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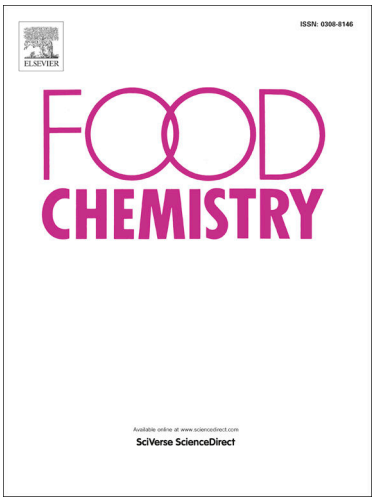

Review

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PROTEIN RECOVERY FROM BREWERY SOLID WASTES

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Abstract

The large amount of agroindustrial wastes produced annually is an important problem in much of the world, mainly due to two factors: an increase in food demand, which requires an increase in production, and the establishment of new environmental regulations that require industries to treat their wastes. On the other hand, the growth of environmental awareness, the use of green technologies, and the recognition of some agro-industrial waste as by-products with nutritional value has motivated researchers to focus on adding value to these by-products. In recent years, research works have been conducted to study the wastes generated during brewing, and how to convert those by-products into useful products with added value. This review will address the feasibility of transforming brewing industry wastes into value-added products to ensure the sustainable reuse of the biological resources, with a focus on protein recovery.

Keywords: brewery waste; proteins; extraction methods; beer spent grain; hot trub; residual yeast[1]

1. INTRODUCTION

Beer is the most widely consumed fermented alcoholic beverage in the world. There are records of its consumption in the Middle East that date back to a millennia before Christ, and it was also used for therapeutic purposes. Natural ingredients such as water, malted barley, yeast and hops have been used in its production since its origins (Mussatto et. al, 2006). Malt is the most significant ingredient of beer, contributing to its color, aroma, flavor and body. In addition, different kind of malts provide different amounts and types of available sugar, which will determine the final alcohol content of the beer. Proteins from the malt also play a fundamental role in the structure and formation of the foam (Deloitte, 2017).

[1] *Abbreviations:* BSG, brewers' spent grains; SBY, spent brewer's yeast; INDEC, National Institute of Statistics and Censuses; SDS, sodium dodecyl sulfate; RSM, response suface methodology; BPC, brewers' spent grains protein concentrate; TCA, tricholoroacetic acid; HIFU, high intensity focused ultrasound probe; HILIC, hydrophilic interation liquid chromatography; UAE, ultrasound-assisted extraction; PLE, pressurized liquid extraction; ACE, angiolensin-converting enzyme; subW, subcritical water; RNA, ribonucleic acid; OAC, oil absorption capacity.

Brewing produces three types of waste: brewer's spent grains (BSG or bagasse), spent hops, and spent brewer's yeast (SBY). About 14-20 kg of depleted grain, 0.2-0.4 kg of depleted hops and 1.5-3 kg of residual yeast are produced for every 100 litres of brewed beer (dos Santos Mathias et al. 2014). Despite technological advances, the amount of waste inherent to beer production has hardly been reduced, largely due to the sequential steps that characterize this process (dos Santos Mathias et. al. 2015).

At industrial-scale, the different types of waste generated at different production stages are brought together into a single waste responsible for the losses (losses of wort, extract and beer) in the brewing process (dos Santos Mathias et. al. 2015). Some authors have reported that several industrial by-products may eventually become pollutants if their use is not directed adequately. Proper disposal is also usually expensive and it leads to loss of potentially useful biomass. Thus it would be more convenient to overcome this issue by developing strategies that valorize these by-products (Puligundla et. al. 2020).

Although brewing waste is used mainly as animal feed, as breweries are increasingly in urbanized areas this strategy has become less profitable. Therefore, more lucrative applications such as biofuel production, building or packaging materials, and adsorbents have been proposed (Lynch et al. 2016). Several attempts have also been made in recent years to use spent grain in the production of value-added compounds (sugars, proteins, acids, antioxidants, xylitol, lactic acid, etc.), cultivation of microorganisms, or the production of enzymes (Mussatto et. al. 2006; Lynch et. al. 2016; Vieira et. al. 2016). However, this innovative perspective has been poorly studied so far. An adequate approach to start improving the value of wastes from the brewing industry is to provide knowledge about the different components through their characterization.

However, previous studies dealing with the composition of wastes have mainly centered on carbohydrates, lignin, p-hydroxycinnamic, and to a lesser extent on lipids and proteins (Salihu et. al. 2011; Lynch et. al. 2016; Ikram et. al. 2020). In the last couple of decades interest in BSG applications has increased especially in areas such as food and biotechnological processes, while research on the uses of SBY and hot trub is still incipient. Recently consumer markets have been attracted by the potential use of plant-based proteins as an alternative to animal proteins. Thus the

recovery of proteins from brewing wastes may represent an innovative and highly valuable source for the industry, not only by adding value, but also by allowing to reduce waste treatment costs (Li et. al. 2017; Salazar Ortega, 2018; Puligundla et. al. 2020).

Despite these studies, we have not found in the literature a systematic summary, discussion or update of the extraction methods of the different by-products obtained from brewing. The present review offers an exhaustive bibliographic search and describes the waste generated during the brewing process, its composition, and the different methods used for the recovery of proteins, considering their advantages and drawbacks to ensure a sustainable reuse.

2. BEER IN ARGENTINA AND THE WORLD

Beer properties such as alcohol content, bitterness, pH, turbidity, color and flavor vary depending on the brewering process, type of ingredients and formulation. While a few multinationals dominate the market of industrial beer, craft beer has appeared as a result of the increasing demand for new varieties and styles of beer, as well as the globalization of information about experiences and beer recipes. Craft beer originated in the late 1970s in the United Kingdom, which pioneered the rise in small breweries, later called microbreweries or "brewpubs" (Garavaglia and Swinner, 2018).

Global beer consumption has increased in the last fifty years to up 150 billion litres per year, exceeding wine consumption by a factor of seven. Countries that have been traditionally brewing countries, such as the United Kingdom, Ireland and Belgium, seen a decrease in their consumption by more than 50%, and fell abruptly in the production ranking. Meanwhile, growth in demand has concentrated in countries such as China and Argentina, where beer consumption increased markedly in the last four decades (Colen and Swinnen, 2016). China is the world’s largest producer of beer, producing up to 38,000 tons of beer, followed by the United States with 21,461 tons, Brazil with 14,138 tons, and Mexico with 11,978 tons per year (FAOSTAT, 2018). This new beer market has intensified over the past two decades, with traditional brewing countries falling far behind in beer production.

The global market of craft beer is changing rapidly. It was valued at USD 85.02 billion in 2015, and it is expected to witness a significant growth in a few years. According to a report by Grand View

Research Inc., it is estimated that by 2025 it will have a value of USD 502.9 billion and an annual growth rate of 19.9%, as a response to the growing demand, the great variety of new styles and flavors, and the entry into new markets (Deloitte, 2017).

In line with the evolution of the lgobal beer market, the Argentinian market has experienced a marked dynamism in the last two decades, characterized by globalization and concentration of the industrial beer market, and also by the emergence of craft beers at the local level and small-scale (Ablin, 2015). According to data from the Argentinian Chamber of Craft Brewers and the National Statistical and Census Institute (INDEC), the beer market showed an average growth of 40% in the last five years. In view of this increase, in March 2017 article 1082 bis of the Argentine Food Code (Código Alimentario Argentino, CAA, 2017) was modified so that, in order to be labelled "Craft Brewing", the brewing process has to meet certain requirements: a) use manual or semi-automatic equipment; b) not use food additives; c) use only natural ingredients, and in the case of fruit juices or extracts, they must be previously pasteurized (Civitaresi et al. 2017; Moren and Sosa, 2019).

3. BREWING PROCESS

The brewing process begins with malting, which involves the controlled germination of barley, in which hydrolytic enzymes are synthesized and the cell walls, proteins and starch of the endosperm are digested, making the grain friable (Celus et al. 2006). Then the grain is dried to stop the process, and roasted. The roasting is be more or less intense depending on the aromas to be achieved. Finally the grain is crushed to expose most of the sugar contained in the cotyledons.

