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Selective extraction of biomolecules from *Saccharomyces cerevisiae* assisted by high-pressure homogenization, pulsed electric fields, and heat treatment: Exploring the effect of endogenous enzymes

J. Marín-Sánchez, A. Berzosa, I. Álvarez, C. Sánchez-Gimeno, J. Raso *

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

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ABSTRACT

Saccharomyces cerevisiae contains valuable compounds sought by the industry, prompting the exploration of effective extraction methods. Three physical methods were compared—High-Pressure Homogenization (HPH), Pulsed Electric Fields (PEF), and Heat Treatment (HT)—for releasing diverse bioproducts (amino acids, glutathione, proteins, and mannoproteins) from *S. cerevisiae*. Treatments (HPH: 2 passes at 100 MPa, PEF: 15 kV/cm for 100 μs (44.9 kJ/kg), HT: 60 °C for 5 min) affected over 90% of *S. cerevisiae* cells. Despite permeabilization, HT exhibited low efficacy in releasing compounds. HPH led to complete cell disruption, extracting maximum glutathione (1.09 ± 0.04 g/100 g_{dw}) and proteins (68.95 ± 0.96 g/100 g_{dw}) after 2 h. PEF facilitated the gradual extraction of low molecular weight compounds while leaving high molecular weight compounds unreleased initially. After 48 h, 72% of the total protein and amino acid content was released through hydrolysis catalyzed by endogenous yeast proteases. Furthermore, 64.1% of mannoproteins were obtained after 72 h of incubation. This study highlights the efficacy, scalability, and industrial potential of HPH and PEF, demonstrating their ability to produce tailored extracts over time and underscoring the significant role of endogenous enzymes in optimizing extraction yields. These findings provide valuable insights for enhancing efficiency and sustainability in bioproduct extraction from *S. cerevisiae*.

1. Introduction

Saccharomyces cerevisiae, a yeast extensively utilized in various industrial processes, possesses fermentative capabilities that have been exploited for centuries in the production of a wide range of fermented products, including bread, wine, and various alcoholic drinks (Gautério et al., 2023). Additionally, these microorganisms contain multiple compounds, such as proteins, amino acids, and other bioactive molecules, with potential applications across diverse sectors including food, feed, and pharmaceuticals (Jaeger et al., 2020). Yeast extracts, for example, have been found to possess a well-balanced amino acid profile that can serve as a protein-rich bioactive ingredient to enhance the nutritional value of foods or to prepare food supplements (Ribeiro-Oliveira et al., 2021; Vieira et al., 2016). Other compounds present in yeast, such as glutathione, a non-protein thiol compound, hold significant promise as antioxidants (Santos et al., 2022). Furthermore, the yeast cell wall represents a potential source of valuable compounds, including mannoproteins, which present numerous opportunities for improving the technological and oenological properties of wine (De Iseppi et al., 2020). In recent years, mannoproteins have also attracted attention due to their alleged health-promoting functions, such as stimulation of angiogenesis and antineoplastic activities (Liu et al., 2011, 2015; Yoon et al., 2019). Additionally, yeasts such as *S. cerevisiae* are among the most widely used microorganisms for producing a variety of recombinant proteins (Parapouli et al., 2020).

Obtaining bioproducts produced by yeasts for different applications requires a release step to extract them from the cell. However, extracting substances of interest faces challenges due to the barrier effect of the cell wall and cytoplasmic membrane (Ganeva et al., 2020; Klis et al., 2006). The yeast cell wall is commonly regarded as an entirely permeable layer, conferring rigidity to the cell while forming a widely open network through which most intracellular compounds can pass. However, the cytoplasmic membrane typically restricts the passage of most solutes unless facilitated by specialized transport mechanisms (Cohen, 2004).

The procedure selected for extracting intracellular compounds not only determines the efficiency of the extraction yield but also affects the

* Corresponding author. 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).

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particle size distribution of the cell debris and the number of released contaminants, which subsequently impact downstream procedures and the economic cost of the process (Balasundaram et al., 2009). Two distinct strategies can be followed for releasing compounds from microbial cells: obtaining a mixture of all compounds, which generally requires subsequent purification processes, or achieving a selective release of the targeted compounds (Gautério et al., 2023). In this study, the advantages and disadvantages of these two strategies were evaluated by comparing the performance of high-pressure homogenization (HPH), which causes mechanical cell disruption, with two procedures—pulsed electric fields (PEF) and heat treatment (HT)—that increase cell permeability without cell lysis or destruction of the cell structure.

HPH is a physical procedure that forces the cell suspension through a narrow nozzle or valve at high pressure, primarily causing cell disruption through fluid shear forces (Nemer et al., 2021). It has demonstrated success in disrupting cells from various yeasts, enabling the recovery of a diverse array of compounds including enzymes, yeast extract, β-glucans, or lipids (Dimopoulos et al., 2020; Gorte et al., 2020; Patil et al., 2016). PEF induces electroporation of the cell membranes because of the electric field generated by the application of very short pulses of high voltage between two electrodes where the cell suspension is located (Mahnič-Kalamiza & Miklavčič, 2022). Pores created by PEF in the cytoplasmic membrane facilitate the release of intracellular compounds from both eukaryote and prokaryote cells (Buchmann et al., 2019; Ganeva et al., 2003; Kotnik et al., 2012). Finally, thermal processing increases the permeability of the cytoplasmic membrane, as evidenced by the loss of intracellular compounds such as ions and UV-absorbing substances (Cebrián et al., 2017).

The effectiveness of PEF, HPH, and HT in releasing target components from various microalgae biomass has been previously assessed (Carullo et al., 2020; Grimi et al., 2014; Safi et al., 2017). However, in these studies, the efficiency of these techniques was compared after a few hours or immediately following the application of the treatments. Under these extraction conditions, the impact of endogenous enzymes released from vacuoles due to the treatments on the extraction of cell components has not been evaluated. Recently, it has been demonstrated that these enzymes play an important role in the extraction of biomolecules from yeast biomass treated with PEF when the extraction time is extended (Berzosa et al., 2023; Yang et al., 2021). Conversely, the effect of these enzymes on the extraction of compounds from yeast treated with HPH and HT remains unknown.

This study aimed to compare the efficiency and performance of three physical procedures—HPH, PEF, and HT—on the release of various bioproducts (amino acids, glutathione, proteins, and mannoproteins) produced by the yeast *S. cerevisiae*. The effect of endogenous enzymes released because of the treatments was also evaluated over the incubation period.

2. Materials and methods

2.1. Microorganism and growth conditions

Saccharomyces cerevisiae SafAle S-04 brewing commercial strain was supplied by Fermentis (Lesaffre, Marcq-en-Barœul, France). Precultures were prepared by inoculating a test tube containing 10 mL of Sabouraud Dextrose Liquid Medium (Oxoid, Basingstoke, UK) and incubation for 24 h at 25 °C. Flasks containing 650 mL of Saboraud Dextrose Liquid Medium (Oxoid) were inoculated with the preculture medium at a concentration of 10^4 CFU/mL. The culture was incubated at 25 °C under orbital shaking until the stationary growth phase was achieved after 48 h.

2.2. Methods for improving the release of compounds

HPH, PEF, and HT were tested for improving the release of compounds from *S. cerevisiae* cells. Before the treatments, the biomass obtained from the culture was centrifuged at 3000 *g* for 10 min at 20 °C and resuspended in citrate-phosphate McIlvaine buffer of pH 7 and a conductivity of 2 mS/cm, to a final concentration of 10^8 CFU/mL measured in a Thoma chamber (ServiQuimia, Constantí, Barcelona).

