Food and Bioproducts Processing 143 (2024) 45–53

Contents lists available at ScienceDirect

Food and Bioproducts Processing

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

Purification of bioactive peptides from spent yeast autolysates

Ana Sofia Oliveira [a], Ana Margarida Pereira [a,b,*], Carlos M.H. Ferreira [a,b,*], Joana Odila Pereira [a,b,*], Manuela Amorim [a], Margarida Faustino [a], Joana Durão [a,b], Manuela E. Pintado [a], Ana P. Carvalho [a]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal
[b] Amyris Bio Products Portugal Unipessoal Lda, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal

ARTICLE INFO

Keywords:
Spent yeast
Functional foods
Ultrafiltration
Size-exclusion chromatography
Bioactive peptides

ABSTRACT

Spent brewer's yeast have been described as an important source for bioactive peptides, with their properties well proven over the years. Besides the brewing sector, growing of large-scale processes on synthetic biology industry has generated a substantial amount of spent engineered yeast with further potential to be valued in a circular economy approach. In line with this perception, in this work, peptide-rich fractions were obtained from spent yeast peptide-rich extract autolysate ("ESY1"), using two different techniques, namely protein ultrafiltration and size-exclusion chromatography (SEC). Both methods allowed the production of different peptide fractions with diverse protein content, molecular weight distribution and peptides sequences. Overall, fractions with higher protein content and more purified reveal higher bioactivities, namely antihypertensive and anti-cholesterolemic, emphasizing that the purification process is an important step to include in production process of a dietary supplement with these specific features. In addition, significant antioxidant capacity values for peptide fractions purified by SEC were obtained, highlighting their potential use in economic sectors where antioxidant capacity has a massive impact, such as cosmetics.

1. Introduction

As one of the main brewing by-products (2–4 kg of spent yeast per 100 L of beer) (Fărcaş et al., 2017) and designation of human consumption-generally recognized as safe (GRAS), spent brewer's yeast have been commercialized in the food market for years. Despite thousands of described yeast species, *Saccharomyces cerevisiae* (*S. cerevisiae*) still dominates this market (Ma et al., 2023; Oliveira et al., 2022a) due to their high protein (40–74% of dry weight) and essential amino acids (70.2–124 g/100 g protein) contents that allow the development not only of dietary supplements, but also of multiple functional foods, such as cooked ham, low-calorie mayonnaise, meat substitutes, yogurt, vegetable juices, toast spreads, soups and ready-to-eat meal (Zeko-Pivač et al., 2023).

In the last decades, the interest for other yeast ingredients has been growing in several economic sectors, due to their established biologic relevance properties, leading to the development of several extraction and purification processes for their production over the years (Marson et al., 2020a). Bioactive peptides are one example of these molecules demonstrating, among other, antimicrobial, antihypertensive and antioxidant properties, mainly in agri-food and cosmetic sectors (Oliveira et al., 2022a). However, aiming their production, yeast extraction processes often resort to physical, chemical or enzymatic extraction methodologies, where proteins are cleaved and freed from cell envelope, thus compromising the obtention of specific bioactive peptide fractions. For that reason, several isolation and purification steps have been added to the extraction process in order to decrease yeast cell debris and other contaminants that can influence the final biological properties. One must, however, bear in mind that the final process cost and selectively are inversely related, meaning that a value-added ingredient needs to be produced towards an overall cost-effective process (Oliveira et al., 2022a).

In addition to its application in food industry, *S. cerevisiae* strains have been engineered in large scale production of commercial target ingredients through their fermentation processes, such as farnesene, propanol and butanol, among others (Oliveira et al., 2022a). Similarly to

* Corresponding authors at: Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal.
E-mail addresses: ammpereira@ucp.pt (A.M. Pereira), chferreira@ucp.pt (C.M.H. Ferreira), jodila@ucp.pt (J.O. Pereira).

https://doi.org/10.1016/j.fbp.2023.10.010
Received 25 July 2023; Received in revised form 23 October 2023; Accepted 28 October 2023
Available online 3 November 2023

what happens in brewing industry, these large-scale fermentation processes generate significant amounts of spent yeast which must be valued in sustainable and circular economy approaches.

Considering the bioactive potential of spent yeast peptides and their significant amount generated as by-products in industrial waste, the aim of the present study was the application of purification processes to a peptide-rich extract from engineered spent yeast autolysate previously produced as detailed in Oliveira, Odila Pereira et al (Oliveira et al., 2022a)., for the obtention of bioactive peptides-rich fractions with targeted biological properties for dietary supplementation. After literature review (Oliveira et al., 2022a), two purification methodologies were chosen: ultrafiltration and gel filtration, using size-exclusion chromatography (SEC). The purified peptide-rich fractions were then characterized and assessed for their potential bioactive properties, namely antimicrobial, anticholesterolemic, antihypertensive and antioxidant properties.

2. Material and methods

2.1. Peptide-rich extract production

The peptide-rich extract used in the present study was chosen from a previous work (Oliveira et al., 2022a) using protein content and molecular weight (MW) profile of extract as criteria, together with protein recovery and sustainability of the extraction processes evaluated. Briefly, a *S. cerevisiae* strain from Amyris, Inc. (Emeryville, California, USA) engineered to produce β-farnesene, identified as "ESY1″, was autolysed for 16 h, at 50 °C and 120 rpm in a New Brunswick™ Innova® 40/40 R Benchtop Orbital Shaker from (Eppendorf, Hamburg, Germany). After yeast treatment, the intrinsic enzymes were inactivated (95 °C, 5 min) and the cell suspension centrifuged (4696g, 10 min, 4 °C). The obtained supernatant, rich in protein and peptides, was collected and freeze-dried (Freeze-dryer Alpha 2–4 LSCbasic, Martin Christ, Osterode am Harz, Germany).

2.2. Purification of bioactive peptides

2.2.1. Ultrafiltration

As illustrated in Fig. 1, peptide-rich extract was resuspended in deionized water (10 g/L) and the solution underwent ultrafiltration in an Amicon® stirred cell model (Merck KGaA, Darmstadt, Germany) using a 1 kDa cut-off Ultracel® regenerated cellulose membrane (Merck KGaA, Darmstadt, Germany) (Oliveira et al., 2022b). Thereafter, the retentate was diafiltrated with 3 volumes of deionized water, resulting in two purified fractions with different peptide MW: "ESY1 > 1 kDa" and "ESY1 < 1 kDa". At the end, samples were freeze-dried for further analysis.

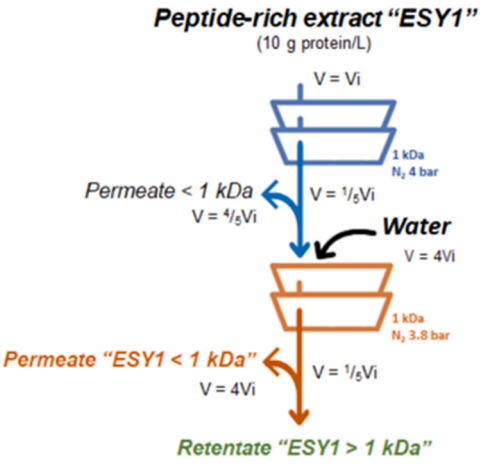

Fig. 1. Diagram of ultrafiltration process for production of peptide-rich fractions with different MW: "ESY1 > 1 kDa" and "ESY1 < 1 kDa".

