Isolation of proteins from by-products and residues of the brewing industry

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Abstract

Proteins are useful biomolecules both for their nutritional value and functional properties. The modern way of living, the necessity to cover the nutritional protein needs of the population, combined with the tendency to adopt a vegan lifestyle, lead to proteins' isolation from alternative sources, such as by-products of agro-industrial sector. By-products of brewing industry are rich in proteins; brewer´s spent grains (BSG) contain about 20% and brewers´ spent yeast (BSY) contain about 50% on dry base. In this work, proteins from BSG were isolated using three methods: conventional (CE), ultrasound assisted (UAE) and enzymatic assisted extraction (EAE), as well as proteins from BSY were isolated using CE and UAE. In CE and UAE, three solvents were tested: NaOH, Ethanol/NaOH and Methanol/NaOH. UAE was optimized regarding biomass to solvent ratio, ultrasound power and extraction time. In EAE, ultrasound pretreated and crude BSG were used as raw materials, the enzyme Alcalase 2.4L was used, and the effect of enzyme loadings and time was examined. The protein content of all the extracts was measured by Bradford method. Considering CE, the highest protein yield was observed for NaOH at 1:20 ratio. For UAE, the use of NaOH led to maximum protein yield when ultrasound power and extraction time were maximized, due to more cavitational effects. Ethanol/NaOH in 1:10 ratio led to higher protein yields in UAE and CE. For EAE, the increase of extraction time contributed to the increase of protein yield, whereas, higher enzyme loading did not affect protein content. The optimum extraction conditions were: UAE using NaOH, 1:20 ratio, ultrasound power 750 W, extraction time 20 min, leading to isolation of 61.39 mg protein/g BSG. Regarding BSY, the highest protein yield was obtained for UAE using NaOH with a protein content of 35.84 mg protein/g BSY. The present study clearly demonstrated that UAE and EAE could successfully replace CE for BSG and BSY protein extraction.

Keywords

brewer' spent grain and yeast, ultrasound assisted extraction, enzyme assisted extraction

1. Introduction

The industrial process of beer manufacturing generates relatively large amounts of by-products and residues, including spent grain, spent hops and yeast. These by-products are agricultural products, and can be easily utilized in order to be recycled and reused. Thus, compared to other industries, brewing industry tends to be more environmentally friendly [1]. The insoluble, outer pericarp-seed coat parts of barley grains, derived from mashing process (hot water extraction at 65–70°C), are the brewers' spent grains (BSG). BSG is the most important by-product generated from breweries, representing ~85% of the total amount of by-products [2]. 100 L of brewed beer produce around 20 kg of wet BSG. Until now, BSG is mainly used as a low-value animal feed [3]. It is a lignocellulosic material, consisting mostly of fibre (hemicellulose and cellulose), protein and lignin. Proteins represent about 15-30% of total dry weight, typically present at levels of 20%. The most important proteins are hordeins, glutelins, globulins and albumins. BSG consists of essential amino acids at around 30% of the total protein content. Among them, lysine, which is often deficient in cereal foods, is the most abundant (14.3%). Lignin is also another vauable compound of BSG, representing about 10–28% of the total dry weight. Finally, BSG contains a variety of minerals, such as silicon, phosphorus, calcium and magnesium [3].

In addition, another significant by-product of the brewing industry is the brewing spent yeast (BSY). Beer production generates large quantities of yeast during fermentation and lagering. It is estimated that

for the production of 100 L of beer, 0.7-1.1 kg of BSY is obtained after fermentation [4, 5]. Spent yeast, after primary fermentation, is used in small quantities to pitch the next batch. At the end of lagering process, 0.5–0.9 kg yeast per hl of finished beer is generated. BSY can be used as a starting material for the production of yeast extract. The separation of yeast compounds for food applications is a crucial issue [4, 5]. Yeast extracts are produced through yeast cell wall degradation using endogenous or exogenous enzymes. Extract preparation can be conducted using either autolysis or hydrolysis method. In autolysis, the intracellular enzymes break down cell constituents like proteins, glycogen and nucleic acids. Cells must be killed without inactivating the yeast enzymes (usually under moderate agitation and temperatures between 30 and 60°C for 12–24 h). Hydrolysis method is divided into acid hydrolysis or enzymatic hydrolysis. Although acid hydrolysis leads to a high production yield, it is less attractive, due to the high salt content and potential presence of carcinogenic compounds such as monochloropropanol and dichloropropanol. The enzymatic hydrolysis of yeast cells is more desirable as it produces extracts with a low salt content [6-8]. Spent brewer's yeast is usually sold as inexpensive animal feed after inactivation by heat. Yeast biomass can be, also, used in food industry for the generation of yeast protein concentrates (and isolates) reserving their nutritive value and functional properties. BSY is low in calories, fat and carbohydrates, however, it can be a valuable source of cheap fibre, mainly β-glucans, nucleotides, vitamins and minerals. The protein content of BSY is present at level of 50% per dry weight.

