Innovative Food Science and Emerging Technologies 94 (2024) 103705

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

Sequential extraction optimization of compounds of interest from spent brewer's yeast biomass treated by Pulsed Electric Fields

Alejandro Berzosa, Javier Marín-Sánchez, Carlota Delso, Jorge Sanz, Ignacio Álvarez, Cristina Sánchez-Gimeno, Javier Raso *

Food Technology, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2, (Universidad de Zaragoza-CITA), Zaragoza, Spain

ARTICLE INFO

Keywords:
Pulsed Electric Fields
Brewer's yeast biomass
Mannoprotein
Glutathione
Protein
Sequential extraction

ABSTRACT

The brewing industry produces significant volumes of spent brewer's yeast (SBY), which presents an intriguing opportunity for valorization. This study aims to optimize the extraction of various compounds of interest from electroporated SBY located both in the cytoplasm (amino acids, glutathione and proteins) and in the cell walls (mannoproteins). The optimization of the extraction time, temperature and pH, allowed obtaining an extract rich in glutathione of 2.31 ± 0.15 mg/g dw after 1 h of incubation (pH 8; 30 °C) and, a second extract rich in amino acids (155.74 ± 7.83 mg/g dw) and proteins (331.70 ± 15.64 mg/g dw) after a second incubation (37 °C, 47 h) of the biomass. To achieve comprehensive valorization of SBY, the exhausted yeast biomass was incubated with lyticase to extract mannoproteins from the cell wall. This study showcases the efficacy of a multiple response function in optimizing valuable compound extraction from electroporated SBY, aligning with circularity principles.

1. Introduction

Maximizing the utilization of by-products through the circular economy strategy is critical for enhancing the sustainability of the food industry (Maqbool, Khan, Haleem, & Khan, 2020). Spent brewer's yeast (SBY) stands as a significant by-product of the brewing industry. The residual yeast biomass generated per hectoliter of beer is estimated to range from 1.5 to 3 kg (85–90% moisture) (Thiago, Pedro, & Eliana, 2014). Considering the global beer production in 2022, which amounted to approximately 1.9 billion hectoliters, an estimated 2.85 to 5.70 million tons of SBY were produced. Disposing of this biomass poses a challenge for breweries, as it is commonly utilized as a protein source for animal feed, yielding no economic benefit (Jaeger, Arendt, Zannini, & Sahin, 2020; Schlabitz, Neutzling Lehn, & Volken de Souza, 2022). However, SBY represents a substantial reservoir of valuable compounds applicable not only in the food industry but also in the pharmaceutical and cosmetic sectors, thereby adding value to this waste. Several studies conducted in recent years aim to valorize this by-product (Amorim, Pinheiro, & Pintado, 2019; Kruk, Varmanen, Edelmann, Chamlagain, & Trząskowska, 2024; Oliveira et al., 2022; Soh et al., 2022; Vieira, Teixeira, & Ferreira, 2016; Zeko-Pivač, Habschied, Kulisic, Barkow, & Tišma, 2023).

Yeast extract, comprising high-quality proteins (ranging from 35% to 60% on a dry basis) containing all essential amino acids, holds remarkable potential as a nutrient source for microorganisms' growth (Ferreira, Pinho, Vieira, & Tavarela, 2010; Saksinchai, Suphantharika, & Verduyn, 2001). Furthermore, these proteins may contribute to stabilization and clarification processes (Gaspar et al., 2019). On the other hand, spent yeast retains bioactive compounds like glutathione, a tripeptide with high antioxidant capacity that has been suggested to reduce the use of sulphur dioxide in the wine industry (Giménez et al., 2023; Martínez & González-Arenzana, 2022). Additionally, in the cosmetic or food industry, it finds applications as a preservative or supplement (Li, Wei, & Chen, 2004). The yeast cell wall (constituting 15%–30% of dry weight) is also a source of valuable compounds, such as mannoproteins (making up 35%–40% of the cell wall dry weight). The literature extensively delineates the key role of mannoproteins in winemaking, underscoring their substantial impact on the organoleptic characteristics of wine (Li, Zhai, Ma, Duan, & Yi, 2023) and in ensuring stability through mechanisms like the reduction of tannin aggregation and prevention of protein precipitation, among other functions (Ribeiro, Fernandes, Nunes, Filipe-Ribeiro, & Cosme, 2014; Rodrigues, Ricardo-Da-Silva, Lucas, & Laureano, 2012). Mannoproteins also exhibit a wide range of other applications, showcasing antifungal, prebiotic, or

* Corresponding author at: Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, c/Miguel Servet, 177, 50013 Zaragoza, Spain.
E-mail address: jraso@unizar.es (J. Raso).

https://doi.org/10.1016/j.ifset.2024.103705
Received 11 March 2024; Accepted 20 May 2024
Available online 21 May 2024

even emulsifying properties (Abdolshahi et al., 2019; Diez, Guadalupe, Ayestarán, & Ruiz-Larrea, 2010).

Significant advances have been made in the extraction of compounds from SBY owing to the implementation of cutting-edge technologies. Various studies have proposed different extraction methodologies, such as bead milling, high pressure homogenization (HPH), ultrasound, autolysis, enzymatic hydrolysis or acid-alkali hydrolysis (Amorim et al., 2019; Chae, Joo, & In, 2001; Oliveira et al., 2022; Vieira, Teixeira, & Ferreira, 2016; Zeko-Pivač et al., 2023). However, the majority of these approaches focus on extracting a single product, leading to drawbacks associated with either their economic cost or the low purity of the extracts.

An effective strategy to enhance by-product recovery efficiency and develop cost-effective processes involves obtaining multiple marketable products from yeast biomass waste, rather than focusing on a singular outcome. Recently, the utilization of Pulsed Electric Fields (PEF) technology as an initial pre-treatment step for yeast biomass, within a cascade processing approach, has demonstrated promise in achieving this objective (Berzosa, Delso, Sanz, Sánchez-Gimeno, & Raso, 2023), as other authors have proposed in microalgae (Gusbeth & Frey, 2022).

PEF technology involves the application of high-intensity electric fields (kV) for brief periods (ms - μs), inducing a phenomenon known as electroporation that disrupts the selective permeability of the cell membrane (Mahnič-Kalamiza & Miklavčič, 2022). Electroporation is characterized by the formation of small pores, allowing uncontrolled movement of molecules across the cell membrane (Teissie, Golzio, & Rols, 2005). This process facilitates the release of intracellular compounds, including proteins, amino acids, small peptides, and ionic substances (Dimopoulos, Stefanou, Andreou, & Taoukis, 2018; Ganeva, Angelova, Galutzov, Goltsev, & Zhiponova, 2020). Moreover, PEF-induced electroporation has been observed to induce autolysis, resulting in the self-degradation of the cell wall by its own enzymes and the subsequent release of mannoproteins (Martínez, Cebrián, Álvarez, & Raso, 2016). Despite the application of high voltages during the process, the short duration of the treatments minimizes the energetic requirements.

The primary objective of the current study was to optimize the extraction conditions following PEF treatment of brewer's yeast biomass, aiming to maximize the extraction of diverse compounds of interest located both in the cytoplasm and the cell walls. This optimization is considered a crucial step in the revalorization of this by-product, facilitating the production of a diversified cascade of marketable products relevant to various industry sectors.

2. Materials and methods

2.1. Brewer's yeast biomass

SBY was supplied by the local brewery, "La Zaragozana" (Zaragoza, Spain). After washing twice with distilled water by centrifugation (1593 *g* for 10 min at 20 °C), yeast biomass was resuspended in citrate-phosphate McIlvaine buffer of 2 mS/cm conductivity and different pH (4.0, 6.0 or 8.0) to reach a final concentration of 3×10^8 UFC/mL (48.3 ± 5.4 g dry weight/L).

2.2. PEF processing

Yeast biomass was subjected to PEF treatment in a continuous flow chamber using a commercial PEF equipment (Vitave, Prague, Czech Republic) capable of delivering monopolar square waveform pulses up to 20 kV with adjustable pulse width ranging from 500 ns to 100 μs at a maximum current intensity of 500 A.

A peristaltic pump (BVP, Ismatec, Wertheim, Germany) was used to circulate the yeast biomass at a rate of 5 ± 0.1 L/h through a titanium parallel electrode chamber of 0.4 cm gap, 3.0 cm length and 0.5 cm width. Monopolar square waveform pulses, with a width of 3 μs, were delivered at electric field strengths of 10, 12 and 15 kV/cm, with frequencies ranging from 15.5 to 116.7 Hz, resulting in total treatment durations ranging from 20 to 150 μs, calculated by multiplying the total number of pulses applied by the pulse width. These treatments corresponded to a total specific energy ranging from 4.2 to 85.3 kJ/kg of yeast suspension. The actual voltage during the treatments was monitored using a high voltage probe (Tektronik, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronik, TDS 220). Simultaneously, the output temperatures (ranging from 21 ± 0.1 to 42.5 ± 0.1 °C) were measured using a K-type thermocouple integrated in the circuit (Ahlborn, Holzkirchen, Germany). Yeast biomass was tempered at 20 °C with a heat exchanger located before the treatment chamber and cooled down to below 20 °C in <5 s via a heat exchanger located just after the treatment chamber after the PEF treatments.