2.2.1. High-pressure homogenization treatment (HPH)

A HPH unit (Niro Soavi Panda, GEA, Düsseldorf, Germany) was used to apply up to 3 passes at 100 MPa at a flow of 10 L/h. Before each treatment, the apparatus underwent disinfection by recirculating a 50% ethanol solution and rinsing it with sterile water. After the treatment the collected sample was cooled in an ice water bath for 5 min. The temperature reached after the treatment that was determined with a type K thermocouple (Ahlborn, Holzkirchen, Germany) did not exceed 40 °C.

The effect of HPH on cells of *S. cerevisiae* was monitored by comparing the number of intact cells before and after the different passes under the microscope (Y-FL, Nikkon, Tokyo, Japan) using a Thoma chamber (ServiQuimia). Cell destruction was expressed as the percentage of fragmented cells.

2.2.2. Pulsed electric fields treatment (PEF)

PEF treatment was performed in a commercial device (Vitave, Prague, Czech Republic). The actual applied voltage was monitored with a high-voltage probe (P6015A, Tektronik, Wilsonville, Oregon, USA) connected to an oscilloscope (TDS 220, Tektronik). The temperature reached after the treatment was determined with a type K thermocouple located at the exit of the treatment chamber A refrigeration circuit was installed after the treatment chamber to cool the samples to 20 °C immediately after the treatment.

A titanium parallel electrode chamber of 0.4 cm gap, 3.0 cm length, and 0.5 cm width was fed with a peristaltic pump (BVP, Ismatec, Wertheim, Germany) at a flow rate of 5 L/h. Treatments at 12, 15, and 18 kV/cm from 20 to 150 μs using monopolar square wave pulses of 3 μs width were applied. The total specific energy of the treatments ranged from 9.66 ± 2.1 to 109.83 ± 3.57 kJ/kg corresponding to exit temperatures from 24.9 ± 0.4 to 48.8 ± 0.8 °C.

The permeabilization of yeast cells following PEF treatment was evaluated by assessing the uptake of propidium iodide (PI) fluorescent dye (Sigma-Aldrich, Barcelona, Spain) inside the cells as previously described (Martínez et al., 2016). Permeabilization was expressed as the percentage of permeabilized cells, calculated from the total number of cells in the field of view of the microscope and the number of fluorescently stained cells in the same field.

2.2.3. Heat treatment (HT)

HT was conducted in test tubes immersed in a thermostatic bath at 40, 50, and 60 °C for times ranging from 1 to 30 min. The range of specific energy required to achieve the treatment temperature varied from 83.6 to 167.2 kJ/kg. The treatment temperature was monitored using a K-type thermocouple probe. Following the treatment, the collected sample was then cooled in an ice water bath for 5 min.

The permeabilization of yeast cells following HT treatment was evaluated by assessing the uptake of PI fluorescent dye as described above.

2.3. Monitoring the release of compounds from S. cerevisiae

After each treatment, the suspensions were incubated at 35 °C, and compound release was monitored at 2, 6, 24, 48, and 72 h. The concentration of free α-amino nitrogen, glutathione, proteins, mannoproteins and protease, β-glucosidase, and β-glucanase activity was determined in the supernatant after centrifugation at 1593*g* for 10 min.

Bead mill treatment (BioSpec Products, Bartlesville, USA) was used to determine the total concentration of the different compounds of interest in the yeast cells assayed following the protocol previously described by Berzosa et al. (2023).

2.4. Analytical methods

2.4.1. Free α-amino nitrogen (FAN) concentration

The quantification of free α-amino nitrogen (FAN) was carried out using the ninhydrin assay, based on the method described by Dimopoulos et al. (2018). The assay relies on the oxidation and decarboxylation of alpha-amino acids by ninhydrin, followed by the reaction of the reduced ninhydrin with unreduced ninhydrin to form a blue complex with strong absorbance at 570 nm. Briefly, 500 μL of the properly diluted extract in distilled water was mixed with 250 μL of Ninhydrin Reagent (Sigma-Aldrich, Missouri, USA) and incubated for 15 min at 100 °C. After cooling the suspensions in an ice-water bath for 5 min, 1.25 mL of stop solution (0.2 g KIO3 in a solution of 60 mL/100 mL water and 40 mL/100 mL ethanol) was added to prevent further color development. The absorbance at 570 nm was measured against a blank prepared with distilled water instead of extract. Results were expressed as g of L-glycine equivalents per 100 g of dry weight.

2.4.2. Reduced glutathione

The colorimetric determination of reduced glutathione (GSH) was performed using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Thermo Fisher Scientific, Rockford, USA), according to the modified method described by Rahman et al. (2007) with modifications described by Berzosa et al. (2023). GSH reacts with DTNB to form the chromophore TNB (5-thio-2-nitrobenzoic acid), which has a maximum absorbance of 412 nm. Briefly, 20 μL of the sample was added to 960 μL of phosphate buffered saline (PBS) pH 7.5 containing 5.6 mmol/L EDTA (Sigma-Aldrich) and 0.4 g/100 mL DTNB solution. After incubation for 2–10 min at room temperature, absorbance was measured at 412 nm. Glutathione concentrations were calculated using a standard curve prepared with reduced L-glutathione (Sigma-Aldrich) in the concentration range of 3.9–2000 μg/mL. Results were expressed as g reduced L-glutathione per 100 g of dry weight.

2.4.3. Proteins

Protein extraction was quantified using the commercial Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) based on the Biuret reaction, which involves the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium, followed by colorimetric detection of the cuprous cation (Cu^{1+}) using a single reagent containing bicinchoninic acid (BCA). Briefly, 25 μL of the sample (properly diluted in distilled water) was mixed with 200 μL of the working reagent, shaken, and incubated at 37 °C for 30 min protected from light. Absorbance was measured at 562 nm after incubation. A standard curve was generated with albumin in a concentration range of 2.00 to 0.06 mg/mL. Results were expressed as g of albumin equivalents per 100 g of dry weight.

2.4.4. Mannoproteins/mannose

Mannoproteins are complex glycoproteins composed of mannose units covalently linked to polypeptide chains. The release of mannoproteins from yeast cells was indirectly assessed by quantifying the concentration of mannose in the supernatant of cell suspensions (Martínez et al., 2016). This was achieved through hydrolysis of mannose chains constituting the mannoproteins into their monomeric form, using sulfuric acid (1.5 mol/L, 100 °C for 90 min) followed by neutralization with NaOH (2 mol/L). Mannose concentration was then measured by an enzymatic method according to Dupin et al. (2000), using a commercially available assay kit (D-Mannose, D-Fructose, D-Glucose Assay kit, Megazyme International, Wicklow, Ireland). The total mannose concentration was determined after hydrolysis of the whole yeast cell. Results were expressed as g mannose per 100 g of dry weight.

2.4.5. Protease activity

The assessment of protease activity was conducted using the commercial Pierce Colorimetric Protease Assay Kit (Thermo Fisher Scientific), where the quantification was based on the measurement of TNB-peptide formation with succinylated casein as the substrate. To prepare the substrate, a 1 g/100 mL solution was heated and stirred at 80 °C until complete dissolution. After cooling to room temperature, the solution underwent centrifugation at 12,000 *g* for 10 min, and the resulting supernatant was stored at 0 °C.