Alkali extraction followed by acid precipitation is used to isolate proteins from plant tissues, although it is disadvantageous because of the increased extraction time and used energy. Ultrasound assisted extraction (UAE) is chosen as an alternative method in protein extraction, aiming to reduce extraction time, energy consumption and use of solvent and is characterized as a green extraction method. The effect of the frequency of ultrasound on the rate and extraction kinetics is important and depends on both the material's structure and the nature of the compound aimed to be extracted. Ultrasounds allow easy mixing, fast energy transfer, low temperature, selective extraction and reduced equipment size. The parameters that can be optimized in UAE are ultrasound power, pressure and temperature, time, type of solvent, and particle size of the biomass. Careful study of these operational parameters is great importance to achieve the highest efficiency. There are many applications of UAE in the extraction of compounds from fruit and vegetables, herbs and spices, oleaginous seeds and microorganisms [9, 10].

Another attractive method for protein recovery that is able to replace successfully conventional extraction (CE) techniques, is the extraction with the use of enzymes. The basic principle of enzyme assisted extraction (EAE) is the use of enzymes as a catalyst, in order to disrupt plant cell walls by hydrolysis, and release the intracellular compounds. The plant cell wall binds to the active site of the enzyme, causing the change of its shape so that the substrate fits onto its active site. Change in the enzyme shape causes breakage of bonds of the cell wall, releasing the active constituents. EAE results in reducing extraction time, minimizes use of solvents and increases protein yield. However, enzyme-assisted extraction of bioactive compounds from plants has, also, commercial and technical limitations: (i) the cost of enzymes is relatively expensive for processing large volumes of raw material; (ii) enzyme-assisted extraction can be difficult to scale up to industrial scale since enzymes behave differently in different environmental conditions (e.g. percentage of dissolved oxygen, temperature, etc.) and nutrient availability [11, 12].

The objective of this work is the isolation of proteins from BSG and BSY streams and the optimization of the environmentally friendly extraction methods of UAE and EAE. Protein content is evaluated using the Bradford method.

2. Materials and Methods

The raw materials used are Brewer's Spent Grain (BSG) and Brewer's Spent Yeast (BSY), kindly provided by Athenian Brewery S.A. After being received, samples were frozen at -30°C.

2.1 Pretreatment

2.1.1 Pretreatment of BSG

Freeze drying: BSG were lyophilized in a freeze-drier (Leybold-Heraeus Freeze Dryer GT2) for 48 h and then stored at -30°C for further use.

Grinding: All samples were milled using a conventional coffee grinder in order to reduce their particle size, and maximize proteins' extraction yield.

Defatting: Lipids have been reported to interfere with hydrophilic extraction procedures. Therefore, BSG was initially submitted to a defatting process before use as starting material for the sequential experiments. Defatted BSG were obtained by mixing dried and milled BSG with n-hexane at a solid-liquid ratio of 1:5 in an ice-bath for 1 h. Then, the hexane phase was removed by centrifugation (3500×g, 20 min). The remaining solids were dried at 42°C until constant weight [13].

2.1.2 Pretreatment of BSY

Debittering: In order to remove beer liquor, BSY is centrifuged at 3500xg speed for 20 min. Then, the solid phase is washed three times with phosphate buffer, pH 7 [14]. BSY were dried at 100°C until constant weight in order to determine their moisture content.

2.2 Protein extraction

For BSG protein isolation, three different extraction methods were implemented: (a) CE (b) UAE (c) EAE, while protein recovery from BSY was performed using (a) CE and (b) UAE. Additionally, the effect of different operational parameters was examined, in relation to extraction yield.

