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Advancing Protein Recovery from Brewery Spent Grain Using Enzyme-Assisted Extraction

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Abstract

Brewers' spent (BSG) grain is the main side stream product of beer production and it is a promising source of proteins and fibre fractions for further food applications. The growing focus on sustainable food production is increasingly highlighting the potential of agri-food by-products, especially BSG, to be reused as a valuable source of high-value ingredients like proteins. This study was performed to analyse enzyme-assisted methods for producing protein concentrates from BSG, focusing on recovery efficiency and functional quality. In the research, dark beer (dark lager) BSG with protein content 30.83 ± 0.25 g 100g^{-1} (DW) were used. Enzymatic hydrolysis was performed using proteases (neutral and acidic) and carbohydrases (cellulase, β -glucanase, xylanase, and α -amylase) individually and in various combinations. Ultrasound pre-treatment was used for enzymatic accessibility, while further optimisation of hydrolysis time and enzyme dosage enhanced yields. The resulting protein concentrates contained different molecular weight fractions (14–40 kDa), typical of hordein and glutelin. The extracted proteins showed high water (5.37 ± 0.91 g g^{-1}) and oil holding capacity (6.82 ± 0.47 g g^{-1}). Foaming ability and foam stability were moderate, but still sufficient for application into food systems. Both soluble and insoluble fractions were obtained in meaningful amounts, suggesting their suitability for functional applications in food.

Keywords: brewers' spent grain, protein recovery, side stream, enzyme-assisted extraction, concentrate.

1. Introduction

Brewer's spent grain is the main by-product of beer manufacturing, and it accounts for nearly 85% of the total solid residues and side streams that are being generated during the brewing process [1]. Globally, the annual volume of brewers' spent grain production is estimated to exceed more than 40 million tons, which positions them as one of the biggest foods and agro-processing side streams in the food sector [2].

The BSG (brewer's spent grain) primarily consists of barley husks, pericarp and residual endosperm, which remains after the step when the wort is extracted. The mass contains large quantities of valuable nutrients such as proteins (15–30%), dietary fibre (50–70%), lipids (2–8%) and phenolic compounds [3]. Still, despite BSG containing these valuable materials, its use is limited due to the low-value applications, because it's used as a low-cost animal feed and for composting; therefore, it's challenging to explore its potential in the food sector.

In contemporary society, there is an increasing tendency of circular bioeconomy principles and sustainable food production systems and it's one of the reasons for the increased interest in BSG valorisation into high-value ingredients and food products. Additionally, plant-based proteins and dietary fibre fractions are appealing to modern consumers because they can serve as functional ingredients for the development of alternative proteins, nutraceuticals and functional foods [4]. However, the efficient recovery of these compounds is still challenging and hardly achievable due to the BSG's complex lignocellulosic matrix, which restricts protein solubilization and limits accessibility to bioactive compounds. The proteins (mostly hordein and glutelin) are encapsulated in this matrix of hemicellulose, cellulose and lignin, which creates a strong physical and chemical barrier that reduces the extraction outcome/efficiency [5].

Conventional extraction methods often rely on alkaline or/and acid hydrolysis, which can be effective but are associated with chemical waste generation, partial denaturation of proteins. In contrast, the enzyme-assisted extraction has shown promising results which at the same time is

green. So, by using specific hydrolytic enzymes such as proteases, cellulases and xylanases to degrade the polysaccharide cell wall and release trapped proteins. [6]. These chemical processes are mild, environmentally friendly and can improve protein recovery yield at the same time preserving functional properties [7]. Previous studies have shown that combined enzyme mixes (particularly protease and carbohydrase mixes) improve protein solubility by breaking hydrogen and glycosidic linkages between protein and fibre components [8].

Moreover, physical pre-treatments have been reported to further enhance protein recovery from plant-based matrices. Ultrasound cavitation disrupts the lignocellulosic structure, increases mass transfer, and improves enzyme accessibility, thereby reducing enzyme dosage and processing time while increasing extraction yields. Also, the integration of ultrasounds perfectly aligns with sustainable and green processing goals by minimising chemical input and promoting energy efficiency [9,10].

Building on the previous research that analysed the compositional variability of brewers' spent grain (BSG) from Latvian breweries of different production scales and beer types [11], this study advances to the next stage of BSG utilisation by focusing on protein recovery. Although interest in enzyme-assisted extraction has increased, optimised extraction strategies and comprehensive understanding of the functional properties of the recovered proteins remain limited.

Therefore, the aim of the present study is to investigate enzyme- and ultrasound-assisted protein extraction from BSG using different enzymes, including neutral and acidic proteases and carbohydrases, applied individually and in various combinations. The study further intended to evaluate chemical composition and functional properties of the resulting protein concentrates and to assess their suitability for food production, including their water and oil holding capacity and foaming ability.

2. Materials and Methods

2.1 Standards and reagents

The list of reagents used in the present study is summarised in Table 1.