2.2.1. Evaluation of the effect of PEF on the electroporation of S. cerevisiae from brewery

The permeabilization of the cytoplasmic membrane due to the electroporation caused by the PEF treatment was evaluated by measuring the uptake of the fluorescent dye propidium iodide (PI; Sigma-Aldrich, Missouri, United States). PI is a hydrophilic molecule of low molecular weight (660 Da) that is only able to cross the cytoplasmatic membrane that has been permeabilized. Into the cytoplasm PI binds nucleic acids to form a fluorescent complex with excitation and emission peaks at 535 nm and 617 nm respectively. After PEF treatments, 50 μL of PI (0.1 mg/mL) were add to 450 μL yeast biomass suspension, resulting in a final concentration of 0.015 mM, suspensions were incubated for 10 min at room temperature, as it has been previously described by Martínez et al., (2016). Yeast biomass was previously resuspended on phosphate-buffered saline (PBS) to yield a concentration of 10^8 UFC/mL. The number of electroporated cell yeast was determine by epifluorescence microscopy (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan). Results are expressed by percentage of electroporated cells.

2.3. Bead mill treatment

The total concentration of glutathione, amino acids and proteins present into the SBY, was determined after the complete destruction of the yeast cells by bead milling (Mini-Beadbeater-Plus; BioSpec, Bartlesville, USA). 1.5 mL of the SBY was added with 0.5 mm diameter glass beads in a 1:5 weight ratio (glass bead/yeast biomass suspension) in a 2.0 mL screw-capped tube. The progress of mechanical disruption was monitored by microscopic observations (Eclipse E400, Nikon, Tokyo, Japan). Fourteen cycles, each lasting 70 s with cooling intervals in an ice water bath, were required to disintegrate >90% of the cells. Subsequently, the suspensions were centrifuged at 1593 *g* for 10 min to obtain the supernatant, in which the total concentration of compounds was analyzed.

2.4. Experimental design and mathematical modelling

A Central Composite Design (CCD) was used for evaluating the influence of temperature, pH and extraction time on the release of cytoplasmatic compounds (proteins, free amino acids and glutathione reduced) after the electroporation by PEF treatment of SBY. Table 1

Table 1
Factors and experimental domain for the optimization of the extraction of glutathione reduced, amino acids and proteins from electroporated cells of brewer's yeast biomass.

Factors	Experimental domain	
	Level (−)	Level (+)
Temperature (°C)	20	40
Extraction time (h)	1	48
pH	4	8

illustrates the factors with their experimental domain of the design. Each combination was assayed with two SBY obtained in two different days. Thus, following PEF treatment and subsequent incubation under the conditions established by the CCD, the suspensions were centrifuged at 1593 *g* for 10 min to obtain the supernatant, in which the concentration of glutathione, amino acids and proteins were analyzed. The experiments were repeated twice for each combination.

Mathematical modelling was applied to fit the concentration of each compound (proteins, amino acids, and glutathione) to the following polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i<j}^{n} \beta_{ij} X_i X_j, \quad (1)$$

where Y denotes the predicted concentration of each compound, with the terms β_0, β_i, β_{ii}, and β_{ij} corresponding to the intercept, linear, quadratic, and cross-product coefficients, respectively. X_i and X_j denote the independent variables, while n signifies the number of independent factors involved in the analysis. Backward regression was employed to eliminate non-significant parameters from the corresponding equations ($p > 0.05$).

2.4.1. Selection of optimal conditions

To determine the optimal incubation conditions, the Design-Expert optimization tool (version 10.0, Stat-Ease Inc., Minneapolis, MN, United States) was utilized. This tool generated multiple solutions by numerically optimizing the factors (temperature, pH, and extraction time) to maximize the responses, which include the release of proteins, amino acids, and glutathione.

2.5. Enzymatic treatment

Lyticase from *Arthrobacter luteus* was used as a commercial enzyme complex (Sigma-Aldrich) to release the mannoproteins from the cell walls. Lyticase activity is due to the combined action of two enzymes: β-1,3-glucanase and alkaline protease. Initially the protease binds to the cell wall releasing some proteins and producing holes in the mannoprotein layer, thus, the β-glucan matrix is exposed to the action of glucanase allowing the release of the mannoproteins. Exhausted electroporated SBY after the extraction of the cytoplasmic compounds were suspended in McIlvane buffer (pH 6.5) and lyticase was added to a final concentration 1700 units of lyticase (LYT) per gram of dry weight of initial yeast biomass (9 U/mL). The enzymatic treatment was carried out at 37 °C for 1, 6, 24 and 48 h. Then the suspensions were centrifuged at 1593 *g* for 10 min to obtain the supernatant, in which the concentration of mannoproteins was determined.

2.6. Analytical measurement

2.6.1. Protein concentration

Protein extraction was quantified using the commercial Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA). This method relies on the reduction of Cu^{2+} to Cu^{+1} by proteins in an alkaline medium, known as the Biuret reaction, followed by colorimetric detection of the cuprous cation (Cu^{+1}) using a single reagent containing bicinchoninic acid (BCA). In brief, 200 μL of the working reagent was combined with 25 μL of the sample, which had been properly diluted in distilled water. The mixture was shaken and then incubated at 37 °C for 30 min. Following the incubation, absorbance was measured at 562 nm. A standard curve was generated using albumin with concentrations ranging from 2.0 to 0.06 mg/mL. The results were reported as milligrams of albumin equivalents per gram of dry weight.

2.6.2. Free α-amino nitrogen (FAN) concentration

The α-amino nitrogen concentration (FAN) was determined using the ninhydrin assay, following the procedure outlined by Dimopoulos et al., 2018, with some modifications. This assay is based on the oxidative decarboxylation of alpha-amino acids induced by ninhydrin. The reduced ninhydrin compound reacts with the unreduced form, producing a blue complex with a strong absorbance at 570 nm. In brief, 500 μL of the extract (properly diluted in distilled water) was combined with 250 μL of Ninhydrin Reagent (Sigma-Aldrich) and incubated for 15 min at 100 °C. The mixtures were then rapidly cooled in an ice-water bath for 5 min, after which 1.25 mL of a stop solution (0.2% KIO_3 in 40% ethanol) was added to halt any further colour development. Absorbance at 570 nm was measured against a blank sample prepared with distilled water in place of the extract. The results were reported as milligrams of Glycine equivalents per gram of dry weight. The FAN represents the concentration of free amino acids and small peptides and serves as an indicator of the degree of proteolysis.

2.6.3. Reduced glutathione concentration

The quantification of the reduced form of glutathione (GSH) was carried out using a colorimetric method with DTNB (5.5'-Dithiobis-(2-nitrobenzoic Acid)) (Thermo Fisher Scientific), following a procedure similar to that described by (Ganeva et al., 2020), with some adaptations. GSH reacts with DTNB, yielding the chromophore TNB (5-thio-2-nitrobenzoic Acid), which exhibits a maximum absorbance at 412 nm. In brief, 960 μL of phosphate-buffered saline (PBS) at pH 7.5 and containing 5.6 mM EDTA (Sigma-Aldrich) were mixed with 20 μL of a 0.4% DTNB solution prepared in the same buffer, along with an additional 20 μL of the sample. After an incubation period of 2–10 min at room temperature, the absorbance was measured at 412 nm. Glutathione concentrations were determined using a standard curve prepared with reduced L-glutathione (Sigma-Aldrich), covering a concentration range from 3.9 to 2000 μg/mL. The results were reported as milligrams of reduced L-glutathione per gram of dry weight.

2.6.4. Mannoproteins - mannose concentration

Mannoproteins are composed of mannose units linked to polypeptide chains. The release of mannoproteins was indirectly assessed by measuring the concentration of mannose in the supernatant of the suspensions (Martínez et al., 2016; Quirós, Morales, Pérez-Través, Barcenilla, & Gonzalez, 2011). This was achieved through hydrolysis with sulfuric acid (resulting in a final concentration of 1.5 M) at 100 °C for 90 min, followed by neutralization with NaOH (2 M). During this process, the mannose chains that make up the mannoproteins are broken down into their monomeric form. The quantitative analysis of mannose concentration was performed using an enzymatic method, specifically the D-Mannose, D-Fructose, D-Glucose Assay kit (Megazyme International, Wicklow, Ireland).