This process is known as barley's malt or malting (Alburqueque et al. 2018). The ground malt is mixed with an appropriate amount of water and gradually heated at different temperatures and times for the hydrolytic enzymes to act. This enzymatic degradation allows the release of fermentable (maltose and maltotriose) and non-fermentable (dextrins) carbohydrates, soluble proteins, polypeptides and amino acids.

This aqueous extract is called wort (Lynch et. al. 2016), and it is the main waste produced by the beer industry. It is separated from the insoluble materials (spent barley grain, BSG or bagasse) by filtration (lautering), and then the wort is boiled with hops, which adds aroma and bitterness to the

beer. In this step, some high molecular weight proteins are denatured due to the heat, and precipitate together with the hop residues, generating another residue (hot trub). Boiling stops the enzymatic activity, sterilizes the wort, and coagulates some proteins that cause turbidity in the product. The wort is cooled and aerated to generate the optimal conditions for yeast to growth. With the addition of yeast (*Saccharomyces* species), most of the carbohydrates present in the wort break down into alcohol and carbon dioxide, while the metabolites generated by yeast provide the characteristic flavor and aroma of the product. The fermentation process is divided into two stages: first, the primary fermentation that involves the removal of the excess yeast, and a secondary fermentation where maturation and natural saturation with carbon dioxide is reached (Ablin, 2015). In Argentina, two types of craft beer are marketed according to the type of fermentation, ale and lager. Ale originated in England and it is made with *Saccharomyces cerevisiae*. The fermentation process occurs in the upper part of the fermenter at 15-25 °C for short periods of time (5-7 days). Lager beer is German, and it is made with low fermentation yeasts (at the bottom of the fermenter), such as *Saccharomyces carlsbergenesis* and *Saccharomyces uvarum*, at low temperatures (less than 10 °C) and longer fermentation times (1-3 months) (Priest and Stewart, 2006, Moren and Sosa, 2019). In this stage, the exhausted yeasts are the final residue of the process (spent brewer's yeast, SBY). At the end of the process, the obtained product is filtered and bottled.

4. BY-PRODUCTS AND WASTES FROM THE BREWING PROCESS

The recovery of compounds with commercial value from the wastes of the brewing process would reduce the volume of generated residues and improve the economic viability of the process. At present there are many studies on the possible application of brewery by-products in the food industry as functional additives or ingredients with beneficial health effects; however, these byproducts have not yet been used in large-scale production, and the search for technologies that allow to scale up the laboratory methodologies to market products is booming (Salihu and Bala, 2011; Rachwał et al, 2020; Wen et al. 2019).

4.1. Brewer’s spent grains (BSG)

Spent grain is the most abundant by-product of the brewing process, corresponding to about 85 % of the total by-products, and it accounts for (in average) 31% of the original malt weight (Salihu and Bala, 2011). The chemical composition of BSG varies according to the barley variety, the harvest, malting and mashing conditions, quality and type of adjuncts added in the brewing process, among other factors (Huige, 2006). BSG is considered a lignocellulosic material rich in proteins, lignin, cellulose, hemicellulose and lipids, with protein and fiber representing about ~90 % of its composition (Mussatto et. al. 2006; Ikram et al. 2017). Table 1 shows the main components of BSG reported in the literature.

The main disadvantage of BSG is its high moisture content, with values between 75 and 90 %, which makes it an unstable material and susceptible to microbial spoilage. This feature is a major limitation to using spent grains in the food industry, because they would need to comply with regulations that ensure the quality and safety of food products for human consumption. At present the use of BSG is promoted for animal feed production or for composting in landfils, although in the long term the latter could cause environmental pollution due to its chemical composition (Yu et al, 2020, Rachwał et al. 2020; Celus et. al. 2007).

The major protein fraction of BSG consists of hordeins (β, C, D y γ), which belong to the prolamin group and constitute over 50 % of the total protein content. The other protein fractions are glutenins (22 %), albumins and globulines (about 2 % each) (Vieira et al. 2014; Ikram et al. 2017). BSG is a good source of essential (30% of the total BSG protein content) and nonessential amino acids, with lysine, leucine, phenylalanine, isoleucine, threonine and tryptophan as the most abundant of the first group, and glutamic acid, proline and valine completing the amino acid profile (Huige, 2006; Mussatto et al. 2006, Rachwał et al, 2020). Although it is known that during the malting process barley proteins are degraded to small peptides and amino acids, it is still necessary to have a better understanding of the changes that take place in this process and how this affects the final composition of BSG (Niemi et al. 2013, Jaeger et al. 2021).

The lignocellulosic material of the BSG is a complex structure mainly composed of three polymeric fractions: cellulose, hemicellulose and lignin. The cellulose fraction is associated with hemicellulose and other structural components, and it is surrounded by a lignin sheath. This substrate around the

cellulose microfibrils is partly covalently linked to hemicellulose, covering some regions of cellulose from access of hydrolytic enzymes and acids (Mussatto et al. 2008). Arabinoxylans are the major hemicelluloses of BSG; they are composed of xylopyranose and arabinofuranose, and they can be extracted by strong alkali solutions (Mandalari et al., 2005). Arabinoxylans are considered dietary fiber due to their resistance to hydrolysis by digestive tract enzymes. They can also present immunomodulatory activity, and some authors consider arabinoxylo-oligosaccharides (obtained by the partial hydrolysis of arabinoxylans) as prebiotics (Broekaertet al. 2010; Vieira et al. 2014). Lignin is a polyphenolic macromolecule from which phenolic acids can be isolated (Musatto et al. 2007). BSG is rich in ferulic and p-coumaric acid, followed by caffeic, sinapic and syringic acids. The high level of phenolic compounds in BSG can be explained by the fact that, during the development of barley seeds, these compounds are specifically accumulated in the grain husk (Zuorro et al. 2019). Phenolic compounds have anti-carcinogenic, anti-inflammatory and antioxidant activities (Mc Carthy et al. 2012).

Lipids are present in BSG in smaller percentages than fibers and proteins. The predominant lipids in BSG are triglycerides (55 %), while phospholipids and diglycerides account for 9.1% and 5.7%, respectively. Compared to unmalted barley, BSG has lower triglyceride and phospholipid contents and more free fatty acids (30 %). Linoleic (18:2), oleic (18:1) and palmitic (16:0) acids are the more common fatty acids present in BSG, and small levels of linolenic (18:3) and stearic (18:0) acids have also been found (Niemi et al. 2012; del Rio et al. 2013).

The incorporation of BSG as meal in the manufacture of bakery products such as bread, biscuits, cookies, muffins, cakes, waffles, pancakes, snacks, doughnuts and brownies has been recently reported (Ainsworth et al. 2007; Ktenioudaki et al. 2012; Mussatto, 2014; Shih et al. 2020; Amoriello et al. 2020). The use of small amounts of this residue (up to 100 g/kg) was recommended to avoid alterations in the flavor and texture of the final product (Lynch et al. 2016). Nocente et al. (2019) analyzed different formulations of dry pasta where three different BSG concentrations were added to durum wheat semolina, and they found that the pasta enriched with BSG showed higher protein and fiber contents with minimal effect on the sensory properties of the cooked pasta. However,

further studies such as microbiological stability testing should be performed to evaluate the shelf life of the BSG-enriched pasta, since this still represents the major constrain for its use.

BSG has also been proposed as a fat substitute for the elaboration of high-fiber and low-fat meat products. The addition of BSG in the manufacture of frankfurters allowed to increase the fiber content without negatively affecting the sensory parameters Özvural et al. (2009). Choi et al. (2014) also evaluated the effects of replacing pork fatback with BSG pre-emulsion on the quality characteristics of reduced-fat chicken sausages. With the addition of BSG, not only did the fat content and energy values decrease, but also their hardness, gumminess and chewiness improved. No significant differences were observed in the overall acceptability between the control and the chicken sausage with added BSG pre-emulsion (20-25%) and lower content of added pork fat (5-10%). Nagy et al. (2017) used BSG to obtain new types of smoked sausages with desirable nutritional properties and acceptable sensory characteristics by partially replacing animal protein with BSG protein.