2.2.1 Conventional Extraction

BSG: During CE, the solid biomass – solvent ratio was studied for a given extraction time and temperature. More specifically, 50^{o}C was selected as extraction temperature, and 2 h with agitation as extraction time [13]. The selected solid biomass – solvent ratios were 1:10 and 1:20. The solvents used were NaOH 0.1 M [13], an Ethanol/ 1M NaOH 45:55 solution [15, 16] and a Methanol/ 1 M NaOH 45:55 solution. After extraction (each repeated twice) the system was removed from the magnetic stirrer and stored for further processing.

BSY: During CE, the solid biomass – solvent ratio, extraction time and temperature were kept constant at 1:20, 24 h and 50°C, respectively, changing, only, the solvent type. All corresponding solvents used for BSG extraction were examined, including phosphate buffer solution pH 7.

2.2.2 Ultrasound Assisted Extraction

BSG: The temperature in all UAE experiments was kept constant, at 25^{o}C, with the aid of refrigerant (set to 12^{o}C), which flows in the outside wall of the extraction vessel, so as to limit the effect of the temperature, which sharply raises up. The different examined operational parameters were: (a) solid biomass – solvent ratio, 1:10 and 1:20 (b) ultrasonic power with selected values 150, 450, 750 W (c) extraction time at 5, 10, 20 min. The extractions were carried out in the presence of magnetic stirring and repeated twice for each examined parameter.

BSY: During UAE, the solid biomass – solvent ratio, extraction time, power and temperature were kept constant at 1:20, 20 min and 750 W, respectively, changing, only, the solvent type. All corresponding solvents used for BSG extraction were examined, including phosphate buffer solution pH 7.

2.2.3 Enzymatic Assisted Extraction

As extraction solvent, a phosphate buffer pH = 7 was used, to which enzyme Alcalase 2.4 L was added [17]. The solid biomass – solvent ratio was kept constant at 1:20, as well as extraction temperature at 50^{o}C. In this temperature enzyme operates to an excellent degree [18]. Examined parameters were time, which was varied between 6, 12 and 24 h, the amount of enzyme, which was selected at 10 and 20 mg, and the use of BSG, which was selected to be either pretreated using ultrasound or without pretreatment. For BSG, that has been pretreated using ultrasound, after UAE, the extracted biomass together with the solvent was centrifuged to recover the BSG, that has been ruptured. Then, transferred in an oven at 42^{o}C, in order to completely remove the solvent entered the biomass.

2.3 Protein recovery

2.3.1 Centrifugation

BSG: To isolate the protein extract, the method of centrifugation (Centrifuge NF400, Nuve, Ankara, Turkey) is applied at 3500×g speed for 20 min. The liquid phase is isolated and stored for further processing, while the solid phase is discarded.

BSY: The extracted biomass from each experiment is centrifuged at 3500×g speed for 20 min, and the supernatant (inner yeast extract) is carefully pipetted out and subjected to Bradford method for a protein content measurement [13].

2.3.2 Protein precipitation

The precipitation of proteins is based on the isoelectric point method. Extract's pH, in which the proteins are dissolved, is adjusted to such a value, that the molecules of the proteins are electrically neutral. The point at which this change occurs is the isoelectric point. At this point, proteins show very little solubility and, for this reason, they form agglomerates, precipitate and maintain their solid structure. Due to such precipitation, the solution is visibly cloudy [19]. In this work, the extract was placed in a stirred beaker and its pH was adjusted at 3.8 using HCl, which is the corresponding value for proteins recovery. In order to facilitate the crystallization of the precipitated proteins, this procedure should be performed in a low temperature, at 4^{o}C. Centrifugation followed, after which, the solid phase - where the precipitated proteins are – was collected, while the supernatant was discarded. The reverse occurs in EAE and supernatant is collected. The solid phase underwent, then, a redissolution process in deionized water until pH adjusted to 7.0, in order to return the proteins in a dissolved form. This occurs adjusting the pH at 7. The protein extracts were frozen at -30°C and lyophilized for 72 h. Freeze-dried protein extracts were re-dissolved in the certain solvent used for the extraction [20].