For the protease activity measurement, a mixture of 400 μL of the sample and 400 μL of the substrate was incubated for 1 h at 40 °C in a thermostatic bath. Following incubation, 500 μL of 5 g/100 mL trichloroacetic acid was added and kept in an ice water bath for 30 min. The samples were subsequently centrifuged at 12,000 *g* for 10 min, and 200 μL of the supernatant was collected in a 96-well plate. The absorbance was then measured at 340 nm. The enzymatic activity was defined in katal (kat), with 1 kat representing the amount of protease that hydrolyzes 1 mol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per second at 40 °C. The results were then expressed as a percentage of the enzymatic activity, relative to the enzymatic activity of the extract obtained after treatment with the bead mill.

2.4.6. β-glucosidase activity

The activity of β-glucosidase was assessed by measuring the release of p-nitrophenol (pNPh) using 4-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate of the enzyme. The pigmented product was detected spectrophotometrically at 400 nm. The assay was carried out according to the method described by Hernández et al. (2003), with some modifications. Briefly, 800 μL of extract (properly diluted in distilled water) was mixed with 1 mL of reaction mixture containing 5 mmol/L of pNPG in McIlvane buffer pH 4.0 and incubated at 50 °C for 25 min. The reaction was stopped by adding 2 mL of 1 mol/L Na_2CO_3. The absorbance at 400 nm was measured against a reaction blank, which was prepared by adding 2 mL of 1 mol/L Na_2CO_3 to 800 μL of sample and then adding 1 mL of reaction mixture. β-glucosidase activity was determined using a p-nitrophenol standard curve. The enzymatic activity was measured in katal (kat), with 1 kat representing the amount of β-glucosidase that hydrolyzes the substrate to generate an absorbance equivalent to 1 mol of p-NPh per second. The results were then expressed as a percentage of the enzymatic activity, relative to the enzymatic activity of the extract obtained after treatment with the bead mill.

2.4.7. β-glucanase activity

The assessment of β-glucanase activity was performed using the commercial β-Glucanase Assay Kit (Megazyme International). In this assay, samples were incubated with Azo-Barley glucan substrate under specific conditions. The dyed substrate undergoes depolymerization by yeast β-glucanase, resulting in fragments that become soluble in the presence of a precipitant solution. Upon centrifugation, the absorbance at 590 nm of the supernatant solution correlates directly with the yeast β-glucanase levels in the samples.

Briefly, 0.5 mL of pre-warmed (40 °C) Azo-Barley glucan substrate solution was added to centrifuge tubes, and the tubes along with their contents were preincubated at 40 °C for 5 min. Subsequently, 1 mL of yeast extract was introduced into each tube, thoroughly mixed, and then incubated at 40 °C for 1 h. Afterward, 3 mL of precipitant solution (provided by the manufacturer) was added, vigorously mixed, and the tubes were allowed to stand at room temperature for 5 min. The tubes underwent centrifugation at 1000*g* for 10 min, and the absorbance of the supernatants was measured against a reaction blank in distilled water. The activity of β-glucanase was measured in katal (kat), where 1 kat corresponds to the amount of enzyme that hydrolyzes the substrate to generate an absorbance increase equivalent to 1 mol of substrate per second. The results were then expressed as a percentage of the enzymatic activity, relative to the enzymatic activity of the extract obtained after treatment with the bead mill.

2.5. SDS-PAGE

Protein detection was conducted using 4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad, California, USA). In brief, 15 μL of undiluted sample was combined with 14.25 μL of Laemmli Sample Buffer (Bio-Rad) and 0.75 μL of 2-Mercaptoethanol (Bio-Rad), followed by heating at 95 °C for 5 min. Subsequently, 20 μL of the mixture was loaded into the gel, and Precision Plus Protein™ Standard (Bio-Rad) was added directly into the gel. SDS-PAGE was performed using a Mini Protean® 3 Cell (Bio-Rad) connected to a PowerPac™ Basic Power Supply (Bio-Rad), with a running buffer consisting of 25 mmol/LTRIS, 0.1 g/100 mL SDS, 250 mmol/L glycine, pH 8.3.

Following electrophoresis, protein bands were visualized by exposing the gel to a Gel Doc™ EZ System with a Stain-Free Tray (Bio-Rad). The gel was irradiated at a wavelength of 302 nm for 5 min, initiating a chemical reaction between the indole of tryptophan and the TCE embedded in the gel. Consequently, bands corresponding to specific proteins became visible under UV light (Ladner et al., 2004).

2.6. Statistical data analysis

The results are presented as mean ± standard deviation of the mean, obtained from at least three replicates analyzed in duplicate (n = 6). Statistical significance among treatments was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using Graph-Pad 8.4.2 (Prism, San Diego, California, United States), considering statistical differences as significant at $p < 0.05$. In addition, the heat maps were performed in Graph-pad 8.4.2 (Prism), with the values of each parameter normalized based on the set of values of the whole column.

3. Results and discussion

3.1. Selection of the HPH, PEF, and HT treatments for improving extraction

The evaluation of the impact of different disruption and permeabilization procedures on yeast cells was conducted based on their respective mechanisms of action. Phase-contrast microscopy images (Fig. 1) illustrate the diverse effects of HPH, PEF, and HT on yeast cell structure. While HPH resulted in complete cell disruption and debris formation, PEF and HT maintained cell structure while rendering the cytoplasmic membrane permeable to PI.

Fig. 2 illustrates the impact of treatment intensity of HPH on cell destruction, and PEF and HT on cytoplasmic membrane permeabilization as assessed by PI uptake. The efficacy of these techniques for obtaining microbial bioproducts has been previously documented by various authors (Dimopoulos et al., 2020; Ganeva et al., 2020; Ren et al., 2007). However, most studies either assay only one of these techniques or fail to define treatment conditions affecting the same proportion of the cell population, thereby complicating the effectiveness of comparisons.

Cells of *S. cerevisiae* exhibited high sensitivity to HPH treatments. One pass at 100 MPa disrupted approximately 83.8 ± 1.8% of the