Another proposed application of BSG in the food industry is the production of tarhana, a fermented product made from wheat meal and yogurt. In the study by Özboy-Özbaș et al. (2010), the effects of sugarbeet fiber and BSG on the quality of tarhana were analysed. The results showed that the addition of BSG significantly increased the protein and crude fat content.

4.2. Spent brewer's yeast (SBY)

Spent brewer's yeast (SBY), also known as residual yeast or surplus yeast, is another by-product generated during brewing, which accounts for up to 15 % of the total by-products generated. SBY is recovered by sedimentation at the final stage of the second fermentation and after maturation. SBY is an abundant source of proteins, minerals and B-complex vitamins. In addition, spent yeasts are a rich source of nutraceuticals such as β-glucans or mono- and oligosaccharides (Ferreira et al. 2010; Rachwał et al. 2020; Puligundla et al. 2020). Due to the yeast qualities during brewering, the biomass grows enormously, so the excess yeast can be collected and reused up to six times (Rachwał et al. 2020). Table 2 shows the main components of SBY.

SBY is generally recognized as safe (GRAS) for human consumption (Ferreira et al., 2010). However, since it contains high levels (6–15 %) of nucleic acids, its consumption can increase uric acid levels in the blood causing hyperuricemia. For this reason, its application in human nutrition as a protein source has been limited (Podpora et al., 2016). SBY is generally used in animal feeds after inactivation by heat (Huige, 2006; Ferreira et al. 2010). Additional disadvantages of this by-product include short shelf life, transportation costs, and requiring further processing (Boateng et al. 2015).

Although spent yeasts are generally disposed of in landfils, recovery can be achieved by extracting and isolating more valuable components such as proteins, amino acids, β-glucans, functional peptides, vitamins, minerals, flavor and other compounds (Pejin et al. 2019; Puligundla et al. 2020).

4.3. Hot trub

Hot trub is a term referring to sediments formed during wort boiling. In general these residues represent 1-2 % of the total by-products and are removed before fermentation. However, in some cases, hops can be added and removed at different stages of the brewing process (Rachwał et al. 2020). It is a slurry consisting of entrained wort, hop particles, and predominantly insoluble high molecular weight proteins. It has also been reported to contain a high carbon concentration due to its large reducing sugar content (Senna et al. 2021).

In general, hot trub is characterized by high moisture and low ash content (Huige, 2006; Rachwał et al. 2020), and by the presence of proteins, non-isomerized hop bitter substances, polyphenols, carbohydrates such as pectins and starch glucans, fatty acids and minerals (dos Santos Mathias et al. 2014; 2015; Vieira et al. 2016). Typical proportions of these compounds are showed in Table 3. The protein content in hot trub depends on several factors and its average values may vary in a wide range according to the brewing process. The formation of trub is desirable since it removes components such as soluble proteins and phenols that would otherwise react and form undesirable insoluble complexes in the beer (Rachwał et al. 2020).

Hot trub is usually disposed of together with BSG and other ingredients, so it is used in the same manner as BSG (Priest and Stewart, 2006). Essential oils obtained from trub have been studied as

insect repellents given their biological properties (Bravi et al. 2021),and and they have significant potential for application in bioprocesses (dos Santos Mathias et al. 2015).

Several studies have emphasized the beneficial and pharmacologically important properties of phenolic and polyphenolic compounds on the cardiovascular system and cancer treatment (Shahidi and Ambigaipalan, 2015, Vieira et al., 2016). An increasing number of scientific publications are focusing on this solid waste as a valuable resource for biotechnological applications, highlighting its composition and antioxidant capacity (Tatullo et al. 2016; Cermak et al. 2017; Bogdanova et al. 2018; Costa et al. 2021).

5. PROTEINS FROM BY-PRODUCTS AND WASTE

Barley proteins have historically been classified according to Osborne fractionation by their solubility in water (albumins), dilute salt solutions (globulins), alcoholic solutions (hordeins), and dilute alkali or acid solutions (glutelins) (Osborne, 1924). In recent years, knowledge about the molecular level of the barley proteins in each fraction has increased, and classifying grain proteins based on functional or physical properties has proved to be more useful (storage, structural, metabolic, and protective proteins) (Finnie and svensson, 2014). Half of the protein content in cereal grains are storage proteins, and thus they are very important for the processing properties and nutritional quality. Storage proteins can be divided into globulins (present in the embryo and aleurone layer), and prolamins or hordeins (present in the endosperm). The most abundant storage proteins are hordeins, which account for 30–50 % of the nitrogen content of the barley grain and play an important role in grain properties due to their chemical composition (Finnie and svensson, 2014). Hordeins are classified into β-, C-, D- and γ-hordeins according to their electrophoretic mobility on SDS-PAGE. Hordeins are also known as prolamins because of their high proline and glutamine contents. This protein group is characterized in general by its highly biased amino acid composition, rich in glutamine, which makes up 30–40 % of the total amino acid residues, while being deficient in lysine, threonine and tryptophan. β-, C- and γ-hordeins contain 20-30 % of proline, whereas D-hordein contains about 10 % of proline and is rich in glycine, serine and threonine; C-hordeins contain no cysteine (Connolly et al. 2013). The influence of β-hordeins on the malting quality

represents a resource to improve its quality. On the other hand, some authors consider that there is no direct correlation between the hordein profile and malting quality (Finnie and svensson, 2014). Some studies showed that during malting, rapid degradation of D-hordeins followed by degradation of β-and C-hordeins occurs (Weiss et al 1992).

After malting and during the brewing process, the content and composition of the original proteins present in the barley change. In the lautering step, a small amount of soluble globulins and albumins are extracted from BSG (7.5 % of total protein content). The most abundant proteins in BSG are still hordeins, which represent 43 % of the total protein content, while glutelins represent about 21.5 % (Ikram et al., 2017). D-hordeins are not found in BSG as a consequence of their rapid degradation during the malting process (Huige, 2006; Mussatto et al., 2006).

SBY can also be considered a source of proteins. However, the yeast cell wall must be broken down before processing to ensure the release of the intracellular components. After the breakage, the proteins can be readily accessed by the enzymes. This breakage is also interesting from a nutritional point of view because it can greatly improve protein digestibility (Marson et al., 2019). As stated above, another limiting factor in the use of yeast biomass as a protein source for human consumption is its high nucleic acid content. However, some reagents and techniques are used to isolate yeast proteins with low RNA (Ferreira et al. 2010).

The protein content in the trub depends on various factors, such as brewing process, type of malt, the addition of other grains, and type of hop used (Bravi et al. 2021). Although proteins from hot trub are thermostable and resistant to proteolysis, they can undergo changes by hydrolysis, the Maillard reaction, or by reduction of disulfide bonds. On the other hand, the high molecular-weight proteins from spent hops precipitate and may form complexes with the tannins, resulting in a bitter flavor (Saraiva et al. 2019). Therefore, despite hot trub having large amounts of water and protein, it is not used directly in the food industry due to its bitterness (Saraiva et al. 2019). Extraction of the proteins is then required to consider its use in food applications.

6. PROTEIN EXTRACTION PROCESS

Although animal proteins (e.g. milk proteins) have traditionally been used in the food industry, recent awareness of the link between food supply, population growth, health and global warming has led to an increasing research interest in plant proteins (Connolly et al. 2014; Qin et al. 2018). This great interest in protein extraction and isolation from brewing by-products could contribute to the increase in possible plant sources for their application in food industry (Qin et al. 2018). In recent years, several methods for the extraction of proteins from brewing wastes have been investigated (González-Garcia et al. 2021; Ibbett et al. 2019; Wen et al. 2019; Qin et al. 2018). However, the costs, time and availability of the process are factors that can influence the selection of the most suitable method, as well as the quality of the proteins and their subsequent application.