2.4 Measurement of protein content

In order to measure the protein content after each extraction experiment, Bradford method was applied. In this method, the dye used as reagent is Brilliant Blue G, which can form protein complex. This protein complex absorbs maximum at 595 nm. Protein concentration is expressed as ratio of absorptions. For this purpose, a calibration curve was constructed. This was achieved by using albumin as standard protein. For each extraction experiment, BSG and BSY freeze dried extracts and BSG-EAE liquid extracts were treated with the respective extraction solvent, in the framework of Bradford method's application. The same was applied to blank samples. In EAE experiments blank sample was produced, after boiling of the buffer-alcalase system for about 20 min, so as to inactivate the enzyme.

2.5 Coding of experimental parameters

The following Table depicts the coding of extraction method and solvent system. Statistical processing was done using Statistica software (version 13.6 StatSoft®Inc., Palo Alto, USA). Statistical analysis of variance (ANOVA) was implemented and the tests were done with ''Tukey'' technique. Differences were considered significant, when $p<0.05$.

Table 1 Coding of experimental parameters

	Method			
	Conventional Extraction	Ultrasound assisted extraction	Enzymatic assisted extraction	
Code	**CE**	**UAE**	**EAE**	
	Solvent system			
	NaOH 0.1 M	Ethanol/NaOH (45:55)	Methanol/NaOH (45:55)	Phosphate buffer pH 7
Code	**A**	**B**	**C**	**D**

3. Results and Discussion

3.1 Recovery of proteins from BSG

The following figure (Fig.1) depicts the BSG protein yield obtained from the conventional extraction, for the three different solvent systems, and the two solid biomass – solvent ratios.

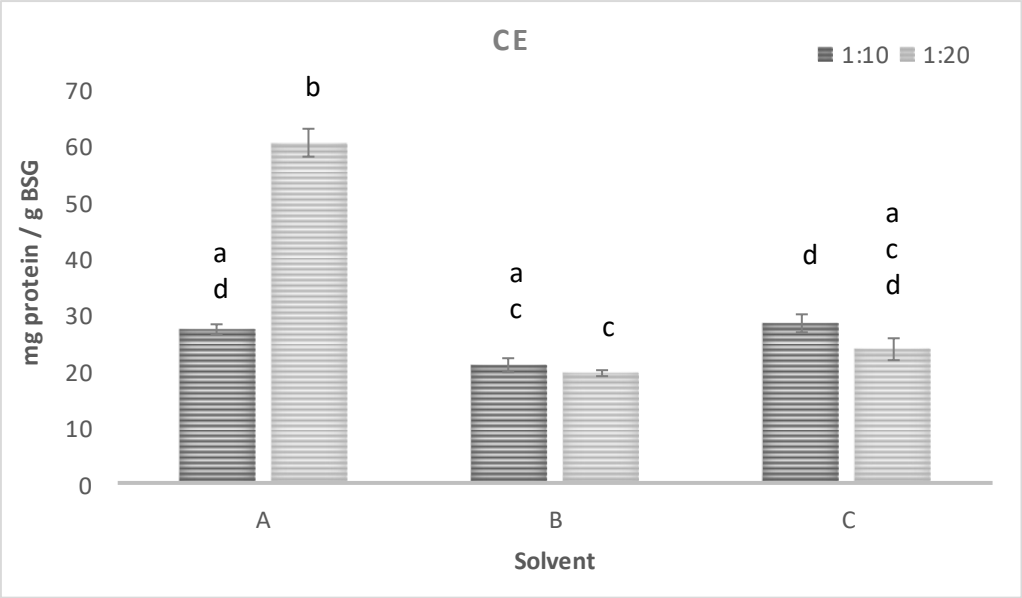

Fig. 1 BSG protein yield of CE for the various tested parameters.

Considering Fig.1, the highest protein yield was observed for 0.1 M NaOH (A) at 1:20 solid biomass – solvent ratio. The use of Methanol/NaOH (45:55) (C) led, also, to higher protein yield, in comparison to Ethanol/NaOH (45:55) (B), but the difference between 1:10 and 1:20 ratio was not significant for both solvent systems. For this reason, the use of 1:10 solid biomass – solvent ratio is preferred in the cases of Ethanol/NaOH (45:55) and Methanol/NaOH (45:55), in terms of cost-effectiveness and environmental friendliness. The use of 0.1 M NaOH solvent targeted the isolation of proteins, such as glutellins which are isolated in the presence of an alkaline phase, while the presence of the aqueous phase contained in this solvent could also lead to the isolation of albumin and globulins dissolved in aqueous systems [17, 20]. As regards the use of Ethanol/NaOH solvent, this was chosen in order to enhance the isolation of proteins, such as prolamins that are dissolved in alcohols, and at the same time to enhance the alkaline phase of the solvent in order to isolate all the proteins contained in the spent barley grains [15-17, 20]. The use of Methanol was chosen for similar reason.