Table 1. List of materials and kits used in the study

Materials, chemicals, kits	Producer	Country
Protein 230 Sample buffer	Agilent Technologies	
Lithium dodecyl sulphate 1-5% (w/w) CAS Number 2044-56-6	Manufacturing GmbH&Co. KG	Germany
Protein 230 ladder Glycerol 7-137% (w/w) CAS number 77-86-1	Agilent Technologies Manufacturing	Germany
Lithium dodecyl sulphate 0.1-17% (w/w) CAS number 2044-56-6	GmbH&Co. KG	
Protein 230 Dye concentrate Dimethyl sulfoxide 80-100% (w/w) CAS number 67-68-5	Agilent Technologies Manufacturing GmbH&Co. KG	Germany
Protein 230 gel matrix	Agilent Technologies	
Trometamol 0.50-1.50% (w/w). CAS number 77- 86-1	Manufacturing GmbH&Co. KG	Germany
Kjeltab tablets Cu/3.5 (3.5g K ₂ SO ₄ , 0.4g CuSO ₄)	Sigma-Aldrich	Germany
Concentrated sulfuric acid, H ₂ SO ₄ , CAS number 7664-93-9	Chempur	Poland
Hydrogen peroxide H ₂ O ₂ (30% concentration) CAS number 7722-84-1	Sigma-Aldrich	Germany
Boric acid H ₃ BO ₃ CAS number 10043-35-3	Chempur	Poland
Sodium hydroxide NaOH CAS number 1310-73-2	Sigma-Aldrich	Germany
Hydrochloric acid HCL CAS number 7647-01-0	Sigma-Aldrich	Germany

Experimental work (Table 2) included the extraction of protein concentrates from brewer's spent grain (BSG) and the evaluation of its chemical and functional properties. The following analyses were performed: determination of crude protein after hydrolysis, moisture, water and oil-

holding capacity, foaming capacity, foam stability, colour and molecular weight of protein concentrates.

Table 2. Experimental work design

Stage	Experimental work
I	Protein recovery from BSG sample using ultrasound-assisted enzymatic hydrolysis. Testing of six enzymes and two enzyme mixtures. Selection of the three most effective enzymes based on Kjeldahl analysis of the pellet.
II	Process optimisation through increased enzyme concentration and hydrolysis time. Final extraction of protein concentrate.
III	Functional and compositional analysis of the protein concentrate powders (colour, solubility, foaming, water- and oil-holding capacity). Amino acid profile determination.

In the present research, dark malt BSG obtained from Latvian brewery (crude protein 30.83 ± 0.25 g 100g^{-1} DW, total fibre $63.61\% \pm 1.33$ g 100g^{-1} DW, fats $(5.43 \pm 0.06\%)$ DW, 82% moisture content) was used for protein extraction. A commercial soy protein isolate (provided by the wholesaler DION) with the following chemical composition: fats 1 g 100g^{-1} , carbohydrates 3 g 100g^{-1} , dietary fibres 0.3 g 100g^{-1} , protein 88 g 100g^{-1} , was used as a control sample for comparison with the extracted BSG protein concentrates.

The research was conducted in several stages to develop an optimised enzymatic extraction method for receiving protein concentrate from BSG.

2.3. Description of used commercial enzymes

The study was performed by using six different commercial enzymes provided by the company Novozymes A/S. Finizym (β -glucanase, endo-1,3(4)-), Neutrase (neutral protease), ProEx (acidic protease), Shearzyme (xylanase, endo-1,4-), Thermamyl (α -amylase), and Ultimase (cellulase). The main characteristics of the enzymes used in the research are summarised in Table 3. Neutral and acidic proteases were chosen to break peptide bonds and directly release proteins, while carbohydrases (cellulase, xylanase and β -Glucanase focus on the lignocellulosic matrix: cellulose, hemicellulose, β -glucans, because the lignocellulosic matrix captures proteins in BSG. α -Amylase was included in the list of used enzymes to remove residual starch that can interfere with mass transfer and protein solubilization. Two mixed enzyme systems were used to combine synergy between proteolysis and cell-wall systems: Neutrase (neutral protease) + Ultimase (cellulase) and ProEx (acidic protease) + Shearzyme (xylanase). Both these systems increase porosity and accessibility of the matrix, which improves protease access to peptide bonds.

Table 3. Description of enzymes

Enzyme type	Optimal Temperature ($^{\circ}\text{C}$)	Optimal pH	Main Activity
Protease neutral	45-50	6.0-6.5	0.8 U mL^{-1}
Protease acidic	40	3.5-4.5	0.8 U mL^{-1}
α -Amylase	85-88	5.6-6.0	24,000 U mL^{-1}
Xylanase (endo-1,4-)	60	5.5	100 U mL^{-1}
β -Glucanase (endo-1,3(4)-)	65	5.0	25 U mL^{-1}
Cellulase	50	5.0	120 U mL^{-1}

The enzymes were stored according to the manufacturer's recommendations and used within their activity-stable period. Enzymatic dosages were optimised based on preliminary trials. The following enzyme concentrations were applied (to 100g of native BSG moisture):

- Neutral protease (Neutrase): 0.987 mL 100g^{-1} BSG
- Acidic protease (ProEx): 0.0346 mL 100g^{-1} BSG
- α -Amylase (Thermamyl): 0.0420 mL 100g^{-1} BSG
- Xylanase (Shearzyme): 0.0923 mL 100g^{-1} BSG
- β -Glucanase (Finizym): 0.0923 mL 100g^{-1} BSG
- Cellulase (Ultimase): 0.3462 mL 100g^{-1} BSG
- Neutral protease (0.987 mL 100g^{-1} BSG) + Cellulase (0.3462 mL 100g^{-1} BSG) (Mix 1)

- Acidic protease (0.0346 mL 100g⁻¹ BSG) + Xylanase (0.0923 mL 100g⁻¹ BSG) (Mix 2)

The deionized water was used in all solution preparations.