2.2.2. Size-exclusion chromatography (SEC)

Size exclusion chromatography, using a Hiprep™ 26/10 Desalting column (GE Healthcare) was performed in an ÄKTA Pure™ 25 M (Cytiva, USA) purification system, and monitored by ultra-violet absorption at 280 nm using a UV detector. Lyophilized peptide-rich extract was resuspended in ultrapure water to a final concentration of 9 g/L and filtered through a 0.45 μm filter (Chromafil® PET-45/25, Macherey-Nagel, Germany) before being loaded into a pre-equilibrated column (injection volumes of 2 mL). Ultrapure water, at a flow rate of 5 mL/min, was used as elution buffer and fractions of 5 mL were collected.

2.3. Peptides chemical characterization

2.3.1. Protein content

2.3.1.1. Dumas. Protein content of ultrafiltration fractions "ESY1 > 1 kDa" and "ESY1 < 1 kDa" was performed as reported by Dumas et al (Dumas, 1831). with a Dumatec™ 8000 system (Foss, Hilleroed, Denmark). Helium and oxygen were used for analysis at flow rates of 195 mL/min and 300 mL/min, respectively, at 1100 mbar. About 50 mg of sample was weighed into aluminium crucibles and protein content was determined from the total nitrogen content, multiplied by a conversion factor of 5.5 (Marson et al., 2020b), using a calibration curve from 10 mg to 150 mg of ethylenediaminetetraacetic acid (EDTA) calibration standard (Foss, Hilleroed, Denmark).

BCA and dry weight.

Protein concentration of SEC fractions was determined using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific Inc., Massachusetts, USA), according to the manufacturer's instructions. Bicinchoninic acid (BCA) interacts with Cu^{1+} resulting from the reduction of Cu^{2+} ions from the copper sulphate by peptide bonds in alkaline pH, forming a purple-coloured complex with a strong linear absorbance at a wavelength of 562 nm with increasing protein concentrations (Smith et al., 1985). Protein content of the fractions was calculated after dry weight determination, performed at 105 °C for 24 h according to standard procedures of the Association of Official Analytical Chemists (Association, 2005).

2.3.2. MW distribution

2.3.2.1. Size exclusion chromatography (SEC). The MW of peptides in SEC and ultrafiltration fractions was evaluated using a HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) with an AdvanceBio SEC 130 A 2.7 μm (7.8 × 300 mm) column (Agilent Technologies Inc., Santa Clara, CA, USA) based on Tian et al (Tian et al., 2020). methodology with some modifications. The elution was carried out using 150 mM sodium phosphate buffer (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 1 mL/min flow rate. The elution was monitored at 280 nm and conalbumin (75 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), lysine peptide (775 Da) and tryptophan (204 Da) were used as standards for the calibration curve.

2.3.3. Proteomics

A nanoLC-MS/MS, namely Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), was used for peptides identification, as described by Osório et al (Osório et al., 2021). Samples were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 μm i.d., 160454, Thermo Scientific, Bremen, Germany) and after 3 min loading, the trap column was switched in-line to a 50 cm × 75 μm inner diameter EASY- Spray column (ES803, PepMap

RSLC, C18, 2 μm, Thermo Scientific, Bremen, Germany). Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

Peptide sequences were obtained using the Proteome Discoverer 2.5.0.400 software (Thermo Scientific, Bremen, Germany), as described by Osório et al (Osório et al., 2021)., using *S. cerevisiae* protein sequence database instead. After identifying the peptide sequences, we conducted an *in silico* search of these sequences against databases that contain information on known peptide activities. The databases used in our analysis included the Database of Antimicrobial Activity and Structure of Peptides (DBAASP) (Pirtskhalava et al., 2021), the Data repository of antimicrobial peptides (DRAMP) (Shi et al., 2022), the Collection of Anti-Microbial Peptides (CAMP) (Gawde et al., 2023), the Antimicrobial Peptide Database (APD) (Wang et al., 2016) and the BIOPEP-UWM (MinkiewiczIwaniakDarewicz, 2019). Unique matches and partial matches were both considered.

2.4. Peptides bioactivity

2.4.1. Antimicrobial Activity

Time-growth inhibition curves of *Escherichia coli* (DSM1576) and *Staphylococcus aureus* (DSM799) (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were performed to assess the potential antimicrobial activity. According to the reported values of antimicrobial peptides in literature (Oliveira et al., 2022a), all samples were prepared at 0.5% (w/v) in Mueller Hinton (MH) growth medium (Biokar Diagnostic, Beauvais, France) and sterilized using 0.22 μm filters (Millipore, Billerica, MA, USA). Both bacterial strains were used as monocultures and grown in Tryptic Soy Agar (TSA; Biokar Diagnostic, Beauvais, France) before the assay, with 3 g/L of yeast extract (Sigma-Aldrich, Munich, Germany) at 37 °C for 24 h under aerobic conditions. One colony was then picked, transferred to a tube with 10 mL of MH and grown at the same abovementioned conditions. On the day of experiment, bacteria inoculums were adjusted to an optical density (OD) at 625 nm of 0.08–1 (corresponding to a cell density of 1×10^8 cells/mL), followed by a 10-times dilution in MH to obtain the working inoculums. For the bacterial growth inhibition assay, 980 μL of each sample were inoculated with 20 μL of the working bacterial inoculums. After mixing, 200 μL of the suspensions were pipetted to a 96-well microtiter plate (Nunc, Darmstadt, Germany), and the OD at 625 nm was assessed during 24 h at 37 °C, with 1 h intervals, using a microplate reader (Epoch, Vermont, USA). Inoculated MH medium was used as a negative control and inoculated MH medium with 0.5% (w/v) ampicillin, a concentration known to inhibit *E. coli* and *S. aureus* growth (Proma et al., 2020) was used as positive control. Blanks of the samples were performed to correct sample colour OD interference.

2.4.2. Angiotensin converting enzyme (ACE) inhibition assay

Amorim et al (Amorim et al., 2019a). methodology was exploited for ACE inhibition assay using o-Abs-Gly-p-nitro-Phe-OH trifluoroacetate salt (Bachem, Bubendorf, Switzerland) as substrate and 42 mU/mL of ACE (peptidyl-dipeptidase A from rabbit lung) (Sigma- Aldrich, St Louis, USA). ACE inhibition (%) was quantified using Eq. 1:

$$ACE \quad inhibition(\%) = \frac{F_{control} - F_{sample}}{F_{control}} \times 100 \tag{1}$$

where $F_{control}$ and F_{sample} are the fluorescence values obtained for the control (maximum ACE activity) and the sample, respectively. The calculation of IC50 values (concentration needed to inhibit 50% of ACE activity) for all samples was performed by non-linear fitting of the data, using a four-parameter logistic regression model. The assay was performed in triplicate.