6.1. BSG protein extraction methods

Protein extraction from BSG would provide another source of protein to meet future demands, and it would lead to an optimal exploitation and valorization of this by-product. Different protein extraction methods from BSG (hydrolysed, concentrated and isolated proteins), including ethanolic, alkali and enzymatic extraction, among others, have been examined. It has been shown that the addition of pretreatments , such as hydrothermal, enzymatic, ultrasound-assisted extraction or microwaves, can improve the performance of traditional protein extraction processes (Vieira et al. 2014; Rommi et al. 2018; Arauzo et al. 2019; He et al. 2019; Ibbett et al. 2019; Wen et al. 2019; Yu et al. 2020). Although there have been several reports on protein extraction procedures from BSG (Wen et al. 2019), a comprehensive comparison of these methods based on their performance and quality of the proteins obtained has not yet been made. Details of different methods for extracting BSG proteins are presented and discussed below.

6.1.1. Ethanolic extraction

The first reports on the extraction of proteins from BSG were carried out by alcoholic extraction. Ervin et al. 1989 studied BSG as raw material for the preparation of a protein concentrate (BPC). The extraction was performed with dried BSG and a solution of 3 % sodium dodecyl sulfate (SDS) and 0.5 % sodium hydrogen phosphate (Na_2HPO_4, pH 7.0), and the mixture was refluxed for 1 h at

different temperatures (between 27 and 100 °C). The extract was then separated from the BSG residue to obtain a clear filtrate Precipitation of the proteins extracted from BSG was carried out by four procedures: 1) adjustment of pH (2.0), 2) addition of 95 % ethanol to the extract (0.7:1 ratio), 3) refrigeration of the extract (4 °C), and 4) addition of 95 % ethanol followed by refrigeration. The precipitated proteins were recovered by centrifugation (9500 g, 0 °C), washed with ethanol, and then lyophilized. The highest yields were obtained at 100 °C with extraction method 4, with 49.2 ± 2.9 % of protein recovery.

Ethanolic protein extraction was also studied by other authors using a similar methodology to that of Ervin et al. (1989). Diptee et al. (1989) optimized factors through response surface methodology (RSM) based on a fractional factorial design and central composite rotatable design. Temperature (50 - 100 °C), time (30-90 minutes), BSG:extractant ratio (1:4-1:8), concentration of Na_2HPO_4 (0-1 %), particle size (1-2 mm), and concentration of SDS (1-3 %) were the optimized factors. They observed a 60 % protein yield from defatted BSG using a concentration of 0.6 % Na_2HPO_4 (in the extractant solution), a BSG:extractant ratio of 1:4 (w:v), with the mixture heated at 90 °C for 95 min, a grain particle size of 1 mm and 0.5 % of SDS.

6.1.2. Ultrafiltration

Ultrafiltration (UF) in the recovery of protein isolates from BSG was studied by Tang et al. (2009). Their results showed that the membrane process improved BSG protein isolation, as the separation by membrane allows the removal of compounds based on molecular size, such as salts or water. First the BSG extract was prepared by ultrasound-assisted extraction using sodium carbonate buffer (pH 10), 1:10 ratio (w/v), for 1 h; then it was filtered through a nylon cloth and the remaining solution was centrifuged (10,000 g) at 4 °C. The supernatant was collected and used as a feed solution. Then the supernatant was filtered. Membranes of MWCO (molecular weight cut off) of 5 and 30 kDa with a surface area of 0.05 m^2 were used in these experiments (~25 ◦C). The results were promising, with over 92 % of the protein being retained by the ultrafiltration membrane with MWCO of 5 kDa.

Although this type of fractionation allows to obtain a high yield of good quality protein, it has some operational disadvantages such as concentration polarization and membrane fouling that reduce the permeate flux well below the theoretical capacity and change the selectivity of the membrane (Tang et al., 2009).

6.1.3. Alkali extraction

Alkaline extraction is one of the most commonly used methods for the extraction of protein isolates and concentrates. Despite the lower costs, alkaline extraction has the lowest profit among all extraction techniques due to its low protein yield. Several studies have been conducted on the effect of the operating conditions of this methodology to improve the protein yield (Connolly et al. 2013; Vieira et al. 2014).

Celus et al. (2007) obtained BPC from BSG by alkaline extraction and acid precipitation.The BPC was then hydrolyzed with commercial enzymes (Alcalase, Flavourzyme, and Pepsin) for improved techno-functional properties. The BPC was prepared with a BSG:alkali ratio of 1.7:10, at 60°C for 1 h, then the samples were filtered (180 μm). The solubilized proteins were precipitated by citric acid and centrifuged at 4 °C. The BPC contained 60 % proteins, 12 % fat, 2.0 % ash and 26 % carbohydrates (on dry basis). After hydrolysis, the protein contents varied between 66 % and 77 % according to the enzyme, and increased solubility was obtained with Flavourzyme (protein yield 92 %). The fractionation of the hydrolysates precipitated by ammonium sulphate or ethanol to obtain an homogeneous fraction considering hydrophobicity and molecular weight was reported by the same research group a few years later (Celus et al. 2009).

Connolly et al. (2013) obtained protein-enriched isolates from sheared pale and black BSG using sequential aqueous and alkaline extraction followed by isoelectric precipitation. In preliminary studies, they analysed different factors such as type and concentration of alkali, temperature and solid:liquid ratio. The best yield was obtained with NaOH 110 mM, weight/volume ratio of 1:20, for 1 h at 50 °C, achieving a maximum of 59 % and 15 % for pale and black BSG, respectively.

Vieira et al. (2014) proposed a three-step sequential extraction of proteins and arabinoxylans from BSG with different alkalissolvents (KOH and NaOH) and concentrations (0.1-4.0 M). They also

studied other factors that could affect the extraction performance such as time (2 and 24 h), solid:liquid ratio (1:2 and 1:5), temperature (room temperature and 40 °C), and agitation (constant and occasional). The optimal conditions were: ratio of 1:2 (w/v), room temperature, for 24 h. Protein precipitation was achieved by lowering the pH to 3 with a saturated solution of citric acid. Additionally, this process simultaneously recovers proteins (79-83 %), arabinoxylans (62-86 %) and solvent.

Alu'datt et al. (2018) studied the isolation and coprecipitation of proteins from BSG and soybean flour using sonication as pre-treatment. They evaluated the effects of sonication on the structure and biological properties of isolated proteins and protein co-precipitates obtained by alkaline extraction, and also the extractability of phenolics from BSG and soybean flour. Although their work focuses on the properties of the obtained proteins, it is important to highlight that the alkali extraction showed similar yield results to those reported by other authors (66.6 % of protein content). The conditions used were 1M NaOH, pH 12 with isoelectric precipitation and heating (1M HCl, pH 4.6, 95 °C/30 min).

Arauzo et al. (2019) performed an alkaline extraction with NaOH (0.1 M) at pH above 11 with agitation, a solid:liquid of ratio 1:10, for 2 h at 40 °C, and centrifugation at 4 °C.The protein-rich supernatant was then precipitated with 1.0 M trichloroacetic acid (TCA) at pH 3. The insoluble proteins precipitated and were recovered after centrifugation at 13,500 rpm and 4 °C for 20 min. The yield values obtained under these conditions are similar to those found by Celus et al. 2007 (60%). It is worth pointing out that the physical and functional properties of the recovered proteins were not affected by the extraction methodology.

Recent studies have examined the fractionation of proteins from BSG, pasture grass, cyanobacteria derivatives of waste material, and industrial by-products of the food sector. Du et al. (2020) studied the protein extraction from BSG with different methods (alkaline treatment, aqueous extraction, and subcritical water extraction). Alkaline extraction was performed as described by Arauzo et al. (2019), and the same conditions were used for the aqueous extraction but replacing the alkali solvent with water. Subcritical extraction was carried out with a semi-continuous reactor at 200 °C, pressure 40 bar, and a flow rate of 6 mL/min, for 20 min. TCA precipitation of proteins was used for the three

extraction methods. The authors showed that the alkaline treatment was the most efficient method in the extraction of proteins from BSG (21.4 % g protein/100 g BSG), compared to the results obtained by Celus et al. (2007). Some functional properties of the proteins such as thermal stability and oil-water interfacial tension were also analyzed.