2.7. Dry cell weight determination

The dry weight of the samples was determined by drying them to constant weight (30 °C, 15 h) using a centrifugal concentrator (miVac DNA-23050-B00, Ipswich, England).

2.8. Statistical analysis

The presented results are expressed as the mean ± standard deviation of a minimum of three replicates of two experiments. To assess significant differences, a one-way analysis of variance (ANOVA) was conducted, followed by a Tukey test, utilizing GraphPad Software (GraphPad Software Inc., San Diego, California, United States). Statistical significance was recognized at a threshold of $p < 0.05$.

The central composite design, multiple regression analysis and model significant evaluations were performed with Design-Expert software (Stat-Ease Inc.).

3. Results and discussion

3.1. Selection of PEF treatment conditions for electroporation of the SBY

Fig. 1 illustrates the impact of electric field strength and treatment duration on the electroporation percentage of SBY cells. Given that the pH of the treatment medium is a crucial factor influencing electroporation via PEF (Delso, Martínez, Cebrián, Álvarez, & Raso, 2020; Wouters, Alvarez, & Raso, 2001), treatment conditions for electroporating yeast cells were investigated at the subsequent extraction pH levels (pH = 4.0, 6.0, 8.0). Across the range of treatment conditions explored, the percentage of permeabilized cells increased with both electric field strength and treatment duration at all three pH levels investigated. While the pH did not affect PEF efficiency within the pH range of 6.0 to 8.0, at pH 4.0, the efficacy of PEF treatments was higher at lower electric fields tested. For instance, to electroporate 75% of the yeast cell population with a 150 μs treatment at pH 6 and 8, it was necessary to increase the electric field from 10 to 12 kV/cm. At 15 kV/cm, no statistically significant differences ($p > 0.05$) in electroporation percentage were observed when experiments were conducted in media of different pH. These findings align with prior studies indicating that electric fields exceeding 10–12 kV/cm are needed to inactivate more than one logarithmic cycle (90% population) of *S. cerevisiae* (Aronsson, Rönner, & Borch, 2005; Cserhalmi, Vidács, Beczner, & Czukor, 2002). Moreover, it has been noted that under acidic conditions (pH 3.5–4), *S. cerevisiae* exhibited higher sensitivity to PEF (Aronsson and Rönner, 2001; Timmermans et al., 2014).

The irreversible electroporation induced by PEF results in permanent permeabilization of the cytoplasmic membrane, leading to uncontrolled transport across the membrane and the release of intracellular compounds. Previous studies have established a correlation between the extraction efficiency of cytoplasmic compounds and the proportion of the population irreversibly electroporated by PEF (Berzosa et al., 2023). However, when 90% of the population is electroporated, further increases in the proportion of electroporated cells do not significantly enhance extraction efficiency. Therefore, the chosen treatment conditions to permeabilize SBY cells for facilitating intracellular compound release in subsequent experiments at pH 4 were 12 kV/cm for 150 μs (61.74 kJ/kg), resulting in around 90% electroporation. However, at higher pH levels, treatment had to be increased to 15 kV/cm for 150 μs (85.26 kJ/kg) to achieve the necessary level of electroporation for maximizing extraction. Consequently, at the lowest pH investigated, the energy requirements for the required PEF treatment were reduced by 27%.

3.2. Extraction of cytoplasmic compounds from electroporated SBY at different times and temperatures in media of different pH

The extraction processes are affected by numerous factors that may interact with one another. Employing mathematical techniques such as experimental design and response surface methodology allows for the generation of models that account for potential interrelationships among these factors. Moreover, these models are very useful for determining the best extraction conditions (Boateng, 2023).

Table 2 presents the amount of amino acids, glutathione, and proteins released from PEF-treated SBY following the extraction conditions established through central composite design. Prolonged extraction time and high temperatures might contribute to the oxidation of the extracted products, especially glutathione. Therefore, based on previous screening experiments (data not shown), 40 °C and 48 h of extraction were selected as maximum temperature and extraction time. The concentration of each compound, expressed in mg per g of dry weight extracted post-complete mechanical disruption of SBY via bead milling, is also detailed in the table.

The concentration of compounds extracted from electroporated SBY ranged from 5.12 to 162.52 mg/g dw for amino acids, 0.57 to 2.94 mg/g dw for glutathione, and 77.24 to 382.27 mg/g dw for proteins. Hence, by adjusting extraction conditions, the release of amino acids, glutathione, and proteins could potentially be improved by 32, 5, and 5 times, respectively. These findings align with those of other researchers showing comparable extraction yields or concentrations of these compounds in yeast extracts using various extraction methods. For reduced glutathione, methods include ultrafiltration and nanofiltration membranes (yielding 1.8–2.5 mg/g dw), aqueous two-phase systems (83% extraction yield), or PEF (with yields between 72 and 84% after 10 min of extraction) (Ganeva et al., 2020; Y. Wang, Xiao, Zhang, & Feng, 2021; Wu, Tang, Du, & Xu, 2010). Commonly used methods for amino acid extraction include autolysis, mechanical disruption, and PEF treatments,

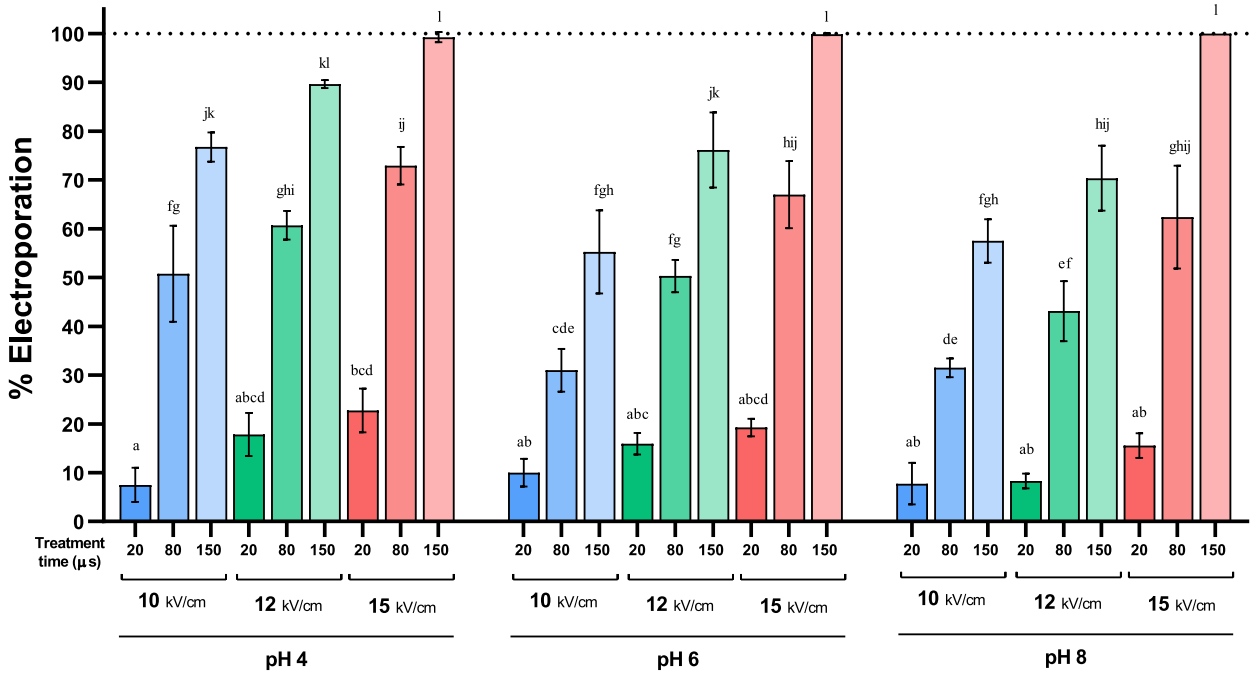

Fig. 1. Influence of electric field strength, treatment time and pH of treatment medium on electroporation of cells of brewer's yeast biomass.