2.4.3. Antioxidant

2.4.3.1. Scavenging activity using 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate)-radical cation ($ABTS^{\bullet+}$). The methodology reported by Gonçalves et al (Gonçalves et al., 2019)., with some modifications for a 96-well plate scale (Oliveira et al., 2022b) was used for $ABTS^{\bullet+}$ scavenging activity. $ABTS^{\bullet+}$ scavenging activity (%) was calculated using Eq. 2:

$$ABTS^{\bullet+} \quad scavenging \quad activity \quad (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \tag{2}$$

where $A_{control}$ and A_{sample} are the absorbances values for the control and the sample, respectively. A Trolox standard curve (with a concentration range of 50–560 μM) (Sigma-Aldrich, Inc., St. Louis, USA) was used to express Trolox Equivalent (TE) antioxidant activity of the samples (μmol/g sample). The assay was performed in triplicate.

Oxygen radical absorbance capacity (ORAC).

ORAC was assessed according to Coscueta et al (Coscueta et al., 2019). Trolox standard curve (with a concentration range of 10–80 μM) allowed the quantification of TE antioxidant activity of the samples (μmol/g sample), after assessing the area under curve (AUC) for each sample. The assay was performed in triplicate.

2.4.4. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibition assay

HMG-CoA reductase inhibition was assessed by the colorimetric HMG-CoA Reductase Activity Assay Kit (Abcam, Cambridge, United Kingdom), according to the manufacturer's guidelines. The lower biocompatible Caco-2 concentration of peptide-rich extracts from spent yeast, previously assessed (Oliveira et al., 2022b), was used in this assay. This method is based on consumption of NADPH by the enzyme which is evaluated by the decrease of absorbance at 340 nm during 15 min. Quantification of the HMG-CoA reductase activity (% inhibition) is performed using absorbance values of two time points within the linear range, and with a minimum of 2 min apart, as recommended by the manufacturer. Atorvastatin was used as a reference drug inhibitor and Pravastatin as a commercial inhibitor (control). The assay was performed in triplicate.

2.5. Statistical analysis

Statistical analysis was performed using the Real Statistics Resource Pack software (Release 7.2) and results were expressed as average ± standard deviation. Outliers were excluded using the interquartile range with multiplier of 2.2 and data normality was checked using the Shapiro-Wilk test. Protein and bioactivities results were subjected to the analysis of variance (ANOVA) followed by Tukey's post hoc test.

3. Results and Discussion

3.1. Protein content (% w/w)

Purification of peptide-rich extract by SEC allowed the separation of four peptidic fraction as observed in the chromatogram (Fig. 2), that were collected and assessed for their protein content. Differently, only two purified fractions, "ESY1 > 1 kDa" and "ESY1 < 1 kDa", were obtained from ultrafiltration purification and quantified for their protein content by Dumas (Dumas, 1831). Protein content in a variety of samples can be rapidly determine by this method, an analytical method for the quantification of nitrogen, that is in accordance with international standards such as AOAC (Dumas, 1831; Association, 2005). However, due to quantity restrictions, protein content in SEC fractions was determined by the combination of BCA and dry weight determinations instead.

Protein content of the different fractions obtained by ultrafiltration

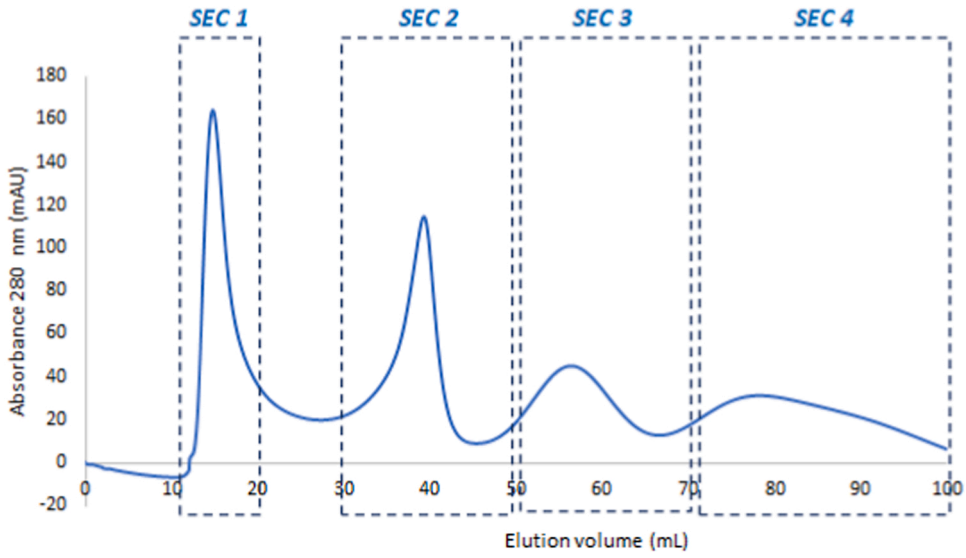

Fig. 2. Elution profile of the SEC purification of peptide-rich extract "ESY1". Four different peptide fractions, corresponding to the four peaks observed, were collected (Sections 1 to 4).

and SEC, as well as protein content of the protein-rich extract obtained after autolysis of spent yeast (16 h, 50 ºC) (Oliveira et al., 2022b) can be seen in Table 1. Results show that protein content significant increased ($p < 0.05$) in ESY1 < 1 kDa (61.7 ± 0.6%) when compared to ESY1 > 1 kDa (22.5 ± 0.1%). The protein content here reported for ESY1 < 1 kDa is in accordance to the previous results of peptide rich-extract obtained from waste streams of mannan extraction from engineered spent yeast (*S. cerevisiae*) purified by ultrafiltration using a 1 kDa cut-off membrane (Oliveira et al., 2022b). Protein content also significantly increases ($p < 0.05$) in Section 4 (89.0 ± 0.8%) when compared to Section 1 (20.2 ± 3.4%), Section 2 (13.1 ± 4.3%) and Section 3 (43.6 ± 3.1%). These results are somewhat expected, since during both filtration processes, molecules are separated based on their sizes. Thus, in the ultrafiltration process, smaller molecules like minor proteins, peptides and amino acids, will pass through the 1 kDa cut-off membrane towards fraction, producing the cleaner ESY1 < 1 kDa fraction, while other larger molecules than proteins that are present in the extract will be retained in fraction ESY1 > 1 kDa, thus decreasing protein content in this fraction. Similarly, during gel filtration by SEC, these molecules with higher MW will be eluted first, while molecules with smaller MW will be trapped within the porous matrix, being eluted later, in fractions where the amount. Furthermore, the protein content determined for the Section 2 fraction, reveals that the higher number of other molecules are in that fraction.

