultaneous action of various enzymes which target different bonds.

The enzymes involved in hydrolysis of arabinoxylan (AX), the main hemicellulose in BSG, are shown in Figure 13 and include: 1) α-L-arabinofuranosidase, 2) endo-1,4-xylanase, 3) β-xylosidase, 4) ferulic acid esterase and 5) acetyl xylan esterase. To attain complete AX conversion, all five enzymes must be present in the enzyme mixture used for enzymatic treatment (Xiros et al., 2011).

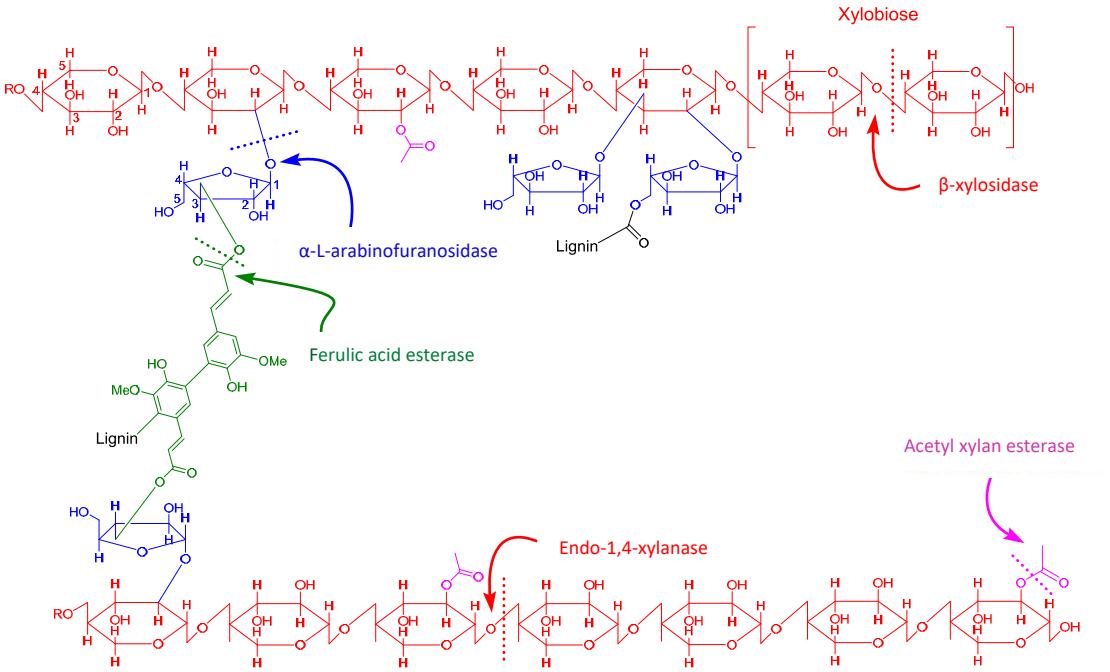


Figure 13. Enzymes involved in the hydrolysis of arabinoxylan (AX) which is composed of a xylose backbone (red) substituted with acetyl (pink) and arabinose (blue) moieties. Adjacent xylose chains are linked via cross-linkages of ferulic acid (green). Dashed lines denote the sites of enzymatic cleavage. The figure is modified from Karlsen and Skov (2022) *(**Paper 1**).*

To obtain an effective enzymatic conversion of cellulose and hemicellulose, the biomass must undergo a pretreatment prior to hydrolysis. During this process, the accessibility of cellulose and hemicellulose to hydrolytic enzymes is increased by lignin removal or structural alterations of lignocellulose. Removal of lignin is possible via the different delignification strategies discussed in **Part II** while the overall structure of lignocellulose may be disintegrated by mechanical pretreatment such as the micronization procedure applied for BSG in this PhD project. Mechanical pretreatment decreases cellulose crystallinity and increases the overall surface area onto which enzymes can act, but it does not remove or modify lignin (Alvira et al., 2010). Therefore, this approach might be less suitable for biomasses of high lignin content.

Numerous factors have been found to influence the enzymatic hydrolysis of cellulose and hemicellulose which must be taken into account when optimizing a hydrolysis process. These factors include type of pretreatment, enzyme dosage, buffer pH, temperature, agitation mode and duration of hydrolysis (Amit et al., 2018). Further, higher enzyme dosages are required for biomasses with a high lignin content due to adsorption of hydrolytic enzymes onto lignin (Lu et al., 2016).

To date, an extensive body of literature has investigated the enzymatic conversion of the cellulose and hemicellulose in BSG employing either commercial enzyme mixtures or enzyme extracts produced by SSF (Forssell et al., 2008; Giacobbe et al., 2019; Mussatto et al., 2008; Paz et al., 2019; Rochelle et al., 2008; Xiros et al., 2008b, 2008a). Hence, the focus of this PhD project was not to develop a fully optimized hydrolysis process for BSG. Instead, enzymatic hydrolysis was employed as a tool for producing bulk volumes of BSG hydrolysate adopting a modified version of the procedure described by Forssell et al. (2008). Initially, this procedure was further optimized with respect to enzyme dosage and hydrolysis duration. The following section provides a brief outline of the main results from the initial optimization experiments.

5.1.2 Optimization of enzymatic hydrolysis of cellulose and hemicellulose in BSG

According to the procedure outlined by Forssell et al. (2008), BSG was suspended in ammonium acetate buffer (50 mM, pH 5.0) to achieve a final BSG concentration of 10% w/v. This suspension was preheated to 50°C and the enzyme preparation Depol 686L was added (Biocatalyst, UK). The resulting reaction mixture was incubated at 50°C for 5 h with intermittent agitation after which the solid and liquid fractions were separated by centrifugation. The solid fractions were washed twice to extract sugars that might be trapped in the BSG matrix. The liquid fractions from washing were combined with the liquid fraction obtained directly from the reaction mixture to produce a dilute hydrolysate. Finally, glucose was quantified in the dilute hydrolysates using the Glucose Colorimetric Detection Kit (ThermoFisher Scientific, Norway). Concentrations of glucose were used as an indirect measure of cellulose hydrolysis efficiency. Likewise, the efficiency of AX hydrolysis can be measured in terms of xylose and arabinose concentrations; however, quantification of these sugars was not

possible due to technical constraints. Because of this, the conversion efficiency of the NSP in BSG was solely based on cellulose hydrolysis. Due to a low lignin content (~3% DM), it was assumed that lignin would have minimal impact on enzyme activity; therefore, micronized BSG was not delignified prior to hydrolysis. To optimize the original procedure of Forssell et al. (2008), three different enzyme dosages were tested: 16, 53 and 106 U/mL. Concurrently, a control treatment was performed to determine the degree of glucose solubilization in buffer solution without enzyme.

Results from the initial optimization experiment showed an increasing trend in glucose concentration with increasing enzyme dosage, thus the highest glucose concentration was observed at an enzyme dosage of 106 U/mL (Figure 14). At the lowest enzyme dosage (16 U/mL), the glucose concentration was comparable to that obtained from the control treatment which implied a lack of enzymatic conversion. Moreover, the glucose concentrations were generally low for all tested enzyme dosages (0.2-0.8 g/L) and therefore further optimization was required. To examine how the nutritional quality of BSG is affected by hydrolysis using different enzyme dosages, protein contents were determined in the BSG residues (BSG_{RES}) with the Kjeldahl method. Results from this analysis revealed an increase in the protein content from ~20% in untreated BSG to ~29% in hydrolysed BSG (data not shown). More interestingly, the protein content seemed unaffected by enzyme dosage which may raise questions about the actual cause of the protein enrichment observed during hydrolysis. Although interesting, these findings were not explored further here but may serve as a base for future research.

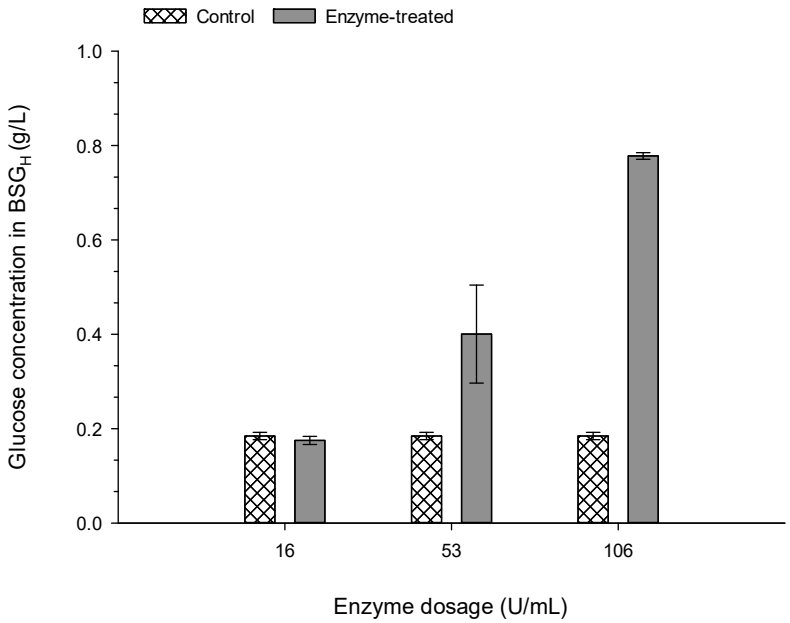


Figure 14. Effect of enzyme (Depol 686L) dosage on the glucose concentration in brewer's spent grain hydrolysate *(BSG_H) produced by enzymatic treatment for 5 h. All treatments were performed in duplicates and results are presented as mean±SD.*

In an attempt to further optimize the hydrolysis of BSG, a second optimization experiment was conducted which compared incubation times of 5 and 24 h using a fixed enzyme dosage of 106 U/mL. A comparison of the results gained from this experiment revealed a two-fold increase in the glucose concentration (~0.8 g/L to ~2.0 g/L) when the incubation time was increased from 5 to 24 h. Even though this finding may seem convincing, the glucose concentration was still too low for preparation of media with a glucose concentration of 2.5 g/L which was used in all fermentation experiments. Furthermore, it is preferable to utilize highly concentrated hydrolysates for media preparation in order to minimize consumption of chemicals and enzymes. This can be illustrated by a hypothetical case in which a fermentation medium is prepared from two hydrolysates with glucose concentrations of 5 g/L (H1) and 300 g/L (H2). To prepare 1 L of medium with a final glucose concentration of 2.5 g/L, 500 mL must be added of the H1 hydrolysate (5 g/L). By comparison, the same medium concentration can be obtained by adding 25 mL of the H2 hydrolysate (300 g/L). This example clearly underpins the importance of utilizing hydrolysates of high sugar concentration in media preparation. Two different approaches might prove useful for increasing the glucose concentration in the BSG hydrolysate produced during the

second optimization experiment. One is based on the evaporation of water to reduce the volume of the hydrolysate while the other omits the washing of solid BSG residues. It was decided that omission of washing would be the best-suited approach since evaporation by oven-or freeze-drying is an energy-and time-consuming process. To verify the appropriateness of the selected approach, glucose was quantified in the liquid fractions produced from hydrolysis and washing of BSG. The liquid fraction obtained from hydrolysis had a glucose concentration of 5-6 g/L which was sufficient, but not ideal, for preparation of fermentation media with a glucose concentration of 2.5 g/L. In contrast, the fractions obtained from washing of the BSG residue contained negligible amounts of glucose which indicated that washing did not improve glucose recovery from BSG. Based on these results, washing was excluded from the procedure and the enzymatic hydrolysis of BSG was carried out using an enzyme dosage of 106 U/mL and a duration of 24 h.

5.2 Submerged fermentation

5.2.1 Basics of submerged fermentation

Submerged fermentation is the cultivation of microorganisms in liquid media containing excess water (>95%) and nutrients (nitrogen, carbon and salts) (Ouedraogo and Tsang, 2021). It represents an important process for industrial production of a diversity of value-added compounds including enzymes, microbial biomass and metabolites such as antibiotics, organic acids, alcohols and amino acids (Martínez-Medina et al., 2018). Submerged fermentations are carried out in stirred bioreactors/fermenters which allow for strict regulation and monitoring of different operating parameters known to influence the performance of the cultivated microorganisms. Among these parameters are medium pH, temperature, dissolved oxygen (DO) and foaming. Furthermore, agitation is crucial for maintaining an even distribution of oxygen, cells and nutrients (Ouedraogo and Tsang, 2021). As depicted in Figure 15, an industrial fermentation process can be divided into six basic components:

1) Formulation of media for fermentation and inoculum production.
2) Sterilization of media, bioreactor and accessory equipment and solutions.
3) Development of an inoculum containing an adequate quantity of active cells.
4) Inoculation of fermentation medium in the bioreactor (addition of inoculum).

5) Cultivation under ideal conditions for product formation.
6) Isolation and purification of the intended product(s).

During the cultivation step (5), three interrelated processes take place: 1) biomass accumulation (growth), 2) substrate consumption and 3) product formation (Stanbury et al., 1995a).

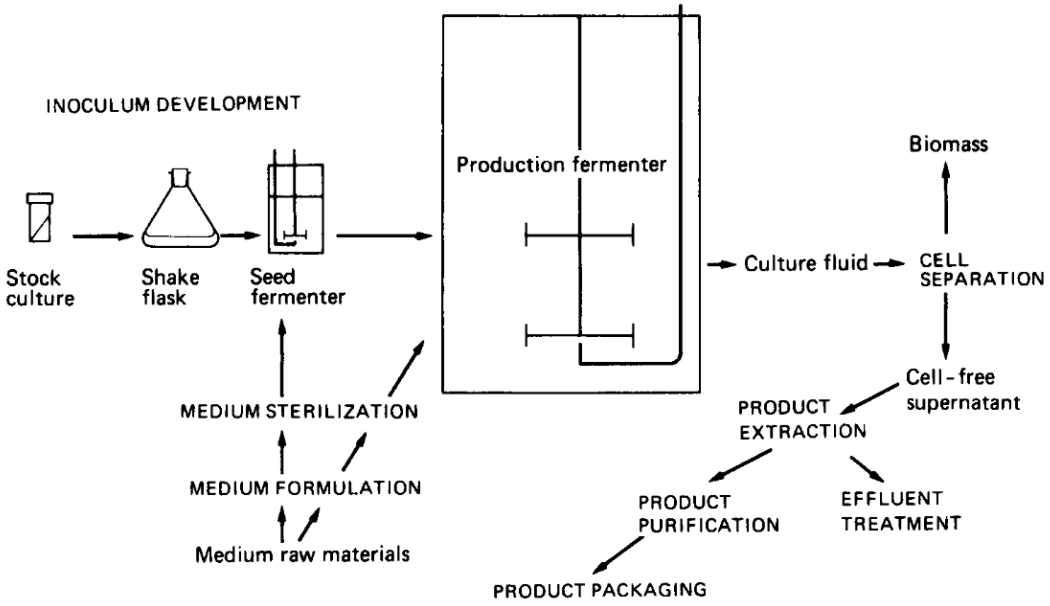


Figure 15. General steps of an industrial fermentation process. Reprinted from Stanbury et al (1995a) *with permission from Elsevier.*

Submerged fermentation may be performed by three different configurations constituting batch, fed-batch and continuous fermentation. The choice of fermentation mode is largely dictated by the producing microorganism as well as the product (Ouedraogo and Tsang, 2021; Stanbury et al., 1995b). Operational details of each configuration are outlined in Figure 16.

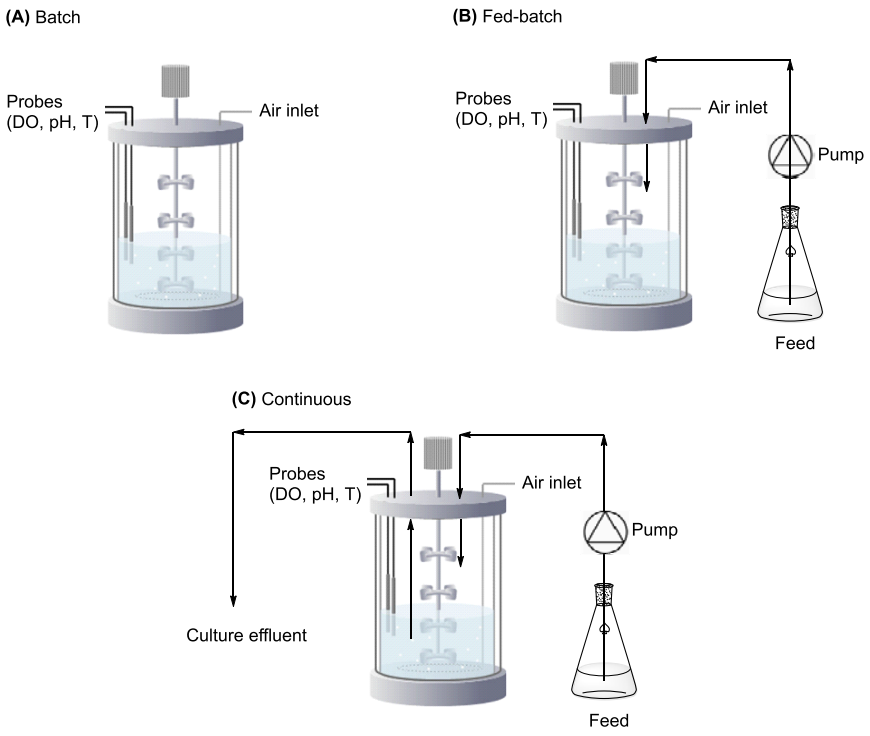


Figure 16. Graphical presentation of the three major fermentation modes. (A) batch, (B) fed-batch and (C) continuous fermentation. Arrows indicate the directions of fluids. The figure was modified from Ochoa (2019)*. T: temperature, DO: dissolved oxygen.*

Batch fermentation occurs in a *closed system* that contains limited amounts of nutrients. During batch fermentation, nutrients are not added and wastes are not removed; thus, growth is restricted by two main factors: 1) nutrient depletion and 2) production of inhibitory compounds. In batch fermentation, the cell population undergoes four phases: (1) the lag phase, which is the period of adaptation; (2) the exponential phase, which is the period of maximum growth; (3) the stationary phase; which involves cessation of growth due to nutrient depletion and (4) the death phase, which is characterized by loss of cell viability (Shanmugam et al., 2022). Fed-batch fermentation is a variant of the batch mode in which a nutrient-rich feed is added to the batch culture without removing waste. This process may prevent nutrient depletion but it does not overcome the growth inhibition caused by secretion of inhibitors (Ochoa, 2019). The problems encountered in batch and fed-batch fermentation may be evaded by using continuous fermentation. This mode is characterized by simultaneous feed addition and waste removal which maintain high nutrient concentrations while eliminating dead cells and toxic compounds (Stanbury et al., 1995b).

5.2.2 Production of value-added compounds for the aquaculture sector by submerged fermentation of BSG-derived sugars

The experimental work of **Part III** intended to develop microbial processes for the production of free amino acids and single cell protein (SCP) using BSG hydrolysate as fermentation substrate. These products were chosen as the primary focus as they could potentially be integrated as alternative ingredients in aquafeed production. Free amino acids may find application as crystalline amino acids (CAA) which are used for fortification of deficient amino acids in formulated diets (Nunes et al., 2014). On the other hand, BSG-derived SCP might be a potential protein source for aquafeeds owing to the fact that microbial SCP is generally rich in high-quality protein (Nangul and Bhatia, 2013). The most prominent results from the research of **Part III** will be presented shortly in the next section with reference to **Manuscript 3** and **Manuscript 4**.

5.2.2.1 Production of free amino acids

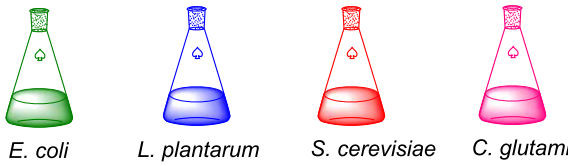
Microbial production of amino acids is obtainable by two types of processes: fermentation and enzymatic conversion of which fermentation is the most widespread method (D'Este et al., 2018). Enzymatic processes occur via hydrolysis of peptides to free amino acids by means of proteases secreted by the cultured microorganism (Toe et al., 2019). Fermentation is closely linked to the central C-metabolism and involves the conversion of sugars into intermediates which serve as precursors for amino acid production (Sanchez et al., 2018). Amino acids are primary metabolites produced at largest quantities during the exponential phase where cells have highest metabolic activity (Stanbury et al., 1995a). The production of amino acids is to a large extent regulated by negative feedback control; a process which ensures that the microbial cells do not produce more amino acids than they need to sustain growth (Currell and Dam-Mieras, 1991). Overproduction of amino acids is a prerequisite for obtaining a successful fermentative process, but it does not occur naturally due to metabolic regulation. However, overproduction of amino acids may be induced by two general approaches: 1) modification of growth media and 2) metabolic engineering where strains are genetically modified to overproduce specific amino acids (Ma et al., 2017; Shakoori et al., 2012). In the literature, amino acid production has been reported for various microorganisms including *Corynebacterium glutamicum, Saccharomyces cerevisiae, Escherichia coli*, *Lactobacillus plantarum* and different *Bacillus* species

(Gopinath and Nampoothiri, 2014; Malaney et al., 1991; Rodriguez et al., 2014; Shakoori et al., 2012; Toe et al., 2019). Amongst these, *C. glutamicum* and *E. coli* are the most widely used microorganisms for industrial production of several amino acids. While *C. glutamicum* is the primary producer of glutamic acid (Glu) and lysine (Lys), *E. coli* accounts for the main production of aromatic amino acids (Gopinath and Nampoothiri, 2014; Rodriguez et al., 2014). Industrial manufacturing of most amino acids employs fed-batch fermentation followed by separation and purification using ion exchange chromatography and crystallisation, respectively (Sanchez et al., 2018).

The major objective of the work presented in **Manuscript 3** was to optimize a microbial process for transforming BSG-derived sugars into amino acids. To do so, an experimental design was adopted which included the following sequence of steps:

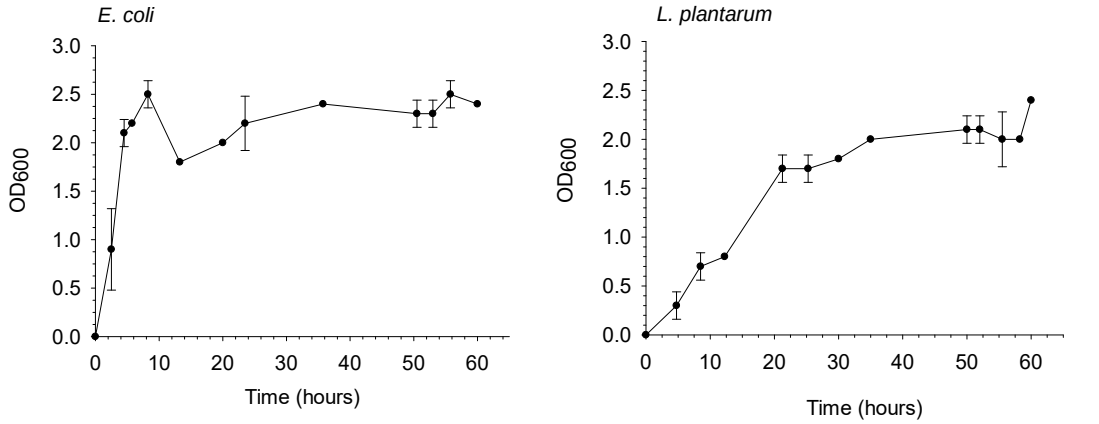
I. Shake flask cultivations: initial screening of the growth performance of different microorganisms in BSG-based media. The screened microorganisms included native strains of *E. coli*, *L. plantarum*, *C. glutamicum* and *S. cerevisiae*. Those of highest growth performance were selected and their amino acid production examined.
II. Batch fermentation: the microorganisms that produced amino acids during shake flask cultivation were cultivated in a bioreactor to investigate the effect of controlled conditions (pH and DO) on growth and amino acid production.
III. Fed-batch fermentation: microorganisms, for which amino acid production was biomass-associated, were cultured by fed-batch fermentation to enhance the productivity.

A graphical summary of the experimental design and the most compelling results is shown in Figure 17.

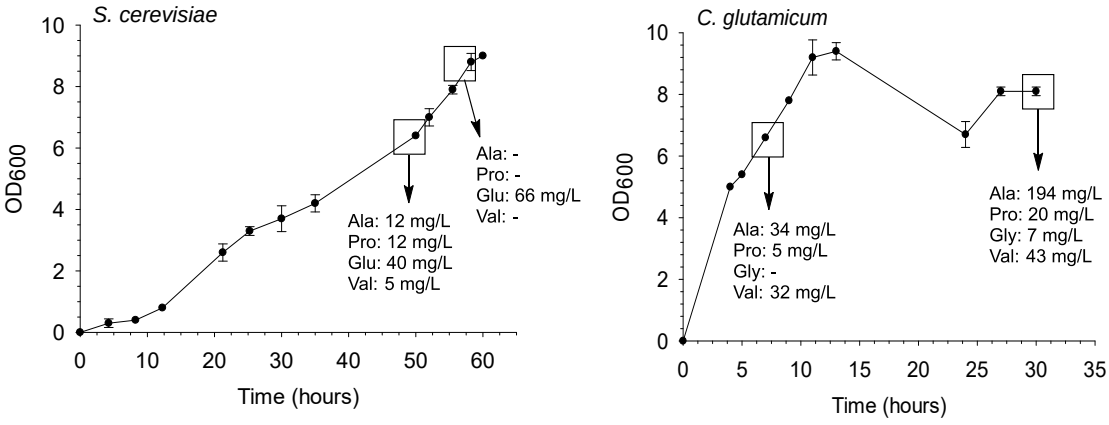


1) Initial shake flask cultivation in BSG-based media

Results - growth and amino acid production



Not investigated further due to low growth performance (OD_{600}<8)



Investigated for amino acid production due to high growth performance (OD_{600}>8)

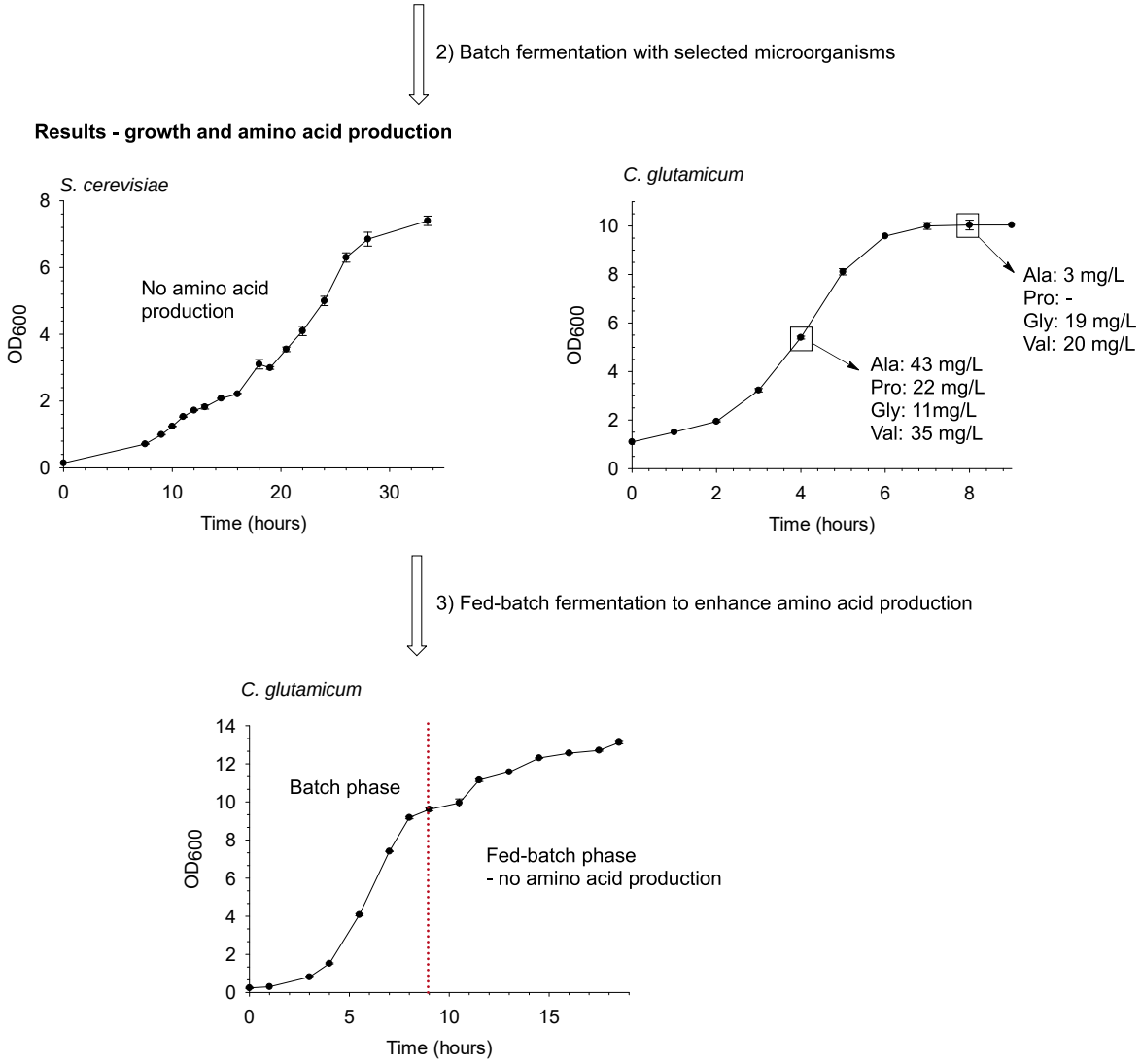


Figure 17. Graphical overview of experimental set-up and the most significant results obtained from the study on amino acid production by submerged fermentation using brewer's spent grain (BSG) as a substrate. More details can be found in ***Manuscript 3****. Ala: alanine, Pro: proline, Gly: glycine, Val: valine, Glu: glutamic acid.*

Based on the results shown in Figure 17, the following conclusions can be drawn:

- *E. coli* and *L. plantarum* exhibited low growth performance (OD_{600}<8), probably due to the drop in pH observed during cultivation. As a result, these microorganisms were not selected for further investigation.
- *C. glutamicum* and *S. cerevisiae* had high growth performance (OD_{600}>8) and therefore it was investigated whether they produced amino acids during shake flask cultivation.

- During shake flask cultivation, *C. glutamicum* produced alanine (Ala), proline (Pro), valine (Val) and small quantities of glycine (Gly). Likewise, *S. cerevisiae* produced Ala, Pro and Val but also Glu when grown in shake flask.
- During batch fermentation, no amino acid production was seen for *S. cerevisiae* while *C. glutamicum* produced the same amino acids as in shake flask cultivation, yet with higher production of Gly.
- Fed-batch fermentation with *C. glutamicum* did not increase amino acid production. Instead, all amino acids, except for Gly, decreased during the fed-batch phase indicating that they were consumed.
- For *C. glutamicum* and *S. cerevisiae*, amino acid production appeared to be dependent on two variables: 1) the growth phase (exponential vs stationary) and 2) the cultivation mode (shake flask vs batch fermentation).

Altogether, these findings were encouraging as they demonstrate the feasibility of utilizing BSG as a substrate for amino acid production by submerged fermentation either in shake flasks or under controlled conditions in a bioreactor. Although this work was promising, its main limitation was a low productivity compared to industrial amino acid production for which productivities are normally found within the range of 10-150 g/L (Lee et al., 2016). Thus, more research is required to optimize amino acid production from BSG-derived sugars by submerged fermentation. It is suggested that future studies focus on *C. glutamicum* due to its industrial significance and the fact that it appeared to be a better amino acid producer compared to *S. cerevisiae* in our research. A natural progression of this work would be to optimize the fed-batch fermentation. This could be realized by increasing the glucose concentration of the feed as the observed amino acid consumption combined with a rapid decline in glucose suggest that lack of productivity is caused by substrate limitation. One possible way to increase the glucose concentration is to optimize the hydrolysis process. Typically, glucose concentrations as high as 500 g/L are required to prevent fast depletion of the growth limiting substrate during feeding (Xu et al., 1999). The hydrolysis method used to produce the BSG hydrolysate led to a glucose concentration of ~5 g/L which is probably too low for feeding. Optimization of BSG hydrolysis may be doable by adopting methods that increase the hydrolysability of cellulose by either increasing the accessibility of cellulose to enzymes or eliminating adsorption of enzymes to lignin. As previously mentioned, hydrolysis of cellulose can

be enhanced by employing a suitable pretreatment method for removing lignin and/or hemicellulose. For instance, sequential treatment with dilute acid and base has proven effective in increasing cellulose hydrolysis (Mussatto et al., 2008). In addition, the BSG hydrolysate contained high concentrations of acetate (8-9 g/L) which could be a contributing factor to the low amino acid production because of the inhibitory effect of acetate (Baek et al., 2008). The acetate present in the BSG hydrolysate may originate from two different sources including the buffer (ammonium acetate) and the hydrolysis of arabinoxylan (AX) during which xylose-linked acetate is released (Figure 13). Minimizing the acetate concentration in the BSG hydrolysate may therefore help increase the productivity. This may be accomplished by using a different buffer solution and employing an enzyme mixture that does not contain AX-degrading enzymes. Furthermore, the use of surfactants may be a useful tool for increasing the cellulose hydrolysis because these compounds stimulate substrate-enzyme interactions by obstructing the adsorption of enzymes to lignin (Amit et al., 2018).

Another interesting area of future research would be to investigate whether it is possible to improve amino acid production by using metabolically engineered strains of *C. glutamicum*. There is a possibility that amino acid production can be enhanced by using strains that lack the natural feedback regulation. In addition, strains with a broadened repertoire of C-sources might also augment the productivity due to the increased C-flux towards amino acid production. Native strains of *C. glutamicum* are not capable of metabolizing xylose; however, the construction of recombinant strains that are able to co-utilize glucose and xylose has been reported by Kawaguchi et al. (2008). In the context of this PhD project, it would be interesting to assess how amino acid production is affected by co-utilization of glucose and xylose from the BSG-based medium.

5.2.2.2 Production of yeast single cell protein (SCP)

Single cell protein is derived from biomass of different unicellular organisms which can be clustered into three generic groups: bacteria, fungi and microalgae (Anupama and Ravindra, 2000). Most sources of SCP might be suitable for aquafeeds due to their high content of protein and essential amino acids (EAAs); though, the protein content is known to vary depending on the SCP source. Often the highest protein content is found in algal (60-70%) and bacterial (50-80%) biomasses while fungal biomass normally has a lower protein content (30-50%). Furthermore, fungal SCP is often

deficient in methionine (Met) which may restrict its incorporation in aquafeeds (Ritala et al., 2017). These generalities, however, must be treated with caution as the protein content of SCP is influenced by several factors including type of species and fermentation substrate (Bratosin et al., 2021).

The research outlined in **Manuscript 4** evaluates the nutritional quality of *S. cerevisiae* biomass produced by submerged fermentation using BSG as substrate. This evaluation was based on a comparison with fishmeal concerning protein content and amino acid composition. The most significant findings of **Manuscript 4** are summarized below:

1. *S. cerevisiae* biomass had a lower protein content (~38%) as compared to fishmeal (50-70%).
2. *S. cerevisiae* biomass was rich in several amino acids; however, most amino acids were found in lower concentrations compared with fishmeal.
3. The most abundant amino acids in *S. cerevisiae* biomass were Glu (~5.3% vs 6.9-9.9% in fishmeal), Asp (~4.1% vs 4.6-7.0% in fishmeal) and Lys (~3.2% vs 3.6-5.9% in fishmeal).
4. *S. cerevisiae* biomass was deficient in Met (~0.6% vs 1.5-2.0% in fishmeal) and His (~0.9% vs 2.3-4.2% in fishmeal).

Collectively, these findings suggest that the nutritional quality of BSG-derived *S. cerevisiae* biomass was high enough that it could partially replace fishmeal in aquafeeds without affecting protein digestion or growth performance. Notwithstanding this, a higher protein content and a more balanced amino acid profile will be required to permit higher inclusion levels (60-90%) of *S. cerevisiae* biomass in aquafeeds.

In the light of these findings, future studies should focus on optimizing the process productivity and the protein content of microbial biomass. One reasonable approach to enhance the productivity would be to adopt continuous fermentation which is the most common fermentation mode for industrial production of SCP (Stanbury et al., 1995b). The major advantage of continuous fermentation is that it enables high biomass production, because growth is not limited by the C-source or the presence of inhibitory compounds produced by the fermenting microorganisms (Lindskog, 2018). There are two major strategies for increasing the protein content of BSG-derived fungal biomass. Firstly, the protein content could be increased by downstream

processing of the biomass which is initiated by cell wall disruption followed by chemical or enzymatic extraction of proteins (García-Garibay et al., 2014). Secondly, a higher protein content could be attained by substituting *S. cerevisiae* with a better-performing microorganism. For instance, *Candida* species may be potential candidates because of their ability to grow on various substrates and produce biomass of high protein content (50-60%) (Gao et al., 2012).

6. Conclusions and future perspectives

The present PhD project has advanced our knowledge regarding the valorization of brewer's spent grains (BSG) for aquafeed applications. This work has made a significant contribution to existing knowledge and created new insight into the refinement of BSG. In essence, the work consisted of three major parts with the overarching objective of refining BSG into high-quality products that might find use as ingredients in aquafeeds. **Part I** aimed to develop an effective process for producing protein isolates from BSG by employing chemical extraction. **Part II** intended to remove lignin from BSG by solid-state fermentation (SSF) in order to increase the protein content of BSG and remove the antinutritional effect of lignin. **Part III** was split into two sections: 1) hydrolysis of cellulose and hemicellulose from BSG to produce a liquid hydrolysate and 2) production of single cell protein (SCP) and free amino acids by submerged fermentation using BSG hydrolysate as a substrate. Both fermentation products have great potential for aquafeed production as they represent sources of protein or crystalline amino acids.

Overall, the results obtained from this PhD project were promising; yet, there is still space for improvement. Main conclusions and general directions for future research are summarized below:

- **Part I** led to development of two procedures for extracting proteins from BSG. One was based on isoelectric precipitation while the other utilized freeze-drying to obtain protein isolates. Both methods had their pros and cons with respect to recovery and protein content of isolates. The precipitation-based method was associated with high protein content of isolates (~55% DM) but poor recovery (~10%). Conversely, freeze-drying combined with multiple extractions resulted in a relatively high recovery (~45%) but low protein content of isolates (~25% DM). Consequently, further research must be done to establish a method that maximizes the recovery as well as the protein content of isolates. Specifically, future studies should focus on optimizing parameters known to influence precipitation efficiency and protein solubilization during extraction.
- **Part II** demonstrated the removal of lignin by SSF using the lignin-degrading fungus *Phanerochaete chrysosporium*; though, the lignin removal rate was low (~0.75%/day). Unfortunately, this process did not cause any meaningful increase

in the protein content of BSG and led to an increase in biomass-bound chitin. To increase the efficacy of lignin removal and circumvent chitin accumulation, it is suggested that future activities explore the potential of enzymatic systems.

- **Part III** was interesting in two ways. First, it established a novel process for converting the cellulosic fraction of BSG into free amino acids by submerged fermentation using the state of the art amino acid producer *Corynebacterium glutamicum*. Second, it demonstrated the production of SCP in the form of *Saccharomyces cerevisiae* biomass by submerged fermentation using BSG as substrate. Although encouraging, both processes had certain limitations that need to be considered in future research. The fermentative production of amino acids suffered from low productivities as compared to industrial production while the drawbacks of SCP production were related to the nutritional constraints of the fungal biomass including a relatively low protein content (~38% DM) and deficiency in some amino acids. These issues could potentially be addressed via optimization of fermentation mode, microbial strain and media composition.

Ultimately, the practicability of using BSG-refined products in aquafeeds must be validated for target fish species focusing on protein digestibility and growth performance at different inclusion levels. However, this presupposes fully optimized valorization processes for BSG which should be the first priority of future studies.

References

Abdel-Hamid, A.M., Solbiati, J.O., Cann, I.K.O., 2013. Insights into Lignin Degradation and its Potential Industrial Applications, Advances in Applied Microbiology. Elsevier. https://doi.org/10.1016/B978-0-12-407679-2.00001-6

Al-Souti, A., Gallardo, W., Claereboudt, M., Mahgoub, O., 2019. Attractability and palatability of formulated diets incorporated with chicken feather and algal meals for juvenile gilthead seabream, Sparus aurata. Aquac. Reports 14, 100199. https://doi.org/10.1016/j.aqrep.2019.100199

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour. Technol. 101, 4851–4861. https://doi.org/10.1016/j.biortech.2009.11.093

Amin, M.N., Katersky Barnes, R., Adams, L.R., 2014. Effects of different protein and carbohydrate levels on growth performance and feed utilisation of brook trout, Salvelinus fontinalis (Mitchill, 1814), at two temperatures. J. Appl. Ichthyol. 30, 340–349. https://doi.org/10.1111/jai.12396

Amit, K., Nakachew, M., Yilkal, B., Mukesh, Y., 2018. A review of factors affecting enzymatic hydrolysis of pretreated lignocellulosic Biomass. Res. J. Chem. Environ. 22, 62–67.

Anupama, Ravindra, P., 2000. Value-added food: Single cell protein. Biotechnol. Adv. 18, 459–479. https://doi.org/10.1016/S0734-9750(00)00045-8

Asurmendi, P., Barberis, C., Dalcero, A., Pascual, L., Barberis, L., 2013. Survey of Aspergillus section Flavi and aflatoxin B1 in brewer's grain used as pig feedstuff in Córdoba, Argentina. Mycotoxin Res. 29, 3–7. https://doi.org/10.1007/s12550-012-0148-5

Atiwesh, G., Parrish, C.C., Banoub, J., Le, T.A.T., 2022. Lignin degradation by microorganisms: A review. Biotechnol. Prog. 38. https://doi.org/10.1002/btpr.3226

Baek, S.W., Kim, J.S., Park, Y.K., Kim, Y.S., Oh, K.K., 2008. The effect of sugar decomposed on the ethanol fermentation and decomposition reactions of sugars. Biotechnol. Bioprocess Eng. 13, 332–341. https://doi.org/10.1007/s12257-007-0161-2

Barakat, A., Monlau, F., Solhy, A., Carrere, H., 2015. Mechanical dissociation and fragmentation of lignocellulosic biomass: Effect of initial moisture, biochemical and structural proprieties on energy requirement. Appl. Energy 142, 240–246. https://doi.org/10.1016/j.apenergy.2014.12.076

Bartolomeé, B., Santos, M., Jiménez, J.J., Del Nozal, M.J., GÓmez-Cordoveés, C., 2002. Pentoses and hydroxycinnamic acids in brewer's spent grain. J. Cereal Sci. 36, 51–58. https://doi.org/10.1006/jcrs.2002.0442

Béguin, P., Aubert, J.-P., 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. 13, 25–58.

Bibiano Melo, J.F., Lundstedt, L.M., Metón, I., Baanante, I.V., Moraes, G., 2006. Effects of dietary levels of protein on nitrogenous metabolism of Rhamdia quelen (Teleostei: Pimelodidae). Comp. Biochem. Physiol. Part A 145, 181–187. https://doi.org/10.1016/j.cbpa.2006.06.007

Blanchette, R.A., 1995. Degradation of the lignocellulose complex in wood. Can. J. Bot. 73, 999–1010. https://doi.org/10.1139/b95-350

Blumenthal, H.J., Roseman, S., 1957. Quantitative estimation of chitin in fungi. J. Bacteriol. 74, 222–224. https://doi.org/10.1128/jb.74.2.222-224.1957

Boyd, C.E., 2015. Overview of aquaculture feeds, Feed and Feeding Practices in Aquaculture. Elsevier Ltd. https://doi.org/10.1016/b978-0-08-100506-4.00001-5

Boyd, C.E., McNevin, A.A., Davis, R.P., 2022. The contribution of fisheries and aquaculture to the global protein supply. Food Secur. https://doi.org/10.1007/s12571-021-01246-9

Bratosin, B.C., Darjan, S., Vodnar, D.C., 2021. Single cell protein: A potential substitute in human and animal nutrition. Sustain. 13, 1–24. https://doi.org/10.3390/su13169284

Byers, M., Miflin, B.J., Smith, S.J., 1983. A quantitative comparison of the extraction of protein fractions from wheat grain by different solvents, and of the polypeptide and amino acid composition of the alcohol-soluble proteins. J. Sci. Food Agric. 34, 447–462. https://doi.org/10.1002/jsfa.2740340506

Camarero, S., Galletti, G.C., Martinez, A.T., 1994. Preferential degradation of phenolic lignin units by two white rot fungi. Appl. Environ. Microbiol. 60, 4509–4516. https://doi.org/10.1128/aem.60.12.4509-4516.1994

Camarero, S., Ibarra, D., Martínez, M.J., Martínez, Á.T., 2005. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl. Environ. Microbiol. 71, 1775–1784. https://doi.org/10.1128/AEM.71.4.1775-1784.2005

Cancel, A.M., Orth, A.B., Tien, M., 1993. Lignin and veratryl alcohol are not inducers of the ligninolytic system of Phanerochaete chrysosporium. Appl. Environ. Microbiol. 59,

2909–2913. https://doi.org/10.1128/aem.59.9.2909-2913.1993

Carvalho, M.A.G. de, Fernandes, L.F.L., Gomes, L. de C., 2017. Digestibility, protein retention rate and ammonia excretion in juvenile fat snook (Centropomus parallelus) fed with different protein levels. Ciência Rural 47, 1–7. https://doi.org/10.1590/0103-8478cr20160369

Casey, E., Sedlak, M., Ho, N.W.Y., Mosier, N.S., 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of Saccharomyces cerevisiae. FEMS Yeast Res. 10, 385–393. https://doi.org/10.1111/j.1567-1364.2010.00623.x

Celus, I., Brijs, K., Delcour, J.A., 2006. The effects of malting and mashing on barley protein extractability. J. Cereal Sci. 44, 203–211. https://doi.org/10.1016/j.jcs.2006.06.003

Chavan, U.D., McKenzie, D.B., Shahidi, F., 2001. Functional properties of protein isolates from beach pea (Lathyrus maritimus L.). Food Chem. 74, 177–187. https://doi.org/10.1016/S0308-8146(01)00123-6

Cho, J.H., Kim, I.H., 2011. Fish meal - nutritive value. J. Anim. Physiol. Anim. Nutr. (Berl). 95, 685–692. https://doi.org/10.1111/j.1439-0396.2010.01109.x

Christopher, L.P., Yao, B., Ji, Y., 2014. Lignin biodegradation with laccase-mediator systems. Front. Energy Res. 2, 1–13. https://doi.org/10.3389/fenrg.2014.00012

Coelho, E., Rocha, M.A.M., Moreira, A.S.P., Domingues, M.R.M., Coimbra, M.A., 2016. Revisiting the structural features of arabinoxylans from brewers' spent grain. Carbohydr. Polym. 139, 167–176. https://doi.org/10.1016/j.carbpol.2015.12.006

Couto, S.R., Longo, M.A., Cameselle, C., Sanromán, A., 1998. Influence of some inducers on activity of ligninolytic enzymes from corncob cultures of Phanerochaete chrysosporium in semi-solid-state conditions. Prog. Biotechnol. 15, 703–708. https://doi.org/10.1016/S0921-0423(98)80106-8

Craig, S., Kuhn, D.D., 2017. Understanding fish nutrition, feeds and feeding. Virginia Coop. Ext., 1–6.

Crowe, N.L., Baker, B.E., Inteaz, A., 1985. Solubilisation of nitrogenous constituents of brewer's spent grain. J. Inst. Brew. 9, 148–150. https://doi.org/10.1002/j.2050-0416.1985.tb04320.x

Currell, B.C., Dam-Mieras, R.C.E., 1991. Amino acids: their production and uses in the food industry, Biotechnological Innovations in Food Processing.

https://doi.org/https://doi.org/10.1016/C2009-0-24973-7

D'Este, M., Alvarado-Morales, M., Angelidaki, I., 2018. Amino acids production focusing on fermentation technologies – A review. Biotechnol. Adv. 36, 14–25. https://doi.org/10.1016/j.biotechadv.2017.09.001

D'Heysselaer, S. de C., Bockstal, L., Jacquet, N., Schmetz, Q., Richel, A., 2022. Potential for the valorisation of brewer's spent grains: A case study for the sequential extraction of saccharides and lignin. Waste Manag. Res. 40, 1007–1014. https://doi.org/10.1177/0734242X211055547

Dalsgaard, J., Ekmann, K.S., Pedersen, P.B., Verlhac, V., 2009. Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (Oncorhynchus mykiss), and on the magnitude and composition of phosphorus waste output. Aquaculture 286, 105–112. https://doi.org/10.1016/j.aquaculture.2008.09.007

De Carvalho, C.V.A., Bianchini, A., Tesser, M.B., Sampaio, L.A., 2010. The effect of protein levels on growth, postprandial excretion and tryptic activity of juvenile mullet Mugil platanus (Günther). Aquac. Res. 41, 511–518. https://doi.org/10.1111/j.1365-2109.2009.02340.x

Deleu, L.J., Lambrecht, M.A., Van de Vondel, J., Delcour, J.A., 2019. The impact of alkaline conditions on storage proteins of cereals and pseudo-cereals. Curr. Opin. Food Sci. 25, 98–103. https://doi.org/10.1016/j.cofs.2019.02.017

Dersjant-li, Y., 2002. The Use of Soy Protein in Aquafeeds, in: Avances En Nutrición Acuícola. Memorias Del VI Simposium Internacional de Nutrición Acuícola. pp. 541–558.

Dessie, W., Xin, F., Zhang, W., Zhou, J., Wu, H., Ma, J., Jiang, M., 2019. Inhibitory effects of lignocellulose pretreatment degradation products (hydroxymethylfurfural and furfural) on succinic acid producing Actinobacillus succinogenes. Biochem. Eng. J. 150, 1–6. https://doi.org/10.1016/j.bej.2019.107263

Diógenes, A.F., Basto, A., Estevão-Rodrigues, T.T., Moutinho, S., Aires, T., Oliva-Teles, A., Peres, H., 2019. Soybean meal replacement by corn distillers dried grains with solubles (DDGS) and exogenous non-starch polysaccharidases supplementation in diets for gilthead seabream (Sparus aurata) juveniles. Aquaculture 500, 435–442. https://doi.org/10.1016/j.aquaculture.2018.10.035

Eriegha, O., Ekokotu, P.A., 2017. Factors affecting feed intake in cultured fish species: A

review. Anim. Res. Int. 14, 2697–2709.

Ervin, V., Alli, I., Smith, J.P., Li, Z., 1989. Extraction and Precipitation of Proteins From Brewer's Spent Grain. Can. Inst. Food Sci. Technol. J. 22, 216–221. https://doi.org/10.1016/s0315-5463(89)70386-2

Faison, B.D., Kirk, T.K., 1985. Factors involved in the regulation of a ligninase activity in Phanerochaete chrysosporium. Appl. Environ. Microbiol. 49, 299–304.