6.1.4. Ultrasound pre-treatment

Ultrasound is an emerging, environmentally friendly and non-thermal technology. Ultrasound-assisted extraction (UAE) could be a cost-effective, simple and efficient method to assist in protein extraction (Yu et al. 2020). Tang et al. (2010[a]) determined the best conditions for the UAE of protein from BSG: pH = 10, solvent-sample weight ratio of 1:80 (w:v), ultrasound power of 180 W, 1 h, and 5 extraction times. The protein yield obtained undern those condition was 50.69 %. Tang et al. (2010[b]) later optimized the factors through response surface methodology (RSM) based on a central composite design. Three independent variables were evaluated: time (26-93 min), ultrasonic power (46-113 W/100 mL of extractant) and solid:liquid ratio (1.3-4.6 g/100 mL). Optimal conditions in this study were: time of 82.4 min, ultrasonic power of 88.2 W/100 mL of extractant, and S/L of 2.0 g/100 mL, which yielded values of 96.4 ± 3.5 mg/g BSG. Although the authors did not report the total protein content extracted from BSG, they considered that the application of UAE improved the protein yield.

Li et al. (2021) examined the effects of UAE on protein yield from BSG. This study offered insight into the functional and structural changes of the proteins induced by sonication. They pretreated BSG with ultrasound at a controlled temperature (25 °C), using 0.1 M NaOH at a solid:liquid ratio of 1:15 (w/v). This process optimized the ultrasonic power (150-350 W), extraction time (5-25 min), and duty cycle (20-100 %). The supernatant was then centrifugated at 8000 g and 10 °C for 20 min, and precipitated to pH 3.8 using HCl (2M) at 4 °C. The results showed that with 0.1 M NaOH, working at 250 W for 20 min, with and a duty cycle of 60 %, UAE could improve the yield up to 86.16 % compared to an extraction without ultrasound pretreatment (45.71 %). In addition, according to this study, UAE can enhance the functional properties of BSG proteins.

González-Garcia et al. (2021) studied the extraction of protein hydrolysates from BSG using a high intensity focused ultrasound probe (HIFU) (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA). After optimization, the values of the HIFU amplitude, the extraction time and the extraction temperature were 70%, 15 min and 60 °C, respectively.The extract was then centrifuged and the proteins in the supernatant were precipitated with HCl at pH 3.8. Under optimal UAE conditions, it was possible to extract 12.6 ± 0.1 g protein/100 g of BSG (~ 43.5 % of proteins). These values were similar to those obtained by Li et al. (2021), but lower than those reported by Tang et al. (2010[a]).

6.1.5. Enzymatic treatment

Several authors have used this methodology for the recovery of proteins. Treimo et al. (2008) studied the use of 15 enzymes (proteinases, carbohydrases, multienzyme complexes, etc.) to solubilize proteins from BSG. The experiments were performed under the corresponding optimal conditions for each type of enzyme. They observed that the protein-rich hydrolysates were produced efficiently with the peptidase treatment, where over 60 % of proteins were obtained. The best results were obtained with Alcalase (at pH 8.0, for 4 h and with 10-20 µL enzyme), which solubilized 77 % of total proteins present in the by-product. They also determined that the processes that combined peptidases did not achieve better results. In contrast, Niemi et al. (2013) worked with a combination of carbohydrases and proteases, obtaining promising results. Ground BSG was treated with Depol 740L (xylanase) with a solid:liquid ratio of 1:10 (w/v), for 5 h at 50 °C with continuous stirring. The liquid and solid phases were separated by centrifugation (4000 rpm, 30 min). Then the solid phase was treated with different proteases (10 % w/v, 40 °C, for 4 h) and the supernatant was recovered again. Protein solubility was improved by the pretreatment with carbohydrases, reaching a solubilization of the total proteins of 86% with both types of enzymes, while 76% solubility was attained when Alcalase was used alone. In addition, Niemi et. al. 2013, suggested that working at alkaline pH with alkaline proteases also improved the yield compared to neutral or acid proteases (21 and 30 %, respectively).

A few years later Qin et al. (2018) compared the enzymatic pretreatments carried out at pH 6.25 and 8.0, with different solid:liquid ratios (6.67, 5, 4 and 2.5% w/v), and 2.4 l 10% Alcalase® (mL/100

g BSG), 250 rpm, 60 °C for 2, 8 and 24 h. Results showed that only 43-50 % of protein could be extracted with this methodology. The best results reported by Qin et al. (2018) were those obtained using a higher solid:liquid ratio and longer times. These yields are significantly lower than those reported by other authors.

In the same year, Rommi et al. (2018) investigated the extraction of proteins and lignin from BSG by alkaline extraction and acid precipitation with enzymatic pretreatment (carbohydrase and protease) and/or thermochemical pretreatment (steam explosion). They reported that alkaline extraction with pretreatments, including steam explosion and the subsequent enzymatic hydrolysis of the cell wall (with alkaline protease), could increase the recovery of protein hydrolysates. However, the extraction of both compounds resulted in a decrease in protein extraction due to the co-precipitation of proteins and lignin, as well as the incomplete removal of proteins from lignin, which suggest a extremely close interaction between these compounds. Technical protein yields were of 46-65 %. These yields improved after steam explosion or carbohydrase pretreatments. However, steam explosion could affect the techno-functional properties of the extracted proteins. These results are in agreement with those reported by Niemi et al. (2013), who also used carbohydrases and proteases, although the protein yields obtained were lower.

He et al. (2019) proposed in their work the simultaneous extraction of fibers and proteins from BSG. They analyzed the effect of different concentrations of chemicals (sodium hydroxide and sodium bisulphite) and enzymes (Alcalase, 5, 20 and 35 µL/g dry BSG) to obtain the highest yield of proteins and fibers using the lowest amountof chemicals and enzymes. The ground BSG was mixed with the reagent used for the extraction at 60 °C for 4 h with constant stirring. Then the suspension was filtered, and fiber was obtained from the solid fraction, while the proteins were obtained from the liquid fraction. A pretreatment with Alcalase followed by alkaline extraction with sodium hydroxide showed promising results for the extraction of proteins and fibers from BSG. Under optimal conditions with Alcalase treatment (20 µL/g of dry BSG), the protein separation efficiency was as high as 83.7 %. Treatment of BSG with sodium bisulphite for protein and fiber separation was overall not effective. When the sodium bisulphite concentration was increased from 0 to 5% (w/w), the protein separation efficiency remained at 68 %.

Yu et al. (2020) developed an ultrasound-assisted enzymatic process to separate proteins from BSG, to produce hydrolysates and determine their physicochemical properties. The conditions they used were an enzyme concentration of 20 μL g^{-1} BSG (Alcalase® 2.4L) with 3 h of incubation, and an ultrasound treatment where the power output (amplitude 40 %) was in pulses of 5 seconds on and 3 seconds off, for 10 min. The ultrasound power density was determined at 227.5 W L^{-1}. They obtained protein yields of 69.8 %. The ultrasound pretreatment reduced the enzyme loading, and therefore the costs for protein separation, contributing to the economic feasibility of the process. Although the enzymatic treatment is used for protein extraction as a technique that reduces the use of chemical substances and can be considered a green process, it has been observed that its yields are not as promising as those obtained with other methodologies. All the same, its application as a pretreatment improves protein extraction yields from BSG.

6.1.6. Hydrothermal pretreatment

A hydrothermal treatment was used as a pretreatment for the extraction of proteins from BSG by Qin et al. (2018). BSG were submitted to a hydrothermal pretreatment under different conditions of solid:liquid (6.67; 5; 4, and 2.5% w/v), temperature (30 up to 135 °C) and time (1 up to 24 h). The best results (64-66 % of protein extraction) were obtained at 60 °C, with a reaction time of 24 h and a solid:liquid of 4 and 2.5 %, respectively. Since the authors only proposed two conditions, it would be interesting to carry out an optimization study to maximize protein extraction. Although hydrothermal pretreatments have shown good yields, it has not yet been sufficiently explored.