2.4. Protein recovery and evaluation of enzyme efficiency

2.4.1 Enzymatic hydrolysis

Precisely 50 g of wet BSG (moisture content 82 ± 3 %) were mixed with 150 mL of distilled water (1:3 w/v ratio) at 20 ± 1 °C and stirred for 10 minutes to obtain a uniform product mixture.

In order to improve protein extraction and enzyme accessibility, the prepared mixture underwent ultrasound pre-treatment in a Witeg WUC-D (Germany) ultrasonic bath operating at 100 W and 40 kHz for 20 minutes at room temperature (20 ± 2 °C) without heating. This pre-treatment was used to disrupt the complex lignocellulosic structure of BSG, facilitating improved enzyme penetration into the cell wall matrix [9,10].

Following ultrasound treatment, six individual enzymes and two enzyme combinations were tested: Finizym (β -glucanase, endo-1,3(4)-), Neutrase (neutral protease), ProEx (acidic protease), Shearzyme (xylanase, endo-1,4-), Thermamyl (α -amylase), and Ultimase (cellulase). The enzyme mixtures included Neutrase + Ultimase and ProEx + Finizym.

Enzymatic hydrolysis was performed using a magnetic stirrer with a hotplate (Witeg, Germany) at a constant speed of 575 rpm. Each sample was hydrolysed for 2 hours at the enzyme's optimal temperature (ranging from 45 °C to 80 °C, Table 2) under continuous stirring. Reaction pH values were adjusted to each enzyme's recommended range using 0.1 M sodium hydroxide (NaOH) and/or 0.1 M hydrochloric acid HCl. Hydrolysis pH was set to the manufacturer's recommended value and checked every 30 minutes with readjustment as required: neutral protease pH 6.0–6.5 (45–50 °C), acidic protease pH 3.5–4.5 (40 °C), α -amylase pH 5.6–6.0 (85–88 °C), xylanase pH 5.5 (60 °C), β -glucanase pH 5.0 (65 °C), cellulase pH 5.0 (50 °C).

After hydrolysis, enzyme activity was stopped by heating the mixtures to 90 °C for 10 minutes. The samples were immediately cooled to room temperature and centrifuged at 3000 rpm ($\approx 2300 \times g$) for 12 minutes using a CM-6MT centrifuge (Latvia). The supernatant containing solubilised proteins was separated from the pellet, which contained the unhydrolyzed residue.

All hydrolysis treatments were performed in triplicate to ensure data reproducibility.

2.4.2. Protein recovery determination

The determination of protein content was carried out using a Kjeltac™ 2100 System (Foss, Denmark) in accordance with the AACC Method 46-20 [12] protocol. During the analysis, samples were digested with concentrated sulfuric acid, which oxidised carbon to carbon dioxide and converted nitrogen compounds to ammonium ions. These ions were transformed into ammonium sulphate through reaction with sulfuric acid.

The crude protein content was calculated by multiplying the nitrogen percentage by a conversion factor of 6.25 (1).

Protein recovery efficiency was expressed as a percentage relative to the initial protein content in untreated BSG, using the formula (2):

$$(1). \%Protein = Nitrogen(\%) * F;$$

Where F is the conversion factor of 6.25

The protein recovery efficiency was expressed as a percentage relative to the initial protein content in untreated BSG, using the formula:

$$(2). \frac{Protein\ recovered}{Total\ protein} \times 100\%.$$

All analyses were performed in triplicate, and the results were reported as mean values $\pm$ standard deviation.

2.5. Functional and compositional analysis of the protein concentrate powders

2.5.1. Water and Oil Holding Capacity

The capacities for binding water and oil were determined based on the method by Mirmoghtadaie et al. (2009), with slight modifications [13], as described [14] by Sargautis, Kince (2023).

To assess these properties, 1.00 g of each obtained protein powder sample was dispersed in 10.0g refined deodorised sunflower oil or 10.0g ultra-pure water. The mixtures were stirred for one minute using a vortex mixer (VXHDDG, Ohaus, USA) at 25 ± 2 °C at speeds of 1200 rpm (for water) and 2500 rpm (for oil), followed by a 30-minute resting phase. During this time, the mixtures were intermittently agitated every 10 minutes for 10 seconds. Afterwards, the dispersions were centrifuged at $3000 \times g$ for 5 minutes using a Hermle Z 206 A centrifuge (Hermle Labortechnik GmbH, Germany). The supernatant was discarded, and the retained pellet was weighed. Water and oil holding capacities were expressed as the amount of water or oil in grams retained per gram of protein concentrate, respectively. The used formula (3) is as follows:

$$(3). \frac{m_{wet\ pellet} - m_{dry\ samples}}{m_{dry\ samples}}$$

All analyses were performed in triplicate, and the results were reported as mean values $\pm$ standard deviation.

2.5.2. Foaming capacity and foam stability

To determine foaming properties, 1.0g of protein concentrate was combined with 33.0g of ultrapure water (pH 7) in a 50 ml graduated cylinder. The sample was stirred magnetically for 30 minutes and then homogenised for 5 minutes at 10000rpm using a high-shear mixer (T10 Ultra Turrax, IKA Werke GmbH & Co. KG, Germany). Foam volume was measured at multiple time points: 5, 10, 30, 60, and 120 minutes. The method calculates foaming capacity using the formula (4):

$$(4.) \text{Foaming capacity} = \frac{V_f}{V_i} \times 100;$$

Where:

V_f – volume of the foam (ml);

V_i – initial sample volume (ml).