When comparing both purification approaches, it is possible to conclude that SEC produced the fraction with the highest ($p < 0.05$) protein amount (Section 4; 89.0 ± 0.8%), supporting reports of being one of the widely techniques for isolation of antimicrobial, antihypertensive and antidementia peptides from *S. cerevisiae* (Oliveira et al., 2022a). However, costs associated to chromatography equipment are substantially higher than those associated with the ultrafiltration methodology, indicating that SEC needs to produce a value-added ingredient in order to justify their use. Indeed, this is the reason why chromatographic techniques are more frequently applied in purification of pharmaceutical proteins and peptides (Pratap et al., 2017). Nevertheless, some authors have already reported their use to produce yeast peptides with important biological properties, such as antihypertensive (Kim et al., 2004; Kohama et al., 1990; Kanauchi et al., 2005; Mirzaei et al., 2015; Ni et al., 2012a, 2012b), antimicrobial (Gddoa Al-sahlany et al., 2020; Branco et al., 2014, 2017; Caldeira et al., 2019) and antioxidant (Mirzaei et al., 2015). In contrast, the use of ultrafiltration with filter membranes offers several advantages for concentrating proteins and peptides. One significant advantage is its low energy consumption. Additionally, it provides a gentle treatment of molecules, preserving their integrity and functionality. These reasons contribute to the widespread adoption of ultrafiltration in food processing industry (Zeko-Pivač et al., 2023; Vollet Marson et al., 2020; Mohammad et al., 2012) or to the production of peptides with important bioactivities for nutraceutical supplementation, including antihypertensive (Oliveira et al., 2022b; Amorim et al., 2019a; Kim et al., 2004; Mirzaei et al., 2015; Ni et al., 2012a; Hu et al., 2014), antimicrobial (Gddoa Al-sahlany et al., 2020; Branco et al., 2014; Caldeira et al., 2019; Comitini et al., 2005; Albergaria et al., 2010), antioxidant (Oliveira et al., 2022b; Amorim et al., 2019a; Mirzaei et al., 2015; Hu et al., 2014; Guo et al., 2020; Jung et al., 2011) and, more recently, anticholesterolemic (Oliveira et al., 2022b). Furthermore, in this work, the ultrafiltration fractions obtained can be classified as "rich in protein" according to the European Commission (European Commission, Nutrition claims, 2012). This designation highlights their potential as ingredients for nutraceutical market, as previously reported (Oliveira et al., 2022a).

All things considered, both techniques appear to be interesting processes to be included in protein-rich extracts production, but their choice requires a cost-effectiveness evaluation directly related to the economic sector to be applied.

Table 1
Protein content (% w/w) of original ESY1 extract and fractions obtained from ultrafiltration (ESY1 >1 and ESY1 <1) and Sections (1 to 4).

		Protein (%)
Original ESY1[a]		54.5 ± 1.0
Ultrafiltration[b]	ESY1 > 1 kDa	22.5 ± 0.1
	ESY1 < 1 kDa	61.7 ± 0.6
SEC fractions[c]	Section 1	20.2 ± 3.4
	Section 2	13.1 ± 4.3
	Section 3	43.6 ± 3.1
	Section 4	89.0 ± 0.8

Results are expressed as average ± standard deviation ($n = 3$). [a] Oliveira et al (Oliveira et al., 2022b)., [b] Dumas (Dumas, 1831) (section 2.1.1.1), [c] BCA (section 2.1.1.2). p-values can be consulted in Supplementary material 1.

3.2. MW distribution

In our previous work (Oliveira et al., 2022a), ESY1 showed a large MW population of peptides under 1 kDa (74.5%), followed by 1–3 kDa

(23.9%), and small percentages of 3–5 kDa (1.72%) and 5–10 kDa (0.15%). For this reason, a 1 kDa cut-off membrane filtration was chosen for peptides separation. However, these results are very different from other works reporting MW of 5–35 kDa in spent yeast autolysates. Indeed, peptides under 3 kDa were only obtained after including enzymatic hydrolysis in process (Amorim et al., 2019b; Marson et al., 2022; Xie et al., 2017).

Protein and peptides MW distribution of the ultrafiltration and SEC fractions was assessed by HPLC-SEC. Unfortunately, the MW of ESY1, and respective ultrafiltration fractions, could not be determined by HPLC-SEC, since a good chromatographic resolution was not obtained with our column, probably related with the complexity of the matrix. For that reason, it was assumed that ESY1 ultrafiltration fractions own MW associated with cut-off membrane separation, that is, peptides above 1 kDa in ESY1 $>$ 1 kDa and peptides under 1 kDa in ESY1 $<$ 1 kDa. Results regarding the protein and peptides MW distribution in SEC fractions (Table 2) show that the most representative population in Section 1 had a MW around 3 kDa (75.4%), while the most representative in fraction Section 2 had approximately 1 kDa (45.9%). Peptides below 300 Da characterized fraction Section 3 (91.0%) and amino acids composed the majority of fraction 4 (92.6% under 200 Da) (Table 2). Comparing the MW distribution of all samples, Section 2 and Section 3 seemed to be similar with the original ESY1, being mostly constituted by $\leq$ 1 kDa peptides, and promising bioactive fractions.

After analysis of protein content and MW determinations, it possible to hypothesise that fractions ESY1 $<$ 1 kDa and Section 4 are the ones with the most promising for bioactive properties, as it has been reported that peptides containing 3–20 amino acids are highly specific in their biological functions and in choosing biological and metabolic targets (Oliveira et al., 2022a).

3.3. Proteomics analysis

After proteomics analysis, several peptide sequences were identified in ESY1 and respective fractions obtained by ultrafiltration and SEC, from approximately 600 to 5000 Da (Supplementary material 2). As expected, the original extract "ESY1″ had the highest number of peptide sequences identified, along with fraction ESY1 $>$ 1 kDa (6343). These were followed by Section 1 (1803) and Section 4 (599), while both less than 300 peptides were identified in Section 2 and Section 3 (Table 3). The main purpose of this analysis was to identify potential bioactivities related to the peptide sequences found in the samples by matching those sequences with bioactive peptides reported in different peptide bioactivity databases (PBD, see section 2.1.3 for details). After conducting a thorough search in the data found in each of the PBD, sequences of our peptides present in all our fractions were found within the sequences of peptides listed in the PBD, with antibacterial, antimicrobial and antifungal activities reported (Table 3). However, it is important to note that only partial matches were identified during our analysis. Specifically, our peptide sequence was found as a component within a larger peptide sequence in the PBD, and an exact match was not found.

Table 2
Protein and peptides MW distribution in SEC fractions represented as percentage of chromatogram total area (% total area).

SEC fractions	MW (kDa)	% total area
Section 1	121	10.6
	3.16	75.4
	0.41	8.32
	0.21	5.67
Section 2	108	9.38
	53.4	4.49
	1.37	45.9
	0.37	21.2
	0.20	19.0
Section 3	0.96	3.31
	0.59	5.64
	0.36	51.3
	0.20	39.7
Section 4	0.56	2.00
	0.39	5.41
	$<$ 0.2	92.6

* PBD: BAASP, DRAMP, CAMP, APD and BIOPEP-UWM.

Several authors already described antimicrobial activities of yeast peptides (Gddoa Al-sahlany et al., 2020; Branco et al., 2014, 2017; Caldeira et al., 2019), and therefore these results are not surprising. However, considering the partial matches observed, it is plausible to argue that there is untapped potential to enhance the antimicrobial activity of these extracts. One potential approach is to reduce autolysis time, thereby minimizing peptide breakdown and achieving a closer match to those peptides described in the PBD. Nonetheless, the antimicrobial potential is still present and, for that reason, the effect upon the growth curves for two important food-related pathogen agents, exposed to our samples, was addressed and detailed in the next section.

3.4. Bioactivities

3.4.1. Antimicrobial

To evaluate the potential antimicrobial activity of the different peptide fractions collected after ultrafiltration and SEC, a growth inhibition assay was carried out, and results of the fractions impact on *E. coli* and *S. aureus* growth can be seen in Fig. 3. Contrary to what was expected from the PBD results, none of the peptide fractions inhibited bacterial growth. As a matter of fact, all fractions (except Section 1 against *S. aureus*) seem to be promoting bacterial growth.