Table 2
Extraction yield (mg per g of dry matter) of glutathione reduced, amino acids and proteins from electroporated cells of brewer's yeast biomass under extraction times, pH and temperatures established by the central composite design. Total content (mg per g of dry weight) of glutathione reduced, amino acids and proteins of brewer's yeast biomass determined after bead milling is also indicated.

pH	Temperature (T[a])	Extraction time (t)	**Amino acids**	**Glutathione**	**Proteins**
	°C	hours	mg/g dw[a]	mg/g dw	mg/g dw
4	20	1	5.12 ± 0.61	1.82 ± 0.10	77.24 ± 7.91
4	40	1	7.57 ± 0.86	1.80 ± 0.12	91.22 ± 5.28
6	30	1	6.50 ± 0.67	1.95 ± 0.02	91.34 ± 16.79
8	20	1	11.00 ± 0.95	2.38 ± 0.01	115.62 ± 2.31
8	40	1	12.30 ± 3.30	2.49 ± 0.10	167.17 ± 9.69
4	30	24	56.83 ± 2.27	2.14 ± 0.04	131.32 ± 13.85
6	20	24	30.54 ± 1.67	2.08 ± 0.24	151.74 ± 2.24
6	30	24	63.18 ± 2.10	2.32 ± 0.11	263.31 ± 18.28
6	40	24	70.25 ± 0.98	1.85 ± 0.14	138.03 ± 24.62
8	30	24	74.82 ± 7.27	2.57 ± 0.13	280.41 ± 22.15
4	20	48	48.01 ± 0.14	2.17 ± 0.18	118.73 ± 17.14
4	40	48	115.91 ± 10.89	0.57 ± 0.12	176.09 ± 0.66
6	30	48	111.87 ± 11.34	2.27 ± 0.46	382.27 ± 22.38
8	20	48	48.31 ± 1.27	2.94 ± 0.14	225.26 ± 9.69
8	40	48	162.52 ± 0.00	0.83 ± 0.30	356.44 ± 12.46
Total concentration (bead mill)			**15.55 ± 2.25**	**4.65 ± 0.54**	**548.32 ± 113.98**

[a] g dw: grams of dry weight.

with concentrations of amino acids in the extracts ranging from 100 to 500 mg/g dw and extractions yields close to 100% just a few hours of incubation after PEF treatment or even higher than 100% with longer incubations (Berzosa et al., 2023; Boonyeun, Shotipruk, Prommuak, Suphantharika, & Muangnapoh, 2011; Ganeva et al., 2020; Jacob, Hutzler, & Methner, 2019). Protein extraction methods also yield variable results (10–76% extraction yields) between extraction methods (bead mill, autolysis, hydrolysis, sonication and high-pressure homogenization) (Chae et al., 2001; Jacob et al., 2019; Oliveira et al., 2022; Tanguler & Erten, 2008), with PEF technology showing yields between 5 and 90% (Ganeva et al., 2020). These findings underscore the variability in extraction yields of yeast compounds, which is influenced by factors such as growth conditions and yeast strains. Moreover, the chosen extraction methodology and the proper extraction conditions significantly impact the outcome.

3.2.1. *Mathematical modelling*

Predictive equations were formulated by fitting a quadratic regression model to experimental values (Table 2) to elucidate the empirical relationship between extraction yield and extraction conditions. The terms of the equation, along with their coefficients, are presented in Table 3 after eliminating non-significant effects ($p > 0.05$) for each analyzed compound. Table 3 also shows the results of the analysis of variance (ANOVA), including the statistical measures used to assess the adequacy of the generated models.

The F-values obtained from the ANOVA analysis underscore the significance of the models ($p < 0.0001$), indicating that the terms in the equations exert a noteworthy impact on the response. The non-significance ($p > 0.05$) of the model's lack of fit test attests to the reliability of the derived equations. Each model exhibited an adequate precision >4, and the values of RMSE, R^2, and R^2_{adj} were reasonable, enabling the use of the models for estimating the influence of the independent variable on the response. To validate the equations, a new set of experiments was conducted under extraction conditions within the experimental domain but different from those established by the CCD. The experimental results were then compared with the estimates from the equations, confirming the suitability of the models in predicting the extraction of amino acids glutathione and proteins from SBY treated by PEF over time in media with varying pH at different temperatures (Suppl. Fig. 1).

For the three compounds analyzed, extraction yield was found to be dependent on pH, temperature, and extraction time. The quadratic factor of temperature was present in all three models, indicating a nonlinear relationship between temperature and the extraction of these compounds. Regarding interactions, while the extraction yield of amino acids was influenced by the interaction between the three factors (pH*T[a]; pH*t and T[a]*t), extraction yield of proteins and glutathione was only affected by the interactions pH and extraction time (pH*t) and temperature and time (T[a]*t), respectively.

The F-values for different terms in the equations revealed that the most significant factors influencing extraction yield varied for each extracted compound. For glutathione extraction yield, temperature (F-value: 41.89) and the interaction of T[a]*t (F-value: 50.79) were the most significant factors. Conversely, for amino acid extraction yield, the most significant factor was extraction time (F-value: 632.49), followed by temperature (F-value: 163.16). In the case of proteins, the most significant variable in the model was extraction time (F-value: 48.8), similar

Table 3
Coefficients and F-values of the mathematical equations to describe the influence of extraction time temperature and pH on extraction of glutathione, proteins and amino acids from electroporated cells of brewer's yeast biomass after multiple regression modelling. Statistics to test the adequacy are also shown.

	Amino Acids		Glutahione		Proteins	
	Coefficient β_n	F-Value	Coefficient β_n	F-Value	Coefficient β_n	F-Value
Model	–	138,42	–	26,66	–	20,26
Intercept	−42,64	–	−1,95	–	−626,49	–
X_1 - **pH**	−7,06	18,27	0,14	20,83	16,27	28,81
X_2 - **Temperature** (T[a])	5,13	163,16	0,23	41,89	43,19	5,50
X_3 - **Extraction Time** (t)	−1,53	632,49	0,05	7,78	0,29	48,8
X_1*X_2 - **pH*T[a]**	0,28	8,18	–	–	–	–
X_1*X_3 - **pH*t**	0,09	5,27	–	–	0,46	3,54
X_2*X_3 – **T[a]*t**	0,09	127,49	−0,002	50,79	–	–
X_2^2 – **T[a]²**	−0,11	14,1	−0,003	12,03	−0,68	14,65
R^2	0,978		0,847		0,809	
R^2 adj.	0,971		0,816		0,767	
Adeq. Precision	39,18		19,00		14,65	
RMSE	5,90		0,20		39,71	

to amino acids, followed by pH (F-value: 28.81).

3.3. Influence of extraction conditions in the extraction yield of amino acids, glutathione and proteins from electroporated SBY

Various graphical representations, utilizing the obtained regression equations within the experimental domain, were generated to elucidate the impact of extraction time (Fig. 2), pH (Fig. 3), and temperature (Fig. 4) on the extraction yield of intracellular compounds from electroporated BYS.

3.3.1. Influence of extraction time

Fig. 2 illustrates the influence of extraction time on the extraction yield of amino acids (**2A**), glutathione (**2B**), and proteins (**2C**) at three different temperatures and a constant pH of 6. The extraction yield of amino acids and proteins exhibits a linear increase with treatment time, regardless of the extraction temperature. Other researchers have previously indicated an increase in protein and amino acids extraction as extraction time increases after PEF treatment, both in yeasts (Dimopoulos et al., 2018; Ganeva et al., 2020) and other microorganisms (t Lam et al., 2017). Although nearly the entire content of free amino acids, as determined by bead milling (15.55 ± 2.25 mg/g dw), was released within the first hour of incubation at all three temperatures, extended extraction times led to further increases in amino acid extraction yields. This notable increase can be attributed to the enzymatic hydrolysis of proteins within the yeast cytoplasm. This hydrolysis would be triggered by the release of proteases from yeast vacuoles due to osmotic imbalances caused by electroporation (Aguilar-Machado et al., 2020; Martínez et al., 2016). Consequently, two simultaneous phenomena occur during extraction: enzymatic protein hydrolysis and the release of amino acids and proteins. The higher protein extraction yield observed at 30 °C compared to 40 °C (Fig. 3C) may be explained by the fact that protein hydrolysis and subsequent amino acid release are more pronounced at 40 °C.

In the case of glutathione, the effect of extraction time on extraction yield was also linear, but while extraction tends to increase slightly with time at 20 °C, it tends to decrease at 30 and 40 °C. The reactivity of glutathione at higher temperatures may explain this behavior. Glutathione is a molecule involved in the oxidative stress of cells, that easily reacts with other molecules, thus oxidizing from reduced (GSH) to oxidized form (glutathione disulphide "GSSG") (Valdivieso Ugarte, 2006). Hence, the reduced glutathione extracted within a few hours of extraction would react over time with the other molecules that would be extracted, this effect being accelerated by the temperature. Wang, Hung, and Lin (2010) showed that extending the incubation period within the temperature range of 10 to 50 °C resulted in a continuous oxidation of GSH to glutathione disulphide, being markedly greater at 50 °C and requiring 395 and 140 h for the complete oxidation of glutathione at 10 and 50 °C respectively. Despite the expected lower total glutathione extraction at lower temperatures, the measured reduced glutathione in the extraction media was higher at 20 °C.

3.3.2. Influence of pH

Fig. 3A, B, and C depict the influence of extraction media pH on the extraction yield of amino acids, glutathione, and proteins at three different temperatures and a constant extraction time of 24 h.