2.5.3. Colour analysis ($L^*a^*b^*$)

Colour evaluation of the received protein concentrate was conducted by using a Colour Tec-PCM/PSM colourimeter and ColorSoft QCW software (Accuracy Microsensors Inc., Pittsford, NY, USA). Colour differences were quantified according to the CIE Lab* colour space. The total colour difference (ΔE) was calculated using the equation (5) below, based on methods CIE [15] (2018). The colour differences were calculated using the equation below :

$$(5). \Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2};$$

Where:

ΔE – overall colour difference;

L^*, a^*, b^* – colour coordinates of the sample;

L_0^*, a_0^*, b_0^* – colour coordinates of the original material.

All measurements were performed in triplicate, and results are reported as mean $\pm$ standard deviation. Soy protein isolate was used as the reference sample for ΔE calculation.

2.5.4. Molecular weight determination of protein

The molecular weight of protein fractions was determined using SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis). In this method, proteins were denatured and separated by electrophoresis based on their molecular weight. As the first step, all samples were solubilised by using 6M urea and 2% SDS buffer and after diluted to 4 mg mL⁻¹ (scales TE-124S-OCE, Sartorius AG, Gottingen, Germany). Following this, they were loaded onto polyacrylamide gels and subjected to an electric field, which allowed proteins to migrate based on size; smaller proteins moved further than larger ones. After separation, the gels were stained (with Coomassie Brilliant Blue) to visualise the protein bands. The molecular weight of the proteins was estimated by comparing the migration distance of sample bands to that of a molecular weight standard marker (Sargautis, Kincaid, 2023).

2.6. Statistical analysis

Statistical evaluation was carried out using JASP Team (2025), JASP 0.95.4 (software). One-way analysis of variance (ANOVA) was used to identify significant differences among treatments, and Tukey's honestly significant difference (HSD) test was used for post hoc comparisons. Normality (Shapiro-Wilk) and homoscedasticity (Levene) were verified. Statistical significance was considered at $p < 0.05$ where different letters indicate significant differences.

2.7. Human and animal rights statement

This article does not contain any studies involving human participants or animals performed by any of the authors.

3. Results

3.1. Evaluation of enzymatic hydrolysis efficiency (1st stage)

The first stage of the study was performed to analyse the effectiveness of different enzyme treatments in recovering proteins from the selected brewer's spent grain sample. A total of eight enzymatic treatments were tested (including 2 mixes). Each one was applied to ultrasound pre-treatment before the hydrolysis. Six individual enzymes and 2 combinations were tested: neutral protease (Neutrase), acidic protease (ProEx), α -amylase (Thermamyl), xylanase (Shearzyme), β -glucanase (Finizym), cellulase (Ultimase), and two combinations — ProEx + Finizym and Neutrase + Ultimase.

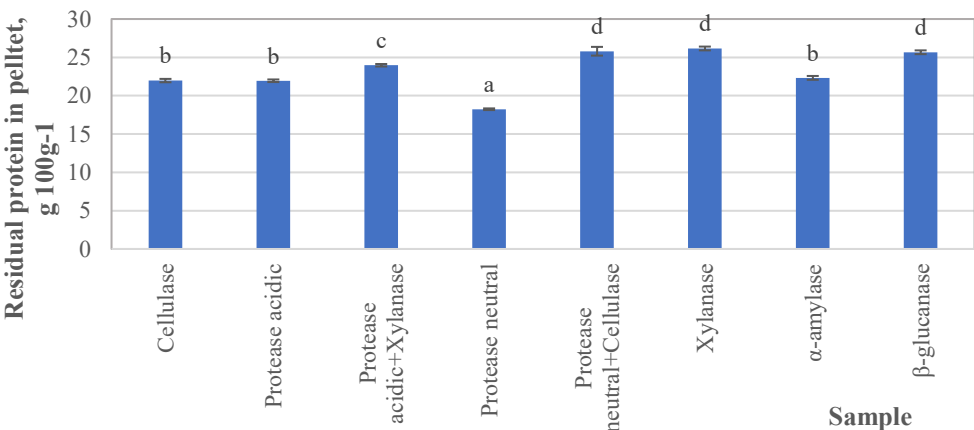


Fig. 1. Residual protein content in BSG pellets after ultrasound-assisted enzymatic hydrolysis. Values are expressed as g 100 g⁻¹ DW and shown as mean ± standard deviation (n = 3). Different lowercase letters indicate significant differences among treatments according to Tukey's HSD test (p < 0.05)

As shown in Figure 1, residual protein content differed significantly among the enzymatic treatments. The residual values ranged from 18.15 to 26.20 g 100g⁻¹, with the lowest content in the individual Protease (Neutral) treatment (18.23 ± 0.1 g 100g⁻¹), which was the most effective at this stage, as this works within neutral pH, similar to BSG slurry, followed by Protease (acidic) (21.94 ± 0.16 g 100g⁻¹), closely followed by the cellulase (21.96 ± 0.21 g 100g⁻¹). Less effective was treatment with xylanase, which achieved (26.15 ± 0.25g 100g⁻¹). Figure 2 shows that the sole protease performs best. Carbohydrases alone (xylanase, β-glucanase) mostly affect hemicellulose and β-glucan network/complex, and because of that, there should be an additional mechanism in order to cleave the peptide bonds, e.g. mix of carbohydrase and protease by Chin et al. [8].