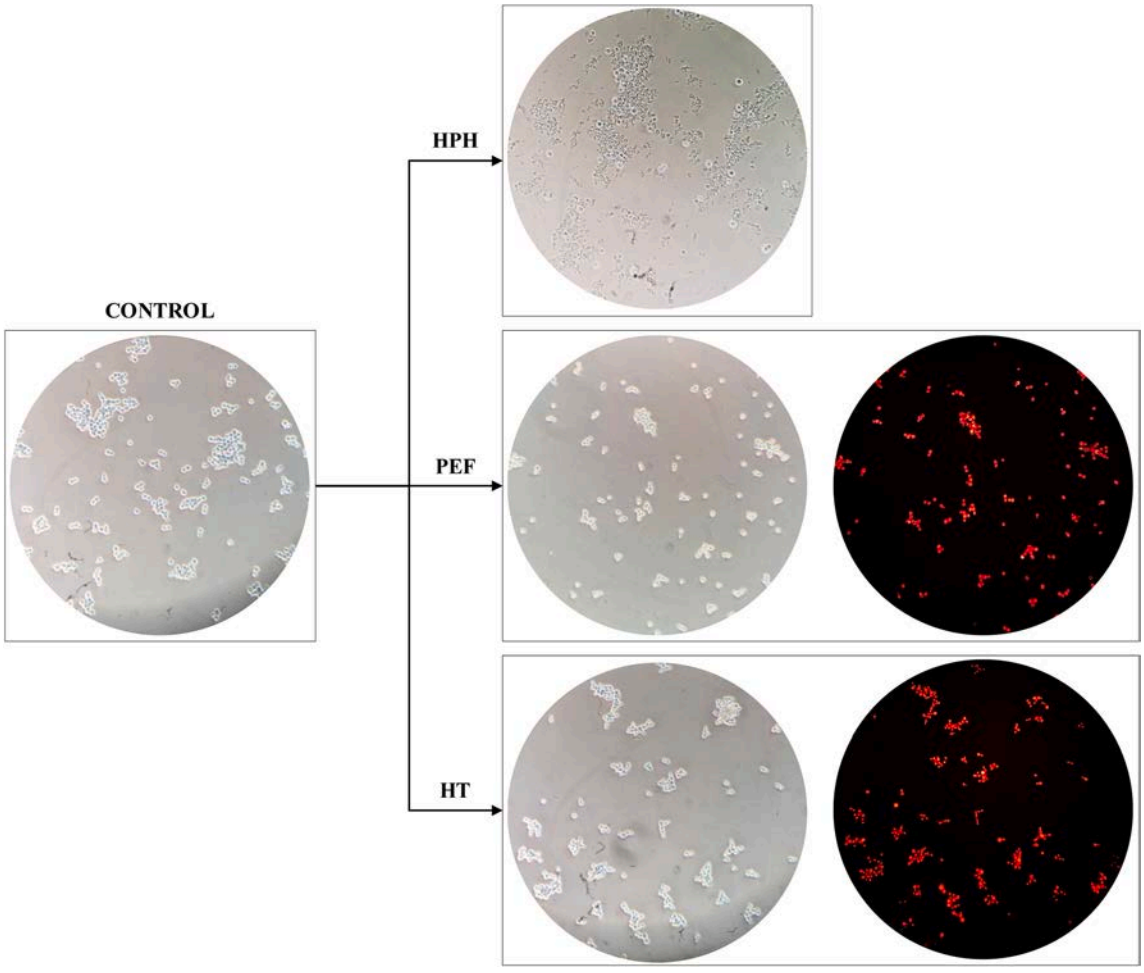

Fig. 1. Images of *S. cerevisiae* cells after high-pressure homogenization (HPH), pulsed electric fields (PEF) and heat treatment (HT) observed under a phase-contrast microscope at 40× magnification. The images corresponding to the cells treated by PEF and HT were also captured after propidium iodide staining.

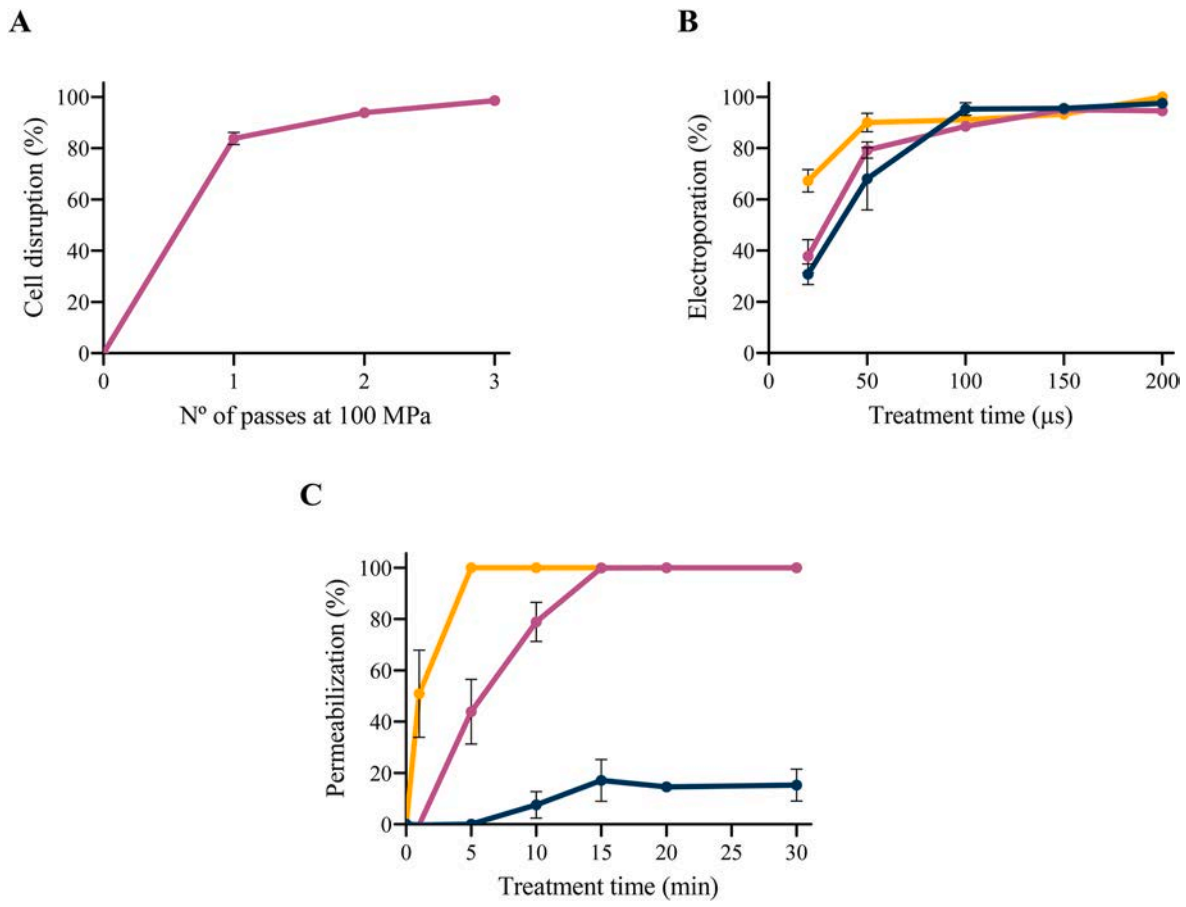

Fig. 2. Percentage of disrupted *S. cerevisiae* cells after HPH (100 MPa) (**A**). Percentage of electroporated *S. cerevisiae* cells after PEF treatment at (●) 12 kV/cm, (●) 15 kV/cm and (●) 18 kV/cm (**B**). Percentage of permeabilized *S. cerevisiae* cells after HT at (●) 40 °C, (●) 50 °C and (●) 60 °C (**C**).

population, while after 2 passes, around 93.9 ± 0.6% of cells were broken (Fig. 2A). HPH has been utilized to disrupt cells from various yeast strains, recovering intracellular compounds such as proteins, lipids, pigments, or enzymes. Studies have shown that treatment efficacy depends on operating variables such as pressure, number of passes, and temperature, as well as cell characteristics influencing disruption effectiveness (Dimopoulos et al., 2020; Gautério et al., 2023; Maresca et al., 2011; Patrignani et al., 2009).

The effect of PEF treatments of different intensities on *S. cerevisiae* cell permeabilization increment is depicted in Fig. 2B. At shorter treatment times, PEF efficacy in permeabilizing *S. cerevisiae* cells was higher at 18 kV/cm. However, with extended treatment duration to 100 µs, the influence of electric field strength diminished. Under these conditions, over 90% of *S. cerevisiae* cells became permeable to PI. These results, coupled with insights from other studies on electroporation of *S. cerevisiae* cells by PEF (Berzosa et al., 2023; Cserhalmi et al., 2002; Martínez et al., 2016; Q. Zhang et al., 1994), suggest an electric field threshold for inducing significant electroporation in *S. cerevisiae* likely above 10 kV/cm, with complete electroporation achievable via treatments of moderate duration between 10 and 20 kV/cm.

Cell permeabilization by HT was dependent on temperature and treatment time. Treatment at 40 °C proved inadequate to permeabilize more than 20% of the population at all treatment times assayed (Fig. 2C). Conversely, while at 50 °C, treatment needed to be extended for 15 min to permeabilize the entire population, the same effect at 60 °C required only 5 min. Although cellular permeabilization assessment was not conducted, several studies have reported enhanced extraction of intracellular compounds from various *S. cerevisiae* strains within the temperature range of 45–60 °C, with extraction yields beginning to decline beyond 60 °C (Alves et al., 2021; Yoshikawa et al., 1994).