The extraction yield of amino acids was independent of pH at 20 °C, with a slight increment occurring at higher temperatures. For glutathione and proteins, extraction yield increases linearly with pH at any extraction temperature, with a similar increment observed at all temperatures. Increasing the pH from 4 to 8 enhances extraction yield between 20 and 30% and 38–58% for glutathione and proteins respectively. This result aligns with previous findings indicating that protein extractability from matrices treated with PEF increased at higher pH (Ganeva et al., 2020).

Considering that most extracted amino acids result from proteolytic

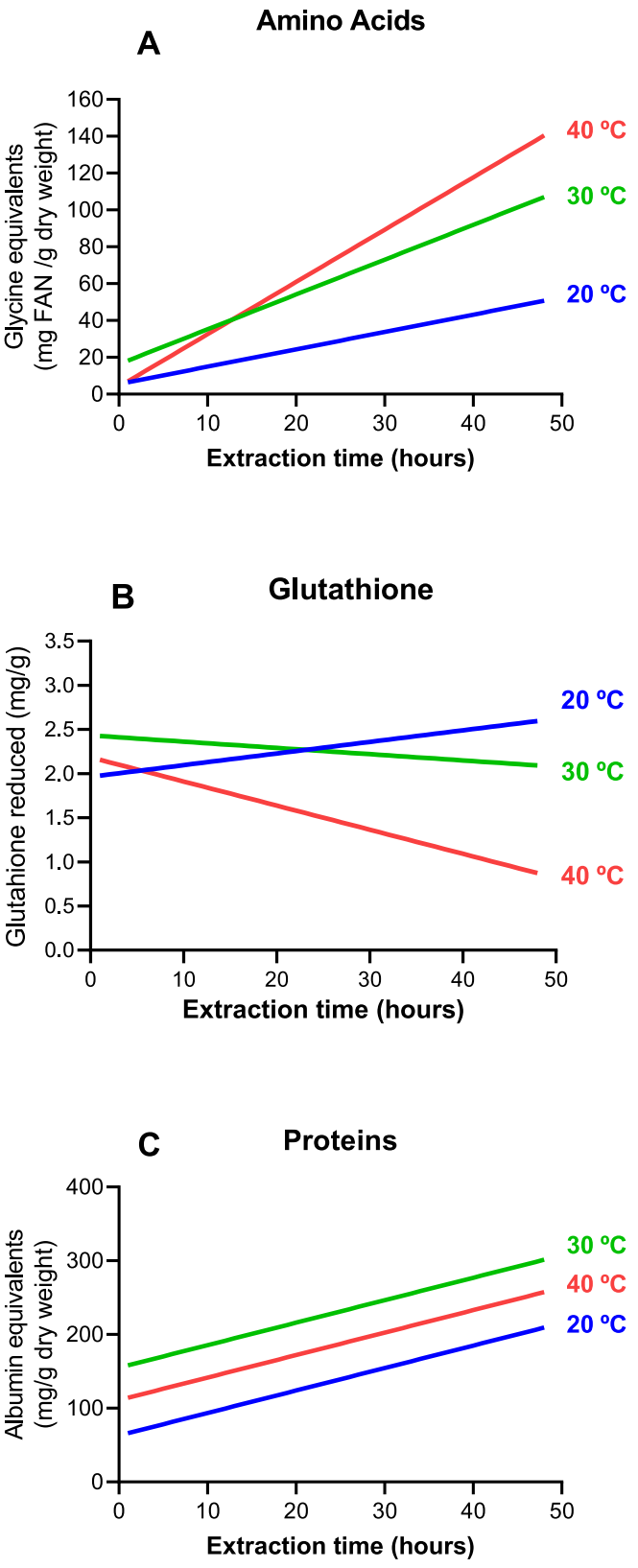

Fig. 2. Graphs illustrating the influence of the extraction time at different temperatures and pH 6 on the release of amino acids (A), glutathione (B) and proteins (C) from electroporated cells of brewer's yeast biomass.

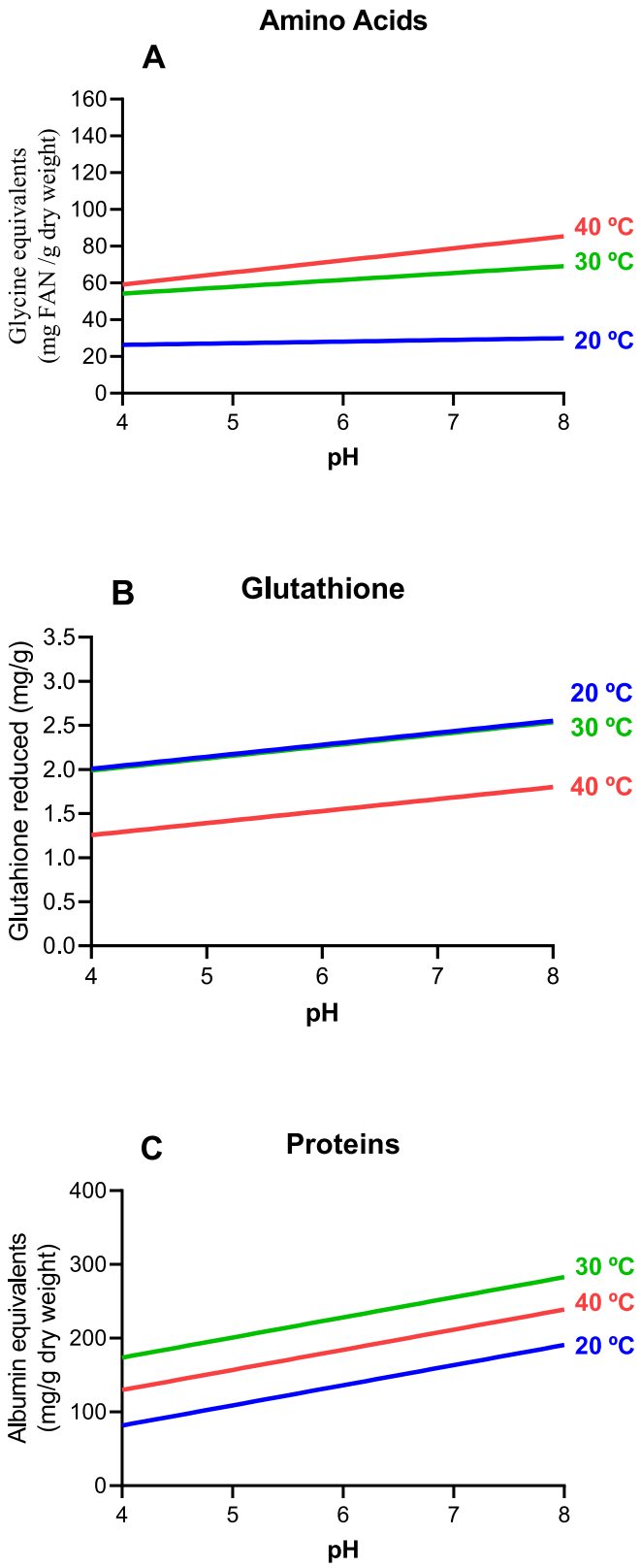

Fig. 3. Graphs illustrating the influence of the pH at different temperatures and 24 h of extraction on the release of amino acids (A), glutathione (B) and proteins (C) from electroporated cells of brewer's yeast biomass.

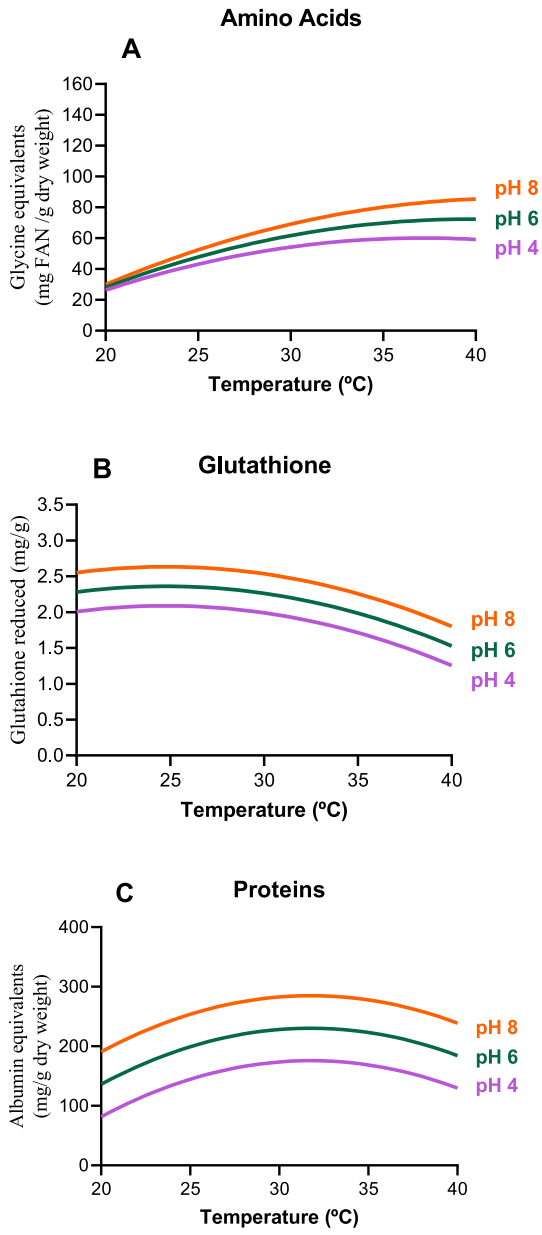

Fig. 4. Graphs illustrating the influence of the temperature at different pH and 24 h of extraction on the release of amino acids (A), glutathione (B) and proteins (C) from electroporated cells of brewer's yeast biomass.