Falco, F., Stincone, P., Cammarata, M., Brandelli, A., 2020. Amino Acids as the Main Energy Source in Fish Tissues. Aquac. Fish. Stud. 2. https://doi.org/10.31038/afs.2020223

Fan, Y., Zhang, Z., Wang, F., Li, J., Hu, K., Du, Z., 2019. Lignin degradation in corn stover catalyzed by lignin peroxidase from Aspergillus oryzae broth: Effects of conditions on the kinetics. Renew. Energy 130, 32–40. https://doi.org/10.1016/j.renene.2018.06.037

FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Fernandes, A.C., Carvalho, P.L.P.F. de, Pezzato, L.E., Koch, J.F.A., Teixeira, C.P., Cintra, F.T., Damasceno, F.M., Amorin, R.L., Padovani, C.R., Barros, M.M., 2016. The effect of digestible protein to digestible energy ratio and choline supplementation on growth, hematological parameters, liver steatosis and size-sorting stress response in Nile tilapia under field condition. Aquaculture 456, 83–93. https://doi.org/10.1016/j.aquaculture.2016.02.001

Fernandes, H., Salgado, J.M., Ferreira, M., Vršanská, M., Fernandes, N., Castro, C., Oliva-Teles, A., Peres, H., Belo, I., 2022. Valorization of Brewer's Spent Grain Using Biological Treatments and its Application in Feeds for European Seabass (Dicentrarchus labrax). Front. Bioeng. Biotechnol. 10, 1–15. https://doi.org/10.3389/fbioe.2022.732948

Fiorella, P.C.-T., Sylvia, C.A.-A., Tânia, F.-C., Meireles, M.A.A., 2015. Obtaining Oligo- and Monosaccharides from Agroindustrial and Agricultural Residues Using Hydrothermal Treatments. Food Public Heal. 4, 123–139. https://doi.org/10.5923/j.fph.20140403.08

Forssell, P., Kontkanen, H., Schols, H.A., Hinz, S., Eijsink, V.G.H., Treimo, J., Robertson, J.A., Waldron, K.W., Faulds, C.B., Buchert, J., 2008. Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. J. Inst. Brew. 114, 306–314. https://doi.org/10.1002/j.2050-0416.2008.tb00774.x

Galanakis, C.M., 2015. Separation of functional macromolecules and micromolecules: From

ultrafiltration to the border of nanofiltration. Trends Food Sci. Technol. 42, 44–63. https://doi.org/10.1016/j.tifs.2014.11.005

Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618–628. https://doi.org/10.1007/s00253-002-1058-9

Gao, Y., Li, D., Liu, Y., 2012. Production of single cell protein from soy molasses using Candida tropicalis. Ann. Microbiol. 62, 1165–1172. https://doi.org/10.1007/s13213-011-0356-9

Gao, Z., Shen, P., Lan, Y., Cui, L., Ohm, J.B., Chen, B., Rao, J., 2020. Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. Food Res. Int. 131, 1–9. https://doi.org/10.1016/j.foodres.2020.109045

García-Garibay, M., Gómez-Ruiz, L., Cruz-Guerrero, A.E., Bárzana, E., 2014. Single Cell Protein: Yeasts and Bacteria, Encyclopedia of Food Microbiology: Second Edition. https://doi.org/https://doi.org/10.1016/B978-0-12-384730-0.00310-4

Garling, D.L., Wilson, R.P., 1976. Optimum dietary protein to energy ratio for channel catfish fingerlings, Ictalurus punctatus. J. Nutr. 106, 1368–1375. https://doi.org/10.1093/jn/106.9.1368

Gassara, F., Brar, S.K., Tyagi, R.D., Verma, M., Surampalli, R.Y., 2010. Screening of agro-industrial wastes to produce ligninolytic enzymes by Phanerochaete chrysosporium. Biochem. Eng. J. 49, 388–394. https://doi.org/10.1016/j.bej.2010.01.015

Gatlin, D., 2010. Principles of Fish Nutrition. South. Reg. Aquac. Cent. SRAC 1–8.

Gerbaldo, G.A., Pereyra, C.M., Cavaglieri, L.R., Ruiz, F., Pascual, L., Dalcero, A.M., Barberis, I.L., 2011. Surveillance of aflatoxin and microbiota related to Brewer's grain destined for swine feed in Argentina. Vet. Med. Int. 2011. https://doi.org/10.4061/2011/912480

Ghaly, A.E., Ramakrishnan, V. V., Brooks, M.S., Budge, S.M., Dave, D., 2013. Fish processing wastes as a potential source of proteins, amino acids and oils: A critical review. J. Microb. Biochem. Technol. 5, 107–129. https://doi.org/10.4172/1948-5948.1000110

Giacobbe, S., Piscitelli, A., Raganati, F., Lettera, V., Sannia, G., Marzocchella, A., Pezzella, C., 2019. Butanol production from laccase-pretreated brewer's spent grain. Biotechnol. Biofuels 12, 1–8. https://doi.org/10.1186/s13068-019-1383-1

Glencross, B., Bermudes, M., 2010. Effect of High Water Temperatures on the Utilisation

Efficiencies of Energy and Protein by Juvenile Barramundi, Lates calcarifer. Fish. Aquac. J. 01. https://doi.org/10.4172/2150-3508.1000014

Glencross, B.D., Baily, J., Berntssen, M.H.G., Hardy, R., MacKenzie, S., Tocher, D.R., 2020. Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. Rev. Aquac. 12, 703–758. https://doi.org/10.1111/raq.12347

Gomez Del Pulgar, E.M., Saadeddin, A., 2014. The cellulolytic system of thermobifida fusca. Crit. Rev. Microbiol. 40, 236–247. https://doi.org/10.3109/1040841X.2013.776512

Gopinath, V., Nampoothiri, K.M., 2014. Corynebacterium glutamicum, Second Edi. ed, Encyclopedia of Food Microbiology. Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00076-8

Green, J.A., Hardy, R.W., Brannon, E.L., 2002a. The optimum dietary essential amino acid pattern for rainbow trout (Oncorhynchus mykiss), to maximize nitrogen retention and minimize nitrogen excretion. Fish Physiol. Biochem. 27, 109–115. https://doi.org/10.1023/B:FISH.0000021877.49848.8f

Green, J.A., Hardy, R.W., Brannon, E.L., 2002b. The optimum dietary essential: Nonessential amino acid ratio for rainbow trout (Oncorhynchus mykiss), which maximizes nitrogen retention and minimizes nitrogen excretion. Fish Physiol. Biochem. 27, 109–115. https://doi.org/10.1023/B:FISH.0000021877.49848.8f

Hammel, K.E., Tien, M., Kalyanaraman, B., Kirk, T.K., 1985. Mechanism of Oxidative Ca - CB Cleavage of a Lignin Model Dimer by Phanerochaete chrysosporium Ligninase. J. Biol. Chem. 260, 8348–8353.

Hanif, M., Khattak, M.K., Sheikh, S., Khan, S., Saeed, M., Khan, A., Saqlain, M., 2014. Impact of Type and Particle Size on the Protein. Sci. Tech. Dev. 33, 107–109.

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: Effects of global demand and supplies of fishmeal. Aquac. Res. 41, 770–776. https://doi.org/10.1111/j.1365-2109.2009.02349.x

Hardy, R.W., Barrows, F.T., 2003. Diet Formulation and Manufacture, in: Fish Nutrition. pp. 505–600. https://doi.org/10.1016/b978-012319652-1/50010-0

He, Y., Galagarza, O.A., Wang, H., Taylor, Z.W., Ferguson, C.S., Ogejo, J.A., O'Keefe, S.F., Fernández Fraguas, C., Yu, D., Poe, N.E., Wiersema, B.D., Kuhn, D.D., Huang, H., 2020. Protein-rich product recovered from brewer's spent grain can partially replace fishmeal in diets of Pacific white shrimp, Litopenaeus vannamei. Aquac. Res. 1–13.

https://doi.org/10.1111/are.14664

He, Y., Kuhn, D.D., O'Keefe, S.F., Ogejo, J.A., Fraguas, C.F., Wang, H., Huang, H., 2021. Protein production from brewer's spent grain via wet fractionation: process optimization and techno-economic analysis. Food Bioprod. Process. 126, 234–244. https://doi.org/10.1016/j.fbp.2021.01.005

Heuzé V., Tran G., 2020. Grape pomace. Feedipedia, a programme by INRAE, CIRAD, AFZ and FAO. https://www.feedipedia.org/node/691. Last updated on October 16, 2020, 10:32

Heuzé V., Tran G., Sauvant D., Lebas F., 2017. Brewers grains. Feedipedia, a programme by INRAE, CIRAD, AFZ and FAO. https://www.feedipedia.org/node/74. Last updated on August 17, 2017, 12:20

Heuzé V., Tran G., Sauvant D., Noblet J., Renaudeau D., Bastianelli D., Lessire M., Lebas F., 2015. Corn distillers grain. Feedipedia, a programme by INRAE, CIRAD, AFZ and FAO. https://www.feedipedia.org/node/71. Last updated on May 11, 2015, 14:32

Hilton, J.W., Cho, C.Y., Slinger, S.J., 1981. Effect of extrusion processing and steam pelleting diets on pellet durability, pellet water absorption, and the physiological response of rainbow trout (Salmo gairdneri R.). Aquaculture 25, 185–194. https://doi.org/10.1016/0044-8486(81)90180-0

Huang, S., Huang, D., Wu, Q., Hou, M., Tang, X., Zhou, J., 2020. Effect of environmental C/N ratio on activities of lignin-degrading enzymes produced by Phanerochaete chrysosporium. Pedosphere 30, 285–292. https://doi.org/10.1016/S1002-0160(17)60391-6

Ibarruri, J., Cebrián, M., Hernández, I., 2019. Solid State Fermentation of Brewer's Spent Grain Using Rhizopus sp. to Enhance Nutritional Value. Waste and Biomass Valorization 10, 3687–3700. https://doi.org/10.1007/s12649-019-00654-5

Jacobsen, H.J., Samuelsen, T.A., Girons, A., Kousoulaki, K., 2018. Different enzyme incorporation strategies in Atlantic salmon diet containing soybean meal: Effects on feed quality, fish performance, nutrient digestibility and distal intestinal morphology. Aquaculture 491, 302–309. https://doi.org/10.1016/j.aquaculture.2018.03.053

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., Dayal, J.S., 2019. Fishmeal availability in the scenarios of climate change: Inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. Aquac. Res. 50, 3493–3506. https://doi.org/10.1111/are.14324

Janusz, G., Pawlik, A., Sulej, J., Świderska-Burek, U., Jarosz-Wilkolazka, A., Paszczyński, A., 2017. Lignin degradation: Microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol. Rev. 41, 941–962. https://doi.org/10.1093/femsre/fux049

Jayant, M., Hassan, M.A., Srivastava, P.P., Meena, D.K., Kumar, P., Kumar, A., Wagde, M.S., 2018. Brewer's spent grains (BSGs) as feedstuff for striped catfish, Pangasianodon hypophthalmus fingerlings: An approach to transform waste into wealth. J. Clean. Prod. 199, 716–722. https://doi.org/10.1016/j.jclepro.2018.07.213

Johnson, P., Paliwal, J., Cenkowski, S., 2010. Issues with utilisation of brewers' spent grain. Stewart Postharvest Rev. 6, 1–8. https://doi.org/10.2212/spr.2010.4.2

Karlsen, F., Skov, P. V., 2022. Review – Potentials and limitations of utilising brewer's spent grain as a protein source in aquaculture feeds. J. Clean. Prod. 357, 131986. https://doi.org/10.1016/j.jclepro.2022.131986

Karlsen, Ø., Amlund, H., Berg, A., Olsen, R.E., 2017. The effect of dietary chitin on growth and nutrient digestibility in farmed Atlantic cod, Atlantic salmon and Atlantic halibut. Aquac. Res. 48, 123–133. https://doi.org/10.1111/are.12867

Kaushik, S.J., Cho, C.Y., Bureau, D.P., 2002. Bioenergetics, Third edit. ed, Fish Nutrition. Elsevier (USA). https://doi.org/10.5005/jp/books/11965_2

Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B., Laroche, M., 1995. Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, Oncorhynchus mykiss. Aquaculture 133, 257–274.

Kawaguchi, H., Sasaki, M., Vertès, A.A., Inui, M., Yukawa, H., 2008. Engineering of an L-arabinose metabolic pathway in Corynebacterium glutamicum. Appl. Microbiol. Biotechnol. 77, 1053–1062. https://doi.org/10.1007/s00253-007-1244-x

Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E., Farrell, R.L., 1986. Production of multiple ligninases by Phanerochaete chrysosporium: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. 8, 27–32. https://doi.org/10.1016/0141-0229(86)90006-2

Kokou, F., Fountoulaki, E., 2018. Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients. Aquaculture 495, 295–310. https://doi.org/10.1016/j.aquaculture.2018.06.003

Kumar, D., Singh, B., Korstad, J., 2017. Utilization of lignocellulosic biomass by oleaginous yeast and bacteria for production of biodiesel and renewable diesel. Renew. Sustain. Energy Rev. 73, 654–671. https://doi.org/10.1016/j.rser.2017.01.022

Kumar, V., Sinha, A.K., Makkar, H.P.S., De Boeck, G., Becker, K., 2012. Phytate and phytase in fish nutrition. J. Anim. Physiol. Anim. Nutr. (Berl). 96, 335–364. https://doi.org/10.1111/j.1439-0396.2011.01169.x

Laca, Amanda, Laca, Adriana, Díaz, M., 2019. Hydrolysis: From cellulose and hemicellulose to simple sugars, in: Second and Third Generation of Feedstocks: The Evolution of Biofuels. pp. 213–240. https://doi.org/https://doi.org/10.1016/B978-0-12-815162-4.00008-2

Laine, C., Kemppainen, K., Kuutti, L., Varhimo, A., Asikainen, S., Grönroos, A., Määttänen, M., Buchert, J., Harlin, A., 2015. Extraction of xylan from wood pulp and brewer's spent grain. Ind. Crops Prod. 70, 231–237. https://doi.org/10.1016/j.indcrop.2015.03.009

Lall, S.P., Dumas, A., 2015. Nutritional requirements of cultured fish: Formulating nutritionally adequate feeds, Feed and Feeding Practices in Aquaculture. Elsevier Ltd. https://doi.org/10.1016/B978-0-08-100506-4.00003-9

Lam, A.C.Y., Can Karaca, A., Tyler, R.T., Nickerson, M.T., 2018. Pea protein isolates: Structure, extraction, and functionality. Food Rev. Int. 34, 126–147. https://doi.org/10.1080/87559129.2016.1242135

Lee, D.J., Putnam, G.B., 1973. The response of rainbow trout to varying protein/energy ratios in a test diet. J. Nutr. 103, 916–922. https://doi.org/10.1093/jn/103.6.916

Lee, J.Y., Na, Y.A., Kim, E., Lee, H.S., Kim, P., 2016. The actinobacterium Corynebacterium glutamicum, an industrial workhorse. J. Microbiol. Biotechnol. 26, 807–822. https://doi.org/10.4014/jmb.1601.01053

Leisola, M.S.A., Ulmer, D.C., Waldner, R., Fiechter, A., 1984. Role of veratryl alcohol in lignin degradation by Phanerochaete chrysosporium. J. Biotechnol. 1, 331–339. https://doi.org/10.1016/0168-1656(84)90024-5

Lewis, M.J., Young, T.W., 2001. Brewing, Brewing. https://doi.org/10.1007/978-1-4615-0729-1

Li, K., Xu, F., Eriksson, K.E.L., 1999. Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. Appl. Environ. Microbiol. 65, 2654–2660. https://doi.org/10.1128/aem.65.6.2654-2660.1999

Li, X., Zheng, S., Wu, G., 2021. Nutrition and Functions of Amino Acids in Fish, Advances in Experimental Medicine and Biology. https://doi.org/10.1007/978-3-030-54462-1_8

Lindskog, E.K., 2018. The Upstream Process: Principal Modes of Operation, Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes. Elsevier Ltd. https://doi.org/10.1016/B978-0-08-100623-8.00031-1

Liu, K.S., 2009. Some factors affecting sieving performance and efficiency. Powder Technol. 193, 208–213. https://doi.org/10.1016/j.powtec.2009.03.027

Lizardi-Jiménez, M.A., Hernández-Martínez, R., 2017. Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. 3 Biotech 7. https://doi.org/10.1007/s13205-017-0692-y

Lovell, T., 1998. Nutrition and feeding of fish: chapter 2, Second. ed. Springer US. https://doi.org/10.1007/978-1-4615-4909-3_6

Lu, X., Zheng, X., Li, X., Zhao, J., 2016. Adsorption and mechanism of cellulase enzymes onto lignin isolated from corn stover pretreated with liquid hot water. Biotechnol. Biofuels 9, 1–12. https://doi.org/10.1186/s13068-016-0531-0

Lynch, K.M., Steffen, E.J., Arendt, E.K., 2016. Brewers' spent grain: a review with an emphasis on food and health. J. Inst. Brew. 122, 553–568. https://doi.org/10.1002/jib.363

Ma, Q., Zhang, Q., Xu, Q., Zhang, C., Li, Y., Fan, X., Xie, X., Chen, N., 2017. Systems metabolic engineering strategies for the production of amino acids. Synth. Syst. Biotechnol. https://doi.org/10.1016/j.synbio.2017.07.003

Malaney, G.W., Tanner, R.D., Rodrigues, A.M., 1991. The production of extracellular and intracellular free amino acids during aerated fermentation of glucose by Baker's yeast (Saccharomyces cerevisiae). Folia Microbiol. (Praha). 36, 468–477. https://doi.org/10.1007/BF02884068

Mambrini, M., Kaushik, S.J., 1994. Partial replacement of dietary protein nitrogen with dispensable amino acids in diets of Nile tilapia, Oreochromis niloticus. Comp. Biochem. Physiol. 109A, 469–477. https://doi.org/10.1016/0300-9629(94)90152-X

Mandalari, G., Faulds, C.B., Sancho, A.I., Saija, A., Bisignano, G., Locurto, R., Waldron, K.W., 2005. Fractionation and characterisation of arabinoxylans from brewers' spent grain and wheat bran. J. Cereal Sci. 42, 205–212.

https://doi.org/10.1016/j.jcs.2005.03.001

Martín, C., Jönsson, L.J., 2003. Comparison of the resistance of industrial and laboratory strains of Saccharomyces and Zygosaccharomyces to lignocellulose-derived fermentation inhibitors. Enzyme Microb. Technol. 32, 386–395. https://doi.org/10.1016/S0141-0229(02)00310-1

Martínez-Medina, G.A., Barragán, A.P., Ruiz, H.A., Ilyina, A., Hernández, J.L.M., Rodríguez-Jasso, R.M., Hoyos-Concha, J.L., Aguilar-González, C.N., 2018. Fungal proteases and production of bioactive peptides for the food industry, Enzymes in Food Biotechnology: Production, Applications, and Future Prospects. Elsevier Inc. https://doi.org/10.1016/B978-0-12-813280-7.00014-1

Melro, E., Filipe, A., Sousa, D., Valente, A.J.M., Romano, A., Antunes, F.E., Medronho, B., 2020. Dissolution of kraft lignin in alkaline solutions. Int. J. Biol. Macromol. 148, 688–695. https://doi.org/10.1016/j.ijbiomac.2020.01.153

Mendis, M., Simsek, S., 2014. Arabinoxylans and human health. Food Hydrocoll. 42, 239–243. https://doi.org/10.1016/j.foodhyd.2013.07.022

Mishra, S.P., 2020. Significance of fish nutrients for human health. Int. J. Fish. Aquat. Res. 5, 47–49.

Misra, C.K., Sahu, N.P., Jain, K.K., 2002. Effect of extrusion processing and steam pelleting diets on pellet durability, water absorption and physical response of Macrobrachium rosenbergii. Asian-Australasian J. Anim. Sci. 15, 1354–1358. https://doi.org/10.5713/ajas.2002.1354

Mongeau, R., Brooks, S.P.J., 2015. Dietary Fiber: Determination, 1st ed, Encyclopedia of Food and Health. Elsevier Ltd. https://doi.org/10.1016/B978-0-12-384947-2.00228-2

Montgomery, L.F.R., Bochmann, G., 2014. Pretreatment of feedstock for enhanced biogas production, IEA Bioenergy.

Mosher, M., Trantham, K., 2017. Brewing Science: A Multidisciplinary Approach, Brewing Science: A Multidisciplinary Approach. https://doi.org/10.1007/978-3-319-46394-0

Mould, E.D., Robbins, C.T., 1981. Evaluation of Detergent Analysis in Estimating Nutritional Value of Browse. J. Wildl. Manage. 45, 937–947.

Mussatto, S.I., Dragone, G., Roberto, I.C., 2006. Brewers' spent grain: Generation, characteristics and potential applications. J. Cereal Sci. 43, 1–14. https://doi.org/10.1016/j.jcs.2005.06.001

Mussatto, S.I., Fernandes, M., Milagres, A.M.F., Roberto, I.C., 2008. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzyme Microb. Technol. 43, 124–129. https://doi.org/10.1016/j.enzmictec.2007.11.006

Mussatto, S.I., Fernandes, M., Roberto, I.C., 2007. Lignin recovery from brewer's spent grain black liquor. Carbohydr. Polym. 70, 218–223. https://doi.org/10.1016/j.carbpol.2007.03.021

Mussatto, S.I., Teixeira, J.A., 2010. Lignocellulose as raw material in fermentation processes, in: Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. pp. 897–907.

Nangul, A., Bhatia, R., 2013. Microorganisms : a Marvelous Source of Single Cell Proteins. J. Microbiol. Biotechnol. Food Sci. 3, 15–18.

Nazzaro, J., Martin, D.S., Perez-Vendrell, A.M., Padrell, L., Iñarra, B., Orive, M., Estévez, A., 2021. Apparent digestibility coefficients of brewer's by-products used in feeds for rainbow trout (Oncorhynchus mykiss) and gilthead seabream (Sparus aurata). Aquaculture 530, 735796. https://doi.org/10.1016/j.aquaculture.2020.735796

Neto, F.S.P.P., De Lucca, E.A., Masarin, F., 2016. Particle size influence on the chemical composition from waste Eucalyptus in the processing for industrial production of cellulose pulp. Chem. Eng. Trans. 50, 337–342. https://doi.org/10.3303/CET1650057

Niju, S., Swathika, M., Balajii, M., 2019. Pretreatment of lignocellulosic sugarcane leaves and tops for bioethanol production, Lignocellulosic Biomass to Liquid Biofuels. INC. https://doi.org/10.1016/B978-0-12-815936-1.00010-1

Nunes, A.J.P., Sá, M.V.C., Browdy, C.L., Vazquez-Anon, M., 2014. Practical supplementation of shrimp and fish feeds with crystalline amino acids. Aquaculture 431, 20–27. https://doi.org/10.1016/j.aquaculture.2014.04.003

Ochoa, S., 2019. Fed-batch fermentation - design strategies, in: Comprehensive Biotechnology. Elsevier, pp. 586–600. https://doi.org/10.1016/B978-0-444-64046-8.00093-8

OECD/FAO (2020), OECD-FAO Agricultural Outlook 2020-2029, FAO, Rome/OECD Publishing, Paris, https://doi.org/10.1787/1112c23b-en

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis. 35, 83–108. https://doi.org/10.1111/j.1365-2761.2011.01333.x

Olsen, R.L., Hasan, M.R., 2012. A limited supply of fishmeal: Impact on future increases in

global aquaculture production. Trends Food Sci. Technol. 27, 120–128. https://doi.org/10.1016/j.tifs.2012.06.003

Ouedraogo, J.P., Tsang, A., 2021. Production of native and recombinant enzymes by Fungi for industrial applications. Encycl. Mycol. 2, 222–232. https://doi.org/10.1016/B978-0-12-819990-9.00046-9

Pace, C.N., Treviño, S., Prabhakaran, E., Scholtz, J.M., Franks, F., Wilson, K., Daniel, R.M., Halling, P.J., Clark, D.S., Purkiss, A., 2004. Protein structure, stability and solubility in water and other solvents. Philos. Trans. R. Soc. B Biol. Sci. 359, 1225–1235. https://doi.org/10.1098/rstb.2004.1500

Page, J.W., Andrews, J.W., 1973. Interactions of dietary levels of protein and energy on channel catfish (Ictalurus punctatus). J. Nutr. 103, 1339–1346. https://doi.org/10.1093/jn/103.9.1339

Paz, A., Outeiriño, D., Pérez Guerra, N., Domínguez, J.M., 2019. Enzymatic hydrolysis of brewer's spent grain to obtain fermentable sugars. Bioresour. Technol. 275, 402–409. https://doi.org/10.1016/j.biortech.2018.12.082

Pelletier, N., Klinger, D.H., Sims, N.A., Yoshioka, J.R., Kittinger, J.N., 2018. Nutritional Attributes, Substitutability, Scalability, and Environmental Intensity of an Illustrative Subset of Current and Future Protein Sources for Aquaculture Feeds: Joint Consideration of Potential Synergies and Trade-offs. Environ. Sci. Technol. 52, 5532–5544. https://doi.org/10.1021/acs.est.7b05468

Peña, E., Badillo-Zapata, D., Viana, M.T., Correa-Reyes, G., 2020. Use of grape pomace in formulated feed for the rainbow trout fry, Oncorhynchus mykiss (Walbaum, 1792). J. World Aquac. Soc. 51, 542–550. https://doi.org/10.1111/jwas.12669

Peres, H., Oliva-Teles, A., 2006. Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (Dicentrarchus labrax). Aquaculture 256, 395–402. https://doi.org/10.1016/j.aquaculture.2006.02.010

Pérez-Jiménez, A., Trenzado Romero, C.E., Hernández, G.C., 2011. Metabolic responses to food deprivation in Fish, Biology of Starvation in Humans and Other Organisms.

Péron, G., François Mittaine, J., Le Gallic, B., 2010. Where do fishmeal and fish oil products come from? An analysis of the conversion ratios in the global fishmeal industry. Mar. Policy 34, 815–820. https://doi.org/10.1016/j.marpol.2010.01.027

Petruccioli, M., Raviv, M., Di Silvestro, R., Dinelli, G., 2011. Agriculture and Agro-Industrial Wastes, Byproducts, and Wastewaters: Origin, Characteristics, and Potential in Bio-Based-Compounds Production, Comprehensive Biotechnology. Elsevier, Second Edition. https://doi.org/10.1016/B978-0-08-088504-9.00389-5

Prado, R., Erdocia, X., Labidi, J., 2013. Lignin extraction and purification with ionic liquids. J. Chem. Technol. Biotechnol. 88, 1248–1257. https://doi.org/10.1002/jctb.3965

Qin, F., Johansen, A.Z., Mussatto, S.I., 2018. Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains. Ind. Crops Prod. 125, 443–453. https://doi.org/10.1016/j.indcrop.2018.09.017

Rachwał, K., Waśko, A., Gustaw, K., Polak-Berecka, M., 2020. Utilization of brewery wastes in food industry. PeerJ 8, 1–28. https://doi.org/10.7717/peerj.9427

Reguengo, L.M., Salgaço, M.K., Sivieri, K., Maróstica Júnior, M.R., 2022. Agro-industrial by-products: Valuable sources of bioactive compounds. Food Res. Int. 152. https://doi.org/10.1016/j.foodres.2021.110871

Reis, S.F., Coelho, E., Coimbra, M.A., Abu-Ghannam, N., 2015. Improved efficiency of brewer's spent grain arabinoxylans by ultrasound-assisted extraction. Ultrason. Sonochem. 24, 155–164. https://doi.org/10.1016/j.ultsonch.2014.10.010

Ritala, A., Häkkinen, S.T., Toivari, M., Wiebe, M.G., 2017. Single cell protein-state-of-the-art, industrial landscape and patents 2001-2016. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.02009

Robertson, J.A., IAnson, K.J.A., Brocklehurst, T.F., Faulds, C.B., Waldron, K.W., 2010a. Effect of storage conditions on the microbial ecology and biochemical stability of cell wall components in brewers spent grain. J. Agric. Food Chem. 58, 7266–7272. https://doi.org/10.1021/jf1001099

Robertson, J.A., I'Anson, K.J.A., Treimo, J., Faulds, C.B., Brocklehurst, T.F., Eijsink, V.G.H., Waldron, K.W., 2010b. Profiling brewers' spent grain for composition and microbial ecology at the site of production. LWT - Food Sci. Technol. 43, 890–896. https://doi.org/10.1016/j.lwt.2010.01.019

Rochelle, L., Sciences, P., Curie, B.M., Crépeau, A.M., Rochelle, L., 2008. A Thermomechanical Process for Improving Enzymatic Hydrolysis of Brewer ' S Spent Grain.

Rodrigues, I.M., Coelho, J.F.J., Carvalho, M.G.V.S., 2012. Isolation and valorisation of

vegetable proteins from oilseed plants: Methods, limitations and potential. J. Food Eng. 109, 337–346. https://doi.org/10.1016/j.jfoodeng.2011.10.027

Rodriguez, A., Martínez, J.A., Flores, N., Escalante, A., Gosset, G., Bolivar, F., 2014. Engineering Escherichia coli to overproduce aromatic amino acids and derived compounds. Microb. Cell Fact. 13, 1–15. https://doi.org/10.1186/s12934-014-0126-z

Rodríguez Couto, S., Rivela, I., Muñoz, M.R., Sanromán, A., 2000. Stimulation of ligninolytic enzyme production and the ability to decolourise Poly R-478 in semi-solid-state cultures of Phanerochaete chrysosporium. Bioresour. Technol. 74, 159–164. https://doi.org/10.1016/S0960-8524(99)00156-X

Ruiz, G.A., Xiao, W., Van Boekel, M., Minor, M., Stieger, M., 2016. Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein isolate from quinoa (Chenopodium quinoa Willd). Food Chem. 209, 203–210. https://doi.org/10.1016/j.foodchem.2016.04.052

Salin, K.R., Arun, V. V, Nair, C.M., Tidwell, J.H., 2018. Sustainable Aquaculture, Sustainable Aquaculture. Springer International Publishing. https://doi.org/10.1007/978-3-319-73257-2

Samtiya, M., Aluko, R.E., Dhewa, T., 2020. Plant food anti-nutritional factors and their reduction strategies: an overview. Food Prod. Process. Nutr. 2, 1–14. https://doi.org/10.1186/s43014-020-0020-5

Sanchez, S., Rodríguez-Sanoja, R., Ramos, A., Demain, A.L., 2018. Our microbes not only produce antibiotics, they also overproduce amino acids. J. Antibiot. (Tokyo). 71, 26–36. https://doi.org/10.1038/ja.2017.142

Santos, M., Jiménez, J.J., Bartolomé, B., Gómez-Cordovés, C., Del Nozal, M.J., 2003. Variability of brewer's spent grain within a brewery. Food Chem. 80, 17–21. https://doi.org/10.1016/S0308-8146(02)00229-7

Sari, Y.W., Mulder, W.J., Sanders, J.P.M., Bruins, M.E., 2015a. Towards plant protein refinery: Review on protein extraction using alkali and potential enzymatic assistance. Biotechnol. J. 10, 1138–1157. https://doi.org/10.1002/biot.201400569

Sari, Y.W., Syafitri, U., Sanders, J.P.M., Bruins, M.E., 2015b. How biomass composition determines protein extractability. Ind. Crops Prod. 70, 125–133. https://doi.org/10.1016/j.indcrop.2015.03.020

Sayooj, P., Ebeneezar, S., 2021. Fish Feed Processing and Production Technology: in the

book The Blue Bonanza: A Manual for on the job Training Programme for VHSE students on Advances in Fisheries & Aquaculture Techniques. ICAR-Central Marine Fisheries Research.

Schoemaker, H.E., Leisola, M.S.A., 1990. Degradation of lignin by Phanerochaete chrysosporium. J. Biotechnol. 13, 101–109. https://doi.org/10.1016/0168-1656(90)90096-T

Shahbandeh, M., 2022. Forecast volume of distiller's dry grain produced in the European Union (EU 27) from 2015 to 2028. Available at: https://www.statista.com/statistics/545737/distillers-dry-grains-production-volume-european-union-28/?fbclid=IwAR3xerhpNrTJvMszjNDGLcrpXtuP788_tj_ESf1UdO3ExDcrrUF01aM9WUk. (Accessed 17 July 2022).

Shakoori, F.R., Butt, A.M., Ali, N.M., Zahid, M.T., Rehman, A., Shakoori, A.R., 2012. Optimization of fermentation media for enhanced amino acids production by bacteria isolated from natural sources. Pak. J. Zool. 44, 1145–1157.

Shanmugam, M.K., Mandari, V., Devarai, S.K., Gummadi, S.N., 2022. Types of bioreactors and important design considerations, Current Developments in Biotechnology and Bioengineering. INC. https://doi.org/https://doi.org/10.1016/C2020-0-04191-5

Shraddha, Shekher, R., Sehgal, S., Kamthania, M., Kumar, A., 2011. Laccase: Microbial sources, production, purification, and potential biotechnological applications. Enzyme Res. 2011. https://doi.org/10.4061/2011/217861

Singh, D., Chen, S., 2008. The white-rot fungus Phanerochaete chrysosporium: Conditions for the production of lignin-degrading enzymes. Appl. Microbiol. Biotechnol. 81, 399–417. https://doi.org/10.1007/s00253-008-1706-9

Singhania, R.R., 2011. Production of celluloytic enzymes for the hydrolysis of lignocellulosic biomass, 1st ed, Biofuels. Elsevier Inc. https://doi.org/https://doi.org/10.1016/B978-0-12-385099-7.00008-5

Sinha, A.K., Kumar, V., Makkar, H.P.S., De Boeck, G., Becker, K., 2011. Non-starch polysaccharides and their role in fish nutrition - A review. Food Chem. 127, 1409–1426. https://doi.org/10.1016/j.foodchem.2011.02.042

Sodhi, H.S., Garcha, H.S., Kiran, U., 1985. Screening of mycoflora of spent-up brewer's grain for aflatoxin production. Agric. Res. J. 22, 331–336.

Sousa, D., Venâncio, A., Belo, I., Salgado, J.M., 2018. Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation. J. Sci. Food Agric. 98, 5248–5256. https://doi.org/10.1002/jsfa.9063

Sousa, R. de C.S., Coimbra, J.S.R., Garcia Rojas, E.E., Minim, L.A., Oliveira, F.C., Minim, V.P.R., 2007. Effect of pH and salt concentration on the solubility and density of egg yolk and plasma egg yolk. LWT - Food Sci. Technol. 40, 1253–1258. https://doi.org/10.1016/j.lwt.2006.08.001

Stanbury, P.F., Whitaker, A., Hall, S.J., 1995a. An Introduction to Fermentation Processes. Princ. Ferment. Technol. 1–11. https://doi.org/10.1016/b978-0-08-036131-4.50006-7

Stanbury, P.F., Whitaker, A., Hall, S.J., 1995b. Microbial Growth Kinetics. Princ. Ferment. Technol. 13–33. https://doi.org/10.1016/b978-0-08-036131-4.50007-9

Steiner, J., Procopio, S., Becker, T., 2015. Brewer's spent grain: source of value-added polysaccharides for the food industry in reference to the health claims. Eur. Food Res. Technol. 241, 303–315. https://doi.org/10.1007/s00217-015-2461-7

Surasani, V.K.R., Khatkar, S.K., Singh, S., 2017. "Effect of process variables on solubility and recovery yields of proteins from pangas (Pangasius pangasius) frames obtained by alkaline solubilization method: Characteristics of isolates." Food Bioprod. Process. 106, 137–146. https://doi.org/10.1016/j.fbp.2017.09.008

Tacon, A.G.J., Hasan, M.R., Metian, M., 2011. Demand and supply of feed ingredients for farmed fish and crustaceans : Trends and prospects, FAO Fisheries and Aquaculture Technical Paper

Tan, Y.X., Mok, W.K., Lee, J., Kim, J., Chen, W.N., 2019. Solid state fermentation of Brewers' spent grains for improved nutritional profile using Bacillus subtilis WX-17. Fermentation 5, 1–13. https://doi.org/10.3390/fermentation5030052

Tang, D.S., Tian, Y.J., He, Y.Z., Li, L., Hu, S.Q., Li, B., 2010. Optimisation of ultrasonic-assisted protein extraction from brewer's spent grain. Czech J. Food Sci. 28, 9–17. https://doi.org/10.17221/178/2009-cjfs

Tang, D.S., Yin, G.M., He, Y.Z., Hu, S.Q., Li, B., Li, L., Liang, H.L., Borthakur, D., 2009. Recovery of protein from brewer's spent grain by ultrafiltration. Biochem. Eng. J. 48, 1–5. https://doi.org/10.1016/j.bej.2009.05.019

Tang, Z., Cenkowski, S., Izydorczyk, M., 2005. Thin-layer drying of spent grains in superheated steam. J. Food Eng. 67, 457–465.

https://doi.org/10.1016/j.jfoodeng.2004.04.040

Teles, A.O., Couto, A., Enes, P., Peres, H., 2020. Dietary protein requirements of fish – a meta-analysis. Rev. Aquac. 12, 1445–1477. https://doi.org/10.1111/raq.12391

Thurston, C.F., 1994. The structure and function of fungal laccases. Microbiology 140, 19–26.

Tien, M., Kirk, T., 1988. Lignin Peroxidase of Phanerochaete chrysosporium. Methods Enzymol. 161, 238–249.

Tocco, D., Carucci, C., Monduzzi, M., Salis, A., Sanjust, E., 2021. Recent Developments in the Delignification and Exploitation of Grass Lignocellulosic Biomass. ACS Sustain. Chem. Eng. 9, 2412–2432. https://doi.org/10.1021/acssuschemeng.0c07266

Toe, C.J., Foo, H.L., Loh, T.C., Mohamad, R., Rahim, R.A., Idrus, Z., 2019. Extracellular proteolytic activity and amino acid production by lactic acid bacteria isolated from malaysian foods. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20071777

Türker, M., Selimoğlu, S.M., Taşpınar-Demir, H., 2021. Waste(water) to feed protein: Effluent characteristics, protein recovery, and single-cell protein production from food industry waste streams, Clean Energy and Resource Recovery: Wastewater Treatment Plants as Biorefineries, Volume 2. https://doi.org/10.1016/B978-0-323-90178-9.00017-2

USDA, 2021. Grain Crushings and Co- Products Production 2020 Summary.

Van Rooijen, C., Bosch, G., Van Der Poel, A.F.B., Wierenga, P.A., Alexander, L., Hendriks, W.H., 2013. The Maillard reaction and pet food processing: Effects on nutritive value and pet health. Nutr. Res. Rev. 26, 130–148. https://doi.org/10.1017/S0954422413000103

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J. Dairy Sci. 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Vandana, T., Kumar, S.A., Swaraj, S., Manpal, S., 2019. Purification, characterization, and biodelignification potential of lignin peroxidase from immobilized phanerochaete chrysosporium. BioResources 14, 5380–5399. https://doi.org/10.15376/biores.14.3.5380-5399

Vareltzis, P.K., Undeland, I., 2012. Protein isolation from blue mussels (Mytilus edulis) using an acid and alkaline solubilisation technique-process characteristics and functionality of the isolates. J. Sci. Food Agric. 92, 3055–3064. https://doi.org/10.1002/jsfa.5723

Veide Vilg, J., Undeland, I., 2017. pH-driven solubilization and isoelectric precipitation of proteins from the brown seaweed Saccharina latissima—effects of osmotic shock, water volume and temperature. J. Appl. Phycol. 29, 585–593. https://doi.org/10.1007/s10811-016-0957-6

Venkatadri, R., Irvine, R.L., 1990. Effect of agitation on ligninase activity and ligninase production of Phanerochaete chrysosporium. Appl. Environ. Microbiol. 56, 2684–2691.

Verardi, A., De Bari, I., Ricca, E., Calabrò, V., 2012. Hydrolysis of Lignocellulosic Biomass: Current Status of Processes and Technologies and Future Perspectives in: Bioethanol. INTECH EUROPE. https://doi.org/10.5772/23987

Vieira, E., Rocha, M.A.M., Coelho, E., Pinho, O., Saraiva, J.A., Ferreira, I.M.P.L.V.O., Coimbra, M.A., 2014. Valuation of brewer's spent grain using a fully recyclable integrated process for extraction of proteins and arabinoxylans. Ind. Crops Prod. 52, 136–143. https://doi.org/10.1016/j.indcrop.2013.10.012

Waters, D.M., Jacob, F., Titze, J., Arendt, E.K., Zannini, E., 2012. Fibre, protein and mineral fortification of wheat bread through milled and fermented brewer's spent grain enrichment. Eur. Food Res. Technol. 235, 767–778. https://doi.org/10.1007/s00217-012-1805-9

Wen, C., Zhang, J., Duan, Y., Zhang, H., Ma, H., 2019. A Mini-Review on Brewer's Spent Grain Protein: Isolation, Physicochemical Properties, Application of Protein, and Functional Properties of Hydrolysates. J. Food Sci. 84, 3330–3340. https://doi.org/10.1111/1750-3841.14906

Willows, R.D., Worden, P., Mirzaei, M., 2017. Barley Grain Proteomics, Proteomics in Food Science: From Farm to Fork. Elsevier Inc. https://doi.org/10.1016/B978-0-12-804007-2.00005-9

Wilson, R.P., 1994. Utilization of dietary carbohydrate by fish. Aquaculture 124, 67–80. https://doi.org/10.1016/0044-8486(94)90363-8

Wilson, R.P., Halver, J.E., 1986. Protein and amino acid requirements of fish. Annu. Rev. Nutr. 6, 225–244. https://doi.org/10.1146/annurev.nu.06.070186.001301

Wong, D.W.S., 2009. Structure and action mechanism of ligninolytic enzymes, Applied Biochemistry and Biotechnology. https://doi.org/10.1007/s12010-008-8279-z

Wool, R.P., 2005. Lignin Polymers and Composites, Bio-Based Polymers and Composites. https://doi.org/https://doi.org/10.1016/B978-012763952-9/50017-4

Wright, P.A., 1995. Nitrogen excretion: Three end products, many physiological roles. J. Exp. Biol. 198, 273–281. https://doi.org/10.1242/jeb.198.2.273

Xiros, C., Katapodis, P., Christakopoulos, P., 2011. Factors affecting cellulose and hemicellulose hydrolysis of alkali treated brewers spent grain by Fusarium oxysporum enzyme extract. Bioresour. Technol. 102, 1688–1696. https://doi.org/10.1016/j.biortech.2010.09.108

Xiros, C., Topakas, E., Katapodis, P., Christakopoulos, P., 2008a. Hydrolysis and fermentation of brewer's spent grain by Neurospora crassa. Bioresour. Technol. 99, 5427–5435. https://doi.org/10.1016/j.biortech.2007.11.010

Xiros, C., Topakas, E., Katapodis, P., Christakopoulos, P., 2008b. Evaluation of Fusarium oxysporum as an enzyme factory for the hydrolysis of brewer's spent grain with improved biodegradability for ethanol production. Ind. Crops Prod. 28, 213–224. https://doi.org/10.1016/j.indcrop.2008.02.004

Xu, B., Jahic, M., Enfors, S.O., 1999. Modeling of overflow metabolism in batch and fed-batch cultures of Escherichia coli. Biotechnol. Prog. 15, 81–90. https://doi.org/10.1021/bp9801087

Yinghuai, Z., Yuanting, K.T., Hosmane, N.S., 2013. Application of ionic liquids in lignin chemistry. In: Ionic Liquids - New Aspects for the Future, IntechOpen. https://doi.org/http://dx.doi.org/10.5772/51161

Yoshimatsu, T., Furuichi, M., Kitajima, C., 1992. Optimum Level of Protein in Purified Experimental Diets for Redlin Mullet. Nippon Suisan Gakkaishi (Japanese Ed. 58), 2111–2117. https://doi.org/10.2331/suisan.58.2111

Appendix

Paper 1: Review – Potentials and limitations of utilising brewer's spent grain as a protein source in aquaculture feeds

Paper 2: Optimisation of alkaline protein extraction of brewer's spent grain

Manuscript 3: Evaluation of brewer's spent grain as a feedstock for amino acid production by submerged fermentation

Manuscript 4: Evaluating brewer's spent grain and Ligno biomass as potential substrates for single cell protein production through submerged fermentation

Paper 1

Review – potentials and limitations of utilising brewer's spent grain as a protein source in aquaculture feeds

Published, Journal of Cleaner Production 357 (2022) 131986

Journal of Cleaner Production 357 (2022) 131986

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Review – Potentials and limitations of utilising brewer's spent grain as a protein source in aquaculture feeds

Freja Karlsen *, Peter V. Skov

Technical University of Denmark, DTU Aqua, Section for Aquaculture, DK, 9850, Hirtshals, Denmark

ARTICLE INFO

Handling Editor: Zhen Leng

Keywords:
Aquaculture
Protein sources
Brewer's spent grain
Anti-nutritional factors
Protein extraction
Lignocellulose conversion

ABSTRACT

On a worldwide basis, aquaculture is among the most important food-producing industries. The steadily growing population has led to an increased demand for aquaculture products, which in turns has intensified the farming of aquatic species along with aquafeed production. Fishmeal and soybean meal represent two of the most widely used protein sources in aquafeeds. Increasing demands and costs have fostered a need for supplementing fishmeal and soybean meal with sustainable alternatives in the coming years. Brewer's spent grain (BSG) is the major by-product generated in beer brewing. With a relatively high protein content, low market price and stable annual availability BSG represents a potential protein source for aquaculture feeds. However, in its untreated form BSG contains high levels of anti-nutritional factors (ANFs) such as lignin and fibre which are known to reduce digestive performance in fish. Furthermore, BSG appears to be perishable under inappropriate storage conditions due to its high moisture content. Therefore, the main objective of this review was to compile an overview of different methods that may help facilitate the implementation of BSG in aquafeed. Before BSG may be utilised as a protein source, it must undergo refinement to remove lignocellulosic material. Here, we focus on two general approaches commonly used: a fractionation-and a conversion-based method. The fractionation-based approach relies on chemical extractions to separate BSG into its constituents, thereby removing the ANFs from the protein component. The conversion-based approach aims to transform ANFs into digestible substances by applying a combination of chemical, biological and enzymatic treatments. Application of appropriate preservation methods can help prevent microbial colonisation of BSG. Three different preservation methods were compared including freeze-drying, oven-drying and lactic acid bacteria (LAB) treatment. Of these, LAB treatment appears to be the most favourable with respect to energy-costs and potential health benefits. At present, BSG has not found any practical use in aquafeed production. However, the collection of methods presented in this review may provide a basis for incorporating BSG in aquafeeds and highlight possible future directions for realising this aim.

IPisoelectric precipitation.
SSsuperheat steam drying.

1. Introduction

Globally, aquaculture constitutes one of the fastest-growing food-producing sectors (OECD/FAO, 2016). Due to a steadily growing population, the demand for food has increased resulting in the intensification of aquaculture production (FAO, 2020). Moreover, the recent COVID-19 pandemic will inevitably influence the global food industries including aquaculture; though, it is still not fully understood how and to which extend food production will be affected. It is expected that the pandemic will lead to increasing demand for healthy food, such as fish products and a need for adopting new practices in food production (Galanakis, 2020). Fishmeal and plant-based meals are, at present, the most commonly applied protein sources in commercial fish feed formulations (Jannathulla et al., 2019). However, these ingredients are associated with environmental and economic concerns which have necessitated the search for alternative protein sources (Boyd, 2015). Brewer's spent grain (BSG) represents ~85% of the total by-products generated during the beer manufacturing process (Rachwał et al., 2020). From 1998 to 2020, the global beer production increased from 1.30 to 1.82 billion tonnes (Conway, 2021). In 2020, the production of BSG was estimated at ~36 million tonnes assuming that about 20 kg BSG is generated per hL of brewed beer. Further, BSG is high in proteins by comparison with other abundant agro-industrial by-products (Sousa et al., 2018). Owing to its immense production volumes, stable annual availability and comparatively high protein content, BSG constitutes a

* Corresponding author.
E-mail address: frkar@aqua.dtu.dk (F. Karlsen).

https://doi.org/10.1016/j.jclepro.2022.131986
Received 11 May 2021; Received in revised form 11 April 2022; Accepted 22 April 2022
Available online 27 April 2022

Abbreviations

AA	amino acid
ADL	acid detergent lignin
ADF	acid detergent fibre
AF	aflatoxin
ANF	anti-nutritional factor
AX	arabinoxylan
BSG	brewer's spent grain
CAA	crystalline amino acid
CP	crude protein
DAA	dispensable amino acid
DES	deep eutectic solvent
DM	dry matter content
EAA	essential amino acid
HMF	hydroxymethylfurfural
IA	indispensable amino acid
IL	ionic liquid
LAB	lactic acid bacteria
MRP	Maillard reaction product
NDF	neutral detergent fibre
NEAA	non-essential amino acid
NSP	non-starch polysaccharide

potential, unexploited protein source for aquaculture feed. Even though the biotechnological potential of BSG has been extensively investigated in the literature, a limited number of studies have focused on its utilisation in aquafeed production. Therefore, the main objective of this review was to address novel approaches that may be suitable for transforming unrefined BSG into a high-quality protein source for aquafeed applications with focus on fish feed.

2. Aquaculture feeds

Protein is the main and most expensive ingredient in fish feeds. Currently, fishmeal and soybean meal are the most widely used protein sources in aquafeeds. Yet, these ingredients are not regarded as economically sustainable due to their excessive prices which result from stagnating production volumes and competitive demands (Salin et al., 2018). Thus, the continued growth of aquaculture production relies on the implementation of alternative protein sources in aquafeeds. This section provides an overview of the general composition of aquaculture feeds with emphasis on the protein source while comparing the advantages and drawbacks of utilising fishmeal, soybean meal, or BSG as protein sources in aquafeeds. Formulation of fish diets requires knowledge of the nutritional requirements of target species. The nutritional content of a feed is essentially divided into two major categories: the micro-and macronutrients (Murai, 1991). The micronutrients are comprised of minerals and vitamins while macronutrients provide an energy source for ATP production and building blocks for growth. The macronutrients are categorised into three distinct nutrient classes: carbohydrates, lipids and proteins which yield different amounts of energy when fully oxidised by the organism. The requirement and utilisation efficiency of these three macronutrients differ considerably between fish species which is reflected in their natural feeding behaviour. Carnivorous fish species are the least efficient in digesting and utilising carbohydrates, whereas herbivorous and omnivorous fish species exhibit high efficiency in carbohydrate exploitation (Wilson, 1994). Furthermore, the protein requirement appears to be positively correlated with the trophic level of the fish with carnivorous species having the highest protein requirement (Teles et al., 2020). It is also well-established in the literature that the protein requirement of fish varies with size, life stage and abiotic factors such as water temperature and salinity (Wilson, 1986). Despite the lower metabolism of ectothermic organisms compared to endotherms such as birds and mammals, fish have a higher dietary protein requirement, ranging from 25 to 70% (Teles et al., 2020). This may appear counterintuitive, because fish, unlike endotherms, do not spend energy on maintaining a high body temperature. Furthermore, fish primarily excrete nitrogen as ammonia which is less costly to produce in terms of energy in comparison to urea and uric acid that are the primary nitrogenous compounds excreted in the urine of terrestrial animals (Pandian and Vivekanandan, 1985). However, fish ingest less feed (in % of body mass) and have a faster growth trajectory than endotherms and therefore require more protein in their diets. In the following section, we compare different dietary protein sources based on their nutritional quality.

2.1. Protein sources

Fish, like other monogastric animals, do not have a true protein requirement. Instead, fish require an appropriate mixture of essential (EAAs) and non-essential amino acids (NEAAs) that proteins are composed of (Teles et al., 2020). While NEAAs are synthesised *de novo* from intermediates of the glycolytic pathway or the citric acid cycle, EAAs cannot be produced by the organism and must therefore be supplied in the diet (Litwack, 2018). In most organisms, NEAAs are used as building blocks in a multitude of essential metabolic functions (Hou et al., 2015). NEAAs production may sometimes be insufficient and therefore dietary supplementation is needed to meet the requirements for maximal growth and health (Peres and Oliva-Teles, 2006). The EAAs and NEAAs for fish, which are also referred to as indispensable (IAAs) and dispensable amino acids (DAAs), respectively, are presented in Table 1 (Murai, 1991).

A high-quality protein source for fish feed must fulfill multiple criteria, namely: high palatability and attractability, a balanced AA profile and low quantities of toxins and anti-nutritional factors (ANFs). Antinutritional factors are defined as biologically active compounds that inhibit nutrient absorption and utilisation when present in feed (Glencross et al., 2020). Employing protein sources of high palatability and attractability is crucial to enhance feed consumption as fish are more willing to ingest feeds that appear attractive and palatable to them (Al-Souti et al., 2019). The level of macronutrients, toxins and ANFs, as well as the presence of stimulants and attractants, largely influence feed palatability and attractability. These chemical factors determine the physical characteristics of the feed including taste, smell and visibility which indirectly affect feed palatability and attractability (Tantikitti, 2014). Feeding fish with palatable and attractive diets is of vital importance to increase feed consumption thus reducing the accumulation of organic waste derived from undigested feed (Al-Souti et al., 2019). High levels of ANFs in feed can be problematic for the health and

Table 1
The two major categories of amino acids (AA) for fish (Murai, 1991).