6.1.7. Hydro-mechanical extraction

Ibbett et al. (2019) applied a physical method for the separation of protein concentrates from BSG. They used a vertical toothed colloid mill for hydro-mechanical processing (fixed speed, 3000 rpm) and subsequent centrifugation. The aim was to generate fine protein fractions under controlled conditions. A slurry of the BSG:water (1:10, w:v) was prepared. Samples were passed through the colloid mill 24 times. Centrifugation was carried out at different conditions (28 g, 30 min; 447 g, 10 min; 2800 g, 5 min), allowing for the separation of the thinner, protein-rich material from the coarse

material and liquid. This method increased the protein concentration in the solid from 27.1 to 51.0 %; however, it also increased the carbohydrate content. This increase is probably a result of the cell wall rupture of the aleurone and endosperm tissue. This protein-enhanced product has useful techno-functional properties, such as a good stability in aqueous suspensions.

6.1.8. Pressurized liquid extraction

Pressurized liquid extraction (PLE) requires solvents at high temperatures and pressures. This methodology is considered a green technology for the extraction of various compounds from plant sources. PLE favors the penetration of the solvent in the sample matrix and the mass transfer of the analytes (Mustafa and Turner, 2011; González-Garcia et al., 2021). González-Garcia et al. (2021) proposed a hydrolyzed protein extraction using an accelerated solvent extractor system (ASE 150, Dionex, Sunnyvale, CA, USA). Parameters affecting the extraction by PLE such as extraction time, extraction temperature, solvent composition and the number of cycles were optimized. Under optimal extraction conditions (4.7 % of EtOH, 155 °C, 10 min, and 5 cycles), the protein content of the extract was 20 ± 1 g/100 g of BSG, which represented 69 % of the total BSG proteins. Then the authors analyzed the quality of those proteins based on the number of peptides. The PLE extracts showed high antioxidant, cholesterol esterase and angiotensin-converting enzyme inhibitory activities. PLE extracts also presented a high number of hydrophobic peptides, which resulted in a high bioactivity. Even though this work proposes a green method as a sustainable alternative to conventional protein extraction methods, studies on the extraction BSG proteins with this methodology are still very few.

6.1.9 Subcritical water

Subcritical water (subW) is pressurized water in its liquid state at temperatures between 100-374 °C. Under these conditions, water presents a higher ionic product and a lower dielectric constant than at room temperature (Alonso-Riaño et al., 2021). These properties confer subW a greater selectivity for the separation of different bioactive compounds, mainly carbohydrates and phenolic compounds (Alonso-Riaño, 2019; Torres-Mayanga et al., 2019). Alonso-Riaño et al. (2021) used

subW for the extraction of proteins from BSG. SubW experiments were carried out in a semi-continuous fixed-bed reactor with an HPLC pump for pressurization and water pumping; water temperature (125-185 °C) was reached with a heat exchanger. The authors obtained a maximum hydrolysed protein extraction of 78 % after 150 min at maximum temperature (185 °C) .

6.1.10 Advantages and disadvantages of the methods used for the extraction of proteins from BSG

Table 4 shows the main advantages and disadvantages of each extraction method mentioned in this work. In addition to the techno-functional properties of the proteins obtained, other conditions must be considered when selecting the extraction method or pretreatment: costs, performance, required equipment, extraction conditions, etc.

6.2. SBY's protein extraction methods

The most commonly used methods to obtain SBY proteins are enzymatic hydrolysis, ultrafiltration and hydrothermal treatment (Huang et al., 2012; Amorim et al., 2019; Marson et al., 2019). However, protein hydrolysates and protein isolates from spent yeast are still underexploited, probably due to their complex composition and high nucleic acid content (Lamoolphak et al., 2006; Marson et al., 2019). Most SBY proteins are found inside the cells, thus it is necessary to break the cell structure to release them. Different cell disruption methods have been used as pretreatments for the release of cellular components, such as autolysis, plasmolysis, ultrasound, and glass beads (Jacob et al., 2019; Mason et al., 2019). Although these methods quantify protein content as a response variable, they have not been applied to obtain proteins. The following subsections explain the cell disruption methods and protein extraction methods applied so far.

6.2.1. Cell disruption methods

Autolysis, ultrasound and glass bead mill are the main methods for cell disruption (Boonyeun et al., 2011; Vieira et al., 2013; Marson et al, 2019). Autolysis is a process in which cellular components are solubilized by inherent degradation within the cells, inducing endogenous enzymes with temperature and pH specific conditions (Amorim et. al., 2019; Marson et al., 2019). This process

can be accelerated by using inorganic salts, such as sodium chloride or non-polar organic solvents. In this case, the process is referred to as plasmolysis (Chae et al., 2001). Ultrasound is based on the implosion of cavitation bubbles, which apply forces and break the cell wall (Jacob et al. 2019). The other method of cell disruption is bead milling, which consists in the cell rupture by friction using glass beads at high speed (Vieira et al., 2013; Marson et al., 2019). Table 5 shows different operating conditions for these methods reported in the literature.

Some authors have compared the different disruption methods to evaluate the components obtained. Jacob et al. (2019) studied the use of bead mill, sonotrode and autolysis and concluded that physical methods are a good alternative to conventional autolysis. However, the protein content recovered by both methods did not differ significantly (t-test p-value > 0.05), with values ranging between 482.01-485.24 mg/g d.w.

Vieira et al. (2013) compared autolysis and milling. They obtained higher yields for milling with glass beads, reaching an extraction yield of 95 % of the total proteins contained in the yeasts, while autolysis yielded only 38 %. Marson et al. (2019) also compared both methods and obtained an autolysate with 36% protein (87% of the total sample). They also reported that after autolysis, the protein content decreased probably due to protein degradation and reaction with other compounds. On the other hand, the control and treated samples did not differ significantly on protein content in relation to the physical method.

6.2.2 Hydrothermal treatment

Hydrothermal decomposition involves the use of subcritical or supercritical water without oxidants. Two main chemical reactions take place: oxidation, which converts organic materials into water, carbon dioxide and nitrogen, and hydrolysis, where carbohydrates and disaccharides are transformed into glucose and organic acids or proteins into organic acids and amino acids, respectively. Lamoophak et al. (2006) studied the effect of the hydrothermal treatment on the decomposition of spent yeast waste from the brewing industry. They analyzed the effect of hydrolysis temperature on the amount of released proteins and amino acids. They used Baker's yeast cells as a model for SBY due to their similarity in composition and easy handling. The reaction

was carried out in a closed batch reactor with a 1:10 solid-liquid ratio, a pressure of 101.35 kPa-3.97 MPa, two different temperatures (100 and 250 °C), and a reaction time of 5-30 min. The study determined that under the best conditions for protein extraction (250 °C, 3.97 MPa for 20 min) 7 % of the total protein was obtained. Although the protein yield was low, it was twice as much as that obtained with autolysis in this work.

6.2.3. Ultrafiltration

Membrane separation is a physical method used for the extraction of different bioactive components. Nowadays this is one of the most generally used methods to obtain isolated proteins from SBY. Huang et al. (2012) used ultrafiltration to separate the protein from dry SBY. They carried out an experimental design to optimize three factors: pH (4, 6, and 8), concentration (1.5, 3.0 and 4.5 %), and operating pressure (10, 15 and 20 psi), considering protein yield as a response variable. The ultrafiltration process was carried out with a hollow fiber membrane system consisting of a constant flow pump, polyethersulfone membranes (nominal pore diameter of 0.2 μm), with 5-kDa molecular weight cut-off, and a surface area of 0.05 m^2 (GE Healthcare, Piscataway, NJ, USA). The optimum conditions were as follows: concentration 2.7%, pH 5.0, and pressure 14 psi. The protein yield was 95.03 %, which was in agreement with the maximum predicted value (96.4 4%), validating the model. As reported by the authors, membrane separation, besides being a physical method that does not damage the protein, could be an effective technique for the separation of other bioactive components such as polysaccharides (Huang et al. 2012).