processes, the low influence of pH on amino acid extraction yield may be attributed to the distinct pH optima of proteases. Approximately 80–90% of the overall proteolytic activity of the yeast is mainly associated with proteases A and B (Van Den Hazel, Kielland-Brandt, & Winther, 1992), however, while the pH optimum of protease A is acidic (3.2), protease B has a pH optimum of action close to neutral (7.6) (Jones, 1991). Hence, Protease A, would be responsible for protein hydrolysis at low pH, while protease B, would be more involved in proteolytic activity at higher pH.

3.3.3. Influence of temperature

Fig. 4 demonstrates the influence of extraction temperature on the extraction yield of amino acids (**4A**), glutathione (**4B**), and proteins (**4C**) at the three different pH levels and a constant time of 24 h.

In comparison with the influence of the other studied factors, the temperature's effect on the extraction yield of the three compounds analyzed was not linear. Temperature increments prove more effective

in the range between 20 and 30 °C for amino acids and proteins extraction. Further temperature increments scarcely increase amino acid extraction and tend to decrease protein extraction. Considering that proteolytic activity increases at higher temperatures, the high proteolysis occurring at high temperatures could explain the decrease in protein concentration. This behavior is supported by the increase in amino acid concentration with increasing extraction time (Fig. 2A) and also with increasing temperature (Fig. 4A). This observation agrees with previous studies that reported a decrease in protein extraction yields as temperature increased, correlated with higher amino acid extraction due to enhanced proteolysis (Tanguler & Erten, 2008).

Fig. 4 also reveals that glutathione extraction yield remains constant in the temperature range of 20 to 30 °C, tending to decline with further temperature increments. The higher reactivity of glutathione at higher temperatures likely contributes to the decrease in reduced glutathione concentration.

3.4. Optimization of extraction conditions

Optimizing treatment conditions to maximize extraction yield is an essential approach for reducing costs in industrial processes. When considering various factors, selecting the optimal alternative becomes complicated. The multiple regression equation obtained in this investigation allows us to describe and quantify the impact of each extraction factor and their interactions on extraction yield. Additionally, it enables us to define optimal conditions for maximizing the extraction of amino acids, glutathione, and proteins from electroporated SBY.

To determine the values of time, temperature, and pH that produce the highest response in the extraction yields of the three compounds investigated, the desirability function described by Derringer and Suich (1980), extensively used to optimize processes with multiple simultaneous responses, was applied (Chandra Roy et al., 2021; Vural, Cavuldak, & Anlı, 2018).

Optimization resulted in the following experimental conditions: t = 48 h, pH = 8, and T = 37 °C for amino acids and proteins, and t = 1 h, pH = 8, and T = 30 °C for glutathione.

The optimization revealed that the conditions required to maximize the extraction of the three compounds were the same concerning pH, but different concerning extraction time and temperature for glutathione. These significant differences in extraction time for these compounds provided an opportunity for the development of a sequential extraction process by prolonging the extraction for 48 h. This approach would allow obtaining a first extract rich in glutathione after an hour of extraction, followed by second extract rich in amino acids and proteins. In the second case, while the first extract could be used as an ingredient with antioxidant power, the second extract could serve as a protein supplement for different applications.

To verify the adequacy of the optimization and the feasibility of performing a sequential extraction, a further extraction trial was conducted under the predicted optimal conditions. In this trial, after 1 h of incubation at pH 8 and 30 °C, an extract with a concentration in glutathione of 2.31 ± 0.15 mg/g dw was obtained. Subsequently, a second extract with concentrations of amino acids and proteins of 155.74 ± 7.83 and 331.70 ± 15.64 mg/g dw respectively, was obtained by resuspending the SBY biomass in a pH 8 buffer and incubating at 37 °C for 47 h. The residual standard error (RSE%) between the predicted and actual responses were 14.2, 4.5 and 8.2% for glutathione, amino acids and proteins. These deviations are within the 95% confidence interval estimated by the model indicating that the actual responses under optimal conditions were consistent with the predicted response values, even when the sequential process was carried out.

3.5. Extraction of mannoproteins from the SBY

Compared to other physical techniques used for extracting compounds found within the cell such as ultrasound or HPH that cause cell wall breakage, PEF allows for the extraction of intracellular compounds while maintaining the integrity of the cell wall. Consequently, after extracting intracellular compounds from SBY treated with PEF, a biomass consisting of exhausted cells without cell breakdown should be obtained. To assess whether, in addition to amino acids, glutathione, and proteins, other compounds of interest located in the cell wall, such as mannoproteins, could be obtained for valorizing SBY, the release of mannose from SBY was monitored for 48 h under optimal conditions selected for sequential intracellular compound extraction (pH 8, 30 °C for 1 h and 37 °C for 47 h).

The determination of mannose released from the cell wall is a typical procedure used as an indirect measure of mannoprotein release from yeast cell walls suspensions (Martínez et al., 2016; Quirós et al., 2011). Since mannoproteins are glycosylated proteins with mannose chains, the concentration of mannose released in the supernatant of yeast suspensions is directly related to the concentration of mannoproteins. Results of this study indicated that after 48 h of incubation, approximately 28% of the total mannose contained in the cell wall was released. The release of mannoproteins requires the enzymatic degradation of the cell wall. Electroporation induced by PEF, besides leading to the release of cytoplasmic compounds, also allows the entry of water into the cellular interior. This phenomenon induces plasmolysis of vacuoles containing hydrolytic enzymes such as proteases and β-glucanases that are involved in the autolytic degradation of the cell wall (Aguilar-Machado et al., 2020; Martínez et al., 2016). Consequently, the hydrolytic activity of these released enzymes during 48 h of incubation was the cause of the liberation of mannoproteins and, consequently, the detection of mannose in the supernatant. The fact that only 28% of the total mannose present in the cellular wall of the yeast biomass after was detected in the supernatants after 48 h of incubation indicates that, after the first stage of intracellular compound extraction, a yeast biomass with a cell wall rich in mannoproteins was available.

In order to achieve comprehensive valorization of SBY, the yeast biomass obtained after 48 h of extraction was incubated with lyticase to hydrolyze the cell walls and subsequently extract mannoproteins. The use of externally added enzymes to accelerate yeast autolysis has been previously demonstrated in several yeast strains (Ganeva & Kranz, 2023; Snyman, Nguela, Sieczkowski, Marangon, & Divol, 2021) and has been shown to be beneficial for accelerating the aging on lees of wines, where mannoproteins play a crucial role (Palomero, Morata, Benito, González, & Suárez-Lepe, 2007). Fig. 5 illustrates the extraction of mannoproteins (expressed as % mannose released) from the exhausted yeast biomass over the incubation time with lyticase. It was observed that after one hour of incubation, around 35% of the total mannose was released, and

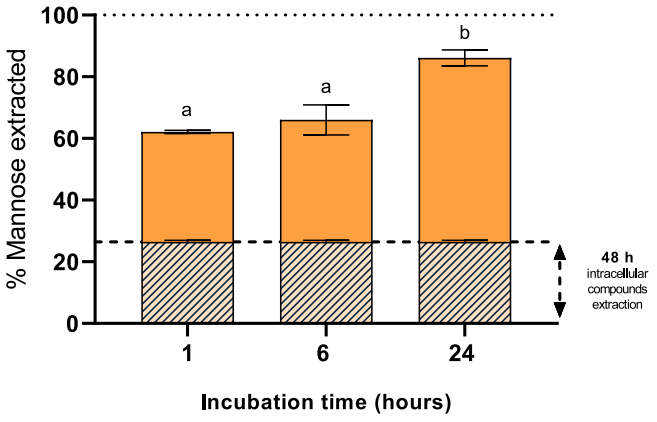

Fig. 5. Release of mannose into the supernatant during incubation at 37 °C for 1, 6 and 24 h, after the addition of lyticase to the exhausted yeast biomass after extraction of cytoplasmic compounds from electroporated cells of brewer's yeast biomass. The concentration of mannose in the extract obtained after 48 h of incubation is also shown.

by extending the incubation to 24 h, the extraction increased to around 60%. Considering that the biomass had released around 28% of the total mannose in the first 48 h of incubation, the mannose that remained in the cell wall after the subsequent incubation with the enzyme was around 10%. Therefore, enzymatic hydrolysis would permit obtaining an extract containing practically the total mannoproteins of the yeast cell wall of the exhausted biomass.