3.2. Extraction of intracellular compounds from S. cerevisiae cells after HPH, PEF, and HT

To effectively compare the efficiency of different selected procedures for extracting compounds from *S. cerevisiae* cells, less intense treatments that impacted more than 90% of the cell population were established. Specifically, these conditions were 2 passes at 100 MPa for HPH, 100 µs at 15 kV/cm for PEF, and 5 min at 60 °C for HT. Fig. 3 illustrates the extraction of various intracellular compounds, such as amino acids, glutathione, and proteins, during a 48-h incubation period following the processing of yeast biomass using the selected HPH, PEF, and HT treatments.

3.2.1. Extraction of amino acids

Fig. 3A demonstrates the effect of different procedures on amino acid extraction during incubation. Amino acids located in the cytosol of *S. cerevisiae* cells (approximately 5 g/100 g_{dw}) were promptly released into the extraction medium regardless of the extraction method used. After 2 h of incubation, no statistically significant differences ($p < 0.05$) were detected in the concentration of amino acids in the extraction media containing yeast biomass treated by HPH, PEF, or HT. The low molecular weight of amino acids enabled similar extraction efficiency in cells disrupted by HPH and those permeabilized by PEF or HT.

With extended incubation time, the amino acid content in the extraction medium notably increased, particularly for yeast treated by HPH and PEF, reaching values of 18.53 ± 0.36 and 16.97 ± 0.21 g/100 g_{dw} after 48 h, respectively. This increase is attributed to protein autolysis catalyzed by endogenous proteases released from yeast vacuoles. To confirm this, the proteolytic activity of endogenous yeast proteases liberated into the extraction medium was evaluated (Table 1).

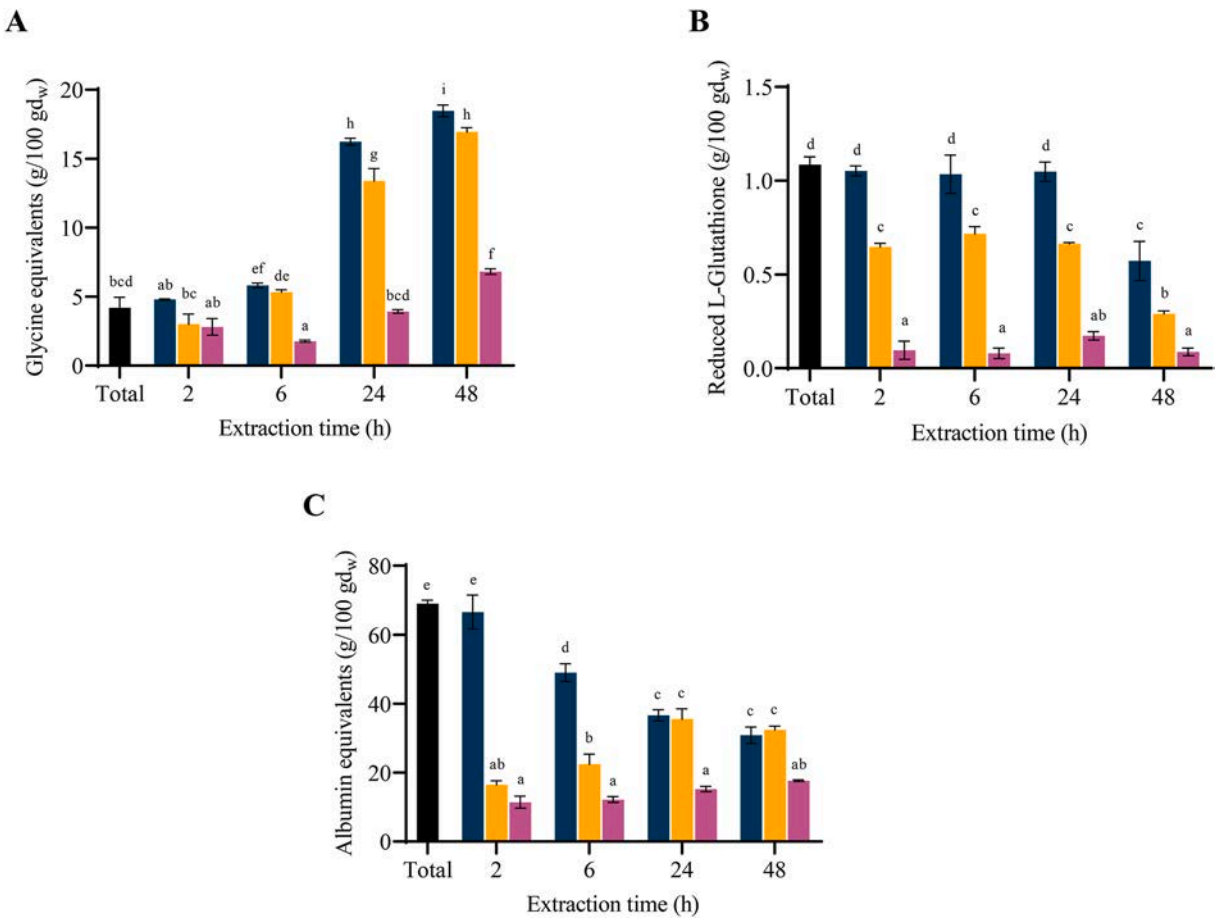

Fig. 3. Extraction of free α-amino nitrogen (**A**), reduced glutathione (**B**) and (**C**) proteins over incubation time at 35 °C from *S. cerevisiae* cells after (●) HPH, (●) PEF and (●) HT. Total: extraction of compounds after (●) bead mill treatment. HPH (2 passes at 100 MPa), PEF (15 kV/cm for 100 μs), and HT (60 °C for 5 min). Bars with different letters indicate significant differences ($p < 0.05$) according to ANOVA and Tukey's post hoc test results.

Table 1
Proteolytic activity of *S. cerevisiae* supernatants subjected to HPH (2 passes at 100 MPa), PEF (15 kV/cm for 100 μs), and HT (60 °C for 5 min) over the extraction time. Values are expressed relative to the activity of the bead mill-treated extract (total) (mean ± SD, $n = 6$). ND: no detected. Different letters indicate significant differences ($p < 0.05$) according to ANOVA and Tukey's post hoc test results. The statistical analysis was conducted by comparing the values across both treatment types and extraction times.

Enzyme relative activity (%)	2 h	24 h	48 h	72 h
HPH	100.2 ± 2.2^{b}	99.5 ± 1.5^{b}	250.4 ± 9.6^{c}	313.1 ± 7.3^{d}
PEF	ND	ND	29.0 ± 0.2^{a}	23.5 ± 2.4^{a}
HT	ND	ND	ND	ND

For samples treated by HPH, the proteolytic activity detected 2 h after treatment was equivalent to the total proteolytic activity detected in yeast biomass. These results confirm that HPH treatment led to the complete disruption of yeast cells and organelles, resulting in the total release of proteases primarily stored in vacuoles (Hecht et al., 2014; Okamoto et al., 2022; Vieira et al., 2016). However, proteolytic activity increased over incubation time, exceeding the initial level by over 2.5-fold after 48 h and over 3-fold after 72 h. This increase is likely related to protective mechanisms involved in preventing yeast autolysis under physiological conditions. Some endogenous yeast proteases are initially found in an inactive state with activation following a gradual controlled process through a cascade of hydrolytic events (Hecht et al., 2014). Therefore, the rise in protease activity over time in extraction media containing yeast biomass treated by HPH suggests the release of both active and initially inactive proteases, gradually activated. This activation of inactive forms could explain the sharp increase in amino acid concentration in the extraction medium after 24 h of incubation.