Essential/indispensable amino acids	Non-essential/dispensable amino acids
Arginine	Aspartate
Histidine	Glutamate
Lysine	Alanine
Leucine	Tyrosine
Isoleucine	Serine
Methionine	Glutamine
Valine	Asparagine
Phenylalanine	Cysteine
Tryptophan	Proline
Threonine	Glycine

nutrition of fish. Impairment of health and nutritional status can be ascribed to reduced protein digestibility and availability resulting from high ANF concentrations (Gilani et al., 2012). To maximise feed utilisation and ensure profitable aquaculture production, the ANF content of the protein sources used in aquaculture feed must be kept at a minimum. Finally, a well-balanced AA profile of the protein source is required for optimal growth, feed utilisation and nitrogen retention of the cultured fish. Several previous studies have demonstrated that the AA composition, as well as the proportion of EAAs and NEAAs, have a significant impact on nitrogen retention and excretion. A study performed by Green et al. (2002a) showed that maximal N retention was achieved when the AA profile of the feed met the AA requirement of the target fish species. By contrast, N excretion increased as levels of dietary AA exceeded the AA requirement of target fish species (Green et al., 2002a). Several studies have investigated how the EAA/NEAA ratio of dietary protein influences protein utilisation efficiency in different fish species including Nile tilapia (*Oreochromis niloticus*) (Mambrini and Kaushik, 1994), rainbow trout (*Oncorhynchus mykiss*) (Green et al., 2002b) and European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles, 2006). Based on the results from these studies, the following generalisations can be made about the EAA/NEAA ratio:

1) N excretion decreases with increasing EAA/NEAA ratio.
2) N retention increases with increasing EAA/NEAA ratio until reaching a plateau value (>50:50) with maximum N retention and minimum N excretion.
3) Feed intake decreases when the EAA/NEAA ratio exceeds 50:50.

An EAA/NEAA ratio of 50:50 appears preferable for aquafeeds as feed intake and growth are maximised at this level. An EAA/NEAA ratio beyond 50:50 is often associated with higher N retention but a lower feed intake (Peres and Oliva-Teles, 2006). Thus, formulating feed with an EAA/NEAA ratio of 50:50 establishes an optimal balance between N retention and feed intake ensuring that N retention is maximised without compromising feed intake.

2.1.1. Fishmeal, plant-based meals and BSG

Current protein sources used in commercial aquafeeds can be divided into two main categories: 1) marine-based meals and 2) plant-based meals. Fishmeal, which is mainly obtained from fisheries production of small pelagic fish species unfavorable for human consumption, constitutes the most common marine-based protein source in aquafeed (Péron et al., 2010).

In 1994, the global production of fishmeal from non-targeted captures peaked at ~7.3 million tonnes (FAO, 2020). Since then, fishmeal production from wild fish stocks had followed a declining trend with minor annual fluctuations reaching a level of ~5.0 million tonnes in 2020 (EUMOFA, 2021). Historically, aquafeed production has relied on fishmeal as the sole protein source because of its excellent nutritional properties including high protein content, high palatability, a well-balanced AA profile and lack of ANFs; the latter two resulting in high protein digestibility (Oliva-Teles, 2012). Beyond that, fishmeal consumption entails low excretion of ammonia and undigested protein in faeces which can be explained by the favourable AA composition and the absence of ANFs, respectively. However, reliance on fishmeal as the sole protein source in aquafeed is not feasible for long-term aquaculture production as increasing demand for aquaculture commodities may cause overexploitation of the wild fish species used to produce fishmeal (Olsen and Hasan, 2012). During the last decades, the rise in fishmeal prices, due to declining catches and increased market demand, has strengthened efforts to transition to plant-based meals in aquafeeds (Jannathulla et al., 2019). Plant-based meals are derived from a variety of different vegetables, predominantly soybean but also rapeseed, cottonseed, groundnut and sunflower (Tacon et al., 2011). Soy protein is available as either meal or protein concentrate, with soybean meal being the most widespread soy product in aquafeed production. Meal and concentrate are distinct in their content of protein, ANFs and AA profile. Soy protein concentrates are rich in proteins (>75%) with negligible amounts of ANFs, while soybean meals have a lower protein content (40–50%) and contain high levels of certain ANFs (Hardy, 2010). Furthermore, soy protein concentrates have an AA profile that resembles fishmeal (Dersjant-li, 2002) whereas soybean meals are deficient in specific AAs, particularly methionine and lysine (Table 2) (Alashi et al., 2013). As a result, soybean meals have lower nutritional value as compared to soy protein concentrates which have restricted their use as fishmeal replacement at high inclusion levels. Besides these nutritional constraints, the incorporation of soybean meal, as well as other plant-based protein sources, in aquafeed may harm the environment mainly due to intensified use of arable land for crop production (Naylor et al., 2021). Pelletier et al. (2018) studied the environmental impact of the production of soybean meal and concentrates for aquafeed in two different geographical regions (US and Brazil). This study found that the production of these soy products was generally associated with great land use and high greenhouse gas emission resulting from deforestation (Pelletier et al., 2018). In addition, the high phytic acid concentration of soybean poses nutritional and environmental constraints for its use in fish feed as phytic acid has been shown to reduce mineral utilisation and

Table 2
Content of crude protein (CP), essential amino acids (EAA) and non-essential amino acids (NEAA) in fishmeal, soybean meal and brewer's spent grain (BSG).

	Fishmeal[a]	Soybean meal[a]	BSG[b]
CP (% DM)	50–70	40–50	20–30
EAA (% DM)			
Tryptophan	1.16	1.30	0.03
Phenylalanine	4.33	5.00	1.03
Valine	5.41	4.80	1.02
Isoleucine	4.53	4.00	0.73
Leucine	7.79	7.80	1.35
Methionine	2.80	1.30	ND
Threonine	4.51	4.00	0.16
Lysine	8.60	6.40	3.17
Arginine	6.43	7.20	1.00
Histidine	3.49	2.60	5.81
NEAA (% DM)			
γ-amino butyric acid	ND	ND	0.06
Alanine	6.52	4.30	0.91
Asparagine	ND	ND	0.33
Aspartate	9.50	11.70	1.06
Glutamate	12.65	18.70	3.67
Glycine	6.89	4.20	0.39
Serine	3.68	5.10	0.83
Tyrosine	3.65	3.20	0.57
Cysteine	0.91	1.60	ND
Glutamine	ND	ND	0.02
Hydroxyproline	1.30	ND	ND
Proline	4.70	5.10	ND
Taurine	0.77	ND	ND
Phenylalanine	4.33	ND	ND
Hydroxylysine	0.29	ND	ND
Ornithine	0.11	ND	ND
EAA/NEAA	0.97	0.82	1.84

DM: dry matter content.
ND: not determined.
[a] EAA and NEAA contents are reported as percentages of fishmeal and soybean meal in the original work (Alashi et al., 2013; Gamboa-Delgado et al., 2017). The contents of individual amino acids in fishmeal were retrieved from Gamboa-Delgado et al. (2017), while the amino acid contents in soybean meal were extracted from Alashi et al. (2013).
[b] EAA and NEAA contents are reported as percentages of total protein content in the original work (Waters et al., 2012). To render values comparable to those reported for fishmeal and soybean meal, the amino acid contents were converted into a percentage of BSG using the following formula: $\frac{X_{AA} * X_{protein}}{100\%}$. X_{AA} is the amino acid content measured as a percentage of the total protein content ($X_{protein}$) which was estimated at 22% by Waters et al. (2012).

increase faecal phosphor excretion (Dalsgaard et al., 2009). The challenges imposed by the utilisation of fishmeal and soybean meal necessitate a search for novel, alternative protein sources with low environmental impact and high nutritional value. From a nutritional point of view, BSG and soybean meal have similar drawbacks: low palatability, high ANF content and deficiency in certain AAs (Table 2). Nonetheless, when comparing their AA profiles it can be argued that BSG provides a better protein source than soybean meal. This can be explained by the higher EAA/NEAA ratio for BSG (1.84) (Waters et al., 2012) compared to soybean meal (0.82) (Alashi et al., 2013) which may enhance N retention in fish and diminish N excretion to the surrounding aquatic environment. When considering the crude protein (CP) content, unprocessed BSG represents a less favourable protein source compared to soybean meal and fishmeal, because it has a significantly lower CP content (Lynch et al., 2016). In a sustainability context, BSG is expected to be superior to both soybean meal and fishmeal owing to its unfluctuating annual availability, vast production volumes and low market price (Mussatto et al., 2006). In terms of circular economy, BSG can be defined as a side stream which is a by-product generated unintentionally from an industrial process. Hence, BSG production does not require any additional energy or resource use other than that needed for beer production. To date, only a few studies have addressed the use of raw and partially refined BSG as a second-generation feedstuff in aquafeeds. Jayant et al. (2018) demonstrated that soybean meal could be substituted with up to 50% of unprocessed BSG without affecting growth and nutrient utilisation in striped catfish (*Pangasianodon hypophthalmus*). In a similar study, Nazzaro et al. (2021) evaluated the influence of partial fishmeal replacement with raw BSG on protein digestibility in rainbow trout and gilthead seabream (*Sparus aurata*). This study revealed that inclusion of 20% BSG did not adversely affect the protein digestibility in these two carnivorous fish species. He et al. (2020) investigated the effect of substituting fishmeal with a BSG-derived product on the feed utilisation and growth performance of Pacific white shrimp (*Litopenaeus vannamei)*. This product was produced by a protease-aided fractionation process as described in He et al. (2019) and had a high content of crude protein (46%). The most interesting finding of this study was that the BSG product could replace up to 50% of fishmeal without affecting the feed utilisation, although higher inclusion levels had a negative influence on the growth performance. Taken together, these studies suggest that the use of unprocessed BSG in aquafeed is feasible at low inclusion levels (20–50%). Due to the low nutritional value, unrefined BSG cannot be employed at high inclusion levels in aquafeed without impairing the growth and N-metabolism of the fish. Thus, BSG must undergo intensive valorisation in order to increase its nutritional value to a level that allows for a higher inclusion (60–90%) in aquafeeds.

2.1.2. Current pros and cons of utilising BSG in aquafeeds

As outlined above, the use of BSG as a protein source in aquafeeds has potential due to a relatively high protein content, a favourable amino acid profile, low market price and stable availability. Despite this, BSG has yet to be implemented in aquafeed formulation, because it has a high content of ANFs and indigestible components which reduces the nutritional value. Before BSG can be incorporated as a protein source in aquafeeds, it must undergo refinement which is an energy and time-consuming process. Several approaches have been considered to utilise BSG in its raw or semi-refined form, including biogas production, enrichment of construction materials such as bricks, substrate for microbial growth and enzyme production and a fibre source in food products (Jackowski et al., 2020). Furthermore, Assandri et al. (2021) reported that unprocessed BSG can be converted into an efficient composting agent by means of a simple procedure in which the composting properties of BSG are improved by addition of manure and a lignocellulosic bulking agent. From a holistic perspective, recycling BSG as a composting agent may lead to a more sustainable cultivation of crops that could potentially be employed in aquafeeds. While this certainly promotes the environmental sustainability in the production of plant-based ingredients for aquafeeds, utilising BSG as a composting agent will not overcome the nutritional challenges associated with plant-based protein sources, nor will it reduce the demand for arable land. The alternative applications of BSG discussed here represent more climate-smart and economic strategies for valorising BSG compared to the extensive refinement needed to enable high inclusion levels of BSG-derived protein in aquafeeds. The implementation of BSG and other protein-rich agro-industrial residues, may help reduce the consumption of fishmeal and soybean meal thereby contributing to a more sustainable aquaculture production which justifies efforts to valorise and implement BSG in aquafeed production.

3. Brewer's spent grain (BSG)

3.1. Production of BSG

Barley (*Hordeum vulgare*) is the core ingredient in the production of beer. Barley grain is composed of three main constituents: the germ (embryo), the endosperm comprising a matrix of starch and protein enclosed by a layer of aleurone cells, and the grain covering (Fig. 1a) (Willows et al., 2017). The grain covering is further divided into three distinct layers constituting the inner seed coat (testa), the middle pericarp and the outer protective husk layer which primarily consists of dead cells with a high content of lignocellulosic material (Fig. 1b) (Mosher and Trantham, 2017).

Beer brewing encompasses three key steps: malting, mashing and fermentation (Fig. 2). During malting, barley grains undergo germination resulting in a chemically modified endosperm with a high concentration of hydrolytic enzymes. The germinated barley (malt) is milled to separate the grain covering from the internal grain parts and to increase the accessibility of the endosperm macromolecules (Lewis and Young, 2001). The milled malt then undergoes mashing; a process in which endosperm starch and proteins are converted to fermentable sugars and AAs, respectively, by the action of enzymes produced during malting. This process generates a sweet liquid known as the wort which constitutes the fermentation medium for beer production. After supplementation with sugars and hops, the wort is filtered through a bed comprising the insoluble and non-degraded parts of malted barley which is deposited at the bottom of the mash tun. The remaining solid fraction obtained after filtration is BSG (Xiros and Christakopoulos, 2012).

3.2. Chemical composition of BSG

Brewer's spent grain is chiefly composed of the grain covering from the original barley seed in addition to a minor portion of endosperm fragments (Lynch et al., 2016). Contents of the main constituents in BSG are presented in Fig. 3.

3.2.1. Lignocellulose

BSG is a heterogeneous, lignocellulosic material comprising high amounts of lignin (~12–28% of dry matter) and non-starch polysaccharides (NSP) (~30–50% of dry matter) derived from the barley husk cell walls. In addition, traces of starch and β-glucans (~1%) derived from the endosperm are also present in BSG (Steiner et al., 2015). Lignin is a complex phenolic polymer synthesised by radical-induced polymerisation of three methoxylated benzene derivatives referred to as phenylpropanoid alcohols (p-coumaryl, coniferyl and sinapyl alcohol). During lignin synthesis, these alcohol monomers are coupled via various types of C–C and ether linkages leading to the formation of a diverse and recalcitrant aromatic network (Zhu et al., 2013). The NSP component consists of cellulose and hemicelluloses in approximately equal amounts, with arabinoxylans (AX) being the most abundant hemicellulose. Arabinoxylans are amorphous, non-cellulosic polysaccharides that function as reservoirs of phenolic acids, primarily ferulic acid, which has antifungal properties (Asiegbu et al., 1996).

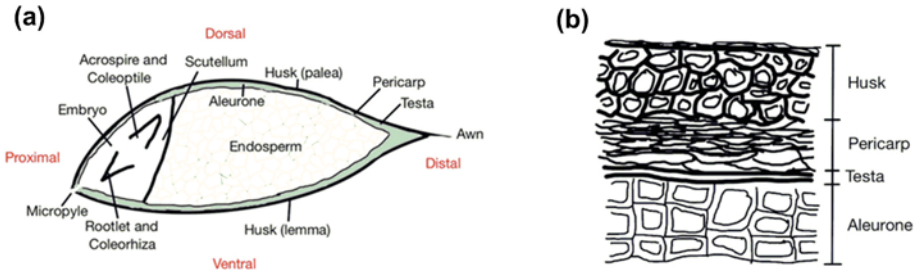


Fig. 1. (a) Longitudinal cross-section of a barley grain showing its primary components (germ, endosperm and grain covering) and (b) structure of the grain covering. Figures are adapted from (Mosher and Trantham, 2017).

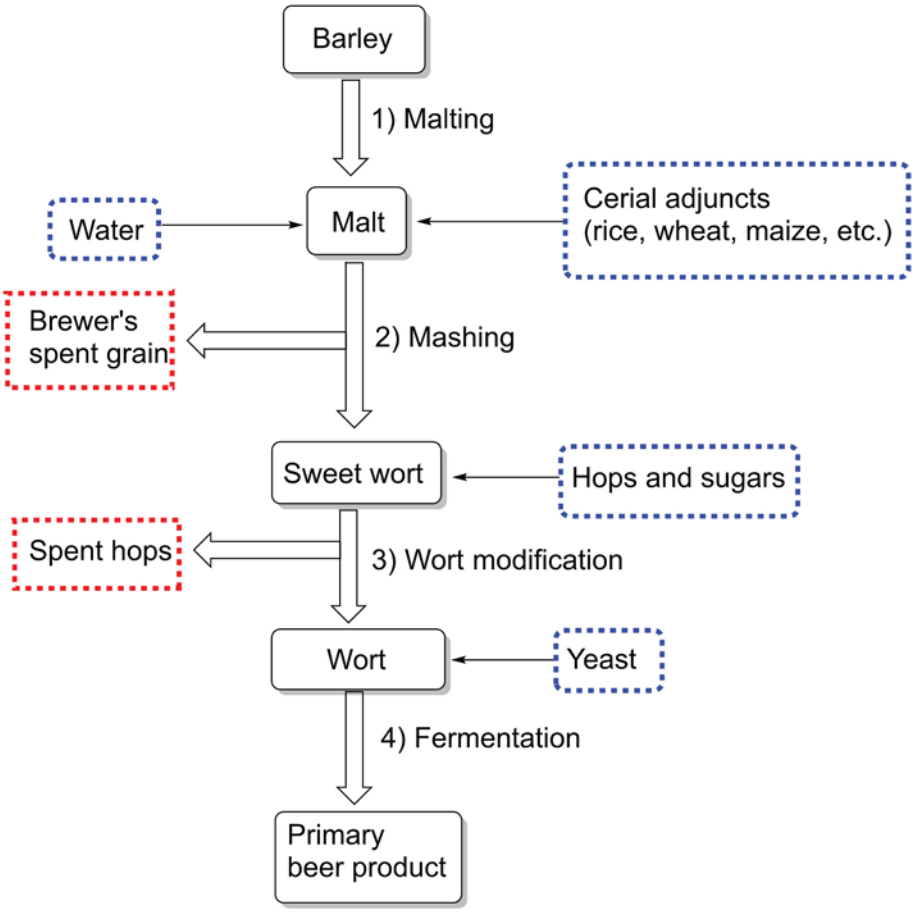


Fig. 2. Schematic representation of key steps in the beer-brewing process. 1) Malting: production of hydrolytic enzymes. 2) Mashing: production of the sweet wort by hydrolysis of endosperm starch and proteins. 3) Wort modification: boiling and clarifying of the sweet wort after addition of hops and sugars. 4) Fermentation: the transformation of modified wort into a primary beer product that requires further processing to obtain the final beer product (not shown). Generated by-products and supplementary ingredients are highlighted in red and blue, respectively.

Structurally, AX consists of a xylose backbone mainly substituted with arabinose at O-2, O-3, or both (Mendis and Simsek, 2014). Some of the arabinose residues may undergo esterification with ferulic acid at O-5 of arabinose which may lead to the formation of diferulic acid cross-linkages between adjacent AX strands under oxidative conditions (Fig. 4) (Mathew and Abraham, 2004). Besides arabinose, the xylose backbone can also be linked to other substituents including uronic acid, methylated uronic acid and acetyl groups (Coelho et al., 2016). The three lignocellulosic components are arranged in such a manner that cellulose forms hydrogen bonds with hemicellulose which in turn is attached to non-core lignin through ether and ester linkages as illustrated in Fig. 4 (Mandalari et al., 2005).

3.2.2. *Protein*

In addition to lignocellulose, protein is the second most predominant constituent of BSG accounting for up to 30% of the dry matter content (Lynch et al., 2016). Cereal proteins, such as those found in BSG, have been divided into four categories based on their solubility in different solvents: 1) albumins (water-soluble), 2) globulins (salt-soluble), 3) prolamins (alcohol-soluble) and 4) glutelins (acid/alkaline soluble) (Byers et al., 1983). The majority of proteins found in BSG are hordeins which are barley prolamins (>50% of total protein) (Vieira et al., 2014). Glutelins represent the second most abundant protein type in BSG while albumins and globulins constitute a smaller fraction of the total amount of proteins in BSG (~2%) (Celus et al., 2006). The proteins of BSG are classified as high-quality proteins because they contain high levels of EAAs. The 10 AAs which are essential to fish account for ~65% of the total protein content of BSG (Table 2). Lysine, which is derived from barley hordeins, constitutes the most abundant EAA followed by leucine, phenylalanine and isoleucine. Other minor constituents present in BSG comprise lipids (~7–10%), minerals (~1%), simple sugars including glucose, fructose and sucrose (<15%), and traces of vitamins (Waters

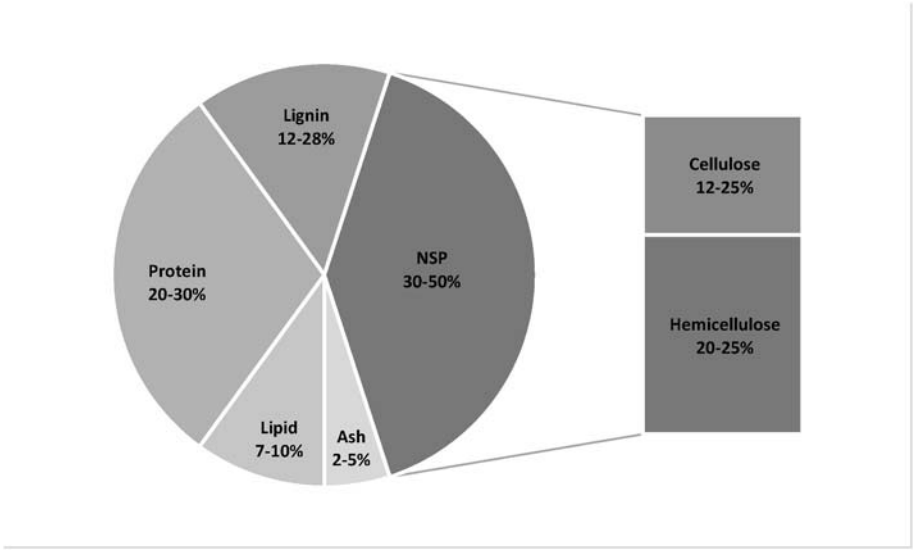


Fig. 3. *Chemical composition of brewer's spent grain (BSG). NSP: non-starch polysaccharides. Percentages of all constituents are reported as ranges because the composition of BSG is known to vary greatly with barley variety, brewing regime and time of harvesting among others as shown by* Santos et al. (2003). *Figures are adapted from* (Lynch et al., 2016).

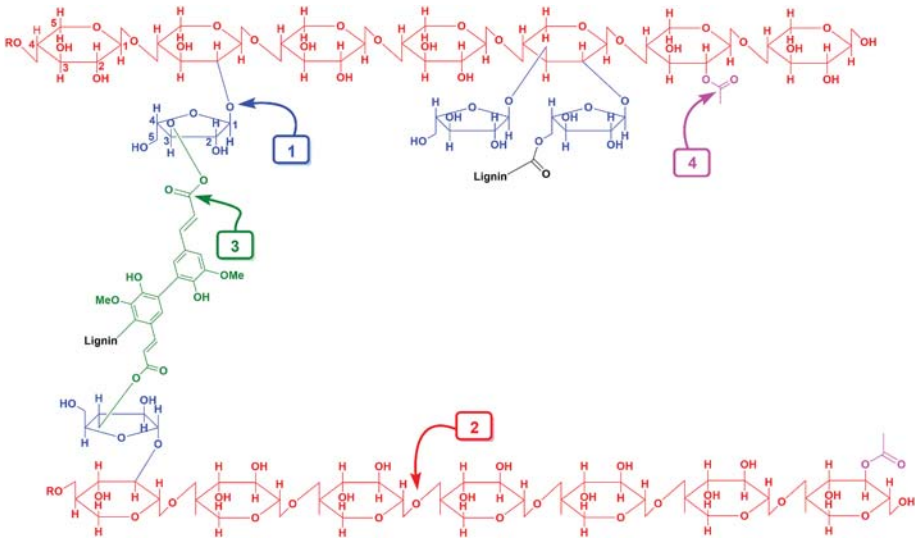


Fig. 4. Generalised structure of arabinoxylans (AX) showing two cross-linked AX strands and two possible sites of lignin coupling. Arabinose (blue) and acetyl groups (pink) are linked to the xylose backbone (red) through ether and ester bonds, respectively. AX strands are cross-linked by C–C coupling between two ferulic acid moieties (green). Enzymatic activities involved in AX hydrolysis are indicated with arrows and numbered as follows: 1; α-L-arabinofuranosidase, 2; endo-1,4-xylanase, 3; ferulic acid esterase and 4; acetyl xylan esterase.

et al., 2012).

4. Prospects of BSG utilisation in aquaculture feed

4.1. Benefits of applying BSG in aquaculture feed

The use of BSG as a protein source in aquaculture feed is expected to be beneficial in several respects. The relatively high content of protein and EAA makes BSG a potential alternative for the conventional meals used in aquafeed formulation which may reduce the reliance on fishmeal and increase the availability of arable land (Salin et al., 2018). As a result, the implementation of BSG as a protein source represents a more environmentally sustainable alternative to current protein sources. Owing to its high annual abundance and low prices, the application of BSG is also expected to be more economically feasible in comparison with conventional protein sources (Mussatto, 2014). Currently, BSG is sold cheaply as feed supplements for livestock, discarded as waste, or incinerated for energy production; the latter two constituting an environmental burden (Johnson et al., 2010). However, employing BSG in animal feed is believed to alleviate the environmental impact resulting from BSG incineration and disposal presuming that appropriate methods are chosen for the storage and processing of BSG (Petit et al., 2020).

4.2. Limitations of applying BSG in aquaculture feed

At present, there are various challenges associated with the utilisation of unrefined BSG in aquafeeds including: 1) a high content of ANFs, 2) a low protein concentration and a deficiency in certain AAs and 3) the choice of adequate preservation methods (Johnson et al., 2010). In the following section, key aspects of these complicating factors will be described in detail. Additionally, we will discuss the potential for using different refinement methods for increasing the nutritional value of BSG to a level that allows for its implementation in aquaculture feed. The practicability of a specific refinement method is highly dependent on its economic performance and environmental implications. At present, the economic costs and environmental impact of the various refinement methods considered in the next chapter are not fully understood and will need further investigation in future research employing

techno-economic analysis and life-cycle assessment. What we know about the economic aspects of BSG valorisation is chiefly based on the work of He et al. (2021) who performed a techno-economic analysis of the production of BSG protein concentrates through wet fractionation. The study found that the production cost of BSG protein concentrates (1043 USD/t) was lower than the average fishmeal price (1449 USD/t) which implies that BSG-derived products may be economically superior to fishmeal in aquafeeds provided that low-cost and time-efficient methods are used to produce them.

4.2.1. Anti-nutritional factors

Anti-nutritional factors encompass a variety of different compounds including lectins, phytic acid, alkaloids, protease inhibitors, tannins and structural components of plant cell walls (NSP and lignin) (Glencross et al., 2020). In unrefined BSG, lignin, cellulose and hemicellulose comprise the main ANFs while tannins and phytic acid account for a minor portion of the ANF content. Comparison of the ANF content in soybean meal and BSG indicates that BSG has higher concentrations of lignin and fibre, whereas the tannin content is considerably higher in soybean meal (Table 3) (Martins et al., 2017). Additionally, BSG and soybean meal contain similar amounts of phytic acid and therefore they may have similar drawbacks concerning the excretion of phosphor (Çantaş and Yildirim, 2020). This review, however, will solely focus on problems related to the anti-nutritional effects of lignocellulose in BSG and how these can be addressed by appropriate refinement strategies.

Previous studies have established that ANFs reduce protein digestibility and bioavailability via different mechanisms depending on the type of ANF in question. For most ANFs, protein digestibility is reduced either by inhibition of digestive enzymes or by chelation with dietary proteins (Gilani et al., 2012). Furthermore, some ANFs may also act as feed deterrents due to their bitter taste resulting in rejection of feed items (Glencross et al., 2020). In a comparative study, Waters et al. (2012) estimated the quantity of phenolic compounds in BSG which include ferulic acid and hydroxycinnamic acids derived from AX and lignin, respectively (Guido and Moreira, 2017). They concluded that BSG contains higher levels of phenolic compounds (131 mg/L) compared to malt (112 mg/L) and barley (90 mg/L) which implies a gradual build-up of lignin and AX during the brewing process. Most literature descriptions have emphasised the discrete effects of various lignocellulosic constituents on protein digestibility of diets fed to different fish species with rainbow trout being the most studied. From these, it can be inferred that the distinct components of lignocellulose have varying impacts on protein digestibility and that their magnitude of impact is commensurate with inclusion levels (Glencross et al., 2012). Cellulose had only a marginal impact on protein digestibility whilst lignin adversely affected this parameter in several species including rainbow trout (Glencross et al., 2012), Nile tilapia (Amirkolaie et al., 2005) and barramundi (*Lates calcarifer*) (Irvin et al., 2016). Conversely, Hansen and Storebakken (2007) found that cellulose inclusion reduced energy and dry matter digestibility although it improved pellet texture in terms of increased durability and hardness. For these reasons, cellulose may prove useful as a filling agent in feed production if applied in quantities that do not negatively affect protein digestion. Furthermore, interactive effects of different lignocellulosic components on protein digestibility have been demonstrated revealing negative synergistic interactions between lignin and certain polysaccharides included in the matrix of lignocellulose (Irvin et al., 2016). From a nutritional point of view, the high content of lignocellulosic material in BSG precludes its utilisation in aquafeed in an unrefined form. As a result, BSG must undergo considerable refinement before it can be considered a suitable protein source for fish. The following section outlines different methods for valorising BSG.

Table 3
Content of anti-nutritional factors (ANFs) in unrefined brewer's spent grain (BSG) and soybean meal.

ANF	Soybean meal	BSG	References
Lignin[a] (% DM)	0.4	5.4[d]	(Heuzé et al., 2017; Heuzé et al., 2020)
Cellulose[b] (% DM)	5.3	16.5[d]	(Heuzé et al., 2017; Heuzé et al., 2020)
Hemicellulose[c] (% DM)	4.8	34.4[d]	(Heuzé et al., 2017; Heuzé et al., 2020)
Phytic acid (% DM)	1.00	1.01[e]	(Ktenioudaki et al., 2015; Martins et al., 2017)
Tannin (mg/g DM)	8.22	1.4 [d]	(Heuzé et al., 2017; Martins et al., 2017)

DM: dry matter content.
[a] Lignin content is measured as acid detergent lignin (ADL) and therefore it does not include acid-soluble lignin.
[b] Cellulose content is calculated from the acid detergent fibre (ADF) content, which is the sum of ADL and hemicellulose, using the formula: cellulose = ADF – ADL.
[c] Hemicellulose content is calculated from the neutral detergent fibre (NDF) and the ADF content by the formula: hemicellulose = NDF – ADF, where NDF includes ADL, cellulose and hemicellulose.
[d] The reported values are based on dehydrated, unground BSG.
[e] The phytic acid content is based on milled BSG flour.

4.2.1.1. Refinement of BSG. An extensive body of literature has described different approaches for addressing the nutritional issues associated with high levels of fibre and lignin in plant-derived protein sources such as those found in BSG. These approaches can be clustered into two main refinement categories: 1) conversion of lignocellulose to digestible components using biological, chemical and enzymatic treatments (Fig. 5), and 2) chemical fractionation (Fig. 6). The overarching goal of both strategies is to produce a protein-rich concentrate containing negligible amounts of fibre and lignin.

4.2.1.1.1. Conversion of lignocellulose

4.2.1.1.1.1. Hydrolysis of cellulose and hemicellulose

Prior studies have mainly focused on the depolymerisation of cellulose and hemicellulose into their respective monosaccharides which consist of glucose, xylose and arabinose using chemical, hydrothermal and enzymatic pretreatment of BSG. As described by D'Este et al. (2018), monosaccharides can serve as precursors for fermentative AA production in the presence of an amino-group donor such as glutamate. Therefore, the application of native or engineered microorganisms, specialised in producing specific AAs by fermentation, can be considered a useful method for increasing certain AAs to a level that matches the nutritional requirement of the fish species concerned. Chemical pretreatment of BSG fibre involves strong alkaline or acidic hydrolysis of cellulose and hemicellulose. Such harsh conditions are likely to induce the production of assorted sugar decomposition products such as furfural, 5-hydroxymethylfurfural (HMF) and acetic acid (Cardenas--Toro et al., 2015). These by-products have proven to be deleterious for most fermenting bacteria and fungi used in the downstream processing of pretreated lignocellulosic material and their production must therefore be minimised. For instance, a study performed by Martín and Jönsson (2003) concluded that the ethanol production of *Saccharomyces* and *Zygosaccharomyces* was inhibited by furfural and HMF. In the same way, Dessie et al. (2019) showed that high concentrations of furfural and HMF had an inhibitory effect on the production of succinic acid production by *Actinobacillus succinogenes*. In addition to the formation of inhibitory by-products, utilising strong alkaline or acidic solution to depolymerise the cellulose and hemicellulose content in BSG may also hydrolyse proteins and other constituents leading to complete deterioration of the material. Some studies have suggested milder hydrolysis conditions for minimising the production of inhibitory by-products. Carvalheiro et al. (2004) investigated the depolymerisation of the NSP component present in BSG and found that the formation of sugar decomposition products could be reduced by application of

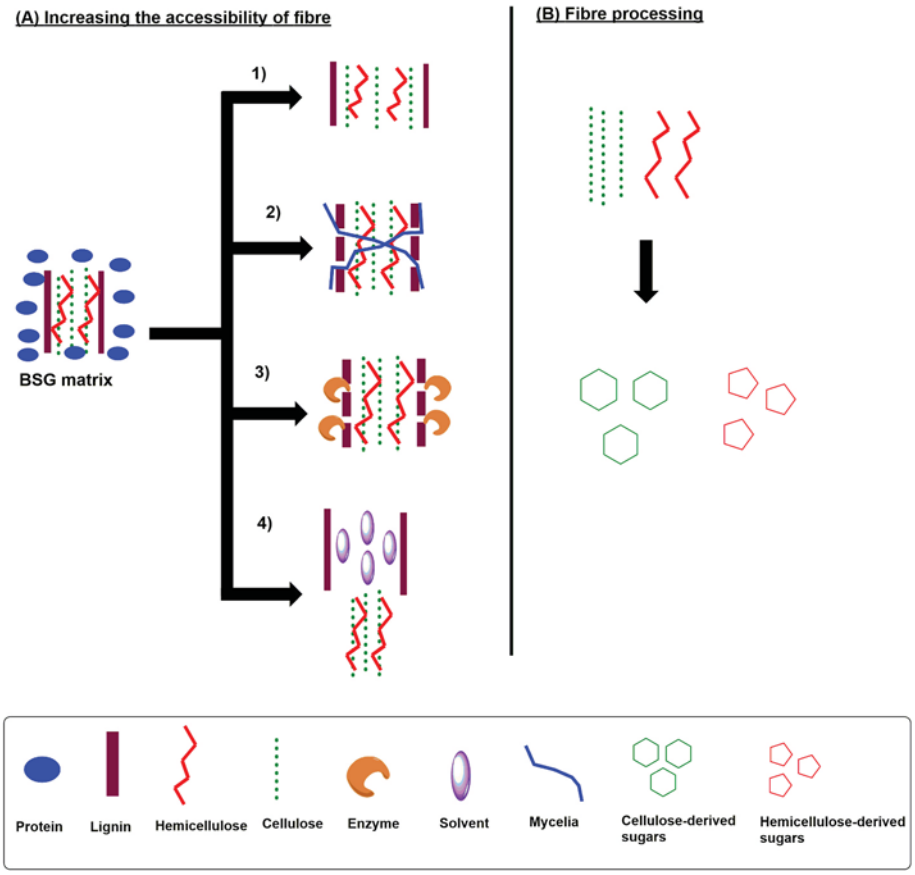


Fig. 5. Conversion of lignocellulose in two steps. (A) Increasing fibre accessibility by 1) disruption of intermolecular forces between cellulose, hemicellulose and lignin, 2) fungal lignin degradation, 3) enzymatic lignin degradation using enzymes excreted during the cultivation of lignin-degrading fungi and 4) lignin extraction with a suitable solvent system. (B) Fibre processing by: 1) production of fermentable monosaccharides by hydrolysis of cellulose and hemicellulose and 2) production of specific amino acids by fermentation of monosaccharides. For the sake of simplicity, proteins are not shown in the downstream processing of lignocellulose and all chemical structures are displayed as pictograms.

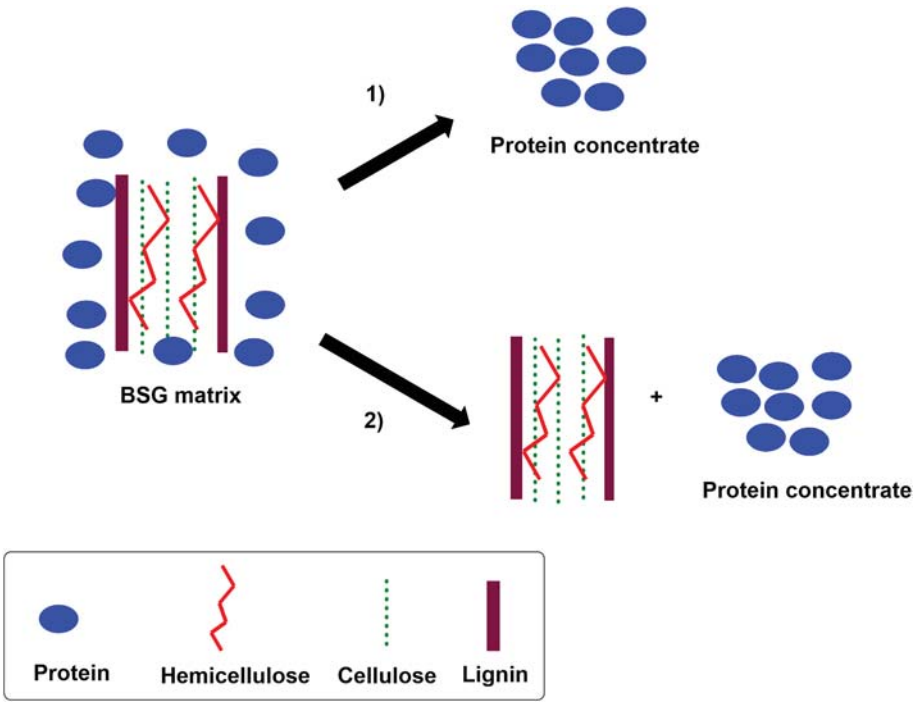


Fig. 6. Chemical fractionation. Protein concentrates can be obtained from brewer's spent grain (BSG) by two different approaches: 1) direct protein extraction using a repertoire of different solvent systems and 2) extraction of fibre and lignin from BSG.

autohydrolysis; a hydrothermal pretreatment in which water is used as the only reagent under elevated temperatures. They found that monosaccharide production was enhanced with prolonged reaction time and increased temperature. At the same time, optimum conditions for monosaccharide production increased the relative content of lignin and by-products. For instance, the lignin content increased from ~29 g/100

g solid to ~58 g/100 g solid after 60 min of incubation at 170 °C. Correspondingly, the content of acetic acid, formic acid, furfural and HMF was increased by 250%, 480%, 490% and 480%, respectively (Carvalheiro et al., 2004). As a result, the findings of this study imply that autohydrolysis is not a feasible method for the complete hydrolysis of cellulose and hemicellulose. The issue of by-product formation encountered by chemical and, to some extent, hydrothermal pretreatment, may be avoided by using an enzymatic approach. In a study performed by Forssell et al. (2008), the solubilisation of BSG fibre was examined with the use of different commercial enzyme mixtures of carbohydrate degrading enzymes. One interesting finding of this study was the incomplete hydrolysis of AX which can be ascribed to the lack of key enzymes involved in the cleavage of the various bonds present in AX. Due to the high structural complexity of AX, complete hydrolysis requires the simultaneous action of several enzymes. On large-scale production, applying multiple enzymes for a single process is infeasible from both an economic and a practical perspective. This issue can be addressed by employing fungal species as multi-enzyme factories. Such systems have been extensively studied using *Fusarium oxysporum* and *Neurospora crassa* as model organisms (Xiros et al., 2008a, 2008b). Application of the crude enzyme extract produced by these species has proven successful in hydrolysing AX from BSG completely. Finally, and perhaps one of the most interesting results from the study conducted by Forssell et al. (2008) was the low hydrolysis efficiency observed for all tested enzyme mixtures. This may be attributable to the restricted enzyme accessibility of cellulose and hemicellulose stemming from the closely interlinked network of the three lignocellulosic components as described by Mussatto et al. (2008). To render the fibre more accessible for enzymatic action and thus facilitate complete depolymerisation, lignin must be removed before fibre hydrolysis. A description of the different delignification strategies will be given in the following section.

4.2.1.1.1.2. Delignification

Generally, two different strategies can be applied to delignify lignocellulosic biomasses—a biological and a chemical approach. The overriding advantage of the biological approach is that it diminishes the formation of by-products. Moreover, biological delignification is believed to be more sustainable in terms of energy consumption and environmental impact because it occurs under milder conditions compared to chemical delignification (Christopher et al., 2014). A compilation of literature has explored various solvent systems suitable for lignin extraction from lignocellulosic biomasses including ionic liquids (ILs), alkaline solutions, ethanol, sodium chlorite and an acetone/water mixture (Denilson, 2010; Melro et al., 2020; Pereira et al., 2016; Prado et al., 2013; Siqueira et al., 2013). However, the majority of presently used extraction solvents are neither environmentally friendly nor safe to handle due to their corrosive and volatile properties. Further, application of these solvent systems is often associated with co-extraction and disintegration of proteins, hemicellulose and cellulose thus preventing further processing of these macromolecules after lignin removal (Prado et al., 2013). Ionic liquids are defined as ionic compounds, composed of organic cations and inorganic/organic counter anions, which exist as dissociated ions at temperatures below the boiling point of water (Singh, 2019). These solvents present novel systems for extracting biodegradable polymers from biomass as they possess several favourable characteristics including low volatility, high thermal stability, safe handling and low environmental impact. Furthermore, ILs have proven to be excellent solvents for lignin extraction because they preferentially dissolve the lignin fraction of lignocellulose. This was validated in a study by Prado et al. (2013) who made a comparative analysis of ethanol, alkaline and IL extraction of lignin from apple tree prunings. Subsequent analysis of the three resulting lignin extracts showed that the greatest purity was achieved when employing IL extraction. In contrast, the lignin extracts obtained from ethanol and alkaline extraction contained high amounts of impurities stemming from co-extraction of other macromolecules in the material (Prado et al., 2013). Studies on the mechanistic aspects of lignin dissolution in ILs suggest that lignin is dissolved due to hydrogen bonding between the hydroxyl groups of lignin and the anion of ILs, which disrupts the network of hydrogen bonds between lignin molecules (Ji et al., 2012). In addition, the study undertaken by Liu et al. (2017) revealed that treatment with deep eutectic solvents (DES), which are a class of ILs comprised of Brønsted acids (proton donors) and bases (proton acceptors), promotes separation of lignin and hemicellulose by disrupting the hydrogen bonds between the two. Despite the evident benefits of using ILs for lignin removal, these solvents are currently not applicable on an industrial scale due to their high production costs. Alternatively, fibre accessibility can be increased by using chemical methods that break the intermolecular forces between the three lignocellulosic components thus separating them from each other. A study by Mussatto et al. (2008) demonstrated that cellulose of BSG could be disentangled from hemicellulose and lignin by a sequence of dilute acid and alkali treatments. This approach, however, does not remove lignin from the BSG matrix and therefore it cannot be considered a suitable refinement method in the context of feed production from plant-based materials. Biological delignification relies on the use of lignin-degrading microbes that possess inducible enzymatic machinery adapted for complete or partial lignin deconstruction of biomasses. Lignin decomposers are classified into two groups comprising fungi and bacteria with fungi generally being more efficient in lignin degradation than bacteria (Janusz et al., 2017). White-rot fungi are believed to be the most efficient lignin degraders as they are capable of oxidising lignin completely to CO_2 and water (Sahadevan et al., 2016). The white-rot fungi *Phanerochaete chrysosporium* constitutes the most well-studied lignin-degrader. Previous studies have unraveled the effect of culture conditions on the growth and enzymatic activity of the ligninolytic enzymes produced by this fungal species. Based on the results from these studies, it has been established that the ligninolytic activity is maximised under high oxygen tension and nitrogen-limited conditions (Kirk et al., 1978). In addition, *P. chrysosporium* grows optimally at a broad range of pH values and at temperatures as high as 40 °C (Richard et al., 1987). In the context of valorising the BSG fibre, *P. chrysosporium*, as well as other white-rot fungi, represent potential candidates for BSG delignification because they selectively degrade lignin and leave the fibre untouched (Wong, 2009). Although white-rot fungi hold great potential for delignification of BSG, their use may be obstructed by the occurrence of antifungal agents such as ferulic acid bound to AX of BSG (Fig. 4). Asiegbu et al. (1996) examined the effect of ferulic acid on the biomass-degrading capacity of several white-rot fungi. An interesting finding from this study was that the growth of *P. chrysosporium* was significantly depressed by ferulic acid, even at low concentrations (0.5 g ferulic acid/L growth medium). Another study performed by Huang et al. (1993) revealed that *Saccharomyces cerevisiae* efficiently transformed ferulic acid into 4-hydroxy-3-methoxystyrene via a decarboxylation pathway. Using yeast-induced transformation of ferulic acid may thus be a useful strategy for eliminating the antifungal effect of ferulic acid ultimately enhancing the efficacy of fungal delignification of BSG. However, to the best of our knowledge, no previous studies have examined how the conversion of ferulic acid affects fungal delignification efficiency. Therefore, further research is required to evaluate the viability of using ferulic acid transformation as a pretreatment method before BSG delignification with white-rot fungi. Another major disadvantage of utilising white-rot fungi in the delignification of BSG is the accumulation of chitin which is the main structural component of fungal cell walls. The presence of fungal-derived chitin in BSG may be an impediment to its application in aquafeeds since high chitin levels have been shown to be correlated with reduced protein digestibility in some fish species (Karlsen et al., 2017). Accumulation of chitin could be prevented by adopting an enzymatic approach for degrading the lignin in BSG. A number of studies have investigated the enzymatic delignification of different lignocellulosic biomass including BSG. For instance, Fan et al. (2019) identified the optimum conditions for small-scale delignification of corn stover catalysed by lignin peroxidase produced by the fungus *Aspergillus oryzae*. Likewise, Giacobbe

et al. (2019) showed that laccase-pretreatment caused efficient delignification of BSG. Although these results are promising, more research is needed in order to develop enzymatic systems that are economically viable for large-scale production.

4.2.1.1.2. Chemical fractionation. The term chemical fractionation describes the process in which a heterogeneous mixture is separated into its different components with the aid of chemical extractions. In general, a fractionation process is characterised by three distinct stages including 1) pretreatment, 2) extraction and 3) purification. The pretreatment involves mechanical processing of the material and serves to increase the accessibility of target compounds, such as proteins, to extraction solvents. During extraction, the target compound is separated from other matrix components by solubilisation in an extraction solvent. For heterogeneous biomasses, such as BSG, simultaneous extraction of other matrix compounds is often unavoidable as these compounds may be soluble in the applied extraction solvent. As a result, the crude extract may contain a mixture of the target compound and various co-extracted compounds. To isolate the target compound from the crude extract, a purification step is required. The most common purification modes used for the isolation of target compounds are precipitation and filtration (Galanakis, 2015). The protein fraction of BSG may be obtained by two distinct fractionation procedures: 1) extraction of lignin and fibre or 2) direct extraction of protein (Fig. 6). Lignin extraction can be accomplished by deploying the solvent systems mentioned in the section "4.2.1.1.1.2 Delignification". Likewise, a variety of procedures for carbohydrate extraction of BSG have been described including sequential alkaline extraction, pressurised hot water extraction, microwave-assisted extraction and hydrothermal treatment (Mandalari et al., 2005). However, the majority of these methods are used to selectively extract AX and are therefore less suitable for extracting other carbohydrates present in the biomass.

4.2.1.1.2.1. Protein extraction

Over the last decades, protein extraction has been extensively investigated for miscellaneous plant-derived biomasses inclusive of BSG. Several methods have been deployed for extracting BSG proteins where alkaline extraction, sequential (Osborne) extraction and ultrafiltration have received the greatest attention (Wen et al., 2019). Table 4 summarises the various protein extraction methods discussed in this section and provides an overview of solvents, protein yields and the extend of ANFs co-extraction for each extraction method. Alkaline extraction, which represents the most common method for protein extraction of plant-based biomass, is based on the high solubility of plant proteins in alkaline solution. Following extraction, solubilised proteins are precipitated by isoelectric precipitation (IP) in which the pH value is lowered to the isoelectric point of the proteins. This process takes advantage of the fact that most plant proteins are highly insoluble at their isoelectric point (Lam et al., 2018). A potential pitfall in alkaline protein extraction of lignocellulosic biomasses is the occurrence of co-extraction of lignin because lignin is partially soluble at high alkalinities (Melro et al., 2020). Nevertheless, deploying IP for protein purification may help prevent lignin contamination as simultaneously extracted lignins and proteins are separated in this process. The efficacy of alkaline protein extraction is influenced by multiple factors including pH value of the aqueous solution, temperature, time, solid to liquid ratio (S:L) and the lipid content of the biomass being extracted (Vei de Vilg and Undeland, 2017). Alkaline protein extraction has several benefits in the form of excellent extraction efficiency, low economic costs and ease of operation (Wen et al., 2019). Notwithstanding, alkaline protein extraction has its limitations. Firstly, the formation of non-endogenous ANFs is promoted by the harsh conditions used in alkaline protein extraction. Some of these ANFs include oxidised sulfur-containing AAs which result from oxidation of thiol (-SH) groups. Under alkaline conditions, extracted L-amino acids or L-peptides may be converted into their D-isomers by racemisation. In the literature, D-amino acids have been defined as non-endogenous ANFs although this definition is problematic because most organisms can absorb and utilise D-amino in absence of L-amino

Table 4
Summary of protein extractions from brewer's spent grain (BSG) reported in the literature.