Other authors have focused their work on the use of ultrafiltration and nanofiltration pilot systems for recovering proteins. Amorim et al. (2016[a]) carried out a protein extraction test with SBY samples by an ultrafiltration process with previously autolyzed yeasts. UF was carried out using an organic membrane (Hydranautics model Dairy 10k 3838-30) with a 7.4 m^2 filtration area and 10 kDa cut-off. They conducted an enzymatic hydrolysis of the retained and filtered extracts, which were then nanofiltrated (organic membrane, model NF 3838/30-FF with an area of 6.9 m^2, and 3 kDa cut-off). Each one of the fractions obtained (with different sizes and molecular weights) was concentrated by reverse osmosis and lyophilized. The fractions turned out to be an important source of concentrated

protein with values ranging between 30 and 70 % db. However, a large amount of carbohydrates was also present in each fraction, being higher in the lower molecular weight fractions.

Recent researches explore the use of ultrafiltration for SBY hydrolysates, considering the impact of pH, fouling of membranes, and low ribonucleic acid (RNA) content (Marson et al. 2021; 2022). The UF process carried out by Marson et al. (2021) was performed with commercial flat sheet membranes made of polyethersulfone and regenerated cellulose (Microdyn-Nadir model, Germany) of 30 kDa of molecular weight cut-off at different pH values (5 and 8). The impact of the feed composition, pH and membrane material on UF performance and fouling was evaluated by examining the membrane-feed interactions and the existing relations between fractions and membrane characteristics. As for pH, a better flow was observed at pH 8; however, it lost effectiveness over time. They also determined that the polyethersulfone membrane was more selective at any pH, and the peptide retention was of 85%, compared to 68% for regenerated cellulose. In addition, it showed a significant effect on RNA adsorption on the regenerated cellulose membranes that did not occur with the polyethersulfone membranes, suggesting that the separation of RNA from the protein fraction of the yeast requires further study.

Marson et al. (2022) based their work on the separation of hydrolysed yeast peptides from sugars and RNA using a membrane fractionation process. Fractionation was carried out using ceramic ultra- and nanofiltration (50, 8, and 1 kDa) and molecular weight cut-off membranes (15, 8, and 1 kDa). After fractionation, the purity of the peptides increased up to 1.7 times in relation to RNA , and 2.7 times in relation to total sugars. Protein-rich extracts were obtained from SBY with a RNA content of less than 1.4%. This low RNA content could advance their use in the food industry.

6.2.4. Enzymatic hydrolysis

Some authors consider hydrolysis as the most effective method to solubilize yeast components, conducted either with proteolytic enzymes or hydrochloric acid (acid hydrolysis) (Huang et al., 2012; Lamoolphak et al., 2006). Chae et al. (2001) studied the effects of different enzymatic treatments on the recovery of protein and solids and the use of SBY in developing flavoring foodstuff. This work consisted in hydrolysing yeast protein into amino acids and low molecular weight peptides and RNA

to obtain nucleotides. The use of multiple enzymes (nuclease and protease), the effects of the type of protease, and the enzymatic concentration were evaluated. They observed that the proteases Protamex (0.6%) and Flavourzyme (2%) exhibited the highest protein recovery (53.6 %) at 12 h of hydrolysis. This increase was produced by the release of intracellular components and the degradation of the yeast protein. Protein recovery mainly depended on the enzyme dosages, highlighting that exoprotease activity was the most important factor in yeast protein hydrolysis. Amorim et al. (2019) proposed an experimental design to optimize two variables: hydrolysis time and enzyme/substrate ratio. The hydrolysis was carried out with proteases from *Cynara cardunculus* at pH 5.2 and 55 °C using the degree of protein hydrolysis as a response. The optimal hydrolysis conditions were: substrate ratio of 4% (v/v) for 4.5 h. It should be noted that the authors suggested that spent brewer's proteins and derived peptides have great potential as ingredients for the prevention and/or control of chronic metabolic diseases such as hypertension. Marson et al. (2019) studied the SBY cell wall disruption using conventional methods, such as autolysis and mechanical disruption (glass beads) in combination with enzymatic hydrolysis (proteolytic enzymes). In the first instance, they compared two conventional methods with hydrolysis by the Brauzin® enzyme (pH 5.5, 60 °C, and 10 % E:S ratio), an enzyme that had not been previously used for this type of study. When comparing the three methods, they observed that autolysis reduced the protein content due to protein degradation and reaction with other components, but it increased protein extractability by 11 % compared to control. On the other hand, as for the mechanical rupture method, there were no differences in protein yield between control and the enzymatically treated samples. This can be attributed to the selected working conditions. Finally, they observed that the use of an enzyme allowed the recovery of 83 % of proteins due to cell disrupture, and concluded that, in addition to obtaining the highest protein yields, protein hydrolysate obtained with Brauzyn® showed the highest antioxidant properties and total solid content.

6.2.5. Advantages and disadvantages of the methods used for the extraction of proteins from SBY

Table 6 presents the advantages and disadvantages of each protein extraction method. Few studies have been carried out on the methods of recovering proteins from spent brewer's yeast.

6.3. Hot trub protein extraction methods

Hot trub is by far the least studied waste for by-product recovery. Its direct application in food products is unlikely due to its bitterness as a result of the large presence of tannins. However, recent studies have focused on reducing the bitter compounds of hot trub for its subsequent application in foods. Saraiva et al. (2019) could remove the bitterness and obtain a protein concentrate suitable for application in the food industry. They carried out the extraction with hot tub and water (1:4 s/l) for 10 min, then the dispersion was heated at 100 °C for 60 minutes to remove most of the tannins. This procedure at high temperatures produced the breakdown of the protein-tannin complexes and the subsequent release of almost all of the tannins. A solid residue with 70 % of the initial protein content of the raw material (40 %) was obtained. They also analyzed different techno-functional properties of the concentrate obtained, observing that the oil absorption capacity (OAC) was not affected, and thus it could be used to enrich high-fat foods and as a new source of vegetable protein.

In another recent work, Saraiva et al. (2021) created an experimental design to optimize the alkaline extraction of proteins from the trub, considering three factors: pH (11-13), concentration (2.5-7.5 g/L), and extraction time (30-90 min). The extraction was carried out at 80 °C, and the protein was then precipitated with 1N HCl, centrifuged, and lyophilized. The optimal conditions were pH 12.3, concentration 5.31 g/L, and an extraction time of 51.78 min. The authors obtained an average percentage of protein isolate of 94.56 ± 0.63 %.

Although no reports could be found in the literature on the protein extraction from hot trub, this residue is a by-product of great interest to be studied in future works.

7. Conclusions

The need to reuse food industry wastes has opened the door to research on extraction methodologies for the recovery of bioactive compounds from food wastes, transforming them into useful by-products. Protein extraction from different sources has been usually carried out using

conventional and obsolete methodologies, but with high proteins yields, and more recently with other new methods that have been less studied, but that show promising results. This range of available methodologies leads to the question of which one is the most suitable for protein extraction from brewing industry wastes. The novel contribution of this work is that the selected methodology will depend on the techno-functional properties of the protein extracts and their subsequent uses. On the other hand, it is important to highlight that not only the pretreatment but also the combination of pretreatment and subsequent extraction process should be considered to improve the protein yield.

Conflict of Interest

The authors declare no conflict of interest.

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Figure 1. Brewing process

Figure 2. Bibliometric analysis on scope of protein extraction research in BSG and spent yeast conducted.

Figure 3. Membrane separation processes: comparison based on the difference in particle size, molecular size and pressure gradient.

Components	**Total content** (% w/w d.b.)	**References**
Hemicellulose	19.2 – 26.0	Mussatto et al. 2006; Moreira et al. 2013; Qin et al. 2018.
Lignin	12.0 – 27.8	Robertson et al. 2010; Moreira et al. 2013; Ikram et al. 2017; Qin et al. 2018.
Cellulose	8.9 – 38.8	Mussatto et al. 2006; Meneses et al. 2013; Moreira et al. 2013; Vieira et al. 2014; Ikram et al. 2017; Patel et al. 2018; Qin et al. 2018;
Proteins	12.5 - 35.4	Mussatto et al. 2006; Treimo et al. 2008; Robertson et al. 2010; Xiros and Chistalopoulos, 2012; Connolly et al. 2013; del Rio et al. 2013; Moreira et al. 2013; Vieira et al. 2014; Kemppainen et al. 2016; Ikram et al. 2017; Rommi et al. 2018; Qin et al. 2018; Patel et al. 2018; Yu et al. 2020.