In the case of yeast cells treated by PEF, a similar increase in proteolytic activity in extraction media would be expected due to the observed similar increase in amino acid extraction over time. However, protease activity was not detected until the first 24 h of extraction, reaching only 29.0 ± 0.2% of activity after 48 h. Despite PEF being applied in the microsecond range not directly affecting cell organelles such as vacuoles, different studies support that PEF triggers enzymatic activity (Berzosa et al., 2023; Canelli et al., 2022; Maza et al., 2020). This effect has been related to vacuole plasmolysis and subsequent enzyme release due to osmotic disequilibrium in the cytoplasm caused by water influx into electroporated cells (Mahnič-Kalamiza & Miklavčič, 2022; Martínez et al., 2016). However, released proteases from vacuoles, probably due to their size, were not able to exit through pores formed by PEF in the cytoplasmic membrane. Consequently, unlike biomass treated by HPH, where protein hydrolysis occurs in the extraction media after complete cell destruction, in the case of yeast cells treated by PEF, proteases would hydrolyze yeast proteins inside the cytoplasm. Amino acids generated and released into the extraction medium through membrane pores. The increase in pore sizes caused by PEF in the cytoplasmic membrane of electroporated cells and/or modification of cell wall integrity over incubation time could explain the detection of proteolytic activity in extraction media after 48 h. To confirm this hypothesis, further analysis of proteolytic activity in the extracellular environment was performed after 1 week, once

degradation of the cell wall by endogenous enzymes was advanced. After this incubation time, proteolytic activity was 4.5 times higher than the initial activity detected in yeast cells (data not shown). Therefore, these results reveal that the increase in amino acid concentration in extraction media containing cell biomass treated by PEF is due to the hydrolysis of intracellular proteins by proteases unable to cross the envelopes surrounding the cytoplasm of electroporated cells. Yang et al. (2021) reported the release of protease activity of *S. cerevisiae* treated by moderate PEF treatments (7 kV/cm for 4 ms), progressively increasing for the first 24 h of incubation, reaching the maximum value at 24 h. However, in this case, the electroporated cells were incubated at 55 °C. This higher incubation temperature could affect the size of the pores generated by PEF and/or accelerate autolysis, facilitating the release of amino acids into the extraction medium.

In comparison with HPH and PEF, the increase in amino acid concentration in extraction medium containing yeast cells treated by HT barely increased with extended incubation time by 24 h, and proteolytic activity was not detected even after 7 days of incubation (data not shown). Despite the similarity in cell permeabilization between yeast treated by HT and those treated by PEF, the thermal denaturation of some active or even inactive proteases gradually activated during autolysis could explain this behavior. Thermal denaturation of yeast endogenous proteases has been reported by different authors at temperatures ranging between 50 and 60 °C (Bilinski & Stewart, 1989; Dreyer et al., 1983; Meussdoerffer et al., 1980).

3.2.2. Extraction of glutathione

Glutathione, a tripeptide composed of L-glutamate, L-cysteine, and glycine, is synthesized in the cytoplasm and plays crucial roles in cellular defense against oxidative stress, detoxification, and maintenance of redox balance (Lu, 2009). Its reduced form exhibits significant antioxidant activity, surpassing even that of ascorbic acid (Foyer & Noctor, 2011). *S. cerevisiae* stands out as a primary source for obtaining glutathione for commercial applications (Bahut et al., 2020; Santos et al., 2007; Schmacht et al., 2017).

The HPH treatment resulted in the complete extraction of glutathione within 2 h post-treatment, yielding a concentration of 1.09 ± 0.04 g/100 g_{dw} (Fig. 3B). This concentration remained stable during the initial 24 h of extraction but decreased by approximately half with extended extraction time. This reduction may stem from the reaction of glutathione with other compounds released from yeast, altering its structure and properties (Santos et al., 2022).

For yeast biomass treated with PEF, the extraction detected after 2 h amounted to approximately 70% of total glutathione. Similar to HPH-treated cells, this concentration remained constant for the initial 24 h of extraction before declining. Although glutathione, with its small molecular weight (0.3 kDa), would be expected to readily exit the cytosol through electroporated cytoplasmic membrane pores, not all detected glutathione was released to the extraction media in this study. The fact that in some yeast strains, glutathione may be stored in different cellular compartments or attached to other molecules could explain the reason why in this study not all the glutathione content was released to the extraction media in the electroporated yeast biomass (Zechmann et al., 2011). The low extraction rate (9%) observed in yeast biomass treated with HT may be attributed to the high reactivity of glutathione at elevated temperatures (Wang et al., 2010).

3.2.3. Extraction of proteins

Similar to amino acids and glutathione, the total protein content (68.95 ± 0.96 g/100 g_{dw}) in yeast cytoplasm was released into the medium following HPH treatment. HPH's efficiency in extracting proteins from microorganisms is well-documented in the literature (Carullo et al., 2020; Comuzzo et al., 2015; Katsimichas et al., 2023). However, unlike previous studies where protein release was only assessed immediately after treatment, a decline in protein concentration in the aqueous extract over incubation time was observed in our study (Fig. 3C). This decline appears associated with protein hydrolysis catalyzed by endogenous proteases. Indeed, after 6 h of incubation, the added protein (49.09 ± 2.44 g/100 g_{dw}) and amino acid (5.81 ± 0.17 g/100 g_{dw}) content in the extraction media almost matched the total protein content in yeast cytoplasm.

For PEF-treated yeast, most of the protein content was retained inside the cells after 2 h of incubation, resulting in a relatively low extraction yield (16.51 ± 0.70 g/100 g_{dw}). However, protein extraction gradually increased over time, stabilizing after 24 h (Fig. 3C), consistent with findings from other studies (Grimi et al., 2014; Ohshima et al., 2000; Scherer et al., 2019). It is established that the yeast cell wall is permeable to proteins with a molecular weight of up to 400 kDa (Ganeva & Galutzov, 1999; Orlean, 2012). Therefore, the initial low protein extraction yield from electroporated yeast cells might be attributed to that pores may not be sufficiently large to release proteins with higher molecular weights or to the electrostatic interactions of intracellular proteins with the negatively charged phosphate groups in the mannane side chains (Ballou, 1976). The increase in free amino acids released from yeast cells treated with PEF during extended incubation suggests simultaneous phenomena occurring in the yeast cytoplasm that affect protein extraction. On one hand, endogenous proteases hydrolyze proteins, releasing amino acids that exit the cell. Simultaneously, this process reduces protein size, facilitating their passage through the cytoplasmic membrane and the cell wall. Consequently, the combined value of free amino acids and proteins extracted from electroporated yeast biomass after 48 h yielded 49.34 ± 0.88 g/100 g_{dw}, representing 72% of the total protein content in the cytoplasm (Fig. 3).

As anticipated from its effectiveness in amino acid and glutathione extraction, HT demonstrated minimal efficacy in protein extraction. Initial protein extraction from HT-treated yeast was notably less efficient (11.48 ± 1.61 g/100 g_{dw}), with a minimal increase over the incubation period (Fig. 3C). Smaller pore sizes formed in the cytoplasmic membrane by HT, potential thermal denaturation of proteases, and protein aggregation due to heating may impede intracellular protein extraction from HT-treated yeast (O'Connell et al., 2014; Tyedmers et al., 2010).