Figs 2-4 present the protein yield obtained from the ultrasound assisted extraction, for the different solid biomass – solvent ratios and ultrasound power, for the different solvent systems, respectively.

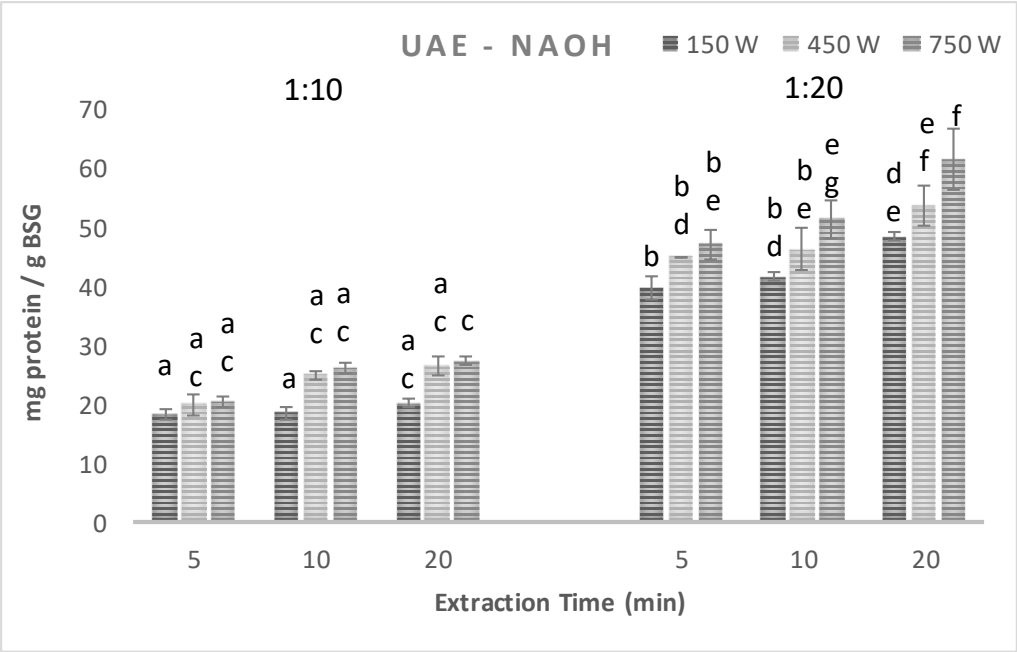

Fig. 2 BSG protein yield of UAE for NaOH 0.1 M for the various tested parameters.

As it can be observed from Fig. 2, the use of 1:20 solid biomass – solvent ratio contributed effectively to protein extraction. Such result can be interpreted based on the availability of the liquid phase. When increased water amount is available, solutes encountered more cavitational effects [21]. Besides that, it was noticed that when both increasing the extraction time and ultrasound power, protein extraction was positively affected. This result can be explained by the fact that higher ultrasound power contributed to cavitation phenomena and, thus, better cell disruption occurred [20]. In the case of 1:20 solid biomass – solvent ratio, the effect of extraction time was more significant, especially when comparing the results of 5-min and 20-min extractions. The optimal conditions of UAE using 0.1 M NaOH were 750W, 20 min and 1:20 solid biomass – solvent ratio, decreasing crucial parameters, such as the extraction time and energy consumption, in relation to CE.