Based on previous findings reported by Li et al. [9], ultrasound pre-treatment has been shown to enhance enzyme-assisted hydrolysis by increasing cell wall porosity and reducing particle aggregation. The cavitation effect generated during ultrasound processing can partially disrupt the lignocellulosic matrix of BSG, thereby improving mass transfer during hydrolysis. In the present study, ultrasound was applied as a processing step following these established principles to support enzymatic extraction, rather than to quantitatively compare its individual contribution.

3.2. Hydrolysis optimisation (2nd stage)

After the identification of neutral protease as the most effective enzyme in Stage 1, additional optimisation was performed to enhance the enzyme efficiency by increasing the enzyme dosage and extending the hydrolysis time for protein recovery. The 2nd stage focused on identifying the enzymatic treatment that resulted in even lower residual protein content in the BSG pellet (as an indicator of overall hydrolysis efficiency).

Three enzyme treatments were compared: Protease (neutral), Protease (acidic), and a combination of Protease (neutral) + Cellulase. Each treatment was applied under optimised conditions determined from preliminary tests, including an increased enzyme concentration (×10) and extended hydrolysis time (4 hours).

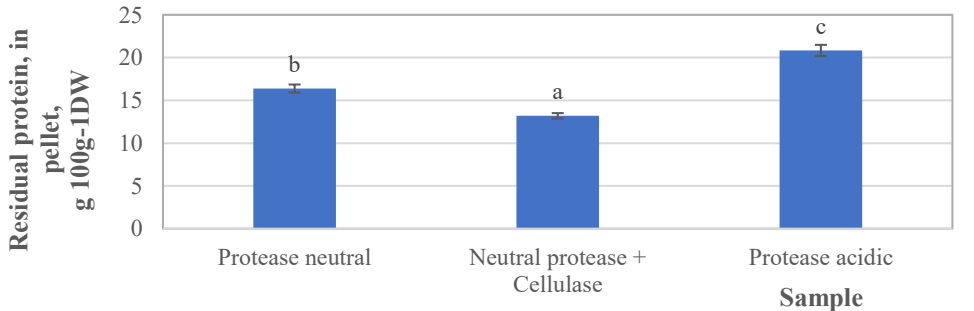


Fig. 2. Residual protein content (g 100 g⁻¹ DW) in BSG after enzymatic hydrolysis adjustment. Different letters indicate significant differences (Tukey HSD, p < 0.05, n=3) between treatments

The lowest residual content (Fig. 2) was observed for the mix of neutral protease and cellulase (13.19 ± 0.22%), followed by neutral protease alone (16.38 ± 0.33%) and acidic protease (20.36 ± 0.2%)

0.47%). The efficiency improved after combining proteolytic and cell-wall degrading enzymes, which helped to achieve more complete hydrolysis of the BSG matrix.

Based on the Chin et al. [8] observations, the synergistic interaction between neutral protease and cellulase likely increased the degradation of both protein and polysaccharide link and structural carbohydrates, which resulted in improved enzyme access to the remaining protein fraction. So, protease-cellulase combined treatment improves protein yields from plant residues.

Protein recovery increased to approximately 57.2% following enzymatic treatment, consistent with previous studies reporting enhanced solubilisation with higher enzyme loading and extended hydrolysis. This improvement is consistent with the findings of Connolly et al. [5]. In their research, the increasing enzyme concentration and prolonged hydrolysis duration improved solubilisation and protein release from brewers' spent grain. The higher residual protein observed with acidic protease is likely due to suboptimal pH conditions caused by the buffering capacity of BSG, which can suppress acidic protease activity. In contrast, neutral protease showed better compatibility with the BSG matrix, particularly when combined with cellulase, which disrupts the lignocellulosic structure and improves protein accessibility [9]. Similar synergistic effects between cellulolytic enzymes and proteases have been reported in the literature [6]. Overall, the combination of neutral protease and cellulase proved most effective, indicating that enzyme selection and process optimisation offer a scalable and chemical-free strategy for improving protein extraction from BSG.

3.3. Functional and compositional characterisation of the obtained protein concentrates (3rd stage)

The final stage of this study was focused on evaluating the functional properties of the protein concentrate, which was obtained after enzymatic hydrolysis optimisation. The goal was to analyse and assess the technological potential of BSG protein concentrate for use in the food sector. The following analyses were performed: water- and oil-holding capacity, foaming capacity and stability, colour parameters (Lab), and molecular weight distribution*, with soy protein isolate (SPI) used as a control reference.

3.3.1. Water and Oil Holding Capacity.

The ability of proteins to retain water and oil is a crucial indicator of their potential suitability for application in the food sector, especially if the target group are baked products.