Extraction method	Conditions	Protein yield	ANFs content[a]	Reference
Extraction using protease treatment	Alcalase: 7.415 U/g BSG, $(NH_4)_2CO_3$ buffer (pH 8.0), S:L = 4.0% w/v, 24 h, 60 °C.	~52%	Not reported	Qin et al. (2018)
Protease treatment of carbohydrolase-pretreated BSG	Shearzyme and Ultraflo: 75 μL/g BSG, demineralised water (pH 5.0), S:L = 1:10, 4 h, 50 °C.	~63%	Not reported	Connolly et al. (2019)
	Alcalase and Flavourzyme, demineralised water, 4 h, 50 °C.			
Water-alkaline-acid sequential extraction	*Water extraction* MiliQ water, S:L = 1:20, 25 °C, 1.5 h. *Alkaline extraction* 110 mM NaOH, S:L = 1:20 w/v, 50 °C, 1 h. Acid extraction 1 M H_2SO_4, S:L = 1:20, 25 °C, 1 h	*Water extraction* 43% *Alkaline extraction* 37% *Acid extraction* 14% Total 94%	Total lignin: ~65% Total cellulose: ~65% Total hemicellulose: ~65%	Qin et al. (2018)
Acidic extraction	40 mg H_2SO_4/g solution, S:L = 1:285, 25 °C, 1 h.	90%	Lignin: ~18% Cellulose + hemicellulose: ~68%	Qin et al. (2018)
Hydrothermal extraction	MiliQ water, S:L = 4.0%, 60 °C, 24 h.	66%	Lignin: ~16–17% Cellulose + hemicellulose: ~20%	Qin et al. (2018)
Extraction with acid detergent (AD) solution	2 g BSG/100 mL AD solution, refluxing, 1 h.	90%	Not reported	Crowe et al. (1985)
Alkaline extraction of cellulase-pretreated BSG	2 g BSG/50 mL citrate buffer (pH 4.9), 3 mg cellulase, 37 °C, 24 h. ~2 g BSG/100 mL NaOH solution (pH 10.5), rt, 2 h.	41%	Not reported	Crowe et al. (1985)
Modified Osborne extraction	25 g BSG/100 mL solvent, 10 min, 25 °C. Solvents: 1) distilled water, 2) NaCl solution, 70% ethanol solution, 3) acetic acid solution and 4) NaOH solution.	12%	Not reported	Crowe et al. (1985)
Ultrasound assisted extraction and protein isolation by ultrafiltration	Ultrasound: Na_2CO_3 buffer (pH 10), S:L = 1:10, for 1 h. Ultrafiltration: membranes: 5 and 30 kDa, 25 °C.	20%	Not reported	Tang et al. (2009)

[a] Anti-nutritional factors (ANFs) include lignin, cellulose and hemicellulose. The ANFs content is calculated as the percentage of extracted ANFs relative to the total ANF amount in untreated BSG and used to measure the purity of protein concentrates. The solid to liquid ratio is denoted as S:L.

acids (Gilani et al., 2012). Yet, the metabolic conversion of D-amino acids may sometimes be too slow to meet the nutritional requirements of the organism and in this case D-amino acids can be classified as non-endogenous ANFs (Friedman and Levin, 2012). Maillard reaction products (MRPs), which are formed in a reaction between a reducing sugar and an AA at high temperatures, comprise another group of non-endogenous ANFs (Gilani et al., 2012). Secondly, alkaline protein extraction has been reported to induce structural and functional changes to isolated proteins thereby affecting their nutritive value. Connolly et al. (2013) found that extraction temperature had a significant impact on AA composition as well as the protein yield. The loss of specific AAs at high temperatures may be explained by their consumption in the Maillard reaction. Furthermore, a positive correlation between extraction temperature and protein yield was reported by Connolly et al. (2013). These observations were consistent with the findings from a study by Ervin et al. (1989) which implied a sizeable loss of glutamate and proline when the extraction temperature was increased from 75 to 100 °C. Notably, this study also revealed an enrichment of several EAA including lysine, leucine and valine, among others, resulting from the temperature increase (Ervin et al., 1989). However, the use of high extraction temperatures is energy-intensive and may therefore not be considered a viable method for large-scale extraction of proteins with a specific AA composition. In addition to this, alkaline extraction is not considered an eco-friendly method as it requires large volumes of solvent. Sequential protein extraction, also referred to as Osborne extraction, is a well-established method for extracting cereal proteins. This extraction method relies on differences in solubility between the four different protein classes found in cereal grains. These protein classes are obtained in separate fractions using a series of different solvent systems (Byers et al., 1983). Celus et al. (2006) deployed a modified Osborne extraction method to identify the different protein types in BSG and to demonstrate that alcohol-soluble hordeins are the most abundant protein class. A study undertaken by Byers et al. (1983) examined the effect of several parameters on the AA composition of protein fractions obtained from milled wheat by Osborne extraction. Results from this study indicated that the AA profile was markedly influenced by the applied extraction conditions including temperature, extraction solvent and the presence of a reducing agent such as mercaptoethanol. These findings suggest that deploying appropriate extraction conditions may help facilitate isolation of proteins with a specific AA composition (Byers et al., 1983). This may serve as a useful strategy for the selective production of protein concentrates with AA profiles that reflect the AA requirements of fish. Similar to alkaline extraction, Osborne extraction is energy consuming and requires large volumes of solvent. Protein recovery by ultrafiltration, which is based on membrane separation processes, offers several advantages over the above-mentioned extraction methods including reduced amounts of solvent, low energy consumption and high purity of protein concentrates (Tang et al., 2009). Tang et al. (2009) examined the applicability of ultrafiltration for obtaining proteins and observed protein retentions greater than 90%. Furthermore, they concluded that ultrafiltration had high efficiency in removing salts originating from the buffer solutions used to solubilise proteins from the BSG matrix. This method is not without drawbacks; the most critical being fouling of membrane surfaces caused by a build-up of a concentrated polarisation layer (Marshall et al., 1993). Recent advances in membrane technology have led to better fouling control and thus increased the efficiency of membrane processes. A systematic review by Mohammad et al. (2012) evaluated the applicability of various abatement strategies for membrane fouling. These include the use of chemically modified membranes, manipulation of operating parameters, such as the transmembrane pressure and temperature, application of ultrasound and vibrating membrane systems for disrupting the fouling layers, backwashing and chemical cleaning of membranes (Mohammad et al., 2012). Chemical modification of membranes is mainly achieved by incorporation of hydrophilic groups as increased hydrophilicity has proven to enhance the antifouling properties of membranes (Asatekin et al., 2007). Employing a combination of these mitigation approaches could potentially increase the economic feasibility of using ultrafiltration as a method for recovering proteins from BSG. It is generally known from the literature that isolated BSG proteins suffer from poor solubility in aqueous media which may impede their use in animal feed applications (Celus et al., 2007). The low water solubility of BSG proteins may be due to the formation of insoluble protein aggregates during mashing as indicated in the work of Celus et al. (2006). Yet, recent research has established that enzymatic hydrolysis of isolated BSG proteins can improve their solubility over a broad range of pH values. This can be explained by the reduction in molecular weight (MW) and the protein unfolding which result in increased exposure of hydrophilic AA residues to the aqueous medium (Celus et al., 2007). Most previous research has reported a relatively poor extractability of BSG proteins. A possible reason for this might be that proteins are tightly encapsulated in the lignocellulosic matrix of BSG. In an attempt to overcome this problem, previous studies have examined the benefits of various pretreatments on protein yield. Most of the previous research has focused on identifying the effect of carbohydrate solubilisation on protein extraction efficiency. For instance, Qin et al. (2018) sought to assess how protein extraction of BSG was effected by different pretreatments including alkaline, acidic, enzymatic, hydrothermal and a combination of those. This study demonstrated a positive relation between carbohydrate solubilisation by the distinct pretreatments and protein extraction yield. Strikingly, the hydrothermal pretreatment appeared to be the most promising in terms of high selectivity for proteins during extraction as the resulting protein concentrates were less contaminated with lignin and carbohydrate residues (Qin et al., 2018). The findings of this study are in agreement with the results from the study of Crowe et al. (1985) who found that protein yield was increased significantly by enzymatic hydrolysis of cellulose prior to extraction of BSG. Moreover, a study by Connolly et al. (2019) concluded that the protein yield increased when BSG proteins were converted into shorter peptides by protease pretreatment. Interestingly, this study found that extraction of the generated peptides did not require a strong alkaline solution and that the resulting peptide-containing isolates exhibited enhanced water solubility in comparison to isolates comprised of intact proteins.

4.2.2. *Quality of BSG proteins*

High protein quality is crucial for the successful implementation of agro-industrial residues in aquafeed production (Nunes et al., 2014). As stated previously, the AA content along with the EAA/NEAA ratio are major determinants used to evaluate the applicability of novel protein sources in animal feed. Ideally, the AA profile and the CP content of the feed must meet the protein and AA requirements of the target species to avoid excretion of excess nutrients and malnutrition. Former studies have documented a deficiency in specific EAA in plant-based products with lysine and methionine generally being the first limiting AAs (Nunes et al., 2014). Within the field of feed formulation, two different methodologies are used to fortify deficient AAs of the diet: 1) ingredient-based formulation and 2) nutrient-based formulation. In ingredient-based formulation, deficient AAs are raised to desirable levels by increasing the inclusion of selected raw materials. In nutrient-based feed formulation, fortification of deficient AAs is achieved by supplementation of crystalline amino acids (CAAs). Despite being more environmentally sound, this method also entails some shortcomings concerning the utilisation efficacy of supplemented CAAs. The nutritional consequences of CAAs supplementation in previous studies appear to be inconsistent (Nunes et al., 2014). Some studies have determined that protein synthesis and growth are depressed when protein-bound AAs are substituted with their CAAs counterparts (Choo et al., 1991; Zarate et al., 1999). A reasonable explanation of this could be that CAAs are readily absorbed from the gastrointestinal tract into the bloodstream from where they are transported quickly to different organ systems of the fish. This theory was supported by Rolland et al. (2015) who found that postprandial plasma concentrations of CAAs in rainbow

trouts peaked transiently after a single meal with a CAA-supplemented diet. Thus, the cellular uptake of protein-bound AAs will be delayed relative to that of CAAs resulting in excess CAAs in the cells; a problem that can be overcome by continuous feeding. In the absence of AAs derived from proteins, CAAs tend to be used as an energy source, which may impede normal protein synthesis in the cells (Zarate et al., 1999). Other studies, however, have found similar or even better utilisation efficiencies of CAAs when compared to protein-bound AAs (Peres and Oliva-Teles, 2005; Rolland et al., 2015, 2016; Williams et al., 2001). Since these studies were conducted for different fish species, it can be argued that the observed inconsistencies in CAA utilisation efficiency originate from inter-species variability in absorption and assimilation rates of CAAs. The significance of different CAA conversion routes was illustrated in a study by Rolland et al. (2016) who found that increasing the dietary levels of crystalline methionine increased AA oxidation in rainbow trout. Surprisingly, the enhanced methionine catabolism did not appear to be correlated with higher ammonia excretion which is possibly due to the conversion of glutamate to glutamine by fixation of free ammonia (Rolland et al., 2016). Thus, for some aquatic species, CAA supplementation provides an adequate and inexpensive method of increasing the dietary content of specific AAs. For unprocessed BSG, lysine and histidine are reported to be the most abundant AAs whereas the sulfur-containing AAs, including methionine and cysteine, appear to be deficient (Waters et al., 2012). However, subjecting BSG to the various chemical and biological refinements outlined in the previous section may induce substantial alterations in the AA composition which are currently unknown. As emphasised previously, unrefined BSG is not a suitable protein source and therefore assessment of protein quality must be undertaken for the BSG-derived protein concentrates rather than for the untreated BSG. When alkaline extraction is employed for achieving protein isolates from BSG, some AAs are enriched whereas others are diminished relative to the original AA composition of raw BSG. This was confirmed by Connolly et al. (2013) who examined the dependence of AA profile on varying extraction temperatures. The most intriguing observation of this study was that concentrations of histidine and lysine decreased during extraction. This finding was supported by He et al. (2019) who also observed a marked decline in the content of histidine and lysine when BSG was subjected to alkaline extraction. Moreover, the content of individual AAs appeared to be more balanced after extraction except for glutamate which was still abundant in the concentrates. This partial equalisation of the various AAs during extraction may prove useful for feed formulation in that it can help abate the need to fortify specific AAs. In addition to the protein extraction approach, fungal pretreatment of BSG may also provide a useful tool for adjusting the AA content as desired. The work of Watson (1976) illustrated how the AA composition of *Saccharomyces cerevisiae* was influenced by the nitrogen source used in the culture medium. Remarkably, the results from this study revealed that the AAs supplied in the culture medium were accumulated proportionally in fungal tissue during the growth period. This trend was observed for all AAs with exception of glutamate that was found in high concentrations irrespective of the AA source (Watson, 1976). For BSG, a more balanced AA profile may be attained by growing *S. cerevisiae* on a BSG medium supplied with one or more CAAs. Furthermore, using fungi with high AA-accumulating capacity in the biological removal of ANFs constitutes a more efficacious and economic refinement strategy for BSG. This can be exemplified by an approach that applies an AA-accumulating fungus to degrade lignin from BSG thereby merging AA optimisation and delignification into a single step. To our current knowledge, yeast are the only fungi reported to accumulate AAs from the culture medium. Therefore, future studies should focus on identifying other fungi with this ability, especially species relevant for the bioconversion of lignocellulosic residues such as BSG.

4.2.3. Preservation of BSG

Owing to the high level of nutrients and moisture, BSG is susceptible to microbial colonisation. Previous studies have established that BSG is microbiologically stable at the point of production; though, under inappropriate storage conditions, it undergoes spoilage (Robertson et al., 2010a, 2010b, 2010a). Microbial proliferation will eventually lead to the deterioration of BSG, which may hinder its use in industrial applications such as aquaculture feed. Therefore, storage and preservation methods are crucial in preventing chemical alteration of BSG (Bartolomeé et al., 2002). The most commonly applied preservation methods for BSG are freezing and drying of which the latter can be subdivided into different types including freeze-drying, oven-drying and superheat steam (SS) drying. Previous studies have focused on describing the chemical alterations that occur when BSG is preserved by freezing, freeze-drying and oven-drying. Bartolomeé et al. (2002) showed that the AX content was considerably reduced when freezing was employed while it was unaffected by the other preservation methods. This is probably due to the enhanced activity of freeze-resistant microbes during the thawing of BSG resulting in depolymerisation of the AX component. In a similar study, Santos et al. (2003) observed a slight decrease in the protein and lipid content when oven-and freeze-drying was applied. This can be explained by the fact that proteins and lipids are partly degraded to form volatile compounds under harsh conditions. These studies suggest that all three methods are less suitable for the preservation of BSG as they give rise to compositional changes of the material. Furthermore, due to the high energy consumption and demand for bulk storage, drying and freezing are not economically feasible on an industrial scale (Johnson et al., 2010). Superheat steam drying provides a means by which these challenges can be circumvented since protein and NSP content remains unaltered (Tang et al., 2005). However, one major disadvantage of this technique is that it requires highly specialised equipment compared to the conventional drying methods.

4.2.3.1. Aflatoxins. Besides compositional deterioration, the production of aflatoxins may also complicate the use of BSG in aquafeeds from a food safety perspective. Aflatoxins comprise a group of 20 structurally related mycotoxins of which only 4 have been described in detail concerning their health effects and toxicity. Based on differences in physical and chemical properties, these aflatoxins are designated AFB_1, AFB_2, AFG_1 and AFG_2 with AFB_1 being the most toxigenic and well-studied (Santacroce et al., 2008). These four aflatoxins have a general structure consisting of a bifuran moiety linked to a cyclopentenone-containing coumarin core. As a consequence of their structural features, aflatoxins are lipophilic compounds that are readily absorbed and metabolised to form highly reactive and toxic intermediates such as aflatoxin-exo-8,9-epoxide. Owing to its strong affinity for various biologically important macromolecules, this intermediate facilitates adduct formation with DNA, RNA and proteins, which may ultimately lead to the onset of organ damage or cancer (Santacroce et al., 2008). Previous studies have confirmed the occurrence of inherent aflatoxin-producing fungi from BSG including several species of *Aspergillus*, *Cladosporium* and *Penicillium* (Gerbaldo et al., 2011). Furthermore, Sodhi et al. (1985) found a positive correlation between fungal proliferation and AFB_1 production under inadequate storage conditions for longer periods. Application of contaminated fish feed in aquaculture may lead to the accumulation of AFB_1 or its more reactive metabolites in fish tissue, particularly in fish species with low aflatoxin tolerance such as rainbow trout (Mwihia et al., 2018). Furthermore, the consumption of fish products containing traces of aflatoxin residues is believed to have severe consequences for human health including aflatoxicosis, nutrient malabsorption, immunosuppression and cancer development (Bbosa et al., 2013). Although a large body of literature has established that the health effects on fish caused by aflatoxin exposure are similar to those observed for humans, there appears to be great interspecific variation in aflatoxin susceptibility. For instance, feeding trials with contaminated feeds have revealed that

rainbow trout is one of the most sensitive species, while Nile tilapia exhibits greater tolerance towards aflatoxin exposure (Mwihia et al., 2018). On the other hand, silver catfish (*Rhamdia quelen*) are highly resistant to high aflatoxins concentrations (Anater et al., 2020). Existing literature suggests that BSG contamination can be minimised largely by proper storage conditions or the removal of aflatoxins from the raw material. Recent studies have focused on AFB_1 elimination through fermentation carried out by lactic acid bacteria (LAB). It has been suggested that LAB can detoxify AFB_1 by reversible binding, thereby abating ingestion of the toxin (Hernandez-Mendoza et al., 2009). The work of Asurmendi et al. (2014) corroborated that improperly stored BSG generally contained low levels of AFB_1 which can be attributed to the activity of native LAB species present in BSG. In addition, Mokoena et al. (2006) have shown that the addition of supplementary LAB cultures to maize-based meals enhanced AFB_1 removal considerably under prolonged storage. These findings may provide a pathway to eliminate AFB_1 and facilitate the implementation of BSG in feed for a broader range of fish species. From a sustainability perspective, preservation of BSG by LAB treatment is preferred over the conventional drying techniques (Petit et al., 2020). However, exposing a feed ingredient to microbial treatments, necessitates thorough evaluation of any pathogenic, or other side-effects that microbial residues may have on fish health. Since there is a general acceptance of the beneficial, probiotic effects of LAB for fish, these bacteria are expected to play a crucial role in protecting fish against harmful pathogens when added to the feed ingredients. Overall, the collection of literature on LAB treatment discussed here, implies that treating BSG with LAB cultures has several advantages including improved preservation, efficient aflatoxin removal and enhanced probiotic effect of the resulting feed.

5. Conclusion

The increasing demand for aquaculture commodities has led to a compelling need for alternative protein sources. Brewer's spent grain (BSG) can be considered a potential protein source in fish feeds because it has a high content of crude protein and essential amino acids (EAA) as compared to other agro-industrial by-products. However, the direct implementation of unrefined BSG in aquafeeds is hindered by high levels of lignin and non-starch polysaccharides (NSP) which constitute anti-nutritional factors (ANFs) for fish and by poor storability due to susceptibility to microbial growth. In this review, we have discussed how these limiting factors can be addressed to facilitate the implementation of BSG in aquafeeds. Different strategies were considered for eliminating ANFs from BSG with emphasis on two major approaches. The first approach aims at removing the ANFs, which may be achieved by chemical extraction of lignin and NSP or by extracting proteins directly from BSG. The second approach, however, relies on the chemical conversion of BSG whereby ANFs are degraded to smaller molecules that can either be digested by the fish or used as starting materials in the synthesis of other substances such as amino acids (AA). Due to a high water content, BSG is susceptible to microbial colonisation. This may cause biomass deterioration and production of aflatoxins which are known to have adverse health effects in several fish species as well as in humans consuming aflatoxin-contaminated fish products. As a result, it is critical to deploy proper storage conditions and preservation of BSG. The most widespread preservation methods include drying and freezing, which are associated with high energy consumption and demand for large storage areas, respectively. Some studies have demonstrated the positive effects of lactic acid bacteria (LAB) including their probiotic properties and efficient aflatoxin removal. The protein quality of refined BSG products, which is determined by their respective AA profiles, must also be taken into consideration. Some studies have shown that the AA profile of BSG is altered considerably by protein extraction while other studies have indicated that certain yeast species are able to change the composition of protein-bound AAs by accumulating crystalline AAs present in the culture medium. These approaches may be applicable for adjusting the AA profile of refined BSG products in such a manner that it satisfies the AA requirement of the target fish species. Presently, none of the discussed methods have been applied to convert BSG into high-quality, protein-rich raw material for aquafeeds. In our opinion, biological refinement of BSG appears to be the most eco-friendly in terms of resource use and energy consumption. From an economic point of view, this approach may be less favourable because most biological conversions are time-consuming and ineffective for short-term production. Therefore, future research might focus on optimising the efficiency of biological systems to facilitate their implementation in the valorisation of BSG as well as other relevant agro-industrial residues. Specifically, enzymatic conversion of lignin, cellulose and hemicellulose should be the primary focus of future studies rather than fungal and bacterial degradation which is often time-consuming and involves a risk of accumulating other ANFs, such as chitin, during the cultivation.

Declaration of competing interest

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

Acknowledgement

This work was funded by Nordforsk from the Nordic Council of Ministers, Norway under grant agreement no. 82342. We would like to thank Manon Eggink, Julie Hansen and Tilo Pfalzgraff for fruitful discussions on fish and animal nutrition. A special thank is also given to Dr. Ivar Lund for providing general recommendations and revisions of the work. Lastly, we express our gratitude to Dr. Carlos Octavio Letelier-Gordo for providing writing assistance and technical knowledge on miscellaneous topics.

References

Al-Souti, A., Gallardo, W., Claereboudt, M., Mahgoub, O., 2019. Attractability and palatability of formulated diets incorporated with chicken feather and algal meals for juvenile gilthead seabream. Sparus aurata. Aquac. Reports 14, 100199. https://doi.org/10.1016/j.aqrep.2019.100199.

Alashi, A.M., Blanchard, C.L., Mailer, R.J., Agboola, S.O., 2013. Technological and bioactive functionalities of canola meal proteins and hydrolysates. Food Rev. Int. 29, 231–260. https://doi.org/10.1080/87559129.2013.790046.

Amirkolaie, A.K., Leenhouwers, J.I., Verreth, J.A.J., Schrama, J.W., 2005. Type of dietary fibre (soluble versus insoluble) influences digestion, faeces characteristics and faecal waste production in Nile tilapia (Oreochromis niloticus L.). Aquacult. Res. 36, 1157–1166. https://doi.org/10.1111/j.1365-2109.2005.01330.x.

Anater, A., Araújo, C.M.T.D., Rocha, D.C.C., Ostrensky, A., Filho, J.R.E., Ribeiro, D.R., Pimpão, C.T., 2020. Evaluation of growth performance, hematological, biochemical and histopathological parameters of Rhamdia quelen fed with a feed artificially contaminated with aflatoxin B1. Aquac. Reports 17, 100326. https://doi.org/10.1016/j.aqrep.2020.100326.

Asatekin, A., Kang, S., Elimelech, M., Mayes, A.M., 2007. Anti-fouling ultrafiltration membranes containing polyacrylonitrile-graft-poly(ethylene oxide) comb copolymer additives. J. Membr. Sci. 298, 136–146. https://doi.org/10.1016/j.memsci.2007.04.011.

Asiegbu, P.O., Paterson, A., Smith, J.E., 1996. Inhibition of cellulose saccharification and glycolignin-attacking enzymes of five lignocellulose-degrading fungi by ferulic acid. World J. Microbiol. Biotechnol. 12, 16–21. https://doi.org/10.1007/BF00327792.

Assandri, D., Pampuro, N., Zara, G., Cavallo, E., Budroni, M., 2021. Suitability of composting process for the disposal and valorization of brewer's spent grain. Agric. For. 11, 1–12. https://doi.org/10.3390/agriculture11010002.

Asurmendi, P., Pascual, L., Dalcero, A., Barberis, L., 2014. Incidence of lactic acid bacteria and Aspergillus flavus in brewer's grains and evaluation of potential antifungal activity of these bacteria. J. Stored Prod. Res. 56, 33–37. https://doi.org/10.1016/j.jspr.2013.11.002.

Bartolomeé, B., Santos, M., Jiménez, J.J., Del Nozal, M.J., GÓmez-Cordoveés, C., 2002. Pentoses and hydroxycinnamic acids in brewer's spent grain. J. Cereal. Sci. 36, 51–58. https://doi.org/10.1006/jcrs.2002.0442.

Bbosa, G.S., Kitya, D., Lubega, A., Ogwal-Okeng, J., Anokbonggo, W.W., Kyegombe, D.B., 2013. Review of the biological and health effects of aflatoxins on body organs and body systems. In: Aflatoxins - Recent Advances and Future Prospects, pp. 239–265. https://doi.org/10.5772/51201.

Boyd, C.E., 2015. Overview of Aquaculture Feeds, Feed and Feeding Practices in Aquaculture. Elsevier Ltd. https://doi.org/10.1016/b978-0-08-100506-4.00001-5.

Byers, M., Miflin, B.J., Smith, S.J., 1983. A quantitative comparison of the extraction of protein fractions from wheat grain by different solvents, and of the polypeptide and amino acid composition of the alcohol-soluble proteins. J. Sci. Food Agric. 34, 447–462. https://doi.org/10.1002/jsfa.2740340506.

Çantaş, İ.B., Yildirim, Ö., 2020. Supplementation of microbial phytase with safflower meal in rainbow trout (Oncorhynchus mykiss): the effects on growth, digestibility, environmental, and serum biological parameters. J. Chem. https://doi.org/10.1155/2020/4634796, 2020.

Cardenas-Toro, F.P., Alcazar-Alay, S.C., Forster-Carneiro, T., M Angela, A.M., 2015. Obtaining Oligo- and monosaccharides from agroindustrial and agricultural residues using hydrothermal treatments. Food Publ. Health 4, 123–139. https://doi.org/10.5923/j.fph.20140403.08.

Carvalheiro, F., Esteves, M.P., Parajó, J.C., Pereira, H., Gírio, F.M., 2004. Production of oligosaccharides by autohydrolysis of brewery's spent grain. Bioresour. Technol. 91, 93–100. https://doi.org/10.1016/S0960-8524(03)00148-2.

Celus, I., Brijs, K., Delcour, J.A., 2007. Enzymatic hydrolysis of Brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates. J. Agric. Food Chem. 55, 8703–8710. https://doi.org/10.1021/jf071793c.

Celus, I., Brijs, K., Delcour, J.A., 2006. The effects of malting and mashing on barley protein extractability. J. Cereal. Sci. 44, 203–211. https://doi.org/10.1016/j.jcs.2006.06.003.

Choo, P.S., Smith, T.K., Cho, C.Y., Ferguson, H.W., 1991. Dietary excesses of leucine influence growth and body composition of rainbow trout. J. Nutr. 121, 1932–1939. https://doi.org/10.1093/jn/121.12.1932.

Christopher, L.P., Yao, B., Ji, Y., 2014. Lignin biodegradation with laccase-mediator systems. Front. Energy Res. 2, 1–13. https://doi.org/10.3389/fenrg.2014.00012.

Coelho, E., Rocha, M.A.M., Moreira, A.S.P., Domingues, M.R.M., Coimbra, M.A., 2016. Revisiting the structural features of arabinoxylans from brewers' spent grain. Carbohydr. Polym. 139, 167–176. https://doi.org/10.1016/j.carbpol.2015.12.006.

Connolly, A., Cermeño, M., Crowley, D., O'Callaghan, Y., O'Brien, N.M., FitzGerald, R.J., 2019. Characterisation of the in vitro bioactive properties of alkaline and enzyme extracted brewers' spent grain protein hydrolysates. Food Res. Int. 121, 524–532. https://doi.org/10.1016/j.foodres.2018.12.008.

Connolly, A., Piggott, C.O., Fitzgerald, R.J., 2013. Characterisation of protein-rich isolates and antioxidative phenolic extracts from pale and black brewers' spent grain. Int. J. Food Sci. Technol. 48, 1670–1681. https://doi.org/10.1111/ijfs.12137.

Conway, J., 2021. Beer Production Wordwide from 1998 to 2020. Available at: https://www.statista.com/statistics/270275/worldwide-beer-production/. (Accessed 17 November 2021).

Crowe, N.L., Baker, B.E., Inteaz, A., 1985. Solubilisation of nitrogenous constituents of brewer's spent grain. J. Inst. Brew. 9, 148–150. https://doi.org/10.1002/j.2050-0416.1985.tb04320.x.

D'Este, M., Alvarado-Morales, M., Angelidaki, I., 2018. Amino acids production focusing on fermentation technologies – a review. Biotechnol. Adv. 36, 14–25. https://doi.org/10.1016/j.biotechadv.2017.09.001.

Dalsgaard, J., Ekmann, K.S., Pedersen, P.B., Verlhac, V., 2009. Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (Oncorhynchus mykiss), and on the magnitude and composition of phosphorus waste output. Aquaculture 286, 105–112. https://doi.org/10.1016/j.aquaculture.2008.09.007.

Denilson, 2010. Selective acetone-water delignification of Eucalyptus urograndis: an alternative towards the biorefinery approach. Open Agric. J. 4, 145–152. https://doi.org/10.2174/1874331501004010145.

Dersjant-li, Y., 2002. The Use of Soy Protein in Aquafeeds. Use Soy Protein Aquafeeds, pp. 541–558.

Dessie, W., Xin, F., Zhang, W., Zhou, J., Wu, H., Ma, J., Jiang, M., 2019. Inhibitory effects of lignocellulose pretreatment degradation products (hydroxymethylfurfural and furfural) on succinic acid producing Actinobacillus succinogenes. Biochem. Eng. J. 150, 1–6. https://doi.org/10.1016/j.bej.2019.107263.

Ervin, V., Alli, I., Smith, J.P., Li, Z., 1989. Extraction and precipitation of proteins from brewer's spent grain. Can. Inst. Food Sci. Technol. J. 22, 216–221. https://doi.org/10.1016/s0315-5463(89)70386-2.

Eumofa, 2021. Fishmeal and Fish Oil: Production and Trade Flows in the EU, EUMOFA - European Market Observatory for Fisheries and Aquaculture Products. https://doi.org/10.2771/062233.

Fan, Y., Zhang, Z., Wang, F., Li, J., Hu, K., Du, Z., 2019. Lignin degradation in corn stover catalyzed by lignin peroxidase from Aspergillus oryzae broth: effects of conditions on the kinetics. Renew. Energy 130, 32–40. https://doi.org/10.1016/j.renene.2018.06.037.

FAO, 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in Action. https://doi.org/10.4060/ca9229en. Rome.

Forssell, P., Kontkanen, H., Schols, H.A., Hinz, S., Eijsink, V.G.H., Treimo, J., Robertson, J.A., Waldron, K.W., Faulds, C.B., Buchert, J., 2008. Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. J. Inst. Brew. 114, 306–314. https://doi.org/10.1002/j.2050-0416.2008.tb00774.x.

Friedman, M., Levin, C.E., 2012. Nutritional and medicinal aspects of D-amino acids. Amino Acids 42, 1553–1582. https://doi.org/10.1007/s00726-011-0915-1.

Galanakis, C.M., 2020. The food systems in the era of the coronavirus (COVID-19) pandemic crisis. Foods 9, 3–10. https://doi.org/10.3390/foods9040523.

Galanakis, C.M., 2015. Separation of functional macromolecules and micromolecules: from ultrafiltration to the border of nanofiltration. Trends Food Sci. Technol. 42, 44–63. https://doi.org/10.1016/j.tifs.2014.11.005.

Gamboa-Delgado, J., Rodríguez Montes de Oca, G.A., Román Reyes, J.C., Villarreal-Cavazos, D.A., Nieto-López, M., Cruz-Suárez, L.E., 2017. Assessment of the relative contribution of dietary nitrogen from fish meal and biofloc meal to the growth of Pacific white shrimp (Litopenaeus vannamei). Aquacult. Res. 48, 2963–2972. https://doi.org/10.1111/are.13129.

Gerbaldo, G.A., Pereyra, C.M., Cavaglieri, L.R., Ruiz, F., Pascual, L., Dalcero, A.M., Barberis, I.L., 2011. Surveillance of aflatoxin and microbiota related to Brewer's grain destined for swine feed in Argentina. Vet. Med. Int. https://doi.org/10.4061/2011/912480, 2011.

Giacobbe, S., Piscitelli, A., Raganati, F., Lettera, V., Sannia, G., Marzocchella, A., Pezzella, C., 2019. Butanol production from laccase-pretreated brewer's spent grain. Biotechnol. Biofuels 12, 1–8. https://doi.org/10.1186/s13068-019-1383-1.

Gilani, G.S., Xiao, C.W., Cockell, K.A., 2012. Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. Br. J. Nutr. 108 https://doi.org/10.1017/S0007114512002371.

Glencross, B., Rutherford, N., Bourne, N., 2012. The influence of various starch and non-starch polysaccharides on the digestibility of diets fed to rainbow trout (Oncorhynchus mykiss). Aquaculture 356 (357), 141–146. https://doi.org/10.1016/j.aquaculture.2012.05.023.

Glencross, B.D., Baily, J., Berntssen, M.H.G., Hardy, R., MacKenzie, S., Tocher, D.R., 2020. Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. Rev. Aquacult. 12, 703–758. https://doi.org/10.1111/raq.12347.

Green, J.A., Hardy, R.W., Brannon, E.L., 2002a. The optimum dietary essential amino acid pattern for rainbow trout (Oncorhynchus mykiss), to maximize nitrogen retention and minimize nitrogen excretion. Fish Physiol. Biochem. 27, 109–115. https://doi.org/10.1023/B:FISH.0000021877.49848.8f.

Green, J.A., Hardy, R.W., Brannon, E.L., 2002b. The optimum dietary essential: nonessential amino acid ratio for rainbow trout (Oncorhynchus mykiss), which maximizes nitrogen retention and minimizes nitrogen excretion. Fish Physiol. Biochem. 27, 109–115. https://doi.org/10.1023/B:FISH.0000021877.49848.8f.

Guido, L.F., Moreira, M.M., 2017. Techniques for extraction of brewer's spent grain polyphenols: a review. Food Bioprocess Technol. 10, 1192–1209. https://doi.org/10.1007/s11947-017-1913-4.

Hansen, J.Ø., Storebakken, T., 2007. Effects of dietary cellulose level on pellet quality and nutrient digestibilities in rainbow trout (Oncorhynchus mykiss). Aquaculture 272, 458–465. https://doi.org/10.1016/j.aquaculture.2007.09.005.

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult. Res. 41, 770–776. https://doi.org/10.1111/j.1365-2109.2009.02349.x.

He, Y., Galagarza, O.A., Wang, H., Taylor, Z.W., Ferguson, C.S., Ogejo, J.A., O'Keefe, S.F., Fernández Fraguas, C., Yu, D., Poe, N.E., Wiersema, B.D., Kuhn, D.D., Huang, H., 2020. Protein-rich product recovered from brewer's spent grain can partially replace fishmeal in diets of Pacific white shrimp, Litopenaeus vannamei. Aquacult. Res. 51, 3284–3296. https://doi.org/10.1111/are.14664.

He, Y., Kuhn, D.D., O'Keefe, S.F., Ogejo, J.A., Fraguas, C.F., Wang, H., Huang, H., 2021. Protein production from brewer's spent grain via wet fractionation: process optimization and techno-economic analysis. Food Bioprod. Process. 126, 234–244. https://doi.org/10.1016/j.fbp.2021.01.005.

He, Y., Kuhn, D.D., Ogejo, J.A., O'Keefe, S.F., Fraguas, C.F., Wiersema, B.D., Jin, Q., Yu, D., Huang, H., 2019. Wet fractionation process to produce high protein and high fiber products from brewer's spent grain. Food Bioprod. Process. 117, 266–274. https://doi.org/10.1016/j.fbp.2019.07.011.

Hernandez-Mendoza, A., Garcia, H.S., Steele, J.L., 2009. Screening of Lactobacillus casei strains for their ability to bind aflatoxin B1. Food Chem. Toxicol. 47, 1064–1068. https://doi.org/10.1016/j.fct.2009.01.042.

Hou, Y., Yin, Y., Wu, G., 2015. Dietary essentiality of "nutritionally non-essential amino acids" for animals and humans. Exp. Biol. Med. 240, 997–1007. https://doi.org/10.1177/1535370215587913.

Huang, Z., Dostal, L., Rosazza, J.P.N., 1993. Microbial transformations of ferulic acid by Saccharomyces cerevisiae and Pseudomonas fluorescens. Appl. Environ. Microbiol. 59, 2244–2250. https://doi.org/10.1128/aem.59.7.2244-2250.1993.

Irvin, S., Blyth, D., Bourne, N., Glencross, B., 2016. A study of the discrete and interactive effects of different polysaccharides on the digestibility of diets fed to barramundi (Lates calcarifer). Aquacult. Nutr. 22, 1047–1054. https://doi.org/10.1111/anu.12321.

Jackowski, M., Niedźwiecki, Ł., Jagiełło, K., Uchańska, O., Trusek, A., 2020. Brewer's spent grains—valuable beer industry by-product. Biomolecules 10, 1–18. https://doi.org/10.3390/biom10121669.

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., Dayal, J.S., 2019. Fishmeal availability in the scenarios of climate change: inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. Aquacult. Res. 50, 3493–3506. https://doi.org/10.1111/are.14324.

Janusz, G., Pawlik, A., Sulej, J., Świderska-Burek, U., Jarosz-Wilkolazka, A., Paszczyński, A., 2017. Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol. Rev. 41, 941–962. https://doi.org/10.1093/femsre/fux049.

Jayant, M., Hassan, M.A., Srivastava, P.P., Meena, D.K., Kumar, P., Kumar, A., Wagde, M. S., 2018. Brewer's spent grains (BSGs) as feedstuff for striped catfish, Pangasianodon hypophthalmus fingerlings: an approach to transform waste into wealth. J. Clean. Prod. 199, 716–722. https://doi.org/10.1016/j.jclepro.2018.07.213.

Ji, W., Ding, Z., Liu, J., Song, Q., Xia, X., Gao, H., Wang, H., Gu, W., 2012. Mechanism of lignin dissolution and regeneration in ionic liquid. Energy Fuel. 26, 6393–6403. https://doi.org/10.1021/ef301231a.

Johnson, P., Paliwal, J., Cenkowski, S., 2010. Issues with utilisation of brewers' spent grain. Stewart Postharvest Rev 6, 1–8. https://doi.org/10.2212/spr.2010.4.2.

Karlsen, Ø., Amlund, H., Berg, A., Olsen, R.E., 2017. The effect of dietary chitin on growth and nutrient digestibility in farmed Atlantic cod, Atlantic salmon and Atlantic halibut. Aquacult. Res. 48, 123–133. https://doi.org/10.1111/are.12867.

Kirk, T.K., Schultz, E., Connors, W.J., Lorenz, L.F., Zeikus, J.G., 1978. Influence of culture parameters on lignin metabolism by Phanerochaete chrysosporium. Arch. Microbiol. 117, 277–285. https://doi.org/10.1007/BF00738547.

Ktenioudaki, A., Alvarez-Jubete, L., Smyth, T.J., Kilcawley, K., Rai, D.K., Gallagher, E., 2015. Application of bioprocessing techniques (sourdough fermentation and technological aids) for brewer's spent grain breads. Food Res. Int. 73, 107–116. https://doi.org/10.1016/j.foodres.2015.03.008.

Lam, A.C.Y., Can Karaca, A., Tyler, R.T., Nickerson, M.T., 2018. Pea protein isolates: structure, extraction, and functionality. Food Rev. Int. 34, 126–147. https://doi.org/10.1080/87559129.2016.1242135.

Lewis, M.J., Young, T.W., 2001. Brewing. https://doi.org/10.1007/978-1-4615-0729-1. Brewing.

Litwack, G., 2018. Human Biochemistry, pp. 63–94. https://doi.org/10.1016/B978-0-12-383864-3.00004-1.

Liu, Yongzhuang, Chen, W., Xia, Q., Guo, B., Wang, Q., Liu, S., Liu, Yixing, Li, J., Yu, H., 2017. Efficient cleavage of lignin–carbohydrate complexes and ultrafast extraction of lignin Oligomers from wood biomass by microwave-assisted treatment with deep eutectic solvent. ChemSusChem 10, 1692–1700. https://doi.org/10.1002/cssc.201601795.

Lynch, Kieran M., Steffen, E.J., Arendt, E.K., 2016. Brewers' spent grain: a review with an emphasis on food and health. J. Inst. Brew. 122, 553–568. https://doi.org/10.1002/jib.363.

Mambrini, M., Kaushik, S.J., 1994. Partial replacement of dietary protein nitrogen with dispensable amino acids in diets of Nile tilapia, Oreochromis niloticus. Comp. Biochem. Physiol. – Part A Physiol. 109, 469–477. https://doi.org/10.1016/0300-9629(94)90152-X.

Mandalari, G., Faulds, C.B., Sancho, A.I., Saija, A., Bisignano, G., Locurto, R., Waldron, K. W., 2005. Fractionation and characterisation of arabinoxylans from brewers' spent grain and wheat bran. J. Cereal. Sci. 42, 205–212. https://doi.org/10.1016/j.jcs.2005.03.001.

Marshall, A.D., Munro, P.A., Trägårdh, G., 1993. The effect of protein fouling in microfiltration and ultrafiltration on permeate flux, protein retention and selectivity: a literature review. Desalination 91, 65–108. https://doi.org/10.1016/0011-9164(93)80047-Q.

Martín, C., Jönsson, L.J., 2003. Comparison of the resistance of industrial and laboratory strains of Saccharomyces and Zygosaccharomyces to lignocellulose-derived fermentation inhibitors. Enzym. Microb. Technol. 32, 386–395. https://doi.org/10.1016/S0141-0229(02)00310-1.

Martins, G.P., Pezzato, L.E., Guimarães, I.G., Padovani, C.R., Mazini, B.S.M., Barros, M. M., 2017. Fatores antinutricionais da soja crua no crescimento e respostas hematológicas da tilápia do nilo. Bol. do Inst. Pesca 43, 322–333. https://doi.org/10.20950/1678-2305.2017v43n3p322.

Mathew, S., Abraham, T.E., 2004. Ferulic acid: an antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Crit. Rev. Biotechnol. 24, 59–83. https://doi.org/10.1080/07388550490491467.

Melro, E., Filipe, A., Sousa, D., Valente, A.J.M., Romano, A., Antunes, F.E., Medronho, B., 2020. Dissolution of kraft lignin in alkaline solutions. Int. J. Biol. Macromol. 148, 688–695. https://doi.org/10.1016/j.ijbiomac.2020.01.153.

Mendis, M., Simsek, S., 2014. Arabinoxylans and human health. Food Hydrocolloids 42, 239–243. https://doi.org/10.1016/j.foodhyd.2013.07.022.

Mohammad, A.W., Ng, C.Y., Lim, Y.P., Ng, G.H., 2012. Ultrafiltration in food processing industry: review on application, membrane fouling, and fouling control. Food Bioprocess Technol. 5, 1143–1156. https://doi.org/10.1007/s11947-012-0806-9.

Mokoena, M.P., Chelule, P.K., Gqaleni, N., 2006. The toxicity and decreased concentration of aflatoxin B1 in natural lactic acid fermented maize meal. J. Appl. Microbiol. 100, 773–777. https://doi.org/10.1111/j.1365-2672.2006.02881.x.

Mosher, M., Trantham, K., 2017. Brewing Science: A Multidisciplinary Approach, Brewing Science: A Multidisciplinary Approach. https://doi.org/10.1007/978-3-319-46394-0.

Murai, T., 1991. Principles of fish nutrition. Aquaculture 92, 291–292. https://doi.org/10.1016/0044-8486(91)90032-3.

Mussatto, S.I., 2014. Brewer's spent grain: a valuable feedstock for industrial applications. J. Sci. Food Agric. 94, 1264–1275. https://doi.org/10.1002/jsfa.6486.

Mussatto, S.I., Dragone, G., Roberto, I.C., 2006. Brewers' spent grain: generation, characteristics and potential applications. J. Cereal. Sci. 43, 1–14. https://doi.org/10.1016/j.jcs.2005.06.001.

Mussatto, S.I., Fernandes, M., Milagres, A.M.F., Roberto, I.C., 2008. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzym. Microb. Technol. 43, 124–129. https://doi.org/10.1016/j.enzmictec.2007.11.006.

Mwihia, E.W., Mbuthia, P.G., Eriksen, G.S., Gathumbi, J.K., Maina, J.G., Mutoloki, S., Waruiru, R.M., Mulei, I.R., Lyche, J.L., 2018. Occurrence and levels of aflatoxins in fish feeds and their potential effects on fish in Nyeri, Kenya. Toxins 10. https://doi.org/10.3390/toxins10120543.

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D. C., Lubchenco, J., Shumway, S.E., Troell, M., 2021. A 20-year retrospective review of global aquaculture. Nature 591, 551–563. https://doi.org/10.1038/s41586-021-03308-6.

Nazzaro, J., Martin, D.S., Perez-Vendrell, A.M., Padrell, L., Iñarra, B., Orive, M., Estévez, A., 2021. Apparent digestibility coefficients of brewer's by-products used in feeds for rainbow trout (Oncorhynchus mykiss) and gilthead seabream (Sparus aurata). Aquaculture 530, 735796. https://doi.org/10.1016/j.aquaculture.2020.735796.

Nunes, A.J.P., Sá, M.V.C., Browdy, C.L., Vazquez-Anon, M., 2014. Practical supplementation of shrimp and fish feeds with crystalline amino acids. Aquaculture 431, 20–27. https://doi.org/10.1016/j.aquaculture.2014.04.003.

OECD/FAO, 2016. OECD-FAO Agricultural Outlook 2016-2025. OECD Publishing, Paris. https://doi.org/10.1787/agr_outlook-2016-en.

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish. Dis. 35, 83–108. https://doi.org/10.1111/j.1365-2761.2011.01333.x.

Olsen, R.L., Hasan, M.R., 2012. A limited supply of fishmeal: impact on future increases in global aquaculture production. Trends Food Sci. Technol. 27, 120–128. https://doi.org/10.1016/j.tifs.2012.06.003.

Pandian, T.J., Vivekanandan, E., 1985. Energetics of Feeding and Digestion. Fish Energ, New Perspect.

Pelletier, N., Klinger, D.H., Sims, N.A., Yoshioka, J.R., Kittinger, J.N., 2018. Nutritional attributes, substitutability, scalability, and environmental intensity of an illustrative subset of current and future protein sources for aquaculture feeds: joint consideration of potential synergies and trade-offs. Environ. Sci. Technol. 52, 5532–5544. https://doi.org/10.1021/acs.est.7b05468.

Pereira, M., Beck, P.H., Muller, D.G., Moreira, J.B., da Silva, J.S., Durigon, A.M.M., 2016. Extraction of Organosolv lignin from rice husk under reflux conditions. Biol. Chem. Res. 87–98.

Peres, H., Oliva-Teles, A., 2006. Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (Dicentrarchus labrax). Aquaculture 256, 395–402. https://doi.org/10.1016/j.aquaculture.2006.02.010.

Peres, H., Oliva-Teles, A., 2005. The effect of dietary protein replacement by crystalline amino acid on growth and nitrogen utilization of turbot Scophthalmus maximus juveniles. Aquaculture 250, 755–764. https://doi.org/10.1016/j.aquaculture.2005.04.046.

Péron, G., François Mittaine, J., Le Gallic, B., 2010. Where do fishmeal and fish oil products come from? An analysis of the conversion ratios in the global fishmeal industry. Mar. Pol. 34, 815–820. https://doi.org/10.1016/j.marpol.2010.01.027.

Petit, G., Korbel, E., Jury, V., Aider, M., Rousselière, S., Audebrand, L.K., Turgeon, S.L., Mikhaylin, S., 2020. Environmental evaluation of new brewer's spent grain preservation pathways for further valorization in human nutrition. ACS Sustain. Chem. Eng. https://doi.org/10.1021/acssuschemeng.0c04236.

Prado, R., Erdocia, X., Labidi, J., 2013. Lignin extraction and purification with ionic liquids. J. Chem. Technol. Biotechnol. 88, 1248–1257. https://doi.org/10.1002/jctb.3965.

Qin, F., Johansen, A.Z., Mussatto, S.I., 2018. Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains. Ind. Crop. Prod. 125, 443–453. https://doi.org/10.1016/j.indcrop.2018.09.017.

Rachwał, K., Waśko, A., Gustaw, K., Polak-Berecka, M., 2020. Utilization of brewery wastes in food industry. PeerJ 8, 1–28. https://doi.org/10.7717/peerj.9427.

Richard, T., Michael, J., Kent, T., John, A., 1987. Growth of the White-Rot Fungus Phanerochaete Chrysosporium in Soil 419–424.

Robertson, J.A., I'Anson, K.J.A., Treimo, J., Faulds, C.B., Brocklehurst, T.F., Eijsink, V.G. H., Waldron, K.W., 2010a. Profiling brewers' spent grain for composition and microbial ecology at the site of production. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 43, 890–896. https://doi.org/10.1016/j.lwt.2010.01.019.

Robertson, J.A., IAnson, K.J.A., Brocklehurst, T.F., Faulds, C.B., Waldron, K.W., 2010b. Effect of storage conditions on the microbial ecology and biochemical stability of cell wall components in brewers spent grain. J. Agric. Food Chem. 58, 7266–7272. https://doi.org/10.1021/jf1001099.

Rolland, M., Larsen, B.K., Holm, J., Dalsgaard, J., Skov, P.V., 2015. Effect of plant proteins and crystalline amino acid supplementation on postprandial plasma amino acid profiles and metabolic response in rainbow trout (Oncorhynchus mykiss). Aquacult. Int. 23, 1071–1087. https://doi.org/10.1007/s10499-014-9865-4.

Rolland, M., Skov, P.V., Larsen, B.K., Holm, J., Gómez-Requeni, P., Dalsgaard, J., 2016. Increasing levels of dietary crystalline methionine affect plasma methionine profiles, ammonia excretion, and the expression of genes related to the hepatic intermediary metabolism in rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 198, 91–99. https://doi.org/10.1016/j.cbpb.2016.04.006.

Sahadevan, L.D.M., Misra, C.S., Thankamani, V., 2016. Characterization of lignin-degrading enzymes (LDEs) from a dimorphic novel fungus and identification of products of enzymatic breakdown of lignin. 3 Biotech 6, 1–16. https://doi.org/10.1007/s13205-016-0384-z.

Salin, K.R., Arun, V.V., Nair, C.M., Tidwell, J.H., 2018. Sustainable Aquaculture, Sustainable Aquaculture. Springer International Publishing. https://doi.org/10.1007/978-3-319-73257-2.

Santacroce, M.P., Conversano, M.C., Casalino, E., Lai, O., Zizzadoro, C., Centoducati, G., Crescenzo, G., 2008. Aflatoxins in aquatic species: metabolism, toxicity and perspectives. Rev. Fish Biol. Fish. 18, 99–130. https://doi.org/10.1007/s11160-007-9064-8.

Santos, M., Jiménez, J.J., Bartolomé, B., Gómez-Cordovés, C., Del Nozal, M.J., 2003. Variability of brewer's spent grain within a brewery. Food Chem. 80, 17–21. https://doi.org/10.1016/S0308-8146(02)00229-7.

Singh, S.K., 2019. Solubility of lignin and chitin in ionic liquids and their biomedical applications. Int. J. Biol. Macromol. 132, 265–277. https://doi.org/10.1016/j.ijbiomac.2019.03.182.

Siqueira, G., Várnai, A., Ferraz, A., Milagres, A.M.F., 2013. Enhancement of cellulose hydrolysis in sugarcane bagasse by the selective removal of lignin with sodium chlorite. Appl. Energy 102, 399–402. https://doi.org/10.1016/j.apenergy.2012.07.029.

Sodhi, Garcha, Kiran, 1985. Screening of mycoflora of spent-up brewer's grains for aflatoxin production. Agric. Res. J. 22, 331–336.

Sousa, D., Venâncio, A., Belo, I., Salgado, J.M., 2018. Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation. J. Sci. Food Agric. 98, 5248–5256. https://doi.org/10.1002/jsfa.9063.

Steiner, J., Procopio, S., Becker, T., 2015. Brewer's spent grain: source of value-added polysaccharides for the food industry in reference to the health claims. Eur. Food Res. Technol. 241, 303–315. https://doi.org/10.1007/s00217-015-2461-7.

Tacon, A.G.J., Hasan, M.R., Metian, M., 2011. Demand and supply of feed ingredients for farmed fish and crustaceans : trends and prospects. FAO Fisheries and Aquaculture Technical Paper.

Tang, D.S., Yin, G.M., He, Y.Z., Hu, S.Q., Li, B., Li, L., Liang, H.L., Borthakur, D., 2009. Recovery of protein from brewer's spent grain by ultrafiltration. Biochem. Eng. J. 48, 1–5. https://doi.org/10.1016/j.bej.2009.05.019.

Tang, Z., Cenkowski, S., Izydorczyk, M., 2005. Thin-layer drying of spent grains in superheated steam. J. Food Eng. 67, 457–465. https://doi.org/10.1016/j.jfoodeng.2004.04.040.

Tantikitti, C., 2014. Feed palatability and the alternative protein sources in shrimp feed. Songklanakarin J. Sci. Technol. 36, 51–55.

Teles, A.O., Couto, A., Enes, P., Peres, H., 2020. Dietary protein requirements of fish – a meta-analysis. Rev. Aquacult. 12, 1445–1477. https://doi.org/10.1111/raq.12391.

Veide Vilg, J., Undeland, I., 2017. pH-driven solubilization and isoelectric precipitation of proteins from the brown seaweed Saccharina latissima—effects of osmotic shock, water volume and temperature. J. Appl. Phycol. 29, 585–593. https://doi.org/10.1007/s10811-016-0957-6.

Vieira, E., Rocha, M.A.M., Coelho, E., Pinho, O., Saraiva, J.A., Ferreira, I.M., Coimbra, M. A., 2014. Valuation of brewer's spent grain using a fully recyclable integrated process for extraction of proteins and arabinoxylans. Ind. Crop. Prod. 52, 136–143. https://doi.org/10.1016/j.indcrop.2013.10.012.

Waters, D.M., Jacob, F., Titze, J., Arendt, E.K., Zannini, E., 2012. Fibre, protein and mineral fortification of wheat bread through milled and fermented brewer's spent grain enrichment. Eur. Food Res. Technol. 235, 767–778. https://doi.org/10.1007/s00217-012-1805-9.

Watson, T.G., 1976. Amino acid pool composition of Saccharomyces cerevisiae as a function of growth rate and amino acid nitrogen source. J. Gen. Microbiol. 96, 263–268. https://doi.org/10.1099/00221287-96-2-263.

Wen, C., Zhang, J., Duan, Y., Zhang, H., Ma, H., 2019. A mini-review on brewer's spent grain protein: isolation, physicochemical properties, application of protein, and functional properties of hydrolysates. J. Food Sci. 84, 3330–3340. https://doi.org/10.1111/1750-3841.14906.