4. Conclusions

PEF-induced electroporation sequentially releases cytoplasmic compounds from SBY, streamlining extraction without damaging the cell wall. This study demonstrates the efficacy of a multi-response function in optimizing the extraction process for PEF-treated SBY. The proposed biorefinery approach integrates the sequential extraction of intracellular compounds and mannoproteins, valorizing a significant proportion of the biomass. This approach not only offers economic benefits to the industry, but is also in line with sustainable development goals. Further studies are required to address challenges related to large-scale extraction and to evaluate the properties of the different extracts obtained for their potential applications in various industrial sectors.

CRediT authorship contribution statement

Alejandro Berzosa: Visualization, Writing – original draft, Conceptualization, Investigation, Methodology. **Javier Marín-Sánchez:** Investigation, Validation, Visualization. **Carlota Delso:** Validation, Visualization. **Jorge Sanz:** Validation, Visualization. **Ignacio Álvarez:** Visualization, Writing – review & editing. **Cristina Sánchez-Gimeno:** Funding acquisition, Supervision, Visualization, Writing – review & editing. **Javier Raso:** Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGTP 3.5 in order to improve readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors affirm that the research was carried out without any affiliations or financial ties that might be perceived as potential conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to express their appreciation for the financial support received from the PEFREV project (Grant number: PID2020-113620RB-I00) of the Spanish Research Agency. Alejandro Berzosa is thankful for the financial assistance granted (Gran Number: FPU20/02527) by the Ministerio de Universidades (Spain), which supported his academic pursuits.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103705.

References

Abdolshahi, A., Marvdashti, L. M., Salehi, B., Sharifi-Rad, M., Ghobakhloo, S., Iriti, M., & Sharifi-Rad, J. (2019). Antifungal activities of coating incorporated with Saccharomyces cerevisiae cell wall mannoprotein on *aspergillus flavus* growth and aflatoxin production in pistachio (*Pistacia vera L.*). *Journal of Food Safety, 39*(2). https://doi.org/10.1111/jfs.12608

Aguilar-Machado, D., Delso, C., Martínez, J. M., Morales-Oyervides, L., Montañez, J., & Raso, J. (2020). Enzymatic processes triggered by PEF for Astaxanthin extraction from *Xanthophyllomyces dendrorhous*. *Frontiers in Bioengineering and Biotechnology, 8*. https://doi.org/10.3389/fbioe.2020.00857

Amorim, M., Pinheiro, H., & Pintado, M. (2019). Valorization of spent brewer's yeast: Optimization of hydrolysis process towards the generation of stable ACE-inhibitory peptides. *LWT, 111*, 77–84. https://doi.org/10.1016/J.LWT.2019.05.011

Aronsson, K., Rönner, U., & Borch, E. (2005). Inactivation of Escherichia coli, listeria innocua and Saccharomyces cerevisiae in relation to membrane permeabilization and subsequent leakage of intracellular compounds due to pulsed electric field processing. *International Journal of Food Microbiology, 99*(1), 19–32. https://doi.org/10.1016/J.IJFOODMICRO.2004.07.012

Berzosa, A., Delso, C., Sanz, J., Sánchez-Gimeno, C., & Raso, J. (2023). Sequential extraction of compounds of interest from yeast biomass assisted by pulsed electric fields. *Frontiers in Bioengineering and Biotechnology, 11*. https://doi.org/10.3389/fbioe.2023.1197710

Boateng, I. D. (2023). Application of graphical optimization, desirability, and multiple response functions in the extraction of food bioactive compounds. *Food Engineering Reviews, 15*(2), 309–328. https://doi.org/10.1007/s12393-023-09339-1

Boonyeun, P., Shotipruk, A., Prommuak, C., Suphantharika, M., & Muangnapoh, C. (2011). Enhancement of amino acid production by two-step autolysis of spent brewer's yeast. *Chemical Engineering Communications, 198*(12), 1594–1602. https://doi.org/10.1080/00986445.2011.560219

Chae, H. J., Joo, H., & In, M.-J. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: Effects of different enzymatic treatments on solid and protein recovery and favor characteristics. *Bioresource Technology, 76*, 253–258.

Chandra Roy, V., Ho, T. C., Lee, H. J., Park, J. S., Nam, S. Y., Lee, H., … Chun, B. S. (2021). Extraction of astaxanthin using ultrasound-assisted natural deep eutectic solvents from shrimp wastes and its application in bioactive films. *Journal of Cleaner Production, 284*, Article 125417. https://doi.org/10.1016/J.JCLEPRO.2020.125417

Cserhalmi, Z., Vidács, I., Beczner, J., & Czukor, B. (2002). Inactivation of *Saccharomyces cerevisiae* and *Bacillus cereus* by pulsed electric fields technology. *Innovative Food Science & Emerging Technologies, 3*(1), 41–45. https://doi.org/10.1016/S1466-8564(01)00052-2

Delso, C., Martínez, J. M., Cebrián, G., Álvarez, I., & Raso, J. (2020). Understanding the occurrence of tailing in survival curves of *salmonella typhimurium* treated by pulsed electric fields. *Bioelectrochemistry, 135*, Article 107580. https://doi.org/10.1016/J.BIOELECHEM.2020.107580

Derringer, G., & Suich, R. (1980). Simultaneous optimization of several response variables. *Journal of Quality Technology, 12*(4), 214–219. https://doi.org/10.1080/00224065.1980.11980968

Diez, L., Guadalupe, Z., Ayestarán, B., & Ruiz-Larrea, F. (2010). Effect of yeast mannoproteins and grape polysaccharides on the growth of wine lactic acid and acetic acid bacteria. *Journal of Agricultural and Food Chemistry, 58*(13), 7731–7739. https://doi.org/10.1021/jf100199n

Dimopoulos, G., Stefanou, N., Andreou, V., & Taoukis, P. (2018). Effect of pulsed electric fields on the production of yeast extract by autolysis. *Innovative Food Science & Emerging Technologies, 48*, 287–295. https://doi.org/10.1016/J.IFSET.2018.07.005

Ferreira, I., Pinho, O., Vieira, E., & Tavarela, J. G. (2010). Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications. In , *Vol. 21. Trends in Food Science and Technology* (pp. 77–84). https://doi.org/10.1016/j.tifs.2009.10.008. Issue 2.

Ganeva, V., Angelova, B., Galutzov, B., Goltsev, V., & Zhiponova, M. (2020). Extraction of proteins and other intracellular bioactive compounds from Baker's yeasts by pulsed electric field treatment. *Frontiers in Bioengineering and Biotechnology, 8*. https://doi.org/10.3389/fbioe.2020.552335

Ganeva, V., & Kranz, A. (2023). Selective extraction of recombinant membrane proteins from *Hansenula polymorpha* by pulsed electric field and lytic enzyme pretreatment. *Microbial Cell Factories, 22*(1), 251. https://doi.org/10.1186/s12934-023-02259-z

Gaspar, L. M., Machado, A., Coutinho, R., Sousa, S., Santos, R., Xavier, A., … Simões, J. (2019). Development of potential yeast protein extracts for red wine clarification and stabilization. *Frontiers in Microbiology, 10*. https://doi.org/10.3389/fmicb.2019.02310

Giménez, P., Just-Borras, A., Pons, P., Gombau, J., Heras, J. M., Sieczkowski, N., … Zamora, F. (2023). Biotechnological tools for reducing the use of sulfur dioxide in white grape must and preventing enzymatic browning: Glutathione; inactivated dry yeasts rich in glutathione; and bioprotection with *Metschnikowia pulcherrima*. *European Food Research and Technology, 249*(6), 1491–1501. https://doi.org/10.1007/s00217-023-04229-6

Gusbeth, C. A., & Frey, W. (2022). Integration of pulsed electric fields in the biorefinery concept to extract microalgae components of interest for food industry. In J. Raso, V. Heinz, I. Alvarez, & S. Toepfl (Eds.), *Pulsed electric fields technology for the food industry: Fundamentals and applications* (pp. 369–384). Springer International Publishing. https://doi.org/10.1007/978-3-030-70586-2_12.