3.3. Extraction of mannoproteins from the cell wall of S. cerevisiae cells after HPH, PEF, and HT

Mannoproteins constitute the outermost layer of the *S. cerevisiae* cell wall, and their extraction necessitates the hydrolysis of the cell wall. Given that mannose in yeast primarily resides in mannoproteins, a straightforward method for quantifying mannoproteins with high reproducibility involves the acid hydrolysis of soluble polysaccharides followed by mannose quantification (Quirós et al., 2012). Fig. 4

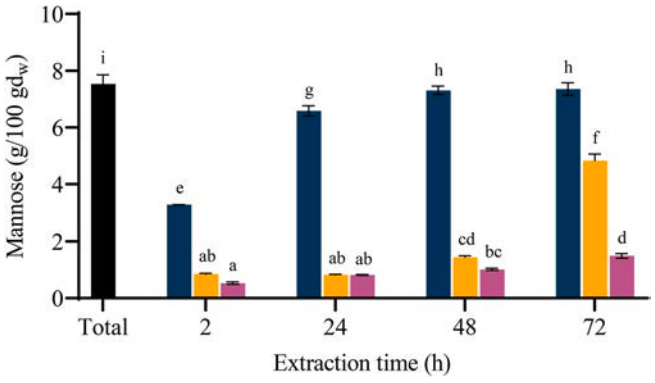

Fig. 4. Extraction of mannose over incubation time at 35 °C from *S. cerevisiae* after (●) HPH, (●) PEF and (●) HT. Total: extraction of compounds after (●) acid hydrolysis of the whole yeast cell. HPH (2 passes at 100 MPa), PEF (15 kV/cm for 100 μs), and HT (60 °C for 5 min). Bars with different letters indicate significant differences ($p < 0.05$) according to ANOVA and Tukey's post hoc test results.

illustrates the mannose levels determined in the extraction medium containing yeast biomass treated by HPH, PEF, and HT, along with the total mannose content in the yeast biomass post-acid hydrolysis.

Following HPH treatment, approximately half of the mannoprotein content in the yeast biomass was released into the extraction media after just 2 h of incubation. Despite the complete destruction of cell structure by HPH, some mannoprotein-containing cell wall fragments remained insoluble and precipitated after centrifugation. Extending the incubation to 24 h, facilitated the extraction of all mannoproteins into the supernatant. Thus, while physical destruction of cell walls due to HPH released roughly half of the mannoproteins, the remainder required the hydrolysis of cell wall fragments by endogenous enzymes like β-glucanases and β-glucosidases (Liberatore et al., 2010; Nguyen, 1982). Table 2 reveals that both β-glucanase and β-glucosidase activities were detected in the extraction medium containing yeast biomass treated by HPH after 2 h of incubation, indicating that the rupture induced by HPH allowed these enzymes access the cell wall fragments to initiate hydrolysis, ultimately leading to complete mannoprotein release after 24 h. Notably, while β-glucosidase showed a pronounced decline, β-glucanase activity increased during the extraction period (Table 2). This increment could be related to the fact that similarly to proteases, some inactive β-glucanases require an activation process (Baladrón et al., 2002).

In contrast, mannoprotein release from PEF-treated biomass was minimal during the initial 48-h incubation period. However, extending incubation to 72 h resulted in 64.1% mannose extraction. Enzymatic activity analysis revealed over 30% of β-glucosidase and β-glucanase activity in the extraction medium after a 2-h incubation of electroporated yeast, indicating enzyme penetration through the cytoplasmic membrane to access the external cell wall region where mannoproteins reside (Kanauchi & Bamforth, 2012; McMahon et al., 1999). Reduced enzyme activity in the extraction medium containing PEF-treated cells, especially β-glucanase, likely contributed to the treatment's lower efficacy in liberating mannoproteins from the cell wall.

HT treatment resulted in consistently low mannoprotein extraction throughout the incubation period, reaching only 19.8% of total mannose after 72 h (Table 2). This inefficiency may be attributed to smaller pore sizes in the cytoplasmic membrane formed by HT compared to PEF, or to potential thermal denaturation of β-glucosidase and β-glucanase, which could explain HT's lower efficacy.

3.4. Comparison of HPH and PEF for extraction of compounds of interest from S. cerevisiae

Fig. 5 presents a succinct and schematic comparison of the efficiency of HPH and PEF in extracting compounds of interest from *S. cerevisiae*.

Table 2
β-glucanase and β-glucosidase activity of *S. cerevisiae* supernatants subjected to HPH (2 passes at 100 MPa), PEF (15 kV/cm for 100 μs), and HT (60 °C for 5 min) over the extraction time. Values are expressed relative to the activity of the bead mill-treated extract (total) (mean ± SD, $n = 6$). ND: no detected. Different letters indicate significant differences ($p < 0.05$) according to ANOVA and Tukey's post hoc test results. The statistical analysis was conducted by comparing the values of β-glucanase and β-glucosidase activity individually across both treatment types and extraction times.

Enzyme relative activity (%)		2 h	24 h	48 h	72 h
HPH	*β-glucanase*	97.2 ± 7.8^{b}	124.2 ± 7.5^{c}	218.2 ± 3.2^{d}	279.5 ± 9.8^{e}
	β-glucosidase	105.3 ± 4.1^{e}	79.1 ± 3.6^{d}	42.6 ± 4.8^{bc}	20.2 ± 0.1^{f}
PEF	*β-glucanase*	34.7 ± 2.8^{a}	90.0 ± 3.8^{b}	107.2 ± 4.7^{bc}	81.3 ± 8.2^{a}
	β-glucosidase	39.3 ± 0.2^{b}	52.1 ± 3.6^{c}	42.4 ± 1.9^{bc}	36.8 ± 0.9^{b}
HT	*β-glucanase*	ND	ND	ND	ND
	β-glucosidase	ND	ND	ND	ND

HT is not included due to its low efficacy in releasing compounds from yeast cells. Values in each cell are row-normalized, representing the relative extraction of each compound, with total compound extraction serving as the reference point.

HPH, causing complete disruption of yeast cells, facilitated the instant and efficient release of intracellular compounds regardless of their molecular weight (Fig. 5). Consequently, this technique serves as the primary strategy for immediate and non-selective extraction of intracellular components without requiring subsequent incubation. The SDS-PAGE of the obtained extracts from yeast cells treated by HPH after 2 h of incubation revealed the presence of many different bands with a molecular weight ranging from 10 to 75 kDa (Fig. 6). These bands correspond to the proteins located in the cytoplasm and probably some proteins of the cell wall. Therefore, HPH selectivity is poor, impacting downstream procedures and the process's economic cost due to cell debris and released contaminants resulting from complete disruption (Balasundaram et al., 2009; Geciova et al., 2002; Katsimichas et al., 2023).

In contrast, the efficacy of the PEF treatment is low for immediate extraction of all intracellular components (Fig. 5). However, PEF enables rapid extraction of low molecular weight compounds such as glutathione without the release of high molecular weight compounds like proteins. As compared with HPH extract obtained after 2 h only two weak bands which correspond to the molecular weight of 37 and 75 kDa were observed in Fig. 6. This suggests a promising approach for enhancing downstream procedures and reducing costs for obtaining low molecular weight intracellular products. It also opens the possibility of sequential extraction of different biomolecules from yeast. This study's results indicate that over time, endogenous enzymes play a pivotal role in extracting components from yeast cells. HPH-induced cell disruption and cytoplasmic electroporation significantly increased the enzymatic activity of various endogenous enzymes such as proteases, β-glucosidase, and β-glucanase. These enzymes contribute to yeast autolysis, degrading biomolecules like proteins and structures such as the cell wall. For HPH-treated yeast cells, these enzymes are immediately released into the extraction medium. However, in PEF-treated cells, some enzymes like proteases likely remain in the cytoplasm due to the inability to penetrate pores caused by PEF treatment. Enzymatic activity in the extraction medium is detected only after a post-PEF treatment period, during which pore size may increase (Bodénès et al., 2019; Saulis, 2010). Therefore, proteins remaining in the cytoplasm of electroporated cells are hydrolyzed into small proteins, peptides, and amino acids increasing the content of these compounds in the extracellular environment. Consequently, due to this proteolytic activity, the heat map corresponding to amino acids and proteins for both extracts as well as the SDS-PAGE profile tend to be similar by extending incubation time (Fig. 6).