Antimicrobial peptides are usually amphipathic and cationic short peptides, whose activity strongly depends on their amino acid sequence, size and charge (Powers and Hancock, 2003; Jenssen et al., 2006). Although their mechanism is not clear yet, the best described mode of action for antimicrobial peptides regards the electrostatic interaction between the cationic peptides and the negatively charged components present on the pathogen membrane bilayers, allowing them to accumulate on the membranes and form pores by 'barrel-stave', 'carpet' or 'toroidal pore' mechanisms (Oliveira et al., 2022a; Mookherjee et al., 2020). For these reasons, the lack of antimicrobial activity, when compared with the peptide sequences in the PBD, might be due to the fact that the peptides identified in the different fractions have an inferior number of amino acids with a positive net charge, such as lysine (K), arginine (R) or tryptophan (W), know to play an important role in the antimicrobial activity of these peptides (Powers and Hancock, 2003; Chan et al., 2006). On the other hand, it is possible that the promotion of bacterial growth in these fractions is attributed to residual sugars. As previously reported, peptide-rich fractions derived from spent yeast contain certain amounts of sugar, potentially including glucose, which could contribute to bacterial proliferation (Oliveira et al., 2022b). Nevertheless, it would be interesting to investigate the potential antimicrobial properties of these peptide fractions against other pathogenic agents in the future. Since different microorganisms have distinct membrane components, their interactions with the peptides in each fraction could vary, leading to diverse outcomes. Exploring such variations could provide valuable insights into the broader antimicrobial potential of these peptide fractions.

3.4.2. Anticholesterolemic, antihypertensive and antioxidant activities

Even though the peptide fractions did not display any antimicrobial activity, other important bioactivities for nutraceutical market, such as antihypertensive, antioxidant and anticholesterolemic activities, were assessed.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a catalyst in the conversion of HMG-CoA to mevalonate, an important intermediate in the cholesterol biosynthesis (DeBose-Boyd, 2008). HMG-CoA reductase inhibitors such as statins have been, thus, widely used in the treatment of hypercholesterolemia. In this work, the original

Table 3
Peptide sequences identified in original ESY1 extract and fractions obtained from ultrafiltration (ESY1 >1 and ESY1 <1) and Sections (1 to 4), together with their bioactivities reported in PBD.

		Total sequences number	Number of matched sequences in PBD*	MW range (Da) of matched sequences in PBD*	PBD* reported bioactivities
Original	ESY1	6343	42	648–1857	Antibacterial, antimicrobial and antifungal
Ultrafiltration	ESY1 > 1	6343	31	816–1720	
	ESY1 < 1	212	3	890–2153	
SEC	Section 1	1803	17	816–1780	
	Section 2	246	2	947–1448	
	Section 3	291	4	831–1448	
	Section 4	599	10	831–1530	

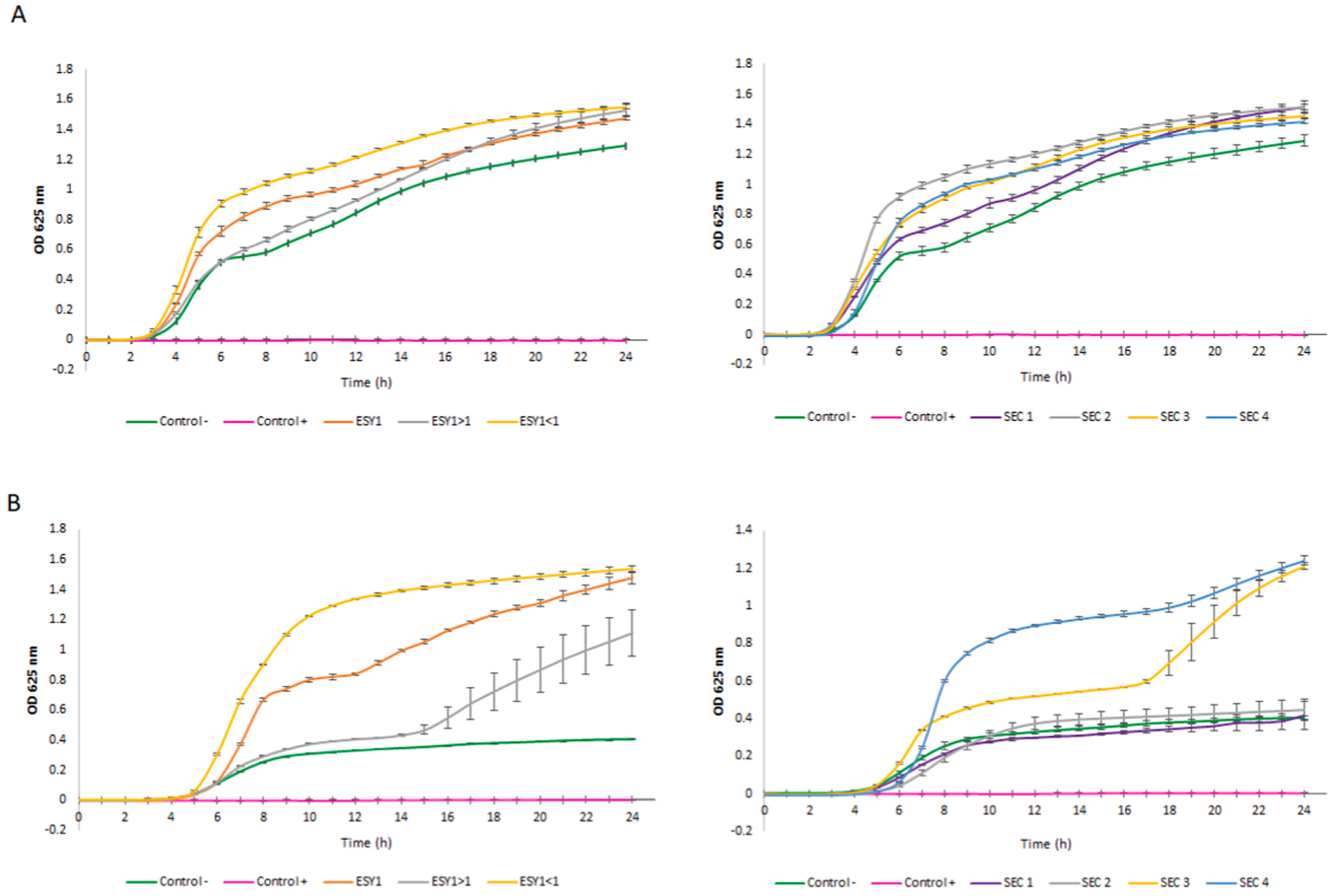

Fig. 3. Growth curves for E. coli (A) and S. aureus (B) exposed to original ESY1 extract and fractions obtained from ultrafiltration (ESY1 >1 and ESY1 <1; on the left) and Sections (1 to 4; on the right).

extract and the different peptide fraction collected after both purification steps were assessed for their anticholesterolemic properties. As expected, after the addition of the ultrafiltration and SEC for purification of the extract autolysate, a significant higher HMG-CoA reductase inhibition ($p < 0.05$) was observed for all the fractions obtained, when compared with the original ESY1, thus increasing from 40.3 to 70.8–77.7%, depending on the fractions (Table 4, Supplementary material 3). In fact, these findings align with our previous study, which documents that spent yeast peptide fractions, both below and above 1 kDa, exhibited a HMG-CoA reductase inhibition range of 62.0–71.3% (Oliveira et al., 2022b). However, these results do not seem to be related with protein content or predominant MW of fractions, since no tendency was observed in this sense.