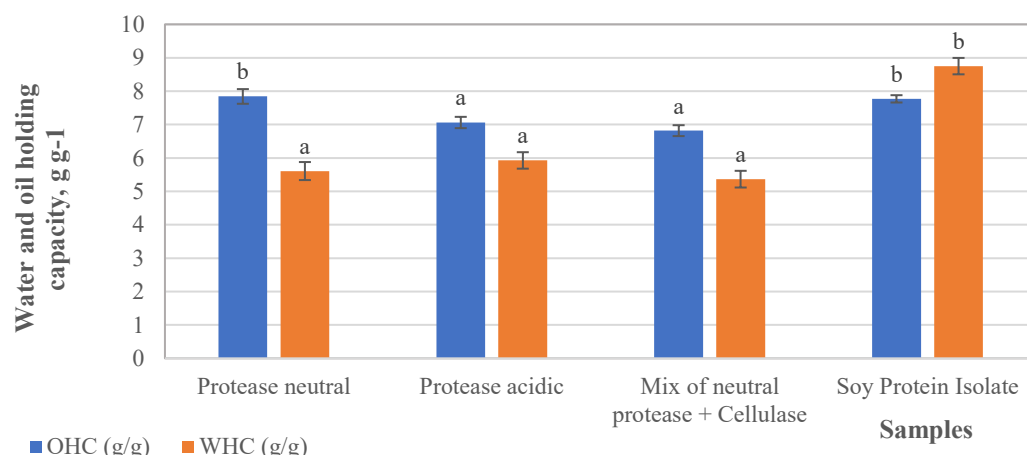


Fig. 3. Water- and oil-holding capacity of BSG protein concentrates obtained after different enzymatic treatments compared with soy protein isolate. Values are expressed as g g⁻¹ and shown as mean ± standard deviation (n = 3). Different lowercase letters indicate significant differences among samples within the same parameter according to Tukey's HSD test (p < 0.05). Letters are assigned from the lowest to the highest mean value

The protein concentrate obtained from the *Neutrase* treatment showed the highest OHC values among all 3 samples, which shows that it has hydration and lipid-binding potential, followed by the Protease (acidic) and the mix of the Protease (neutral) and Cellulase treatments (Fig 3). In contrast, the highest WHC was observed from the acidic protease treatment. This result might be because of the partial hydrolysis and exposure of hydrophilic and hydrophobic sites on the protein surface. In a similar study but with protein concentrate from cereal and legume by-products, the increase in WHC and OHC after enzymatic treatment has been reported [16,17]. In contrast, the samples treated with the neutral protease + cellulase mixture displayed less defined protein bands (presented below) and the appearance of very low molecular weight fragments (~7 kDa), suggesting more

extensive degradation. This higher degree of hydrolysis may explain the reduced WHC observed for this treatment, as excessive breakdown can limit the formation of a protein network capable of effective water retention.

Among the BSG-derived protein extracts, the sample treated with Protease (acidic) showed the highest WHC ($5.93 \pm 0.45 \text{ g g}^{-1}$), followed by Protease (neutral) (5.61 g g^{-1}), while the Protease neutral + Cellulase mix had the lowest ($5.37 \pm 0.21 \text{ g g}^{-1}$). In terms of OHC, Protease (neutral)-treated extract outperformed the other BSG samples, reaching $7.84 \pm 0.98 \text{ g g}^{-1}$, slightly higher than the soy protein isolate ($7.77 \pm 0.87 \text{ g g}^{-1}$). The increased WHC shows improved solubility and swelling of extracted proteins, while the increased OHC shows an increase in surface-active amino acids that can interact with non-polar molecules.

3.3.2. Foaming capacity and stability

Foaming capacity and stability were analysed as indicators of protein surface activity and structural flexibility and were evaluated at 5, 10, 30 and 60 min in comparison Soy protein isolate. In addition to this, foam stability indicates the ability of proteins to stabilise the air-water interface.

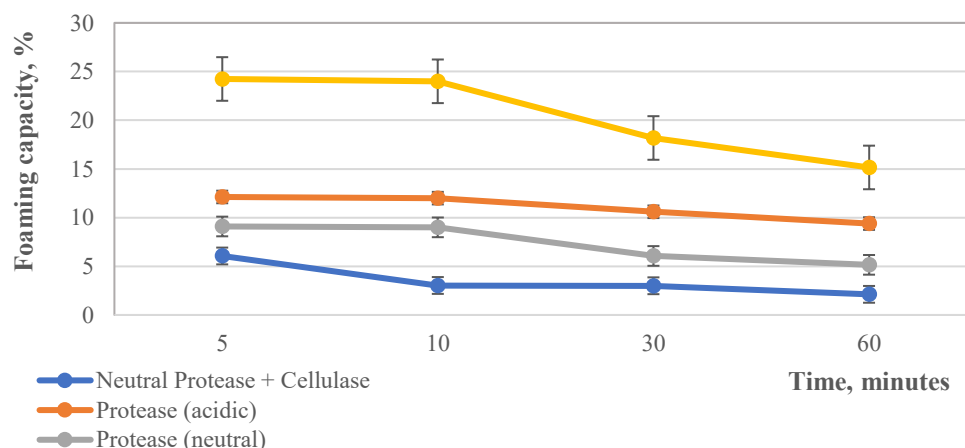


Fig. 4. Protein concentrates' foaming capacity after different enzymatic treatments compared with soy protein isolate over 60 min. Values are expressed as percentage foam volume and are shown as mean $\pm$ standard deviation ($n = 3$)

Across analysed samples, soy protein isolate showed the highest foaming stability and moderate foam stability. This result was followed by the acidic protease (12.12%). This means that there was a partial denaturation and hydrolysis of higher kDa proteins, and this improved solubility. The foaming capacity (Figure 4) of the protein concentrates from a mix of neutral protease and cellulase reached 6.06% after 5 minutes, showing moderate surface activity (Figure 4). Although its initial foam volume was the lowest of the three BSG treatments, the mixed treatment held foam slightly better at later time points. Li et al. [9] noted that smaller peptides and hydrolysed proteins form a lighter, less cohesive foam structure that collapses quickly over time. Still, the overall functionality remained stable in comparison to commercial proteins.

3.3.3. Colour properties ($L^*a^*b^*$)

The BSG protein concentrate was slightly darker and more yellowish compared to the control sample – soy protein isolate (lower L^* , higher b^*). Because BSG is rich in polyphenolic compounds, and even after drying and enzymatic processing, there were some residual polyphenolic compounds. Additionally, there were Maillard reaction products that were formed during drying and enzymatic processing.