The proteolytic phenomenon catalyzed by endogenous enzymes can be leveraged to obtain extracts with different properties. Promoting proteolysis during extraction by incubating cells is required for obtaining protein-hydrolyzed compounds like essential amino acids or peptides. Yeast proteins are rich in essential amino acids with high biological value, while peptides from protein hydrolysis possess biofunctional properties such as antihypertensive, antioxidant, and antimicrobial effects (Mirzaei et al., 2021; Oliveira et al., 2022; S. Zhang et al., 2021). Utilizing yeast's own enzymes for proteolysis can avoid the cost of commercial enzyme additions (Mirzaei et al., 2021; San Martin et al., 2021). As amino acids and peptides from protein hydrolysis can cross the electroporated yeast membrane due to their small size, electroporation of yeast cells should be preferred over complete cell disruption to reduce cell debris and obtain a more purified extract with less downstream procedure impact.

Mannoprotein release from HPH- or PEF-treated yeast cells required enzymatic cell wall hydrolysis. Therefore, to obtain a mannoprotein-rich extract from HPH-treated cells, an incubation of at least 24 h was necessary (Fig. 5). Longer incubations are needed for PEF-treated cells to

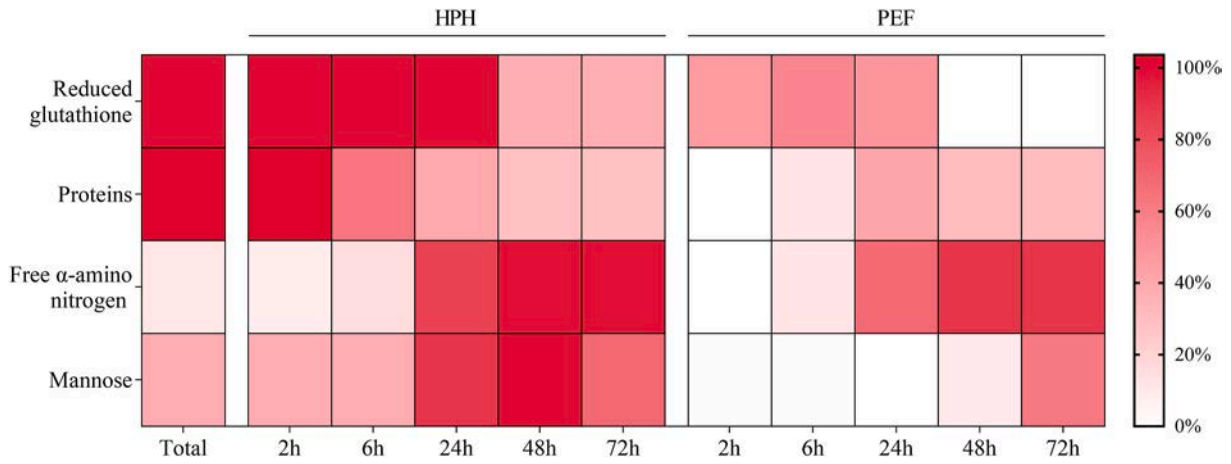

Fig. 5. Heat map of the extraction of compounds over incubation time at 35 °C from *S. cerevisiae* after HPH (2 passes at 100 MPa) and PEF (15 kV/cm for 100 μs) treatments. Values in each cell are row-normalized, representing the relative extraction of each compound. Total: extraction of compounds after bead mill treatment.

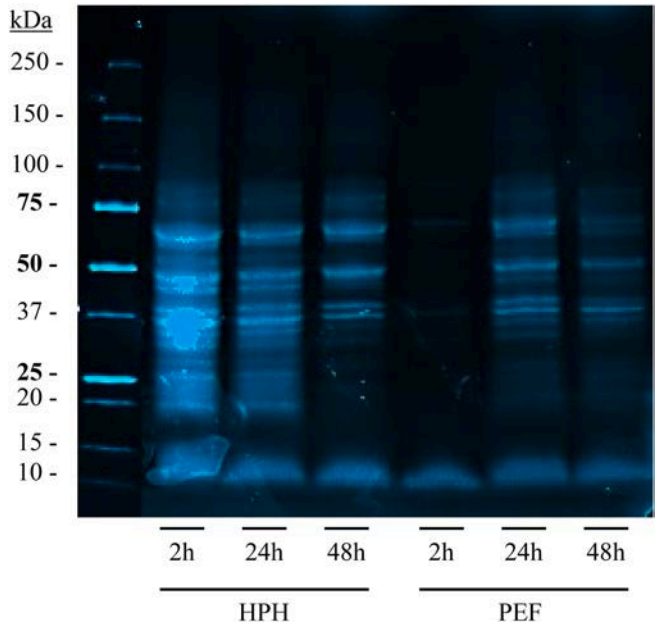

Fig. 6. SDS-PAGE analysis of the extracts obtained from *S. cerevisiae* after HPH (2 passes at 100 MPa) and PEF (15 kV/cm for 100 μs) treatments over incubation time. Separation was performed using a 4–15% protein gel. Lane 1: Precision Plus Protein™ Standard (Bio-Rad).

achieve a mannoprotein-rich extract. Although the mannoprotein concentration in prolonged incubation extracts is lower than in HPH-treated extracts, Fig. 5 shows that electroporation enables sequential extraction of different *S. cerevisiae* cell components. Within 2 h, a glutathione-rich extract with low concentrations of other compounds can be obtained. Most autolytic enzyme activity remains within the cytoplasm at this stage. Suspending the cells again and prolonging the incubation for 24–48 h allows extraction of a hydrolyzed extract rich in peptides and amino acids. Further extending the incubation to 72 h results in an extract enriched in mannoproteins.

4. Conclusion

This study evaluated two distinct approaches for releasing compounds from microbial cells. The performance of HPH, which induces mechanical cell disruption, was compared with PEF and HT, which increase cell permeability without causing lysis.

HT showed limited efficiency in extracting compounds from *S. cerevisiae*, likely due to the degradation of thermolabile compounds, thermal denaturation of enzymes, and small pore sizes in the cytoplasmic membrane.

Conversely, HPH and PEF were effective in extracting valuable molecules from *S. cerevisiae*. Endogenous enzymes play a crucial role in both techniques. HPH is more efficient for rapid, non-selective extraction, while PEF facilitates gradual, targeted release with fewer contaminants.

Both HPH and PEF are scalable, but further studies are needed to optimize conditions, evaluate impacts on purification, and characterize the functional and nutritional properties of the extracts for industrial applications.

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CRediT authorship contribution statement

J. Marín-Sánchez: Writing – original draft, Investigation, Formal analysis, Conceptualization. **A. Berzosa:** Investigation. **I. Álvarez:** Supervision. **C. Sánchez-Gimeno:** Writing – review & editing, Supervision, Conceptualization. **J. Raso:** Writing – review & editing, Supervision, Conceptualization.

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

During the preparation of this work, the authors used ChatGTP 3.5 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 declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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