Lipids	3.0 – 13.5	Connolly et al. 2013; del Rio et al. 2013; Vieira et al. 2014; Kemppainen et al. 2016; Rommi et al. 2018; Qin et al. 2018; Yu et al. 2020.
Minerals	3.2 – 5.0	Conolly et al. 2013; del Rio et al. 2013; Vieira et al. 2014; Rommi et al. 2018; Qin et al. 2018; Yu et al. 2020.

Table 1: Main constituents (% w/w d.b.) of the brewers' spent grain according to references.

Components	**Total content** (% w/w d.b.)	**References**
Proteins	45-60	Amorim et al. 2016; Podpora et al. 2016; Vieira et al. 2016; Jacob et al. 2019; Puligundla et al. 2020;
Minerals	2-8.5	Podpora et al. 2016; Vieira et al. 2016; Jacob et al. 2019; Puligundla et al. 2020; Rachwal et al. 2020;
Carbohydrates	~ 40	Podpora et al. 2016; Amorim et al. 2016[a]; Rachwal et al. 2020
Lipids	~ 4	Rachwal et al. 2020
Vitamins (mg/100g db)		Puligundla et al. 2020; Amorim et al. 2016[a]; Vieira et al. 2016; Huang et al. 2012

Pyridoxine (B6)	55.1
Folic acid (B9)	3.01
Riboflavin (B2)	<0.32
Cyanocobalamin (B12)	<0.25
Nicotinic acid (B3)	77.2

Table 2: Main constituents (% w/w d.b.) of the spent brewer's yeast SBY according to references.

Components	**Total content** (% w/w d.b.)	**References**
Proteins	40 - 70	Barchet, 1993; Priest and Stewart 2006; Kühbek et al. 2007; dos Santos Mathias et al. 2014, 2017; Rachwał et al. 2020.
Hops	10 - 20	Barchet, 1993; Priest and Stewart 2006; dos Santos Mathias et al. 2014;
Polyphenols	5 - 10	Barchet, 1993; Priest and Stewart 2006; dos Santos Mathias et al. 2014. Vieira et al. 2016.
Carbohydrates	4 - 8	Barchet, 1993; Priest and Stewart 2006; dos Santos Mathias et al. 2014, 2017.

Minerals	3 – 5	Barchet, 1993; Priest and Stewart 2006; dos Santos Mathias et al. 2014, 2017.
Fatty acids	1 - 2	Barchet, 1993; Priest and Stewart 2006; dos Santos Mathias et al. 2014;

Table 3: Main constituents (% w/w d.b.) of the hot trub according to references.

Methodology	**Advantages**	**Disadvantages**	**References**
Ethanolic extraction	- Short times. - Low cost.	- Low yield - High temperatures - Addition of chemicals.	Ervin et al. 1989; Diptee et al. 1989.
Ultrafiltration	- High performance. - Good ability in the removal of salts.	- Concentration polarization and fouling.	Tang et al. 2009.
Alkali extraction	- High performance. - Favorable techno-functional properties	- Some amino acids are destroyed.	Celus et al. 2007; Sonawane and Arya,

			2018; Arauzo et al. 2019; Du et al. 2020.
Ultrasound pre-treatment	- Physical method - Breaks down cell structures without exposure to temperature and prolonged times.	- Difficult to apply on a larger scale.	Tang et al. 2010[a]; Tang et al. 2010[b]; Li et al. 2021; Yu et al. 2020; González-Garcia et al. 2021.
Enzymatic treatment	- High Selectivity. - Obtaining different components simultaneously	- Yield and quality vary depending on the enzyme used. - Enzymes increase the cost. - Difficult to apply on a larger scale.	Niemi et al. 2013; Rommi et al. 2018; Qin et al. 2018; He et al. 2019; Paz et al. 2019; Shen et al. 2019; Yu et al. 2020.
Hydrothermal pre-treatment	- High Selectivity. - Low temperature. - No addition of chemicals.	- Better yields at long extraction times (over 24 h).	Qin et al. 2018
Hydromechanical treatment	- Physical method, does not damage proteins.	- Low selectivity. - Low yield.	Ibbett et al. 2019
Pressurized liquid extraction	- Green technology, environmentally friendly.	- It requires subsequent drying, depending on the component to be extracted.	Schantz et al. 2006; Mustafa and Turner, 2011; González-Garcia et al. 2021.
Subcritical water	- Obtaining different bioactive compounds simultaneously, modifying the extraction conditions. - Short extraction times. - Low solvent consumption.	- Difficult to remove moisture. - Thermal degradation may occur at higher temperatures. - Equipment is not easy to clean.	Zhang et al. 2020; Alonso-Riaño et al. 2021.

Table 4: Main advantages and disadvantages of protein extraction methods.

Cell disruption method	Operating conditions	References
Autolysis/Plasmolysis	- In reactor 19 h, 50 °C, with HCl 0.5N, pH 5.2.	Lamoolphak et al. 2006
	- In reactor 3 to 49 h, 50 °C, constant stirring (100 rpm), pH 5.5	Boonyeun et al. 2011
	- In reactor 24 h, 50 °C	Tanguler et al. 2008; Vieira et al. 2013
	- In reactor, 24 h, 50 °C, constant stirring and addition of sodium chloride (0.086 M) and ethyl acetate (0.051 M).	Jacob et al. 2019

	- Temperature 50 °C, pH 6, 24 h under magnetic stirring (700 rpm).	Marson et al. 2019
Mechanical		
Glass Beads	- Diameter of glass beads 0.6 mm, weight ratio of 1:2 (beads/suspension and mix), temperature 4 °C.	Vieira et al. 2013
	- Diameter of glass beads 2.64, 2.96 and 3.86 mm, ratio of 1:2 (beads:suspension), temperature 4 °C.	Marson et al. 2019
	- Diameter of glass beads 0.5 mm, weight ratio of 1:2 (glass beads/ yeast suspension), temperature 7 °C, 15 min.	Jacob et al. 2019
Ultrasound	- Sonotrode diameter 25 mm, operating frequency of 20 kHz, 400 W, temperature 7 °C.	Jacob et al. 2019

Table 5: Operating conditions of cell disruption methods, according to references.

Methodology	**Advantages**	**Disadvantages**	**References**
Autolysis	- Milder method - Cheap - Non-toxic	- Long processing time. - Low extraction yield - Difficulty in solid/liquid separation. - It depends on the autolytic properties of the cell.	Chae et al. 2001; Lamoolphak et al. 2006; Huang et al. 2012; Amorim et al. 2019; Marson et al. 2019.
Plasmolysis	- Modification of the autolysis process - Simple process	- Use of inorganic salts.	Lamoolphak et al. 2006; Huang et al. 2012.

Enzymatic hydrolysis	- High yields	- Expensive to be practical at large scale.	Chae et al. 2001; Lamoolphak et al. 2006; Huang et al. 2012; Amorim et al. 2016; Amorim et al. 2019; Marson et al. 2019.
Hydrothermal treatment	- No addition of chemicals.	- High temperatures	Lamoolphak et al. 2006; Huang et al. 2012.
Ultrafiltration	- Physical separation process - Molecular-level separation - Conservation of biological activity - Low cost - Easy scale-up	- Lack of membrane selectivity - Membrane fouling.	Saxena et al. 2009; Huang et al. 2012; Amorim et al. 2016[a]. Marson et al. 2021; Marson et al. 2022.

Table 6: Advantages and disadvantages of each SBY protein extraction method.

- Brewing produces three wastes: bagasse (BSG), spent hops, and spent brewer’s yeast.
- Review on protein extraction using conventional and more innovative technologies
- The BSG is a source of valuable compounds like proteins.
- The recovery method will depend on the final uses of the protein extracted.
- Proteins' recovery from BSG could be an alternative to valorize this by-product.