ACE is a zinc metallopeptidase that catalyses the conversion of angiotensin I to angiotensin II, a strong vasoconstrictor with direct effect on the raising of blood pressure (Zhao, 2008). For this reason, molecules with ACE inhibition activity can help in the management of hypertension. The peptide-rich extract and the different fractions were evaluated for their antihypertensive activity using an ACE inhibition assay and by calculating their IC50 (Table 4, Supplementary material 3). Original ESY1 and fractions Section 4 and ESY1 < 1 kDa, showed the highest activity, corresponding to the lowest IC50 values, followed by Section 2, Section 3 and ESY1 > 1 kDa, and, finally, Section 1 ($p < 0.05$). While the higher ACE inhibitions of fractions ESY1 < 1 kDa and Section 4 are in accordance with their low MW distribution and protein content/purity, the high ACE inhibition value obtained for original ESY1 indicates the contribution not only of all proteins and peptides, but also of some other components of the extract. Section 1 was characterized with the highest MW distribution (~3 kDa) which can justify its lowest bioactivity. Indeed, small peptides (~ 2–20 amino acids) have been hypothesised have ACE inhibition activity by binding to the hydrophobic C-terminal tripeptide of the enzyme substrate, preventing

Table 4
IC50 values (mg/mL) obtained in ACE-inhibition assay, $ABTS^{\bullet+}$ and ORAC activities (μmol TE/g sample), and HMG-CoA reductase inhibition (%) of original ESY1 extract and fractions obtained from ultrafiltration (ESY1 >1 and ESY1 <1) and Sections (1 to 4).

		HMG-CoA reductase inhibition* (%)	ACE – IC50 (mg/mL)	$ABTS^{\bullet+}$ (μmol TE/g sample)	ORAC (μmol TE/g sample)
Original	ESY1	40.3 ± 2.0	1.69 ± 0.16	157 ± 60	626 ± 22
Ultrafiltration	ESY1 < 1	74.0 ± 4.6	1.80 ± 0.12	255 ± 56	708 ± 109
	ESY1 > 1	70.8 ± 2.4	2.59 ± 0.10	181 ± 44	237 ± 85
SEC	Section 1	77.5 ± 0.3	3.21 ± 0.33	90.5 ± 16.9	2036 ± 277
	Section 2	75.9 ± 0.3	2.28 ± 0.15	77.4 ± 5.1	2608 ± 330
	Section 3	77.7 ± 1.8	2.47 ± 0.09	173 ± 51	43610 ± 3532
	Section 4	71.3 ± 1.2	1.43 ± 0.08	300 ± 17	41091 ± 1021

Results are expressed as average ± standard deviation ($n = 3$). *For Caco-2 biocompatible concentrations (Oliveira et al., 2022b).

ACE-substrate binding, and, consequently, the conversion of angiotensin I to the angiotensin II (Kim et al., 2004). Overall results, mainly those obtained for ESY1, ESY1 < 1 kDa and Section 4, are in accordance with our previous work (Oliveira et al., 2022b) where IC50 values from 0.99 to 1.72 mg/mL were reported.

Oxidative stress, caused by high intracellular levels of reactive oxygen species (ROS) has been frequently associated with aging and aging-related diseases and antioxidant molecules can delay the oxidation by scavenging ROS through different reactions, thus reducing ROS to less reactive forms (Shields et al., 2021). Aromatic amino acids present in the peptide sequences have been presumed to be capable to quench the free radicals through a direct electron transfer mechanism, while the pyrrolidine ring of proline residues may interact with secondary structure of peptides, thus increasing their flexibility and ability to quench singlet oxygen (Amorim et al., 2019a). The potential antioxidant activity of the original extract and the peptide fractions were determined using two scavenging-based assays: $ABTS^{\bullet+}$ and ORAC. While the $ABTS^{\bullet+}$ assay measures the relative ability of antioxidants to reduction of cation radical $ABTS^{\bullet+}$ into $ABTS^{\bullet}$ as compared with a Trolox, a water-soluble analogous of vitamin E and a known antioxidant, the ORAC assay is based on generation of the radical peroxyl (ROO•), a radical with biological importance (Prior et al., 2003).

In the $ABTS^{\bullet+}$ assay, fractions Section 4 and ESY1 < 1 kDa showed the highest activity, followed by ESY1, ESY1 > 1 kDa and Section 3. Section 1 and Section 2 demonstrated to have the lowest activity ($p < 0.05$) (Table 4, Supplementary material 3). Overall, the antioxidant activities are lower than those previously obtained for other peptide-rich extracts (Oliveira et al., 2022b) which might be related with differences in protein content of fractions. On the other hand, the SEC fractions demonstrated the highest ORAC vales, especially Section 3 and Section 4, followed by original ESY1 and ultrafiltration fractions ($p < 0.05$) (Table 4, Supplementary material 3). Similar antioxidant activity results to the ones obtained for the original ESY1, and respective ultrafiltration fractions, were reported by Costa et al (Costa et al., 2023). for spent yeast waste streams fractions (654–1041 μmol TE/g extract). However, SEC fractions showed a substantial increase in the ORAC values to ~ 2000 μmol TE/g extract for Section 1 and Section 2, and ~ 40000 μmol TE/g extract for SEC fractions with low MW peptides and high protein content/purity (Section 3 and SEC4) thus demonstrating their application for antioxidant value-added products on nutraceutical market, or even in the cosmetic sector, as previously suggested by Costa et al (Costa et al., 2023).

When comparing the results obtains by the two antioxidant assays, it is possible no see some differences between them. When working with peptide-rich extract, or even peptide fraction, data interpretation can be tricky due to their complex nature and composition. Moreover, different assays have been used to evaluate antioxidant properties, and the results obtained in those assays are frequently different among them, as all methodologies present advantages and limitations (Schaich et al., 2015). Nevertheless, it is clear that both ultrafiltration and SEC originate some fraction with higher biological activities than the original peptide-rich extract.

4. Conclusion

The present work reports the production of peptide fractions derived from a spent yeast extract autolysate. The fractions were obtained using 1 kDa ultrafiltration or gel filtration, using SEC. These processes allowed to generation of fractions with distinct characteristics, mainly in terms of protein content, MW distribution and peptide sequences. While the original extract autolysate and respective ultrafiltration and SEC fractions did not show any antimicrobial effect, interesting results regarding their antihypertensive, anticholesterolemic and antioxidant properties were observed. These results suggest that these fractions hold promise as potential candidates for dietary supplementation in the form of a single product. All purified peptide fractions showed higher HMG-CoA reductase inhibition values than the original extract. These results demonstrate these processes as crucial methodologies for the development of new anticholesterolemic ingredients using yeast autolysates. The peptide fractions with the highest protein content/purity (ESY1 <1 kDa and Section 4) revealed potential for the formulation of antihypertensive ingredients, as these were the ones with the higher ACE inhibition values. Regarding the antioxidant activity, significantly superior ORAC values were observed in fractions Section 4 and Section 3. This suggests their advantageous use in other economic sectors such as cosmetics, where antioxidant molecules have a large anti-aging impact.