Williams, K., Barlow, C., Rodgers, L., 2001. Efficacy of crystalline and protein-bound amino acids for amino acid enrichment of diets for barramundi/Asian seabass (Lates calcarifer Bloch). Aquacult. Res. 32, 415–429. https://doi.org/10.1046/j.1355-557x.2001.00032.x.

Willows, R.D., Worden, P., Mirzaei, M., 2017. Barley grain proteomics. In: Proteomics in Food Science: from Farm to Fork. Elsevier Inc. https://doi.org/10.1016/B978-0-12-804007-2.00005-9.

Wilson, R., 1986. Protein and amino acid requirements of fishes. Annu. Rev. Nutr. 6, 225–244. https://doi.org/10.1146/annurev.nutr.6.1.225.

Wilson, R.P., 1994. Utilization of dietary carbohydrate by fish. Aquaculture 124, 67–80. https://doi.org/10.1016/0044-8486(94)90363-8.

Wong, D.W.S., 2009. Structure and action mechanism of ligninolytic enzymes. Appl. Biochem. Biotechnol. https://doi.org/10.1007/s12010-008-8279-z.

Xiros, C., Christakopoulos, P., 2012. Biotechnological potential of brewers spent grain and its recent applications. Waste and Biomass Valorization 3, 213–232. https://doi.org/10.1007/s12649-012-9108-8.

Xiros, C., Topakas, E., Katapodis, P., Christakopoulos, P., 2008a. Evaluation of Fusarium oxysporum as an enzyme factory for the hydrolysis of brewer's spent grain with improved biodegradability for ethanol production. Ind. Crop. Prod. 28, 213–224. https://doi.org/10.1016/j.indcrop.2008.02.004.

Xiros, C., Topakas, E., Katapodis, P., Christakopoulos, P., 2008b. Hydrolysis and fermentation of brewer's spent grain by Neurospora crassa. Bioresour. Technol. 99, 5427–5435. https://doi.org/10.1016/j.biortech.2007.11.010.

Zarate, D.D., Lovell, R.T., Payne, M., 1999. Effects of feeding frequency and rate of stomach evacuation on utilization of dietary free and protein-bound lysine for growth by channel catfish Ictalurus punctatus. Aquacult. Nutr. 5, 17–22. https://doi.org/10.1046/j.1365-2095.1999.00083.x.

Zhu, Y., Karen, T.Y., Narayan, S.H., 2013. Application of ionic liquids in lignin chemistry. In: Ionic Liquids - New Aspects for the Future. IntechOpen, pp. 315–346. https://doi.org/10.5772/51161.

Paper 2

Optimisation of alkaline extraction of protein from brewer's spent grain

Published, Journal of the Institute of Brewing (2022)

Research article

Received: 14 February 2022 Revised: 1 June 2022 Accepted: 18 August 2022 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jib.703

Optimisation of alkaline extraction of protein from brewer's spent grain

Freja Karlsen,* Ivar Lund and Peter V. Skov

Extraction of proteins from industrial residues is a potential source for animal feed. Alkaline extraction combined with isoelectric precipitation may be a useful method for isolating protein from brewer's spent grain (BSG), an abundant by-product from the brewing industry. The objective of this study was to examine the effect of temperature (30, 45 and 60°C), time (30, 60, 120 and 180 minutes), agitation (shaking and stirring), precipitation salinity (0.5 M sodium chloride), isolation mode (freeze drying and precipitation), pre-treatment (defatting and delignification) and repeated extraction on the protein yield and protein content from BSG. Generally, the protein content decreased while protein yield increased with increasing extraction temperatures. Yield and content were maximised after 30 minutes reaching the highest protein content at 30°C (54.8%) and the highest protein yield at 60°C (10.5%). Precipitation with 0.5 M sodium chloride reduced the protein content to 36.4% and the yield to 4.2%. Freeze drying combined with triple extraction yielded a protein content of 20-25%, together with a relatively high protein yield (ca. 45-50%). Delignification increased the protein content from 27% to 32% whereas defatting reduced the overall protein yield from 45% to 38%. Combining isoelectric precipitation and single extraction was associated with a higher protein content (40-55%) but poor protein yields (5-10%). In the light of these findings, further research is required to establish conditions that maximise both the protein content and yield.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords: Brewer's spent grain; protein; amino acids; alkaline extraction; extraction yield

Introduction

Protein is the costliest ingredient in formulated diets for farmed animals. Currently, fishmeal and plant derived meals are the most widespread protein sources in the production of animal feeds, particularly aquafeed (Tacon et al, 2011). These protein sources have a high nutritional value; however, sourcing them is associated with economic and environmental concerns (Olsen and Hasan, 2012). Therefore, there is a need for more sustainable protein sources in animal feeds.

Brewer's spent grain (BSG) is the most abundant by-product from the brewing industry with an annual production of more than 40 million tonnes of wet BSG (Petit et al, 2020). At present, the main uses of BSG include supplementation of feed for livestock animals, composting and energy production through incineration (Petit et al, 2020). In recent years, increasing attention has been directed at alternative uses of BSG in food and non-food applications as BSG represents a source of different bioactive compounds and is a favourable substrate for microbial cultivation. As a result, several approaches to valorisation have been investigated for BSG including chemical extraction, enzymatic conversion and microbial production of value added compounds and enzymes (Crowe et al, 1985; Forssell et al, 2008; Pejin et al, 2017; Tišma et al, 2018). BSG has a relatively high protein content of dry matter (20-25% DM) and its availability from the brewing industry is stable (Lynch et al, 2016). As such, BSG constitutes a potential protein source for animal and fish feeds. However, BSG contains high levels of lignin and non-starch polysaccharides (NSP), mainly cellulose and hemicellulose, which has prevented the direct use of unprocessed BSG as a protein source in feed applications (Sousa et al, 2018). To facilitate implementation in animal feeds, BSG must undergo refinement where lignin and NSP are reduced, and the protein content is increased. This may be achieved by either chemical fractionation or conversion of BSG. During chemical fractionation, the protein fraction is isolated by extraction whereas the conversion strategy is based on a chemical transformation of lignin and NSP into digestible compounds. Furthermore, the digestibility of lignin and NSP differs between different animal groups. While ruminants are capable of digesting large amounts of cellulose, a high cellulose content will reduce digestion in most fish species. As a result, the levels of lignin and NSP vary depending on their digestibility by the target animal which must be considered when designing a refinement strategy for BSG.

Protein extraction may offer an efficient and convenient method for obtaining protein rich material while eliminating indigestible components. Extraction of protein from BSG has been explored in previous studies to assess different methods including sequential extraction, alkaline and acidic extraction, salt and detergent mediated extraction and ultrasound assisted extraction (Crowe et al, 1985; Diptee et al, 1989; Tang et al, 2010; Qin et al, 2018; Connolly et al, 2019; Wen et al, 2019). In addition, several procedures for precipitation of solubilised protein have been evaluated. For instance, Ervin et al (1989) reported that ethanol precipitation was more effective than isoelectric precipitation when recovering protein from BSG. Earlier research has focused on optimising the extraction of crude proteins from BSG and characterising their

* Correspondence to: Freja Karlsen, Technical University of Denmark, DTU Aqua, Section for Aquaculture, DK-9850, Hirtshals, Denmark. Email: frkar@aqua.dtu.dk.

DTU Aqua, Section for Aquaculture, Technical University of Denmark, DK-9850, Hirtshals, Denmark

amino acid profile and physicochemical properties such as water solubility and proteolytic digestibility. For successful protein extraction, two criteria must be satisfied: 1) high recovery of proteins from the original material and 2) high protein content and purity. To be considered a suitable protein source for feed applications, particularly aquafeeds, the protein content should be similar to that of fishmeal (70-85%) and plant based protein meals (50-80%) (Gorissen et al, 2018).

This study was undertaken with the objective of evaluating the impact of duration, temperature, precipitation salinity, protein isolation mode, agitation method and chemical pre-treatment on the recovery of crude protein and the true protein and amino acid content after alkaline extraction from BSG. Furthermore, the effectiveness of isoelectric precipitation in terms of protein content and recovery was assessed. In this study, all extractions were performed with micronised BSG, which reduces the particle size and leads to increased surface area and exposure of protein to solvent molecules. Accordingly, micronisation would be expected to increase the extraction efficiency of protein from BSG.

Materials and methods

Spent grain

Brewer's spent grain was provided by Carlsberg Denmark two different batches - batch 1 (B1) and batch 2 (B2). The BSG was autoclaved at 121°C for 15 min and dried at 60°C until the dry matter content (DM) was above 90%. Subsequently, BSG was mechanically processed at the Danish Technological Institute and was autoclaved to prevent any microbial colonisation.

BSG - mechanical processing

Before protein extraction, BSG from B1 and B2 was processed by micronisation to increase the surface area. This process combines milling and sifting to fractionate the raw material into portions with a range of different particle sizes. The micronisation of BSG was performed using a Rotor FITZ®MILL size-reduction instrument and a SCAN-Vibro VÅV vibrational sifter. During the micronisation process, different size fractions were generated where the most abundant fraction accounted for 50-70% of the micronised material and had a particle size of 125-250μm. This fraction was used for protein extraction with the other fractions discarded.

BSG - defatting

BSG samples were defatted using a modification of the method described by Bligh and Dyer (1959). Methanol and chloroform at a ratio of 1:2 (v/v) was added to BSG at a ratio of 15:1 (w/v). The suspension was stirred for 1 hour at room temperature. The extracted lipids were removed by vacuum filtration and the BSG residue was oven dried at 60°C. This procedure resulted in the removal of 90-95% of the lipids in BSG. Defatted BSG was split into triplicate for protein extraction.

BSG - delignification

Delignification of BSG was performed according to the method of Prado et al. (2013). BSG was mixed with a 60% aqueous ethanol solution at a ratio of 1:4 (w/v) and the suspension heated under reflux at 180°C for 90 min. The delignified BSG fraction was recovered by vacuum filtration and the solid residue was washed three times with 60% ethanol and oven dried at 60°C. The delignified BSG was divided into triplicate and used for protein extraction.

Alkaline protein extraction of BSG

Protein from BSG was solubilised and recovered as protein rich isolates using alkaline extraction followed by isoelectric precipitation (Lam et al. 2018). Sodium hydroxide (pH 12.4, 0.01 M) was used for extraction, and the solubilised proteins were precipitated at pH 4.5. To separate the liquid and solid fraction, the extract was centrifuged for 15 min at 3871 x *g* at 25°C (3-18 KS, Sigma, Germany).

The experimental work was divided into two parts to optimise the extraction conditions and to determine the influence of several factors on protein yield and content. The first part investigated the following parameters on the protein content and yield: temperature and time, agitation mode during extraction, precipitation salinity and isolation mode of extracted proteins (Figure 1A). The second part considered how the protein content and yield were affected by repeated extraction and different pre-treatments including delignification, defatting and a combination of these (Figure 1B). All protein extractions were performed in triplicate.

Optimisation - temperature and time

A two factorial experiment was designed with three temperatures (30, 45 and 60°C) and four extraction times (30, 60, 120 and 180 min). For each temperature, the effect of the different extraction times was evaluated.

Optimisation - salinity and precipitation

The influence of salinity on isoelectric precipitation was evaluated by comparing protein isolates obtained from precipitation of proteins from a sodium chloride (0.5 M) enriched supernatant against a control (water). Prior to precipitation, the salinity of the supernatant was adjusted by addition of NaCl.

Protein isolation

Two methods for obtaining protein isolates from the liquid extracts were evaluated: freeze drying and isoelectric precipitation. Besides quantifying the protein content and yield, the recovery of non-protein nitrogen (NPN) and amino acids was determined for both approaches.

Agitation

Protein extractions were carried out using orbital shaking or magnetic stirring. Orbital shaking was in a SW22 shaking water bath (Julabo, Germany) at a speed of 150 rpm. Magnetic stirring was performed on an AREX Digital PRO Hot Plate Stirrer (VELP Scientifica, Italy) at a speed of 1000 rpm.

Repeated extraction and pre-treatment

Before protein extraction, BSG was subject to three chemical pre-treatments including delignification, defatting and a combination of the two. Additionally, extraction of the pretreated material

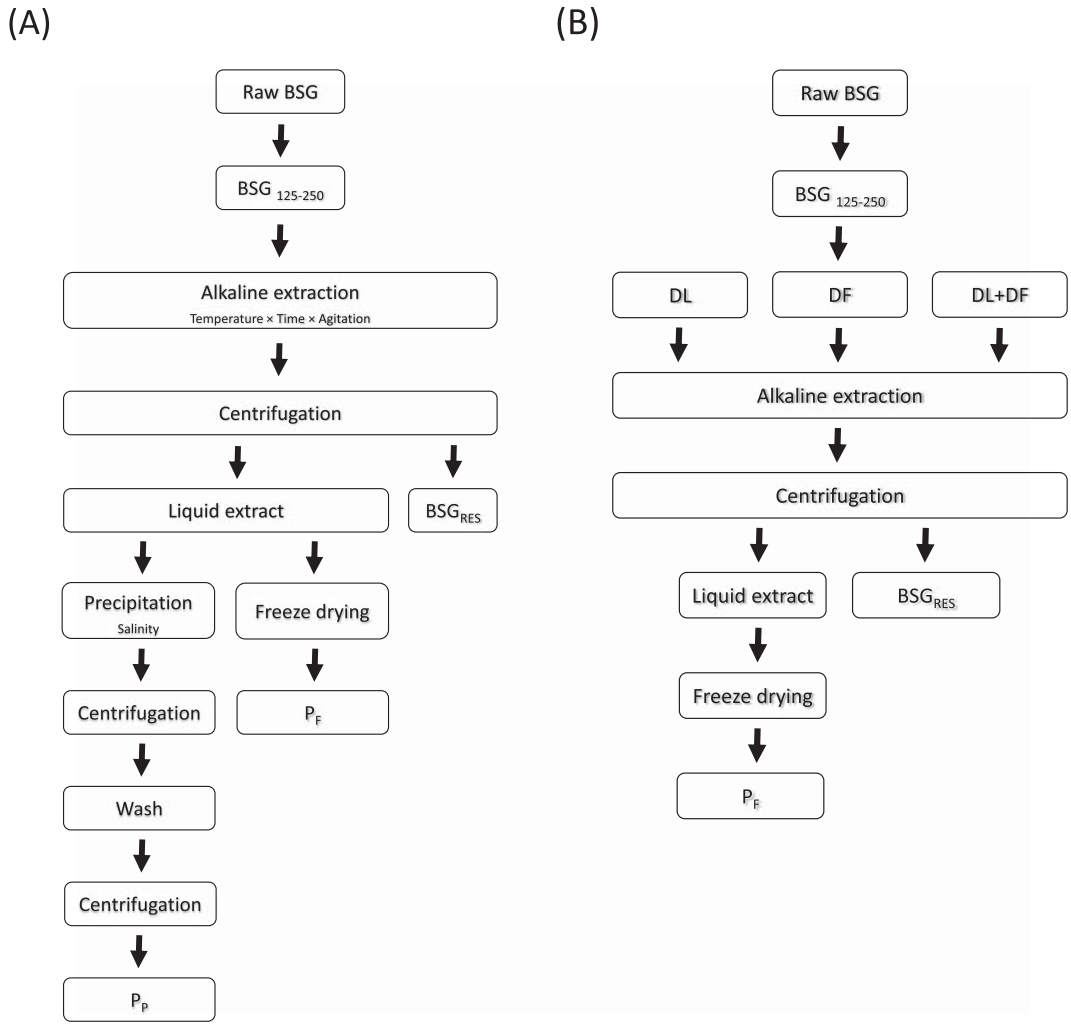


Figure 1. Experimental design of part 1 (panel A) and part 2 (panel B). BSG_{RES}: solid residue of extracted brewer's spent grain (BSG) obtained after alkaline extraction, centrifugation and drying. P_P: protein isolate obtained by isoelectric precipitation. P_F: protein isolate obtained by freeze-drying of the liquid extract. DL: delignification of BSG. DF: defatting of BSG. $BSG_{125\text{-}250}$: micronised BSG fraction used in protein extraction where the numbers in lowercase refer to the particle size of BSG.

and control (untreated BSG) was performed three times to assess how repeated extraction affects the protein yield and content.

Protein content

The concentration of protein produced by extraction of BSG is expressed as a percentage of the dry matter. Crude protein content (C_P) was determined from the nitrogen content measured with the Kjeldahl method while total amino acid protein (AA_P) - also referred to as the true protein content - was based on the sum of the amino acids. The protein content was calculated by multiplying the Kjeldahl nitrogen by a conversion factor of 6.25.

Amino acid content

Amino acids were quantified using the standard procedure described by Otter (2012). Amino acids were liberated from protein by acid hydrolysis with 1.8 mL 6M HCl with 0.2% phenol, for 24 hours at 110°C. Following hydrolysis, 100 µL 30 mM norvalin (internal standard) was added and HCl was removed by evaporation in a RVC 2-18 vacuum centrifuge from CHRIST (Germany) at 210 x *g*. The concentrated sample were resuspended in 3 mL 0.01 M HCl solution and 0.7 mL centrifuged for 10 min at 3000 x *g*. The supernatant was diluted 10-fold and the amino acids were derivatised (Cohen, 1994) with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) to yield short term stable, UV-active amino acid-AQC adducts. Amino acid derivatisation was performed using the AccQ•Tag Ultra Derivatization Kit (Waters, Milford, Massachusetts). The amino acid derivatives were separated and quantified using reverse-phase liquid chromatography with UV detection at 260 nm (Waters ACQUITY UltraPerformance liquid LC (UPLC)). Separation was performed on an ACCQ-TAG ULTRA C18, 2.1x100 mm, 1.7 µm column using water and acetonitrile as eluent systems. A gradient of 0-60% acetonitrile over 10 min and a flow rate of 0.7 mL/s was applied for separation of amino acids.

Protein yield

Protein yield (Y_P) measures the proportion of protein recovered from the original material after extraction. In this study, two Y_P types have been integrated for assessment of protein extraction under different conditions: a Kjeldahl based yield ($Y_{P,\ Kjeldahl}$) and an amino acid-based yield ($Y_{P,\ AA}$). The $Y_{P,\ Kjeldahl,}$ which includes

protein derived nitrogen and non-protein nitrogen (NPN) is calculated by formula (1):

$$Y_{P,\ Kjeldahl} = \frac{m_{Kjeldahl\ protein,\ isolate\ (g)}}{m_{Kjeldahl\ protein,\ BSG\ (g)}} \cdot 100\%$$

$m_{Kjeldahlprotein}$ is the mass of Kjeldahl protein in isolates or the micronised BSG sample before extraction.

The $Y_{P,\ AA}$ is the sum of amino acids present in the isolate and excludes the NPN component. In this work, the two different amino acid-based yields are designated $Y_{P,\ AA1}$ and $Y_{P,\ AA2}$. The $Y_{P,\ AA1}$ estimates the amount of extracted protein nitrogen relative to the total amount of extractable nitrogen in micronised BSG according to formula (2):

$$Y_{P,\ AA1} = \frac{m_{AA\ protein,\ isolate\ (g)}}{m_{Kjeldahl\ protein,\ BSG\ (g)}} \cdot 100\%$$

The $Y_{P,\ AA2}$ is used to quantify the proportion of extractable protein nitrogen recovered from micronised BSG during extraction and is calculated from formula (3):

$$Y_{P,\ AA2} = \frac{m_{AA\ protein,\ isolate\ (g)}}{m_{AA\ protein,\ BSG\ (g)}} \cdot 100\%$$

Amino acid yield

The yield of individual amino acids (Y_{AA}) was calculated using the formula (4) as the amount of extracted amino acid in the isolate relative to the amount that can be extracted from micronised BSG:

$$Y_{AA} = \frac{m_{AA,\ isolate\ (g)}}{m_{AA,\ BSG\ (g)}} \cdot 100\%$$

Statistics

All statistical analyses were performed using the open source software R version 3.5.1 (R Core Team, 2018) while graphical illustrations were constructed using the platform SigmaPlot 14.0. A significance level of 0.05 was applied to all statistical tests in this study. To evaluate the impact of time, temperature and their interactions, the data was subjected to a two-way analysis of variance while the different treatment combinations were compared by a pairwise t-test. The influence of salinity on isoelectric precipitation was evaluated using a t-test. Statistical evaluation of the impact of isolation method on protein content and yield was performed using a t-test whereas the effect on amino acid yields was evaluated by a two-way analysis of variance with amino acid type and isolation mode as the explanatory variables. The effect of pre-treatment was evaluated by a one-way analysis of variance. In addition, the amino acid recovery was examined for protein isolates produced by extraction of defatted BSG and a control to determine the effect of defatting on amino acid extractability. A two-way analysis of variance was performed using amino acid type and pre-treatment (control and defatting) as independent variables.

Results and discussion

Chemical composition of BSG

The amino acid composition was determined for non-micronised and micronised BSG (125-250 μm) to validate the compositional similarities between these BSG fractions. This analysis revealed that micronised and non-micronised BSG from the same batch (B1) were similar in protein and lipid content (Table 1). Although micronisation was employed to increase the extraction efficiency of proteins from BSG, separation of the different size fractions led to losses of micronised material (30-50%). Although this could be overcome by extracting micronised BSG containing all size fractions from the micronisation process, this was not progressed any further. A comparison of the three size fractions suggested that the protein content decreased with increasing particle size. For the smallest fraction (<125 μm), a protein content of about 40% was measured whereas the largest fraction (>250 μm) had a protein content of about 15%. Because of the high dust of the small size fraction and low protein content of the large size fraction, these fractions were less suitable for protein extraction. Accordingly, the medium size fraction (125-250 μm) was use for extractions in this study.

The amino acid composition of BSG reflects its nutritional quality as a protein source for feed applications, and it may prove useful in predicting the amino acid content of BSG derived protein isolates. As shown in Table 1, the micronised BSG contains high levels of glutamic acid (5.31 ± 0.33 g/100 g DM), proline (2.46 ± 0.13 g/100 g DM) and aspartic acid (1.75 ± 0.11 g/100 g DM) while it is deficient in methionine (0.31 ± 0.04 g/100 g DM). Consequently, alkaline extraction of micronised BSG may generate protein isolates rich in glutamate, proline and aspartate but may not be an ideal source of methionine. Moreover, the amino acid composition reported here is consistent with the previous studies presented in Table 1 (Gmoser et al, 2020; Gutiérrez-Barrutia et al, 2022; Oluseyi et al, 2011; Waters et al, 2012; Yu et al, 2020). Due to its thermal instability, tryptophan is susceptible to breakdown when exposed to high temperatures (Cuq et al, 1983) and therefore lost in the hydrolysis conditions used for amino acid analysis. As a result, the true protein content (AA_P) will be underestimated while the NPN content will be overestimated. Waters et al (2012) found that BSG had a relatively low content of tryptophan and therefore the underestimation of AA_P may be of minor concern. Proteins and amino acids may also degrade with autoclaving of the raw material owing to the high temperature. To determine whether these components deteriorate during autoclaving, the composition of autoclaved BSG were compared with that without autoclaving. The results showed that autoclaved and non-autoclaved BSG had a similar content of protein and amino acids indicating that autoclaving did not result in any compositional changes in the BSG (data not shown).

Optimisation of protein extraction

Temperature and time. A nested design was employed to explore the single and combined effects of time and temperature on Kjeldahl based protein yield ($Y_{P,\ Kjeldahl}$) and content (C_P). The two-way analysis of variance indicated that time and temperature significantly affected both protein yield and content ($p<0.001$) while the combined effect of these variables appeared to be insignificant ($p_{yield} = 0.256$, $p_{content} = 0.729$). Protein content decreased with increasing temperature for all extraction times (Figure 2A).

Table 1. Chemical composition of micronised and non-micronised brewer's spent grain (BSG) and comparison of amino acid composition from this study with that from previous publications. All measurements were in duplicate, and results reported as mean ± SD.

	Micronised BSG*	Non-micronised BSG*	Previous studies‡
Composition			
Dry matter (DM) (%)	93.5 ± 0.00	95.9 ± 0.00	-
Ash (%)	3.67 ± 0.03	3.66 ± 0.00	-
Lipids (%)	10.5 ± 0.08	9.90 ± 0.06	-
C_P (%)[a]	25.2 ± 0.11	24.1 ± 0.31	-
AA_P (%)[b]	22.2 ± 1.03	20.9 ± 0.61	-
NPN (%)[c]	3.03 ± 0.92	3.10 ± 0.91	-
Essential amino acids (g/100 g DM)			
Phenylalanine (Phe)	1.24 ± 0.07	1.23 ± 0.04	1.03 - 1.59
Histidine (His)	0.48 ± 0.03	0.45 ± 0.01	0.50 - 5.81
Valine (Val)	1.22 ± 0.06	1.13 ± 0.03	1.02 - 1.45
Isoleucine (Ile)	0.96 ± 0.07	0.90 ± 0.03	0.73 - 1.11
Leucine (Leu)	1.76 ± 0.08	1.64 ± 0.04	1.35 - 3.19
Methionine (Met)	0.31 ± 0.04	0.26 ± 0.03	0.40 - 0.59
Threonine (Thr)	0.87 ± 0.04	0.79 ± 0.01	0.16 - 1.11
Lysine (Lys)	0.93 ± 0.04	0.75 ± 0.00	0.67 - 3.17
Non-essential amino acids (g/100 g DM)			
Arginine (Arg)	1.12 ± 0.01	1.01 ± 0.01	0.50 - 1.22
Alanine (Ala)	1.10 ± 0.49	0.97 ± 0.01	0.80 - 2.05
Aspartate (Asp)	1.75 ± 0.11	1.54 ± 0.01	1.06 - 1.95
Glutamate (Glu)	5.31 ± 0.33	5.28 ± 0.25	3.67 - 5.36
Glycine (Gly)	0.89 ± 0.04	0.80 ± 0.00	0.39 - 1.08
Serine (Ser)	1.04 ± 0.06	0.98 ± 0.03	0.83 - 1.46
Tyrosine (Tyr)	0.70 ± 0.00	0.67 ± 0.03	0.57 - 0.94
Cysteine (Cys)	0.00 ± 0.00	0.00 ± 0.00	0.00 - 0.49
Proline (Pro)	2.46 ± 0.13	2.48 ± 0.11	1.40 - 2.99
Cystine (Csn)	0.10 ± 0.00	0.10 ± 0.01	-

* Values are based on micronised or non-micronised brewer's spent grain (BSG) from batch 1. The micronised BSG had a particle size of 125-250 μm.

‡ Amino acid composition of BSG from previous studies, presented as a range of the values reported by Gmoser et al (2020), Gutiérrez-Barrutia et al (2022), Oluseyi et al (2011), Waters et al (2012) and Yu et al (2020).

[a] Kjeldahl protein calculated by multiplying the Kjeldahl nitrogen by 6.25.

[b] True protein content calculated as the sum of all measured amino acids and excludes any non-protein nitrogen (NPN) found in the Kjeldahl protein.

[c] NPN is the non-protein nitrogen calculated as the difference between Kjeldahl protein and true protein.

Conversely, a positive correlation was observed between protein yield and temperature at 30 minutes, although not statistically significant, while no general trend was found at 60, 120 or 180 minutes (Figure 2B). The inferred relationship between temperature and protein yield could suggest that protein solubilisation increased as the temperature was raised. Within all temperature groups, a declining tendency was observed in protein content and yield when extraction times were prolonged from 30 to 120 and 180 minutes. For protein yield, this trend may reflect a higher degree of complete protein hydrolysis resulting in the loss of protein derived amino acids during isoelectric precipitation. In contrast, the tendency of reduced protein content may stem from the enhanced solubilisation of lignin at higher temperatures and longer extraction times as lignin is partly soluble in dilute alkaline solutions (Melro et al, 2020). This was supported by the visual appearance of the protein isolates as increased extraction time and temperature yielded darker material. These impurities may originate from co-isolated lignin and/or lignin-hemicellulose adducts.

The impact of time and temperature on protein yield are in agreement with Qiaoyun et al (2017) and Surasani et al (2017), where protein yield was positively correlated with time and temperature within a specific range, above which the yield would decrease or remain constant. Further, much of the variability was attributable to factors that were not accounted for in the statistical model. One possible explanation is that protein isolates are produced in a multiple step process which may increase the degree of uncertainty. Moreover, the high residual variation can also be explained by minor fluctuations in pH during isoelectric precipitation which may influence the amount of precipitated protein. This is supported by Chavan et al (2001) who reported that small pH deviations from the isoelectric point affect the protein solubility and the efficiency of isolation. With respect to the protein yield, a combination of 60°C and 30 minutes was the most efficient while the

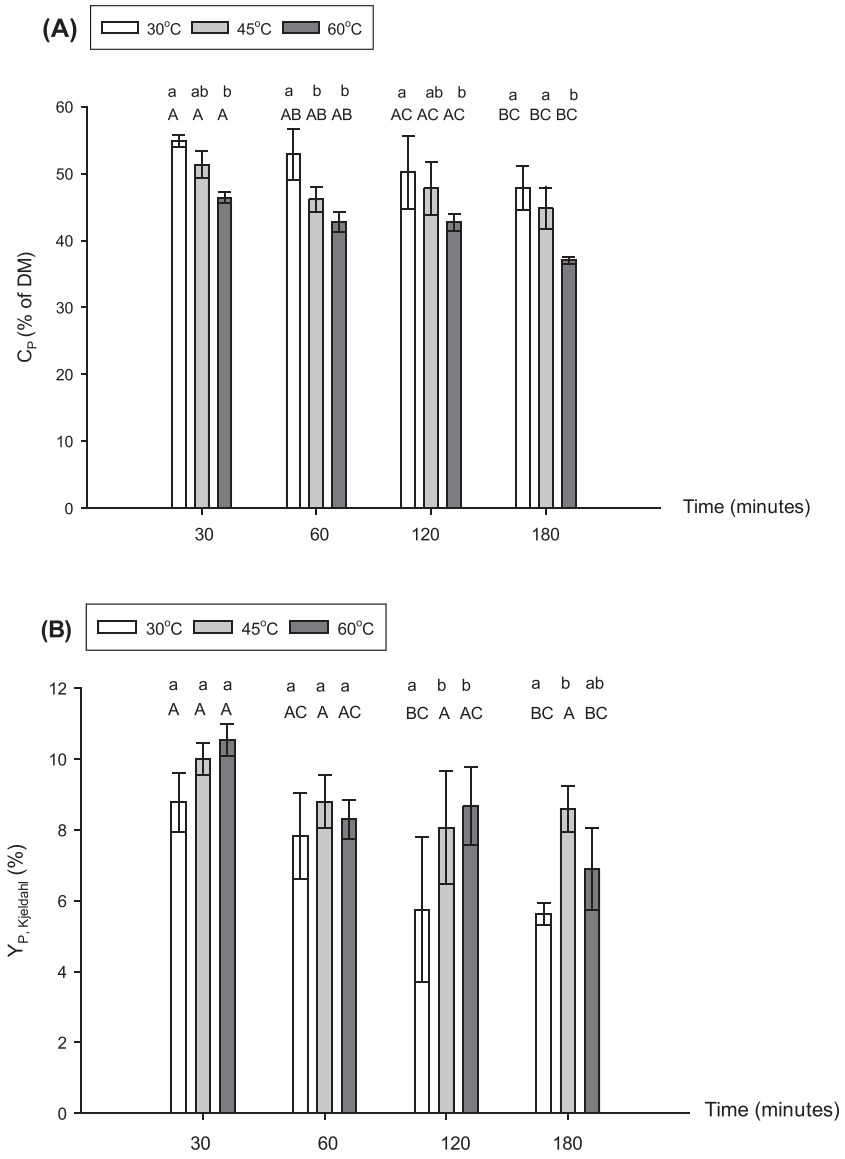


Figure 2. Effect of temperature and time on Kjeldahl-based protein content of isolates (C_P) (A) and Kjeldahl-based protein yield ($Y_{P, Kjeldahl}$) (B) of brewer's spent grain (BSG). Within each time group, three different temperatures were evaluated. Uppercase and lowercase letters are used to designate significant differences between and within different time groups, respectively. C_P = Kjeldahl N (%)·6.25 and $Y_{P, Kjeldahl} = m_{Kjeldahl\ protein,\ isolate\ (g)}/m_{Kjeldahl\ protein,\ BSG\ (g)}\cdot 100\%$. All treatment combinations were tested in triplicate and the results reported as mean±SD.

protein content in the extract was maximised using a temperature of 30°C and an extraction time of 30 minutes. Interestingly, all combinations of temperature and time led to low protein yields (6-10%), although the protein content was relatively high in all isolates (37-55%). There are several explanations for this including: 1) loss of water soluble proteins during washing of the precipitate, 2) complete hydrolysis of proteins to amino acids resulting in a loss of protein derived amino acids during isoelectric precipitation or 3) poor protein extractability caused by interactions with other macromolecules in the BSG matrix. To optimise the protein recovery and identify possible causes of the low protein yield, five optimisation experiments were carried out at a common temperature of 60°C and an extraction time of 30 minutes.

Agitation and salinity. To examine the effect of agitation on protein yield and content, extractions were performed with stirring or orbital shaking. Initially, it was thought that stirring would be more effective than orbital shaking, as stirring may ensure constant exposure of proteins to the extraction solvent. However, the results show that both protein yield and content were unaffected by the type of agitation (p_{yield}=0.629 *and* $p_{content}$=0.847) (Supporting Information, Figure S1). This is in agreement with the findings from Crowe et al (1985) who reported that equal amounts of nitrogen were solubilised from BSG when shaking and stirring were employed. Also, Surasani et al (2017) reported that the velocity and time of stirring did not adversely affect the protein yield and content; although protein solubilisation was maximised with continuous stirring. Based on the principles of the salting-out technique, which takes advantage of the reduced solubility of proteins at higher salt concentrations, it was thought that protein precipitation could be enhanced by increasing the salinity of the liquid extract. This was tested by precipitating out proteins from the liquid extract by addition of 0.5 M sodium chloride. Contrary to the initial hypothesis, this approach showed a marked

reduction in protein yield and content when isoelectric precipitation was performed with a saline extract as compared to the control (Figure 3). It is possible that protein solubility is influenced by an interplay of external factors including medium pH and salinity, protein structure and the amino acid composition of the proteins. This was demonstrated by Sousa et al (2007) who reported that the protein solubility of egg derived proteins was largely dependent on salinity, pH and the type of salt. Similarly, Chavan et al (2001) reported that the solubility of proteins extracted from peas increased with increasing NaCl concentrations near the isoelectric point. This may explain the reduced protein yield observed when proteins were precipitated from a saline extract.

Protein isolation. The low protein yield observed could also be due to a loss of water soluble proteins during the washing step or to the generation of free amino acids by protein hydrolysis. Owing to differences in the isoelectric points, amino acids and proteins may have different solubilities at the pH value (4.5-5) used for precipitation. As a result, the released amino acids may be retained in the liquid extract during precipitation. To establish whether the poor recovery was due to protein loss or protein hydrolysis, proteins were concentrated from the liquid extract by freeze drying (Figure 1B). The protein isolates generated by precipitation and freeze drying were compared with respect to their protein content, protein yields and recovery of NPN (Figure 4). The crude protein content reported in Figure 4A, showed that freeze drying led to a significantly lower protein content compared to precipitation

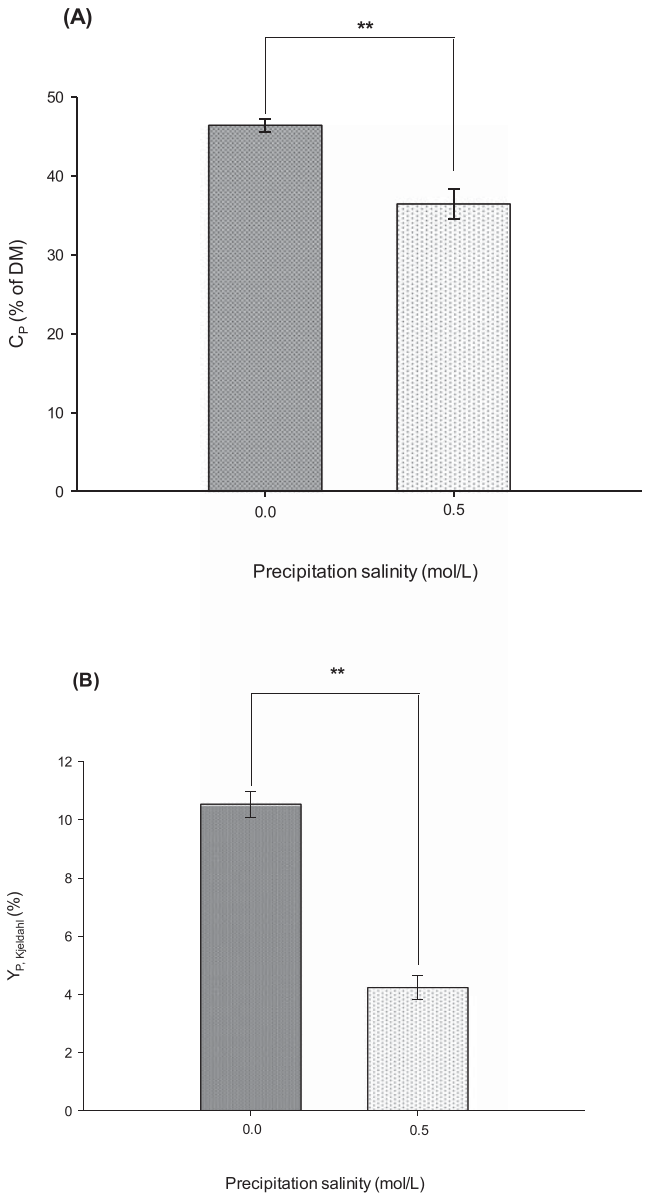


Figure 3. Effect of precipitation salinity on Kjeldahl-based protein content of isolates (C_P) (A) and yield ($Y_{P,\ Kjeldahl}$) (B). Double asterisks (**) indicate that groups are significantly different. Results are presented as mean±SD.

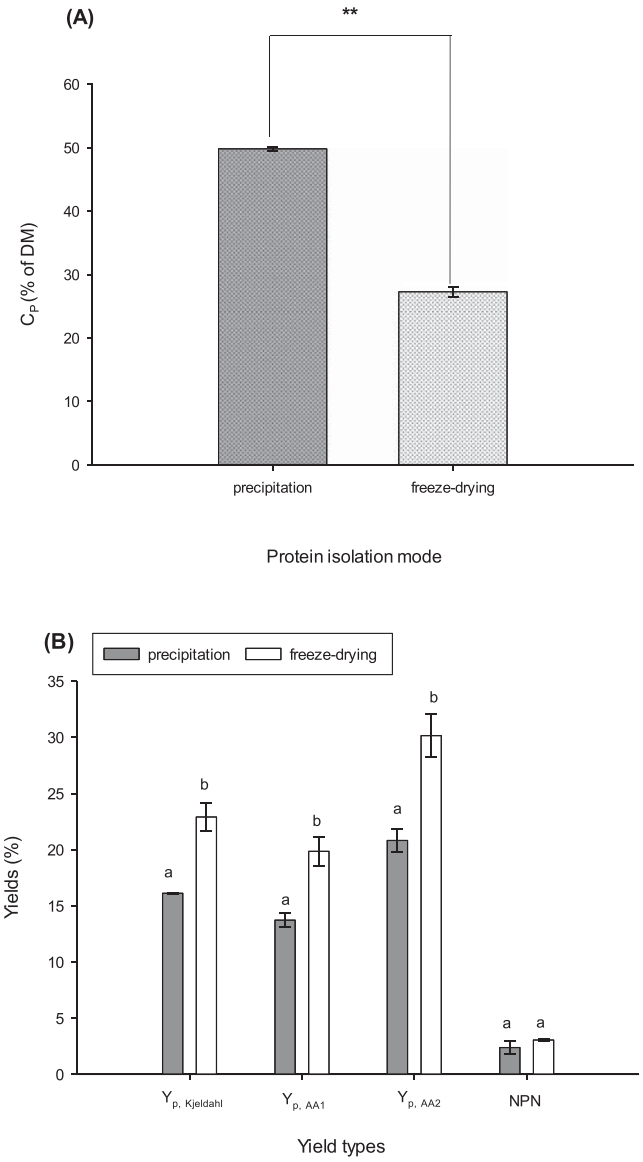


Figure 4. Effect of protein isolation mode on protein content of isolates and extraction yield from brewer's spent grain (BSG). A: Kjeldahl-based protein content (C_P) of isolates obtained by isoelectric precipitation and freeze-drying. B: Three different protein yields and recovery of non-protein nitrogen (NPN) for isolates generated by isoelectric precipitation and freeze-drying. Protein yields and NPN were calculated by the following formulas: $Y_{P,\ Kjeldahl} = m_{Kjeldahl\ protein,\ isolate\ (g)}/m_{Kjeldahl\ protein,\ BSG\ (g)} \cdot 100\%$, $Y_{P,\ AA1} = m_{AA\ protein,\ isolate\ (g)}/m_{Kjeldahl\ protein,\ BSG\ (g)} \cdot 100\%$, $Y_{P,\ AA2} = m_{AA\ protein,\ isolate\ (g)}/m_{AA\ protein,\ BSG\ (g)} \cdot 100\%$, $NPN = Y_{P,\ Kjeldahl} - Y_{P,\ AA1}$. Lowercase letters indicate significant differences in protein and NPN recovery obtained by the two different methods. Data is from triplicates and presented as mean± SD.

($p<0.001$). A likely cause of this is the occurrence of co-extracted impurities in the liquid extract which remain in the isolate after freeze drying.

To evaluate the effect of isolation method on protein and NPN recovery, two different yield types were determined (Figure 4B: 1) with the crude protein yield ($Y_{P,\ Kjeldahl}$) and 2) the true protein yield ($Y_{P,\ AA}$). Further, the true protein yield was measured as the extracted amount of protein relative to the total nitrogen ($Y_{P,\ AA1}$) and the total protein ($Y_{P,\ AA2}$) in BSG. The results presented in Figure 4B indicated that the crude protein yield was significantly higher for isolates produced by freeze drying compared with precipitation ($p_{Y_{P,\ Kjeldahl}} = 0.0113$).

To establish whether this observation was due to the enhanced extraction of proteins, NPN or both, the true protein yield ($Y_{P,\ AA1}$ and $Y_{P,\ AA2}$) and NPN recovery were determined and compared for isolates obtained from both isolation methods. With the crude protein yield, the true protein yield increased significantly when freeze drying was used instead of precipitation ($p_{Y_{P,\ AA1}} = 0.00548$ and $p_{Y_{P,\ AA2}} = 0.00453$). Another interesting finding was that the NPN recovery did not differ significantly between the two isolation modes ($p_{NPN} = 0.193$) which implies that the isolation method only influences the recovery of protein derived nitrogen.

The amino acid yield (Y_{AA}) was used for assessing the degree of protein hydrolysis under the extraction conditions (Figure 5). Here, a substantial increase in Y_{AA} was detected for amino acids when freeze drying was used for protein isolation. This suggests that a greater proportion of proteins are hydrolysed to amino acids when extraction was at 60°C for 30 minutes. Increasing the temperature above 60°C is thought to enhance the hydrolysis of proteins, leading to a further reduction in protein recovery. Hence, increased protein hydrolysis may be an underlying cause of the decreasing protein yield observed at prolonged extraction times (Figure 2B). In the light of this, future research should focus on the effect of time and temperature on protein depolymerisation during extraction. Although freeze drying is associated with higher protein and amino acid recoveries, this method is not suitable for producing protein isolates for feed applications due to the low protein content of the resulting isolates. However, for the purpose of optimising protein recovery, this approach was used in subsequent experiments in this study.

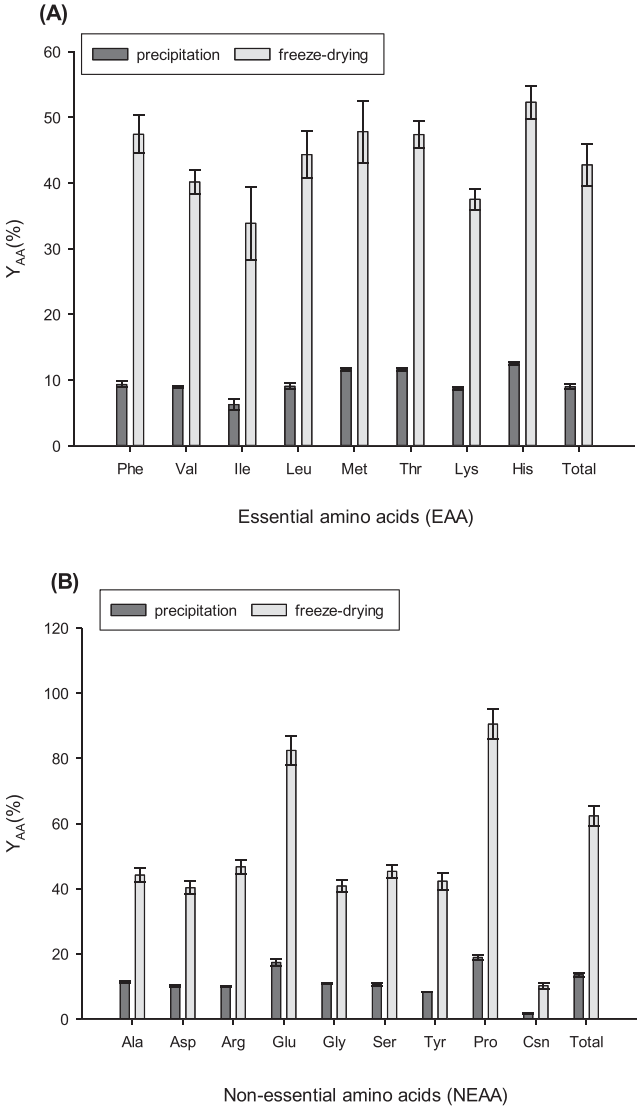


Figure 5. Yields of essential (A) and non-essential (B) amino acids recovered by isoelectric precipitation and freeze-drying of liquid extracts from brewer's spent grain (BSG). The yield of all amino acids differed significantly between the two isolation methods ($p<0.05$) (indicators of significance were omitted for clarity). The total amino acid recovery, which reflects the recovery of the true protein, is calculated by the formula: $Y_{AA,\ total} = \Sigma m_{AA,\ isolate\ (g)} / \Sigma m_{AA,\ BSG\ (g)} \cdot 100\%$. Extractions were carried out in triplicate and the results are presented as mean±SD.

Repeat extraction and pre-treatment. Extraction of proteins from a heterogeneous biomass such as BSG may be restricted by intermolecular interactions between proteins and lipids, carbohydrates and lignin. These associations can lead to the formation of clusters which may impair protein extraction as the proteins are less accessible to solvent molecules. Previous studies have focused on describing the effect of carbohydrate-protein interactions on the extractability of BSG proteins. For instance, Crowe et al (1985) found that cellulase aided degradation of cellulose led to a seven-fold increase in the protein yield from alkaline extraction suggesting that proteins form clusters with the structural carbohydrates of BSG.

In the current study, possible interactions of protein with lipid and lignin were examined to determine whether the low protein recovery resulted from macromolecular interactions. Accordingly, BSG was subjected to three pre-treatments before protein extraction: 1) defatting, 2) delignification and 3) defatting and delignification. In addition, the effect of repeat extraction was evaluated for pretreated and untreated BSG by exposing the same biomass to three consecutive extractions. When repeated extraction was applied, the total protein yields were increased by about 50% compared to a single extraction (Figure 6B). Moreover, similar amounts of biomass were extracted in all three treatments although the yield of crude protein, true protein and NPN decreased with each extraction (Figure S2). It is likely that greater amounts of lignin and carbohydrate are co-extracted by the second and third extraction as most of the protein was recovered during the first extraction. This work was qualitative, and more studies are needed to determine the magnitude of co-extraction.

The one-way analysis of variance indicated that pre-treatment had significant impact on the crude protein yield and content ($p<0.05$). Furthermore, a pairwise analysis revealed that the three pre-treatments had different effects on protein yield and content (Figure 6). The effect of delignification on the total protein yield was insignificant while the protein content was significantly higher in the extract from delignified BSG compared to the control. That protein yields are unaffected by delignification, suggests that

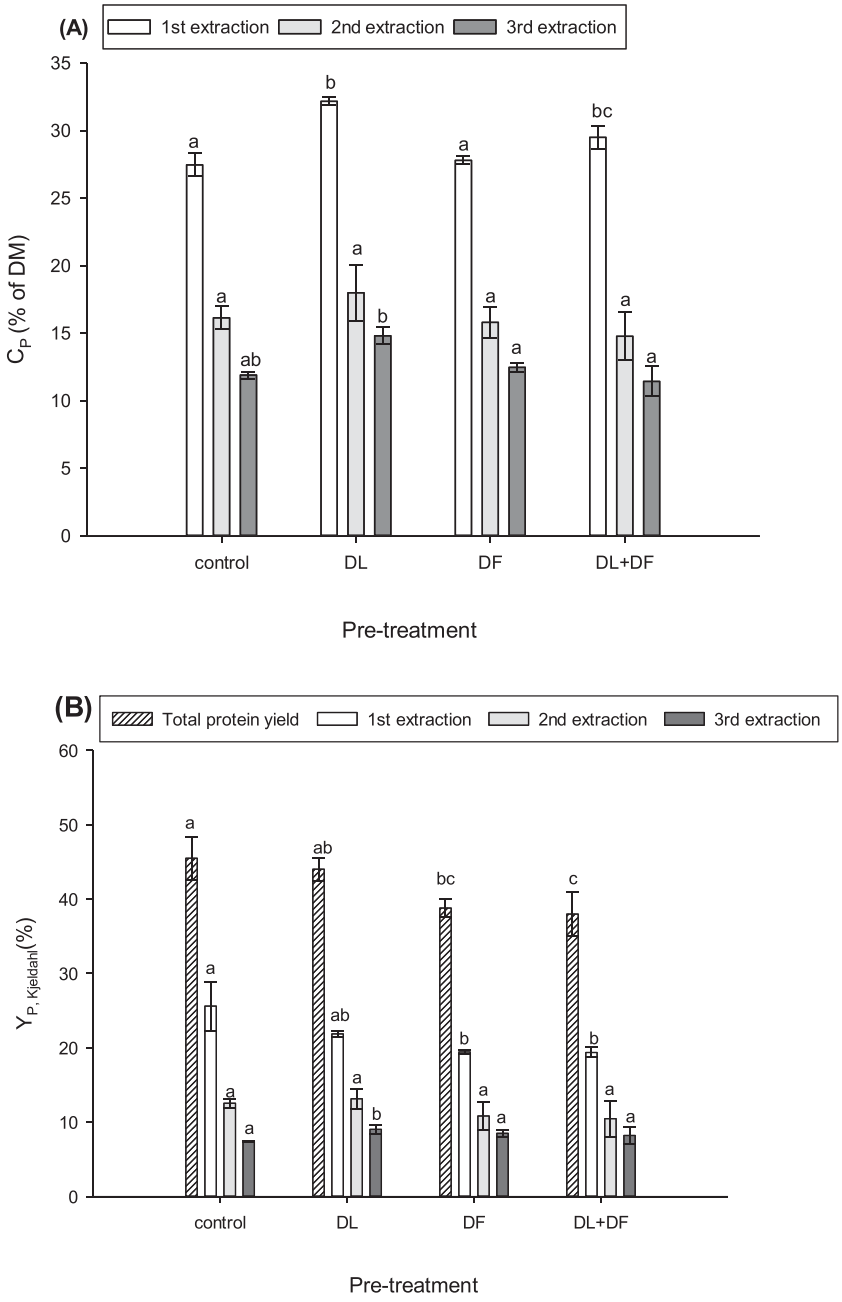


Figure 6. Effect of pre-treatment and repeated extraction on Kjeldahl-based protein content of isolates (C_P) (A) and Kjeldahl-based protein yield ($Y_{P,\ Kjeldahl}$) (B). For each pre-treatment group, extractions were performed three times and compared to a control experiment in which untreated brewer's spent grain (BSG) was extracted three times. Lowercase letters are used to denote significant differences between the pre-treatment groups within the same extraction. Abbreviations: DL = delignification, DF = defatting, DL+DF = defatting and delignification. Results are presented as mean±SD.

lignin and proteins do not interact through intermolecular forces. The lack of protein-lignin interactions is probably a result of protein denaturation during alkaline extraction; a theory supported by Salas et al. (2013) who demonstrated that interactions between lignin and proteins are weakened when proteins are denatured. In contrast, the higher protein content may result from reduced co-extraction of lignin and lignin-hemicellulose adducts, as most of these compounds have been removed during the delignification process. The protein content appeared to be unaffected by defatting suggesting that BSG-bound lipids are not solubilised during alkaline extraction. In addition, a significant reduction was observed in the total protein yield when proteins were extracted from defatted BSG. An explanation for this is that water soluble proteins in BSG are dissolved by the extraction solvent due to their moderate solubility in methanol. Alternatively, the chloroform-methanol solvent for defatting may induce structural changes in the protein which may reduce protein extraction in alkaline solution.