Table 4. Colour of analysed protein concentrate samples

Sample	L^* (Lightness)	a^* (Red/Green)	b^* (Yellow/Blue)
Neutral protease	$81.91 \pm 0.41c$	$4.10 \pm 0.14a$	$19.51 \pm 0.37b$
Mix neutral protease and cellulase	$74.14 \pm 0.32b$	$5.80 \pm 0.12c$	$20.28 \pm 0.24c$
Acidic protease	$70.60 \pm 0.31a$	$6.12 \pm 0.21d$	$23.00 \pm 0.34d$
Soy protein isolate	$89.90 \pm 0.02d$	$4.30 \pm 0.11b$	$18.80 \pm 0.38a$

Different letters within the same column indicate significant differences across samples according to Tukey's HSD test ($p < 0.05$). Letters are assigned from the lowest to the highest mean value.

Mix neutral protease and cellulase, and especially acidic protease, yielded darker products (lower L^*), with higher redness (a^*) and yellowness (b^*) values. Based on the obtained results, lower L^* value is less suitable for light-coloured applications, such as beverages and alternative dairy products. In case of using this protein concentrate in a darker food system, this product will be more acceptable due to the base colour masking effect. This is likely due to increased exposure of amino groups and reducing sugars during ultrasound and prolonged enzymatic hydrolysis, which can enhance Maillard-type reactions even at moderate temperatures. These findings are consistent with Li W et al. [9], who reported similar effects in BSG protein extraction, where ultrasound and heating influenced colour due to pigment interactions and non-enzymatic browning.

3.3.4. Molecular weight of obtained BSG

The molecular weight of the extracted protein fractions was analysed by SDS-PAGE, and the range was between 15 and 70 kDa. The Neutrase sample showed a greater range of bands with lower molecular weight (25–40 kDa), which showed that there was partial hydrolysis with the subsequent formation of smaller peptides. These bands may correspond to β -hordeins, known for their higher molecular mass and contribution to protein functionality such as foaming or emulsifying properties. Sample 1 was used as control, untreated BSG powder.

In contrast, samples treated with a mix of neutral protease and cellulase showed markedly less defined banding patterns, characterised by weaker intensity of middle molecular weight bands at the range of 7–45 kDa and the prominent appearance of low molecular weight fragment below 7 kDa fractions suggests a higher degree of protein degradation, indicating greater protein degradation, potentially due to over-hydrolysis or low extraction efficiency of higher molecular weight proteins.

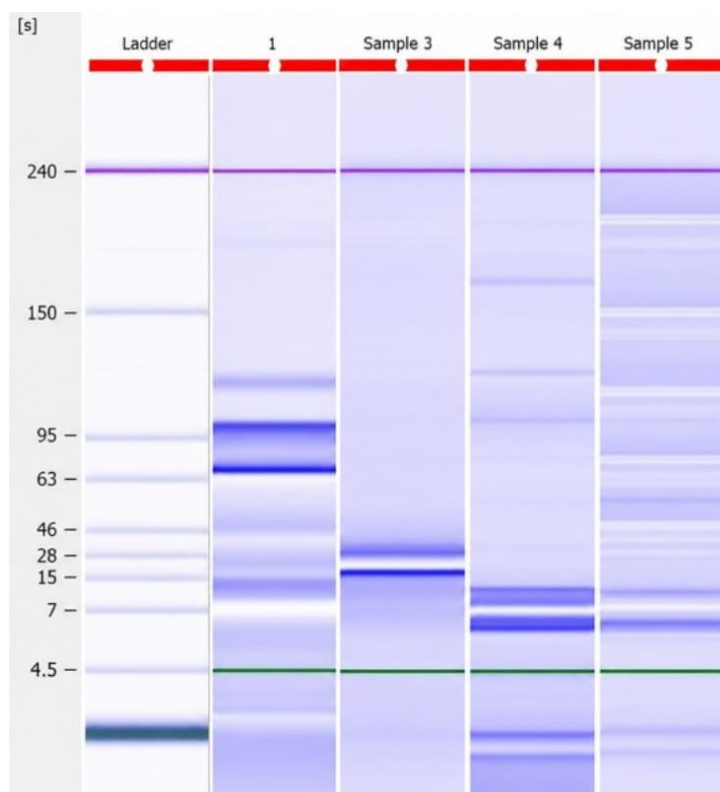


Fig 5. SDS-PAGE image of obtained protein powder (Column 1 – control, untreated BSG powder, Neutral protease sample 3, acidic protease sample 4, mix neutral protease and cellulase sample 5, Ladder – molecular weight marker.)

The presence of low molecular weight fragments (~14–17 kDa) the response to specific enzymatic actions of enzymatic hydrolysis of BSG proteins reported by Celus et al [18].

4. Discussion

This study showed that brewers' spent grain can be used as a promising source of alternative protein concentrate when it is processed through enzyme-assisted extraction/hydrolysis. Based on the chemical composition of 11 BSG samples from different Latvian breweries, one sample with the highest amount of proteins (30.8 ± 0.25 g 100 g⁻¹ DM) protein content was chosen for further

research. This protein content is within the upper range reported for BG protein content (20-32%). The difference in malt type, mashing regime affects the spent grain composition and subsequently the protein content.