In addition, results presented in this study suggests that the addition of a purification step in the production of bioactive peptides from spent yeast autolysates can be advantageous for generating ingredients with specific targeted bioactivities for the nutraceutical market. These purification processes enable the isolation and concentration of bioactive peptides, enhancing their potential application in the development of functional food and dietary supplements. These findings emphasize the importance of implementing purification strategies in order to optimize the production of bioactive peptide ingredients for the nutraceutical industry.

CRediT authorship contribution statement

Ana Sofia Oliveira: Conceptualization, Investigation, Writing – original draft. **Ana Margarida Pereira**: Investigation, Writing – review & editing. **Carlos M.H. Ferreira**: Investigation, Writing – review & editing. **Joana Odila Pereira**: Investigation, Writing – review & editing. **Manuela Amorim**: Investigation. **Margarida Faustino**: Investigation. **Joana Durão**: Investigation. **Manuela E. Pintado**: Resources, Supervision, Project administration, Funding acquisition. **Ana P. Carvalho**: Writing – review & editing, Supervision.

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.

Acknowledgements

This work was co-financed by European Regional Development Fund

(ERDF), through the Operational Program for Competitiveness and Internationalization (POCI) supported by Amyris Bio Products Portugal, Unipessoal Lda and Escola Superior de Biotecnologia—Universidade Católica Portuguesa through Alchemy project 'Capturing High Value from Industrial Fermentation Bio Products (POCI-01–0247-FEDER-027578)'. The authors also thank Doctor Hugo Osório from Instituto de Investigaç ão e Inovaç ão em Saúde (i3s), for proteomics analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2023.10.010.

References

Fărcaş, A.C., Socaci, S.A., Mudura, E., Dulf, F.V., Vodnar, D.C., Tofană, M., Salanță, L.C., 2017. Exploitation of brewing industry wastes to produce functional ingredients. Brew. Technol. https://doi.org/10.5772/intechopen.69231.

Ma, J., Sun, Y., Meng, D., Zhou, Z., Zhang, Y., Yang, R., 2023. Yeast proteins: the novel and sustainable alternative protein in food applications. Trends Food Sci. Technol. 135, 190–201. https://doi.org/10.1016/j.tifs.2023.04.003.

Oliveira, A.S., Ferreira, C., Pereira, J.O., Pintado, M.E., Carvalho, A.P., 2022a. Valorisation of protein-rich extracts from spent brewer's yeast (Saccharomyces cerevisiae): an overview. Biomass Convers. Biorefinery. https://doi.org/10.1007/s13399-022-02636-5.

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, 208. https://doi.org/10.3390/fermentation9030208.

Marson, G.V., de Castro, R.J.S., Belleville, M.-P.P., Hubinger, M.D., 2020a. Spent brewer's yeast as a source of high added value molecules: a systematic review on its characteristics, processing and potential applications. World J. Microbiol. Biotechnol. 36, 95 https://doi.org/10.1007/s11274-020-02866-7.

Oliveira, A.S., Ferreira, C., Pereira, J.O., Pintado, M.E., Carvalho, A.P., 2022a. Spent brewer's yeast (Saccharomyces cerevisiae) as a potential source of bioactive peptides: An overview. Int. J. Biol. Macromol. 208, 1116–1126. https://doi.org/10.1016/j.ijbiomac.2022.03.094.

Oliveira, A.S., Odila Pereira, J., Ferreira, C., Faustino, M., Durão, J., Pereira, A.M., Oliveira, C.M., Pintado, M.E., Carvalho, A.P., 2022a. Spent yeast valorization for food applications: effect of different extraction methodologies. Foods 11, 4002. https://doi.org/10.3390/foods11244002.

Oliveira, A.S., Pereira, J.O., Ferreira, C., Faustino, M., Durão, J., Pintado, M.E., Carvalho, A.P., 2022b. Peptide-rich extracts from spent yeast waste streams as a source of bioactive compounds for the nutraceutical market. Innov. Food Sci. Emerg. Technol. 81, 103148 https://doi.org/10.1016/j.ifset.2022.103148.

Dumas, J.B.A., 1831. Procedes de l'analyse organique. Ann. Chim. Phys. 198–205.

Marson, G.V., de Castro, R.J.S., da, M.T., Machado, C., da Silva Zandonadi, F., de, H.D., Barros, F.Q., Maróstica Júnior, M.R., Sussulini, A., Hubinger, M.D., 2020b. Proteolytic enzymes positively modulated the physicochemical and antioxidant properties of spent yeast protein hydrolysates. Process Biochem. 91, 34–45. https://doi.org/10.1016/j.procbio.2019.11.030.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85. https://doi.org/10.1016/0003-2697(85)90442-7.

Association of Official Analysis Chemists, Official Methods of Analysis of AOAC International, 2005.

Tian, R., Feng, J., Huang, G., Tian, B., Zhang, Y., Jiang, L., Sui, X., 2020. Ultrasound driven conformational and physicochemical changes of soy protein hydrolysates. Ultrason. Sonochem. 68, 105202 https://doi.org/10.1016/j.ultsonch.2020.105202.

Osório, H., Silva, C., Ferreira, M., Gullo, I., Máximo, V., Barros, R., Mendonça, F., Oliveira, C., Carneiro, F., 2021. Proteomics analysis of gastric cancer patients with diabetes mellitus. J. Clin. Med. 10, 1–14. https://doi.org/10.3390/jcm10030407.

Pirtskhalava, M., Amstrong, A.A., Grigolava, M., Chubinidze, M., Alimbarashvili, E., Vishnepolsky, B., Gabrielian, A., Rosenthal, A., Hurt, D.E., Tartakovsky, M., 2021. DBAASP v3: database of antimicrobial/cytotoxic activity and structure of peptides as a resource for development of new therapeutics. Nucleic Acids Res. 49, D288–D297. https://doi.org/10.1093/nar/gkaa991.

Shi, G., Kang, X., Dong, F., Liu, Y., Zhu, N., Hu, Y., Xu, H., Lao, X., Zheng, H., 2022. DRAMP 3.0: an enhanced comprehensive data repository of antimicrobial peptides. Nucleic Acids Res. 50, D488–D496. https://doi.org/10.1093/nar/gkab651.

Gawde, U., Chakraborty, S., Waghu, F.H., Barai, R.S., Khanderkar, A., Indraguru, R., Shirsat, T., Idicula-Thomas, S., 2023. CAMPR4: a database of natural and synthetic antimicrobial peptides. Nucleic Acids Res. 51, D377–D383. https://doi.org/10.1093/nar/gkac933.

Wang, G., Li, X., Wang, Z., 2016. APD3: the antimicrobial peptide database as a tool for research and education. Nucleic Acids Res. 44, D1087–D1093. https://doi.org/10.1093/nar/gkv1278.

Minkiewicz, Iwaniak, Darewicz, 2019. BIOPEP-UWM database of bioactive peptides: current opportunities. Int. J. Mol. Sci. 20, 5978. https://doi.org/10.3390/ijms20235978.