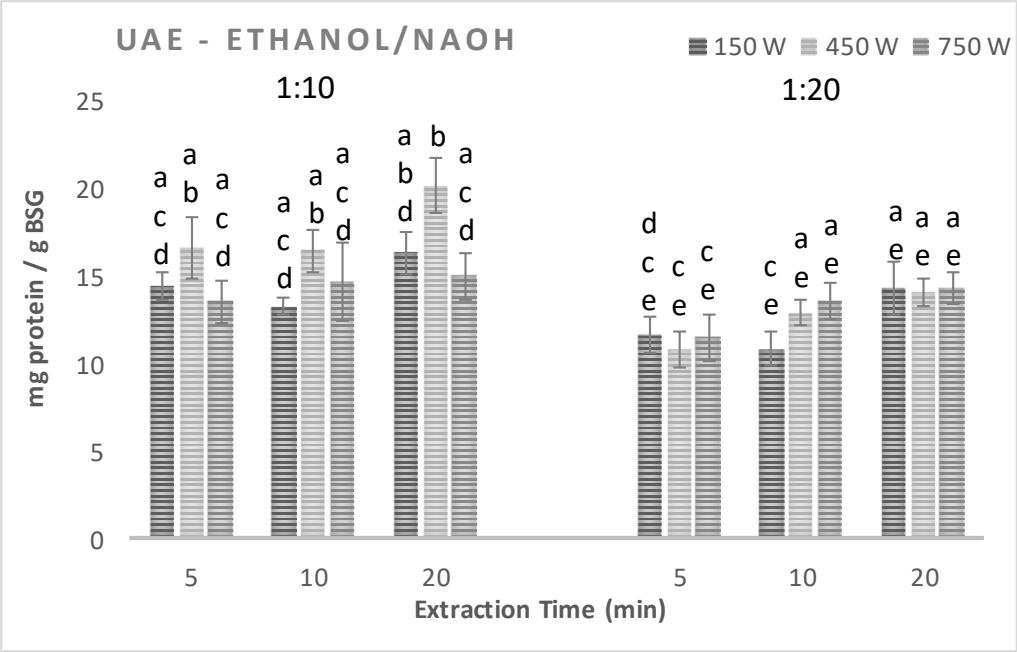

Fig. 3 BSG protein yield of UAE for Ethanol/NaOH (45:55) for the various tested parameters.

As shown in Fig. 3, protein yield was significantly lower for Ethanol/NaOH solvent compared to 0.1 M NaOH. In addition, protein yield was lower in the case of 1:20 solid biomass – solvent ratio. This is a result based on the effect of solvent polarity in the UAE. Decreasing the polarity (use of Ethanol) prevents the cell wall rupture and the release of protein content in the liquid, thus decreasing the extraction efficiency [22]. In the case of 1:10 solid biomass – solvent ratio, the effect of increasing of ultrasound power was more clear. The increase of ultrasound power over 450 W led to lower protein yields. Moreover, extraction time increased the yield in protein content. The optimum operating parameters of UAE using Ethanol/ 1M NaOH (45:55) were 450W, 20 min and 1:10 solid biomass – solvent ratio.

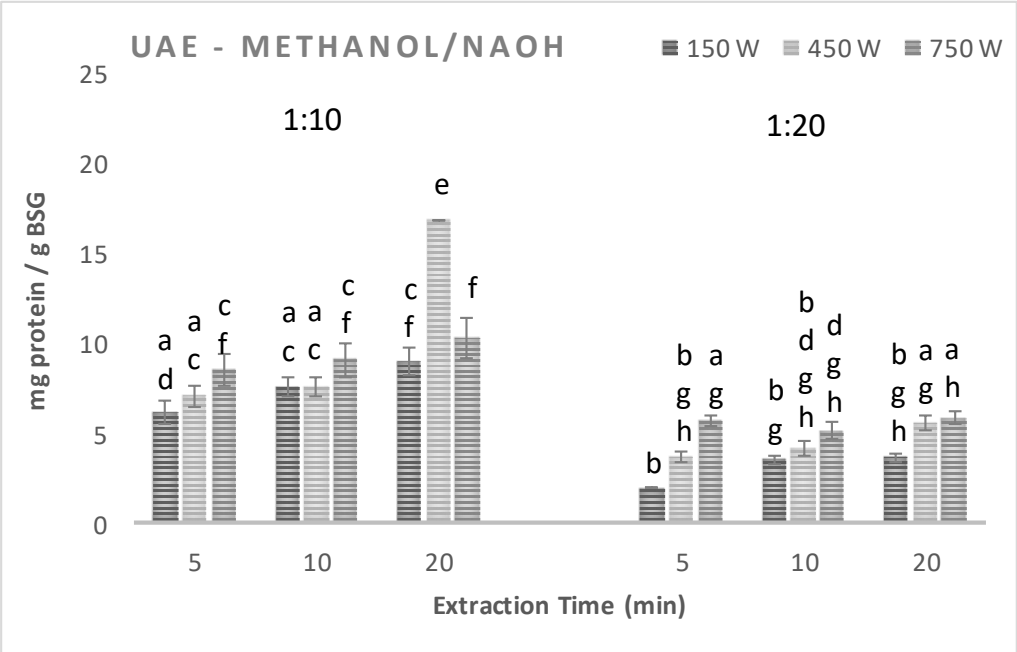

Fig. 4 BSG protein yield of UAE for Methanol/NaOH (45:55) for the various tested parameters.