The first stage of this study was focused on the enzymatic hydrolysis potential for improving protein extraction yield from brewers' spent grain. Among 6 individual enzymes and 2 combinations. Neutral protease (Neutrase) showed the highest efficiency individually and in combination with cellulase (Ultimase), which confirms that proteolytic enzymes that work within a neutral pH range and mild temperature can effectively solubilise proteins from BSG.

In comparison, acidic protease (ProEx) showed lower extraction efficiency, which can be associated with the lower pH optimum range for the enzyme activation (3.5-4.5), which isn't fully compatible with the buffering capacity of the BSG.

In the 2-stage, by increasing enzyme dosage and hydrolysis duration, the new milestone was achieved. This led to a further reduction in residual protein content from 18.2% to 13.2%, which has led to an estimated protein recovery of approximately 58%. These findings are similar to research from Forssell [7] and Chin [8], who also focused on optimising reaction time and enzyme dosage in order to maximise yield and try to maintain economic feasibility. Based on the results from the combination of Neutrase and Cellulase, we can suggest that higher enzyme availability accelerates substrate (BSG) breakdown and helps the enzyme to work deeper into the fibrous structure. This is known as cascade bioprocessing, where the enzyme targets different substrate fractions to achieve better yield.

Among the single enzymes, neutral protease (Neutrase) showed the highest efficiency in reducing residual protein, which supports findings by Connolly et al [5], who performed several experiments where they demonstrated the suitability of neutral proteases for protein recovery from barley-derived by-products at mild pH and temperature conditions. The combination of neutral protease and cellulase also performed well and showed that potential synergy between proteolytic and carbohydrases improves the release of proteins from the plant matrix.

The enzyme combination with β -glucanase also showed promising results, following what was achieved with studies by Forssell et al. [7], who reported that β -glucanase can weaken the cell wall structure and improve protein solubility in cereal by-products. This supports the concept that multiple enzyme classes act better to maximise protein yield. Future work will focus on enzyme (protease-carbohydrase) ratio and dosing optimisation in order to maximise protein recovery and functional performance for techno-functional characteristics.

In contrast, acidic protease (ProEx) demonstrated lower hydrolysis efficiency. This also corresponds with results by Celus et al. [18], who found that acidic proteases were less effective for BSG hydrolysis under mild hydrolysis conditions compared to neutral and alkaline proteases. The lower results, even with the enzyme combinations, suggest that not all enzyme combinations work synergistically, so enzyme compatibility and substrate specificity play a critical role in overall hydrolysis performance.

The obtained protein concentrates recovery values out of the supernatant correspond well with the data reported in the literature, where these results typically range between 45-65% protein yield depending on process intensity [6]. These data confirmed that neutral protease is the most effective single enzyme for protein recovery from BSG in this study.

The critical finding of the optimisation process is that the observed improvements can be achieved without using chemical solubilisation or other extreme process conditions, and this is crucial for the circular bioeconomy.

As for the functional characterisation of the protein concentrate, this study showed promising results in water and oil holding capacities, moderate foaming capacity and color parameters in comparison to the control sample – soy protein isolate. Again, better results were in the Neutrase+Cellulase combination, and this is due to the partial hydrolysis that has affected polar and nonpolar residues; therefore, this helped to improve water binding and emulsification capabilities.

Although foaming stability was slightly lower than in the control sample (soy protein isolate), the foaming capacity was competitive. Partial hydrolysis reduces molecular size and increases the ability of proteins to migrate and stabilise air-water interfaces. At the same time, this can affect long-term foam stability in a negative way [19].

The SDS-PAGE analysis confirmed that enzymatically extracted proteins ranged from 14kDa to 45kDa, which corresponds to major BSG proteins such as hordein and glutelin. Stronger bands were noticed within the range of 30-40kDa in samples that underwent neutral and acidic proteases. This shows that these medium-weight protein fractions were partially hydrolysed, which aligns with the previous information about improved solubility and emulsifying properties.

The main question is about valorisation potential. From a sustainable perspective, the present study supports the use of enzyme-assisted extraction as an environmentally friendly and scalable method for using BSG and converting it into protein ingredients/other functional food ingredients. By avoiding harsh chemicals, using optimised conditions for enzymes, it's possible to work within

the framework of circular bioeconomy, as breweries generate large volumes of spent grain (up to 85%) of total by-products, which are used mostly as a low-value animal feed or are just being discarded. With the implementation of enzyme-assisted extraction, the food producers can diversify their product portfolio, reduce waste.

Moreover, the demonstrated functionality of BSG's protein concentrate is opening the door for its incorporation as an emulsifier's functional food ingredient. That's why future studies should focus on the process scale-up.

5. Conclusions

The present study demonstrates that brewers' spent grain (BSG) from Latvian breweries represents a valuable and yet underutilised source of alternative and functional plant proteins. The results showed that enzyme-assisted extraction, particularly neutral protease and cellulase, significantly improved protein recovery, especially when combined with ultrasound pre-treatment.

Optimisation through increased enzyme concentration and extended hydrolysis time further improved extraction efficiency, reducing residual protein content to as low as 13.19%. The protein concentrates obtained showed different functional properties, including high water- and oil-holding capacities and moderate foaming behaviour, which were comparable or superior to soy protein isolate. SDS-PAGE analysis confirmed the presence of protein fractions within the 14–40 kDa range, corresponding to major BSG storage proteins such as hordein and glutelin.

Overall, the findings demonstrate that BSG protein concentrates can be effectively produced using eco-friendly, enzyme-assisted processes. Future research should focus on scaling up the process, assessing sensory properties, and evaluating functional stability in real food matrices to facilitate industrial application and consumer acceptance.

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