To establish whether the reduced protein yield was due to impaired protein extraction or protein loss during defatting, the amino acid yields (Y_{AA}) were quantified for each of the three extractions (Table 2). Data from the first and second extraction showed that the total amino acid yield, as well as those of individual amino acids, were reduced by defatting. Following the third

Table 2. Comparison of amino acid yields (Y_{AA}) by extraction of defatted and untreated (control) brewer's spent grain (BSG). All extractions were carried out in triplicate and yields are reported as mean ± SD*.

Amino acid **	First extraction		Second extraction		Third extraction	
	$Y_{AA(control)}$ (%)	$Y_{AA(defatted)}$ (%)	$Y_{AA(control)}$ (%)	$Y_{AA(defatted)}$ (%)	$Y_{AA(control)}$ (%)	$Y_{AA(defatted)}$ (%)
Phe	21.9 ± 3.0^a	15.8 ± 2.6^a	9.5 ± 0.5^a	9.0 ± 1.2 ^{a}	5.9 ± 0.2^a	7.0 ± 0.3^b
Val	26.9 ± 4.7^a	20.3 ± 1.0^a	14.1 ± 0.9^a	11.7 ± 2.6^a	9.3 ± 0.2^a	11.0 ± 0.5^b
Ile	20.5 ± 1.0^a	17.5 ± 0.5^b	7.6 ± 0.5^a	8.5 ± 0.9^a	4.8 ± 0.2^a	5.8 ± 0.2^b
Leu	25.6 ± 4.5^a	17.7 ± 2.6^a	12.3 ± 0.7^a	10.9 ± 2.1^a	8.1 ± 0.2^a	9.5 ± 0.5^b
Met	33.5 ± 3.1^a	36.8 ± 10.7^a	13.7 ± 0.7^a	11.5 ± 2.4^a	8.6 ± 0.5^a	10.4 ± 1.2^a
Thr	31.6 ± 7.3^a	20.0 ± 4.5^a	15.3 ± 1.4^a	13.0 ± 2.4^a	9.5 ± 0.2^a	10.8 ± 0.5^b
Lys	26.2 ± 4.2^a	16.8 ± 2.3^b	11.7 ± 1.0^a	9.7 ± 1.9^a	7.1 ± 0.2^a	8.1 ± 0.3^b
Arg	27.6 ± 6.4^a	16.7 ± 3.8^a	12.4 ± 0.9^a	10.4 ± 1.6^a	8.4 ± 0.5^a	9.0 ± 0.7^a
His	33.6 ± 7.8^a	22.0 ± 5.4^a	17.5 ± 2.6^a	15.0 ± 3.1^a	10.9 ± 0.3^a	12.8 ± 0.5^b
Ala	32.9 ± 7.6^a	20.4 ± 4.8^a	17.2 ± 1.4^a	14.1 ± 1.7^a	10.8 ± 0.2^a	12.6 ± 0.5^b
Asp	29.8 ± 7.1^a	18.2 ± 4.1^a	14.9 ± 1.0^a	12.3 ± 2.6^a	9.4 ± 0.2^a	10.8 ± 0.5^b
Glu	44.4 ± 10.4^a	27.7 ± 5.7^a	21.1 ± 0.9^a	17.2 ± 2.8^a	11.7 ± 0.5^a	12.8 ± 0.7^a
Gly	29.6 ± 6.8^a	19.8 ± 5.0^a	15.5 ± 1.4^a	13.6 ± 2.8^a	10.0 ± 0.3^a	12.0 ± 0.5^b
Ser	29.0 ± 6.8^a	18.1 ± 3.6^a	14.8 ± 1.2^a	12.6 ± 2.6^a	9.7 ± 0.2^a	11.0 ± 0.5^b
Tyr	21.8 ± 2.6^a	16.5 ± 0.7^b	9.5 ± 0.7^a	8.3 ± 1.6^a	6.2 ± 0.2^a	7.1 ± 0.3^b
Pro	44.4 ± 9.5^a	28.6 ± 6.1^a	20.5 ± 1.2^a	17.1 ± 2.3^a	10.7 ± 0.5^a	11.8 ± 0.7^a
Csn	6.9 ± 0.2^a	6.9 ± 2.8^a	2.0 ± 0.4^a	1.8 ± 0.5^a	1.0 ± 0.5^a	1.0 ± 0.5^a
Total***	**31.0^a**	**20.8^a**	**14.6^a**	**12.5^a**	**8.7^a**	**10.0 ^{b}**

* Yields of individual amino acids (Y_{AA}) are calculated by the equation: $Y_{AA} = m_{AA,\ isolate\ (g)}/m_{AA,\ BSG}\cdot 100\%$.

** Lowercase letters indicate significant differences between the two pre-treatment groups (defatted and control) within the same extraction.

*** The total amino acid yield from each extraction is calculated using the formula: $Y_{AA,\ total} = \Sigma m_{AA,\ isolate\ (g)}/\Sigma m_{AA,\ BSG\ (g)}\cdot 100\%$.

extraction, the total and individual amino acid yields appeared higher for isolates originating from defatted BSG. Overall, these results imply that defatting had a negative impact on the total amino acid yield from all three extractions, supported by the corresponding reduction in the crude protein yield. Comparing the amino acid composition of defatted and untreated BSG showed a lower amino acid content of defatted BSG compared to untreated BSG (Supporting Information, Table S1). This suggests that protein may be solubilised and lost during defatting with chloroform-methanol. When defatting and delignification were combined, a significant decrease was observed in the total protein yield which may be explained by defatting. On the other hand, the combined treatment had a positive impact on the crude protein content, probably due to delignification.

Conclusions

In the present study, solubilised proteins were either recovered by isoelectric precipitation or freeze drying. Each method had both shortcomings and benefits that need to be addressed in future work. When using isoelectric precipitation, the protein yield was generally low (5-10%) while the protein content in the isolates were high enough to be suitable for feed applications (40-55% DM) (Figure 2). Freeze drying resulted in a higher protein yield (25-30%) with the protein content similar to that of BSG before extraction (20-25%). Furthermore, when the same biomass was extracted three times, the total protein yield increased to 45% compared to 25% from the single extraction; though, the protein content from the second and third extraction were significantly lower compared to the first extraction (Figure 6A). Consequently, the isolates produced by freeze drying are likely to be of low nutritional quality and unsuitable for feed applications.

Previously, protein yields higher than 70% have been reported for alkaline extraction of BSG (Qin et al, 2018). Although the protein yield increased with freeze drying instead of precipitation, the maximum protein yield obtained in this study was significantly lower than the yields obtained in previous study. This suggests that the extraction efficiency is low and needs to be optimised. A likely cause of the low extraction efficiency is that the extraction pH (ca. 12.4) was not optimal for the solubilisation of protein from the BSG batches used here. According to previous work, extraction pH affects protein solubility, and therefore the extraction efficiency could be improved by raising the pH of the alkaline solution (Ruiz et al, 2016; Gao et al, 2020). Initially, a pH range of 8.3-12.4 was evaluated to determine the optimum extraction pH (data not shown). When the pH of the extraction solution was below 12.4, a rapid decline was observed in pH of the BSG suspension which led to low protein solubilisation and a low yield. This decrease may have been caused by lactic acid produced by native lactic acid bacteria in the BSG before autoclaving and suggests that better pH control is required to maintain pH at the desired level during protein extraction (Asurmendi et al, 2014). Furthermore, more alkaline conditions during extraction are associated with reduced purity of protein isolates which may have adverse implications for their use in feed (Ruiz et al, 2016). Therefore, an extraction pH $>$ 12.4 may reduce the nutritional quality of BSG protein.

The solid to liquid ratio (S/L) is also known to influence protein solubilisation during alkaline extraction. Previous studies have employed S/L ratios of 1:5 to 1:60 in the alkaline extraction of protein from different sources of biomass and have shown the optimum

S/L varies with the type of biomass (Vareltzis and Undeland, 2012; Surasani et al, 2017; Vilg and Undeland, 2017). In this study, a S/L ratio of 1:15 was utilised, however the protein yield could possibly be improved by increasing the extraction volume. The low protein yield obtained by isoelectric precipitation could be a result of inadequate precipitation at pH 4.5, as a slight shift in pH during precipitation may cause a significant loss of protein in the liquid fraction (Vilg and Undeland, 2017). Based on the findings of this study, future research should focus on identifying a combination of extraction pH, precipitation pH and S/L ratio that maximises the efficiency of extraction without reducing the quality of protein from BSG.

Author contributions

Freja Karlsen: experimental work, writing (original draft), visualisation and conceptualisation.
Peter Vilhelm Skov: writing (review and editing), funding acquisition and conceptualisation.
Ivar Lund: writing (review and editing).

Acknowledgements

This work was funded by NordForsk under the grant no. 82342. We would like to thank laboratory technicians Ulla Høgh Sproegel and Brian Møller for their great expertise on the experimental set-up and chemical analysis. Further, special thanks are given to Dr. Carlos Octavio Letelier-Gordo and PhD students Julie Hansen, Manon Eggink and Wanhe Qi for proofreading and feedback. Lastly, we thank Dr. Esther Savina for her advice on the statistical analysis and graphical presentation of data.

Conflict of interest

The authors declare there are no conflicts of interest.

References

Asurmendi P, Pascual L, Dalcero A, Barberis L. 2014. Incidence of lactic acid bacteria and *Aspergillus flavus* in brewer's grains and evaluation of potential antifungal activity of these bacteria. *J Stored Prod Res* 56:33-37. https://doi.org/10.1016/j.jspr.2013.11.002

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911-917.

Chavan UD, McKenzie DB, Shahidi F. 2001. Functional properties of protein isolates from beach pea (*Lathyrus maritimus* L.). *Food Chem* 74:177-187. https://doi.org/10.1016/S0308-8146(01)00123-6

Cohen SA. 1994. Applications of amino acid derivatization with 6-carbamate: analysis of feed grains, intravenous solutions and glycoproteins. *J Chromatogr A* 661:25–34. https://doi.org/10.1016/0021-9673(93)E0821-B

Connolly A, Cermeño M, Crowley D, O'Callaghan Y, O'Brien NM, FitzGerald RJ. 2019. Characterisation of the in vitro bioactive properties of alkaline and enzyme extracted brewers' spent grain protein hydrolysates. *Food Res Int* 121:524-532. https://doi.org/10.1016/j.foodres.2018.12.008

Crowe NL, Baker BE, Inteaz A. 1985. Solubilisation of nitrogenous constituents of brewer's spent grain. *J Inst Brew* 91:148–150. https://doi.org/10.1002/j.2050-0416.1985.tb04320.x

Cuq JC, Vié M, Cheftel JC. 1983. Tryptophan degradation during heat treatments: part 2-degradation of protein-bound tryptophan. *Food Chem* 12:73–88. https://doi.org/10.1016/0308-8146(83)90019-5

Diptee R, Smith JP, Alli I, Khanizadeh S. 1989. Application of response surface methodology in protein extraction studies from brewer's spent grain. *J Food Preserv* 13:457-474.

Ervin V, Alli I, Smith JP, Li Z. 1989. Extraction and precipitation of proteins from brewer's spent grain. *Can Inst Food Sci Technol J* 22:216-221. https://doi.org/10.1016/S0315-5463(89)70386-2

Forssell P, Kontkanen H, Schols HA, Hinz S, Eijsink VGH, Treimo J, Robertson JA, Waldron KW, Faulds CB, Buchert J. 2008. Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. *J Inst Brew* 114:306-314. https://doi.org/10.1002/j.2050-0416.2008.tb00774.x

Gao Z, Shen P, Lan Y, Cui L, Ohm JB, Chen B, Rao J. 2020. Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Res Int* 131:1-9. https://doi.org/10.1016/j.foodres.2020.109045

Gmoser R, Fristedt R, Larsson K, Undeland I, Taherzadeh MJ, Lennartsson PR. 2020. From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered* 11:582-598. https://doi.org/10.1080/21655979.2020.1768694

Gorissen SHM, Crombag JJR, Senden JMG, Waterval WAH, Bierau J, Verdijk LB, Loon LJC. 2018. Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino Acids* 50:1685-1695. https://doi.org/10.1007/s00726-018-2640-5

Gutiérrez-Barrutia MB, Castillo MD, Arcia P, Cozzano S. 2022. Feasibility of extruded brewer's spent grain as a food ingredient for a healthy, safe, and sustainable human diet. *Foods* 11:1-20. https://doi.org/10.3390/foods11101403

Lam ACY, Karaca AC, Tyler RT, Nickerson MT. 2018. Pea protein isolates: structure, extraction, and functionality. *Food Rev Int* 34:126-147. https://doi.org/10.1080/87559129.2016.1242135

Lynch KM, Steffen EJ, Arendt EK. 2016. Brewers' spent grain: a review with an emphasis on food and health. *J Inst Brew* 122: 553-568. https://doi.org/10.1002/jib.363

Melro E, Filipe A, Sousa D, Valente AJM, Romano A, Antunes FE, Medronho B. 2020. Dissolution of kraft lignin in alkaline solutions. *Int J Biol Macromol* 148:688-695. https://doi.org/10.1016/j.ijbiomac.2020.01.153

Olsen RL, Hasan MR. 2012. A limited supply of fishmeal: impact on future increases in global aquaculture production. *Trends Food Sci Technol* 27:120-128. https://doi.org/10.1016/j.tifs.2012.06.003

Oluseyi AK, Dawodu FA, Ajanaku CO, Nwinyi OC. 2011. Functional and nutritional properties of spent grain enhanced cookies. *Am J Food Technol* 6:763-771. https://doi.org/10.3923/ajft.2011.763.771

Otter DE. 2012. Standardised methods for amino acid analysis of food. *Br J Nutr* 108:230–237. https://doi.org/10.1017/S0007114512002486

Pejin J, Radosavljević M, Kocić-Tanackov S, Mladenović D, Djukić-Vuković A, Mojović L. 2017. Fed-batch L-(+)-lactic acid fermentation of brewer's spent grain hydrolysate. *J Inst Brew* 123:537-543. https://doi.org/10.1002/jib.452

Petit G, Korbel E, Jury V, Aider M, Rousselière S, Audebrand L, Turgeon SL, Mikhaylin S. 2020. Environmental evaluation of new brewer's spent grain preservation pathways for further valorization in human nutrition. *ACS Sustain Chem Eng* 8:17335-17344. https://doi.org/10.1021/acssuschemeng.0c04236

Prado R, Erdocia X, Labidi J. 2013. Lignin extraction and purification with ionic liquids. *J Chem Technol Biotechnol* 88:1248–1257. https://doi.org/10.1002/jctb.3965

Qiaoyun C, Xinghong N, Liang Z, Zheng T, Jin L, Kang S, Xuan C, Xinghui L. 2017. Optimization of protein extraction and decoloration conditions for tea residues. *Hortic Plant J* 3:172–176. https://doi.org/10.1016/j.hpj.2017.06.003

Qin F, Johansen AZ, Mussatto SI. 2018. Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains. *Ind Crops Prod* 125:443-453. https://doi.org/10.1016/j.indcrop.2018.09.017

R Core Team, 2018: a language environment for statistical computing. Vienna, Austria. https://www.r-project.org/

Ruiz GA, Xiao W, Boekel M, Minor M, Stieger M. 2016. Effect of extraction pH on heat-induced aggregation, gelation and microstructure of protein isolate from quinoa (*Chenopodium quinoa* Willd). *Food Chem* 209:203–210. https://doi.org/10.1016/j.foodchem.2016.04.052

Salas C, Rojas OJ, Lucia LA, Hubbe MA, Genzer J. 2013. On the surface interactions of proteins with lignin. *ACS Appl Mater Interfaces* 5:199-206. https://doi.org/10.1021/am3024788

Sousa D, Venâncio A, Belo I, Salgado JM. 2018. Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation. *J Sci Food Agric* 98:5248-5256. https://doi.org/10.1002/jsfa.9063

Sousa R, Coimbra JSR, Rojas EEG, Minim LA, Oliveira FC, Minim VPR. 2007. Effect of pH and salt concentration on the solubility and density of egg yolk and plasma egg yolk. *LWT - Food Sci Technol* 40:1253-1258. https://doi.org/10.1016/j.lwt.2006.08.001

Surasani VKR, Khatkar SK, Singh S. 2017. Effect of process variables on solubility and recovery yields of proteins from pangas (*Pangasius pangasius*) frames obtained by alkaline solubilization method: Characteristics of

isolates. *Food Bioprod Process* 106:137-146. https://doi.org/10.1016/j.fbp.2017.09.008
Tacon AGJ, Hasan MR, Metian M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: Trends and prospects. *FAO Fisheries and Aquaculture Technical Paper* 564:1-87. http://www.fao.org/docrep/015/ba0002e/ba0002e.pdf
Tang D, Tian Y, He Y, Li L, Hu S, Li B. 2010. Optimisation of ultrasonic-assisted protein extraction from brewer's spent grain. *Czech J Food Sci* 28:9-17. https://doi.org/10.17221/178/2009-CJFS
Tišma M, Jurić A, Bucić-Kojić A, Panjičko M, Planinić M. 2018. Biovalorization of brewers' spent grain for the production of laccase and polyphenols. *J Inst Brew* 124:182-186. https://doi.org/10.1002/jib.479
Vareltzis PK, Undeland I. 2012. Protein isolation from blue mussels (*Mytilus edulis*) using an acid and alkaline solubilisation technique-process characteristics and functionality of the isolates. *J Sci Food Agric* 92:3055–3064. https://doi.org/10.1002/jsfa.5723
Vilg JV, Undeland I. 2017. pH-driven solubilization and isoelectric precipitation of proteins from the brown seaweed *Saccharina latissima*—effects of osmotic shock, water volume and temperature. *J Appl Phycol* 29:585-593. https://doi.org/10.1007/s10811-016-0957-6
Waters DM, Jacob F, Titze J, Arendt EK, Zannini E. 2012. Fibre, protein and mineral fortification of wheat bread through milled and fermented brewer's spent grain enrichment. *Eur Food Res Technol* 235:767-778. https://doi.org/10.1007/s00217-012-1805-9
Wen C, Zhang J, Duan Y, Zhang H, Ma H. 2019. A mini-review on brewer's spent grain protein: isolation, physicochemical properties, application of protein, and functional properties of hydrolysates. *J Food Sci* 84:3330-3340. https://doi.org/10.1111/1750-3841.14906
Yu D, Sun Y, Wang W, O'Keefe SF, Neilson AP, Feng H, Wang Z, Huang H. 2020. Recovery of protein hydrolysates from brewer's spent grain using enzyme and ultrasonication. *Int J Food Sci Technol* 55:357-368. https://doi.org/10.1111/ijfs.14314

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Information

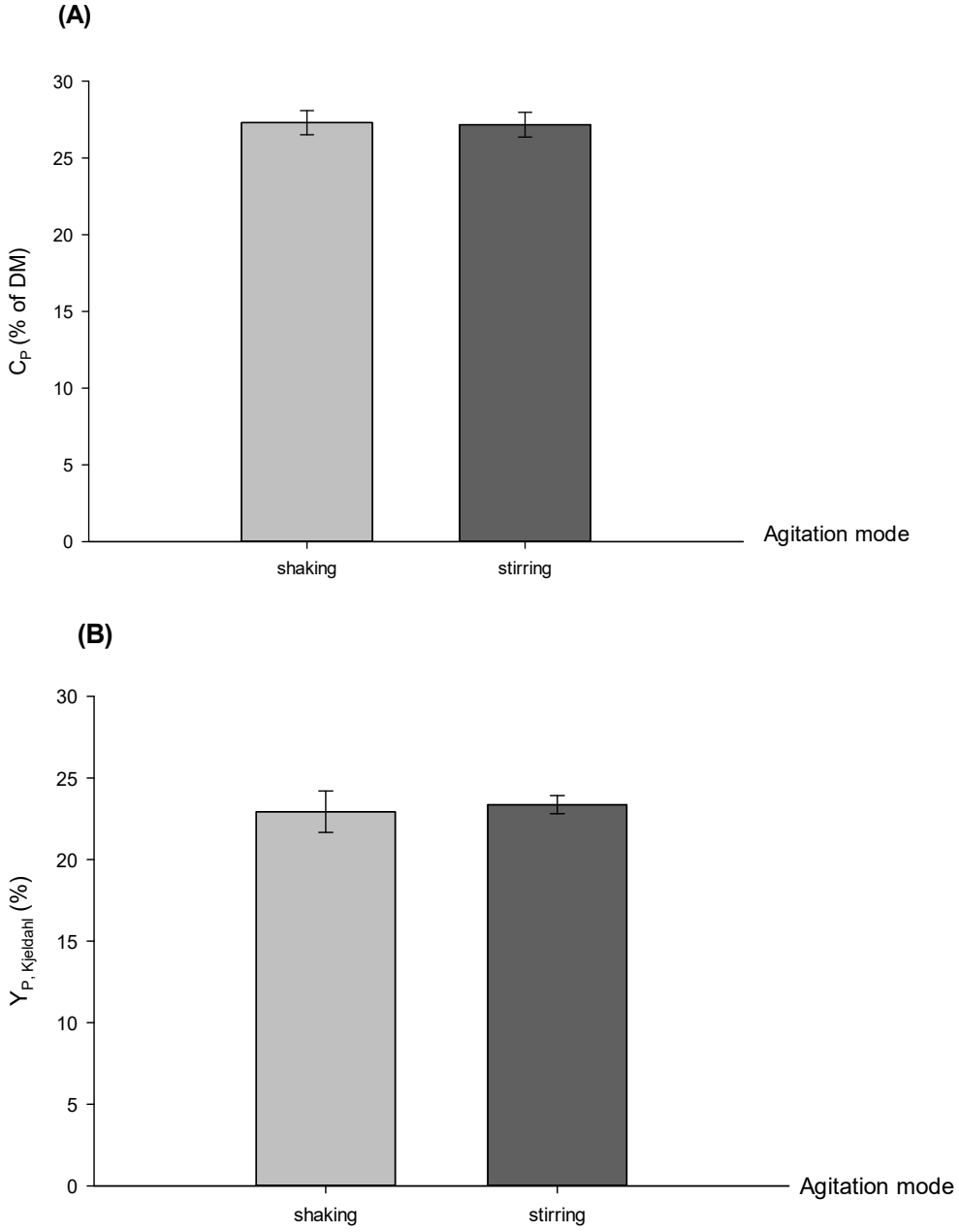


Figure S1. (A): Effect of agitation mode on Kjeldahl-based protein content of isolates (C_P). (B): Effect of agitation mode on Kjeldahl-based protein yield ($Y_{P, Kjeldahl}$). The t-tests showed no significant differences between the two groups neither for the protein content (p=0.847) nor for the protein yield (p=0.629). C_P = Kjeldahl N (%) ·6.25 and $Y_{P, Kjeldahl}$ = $m_{Kjeldahl\ protein,\ isolate}$ (g)/ $m_{Kjeldahl\ protein,\ BSG}$ (g)·100%. Extractions were carried out in triplicates (n=3) and the results are presented as means±SD. Freeze-drying was used to isolate solubilised proteins.

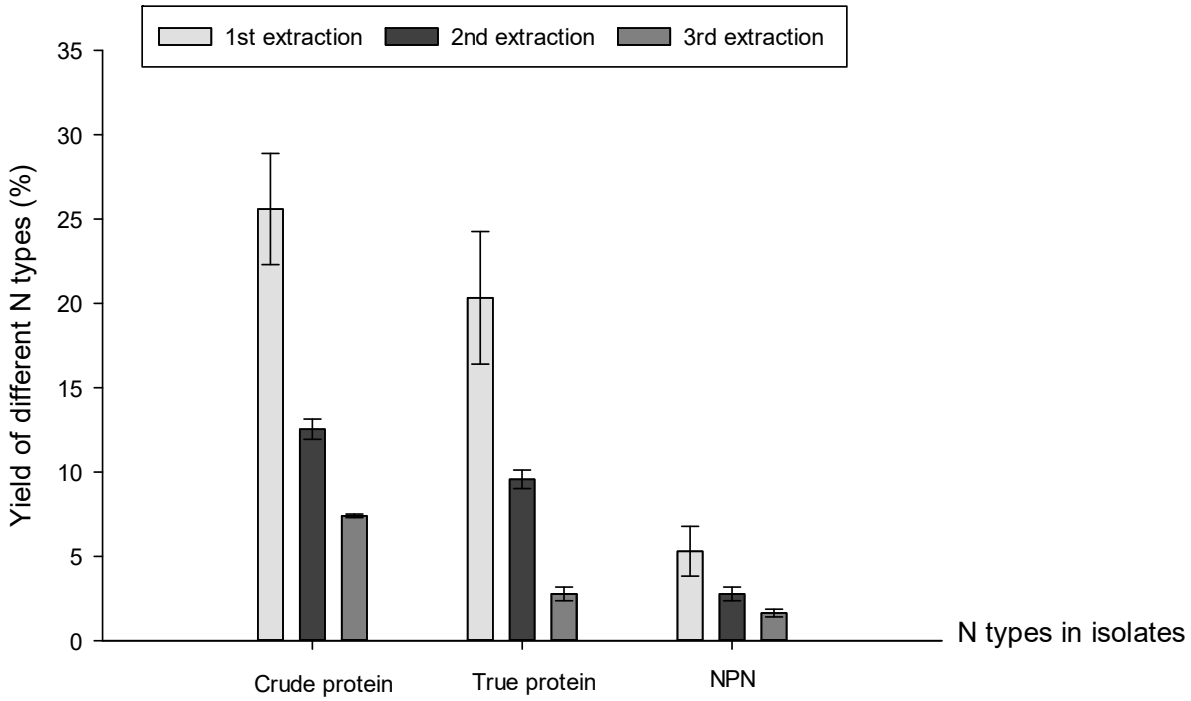


Figure S2. Yields of crude protein ($Y_{P, Kjeldahl}$), true protein ($Y_{P, AA1}$) and non-protein nitrogen (NPN) obtained after three successive extractions. Extractions were done in triplicates (n=3) and data are reported as means±SD. All yield types were significantly different between the three extractions and for clarity indicators of significance were omitted ($p<0.05$).

Table S1. Amino acid composition of untreated and defatted BSG*. All determinations were carried out in duplicates (n=2) and the results are reported as means±SD.

	Untreated BSG	Defatted BSG
Essential amino acids (g/100 g DM)		
Phenylalanine (Phe)	1.45 ± 0.23	1.07 ± 0.33
Histidine (His)	0.23 ± 0.08	0.16 ± 0.01
Valine (Val)	0.71 ± 0.06	0.51 ± 0.25
Isoleucine (Ile)	1.03 ± 0.71	0.75 ± 0.71
Leucine (Leu)	1.10 ± 0.06	0.79 ± 0.28
Methionine (Met)	0.19 ± 0.18	0.19 ± 0.11
Threonine (Thr)	0.41 ± 0.16	0.31 ± 0.03
Lysine (Lys)	0.48 ± 0.01	0.38 ± 0.11
Non-essential amino acids (g/100 g DM)		
Arginine (Arg)	0.55 ± 0.21	0.39 ± 0.03
Alanine (Ala)	0.49 ± 0.18	0.38 ± 0.04
Aspartate (Asp)	0.90 ± 0.35	0.67 ± 0.08
Glutamate (Glu)	2.44 ± 0.82	1.75 ± 0.18
Glycine (Gly)	0.48 ± 0.17	0.37 ± 0.03
Serine (Ser)	0.55 ± 0.21	0.39 ± 0.04
Tyrosine (Tyr)	0.73 ± 0.11	0.46 ± 0.23
Cysteine (Cys)	0.00 ± 0.00	0.00 ± 0.00
Proline (Pro)	1.29 ± 0.41	0.88 ± 0.07
Cystine (Csn)	0.26 ± 0.08	0.17 ± 0.11

*Values are reported for brewer's spent grain (BSG) from batch 2 (see the section Materials and Methods).

Manuscript 3

Evaluation of brewer's spent grain as a feedstock for amino acid production by submerged fermentation

Manuscript will be submitted to Bioresource Technology

Evaluation of brewer's spent grain as a feedstock for amino acid production by submerged fermentation

Freja Karlsen[a*], Peter V. Skov[a], Catherine Boccadoro[b] and Sushil S. Gaykawad[b]

[a]Technical University of Denmark, DTU Aqua, Section for Aquaculture, Niels Juelsvej 30, 9850 Hirtshals, Denmark; pvsk@aqua.dtu.dk

[b]Norwegian Research Centre AS (NORCE), Prof. Olav Hanssensvei 15, 4021 Stavanger, Norway; susg@norceresearch.no; cabo@norceresearch.no

[*]Corresponding author: frkar@aqua.dtu.dk

Abstract

Brewer's spent grain (BSG) was investigated as a fermentation substrate for production of amino acids using *Corynebacterium glutamicum* and *Saccharomyces cere*visiae. *C. glutamicum* produced alanine (Ala), proline (Pro), valine (Val) and glycine (Gly) during shake flask and batch cultivation. The highest Ala production of 193.6±0.09 mg/L was found after 30 h of shake flask cultivation. In the batch fermentation mode, the maximum production of Pro and Val achieved after 4 h was 22.5±1.03 and 34.8±0.11 mg/L, respectively, while Gly production was highest at 18.7±1.30 mg/L after 8 h. *S. cerevisiae* produced Ala, Pro, Val and glutamic acid (Glu) in shake flask cultivation. The highest production of Ala (11.8±1.25 mg/L), Pro (11.8±1.06 mg/L), and Val (4.94±1.01 mg/L) was achieved after 50 h while highest Glu production (66.2±0.49 mg/L) was observed after 60 h. This study successfully demonstrates the production of several amino acids from BSG by submerged fermentation processes.

Key words

Brewer's spent grain; amino acids; submerged fermentation; *Corynebacterium glutamicum*; *Saccharomyces cerevisiae*

1. Introduction

Amino acids are compounds of immense industrial importance. They are utilized in several industrial applications including pharmaceuticals, human food and animal

feed (Mahmood, 2015). A growing demand for amino acids has led to an elevated global production from estimated 6.7 million tonnes in 2014 to projected 10 million tonnes in 2022 (Sanchez et al., 2018). This has fuelled a need for developing new and more sustainable technologies for amino acid production. Presently, amino acids are synthesized by three different routes: 1) chemical synthesis, 2) extraction from protein hydrolysates and 3) microbial processes (Stoimenova et al., 2013). Microbial amino acid production occurs under submerged conditions by fermentation or enzymatic conversion. During enzymatic processes, medium sized peptides are hydrolysed to form free amino acids. In fermentation, sugars are converted to amino acids via intermediates of the central C-metabolism by using different microorganisms (D'Este et al., 2018; Toe et al., 2019). Microbial amino acid production offers several advantages over the chemical and extraction-based methods including mild conditions, low by-product formation and selective production of L-amino acids (D'Este et al., 2018). Besides these benefits, microbial production has several shortcomings including high energy demand, risk of contamination and high substrate consumption, mainly glucose. Glucose is commonly produced from hydrolysis of starch which is also widely used in the food industry (Glittenberg, 2012). Due to an increasing demand for food, the use of starch-derived glucose in microbial processes is not sustainable and as a result, there is a need to find alternative substrates. Recently, lignocellulosic biomasses have received considerable attention as renewable feedstock for microbial production due to their high content of cellulose and hemicellulose which represent sources of fermentable sugars (Zhang et al., 2020). Brewer's spent grain (BSG) is an abundant lignocellulosic by-product from the beer brewing industry (Lynch et al., 2016). Owing to its high content of bioactive compounds, such as protein (20-30%), hemicellulose (30-35%), cellulose (25-30%) and lignin (~10-20), BSG is considered a potential feedstock for several applications (Aliyu and Bala, 2011). Previous research into the valorization of BSG has mainly focused on chemical extractions and production of various value-added compounds by submerged or solid-state fermentations (Mandalari et al., 2005; Mitri et al., 2022; Sousa et al., 2018; Wen et al., 2019).

In this study, we examined the production of amino acids from BSG by submerged fermentation. Selected microorganisms were cultured in BSG hydrolysate-based media to evaluate the suitability of BSG as a fermentation substrate for amino acid

production. Furthermore, we studied the impact of cultivation mode and growth phase on amino acid production. To our knowledge, this is the first study to demonstrate the potential of BSG as a substrate for amino acid production by submerged fermentation.

2. Materials and methods

2.1 Brewer's spent grain (BSG)

Brewer's spent grain was supplied by Carlsberg, Denmark. The raw BSG was autoclaved at 121°C for 15 min and dried at 60°C until the dry matter content was higher than 90%. Dried BSG was micronized at the Danish Technological Institute (Kolding) to obtain a size fraction of 125-250 µm.

2.2 Hydrolysis of brewer's spent grain

A BSG hydrolysate was produced by enzymatic hydrolysis of micronized BSG using a modified version of the procedure outlined by Forssell et al. (2008). Briefly, the enzyme mixture Depol 686L (Biocatalyst, UK) was added to a preheated suspension of BSG (10% w/v) in ammonium acetate buffer (50 mM, pH 5.0) to obtain a final enzyme activity of 106 U/mL. This mixture was incubated at 50°C for 24 h with intermittent agitation. The solid and liquid fractions were separated by centrifugation at 4000 rpm and 21°C for 25 min (5810R Refrigerated Benchtop Centrifuge, Eppendorf, Germany). The liquid fraction was neutralized using a 10 M NaOH solution which resulted in formation of a white precipitate. This precipitate was removed by centrifugation at 4000 rpm and 4°C for 25 min. Finally, the liquid fraction was filter sterilized (0.22 µm Sterivex-GV Pressure Filter, Merck Millipore). For media with a pH below 7, the liquid fraction was acidified using dilute H_2SO_4 before the filtration step.

2.3 Microorganisms

Four microorganisms were studied: *E. coli* ATTC 49161, *Lactobacillus plantarum* Lp 39, *Saccharomyces cerevisiae* (unknown strain) and *Corynebacterium glutamicum* DSMZ 1412.

2.4 Media composition

E. coli, *L. plantarum* and *S. cerevisiae* were cultivated in media with the following composition: KH_2PO_4, 0.052 g/L; $MgSO_4 \cdot H_2O$, 0.036 g/L; Na_2HPO_4, 0.0164 g/L; $MnSO_4 \cdot H_2O$, 0.00384 g/L; CH_3COONa, 0.0384 g/L; yeast extract, 1.0 g/L; BSG hydrolysate as the glucose source, 2.5 g/L. The media was prepared from a salt-yeast extract stock solution and the BSG hydrolysate. The medium pH was adjusted to 7.0±0.1 for *E. coli,* while a pH of 6.5±0.1 was used for *L. plantarum* and *S. cerevisiae*.

The medium used to culture *C. glutamicum* was prepared according to Venkata Narayana et al. (2013) with few modifications: $MgSO_4 \cdot H_2O$, 0.082 g/L; $(NH_4)_2SO_4$, 11.8 g/L; K_2HPO_4, 1.18 g/L; 3,4-dihydroxybenzoic acid, 0.35 mg/L; glucose added as BSG hydrolysate, 2.5 g/L. The medium pH was adjusted to 7.0±0.1. Three stock solutions were used to prepare the medium: 1) BSG hydrolysate, 2) a salt solution and 3) a 3,4-dihydroxybenzoic acid solution. The BSG hydrolysate and the 3,4-dihydroxybenzoic acid solution were sterilized by filtration while all salt solutions were autoclaved at 121°C for 30 min.

2.5 Inoculum preparation

Inoculum for bioreactor cultivations was prepared as follows: 500 µL 15% glycerol stock culture was added to 100 mL sterile medium in a shake flask. The shake flask culture was incubated in an orbital shaker (MaxQ 8000, Thermo Fisher Scientific, Marietta, USA) using the speed and temperature specified for each microorganism.

2.6 Shake flask cultivation

E. coli, *S. cerevisiae*, *L. plantarum* and *C. glutamicum* were grown in 250 mL shake flasks containing 50 mL of sterile medium. Cultivations of *S. cerevisiae* and *L. plantarum* were performed at 160 rpm and 30°C while *E. coli* was cultured at 250 rpm and 37°C. Shake flask experiments with *C. glutamicum* were carried out at 130 rpm and 30°C.

2.7 Bioreactor cultivations

Fermentations with *C. glutamicum* and *S. cerevisiae* were performed in a 3.5 L stirred bioreactor (Chemap, Switzerland) using an over pressure of 0.3 bar and constant aeration through the bottom sparger. The cultivation of both microorganisms was carried out at 30±0.1°C. Dissolved oxygen (DO) was monitored

with a DO probe (InPro6800, Mettler Toledo) and maintained at a minimum level of 40% air saturation by regulating agitation speed or airflow. The pH was measured using a pH probe (InPro3253, Mettler Toledo). For *C. glutamicum*, pH was maintained at 7.0±0.1 with 10 M NaOH and 2 M H_2SO_4. For *S. cerevisiae*, pH was kept at 5.0±0.1 with 1 M NaOH and 2 M H_2SO_4. Foaming was controlled by dropwise addition of Glanapon 2000. Percentages of O_2 and CO_2 in the off-gas were measured continuously using a mass spectrometer (Prima Pro Process Mass Spectrometer, ThermoFisher) and the experimental data was acquired from Labview.

During the batch fermentations with *S. cerevisiae* and *C. glutamicum*, 1.5 L medium was inoculated with 100 mL inoculum. The batch phase was considered to be finished when off-gas CO_2 dropped. In the fed-batch fermentation with *C. glutamicum*, feeding was initiated at the end of the batch phase using an exponential feeding rate, Q(t), calculated by equation (1):

$$Q(t) = \frac{\mu_{int} \cdot M_{b,0} \cdot C_{x,0}}{C_{s,in} \cdot Y_{sx}} \cdot \exp(\mu_{int} \cdot t) \quad (1)$$

Here, $C_{s,in}$ is the glucose concentration in the feed (g/L), $M_{b,0}$ is the initial weight of the fermentation broth (g), $C_{x,0}$ is the initial biomass of the fed-batch phase (g/L) and μ_{int} is the intended growth rate during the fed-batch phase (h^{-1}) which was estimated as 1/3 of the maximum growth rate, μ_{max}, obtained during the batch phase. Y_{sx} is the yield coefficient of biomass on substrate which was determined by equation (2):

$$Y_{sx} = \frac{\Delta_{OD_{600}} \cdot F\ (=\text{biomass produced})}{\Delta C_{s,\ batch}\ (=\text{substrate consumed})} (2)$$

where $\Delta_{OD_{600}}$ is the change in optical density during the batch phase, $\Delta C_{s,\ batch}$ is the substrate consumption during the batch phase and F is a factor used to convert optical density into cell dry weight (CDW). A conversion factor of 0.3 g $CDW \cdot L^{-1} \cdot OD_{600}^{-1}$ for *C. glutamicum* reported by Blombach et al. (2011) was applied to calculate Y_{sx} in this study.

2.8 Sampling procedure

In the fermentation experiments, approximately 7 mL of sample was collected at each sample point regularly. Out of this volume, 2x1 mL was used to determine optical density (OD_{600}) and the rest was centrifuged at 2500 rpm and 4°C for 15 min.

After centrifugation, cell pellets were discarded whereas supernatants were filtered through a sterile filter (0.22 µm Sterivex-GV Pressure Filter unit, Merck Millipore) and used for amino acid and sugar quantification. In the shake flask experiments, the same procedure was deployed except for the filtration step due to the small sampling volumes (1 mL).

2.9 Analytical procedures

2.9.1 Optical density

Biomass was measured as optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 2100 pro spectrophotometer, Amersham Biosciences, Uppasala, Sweden).

2.9.2 Amino acids

The amino acid analysis was carried out in two steps, namely purification and quantification. In the first step, the samples were purified by Solid Phase Extraction (SPE) to eliminate interfering compounds. The SPE purification step was carried out using a strong cation exchange (SCX) cartridge (40-75 µm, 70 Å, 100 mg/1mL, Hawach Scientific, China). Initially, the solid phase was equilibrated with 1 mL of 100% methanol and acidified with 1 mL of 1% (v/v) aqueous formic acid. Samples were diluted two-fold in 1% (v/v) aqueous formic acid and norvalin was added as internal standard (IS) as to obtain a final concentration of 100 µM in the diluted samples. Diluted samples (500 µL) were loaded onto the solid phase and washed twice with 500 µL of 1% (v/v) formic acid in methanol. Then, amino acids were eluted from the solid phase with 2x250 µL 5% ammonium hydroxide in methanol.

In the second step, concentrations of amino acids were determined according to the method described by Cohen (1994). The amino acids were converted into UV-active derivatives by reaction with the substrate 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) using a commercial derivatization kit (AccQ•Tag Ultra Derivatization Kit, Waters, Milford, Massachusetts). Amino acid derivatives were then quantified using reversed-phase liquid chromatography with UV detection at 260 nm (Waters ACQUITY UltraPerformance liquid LC, ACCQ-TAG ULTRA C18, 2.1x100 mm, 1.7 µm column). Water (eluent A) and acetonitrile (eluent B) were used as eluents. A gradient of 0-60% eluent B over 10 min and a flowrate of 0.7 mL/s were applied to separate the amino acids.

2.9.3 Sugars

Sugars (glucose, arabinose and xylose) and organic acids (lactate and acetate) were quantified by HPLC analysis (Shimadzu Nexera XR) using refractive index detection (RID-20A) Compounds were separated by reversed-phase mode (Biorad Aminex HPX-87H column) using the following conditions: 0.6 mL/min, 30°C and a 4 mM aqueous sulfuric acid eluent. Filtrated samples were prepared for injection (1 µL) by two-fold dilution in 0.2% aqueous sodium azide. Quantification was performed by comparing samples with 10 g/L standard solutions of each compound.

2.10 Data analysis

All variables (OD_{600}, amino acids and sugars) were determined in duplicates and results were reported as mean±SD. All graphs were created in the software GraphPad Prism 9.3.1.

3. Results and discussion

3.1 Selection of microorganisms for amino acid production

The four microorganisms, listed in Table 1, were cultured in shake flasks to evaluate their ability to utilize BSG hydrolysate as a substrate for amino acid production. These microorganisms were chosen for the initial screening because they have previously been demonstrated to produce amino acids by submerged processes (Gopinath and Nampoothiri, 2014; Malaney et al., 1991; Rodriguez et al., 2014; Toe et al., 2019).

Table 1

Screening of different microorganisms

Microorganism	Growth performance[a]	Major amino acids produced
E. coli	+	ND
L. plantarum	+	ND
S. cerevisiae	++	Ala, Pro, Glu, Val, Leu, Phe
C. glutamicum	++	Ala, Pro, Val, Gly

[a]Growth performance was based on optical density measured at 600 nm (OD_{600}). +: low growth (OD_{600}<8). ++: high growth (OD_{600}>8).

Initially, their growth performance was investigated and the best-performing microorganisms (OD_{600}>8) were studied further for amino acid production. *E. coli* and

L. plantarum showed low growth performance and therefore amino acid production was not assessed for these microorganisms. The low growth of these microorganisms might be due to drop in medium pH during the growth. The pH dropped, from 7.0 to 4.9 and from 6.5 to 3.8, during cultivation of *E. coli* and *L. plantarum*, respectively. This decrease might stem from production of acetic acid by *E. coli* and lactic acid by *L. plantarum*, although the presence of these metabolites was not confirmed by chemical analysis. A similar pH decline from 6.5 to 5.4 was found for *S. cerevisiae*. However, the growth was not influenced as the optimum pH for growth ranges from 4.0 to 6.0 (Liu et al., 2015). Contrarily, pH increased from 7.0 to 8.1 after 48 h cultivation of *C. glutamicum* which was also found to be within the optimum pH range (6.0-9.0). This explains why growth of *C. glutamicum* was unaffected (Follmann et al., 2009). Based on these results, *C. glutamicum* and *S. cerevisiae* were selected and explored for amino acid production in this study.

3.2 Cultivation of *Corynebacterium glutamicum* and *Saccharomyces cerevisiae*

To develop a microbial process for transforming BSG-derived sugars into amino acids, *C. glutamicum* and *S. cerevisiae* were cultured in shake flasks and fermenter. One shake flask experiment and two batch fermentations were carried out for both microorganisms. Additionally, one fed-batch fermentation was performed for *C. glutamicum*. The term amino acid profile refers to the concentration of free amino acids present in the fermentation broth at a specific time. Based on the changes in amino acid concentrations during growth, their consumption or production was determined.

3.2.1 Shake flask experiments

The aim of these experiments was two-fold, first to select and optimize the media and secondly to study the amino acid formation.

The shake flask experiment with *S. cerevisiae* was performed using BSG hydrolysate as C-source and yeast extract as N-source. Yeast extract also contains carbohydrates (~10% of dry matter) which might be utilized as C-source. To examine whether the BSG hydrolysate comprises the primary C-source for *S. cerevisiae*, media with high (1 g/L) and low (0.5 g/L) concentrations of yeast extract were tested. Reducing the concentration of yeast extract from 1 g/L to 0.5 g/L led to a slight decrease in the growth performance as OD_{600} dropped from ~8 to ~6 after 20 h (data

not shown). This suggests that the carbohydrates in yeast extract support growth of *S. cerevisiae*; however, yeast extract is not the main C-source. Furthermore, it is important to note that the observed decrease in OD_{600} could also be due to limitations of the N-source.

The growth medium for *C. glutamicum* does not contain yeast extract but ammonium sulphate as N-source. Quantification of amino acids in this medium revealed a relatively high background concentration of all amino acids. This may result from solubilization of amino acids during the enzymatic hydrolysis of BSG or simultaneous protein hydrolysis. However, low proteolytic activity has been reported for the Depol 686L mixture and therefore the degree of protein hydrolysis is expected to be insignificant (Forssell et al., 2008). The effect of N-source on *C. glutamicum* growth and amino acid formation was investigated by using media with low (12 g/L) and high (20 g/L) concentrations of ammonium sulfate referred to as M_{Low} and M_{High}, respectively (Figure S1). The growth curves obtained from cultivation of *C. glutamicum* in M_{Low} and M_{High} followed the same trend, although biomass production appeared to be slightly higher when the bacterium was cultured in the M_{High} medium (Figure S1A). On the other hand, *C. glutamicum* had highest production of alanine (Ala) in M_{Low} medium (~194 mg/L) as compared to M_{High} medium (~135 mg/L) (Figure S1B). As a result, M_{Low} medium was chosen for all subsequent cultivations of *C. glutamicum*.

A comparison of the growth data obtained for each microorganism reveals that *C. glutamicum* grows faster than *S. cerevisiae* on BSG hydrolysate (Figure 1). For *C. glutamicum* an unusual growth pattern was observed during the stationary phase where OD_{600} first declined rapidly (~13 h) and then increased (~24 h) (Figure 1A). The underlying cause of this observation is unclear; yet, it may be attributed to changes in the pH and DO as these parameters were not regulated during the shake flask experiments. Alternatively, it could be explained by an outlying data point; though, this scenario is unlikely because the same trend was obtained from cultivations in M_{Low} and M_{High} media (Figure S1A). For *S. cerevisiae*, the shape of the growth curve suggests that it grows diauxically on the BSG hydrolysate (Figure 1B).This observation is supported by Tsao and Vegas (1982) who demonstrated that the growth of *S. cerevisiae* can be sustained by sequential utilization of different sugars when the medium contains more than one sugar.

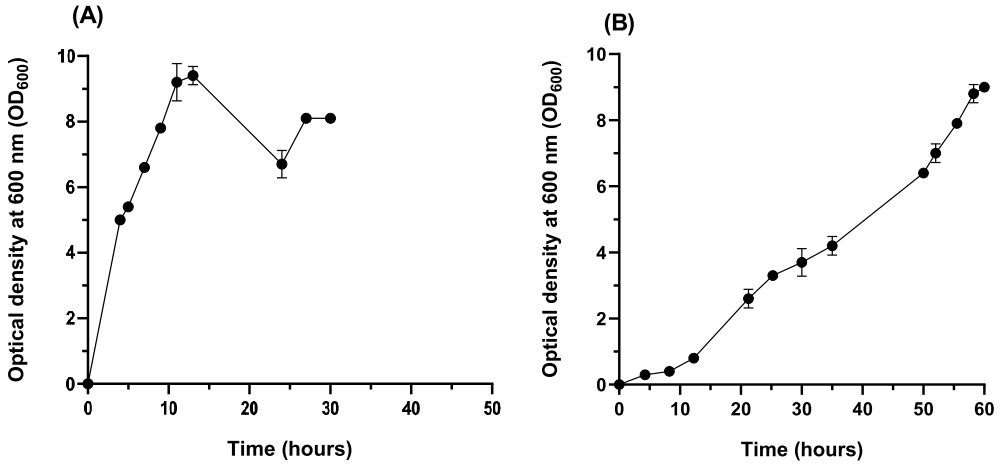
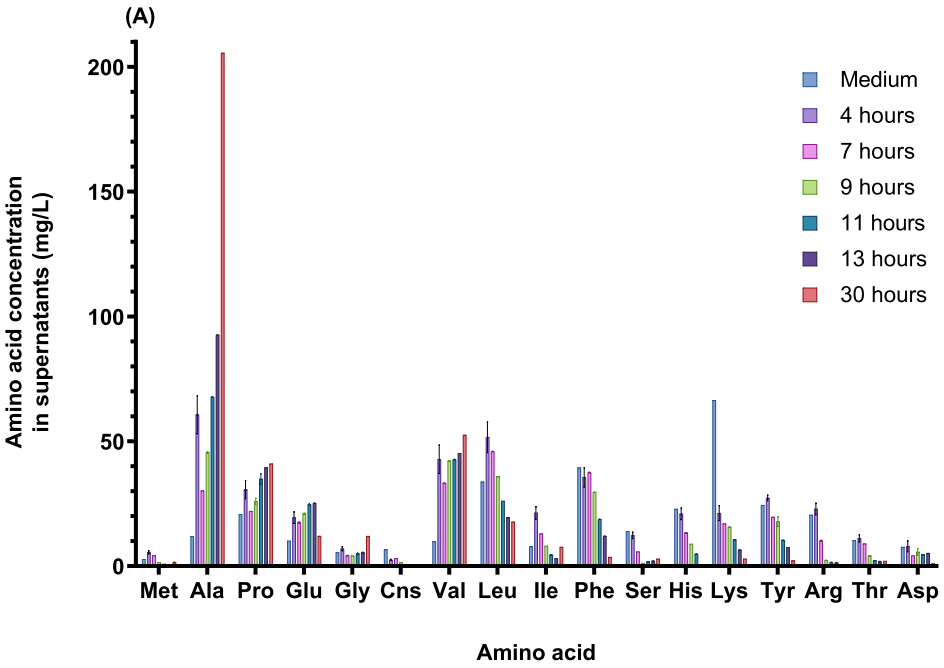


Figure 1. Growth curves obtained from shake flask cultivation with Corynebacterium glutamicum (A) and Saccharomyces cerevisiae (B). Data is reported as the mean±SD of two technical replicates.

Different amino acid profiles were gained from shake flask cultivations of *C. glutamicum* and *S. cerevisiae* (Figure 2). *C. glutamicum* showed highest increase in Ala, valine (Val) and proline (Pro) (Figure 2A). The maximum concentrations achieved for Ala, Val and Pro, after 30 h of cultivation, was 205.5, 52.3 and 41.0 mg/L. This corresponds to a 17, 5 and 2 fold increase in the concentration that was present in medium. For *S. cerevisiae*, several amino acids were produced with maximum production observed at different times (Figure 2B). These amino acids include Ala (45.1 mg/L to 56.9 mg/L), Pro (24.7 to 36.5 mg/L), glutamic acid (Glu) (77.8 to 144.1 mg/L), leucine (Leu) (23.2 to 37.0 mg/L) and phenylalanine (Phe) (18.4 to 31.8 mg/L). All amino acids were produced in small quantities except for Glu that nearly doubled during a growth period of 60 h.