Considering Fig. 4, it is observed that the use of Methanol/NaOH (45:55) had a negative effect on the protein extraction, in the case of 1:20 solid biomass – solvent ratio. It is worth mentioning that, even compared to CE, the extraction efficiency of UAE for the Methanol/NaOH solvent is lower. The optimum operating parameters of UAE using this solvent system were 450W, 20 min and 1:10 ratio, which give a satisfying result.

The evaluation of EAE is presented in Table 2, both of the crude and the pretreated BSG.

Table 2 BSG protein yield for EAE

Extraction time (h)	***Alcalase (mg)***	***mg protein / g pretreated BSG***	***mg protein / g crude BSG***
6	10	5.41	9.23
	20	6.90	8.96
12	10	15.38	17.10
	20	14.80	17.88
24	10	17.78	20.15
	20	18.53	20.15

According to Table 2, the increase in extraction time led to an increase in protein yield which is in agreement with other studies [15]. A rapid increase in protein yield was observed by increasing the extraction time from 6 to 12 h. The increase of extraction time from 12 to 24 h, did not have significant effect on protein yield. Additionally, it was observed that increasing the loading of the enzyme did not

lead to significant increase of protein yield. For each extraction time, the use of ultrasound pretreated BSG led to lower yields of protein content.

3.2 Recovery of proteins from BSY

Recovery of proteins was also investigated using BSY as raw material. The optimum conditions obtained for the case of BSG were applied for four different solvent systems.

Table 3 BSY protein yield for UAE and CE for the different solvent systems

	mg protein / g BSY			
	A	**B**	**C**	**D**
UAE	35.84	25.08	32.39	17.78
CE	29.87	19.39	28.63	6.69

Regarding Table 3, it is observed that in all solvents studied, CE led to lower protein yield / g BSY, compared to UAE. This is due to the biomass depletion, which resulted from the high treatment time (24 h) and led to a degradation of the protein content. The highest protein yield was obtained using 0.1 M NaOH solvent. CE using phosphate buffer (D) as solvent led to the lowest protein yield. It is therefore concluded that the highest selectivity for the isolation of proteins from spent yeast residues was given by NaOH 0.1 M solvent, while methanol also led to satisfactory results, being a promising solvent for the isolation of proteins from BSY.

4. Conclusions

Solid waste management is a major issue for the scientific community. The brewing industry produces large amounts of solid by-products including BSG and BSY which are rich in nutrients. The present study examined the isolation of proteins from BSG and BSY using green extraction methods (UAE, EAE). It was clearly demonstrated that UAE and EAE could successfully replace the CE for BSG and BSY protein extraction. Regarding CE, the highest BSG protein yield was observed for 0.1 M NaOH at 1:20 solid biomass – solvent ratio. UAE using 0.1 M NaOH gives maximum protein yield, when extraction time is extended, solid – liquid ratio is low and, when ultrasonic power is maximized, due to cavitation phenomena. The optimum conditions of UAE using Ethanol/NaOH were 450W, 20 min and 1:10 solid-liquid ratio, based on the solvent's low polarity. The extraction efficiency of UAE for the Methanol/NaOH solvent is lower even compared to CE. However, a combination of conditions that needs further investigation is that of 1:10 solid-liquid ratio, on 450W for 20 min. In EAE a rapid increase in BSG protein yield was observed by increasing the extraction time from 6 to 12 h. Concerning BSY protein extraction, for all four solvents studied, UAE led to higher protein yield/ g BSY, compared to CE, based on biomass depletion. The better results were noticed in the case of 0.1 M NaOH solvent. In general, the use of solvent Methanol/1 M NaOH could be further studied since the protein yield of BSG in the CE and of BSY in the UAE, were higher than of them in which Ethanol/ 1M NaOH used.

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