Jacob, F. F., Hutzler, M., & Methner, F. J. (2019). Comparison of various industrially applicable disruption methods to produce yeast extract using spent yeast from top-fermenting beer production: Influence on amino acid and protein content. *European Food Research and Technology, 245*(1), 95–109. https://doi.org/10.1007/s00217-018-3143-z

Jaeger, A., Arendt, E. K., Zannini, E., & Sahin, A. W. (2020). Brewer's spent yeast (BSY), an underutilized brewing by-product. *Fermentation, 6*(4), 1–23. https://doi.org/10.3390/FERMENTATION6040123

Jones, E. W. (1991). Tackling the protease problem in *Saccharomyces cerevisiae. Methods in Enzymology, 194*(C), 428–453.

Kruk, M., Varmanen, P., Edelmann, M., Chamlagain, B., & Trząskowska, M. (2024). Food by-product valorisation in nutrients through spent brewer's yeast bioprocessing with Propionibacterium freudenreichii. *Journal of Cleaner Production, 434*, Article 140102. https://doi.org/10.1016/J.JCLEPRO.2023.140102

Li, S., Zhai, H., Ma, W., Duan, C., & Yi, L. (2023). Yeast mannoproteins: Organoleptic modulating functions, mechanisms, and product development trends in winemaking. *Food Frontiers, 1–36*. https://doi.org/10.1002/fft2.256

Li, Y., Wei, G., & Chen, J. (2004). Glutathione: A review on biotechnological production. In *, Vol. 66. Applied Microbiology and Biotechnology* (pp. 233–242). https://doi.org/10.1007/s00253-004-1751-y. Issue 3.

Mahnič-Kalamiza, S., & Miklavčič, D. (2022). The phenomenon of electroporation. In V. and A. I, T. S. R. Javier, & Heinz (Eds.), *Pulsed electric fields technology for the food industry: Fundamentals and applications* (pp. 107–141). Springer International Publishing. https://doi.org/10.1007/978-3-030-70586-2_3.

Maqbool, A., Khan, S., Haleem, A., & Khan, M. I. (2020). Investigation of drivers towards adoption of circular economy: A DEMATEL approach. *Lecture Notes in Mechanical Engineering, 147–160*. https://doi.org/10.1007/978-981-15-1071-7_14

Martínez, J., & González-Arenzana, L. (2022). Use of glutathione in the winemaking of white grape varieties. *White Wine Technology, 29–38*. https://doi.org/10.1016/B978-0-12-823497-6.00021-1

Martínez, J. M., Cebrián, G., Álvarez, I., & Raso, J. (2016). Release of mannoproteins during *Saccharomyces cerevisiae* autolysis induced by pulsed electric field. *Frontiers in Microbiology, 7*(SEP). https://doi.org/10.3389/fmicb.2016.01435

Oliveira, A. S., Odila Pereira, J., Ferreira, C., Faustino, M., Durão, J., Pereira, A. M., … Carvalho, A. P. (2022). Spent yeast valorization for food applications: Effect of different extraction methodologies. *Foods, 11*(24). https://doi.org/10.3390/foods11244002

Palomero, F., Morata, A., Benito, S., González, M. C., & Suárez-Lepe, J. A. (2007). Conventional and enzyme-assisted autolysis during ageing over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chemistry, 105*(2), 838–846. https://doi.org/10.1016/J.FOODCHEM.2007.01.062

Quirós, M., Morales, P., Pérez-Través, L., Barcenilla, J. M., & Gonzalez, R. (2011). A new methodology to determine cell wall mannoprotein content and release in wine yeasts. *Food Chemistry, 125*(2), 760–766. https://doi.org/10.1016/J.FOODCHEM.2010.08.066

Ribeiro, T., Fernandes, C., Nunes, F. M., Filipe-Ribeiro, L., & Cosme, F. (2014). Influence of the structural features of commercial mannoproteins in white wine protein stabilization and chemical and sensory properties. *Food Chemistry, 159*, 47–54. https://doi.org/10.1016/J.FOODCHEM.2014.02.149

Rodrigues, A., Ricardo-Da-Silva, J. M., Lucas, C., & Laureano, O. (2012). Effect of commercial mannoproteins on wine colour and tannins stability. *Food Chemistry, 131*(3), 907–914. https://doi.org/10.1016/J.FOODCHEM.2011.09.075

Saksinchai, S., Suphantharika, M., & Verduyn, C. (2001). Application of a simple yeast extract from spent brewer's yeast for growth and sporulation of *Bacillus thuringiensis* subsp. *kurstaki*: A physiological study. *World Journal of Microbiology and Biotechnology, 17*, 307–316.

Schlabitz, C., Neutzling Lehn, D., & Volken de Souza, C. F. (2022). A review of *Saccharomyces cerevisiae* and the applications of its byproducts in dairy cattle feed: Trends in the use of residual brewer's yeast. *Journal of Cleaner Production, 332*, Article 130059. https://doi.org/10.1016/J.JCLEPRO.2021.130059

Snyman, C., Nguela, J. M., Sieczkowski, N., Marangon, M., & Divol, B. (2021). Optimised extraction and preliminary characterisation of mannoproteins from non-*Saccharomyces* wine yeasts. *Foods, 10*(5). https://doi.org/10.3390/foods10050924

Soh, E. Y. S., Lim, S. S., Chew, K. W., Phuang, X. W., Ho, V. M. V., Chu, K. Y. H., … Tiong, T. J. (2022). Valorization of spent brewery yeast biosorbent with sonication-assisted adsorption for dye removal in wastewater treatment. *Environmental Research, 204*, Article 112385. https://doi.org/10.1016/J.ENVRES.2021.112385

t Lam, G. P., Van Der Kolk, J. A., Chordia, A., Vermuë, M. H., Olivieri, G., Eppink, M. H. M., & Wijffels, R. H. (2017). Mild and selective protein release of cell wall deficient microalgae with pulsed electric field. *ACS Sustainable Chemistry and Engineering, 5*(7), 6046–6053. https://doi.org/10.1021/acssuschemeng.7b00892

Tanguler, H., & Erten, H. (2008). Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. *Food and Bioproducts Processing, 86*(4), 317–321. https://doi.org/10.1016/j.fbp.2007.10.015

Teissie, J., Golzio, M., & Rols, M. P. (2005). Mechanisms of cell membrane electropermeabilization: A minireview of our present (lack of ?) knowledge. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1724*(3), 270–280. https://doi.org/10.1016/J.BBAGEN.2005.05.006

Thiago, R. S. M., Pedro, P. M. M., & Eliana, F. C. S. (2014). Solid wastes in brewing process: A review. *Journal of Brewing and Distilling, 5*(1), 1–9. https://doi.org/10.5897/jbd2014.0043

Valdivieso Ugarte, M. (2006). *Obtención y caracterización de cepas de Saccharomyces cerevisiae superproductoras de glutation*.

Van Den Hazel, H. B., Kielland-Brandt, M. C., & Winther, J. R. (1992). Autoactivation of proteinase A initiates activation of yeast vacuolar zymogens. *European Journal of Biochemistry, 207*.

Vieira, E., Teixeira, J., & Ferreira, I. (2016). Valorization of brewers' spent grain and spent yeast through protein hydrolysates with antioxidant properties. *European Food Research and Technology, 242*(11), 1975–1984. https://doi.org/10.1007/s00217-016-2696-y

Vural, N., Cavuldak, A., & Anlı, R. E. (2018). Multi response optimisation of polyphenol extraction conditions from grape seeds by using ultrasound assisted extraction (UAE). *Separation Science and Technology (Philadelphia), 53*(10), 1540–1551. https://doi.org/10.1080/01496395.2018.1442864

Wang, S. S. S., Hung, Y. T., & Lin, Y. C. (2010). Kinetic studies of the oxidation of glutathione in protein refolding buffer. *Bioprocess and Biosystems Engineering, 33*(2), 277–286. https://doi.org/10.1007/S00449-009-0322-0

Wang, Y., Xiao, T., Zhang, Z., & Feng, X. (2021). Extraction and concentration of glutathione from yeast by membranes. *Canadian Journal of Chemical Engineering, 100*(S1), S195–S204. https://doi.org/10.1002/cjce.24084

Wouters, P. C., Alvarez, I., & Raso, J. (2001). Critical factors determining inactivation kinetics by pulsed electric field food processing. *Trends in Food Science & Technology, 12*, 112–121.

Wu, X., Tang, L., Du, Y., & Xu, Z. (2010). Improving glutathione extraction from crude yeast extracts by optimizing aqueous two-phase system composition and operation conditions. *Korean Journal of Chemical Engineering, 27*(6), 1829–1835. https://doi.org/10.1007/s11814-010-0308-2

Zeko-Pivač, A., Habschied, K., Kulisic, B., Barkow, I., & Tišma, M. (2023). Valorization of spent Brewer's yeast for the production of high-value products, materials, and biofuels and environmental application. *Fermentation, 9*(3), 208. https://doi.org/10.3390/FERMENTATION9030208/S1