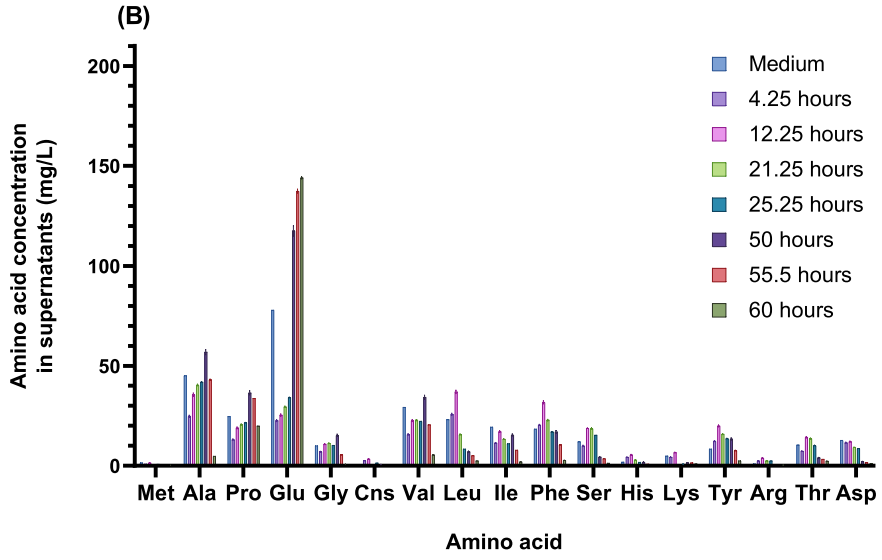


Figure 2. Trends in amino acid concentrations (mg/L) during shake flask cultivations with Corynebacterium glutamicum (A) and Saccharomyces cerevisiae (B). Data is reported as the mean±SD of two technical replicates.

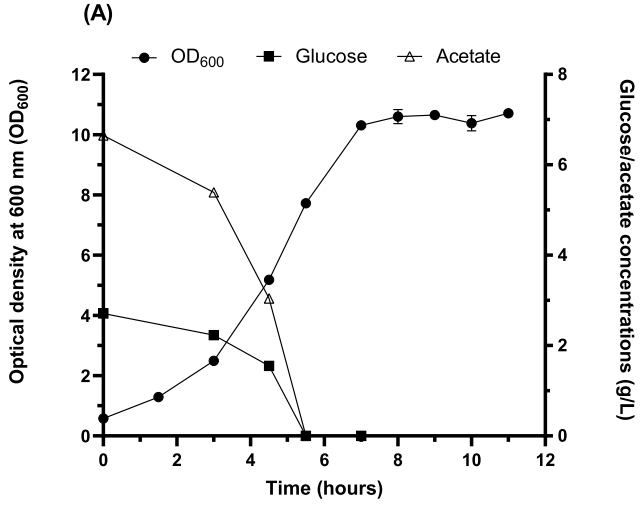
3.2.2 Fermentation experiments

To examine the effect of controlled conditions (pH, aeration, agitation etc.) on growth and amino acid production, fermentations were conducted with *S. cerevisiae* and *C. glutamicum* in a bioreactor.

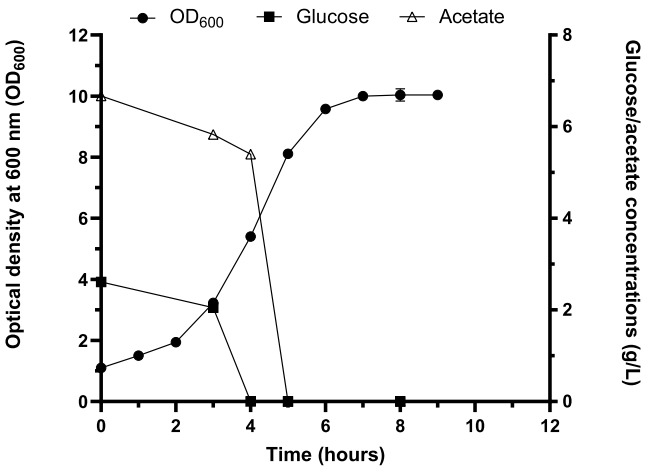
3.2.2.1 Batch fermentations

Batch fermentations were performed in a 3.5 L Chemap fermenter with media volume of 1.5 L using *C. glutamicum* and *S. cerevisiae*. The cultivations of both microorganisms were carried out at 30°C. The pH of 7.0±0.1 and 5.0±0.1 was maintained during batch fermentation experiments for *C. glutamicum* and *S. cerevisiae*, respectively. Batch fermentation experiments were considered finished based on off-gas CO_2 profile. To verify the reproducibility, duplicate batch fermentations were done for each microorganism, and trends in amino acid and growth profiles were studied (Figure 3 and Figure 4).

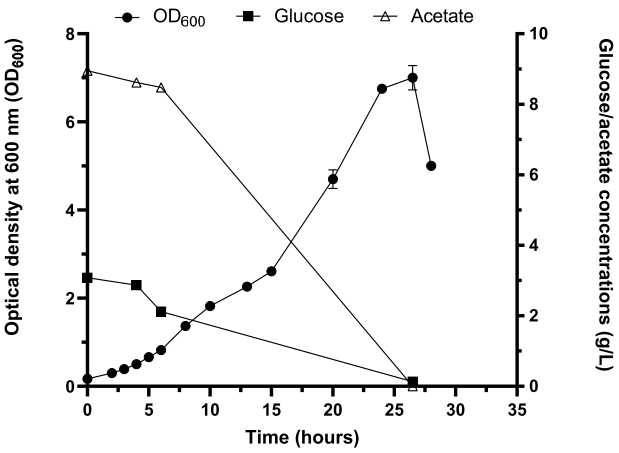
The growth curves and substrate profiles achieved for each microorganism are compared in Figure 3. For *C. glutamicum*, the stationary growth phase was observed after 7-8 h with a maximum OD_{600}~10 (Figure 3A and 3B). No decrease in OD_{600} was detected during the stationary phase as seen in the shake flask experiment (Figure 1A). This finding supports our hypothesis that the decrease in OD_{600} observed during the shake flask experiment resulted from fluctuations in pH and/or DO. The maximum OD_{600} values obtained for *C. glutamicum* in batch fermentation and shake flask cultivation were similar (OD_{600}: 9-10) while the growth period was shorter in the batch fermentations (7-8 h) as compared to shake flask cultivation (30 h). *S. cerevisiae* showed the stationary growth phase after 26-28 h which was shorter as compared to the shake flask experiment (60 h). Comparable OD_{600} values (OD_{600}: 7-9) were achieved in both cultivation modes (Figure 3C and 3D).



(B)


OD600
Glucose
Acetate
Optical density at 600 nm (OD600)
Glucose/acetate concentrations (g/L)
Time (hours)

(C)


OD600
Glucose
Acetate
Optical density at 600 nm (OD600)
Glucose/acetate concentrations (g/L)
Time (hours)

(D)

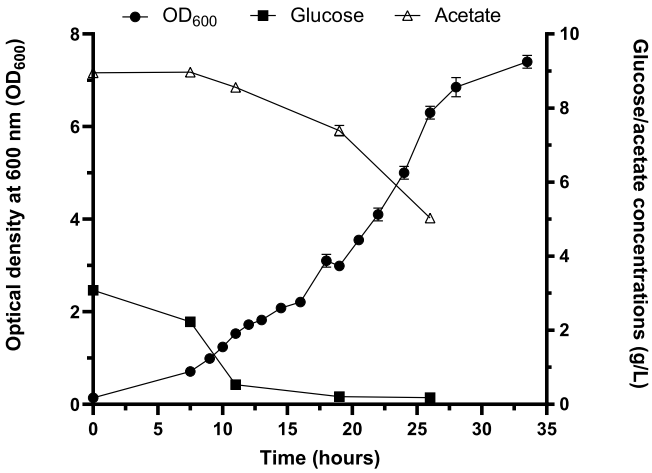
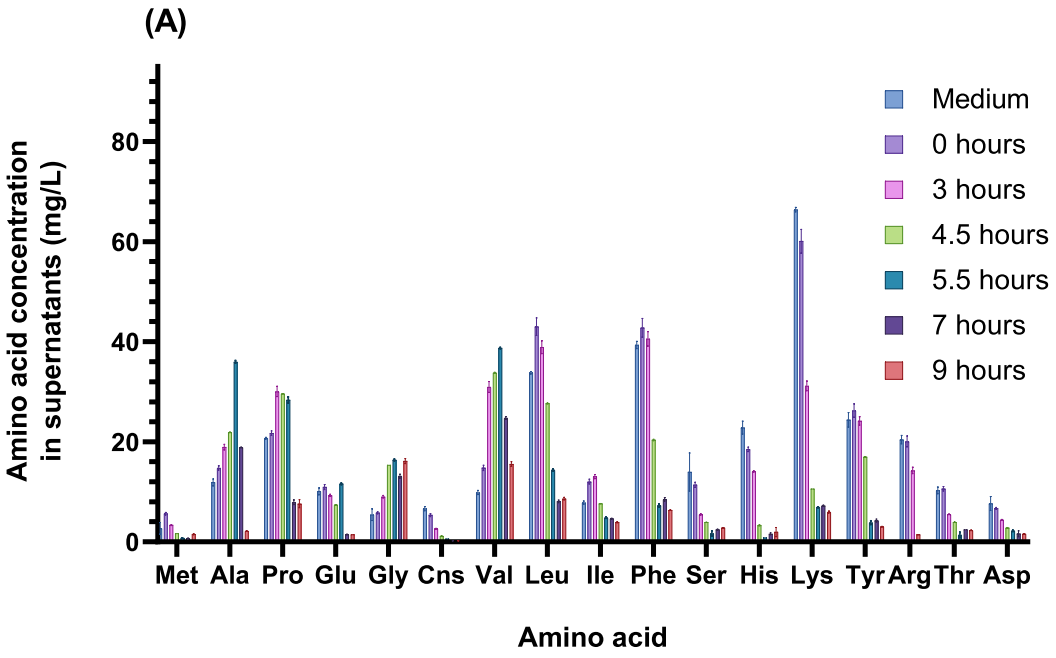
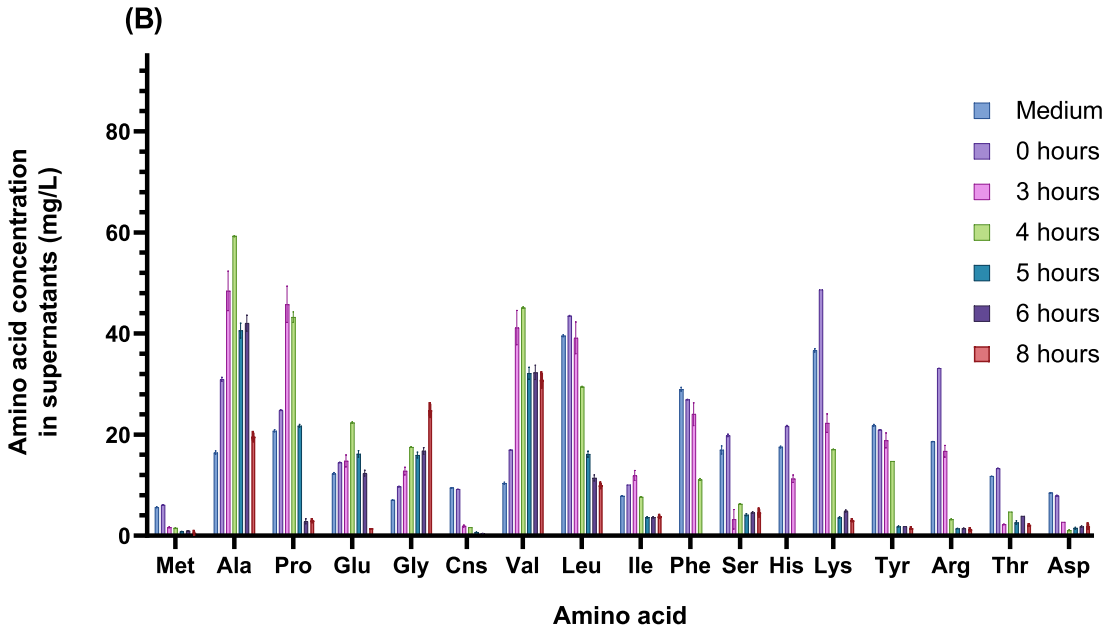

OD600
Glucose
Acetate
Optical density at 600 nm (OD600)
Glucose/acetate concentrations (g/L)
Time (hours)

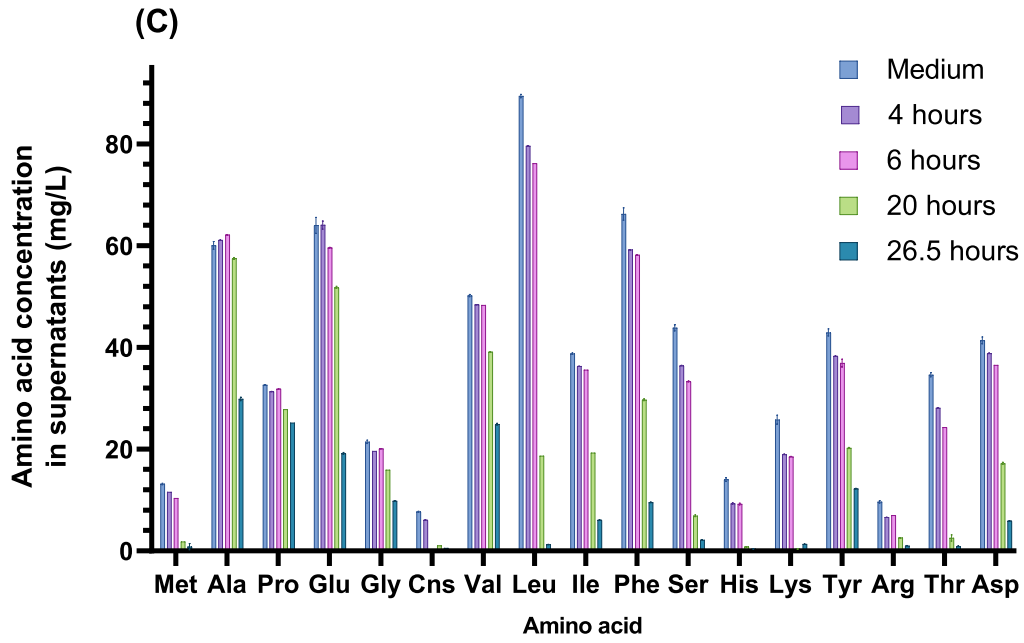
Figure 3. Growth curves and consumption of glucose and acetate during two repeated batch fermentations with Corynebacterium glutamicum (A, B) and Saccharomyces cerevisiae (C, D). Data is reported as mean±SD of two technical replicates.

Chemical analysis showed that the BSG hydrolysate-based media contained different concentrations of a variety of sugars such as glucose (2.5-3.0 g/L), arabinose (1.5-2.0 g/L), xylose (2.6-3.5 g/L), acetate (6.7-8.9 g/L) and lactate (1.0-1.3 g/L). Glucose originates from the hydrolysis of cellulose, and to a lesser extent β-glucans, found in BSG. On the other hand, arabinose, xylose and acetate are major products from hydrolysis of arabinoxylan, the most predominant hemicellulose in BSG (Coelho et al., 2016). Further, the buffer solution (ammonium acetate) used for BSG hydrolysis also contributes to the high media concentrations of acetate. The results presented in Figure 3 indicate that both microorganisms utilize glucose and acetate as major substrates, while neither of them consumed xylose and arabinose. For both microorganisms, lactate concentration remained stable during the exponential phase but was depleted by the end of the cultivations. A possible explanation for this could be that lactate is consumed after the depletion of glucose and acetate (Figure S2). Furthermore, the substrate curves imply that *C. glutamicum* and. *S. cerevisiae* utilize glucose and acetate by two distinct mechanisms. For *C. glutamicum*, overlapping curves indicate simultaneous consumption of glucose and acetate (Figure 3A and 3B). This result is consistent with findings of previous studies which demonstrated that *C. glutamicum* is capable of co-metabolizing glucose with other substrates such as acetate, lactate and various sugars (Gerstmeir et al., 2003). For *S. cerevisiae*, consumption of glucose was observed within the first 10 h after which acetate concentrations began to decrease (Figure 3D). These results support the hypothesis that *S. cerevisiae* grows diauxically with preference for glucose. The decreasing concentrations of acetate strongly suggest that acetate is the second most preferred substrate. To confirm this, however, further analysis is required since *S. cerevisiae* is known to utilize a multitude of other substrates including maltose, sucrose, galactose, ethanol and lactate (Turcotte et al., 2010). Based on the chemical analysis performed in the present study, the possibility that *S. cerevisiae* utilizes ethanol or lactate as substrates after glucose depletion can be ruled out because these compounds started to decline during the stationary phase (data not shown).

Amino acid profile obtained showed that Ala, Pro, Val and Gly were the major amino acids produced by *C. glutamicum* (Figure 4A and 4B). Production of these amino acids appeared to be growth-dependent as it increased with increasing OD_{600} values during the exponential phase. After 4-5 h, the production of Ala, Pro and Val started to decline while no general trend could be inferred for Gly. The decreased productivity may be due to C-source limitation as both glucose and acetate were depleted after 4-5 h; therefore, supplementation with these substrates may promote further growth and potentially increase amino acid production by *C. glutamicum*. All amino acids were consumed by *S. cerevisiae* during batch fermentation except for Ala which increased slightly during the first 10-11 h (Figure 4C and 4D).




(B)
Amino acid concentration in supernatants (mg/L)
0
20
40
60
80
Medium
0 hours
3 hours
4 hours
5 hours
6 hours
8 hours
Met Ala Pro Glu Gly Cns Val Leu Ile Phe Ser His Lys Tyr Arg Thr Asp
Amino acid


(C)
Amino acid concentration in supernatants (mg/L)
0
20
40
60
80
Medium
4 hours
6 hours
20 hours
26.5 hours
Met Ala Pro Glu Gly Cns Val Leu Ile Phe Ser His Lys Tyr Arg Thr Asp
Amino acid

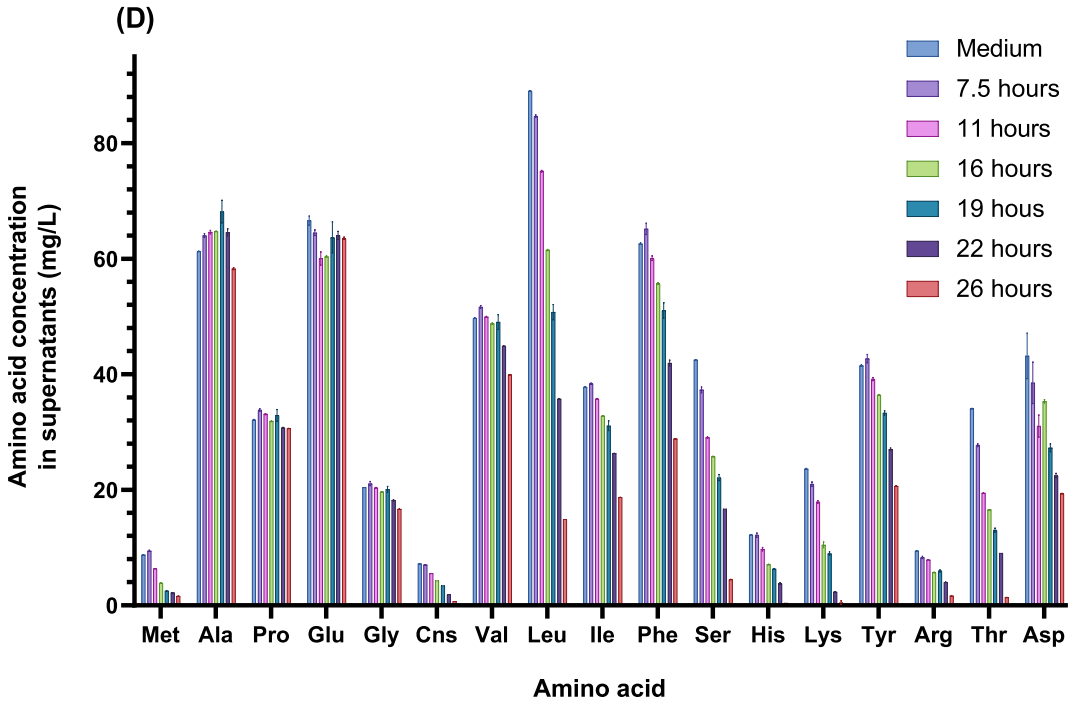


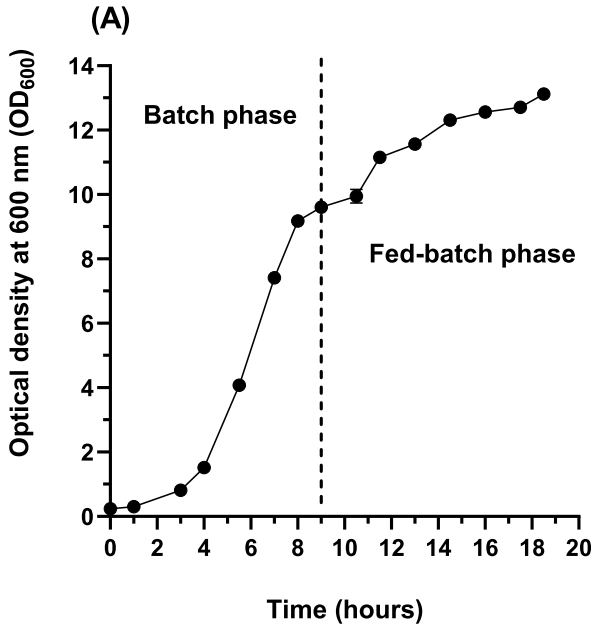
Figure 4. Trends in amino acids concentrations (mg/L) during two repeated batch fermentations with Corynebacterium glutamicum (A, B) and Saccharomyces cerevisiae (C, D). Data is reported as mean±SD of two technical replicates.

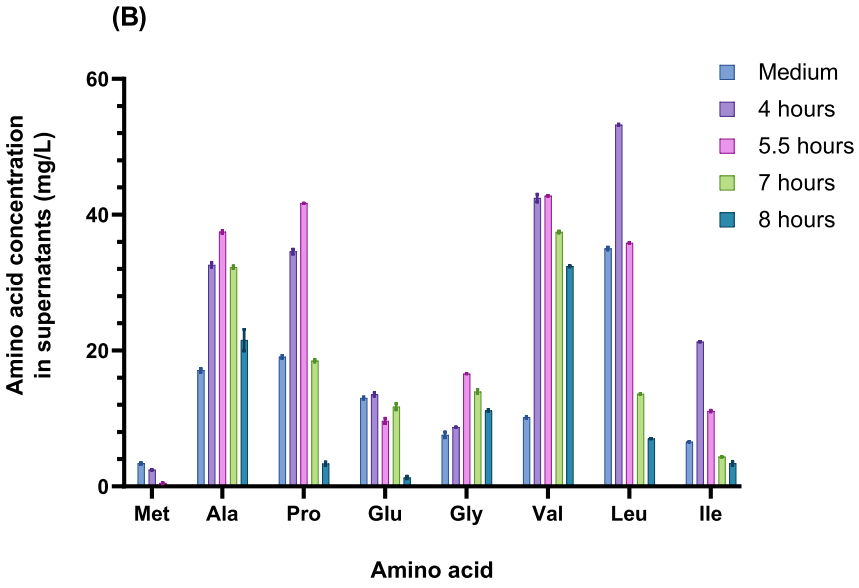
3.2.2.2 Fed-batch fermentation

As the amino acid production showed growth dependency in *C. glutamicum*, the effect of exponential feeding on amino acid production was investigated in an attempt to achieve high productivity. To do so, one fed-batch fermentation experiment was performed using exponential feeding. A fed-batch fermentation consists of two phases: a batch phase and a fed-batch phase. When the batch phase was finished (9 h), indicated by drop in off-gas CO_2, the fed-batch phase was commenced by addition of feed exponentially. The feed was composed of BSG hydrolysate and salts. To achieve high biomass concentration during the fed-batch phase, a higher glucose concentration (4 g/L) was used in the feed as compared to the initial medium (2.5 g/L). The results from the fed-batch fermentation with *C. glutamicum* are presented in Figure 5. It is evident from the growth curve that the culture was not growing exponentially during the fed-batch phase indicating that growth was limited by feed media component (Figure 5A). Contrary to our expectations, beside Gly all amino acid concentrations were found to be low

throughout the fed-batch phase (Figure 5C). The lack of amino acid production could be ascribed to an increasing consumption of amino acids from the medium which may occur under C-limited conditions (Paczia et al., 2012). This hypothesis was corroborated by sample analysis indicating a complete depletion of glucose, acetate and lactate during the fed-batch phase. Similar to the batch phase, xylose and arabinose were not consumed during the fed-batch phase. This suggested that *C. glutamicum* is incapable of metabolizing these sugars, even under severe nutrient deprivation (data not show). Besides, the observed accumulation of Gly could be due to selective degradation of intracellular proteins stemming from nutrient limitation (Gur et al., 2017).

Additional studies are needed to investigate the low or no production of amino acids during the fed-batch phase. Future research should focus on optimizing the process conditions with emphasis on high feed substrate/glucose concentration as it seems to be the limiting factor. To maximize amino acid production, the feed should be formulated in such a way to match the intended growth rate during exponential feeding. This could potentially be achieved by increasing the concentration of BSG-derived glucose in the feed which requires further optimization of the hydrolysis process. Such efforts should concentrate on optimizing the enzymatic hydrolysis of cellulose rather than hemicellulose since *C. glutamicum* did not utilize the hemicellulosic monosaccharides (xylose and arabinose) from the BSG hydrolysate (Figure S2). One possible strategy for optimizing enzymatic cellulose hydrolysis of BSG could be to remove hemicellulose and lignin using a sequential pretreatment with dilute acid and base as reported by Mussatto et al (2008). The idea of this approach is to increase the accessibility of cellulose to cellulases by removing the protective layers of lignin and hemicellulose.


(A)
Optical density at 600 nm (OD_{600})
Batch phase
Fed-batch phase
Time (hours)
0
2
4
6
8
10
12
14
16
18
20


(B)
Amino acid concentration in supernatants (mg/L)
Medium
4 hours
5.5 hours
7 hours
8 hours
Met
Ala
Pro
Glu
Gly
Val
Leu
Ile
Amino acid

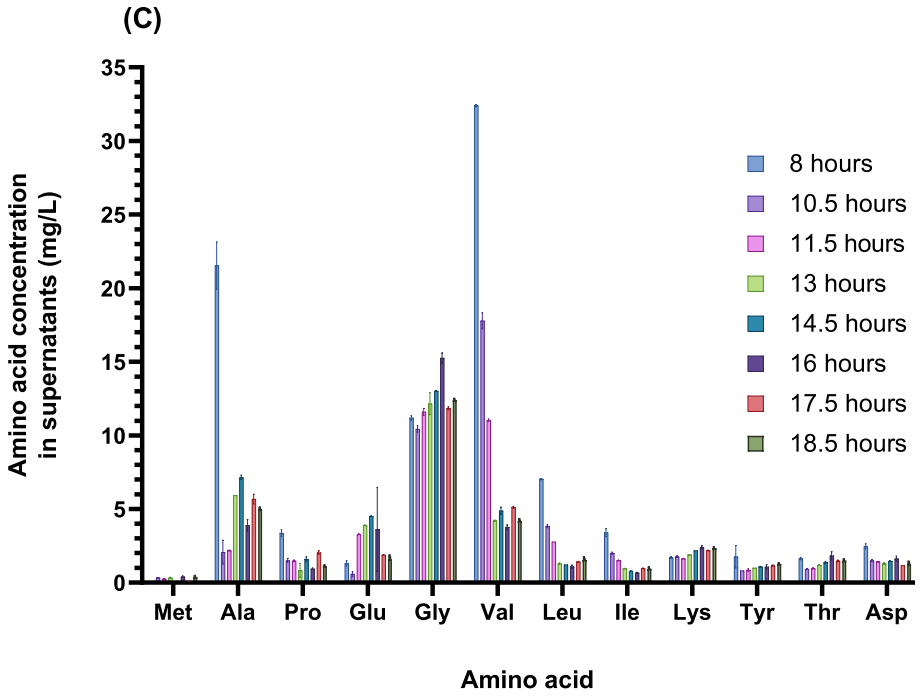


Figure 5. Fed-batch fermentation with Corynebacterium glutamicum. (A): growth curve of the batch phase (0-9 h) and fed-batch phase (9-18.5 h). Feeding was initiated after 9 h of cultivation. *(B): trends in amino acid concentrations during the batch phase. (C): trends in amino acid concentrations during the fed-batch phase where concentrations measured at 8 h were used as the reference point. Concentrations are given in mg/L and all data is reported as mean±SD of two technical replicates.*

3.3 General discussion

In the previous section, the overall performance of *S. cerevisiae* and *C. glutamicum* was compared with respect to growth, substrate consumption and amino acid production. This section will focus on the effect of cultivation mode and growth phase on the production of selected amino acids by *S. cerevisiae* and *C. glutamicum*.

3.3.1 Effect of cultivation mode and growth phase on amino acid production

3.3.1.1 *Corynebacterium glutamicum*

The changes in Ala, Pro, Val and Gly concentrations observed during the exponential and stationary phases of shake flask cultivation and batch fermentation with *C. glutamicum* are shown in Figure 6. These results indicate that amino acid production depends on the growth phase and cultivation mode.

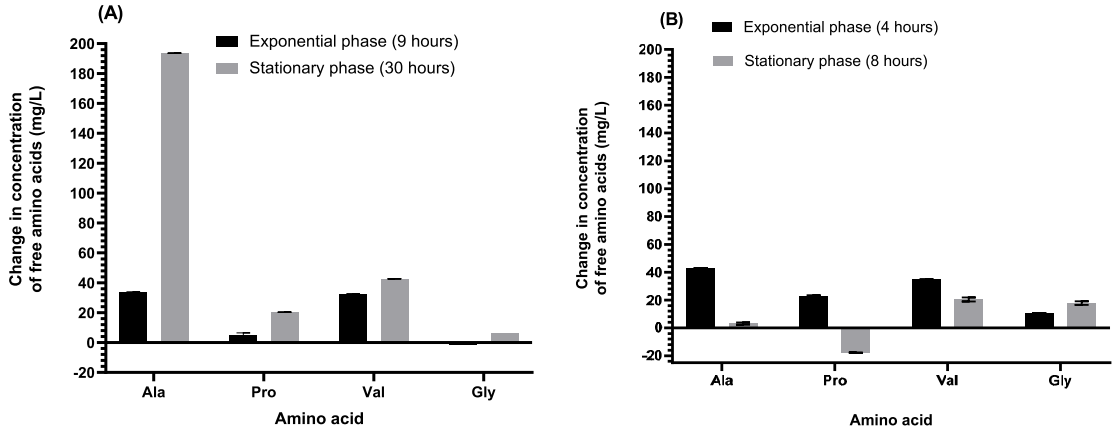


Figure 6. Effect of cultivation mode and growth phase on amino acid production by Corynebacterium glutamicum during (A): shake flask cultivation and (B): batch fermentation.

In the shake flask experiment, Ala, Pro and Val were produced during both growth phases whereas Gly was produced only during the stationary phase (Figure 6A). The production of Ala, Pro and Val was higher in the stationary phase at 30 h (Ala: 193.6 mg/L, Pro: 20.3 mg/L, Val: 42.7 mg/L) as compared to the exponential phase (9 h) (Ala: 33.7 mg/L, Pro: 5.21 mg/L, Val: 32.3 mg/L). This finding was surprising as the productivity is expected to be higher during the exponential phase where the cells possess the maximum metabolic activity. It is likely that the high amino acid concentrations observed in the stationary phase are caused by cell lysis rather than production as suggested by Paczia et al. (2012).

During batch fermentation, the exponential phase (4 h) was associated with the highest production of Ala, Pro and Val (Ala: 42.8 mg/L, Pro: 22.4 mg/L, Val: 34.8 mg/L) (Figure 6B). In the stationary phase (8 h), however, production of Ala and Val was lower than in the exponential phase while Pro was consumed (Ala: 3.16 mg/L, Pro: -17.8 mg/L, Val: 20.4 mg/L). For Gly, the opposite trend was observed as the production appeared to be higher in the stationary phase (Gly: 18.8 mg/L) and lower in the exponential phase (Gly: 10.5 mg/L).

Also, it was evident that Ala production during the stationary phase was substantially higher in the shake flask experiment as compared to that in batch fermentation. This might be related to the different sample processing method applied, depending on the cultivation mode. In shake flask experiment, the cell-free supernatants were produced by centrifugation only whereas in fermentation experiments supernatants

were obtained by a combination of centrifugation followed by filtration. As demonstrated by Paczia et al. (2012), filtration could increase the loss of metabolites, which might be the reason for the lower Ala production observed in the stationary phase of batch fermentation.

3.3.1.2 *Saccharomyces cerevisiae*

Changes in amino acid concentrations observed in shake flask cultivation and batch fermentation with *S. cerevisiae* are summarized in Figure 7. In the shake flask experiments, Ala, Pro, Glu and Val were produced during the exponential phase (50 h). In contrast, Ala, Pro and Val were consumed while Glu was produced during the stationary phase (60 h) (Figure 7A). Interestingly, the production Glu was nearly 50% higher during the stationary phase when compared to the exponential phase. As explained for Ala production by *C. glutamicum*, the high Glu concentration might be due to cell lysis.

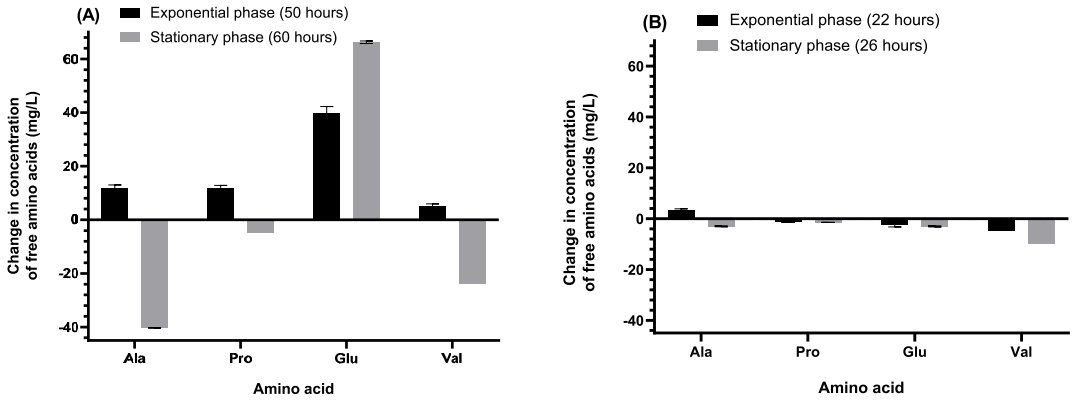


Figure 7. Effect of cultivation mode and growth phase on amino acid production by Saccharomyces cerevisiae. (A): shake flask cultivation. (B): batch fermentation.

Results from the batch fermentations with *S. cerevisiae* showed a clear absence of amino acid production except for Ala which increased slightly during the exponential phase (Figure 7B). One plausible explanation for this could be that the initial pH values differed between the shake flask (pH 6.5) and the batch fermentation (pH 5.0) experiments. Besides medium pH, the two cultivation modes are different with regard to agitation type, aeration and scaling. Considering these differences, it is more likely that variations in amino acid production are caused by a combination of several parameters rather than the single effect of medium pH. To investigate the effect of pH on amino acid production by *S. cerevisiae*, further study is required. Furthermore,

amino acid production by *S. cerevisiae* could possibly be enhanced by increasing the salinity as demonstrated by Malaney et al. (1991) who found elevated extracellular concentrations of certain amino acids at high NaCl concentrations.

3.3.2 General discussion

Although promising, the fermentative processes presented here are not without limitations. First, the productivities were considerably lower as compared to those reported for industrial production of amino acids by fermentation. Lee et al. (2016) reported production of 98 g/L Ala, 150 g/L Val and 13 g/L Pro from large-scale fermentations with metabolically engineered *C. glutamicum* strains. In the present study, a native strain of *C. glutamicum* was employed which yielded 0.043 g/L Ala, 0.035 g/L Val and 0.022 g/L Pro when cultured in a BSG hydrolysate-based medium. This difference may be related to the properties of the producing strain since industrial processes typically employ strains that have been metabolically engineered to overproduce certain amino acids. Another reason could be the presence of lignocellulose-derived inhibitors originating from the hydrolysis of BSG. Recent research has suggested that compounds derived from lignocellulose, such as furfural, 5-hydroxymethylfurural (HMF), vanillin, syringaldehyde and acetate, inhibit the production of value-added compounds by *C. glutamicum* including ethanol, succinic acid and amino acids (Sakai et al., 2007; Wang et al., 2018; Xu et al., 2015). As proposed by Wang et al. (2018), this problem could be overcome be applying strains of high inhibitor tolerance. In addition, any inhibitory effects caused by acetate could be eliminated by performing the hydrolysis in a different buffer solution as the ammonium acetate buffer may be the main contributing factor for the high media concentrations of acetate (8-9 g/L). Second, *C. glutamicum* did not produce any Glu. This finding was surprising as *C. glutamicum* is the most well-studied producer of Glu. The absence of Glu accumulation may be due to excessive biotin levels in the hydrolysate. High biotin levels are associated with reduced C-flux towards Glu synthesis and strengthened cell walls resulting in reduced secretion of Glu to the medium (Wen et al., 2018). Even though biotin was not quantified in the present study, it is reasonable to assume that the BSG hydrolysate contains significant biotin concentrations as Karlović et al. (2020) reported a biotin content of ~100 µg/kg dry weight of BSG. Previous studies have shown that Glu secretion can be induced under biotin-rich conditions by addition of antibiotics which inhibit cell wall

synthesis (Wang et al., 2018; Wen et al., 2018). The use of antibiotics poses environmental and health challenges and should therefore be avoided. A better approach to tackle this issue was described by Wen and Bao (2019) who showed that high Glu production can be achieved by using a metabolically engineered strain of *C. glutamicum* designed to overproduce Glu under biotin-rich conditions. In all, these strategies may prove useful in optimizing Glu production from BSG by *C. glutamicum*. With respect to *S. cerevisiae*, a comparably high Glu production was found in the shake flask experiment. This finding suggests that Glu production by *S. cerevisiae* was not affected by biotin.

Evaluation of other amino acid producing microorganisms would also be viable topic for future research. Besides *E. coli*, *C. glutamicum*, *L. plantarum* and *S. cerevisiae*, certain *Bacillus* species have been reported to be excellent amino acid producers (Shakoori et al., 2012). Previous studies have demonstrated the fermentative production of several amino acids by *B. cereus*, *B. anthracis* and *B. spp*. using either pure glucose or molasses as substrates (Abou-taleb, 2015; Shakoori et al., 2012). Yet, no prior studies have considered the utilization of lignocellulosic feedstock as substrates for amino acid production by *Bacillus* species. Therefore, a future study investigating amino acid production from BSG by different *Bacillus* species would be interesting.

4. Conclusions

The use of brewer's spent grain as substrate for amino acid production using selected microorganisms was demonstrated successfully for the first time. *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* were selected for amino acid production. Cultivation mode and growth phase affected amino acid production in both microorganisms. In shake and batch fermentations, C. *glutamicum* produced alanine, proline, valine and glycine where highest production was found for alanine in shake flasks (193-194 mg/L). In shake flask, *S. cerevisiae* produced alanine, proline, valine and glutamic acid with highest production of glutamic acid (65-66 mg/L). In batch fermentation mode, *S. cerevisiae* produced no amino acids.

Acknowledgements

This work was supported by NordForsk under the Nordic Council of Ministers of Norway [grant number 82342]. We would like to thank researcher Sreerekha Ramanand for generously sharing her knowledge and assisting with the shake flask cultivations. Further, we want to thank chief engineer Elin Austerheim for her indispensable help on miscellaneous practical matters in the laboratory. Finally, we would like to express our gratitude to laboratory technician Ulla Høgh Sproegel and engineer Eystein Opsahl for offering their help with the sample analysis and data acquisition.

References

Abou-taleb, K., 2015. Enhancing Production of Amino Acids from Bacillus spp. Using Batch and Fed-batch Fermentation Strategies. Br. Microbiol. Res. J. 5, 257–272. https://doi.org/10.9734/bmrj/2015/12447

Aliyu, S., Bala, M., 2011. Brewer's spent grain: A review of its potentials and applications. African J. Biotechnol. 10, 324–331. https://doi.org/10.5897/AJBx10.006

Blombach, B., Riester, T., Wieschalka, S., Ziert, C., Youn, J.W., Wendisch, V.F., Eikmanns, B.J., 2011. Corynebacterium glutamicum tailored for efficient isobutanol production. Appl. Environ. Microbiol. 77, 3300–3310. https://doi.org/10.1128/AEM.02972-10

Coelho, E., Rocha, M.A.M., Moreira, A.S.P., Domingues, M.R.M., Coimbra, M.A., 2016. Revisiting the structural features of arabinoxylans from brewers' spent grain. Carbohydr. Polym. 139, 167–176. https://doi.org/10.1016/j.carbpol.2015.12.006

Cohen, S.A., 1994. Applications of amino acid derivatization with 6- carbamate Analysis of feed grains , intravenous glycoproteins solutions and. J. Chromatogr. A 661, 25–34.

D'Este, M., Alvarado-Morales, M., Angelidaki, I., 2018. Amino acids production focusing on fermentation technologies – A review. Biotechnol. Adv. 36, 14–25. https://doi.org/10.1016/j.biotechadv.2017.09.001

Follmann, M., Ochrombel, I., Krämer, R., Trötschel, C., Poetsch, A., Rückert, C., Hüser, A., Persicke, M., Seiferling, D., Kalinowski, J., Marin, K., 2009. Functional genomics of pH homeostasis in Corynebacterium glutamicum revealed novel links between pH response, oxidative stress, iron homeostasis and methionine synthesis. BMC Genomics 10. https://doi.org/10.1186/1471-2164-10-621

Forssell, P., Kontkanen, H., Schols, H.A., Hinz, S., Eijsink, V.G.H., Treimo, J., Robertson, J.A., Waldron, K.W., Faulds, C.B., Buchert, J., 2008. Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. J. Inst. Brew. 114, 306–314. https://doi.org/10.1002/j.2050-0416.2008.tb00774.x

Gerstmeir, R., Wendisch, V.F., Schnicke, S., Ruan, H., Farwick, M., Reinscheid, D., Eikmanns, B.J., 2003. Acetate metabolism and its regulation in Corynebacterium glutamicum. J. Biotechnol. 104, 99–122. https://doi.org/10.1016/S0168-1656(03)00167-6

Glittenberg, D., 2012. Starch-Based Biopolymers in Paper, Corrugating, and Other Industrial Applications, Polymer Science: A Comprehensive Reference, 10 Volume Set. Elsevier B.V. https://doi.org/10.1016/B978-0-444-53349-4.00258-2

Gopinath, V., Nampoothiri, K.M., 2014. Corynebacterium glutamicum, Second Edi. ed, Encyclopedia of Food Microbiology. Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00076-8

Gur, E., Korman, M., Hecht, N., Regev, O., Schlussel, S., Silberberg, N., Elharar, Y., 2017. How to control an intracellular proteolytic system: Coordinated regulatory switches in the mycobacterial Pup-proteasome system. Biochim. Biophys. Acta - Mol. Cell Res. 1864, 2253–2260. https://doi.org/10.1016/j.bbamcr.2017.08.012

Karlović, A., Jurić, A., Ćorić, N., Habschied, K., Krstanović, V., Mastanjević, K., 2020. By-products in the malting and brewing industries-re-usage possibilities. Fermentation 6, 1–17. https://doi.org/10.3390/FERMENTATION6030082

Lee, J.Y., Na, Y.A., Kim, E., Lee, H.S., Kim, P., 2016. The actinobacterium Corynebacterium glutamicum, an industrial workhorse. J. Microbiol. Biotechnol. 26, 807–822. https://doi.org/10.4014/jmb.1601.01053

Liu, X., Jia, B., Sun, X., Ai, J., Wang, L., Wang, C., Zhao, F., Zhan, J., Huang, W., 2015. Effect of Initial PH on Growth Characteristics and Fermentation Properties of Saccharomyces cerevisiae. J. Food Sci. 80, M800–M808. https://doi.org/10.1111/1750-3841.12813

Lynch, K.M., Steffen, E.J., Arendt, E.K., 2016. Brewers' spent grain: a review with an emphasis on food and health. J. Inst. Brew. 122, 553–568. https://doi.org/10.1002/jib.363

Mahmood, Z., 2015. Microbial amino acids production, Microbial Biotechnology. https://doi.org/10.13140/2.1.2822.2245

Malaney, G.W., Tanner, R.D., Rodrigues, A.M., 1991. The production of extracellular and intracellular free amino acids during aerated fermentation of glucose by Baker's yeast (Saccharomyces cerevisiae). Folia Microbiol. (Praha). 36, 468–477. https://doi.org/10.1007/BF02884068

Mandalari, G., Faulds, C.B., Sancho, A.I., Saija, A., Bisignano, G., Locurto, R., Waldron, K.W., 2005. Fractionation and characterisation of arabinoxylans from brewers' spent grain and wheat bran. J. Cereal Sci. 42, 205–212. https://doi.org/10.1016/j.jcs.2005.03.001

Mitri, S., Salameh, S.J., Khelfa, A., Leonard, E., Maroun, R.G., Louka, N., Koubaa, M., 2022. Valorization of Brewers' Spent Grains: Pretreatments and Fermentation, a Review. Fermentation 8. https://doi.org/10.3390/fermentation8020050

Mussatto, S.I., Fernandes, M., Milagres, A.M.F., Roberto, I.C., 2008. Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain. Enzyme Microb. Technol. 43, 124–129. https://doi.org/10.1016/j.enzmictec.2007.11.006

Paczia, N., Nilgen, A., Lehmann, T., Gätgens, J., Wiechert, W., Noack, S., 2012. Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. Microb. Cell Fact. 11, 1–14. https://doi.org/10.1186/1475-2859-11-122

Rodriguez, A., Martínez, J.A., Flores, N., Escalante, A., Gosset, G., Bolivar, F., 2014. Engineering Escherichia coli to overproduce aromatic amino acids and derived compounds. Microb. Cell Fact. 13, 1–15. https://doi.org/10.1186/s12934-014-0126-z

Sakai, S., Tsuchida, Y., Okino, S., Ichihashi, O., Kawaguchi, H., Watanabe, T., Inui, M., Yukawa, H., 2007. Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested Corynebacterium glutamicum R. Appl. Environ. Microbiol. 73, 2349–2353. https://doi.org/10.1128/AEM.02880-06

Sanchez, S., Rodríguez-Sanoja, R., Ramos, A., Demain, A.L., 2018. Our microbes not only produce antibiotics, they also overproduce amino acids. J. Antibiot. (Tokyo). 71, 26–36. https://doi.org/10.1038/ja.2017.142

Shakoori, F.R., Butt, A.M., Ali, N.M., Zahid, M.T., Rehman, A., Shakoori, A.R., 2012. Optimization of fermentation media for enhanced amino acids production by bacteria isolated from natural sources. Pak. J. Zool. 44, 1145–1157.

Sousa, D., Venâncio, A., Belo, I., Salgado, J.M., 2018. Mediterranean agro-industrial wastes as valuable substrates for lignocellulolytic enzymes and protein production by solid-state fermentation. J. Sci. Food Agric. 98, 5248–5256. https://doi.org/10.1002/jsfa.9063

Stoimenova, A., Ivanov, K., Obreshkova, D., Saso, L., 2013. Biotechnology in the production of pharmaceutical industry ingredients: Amino acids. Biotechnol. Biotechnol. Equip. 27, 3620–3626. https://doi.org/10.5504/bbeq.2012.0134

Toe, C.J., Foo, H.L., Loh, T.C., Mohamad, R., Rahim, R.A., Idrus, Z., 2019. Extracellular proteolytic activity and amino acid production by lactic acid bacteria isolated from malaysian foods. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20071777

Tsao, G.T., Vegas, L., 1982. Sequential Utilization 43, 840–845.

Turcotte, B., Liang, X.B., Robert, F., Soontorngun, N., 2010. Transcriptional regulation of nonfermentable carbon utilization in budding yeast. FEMS Yeast Res. 10, 2–13. https://doi.org/10.1111/j.1567-1364.2009.00555.x

Venkata Narayana, A., Vamsi Priya, A., Venkata Nadh, R., Swami, A.V.N., Sumalatha, B., Vijaya Leela, M., 2013. Methionine production by coryneform bacteria through fermentation. Res. J. Pharm. Biol. Chem. Sci. 4, 1489–1498.

Wang, X., Khushk, I., Xiao, Y., Gao, Q., Bao, J., 2018. Tolerance improvement of Corynebacterium glutamicum on lignocellulose derived inhibitors by adaptive evolution. Appl. Microbiol. Biotechnol. 102, 377–388. https://doi.org/10.1007/s00253-017-8627-4

Wen, C., Zhang, J., Duan, Y., Zhang, H., Ma, H., 2019. A Mini-Review on Brewer's Spent Grain Protein: Isolation, Physicochemical Properties, Application of Protein, and Functional Properties of Hydrolysates. J. Food Sci. 84, 3330–3340. https://doi.org/10.1111/1750-3841.14906

Wen, J., Bao, J., 2019. Engineering Corynebacterium glutamicum triggers glutamic acid accumulation in biotin-rich corn stover hydrolysate. Biotechnol. Biofuels 12, 1–11. https://doi.org/10.1186/s13068-019-1428-5

Wen, J., Xiao, Y., Liu, T., Gao, Q., Bao, J., 2018. Rich biotin content in lignocellulose biomass plays the key role in determining cellulosic glutamic acid accumulation by Corynebacterium glutamicum. Biotechnol. Biofuels 11, 1–13. https://doi.org/10.1186/s13068-018-1132-x

Xu, H.T., Wang, C., Zhou, Z.H., Chen, Z.J., Cai, H., 2015. Effects of lignocellulose-derived inhibitors on growth and succinic acid accumulation by Corynebacterium glutamicum. Biotechnol. Bioprocess Eng. 20, 744–752. https://doi.org/10.1007/s12257-015-0201-2

Zhang, B., Jiang, Y., Li, Z., Wang, F., Wu, X.Y., 2020. Recent Progress on Chemical Production From Non-food Renewable Feedstocks Using Corynebacterium

glutamicum. Front. Bioeng. Biotechnol. 8, 1–11.
https://doi.org/10.3389/fbioe.2020.606047

Supplementary information

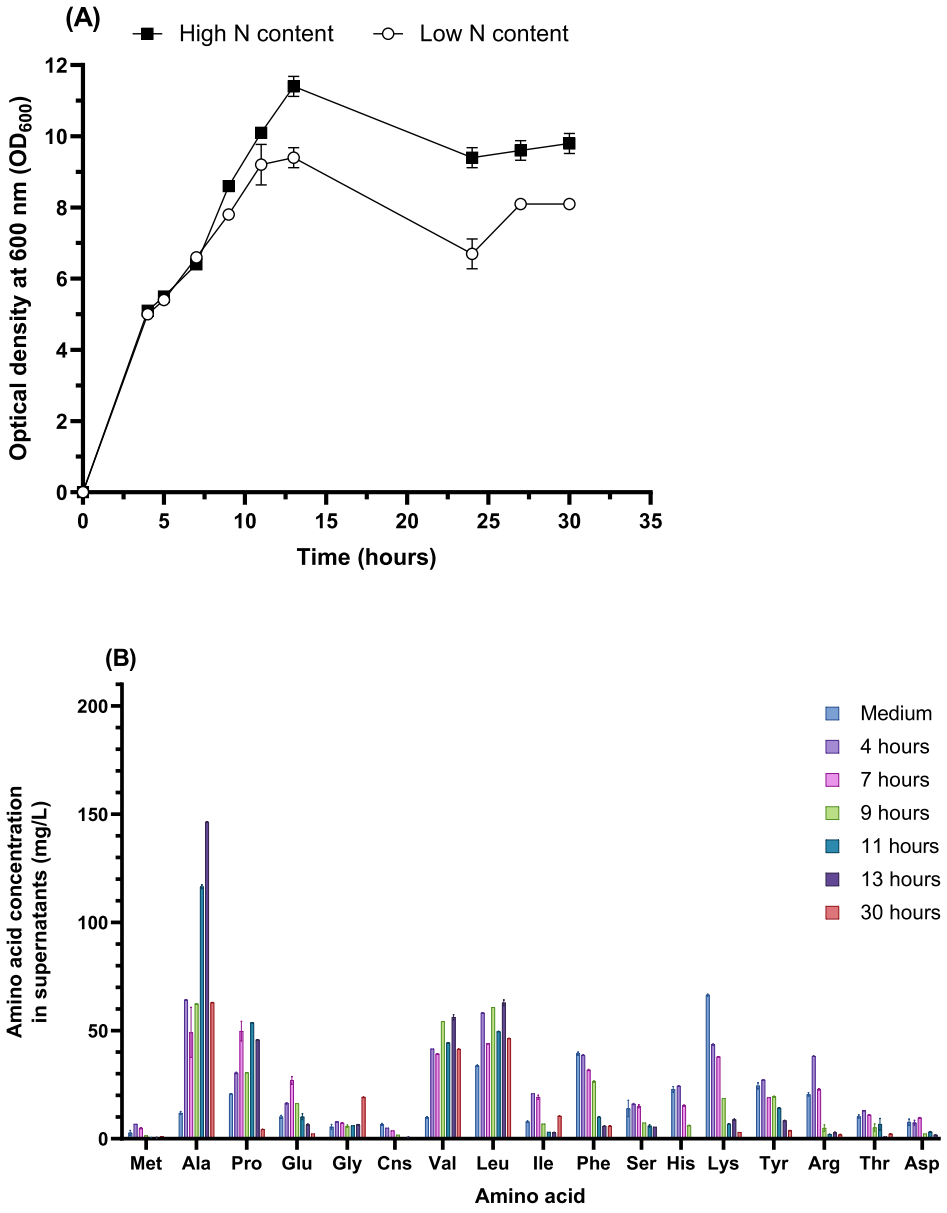


Figure S1. Shake flask experiments with Corynebacterium glutamicum: evaluation of media with different ammonium sulfate concentrations. (A) growth curves and (B) amino acid profiles (mg/L) obtained from cultivation in medium of low ammonium sulfate concentration. Concentrations of ammonium sulfate were 20 g/L (high ammonium sulfate) and 12 g/L (low ammonium sulfate) in the two tested media.

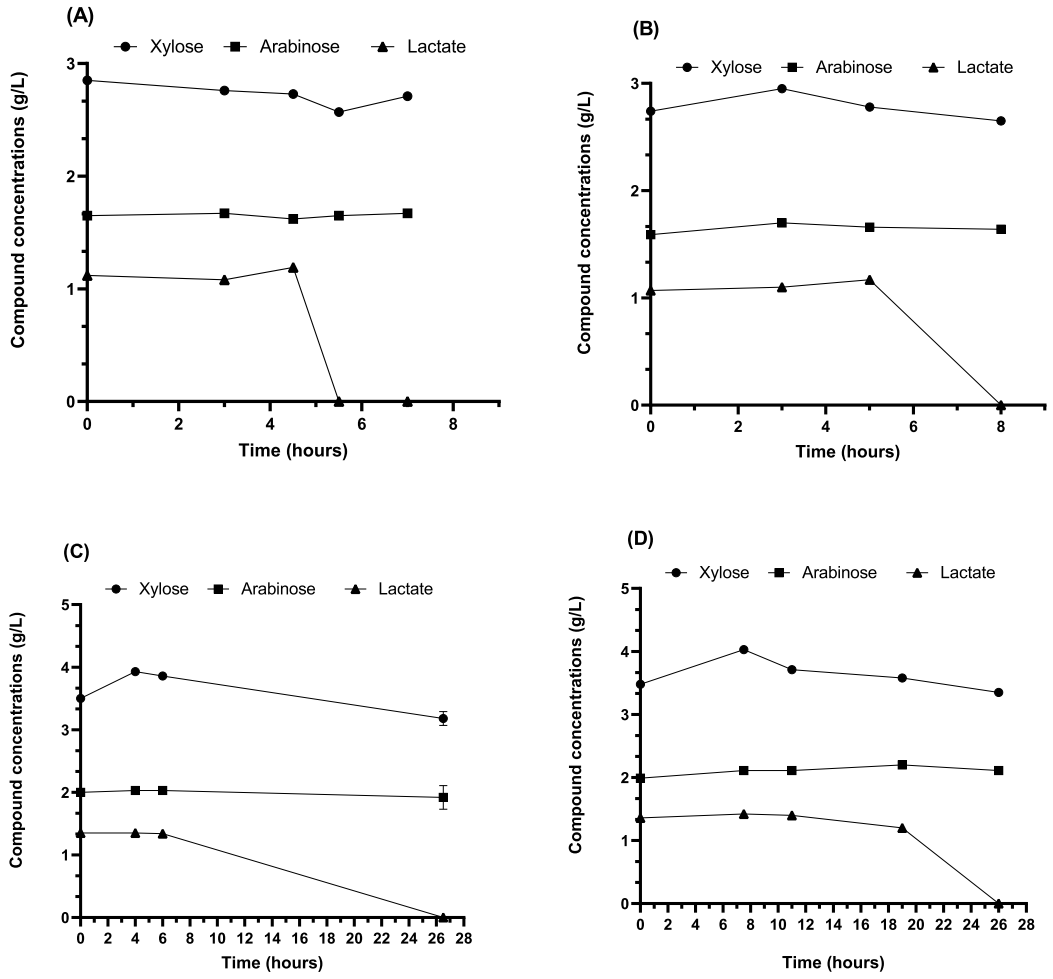


Figure S2. Trends in concentrations of xylose, arabinose and lactate (g/L) during batch fermentations with Corynebacterium glutamicum (A, B) and Saccharomyces cerevisiae (C, D). Data is reported as mean±SD of two technical replicates.

Manuscript 4

Evaluating brewer's spent grain and Ligno biomass as potential substrates for single cell protein production through submerged fermentation

Manuscript will be submitted to Fermentation

Evaluating brewer's spent grain and Ligno biomass as potential substrates for single cell protein production through submerged fermentation

Freja Karlsen[a*], Peter V. Skov[a], Catherine Boccadoro[b] and Sushil S. Gaykawad[b]

[a]Technical University of Denmark, DTU Aqua, Section for Aquaculture, Niels Juelsvej 30, 9850 Hirtshals, Denmark; pvsk@aqua.dtu.dk (P.V.S.)

[b]Norwegian Research Centre AS (NORCE), Prof. Olav Hanssensvei 15, 4021 Stavanger, Norway; susg@norceresearch.no (S.S.G.); cabo@norceresearch.no (C.B.)

[*]Corresponding author: frkar@aqua.dtu.dk (F.K.)

Abstract

Lignocellulosic waste represents valuable feedstock for single cell protein (SCP) production by submerged fermentation. In this study, brewer's spent grain (BSG) and Ligno biomass were evaluated as potential lignocellulosic substrates for production of SCP in the form of yeast biomass using *Saccharomyces cerevisiae*. *S. cerevisiae* was grown in BSG and Ligno biomass-based media and the nutritional quality of the harvested yeast biomasses was assessed based on their protein content and amino acid profile. The growth of *S. cerevisiae* was higher in the BSG-based medium ($OD_{600,\ max}$~7.0) as compared to the Ligno biomass-based medium ($OD_{600,\ max}$~2.5). BSG-derived yeast biomass had a higher protein content (37.9±1.08% DM) than the yeast biomass produced from Ligno biomass (27.9±0.18% DM). The yeast biomasses contained high levels of amino acids except for methionine (Ligno: 0.50±0.01% DM, BSG: 0.62±0.00% DM) and cystine (Ligno and BSG: 0.09±0.00% DM). Glutamic acid was the most abundant amino acid in yeast biomass derived from BSG (5.34±0.23% DM) and Ligno biomass (3.77±0.06% DM). This study demonstrated the production of high-quality SCP products from two lignocellulosic substrates by submerged fermentation. From an economic and nutritional perspective, BSG appeared to be the best substrate for SCP production.

Key words

Lignocellulosic biomass; Brewer's spent grain; Single cell protein; Submerged fermentation; *Saccharomyces cerevisiae*

1. Introduction

Protein is an essential source of amino acids for all living organisms. A steadily growing population has caused an increased global demand for animal protein which is chiefly supplied by the agriculture and aquaculture industries [1]. In 2020, aquaculture and capture fisheries accounted for ~17% of the global production of animal protein for human nutrition [2]. Aquaculture is one of the fastest growing industries and as a result, its contribution to the global supply of animal protein is expected to increase in the future. Fishmeal represents the most common protein source in formulated aquaculture feeds due to its high protein content (50-70%) and well-balanced amino acid profile [3]. The aquaculture sector is the primary consumer of fishmeal (75-80%) while a minor share is consumed by the pig and poultry industry (20-25%) [4]. Notwithstanding its high nutritional value, fishmeal is associated with economic and ecological concerns owing to high prices and declining stocks of forage fish used in the manufacturing of fishmeal. To meet the growing demand for animal protein and reduce the consumption of fishmeal by the food-producing sectors, alternative protein sources must be identified and incorporated in feeds—particularly aquaculture feeds. Single cell protein (SCP) is a promising new protein source because of a high protein content and a favourable amino acid profile that resembles fishmeal [5]. Single cell protein is sourced from different biomasses, mainly bacteria, fungi and microalgae [6]. The protein content is known to vary depending on the SCP source and it is typically highest in SCP from algae (60-70%) and bacteria (50-80%) while fungal SCP often has a lower protein content (30-50%) [7]. At present, various substrates have been utilized to produce fungal and bacterial SCP by fermentation [8]. Among them, lignocellulosic residues represent sustainable substrates for SCP production owing to their high abundance, inexpensive production and high content of cellulose and hemicellulose-bound sugars [9]. Previously, the production of SCP from lignocellulosic by-products has mainly been described for filamentous fungi and yeasts with *Saccharomyces cerevisiae* being the most well-studied and widely used yeast species [10–12].

Incorporation of lignocellulosic by-products as fermentation substrates for SCP production may alleviate the environmental burden posed by these residues and pave the way for a more sustainable production of protein for animal feeds or direct human consumption.

In this study, two lignocellulosic biomasses were evaluated as feedstock for SCP production by *S. cerevisiae* using submerged fermentation. The studied biomasses were brewer's spent grain (BSG), the major by-product from beer brewing, and Ligno biomass. *S. cerevisiae* was cultivated in media containing BSG or Ligno biomass hydrolysates as the C-source. The amino acid profiles and protein content of BSG and Ligno biomass-derived *S. cerevisiae* biomass were determined to evaluate their suitability as substrates for production of high-quality proteins. This study demonstrates the conversion of two lignocellulosic residues into yeast biomass of relatively high nutritional value using submerged fermentation. To our knowledge, this is the first study describing the nutritional quality of *S. cerevisiae* biomass from BSG and Ligno biomass obtained by submerged fermentation. Previously, Casas-Godoy et al. (2020) demonstrated the production of *S. cerevisiae* biomass using BSG; however, they did not determine the amino acid profile and protein content of the harvested yeast biomass [11].

2. Materials and methods

2.1 Materials

Brewer's spent grain (BSG) was supplied by Carlsberg, Denmark. It was processed as follows: autoclaving at 121°C for 15 min, drying at 60°C and micronization. The micronization of BSG was performed at the Danish Technological Institute (Kolding) to produce a size fraction of 125-250 µm. Hydrolyzed Ligno biomass was supplied by Borregaard (Norway).

2.2 Production of hydrolysates

The BSG hydrolysate was prepared from dried, micronized BSG according to the procedure described in Karlsen et al. (2022a) [13]. In brief, this procedure consists of three steps: enzymatic hydrolysis, neutralization of liquid fractions and sterile filtration. The hydrolysis was performed using the following conditions: 10% (w/v) BSG suspension in ammonium acetate buffer (50 mM, pH 5.0) and 24 h of

incubation at 50°C. The enzymatic conversion was initiated by addition of Depol 686L (Biocatalyst, UK) to a preheated buffer-BSG suspension in order to achieve a final enzyme activity of 106 U/mL. After hydrolysis, the liquid fractions were separated from the solid residues by centrifugation (4000 rpm, 21°C and 25 min). Neutralization of the liquid fractions led to production of a white precipitate which was removed by centrifugation (4000 rpm, 4°C and 25 min). In the last step, the liquid fractions were sterilized by filtration through a sterile filter (0.22 µm Sterivex-GV Pressure Filter unit, Merck Millipore).

2.3 Growth experiments

The yeast *Saccharomyces cerevisiae* (unknown strain) was employed for production of SCP from Ligno biomass and BSG hydrolysates. Previous studies have confirmed that *S. cerevisiae* is capable of utilizing both lignocellulosic hydrolysates as substrates for biomass production [13,14]. Therefore, initial screening in shake flasks was not performed prior to bioreactor cultivations.

2.3.1 Media composition

For all cultivations, the following lignocellulosic media were applied: KH_2PO_4, 0.052 g/L; $MgSO_4 \cdot H_2O$, 0.036 g/L; Na_2HPO_4, 0.0164 g/L; $MnSO_4 \cdot H_2O$, 0.00384 g/L; CH_3COONa, 0.0384 g/L; yeast extract, 1.0 g/L; BSG or Ligno biomass hydrolysate as the glucose source using concentrations of 2.5 g/L and 5 g/L, respectively. To prepare these media, a sterile solution of salts and yeast extract was mixed with BSG or Ligno biomass hydrolysates. The BSG hydrolysate was sterilized by filtration while the Ligno biomass hydrolysate was autoclaved at 121°C for 15 min. The salt-yeast extract solution was autoclaved at 121°C for 30 min. For all solutions, pH was adjusted to 5.00 ± 0.1 before sterilization.

2.3.2 Inoculum preparation

Inoculum was produced in shake flasks by inoculating 100 mL sterile medium with 500 µL 15% glycerol stock culture of *S. cerevisiae*. Cells were propagated at 160 rpm and 30°C in an orbital shaker (MaxQ 8000 orbital shaker, Thermo Fisher Scientific, Marietta, USA). The shake flask cultivations were terminated at an optical density of 2.0-2.5 (16-20 hours) and the resulting cell suspension was used for inoculation of the fermentation broth.

2.3.3 Batch fermentations

Batch fermentations were carried out in a 3.5 L stirred bioreactor (Chemap, Switzerland). For BSG and Ligno biomass, two batch fermentations were performed using a medium volume of 1.5 L. During the fermentations, the pressure was kept constant at 0.3 bar and the growing culture was aerated through the bottom sparger connected to the air supply. The temperature was maintained at 30±0.1°C. Dissolved oxygen (DO) was monitored using a DO sensor (InPro6800, Mettler Toledo) and kept above 40% of air saturation by regulating agitation speed and airflow. Medium pH was monitored by a pH sensor (InPro3253, Mettler Toledo) and maintained at 5.00±0.1 by addition of acid (2 M H_2SO_4) and base (1 M NaOH). Foaming was controlled by addition of the antifoaming agent Glanapon 2000. Off-gas percentages of CO_2 and O_2 were determined with one-minute intervals using a mass spectrometer (Prima Pro Process Mass Spectrometer, ThermoFisher Scientific) and all off-gas data was acquired from Labview. When off-gas CO_2 dropped, the experiments were terminated.

2.4 Analytical methods

Samples were collected aseptically with frequent intervals and used for duplicate analysis of biomass, amino acids and sugars. The optical density was determined immediately after each sampling. The rest of the sample was centrifuged (2500 rpm, 4 °C and 15 min) to separate the liquid fraction from the biomass. Biomasses from the same growth phase were combined and freeze-dried, and the supernatants were filtered to remove cell residues (0.22 µm Sterivex-GV Pressure Filter unit, Merck Millipore).

2.4.1 Optical density

Biomass was measured as optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Uppasala, Sweden).

2.4.2 Amino acid composition of biomass

The freeze-dried biomass was analyzed for amino acids as previously reported by Karlsen et al. (2022b) [15]. Initially, samples (~10 mg) were hydrolyzed to liberate protein-bound amino acids under the following conditions: 6 M HCl with 0.2% phenol

(1.8 mL), 24 h and 110°C. After hydrolysis, norvalin was added as an internal standard to reach a final concentration of 100 µM and HCl was removed by evaporation in a vacuum centrifuge (RVC 2-18 vacuum, CHRIST, Germany). The samples were resuspended in dilute HCl (3 mL, 0.01 M) and centrifuged at 3000*g* for 10 min to separate solid and liquid fractions. Then, the liquid fractions were diluted 10-fold and used for amino acid analysis. Amino acids were quantified according to the method described by Cohen (1994) in which amino acids react with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) to form UV-active amino acid-AQC derivatives. This reaction was performed using a derivatization kit (AccQ•Tag Ultra Derivatization Kit, Waters, Milford, Massachusetts). The amino acid derivatives were then separated by reversed-phase liquid chromatography and quantified by UV detection at 260 nm (Waters ACQUITY UltraPerformance liquid LC (UPLC)). Water (B) and acetonitrile (A) were used as eluents. For optimum separation of amino acids, a gradient 0-60% eluent B over 10 min and a flowrate of 0.7 mL/s were applied. Finally, the protein content of fungal biomass was estimated as the sum of all quantified amino acids.

2.4.3 Sugar concentrations of supernatants

Supernatants were analyzed for sugars and organic acids (glucose, xylose/mannose, arabinose, acetate and lactate) as previously reported by Karlsen et al. (2022a) [13]. Briefly, these compounds were quantified by HPLC analysis (Shimadzu Nexera XR) using reversed-phase separation mode (Biorad Aminex HPX-87H column) and refractive index detection (RID-20A). The following conditions were employed for chromatographic separation: a flowrate of 0.6 mL/min, a temperature of 30°C and a 4 mM aqueous sulfuric acid eluent. Prior to analysis, filtrated samples were diluted two-fold in a 0.2% aqueous sodium azide solution. Sample analysis was performed semi-quantitatively by comparing samples with 10 g/L standard solutions of each compound.

2.5 Data analysis

All samples were analyzed in duplicates and results presented as mean±SD of two technical replicates. All figures were prepared in the software SigmaPlot version 14.0.

3. Results and discussion

3.1 Concentrations of amino acids, sugars and organic acids in Ligno biomass and BSG-based media

S. cerevisiae was cultured in two lignocellulosic media based on BSG and Ligno biomass hydrolysates. It is well-known that *S. cerevisiae* accumulates amino acids from the medium; hence, high concentrations of amino acids in the medium may be reflected in the biomass of *S. cerevisiae* [17]. To better explain any potential differences in the amino acid profiles of SCP from BSG and Ligno biomass, the amino acid compositions of both media were determined and compared in Figure 1.

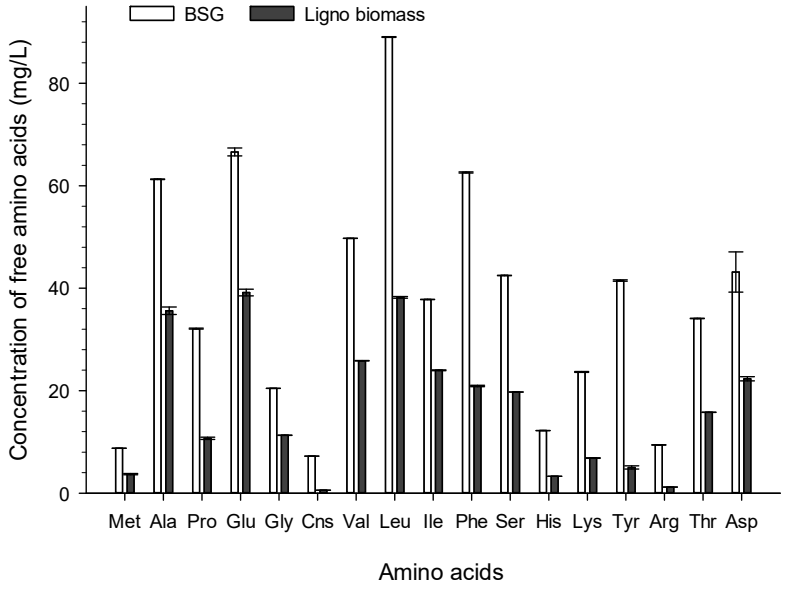


Figure 1. Amino acid concentrations of media with brewer's spent grain (BSG) and Ligno biomass hydrolysates. Data for the BSG-based medium is acquired from Karlsen et al. (2022a) [13].

A comparison of the two media revealed a markedly higher concentration of all amino acids in the BSG-based medium. This result could be related to compositional differences between Ligno biomass and BSG or the hydrolysis method. The relatively high amino acid concentrations found in the BSG-based medium may be due to solubilization of free amino acids during hydrolysis. Alternatively, it is possible that a minor part of the protein in BSG has been hydrolyzed to amino acids because of a low proteolytic activity reported for Depol 686L [18]. The occurrence of amino acids in the Ligno biomass-based medium may stem from the yeast extract as no free amino acids were measured in the Ligno biomass hydrolysate (data not shown).

Based on these results, BSG-derived SCP is expected to have a higher content of all amino acids as compared to SCP from Ligno biomass.

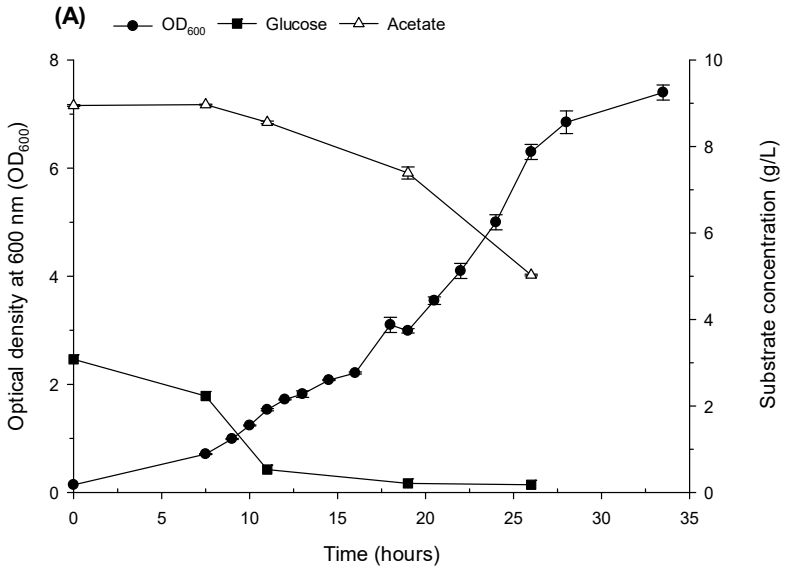
Chemical analysis of sugars and organic acids confirmed that the BSG-based medium contained a broad spectrum of potential substrates for fermentation including glucose (~3.1 g/L), xylose (~3.5 g/L), arabinose (~2.0 g/L), lactate (~1.4 g/L) and acetate (~9.0 g/L), with acetate being the most abundant compound. While glucose primarily originates from cellulose hydrolysis and yeast extract, xylose, arabinose and acetate are generated by hydrolysis of arabinoxylan, the most predominant hemicellulose in BSG [19]. Furthermore, the high acetate concentration can partly be ascribed to the buffer (ammonium acetate) used for BSG hydrolysis which may contribute significantly to the final concentration of acetate in the BSG-based medium. Lactate might result from solubilization of lactic acid, which is produced by indigenous lactic acid bacteria (LAB) present in raw BSG [20]. In contrast, Ligno biomass-based medium contained glucose as the main sugar as well as low levels of a compound which was identified as xylose based on its retention time. As xylose did not occur in the Ligno hydrolysate (data not shown), the measured concentrations must stem from another compound. This compound is probably mannose from the yeast extract released from manoproteins of yeast cell walls during the production of yeast extract [21].

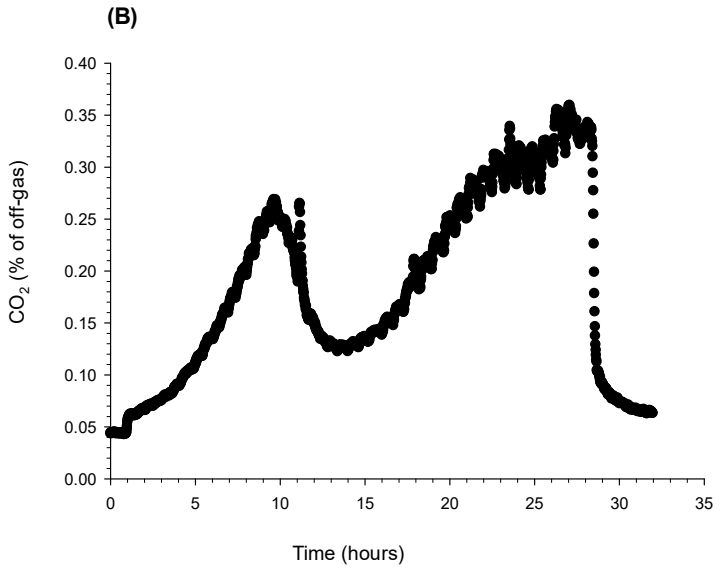
3.2 Single cell protein production by batch fermentation

This section describes the overall performance of *S. cerevisiae* for the two lignocellulosic media focusing on growth and amino acid composition of the biomass. To elucidate how the intracellular pool of amino acids evolves over time, the content of individual amino acids in biomass from different growth phases was compared.

3.2.1 Brewer's spent grain

Figure 2 presents the results obtained from batch fermentation with *S. cerevisiae* using BSG hydrolysate as the C-source.


(A)
OD600
Glucose
Acetate
Optical density at 600 nm (OD600)
Substrate concentration (g/L)
Time (hours)


(B)
CO2 (% of off-gas)
Time (hours)

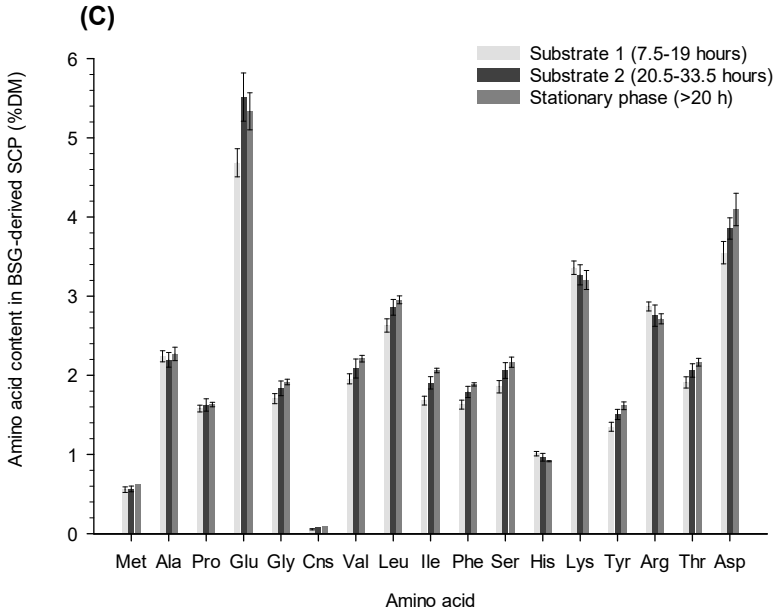


Figure 2. Biomass production (OD_{600}) and substrate consumption (A), off-gas CO_2 (B) and amino acid composition of single cell protein (SCP) (C) obtained from batch fermentation with Saccharomyces cerevisiae using brewer's spent grain (BSG) hydrolysate as C-source. Figure A is acquired from Karlsen et al. (2022a) [13]

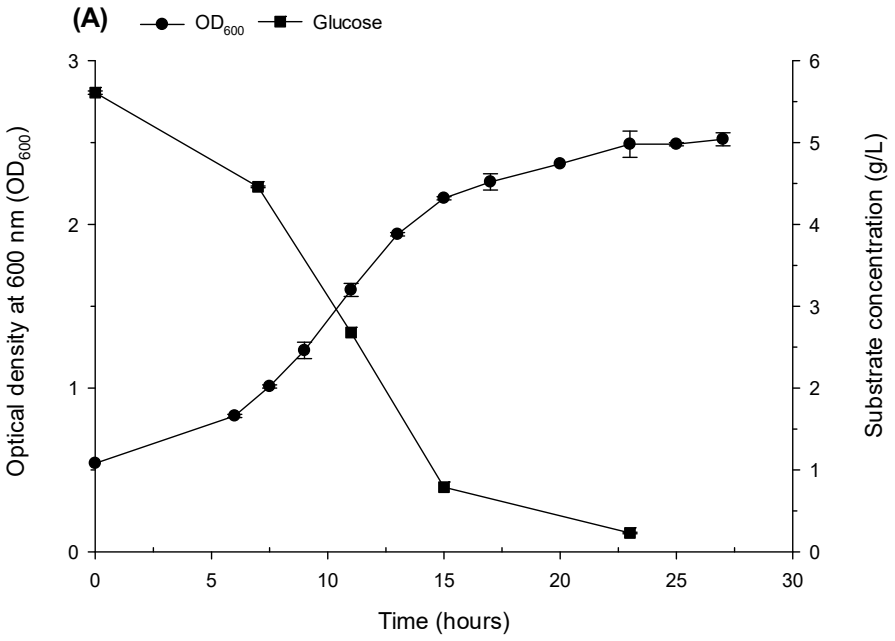
The growth curve revealed a high growth performance with a maximum OD_{600} around 7 (Figure 2A). A decrease in off-gas CO_2 after 28 h indicated that the stationary phase had been reached, although OD_{600} was still increasing at this point (Figure 2B). The curvature of the growth curve is indicative of diauxic growth when cultivating *S. cerevisiae* in BSG-based medium. This observation was further supported by the appearance of the off-gas CO_2 profile which consists of two clearly defined curves, each corresponding to the consumption of one substrate (Figure 2B). Furthermore, the substrate consumption pattern provides strong evidence that *S. cerevisiae* is growing diauxically in BSG-based medium as glucose is consumed fast during the initial period of cultivation followed by slower consumption of acetate (Figure 2A). These results confirm that glucose is the most preferred substrate and suggest that acetate is the second most preferred substrate.

The majority of intracellular amino acids were found in high concentrations except for methionine (Met) (~0.60% DM), cystine (Csn) (~0.10% DM) and histidine (His) (~0.90% DM) (Figure 2C). With concentrations of 4.80-5.50% DM, glutamic acid (Glu) was the most abundant amino acid followed by aspartic acid (Asp) (3.80-4.10% DM) and lysine (3.20-3.30% DM). For most amino acids, minor fluctuations were

observed between different growth phases; however, no general trend could be inferred. Leucine (Leu) was expected to be the most abundant amino acid in the biomass because it had the highest medium concentration (Figure 1). However, the high medium concentration of Leu was not reflected in the biomass. This finding is supported by Watson (1976) who found that some amino acids, including Leu, are not accumulated in biomass despite high concentrations in the medium [17].

3.2.2 Ligno biomass

The results obtained from cultivation of *S. cerevisiae* in Ligno biomass-based medium are summarized in Figure 3.



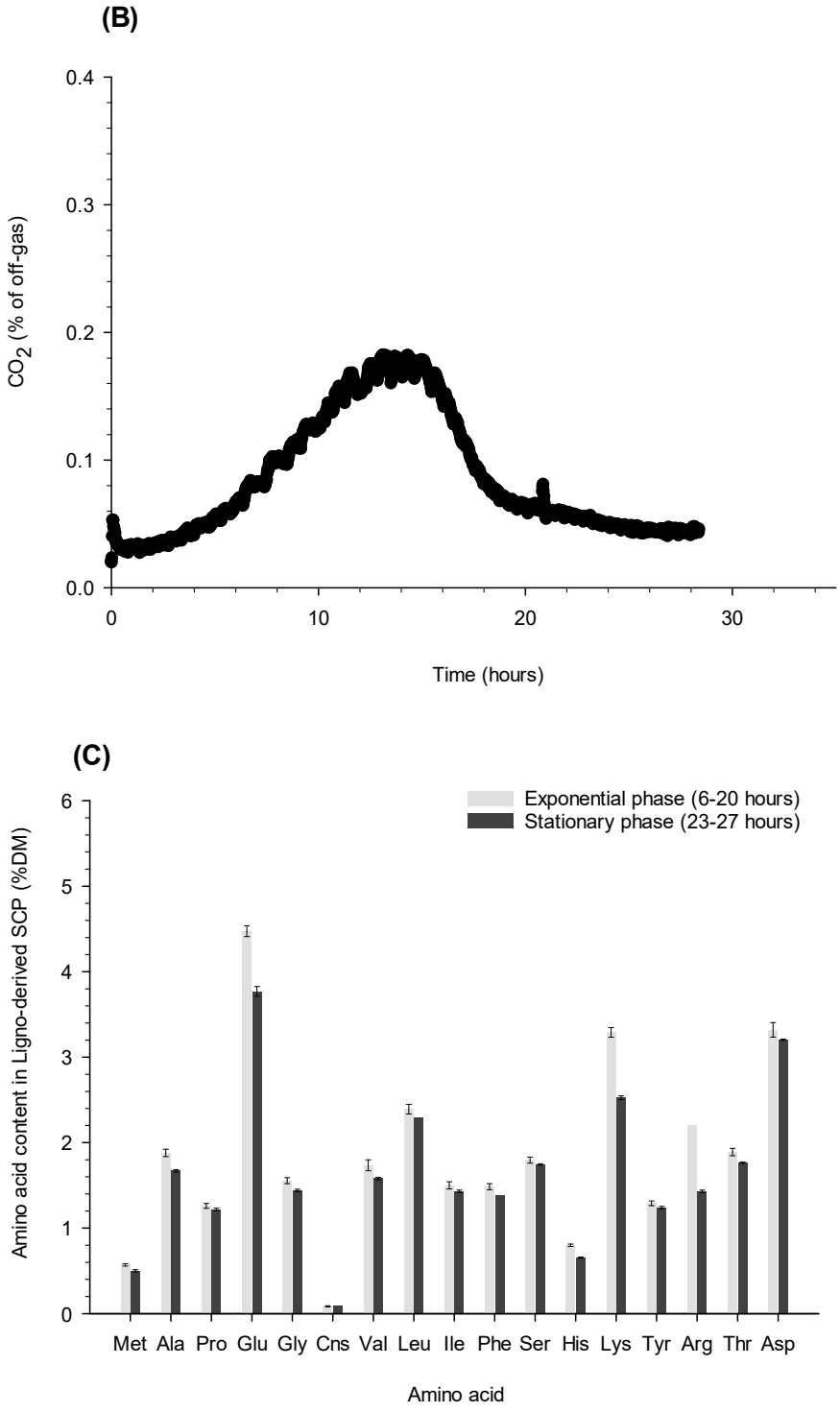


Figure 3. Biomass production (OD_{600}) and substrate (glucose) consumption (A), off-gas CO_2 (B) and amino acid composition of single cell protein (SCP) (C) obtained from batch fermentation with Saccharomyces cerevisiae using Ligno biomass hydrolysate as C-source.

Compared to the BSG based-medium, the growth performance of *S. cerevisiae* was markedly lower in the Ligno biomass-based medium. This difference was evident

from the lower maximum OD_{600} (~2.5) observed for Ligno biomass as compared to BSG (~7.0) (Figure 2A and Figure 3A). Furthermore, a decrease in off-gas CO_2 indicated that *S. cerevisiae* entered the stationary phase after ~16 h when grown on Ligno biomass whereas the growth started to cease after ~28 h in the BSG-based medium (Figure 2B and Figure 3B). The relatively low growth of *S. cerevisiae* in Ligno biomass-based medium is probably due to the presence of furfural and 5-hydroxymethylfurural in the Ligno biomass hydrolysate which were determined at 24.7 ppm and 23.3 ppm, respectively (Eurofins, Norway). These compounds originate from the decomposition of lignocellulosic sugars during hydrolysis and are known to have a strong inhibitory effect on growth and productivity of *S. cerevisiae*, even at low concentrations [22]. Further examination of the growth curve and off-gas CO_2 profile suggested that the growth of *S. cerevisiae* is sustained by a single substrate rather than sequential utilization of two substrates as was seen for the BSG-based medium. Investigation of the substrate consumption pattern corroborated the results from the growth and off-gas CO_2 profiles as a declining trend in the glucose concentration confirmed that glucose was utilized as the sole substrate during cultivation of *S. cerevisiae* in Ligno biomass-based medium (Figure 3A).

The amino acid composition of *S. cerevisiae* grown on Ligno biomass was comparable to that obtained from cultivation on BSG (Figure 3C). As for BSG, Glu (~4.50% DM), Asp (~3.30% DM) and Lys (~3.30% DM) were the most abundant amino acids while Met (~0.60% DM), Csn (~0.10% DM) and His (~0.80% DM) were found in lowest intracellular concentrations. For most amino acids, concentrations were higher during the exponential phase (6-20 h) as compared to the stationary phase (23-27 h) with the greatest difference observed for Glu, Lys and Arg. A possible explanation for this result may be that amino acids from the intracellular pool of *S. cerevisiae* are consumed during the stationary phase due to depletion of amino acids in the medium. This theory was supported by the fact that the medium contained negligible concentrations of all amino acids by the end of the fermentation experiment (data not shown).

3.3 Evaluation of the nutritional quality of *S. cerevisiae* biomass produced from brewer's spent grain and Ligno biomass

Having discussed the characteristics of *S. cerevisiae* biomass obtained from Ligno biomass and BSG, this section will consider the suitability of these products as alternative protein sources focusing on fish feeds. In this context, two major aspects are taken into account: the nutritional quality of the yeast biomasses and the fulfillment of key criteria for SCP sources.

To be considered suitable for fish feed, a protein source must have a high protein content (50-70% DM), an amino acid profile that matches the requirement of the cultured fish species and an appropriate ratio of essential to non-essential amino acids (EAA/NEAA), preferably around one (50/50) [23,24]. Fishmeal satisfies all the above-mentioned criteria and is therefore an ideal protein source for fish [25]. As a result, the evaluation of Ligno biomass and BSG-derived biomasses of *S. cerevisiae* will be based on a comparison with fishmeal. Table 1 compares the nutritional parameters of *S. cerevisiae* biomass from BSG and Ligno biomass with those reported for fishmeal. It can be seen that both yeast biomasses were inferior to fishmeal because of a comparably low protein content (28-38% DM). On the other hand, both biomasses had relatively high levels of all amino acids except for Met and His, and, like fishmeal, they had an EAA/NEEA ratio close to one. A comparison between the two yeast biomasses reveals that BSG-derived *S. cerevisiae* biomass had higher nutritional value than *S. cerevisiae* biomass from Ligno biomass due to higher content of protein (37.9% vs 27.9%) and amino acids as well as a more favorable EAA/NEEA ratio (0.98 vs 0.94). Results for the BSG-derived *S. cerevisiae* biomass are in agreement with those of Lapeña et al. (2020) who reported a protein content ranging from 39.4 to 44.1% for different yeast species grown on spruce wood [12].

Table 1. Amino acid compositions and protein contents of *S. cerevisiae* biomass produced by batch fermentation with Ligno biomass and brewer's spent grain (BSG) compared to fishmeal. The analyzed yeast biomasses were harvested during the stationary phase for BSG and Ligno biomass.

	BSG[a]	Ligno biomass[a]	Fishmeal[b]
Protein content (% DM)[#]	37.9 ± 1.08	27.9 ± 0.18	49.5-70.7
EAA (% DM)[*]			
Phenylalanine (Phe)	1.89 ± 0.02	1.38 ± 0.00	2.50-3.10
Tryptophan (Trp)	ND	ND	0.65-0.90
Histidine (His)	0.92 ± 0.01	0.66 ± 0.01	2.34-4.19
Valine (Val)	2.21 ± 0.04	1.58 ± 0.01	2.56-3.80
Isoleucine (Ile)	2.06 ± 0.03	1.43 ± 0.01	1.74-3.10
Leucine (Leu)	2.96 ± 0.05	2.29 ± 0.00	4.42-5.60
Methionine (Met)	0.62 ± 0.00	0.50 ± 0.01	1.50-2.00
Threonine (Thr)	2.17 ± 0.05	1.77 ± 0.01	2.62-3.30
Lysine (Lys)	3.21 ± 0.12	2.53 ± 0.02	3.60-5.90
Arginine (Arg)	2.72 ± 0.06	1.43 ± 0.01	3.49-4.99
NEAA (% DM)[‡]			
Alanine (Ala)	2.27 ± 0.08	1.67 ± 0.01	1.97-4.90
Aspartic acid (Asp)	4.10 ± 0.21	3.21 ± 0.01	4.55-7.00
Glutamic acid (Glu)	5.34 ± 0.23	3.77 ± 0.06	6.92-9.90
Glycine (Gly)	1.92 ± 0.04	1.44 ± 0.01	3.83-6.78
Serine (Ser)	2.17 ± 0.06	1.75 ± 0.01	2.16-3.14
Tyrosine (Tyr)	1.62 ± 0.05	1.24 ± 0.01	1.88-3.30
Proline (Pro)	1.63 ± 0.03	1.22 ± 0.01	1.93-3.10
Cystine (Csn)	0.09 ± 0.00	0.09 ± 0.00	-
EAA/NEAA	0.98	0.94	0.96-1.34

[a]Data is reported as mean±SD of two technical replicates. Samples from cultivation on Ligno biomass and BSG were collected and merged after 23-27 and >28 hours, respectively. [b]References: [26–28]. [#]Protein contents are calculated as the sum of individual amino acids; therefore, any non-protein nitrogen is excluded. [*]Essential amino acids (EAA) for fish. [‡]Non-essential amino acids (NEAA) for fish.

3.4 Future perspectives

For an adequate SCP source, the following key criteria must be satisfied: 1) high nutritional quality, 2) low toxin concentrations and 3) economically viable production [5]. Economic production of SCP involves the use of fast growing microorganisms capable of converting cheap by-products into microbial biomass.

Due to their relatively low protein content, *S. cerevisiae* biomasses from BSG and Ligno biomass might be suitable for partial replacement of fishmeal at low inclusion levels (30-50%). To allow for higher inclusion (60-90%) of these products, however, the protein content must be increased which can be achieved through downstream processing. The most common approach to increase the protein content of yeast biomass is to disrupt the cell walls and subsequently extract the protein. Disruption of cell walls makes the protein more accessible for extraction and is typically performed using chemical, enzymatic or mechanical treatments [29].

The production of *S. cerevisiae* biomass from BSG seemed economically feasible due to high biomass accumulation. In contrast, Ligno biomass appeared to be a less favorable substrate for *S. cerevisiae* due to lower biomass production. As previously stated, the growth of *S. cerevisiae* is probably impaired by the presence of furfural and 5-hydroxymethylfurfural in the Ligno biomass hydrolysate. Thus, faster growth of *S. cerevisiae* in Ligno biomass-based medium might be facilitated by employing hydrolysis conditions that minimize production of these compounds or by detoxification of the Ligno biomass hydrolysate. Finally, continuous fermentation should be investigated for both lignocellulosic biomasses since this cultivation mode is known to be the most suitable for microbial biomass production as compared to batch and fed-batch fermentation modes [8].

4. Conclusions

The use of brewer's spent grain (BSG) and Ligno biomass as lignocellulosic substrates for production of single cell protein (SCP) in the form of yeast biomass was reported using *Saccharomyces cerevisiae*. *S. cerevisiae* was able to grow on both substrates but displayed the highest growth on BSG. Yeast biomass from BSG had a higher protein content (~38% DM) as compared to that derived from Ligno biomass (28% DM). Amino acid concentrations were generally high in the yeast biomass from either substrate except for methionine and histidine. For both substrates, glutamic acid was the most abundant amino acid in the yeast biomass.

Author Contributions

Conceptualization, S.S.G., F.K. and C.B.; methodology, S.S.G. and F.K.; validation, S.S.G. and F.K.; formal analysis, S.S.G. and F.K.; investigation, S.S.G. and F.K.; writing—original draft preparation, F.K.; writing—review and editing, S.S.G., C.B. and P.V.S.; visualization, F.K.; supervision, S.S.G. and C.B.; project administration, P.V.S. and C.B.; funding acquisition, P.V.S. and C.B. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by Nordforsk from the Nordic Council of Ministers (Norway), grant number 82342.

Acknowledgement

We want to thank researcher Sreerekha Ramanand for helping with the commencement of the experimental work. Furthermore, a great thanks to chief engineer Elin Austerheim for her assistance with practical issues in the lab. Lastly, thanks to lab technician Ulla Høgh Sproegel for and engineer Eystein Opsahl for offering their help with the sample analysis.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

This study did not report any data

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Olsen, Y. How can mariculture better help feed humanity? *Front. Mar. Sci.* **2015**, *2*, 2009–2012, doi:10.3389/fmars.2015.00046.

2. Boyd, C.E.; McNevin, A.A.; Davis, R.P. The contribution of fisheries and aquaculture to the global protein supply. *Food Secur.* **2022**, doi:10.1007/s12571-021-01246-9.

3. Oliva-Teles, A. Nutrition and health of aquaculture fish. *J. Fish Dis.* **2012**, *35*, 83–108, doi:10.1111/j.1365-2761.2011.01333.x.

4. EUMOFA *Fishmeal and fish oil: production and trade flows in the EU*; 2021;

5. Nangul, A.; Bhatia, R. Microorganisms : a Marvelous Source of Single Cell Proteins. *J. Microbiol. Biotechnol. Food Sci.* **2013**, *3*, 15–18.

6. Anupama; Ravindra, P. Value-added food: Single cell protein. *Biotechnol. Adv.* **2000**, *18*, 459–479, doi:10.1016/S0734-9750(00)00045-8.

7. Ritala, A.; Häkkinen, S.T.; Toivari, M.; Wiebe, M.G. Single cell protein-state-of-the-art, industrial landscape and patents 2001-2016. *Front. Microbiol.* **2017**, *8*, doi:10.3389/fmicb.2017.02009.

8. Ugalde, U.O.; Castrillo, J.I. Single Cell Proteins from Fungi and Yeasts. *Appl. Mycol. Biotechnol.* **2002**, *2*, 123–149.

9. Bajpai, P. *Single Cell Protein Production from Lignocellulosic Biomass*; 2017; ISBN 9789811058738.

10. Tropea, A.; Ferracane, A.; Albergamo, A.; Potortì, A.G.; Turco, V. Lo; Di Bella, G. Single Cell Protein Production through Multi Food-Waste Substrate Fermentation. *Fermentation* **2022**, *8*, 1–11, doi:10.3390/fermentation8030091.

11. Casas-Godoy, L.; González-Escobar, J.L.; Mathis, A.G.; Barrera-Martínez, I. Revalorization of untreated Brewer's spent grain: novel and versatile feedstock to produce cellulases, lipases, and yeast biomass in a biorefinery approach. *Biomass Convers. Biorefinery* **2020**, doi:10.1007/s13399-020-01157-3.

12. Lapeña, D.; Kosa, G.; Hansen, L.D.; Mydland, L.T.; Passoth, V.; Horn, S.J.; Eijsink, V.G.H. Production and characterization of yeasts grown on media

composed of spruce-derived sugars and protein hydrolysates from chicken by-products. *Microb. Cell Fact.* **2020**, *19*, 1–14, doi:10.1186/s12934-020-1287-6.

13. Karlsen, F.; Skov, P. V; Boccadoro, C.; Gawkawad, S. Evaluation of brewer's spent grain as a feedstock for amino acid production by submerged fermentation [manuscript in preparation]. **2022**, 1–28.

14. Gaykawad, S.S.; Zha, Y.; Punt, P.J.; van Groenestijn, J.W.; van der Wielen, L.A.M.; Straathof, A.J.J. Pervaporation of ethanol from lignocellulosic fermentation broth. *Bioresour. Technol.* **2013**, *129*, 469–476, doi:10.1016/j.biortech.2012.11.104.

15. Karlsen, F.; Skov, P. V.; Lund, I. Optimisation of alkaline protein extraction of brewer's spent grain. *J. Inst. Brew.* **2022**, 1–17, doi:DOI 10.1002/jib.703.

16. Cohen, S.A. Applications of amino acid derivatization with 6- carbamate Analysis of feed grains , intravenous glycoproteins solutions and. *J. Chromatogr. A* **1994**, *661*, 25–34.

17. Watson, T.G. Amino acid pool composition of Saccharomyces cerevisiae as a function of growth rate and amino acid nitrogen source. *J. Gen. Microbiol.* **1976**, *96*, 263–268, doi:10.1099/00221287-96-2-263.

18. Forssell, P.; Kontkanen, H.; Schols, H.A.; Hinz, S.; Eijsink, V.G.H.; Treimo, J.; Robertson, J.A.; Waldron, K.W.; Faulds, C.B.; Buchert, J. Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. *J. Inst. Brew.* **2008**, *114*, 306–314, doi:10.1002/j.2050-0416.2008.tb00774.x.

19. Coelho, E.; Rocha, M.A.M.; Moreira, A.S.P.; Domingues, M.R.M.; Coimbra, M.A. Revisiting the structural features of arabinoxylans from brewers' spent grain. *Carbohydr. Polym.* **2016**, *139*, 167–176, doi:10.1016/j.carbpol.2015.12.006.

20. Asurmendi, P.; Pascual, L.; Dalcero, A.; Barberis, L. Incidence of lactic acid bacteria and Aspergillus flavus in brewer's grains and evaluation of potential antifungal activity of these bacteria. *J. Stored Prod. Res.* **2014**, *56*, 33–37, doi:10.1016/j.jspr.2013.11.002.

21. Ahranjani, R.N.; Vaghari, H.; Jafarizadeh, H. Yeast Extracts : Production ,

Properties and Application. *6th Natl. Conf. Strateg. Res. Chem. Chem. Eng. With An Emphas. Indig. Technol. Iran* **2020**, 1–6.

22. Delgenes, J.P.; Moletta, R.; Navarro, J.M. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. *Enzyme Microb. Technol.* **1996**, *19*, 220–225, doi:10.1016/0141-0229(95)00237-5.

23. Peres, H.; Oliva-Teles, A. Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (Dicentrarchus labrax). *Aquaculture* **2006**, *256*, 395–402, doi:10.1016/j.aquaculture.2006.02.010.

24. Teles, A.O.; Couto, A.; Enes, P.; Peres, H. Dietary protein requirements of fish – a meta-analysis. *Rev. Aquac.* **2020**, *12*, 1445–1477, doi:10.1111/raq.12391.

25. Courtenay, W.R.; Bardach, J.E.; Ryther, J.H.; McLarney, W.O. Aquaculture, the Farming and Husbandry of Freshwater and Marine Organisms. *Copeia* **1973**, *1973*, 826, doi:10.2307/1443094.

26. Zhang, H.Y.; Piao, X.S.; Li, P.; Yi, J.Q.; Zhang, Q.; Li, Q.Y.; Liu, J.D.; Wang, G.Q. Effects of Single Cell Protein Replacing Fish Meal in Diet on Growth Performance, Nutrient Digestibility and Intestinal Morphology in Weaned Pigs. *Asian Australas. J. Anim. Sci.* **2013**, *26*, 1320–1328.

27. Ido, A.; Kaneta, M. Fish oil and fish meal production from urban fisheries biomass in Japan. *Sustain.* **2020**, *12*, 1–13, doi:10.3390/SU12083345.

28. Hamid, S.N.I.N.; Abdullah, M.F.; Zakaria, Z.; Yusof, S.J.H.M.; Abdullah, R. Formulation of Fish Feed with Optimum Protein-bound Lysine for African Catfish (Clarias Gariepinus) Fingerlings. *Procedia Eng.* **2016**, *148*, 361–369, doi:10.1016/j.proeng.2016.06.468.

29. García-Garibay, M.; Gómez-Ruiz, L.; Cruz-Guerrero, A.E.; Bárzana, E. *Single Cell Protein: Yeasts and Bacteria*; 2014; Vol. 3; ISBN 9780123847331.

Technical
University of
Denmark

DTU Aqua
Kemitorvet
DK-2800 Kgs. Lyngby

www.aqua.dtu.dk