Proma, F.H., Shourav, M.K., Choi, J., 2020. Post-antibiotic effect of ampicillin and levofloxacin to Escherichia coli and Staphylococcus aureus based on microscopic imaging analysis. Antibiotics 9, 1–13. https://doi.org/10.3390/antibiotics9080458.

Amorim, M., Marques, C., Pereira, J.O., Guardão, L., Martins, M.J., Osório, H., Moura, D., Calhau, C., Pinheiro, H., Pintado, M., 2019a. Antihypertensive effect of spent brewer yeast peptide. Process Biochem. 76, 213–218. https://doi.org/10.1016/j.procbio.2018.10.004.

Gonçalves, G.A., Corrêa, R.C.G., Barros, L., Dias, M.I., Calhelha, R.C., Correa, V.G., Bracht, A., Peralta, R.M., Ferreira, I.C.F.R., 2019. Effects of in vitro gastrointestinal digestion and colonic fermentation on a rosemary (*Rosmarinus officinalis L*) extract rich in rosmarinic acid. Food Chem. 271, 393–400. https://doi.org/10.1016/j.foodchem.2018.07.132.

Coscueta, E.R., Campos, D.A., Osório, H., Nerli, B.B., Pintado, M., 2019. Enzymatic soy protein hydrolysis: a tool for biofunctional food ingredient production. Food Chem. X 1, 100006. https://doi.org/10.1016/j.fochx.2019.100006.

Pratap, G., Jadaun, S., Dixit, S., Saklani, V., Mendiratta, S., Jain, R., Singh, S., 2017. HPLC for peptides and proteins: principles, methods and applications, 8, 1–6. https://doi.org/10.5530/phm.2017.8.1.

Kim, J., Dae-Hyoung, L., Jong-Soo, L., Chung, K., Jeong, S., 2004. Characterization of antihypertensive angiotensin I-converting enzyme inhibitor from *Saccharomyces cerevisiae*. J. Microbiol. Biotechnol. 14, 1318–1323.

Kohama, Y., Nagase, Y., Oka, H., Nakagawa, T., Teramoto, T., Murayama, N., Tsujibo, H., Inamori, Y., Mimura, T., 1990. Production of angiotensin-converting enzyme inhibitors from baker's yeast glyceraldehyde-3-phosphate dehydrogenase. J. Pharm. 13, 766–771. https://doi.org/10.1248/bpb1978.13.766.

Kanauchi, O., Igarashi, K., Ogata, R., Mitsuyama, K., Andoh, A., 2005. A yeast extract high in bioactive peptides has a blood-pressure lowering effect in hypertensive model. Curr. Med. Chem. 12, 3085–3090. https://doi.org/10.2174/092986705774933461.

Mirzaei, M., Mirdamadi, S., Ehsani, M.R., Aminlari, M., Hosseini, E., 2015. Purification and identification of antioxidant and ACE-inhibitory peptide from Saccharomyces cerevisiae protein hydrolysate. J. Funct. Foods 19, 259–268. https://doi.org/10.1016/j.jff.2015.09.031.

Ni, H., Li, L., Guo, S.-S., Li, H.-H., Jiang, R., Hu, S.-Q., 2012a. Isolation and identification of an angiotensin-I converting enzyme inhibitory peptide from yeast (*Saccharomyces cerevisiae*). Curr. Anal. Chem. 8, 180–185. https://doi.org/10.2174/157341112798472224.

Ni, H., Li, L., Liu, G., Hu, S., 2012b. Inhibition Mechanism and Model of an Angiotensin I-Converting Enzyme (ACE)-Inhibitory Hexapeptide from Yeast (*Saccharomyces cerevisiae*). PLoS One 7, 1–7. https://doi.org/10.1371/journal.pone.0037077.

Gddoa Al-sahlany, S.T., Altemimi, A.B., Abd Al-Manhel, A.J., Niamah, A.K., Lakhssassi, N., Ibrahim, S.A., 2020. Purification of bioactive peptide with antimicrobial properties produced by *Saccharomyces cerevisiae*. Foods 9, 1–11. https://doi.org/10.3390/foods9030324.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M.G., Caldeira, J., Albergaria, H., 2014. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl. Microbiol. Biotechnol. 98, 843–853. https://doi.org/10.1007/s00253-013-5411-y.

Branco, P., Francisco, D., Monteiro, M., Almeida, M.G., Caldeira, J., Arneborg, N., Prista, C., Albergaria, H., 2017. Antimicrobial properties and death-inducing mechanisms of saccharomycin, a biocide secreted by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 101, 159–171. https://doi.org/10.1007/s00253-016-7755-6.

J. Caldeira G. Almeida A.L. Macedo J.P.M. Silva H. Albergaria. Saccharomycin, a biocide from S. cerevisiae that kill-off other yeasts Ann. Med. 51 2019 94 95 doi: 10.1080/07853890.2018.1562694.

Vollet Marson, G., Belleville, M., Lacour, S., Dupas Hubinger, M., 2020. Membrane fractionation of protein hydrolysates from by-products: recovery of valuable compounds from spent yeasts. Membranes 11, 23. https://doi.org/10.3390/membranes11010023.

Mohammad, A.W., Ng, C.Y., Lim, Y.P., Ng, G.H., 2012. Ultrafiltration in food processing industry: review on application, membrane fouling, and fouling control. Food Bioprocess Technol. 5, 1143–1156. https://doi.org/10.1007/s11947-012-0806-9.

Hu, S., Wang, X., Guo, S., Li, L., Hou, Y., 2014. Preparation and properties of bioactive peptides from yeast protein. Focus. Mod. Food Ind. 3, 52. https://doi.org/10.14355/fmfi.2014.03.007.

Comitini, F., Ferretti, R., Clementi, F., Mannazzu, I., Ciani, M., 2005. Interactions between Saccharomyces cerevisiae and malolactic bacteria: preliminary characterization of a yeast proteinaceous compound(s) active against *Oenococcus oeni*. J. Appl. Microbiol. 99, 105–111. https://doi.org/10.1111/j.1365-2672.2005.02579.x.

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., Gírio, F., 2010. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-Saccharomyces wine-related strains. Appl. Microbiol. Biotechnol. 86, 965–972. https://doi.org/10.1007/s00253-009-2409-6.

Guo, H., Guo, S., Liu, H., 2020. Antioxidant activity and inhibition of ultraviolet radiation-induced skin damage of Selenium-rich peptide fraction from selenium-rich yeast protein hydrolysate. Bioorg. Chem. 105, 104431 https://doi.org/10.1016/j.bioorg.2020.104431.

Jung, E.Y., Lee, H.S., Choi, J.W., Ra, K.S., Kim, M.R., Suh, H.J., 2011. Glucose tolerance and antioxidant activity of spent brewer's yeast hydrolysate with a high content of cyclo-his-pro (CHP). J. Food Sci. 76, 272–278. https://doi.org/10.1111/j.1750-3841.2010.01997.x.

European Commission, Nutrition claims, (2012). https://food.ec.europa.eu/safety/labelling-and-nutrition/nutrition-and-health-claims/nutrition-claims_en (accessed September 26, 2022).

Amorim, M., Pinheiro, H., Pintado, M., 2019b. Valorization of spent brewer's yeast: optimization of hydrolysis process towards the generation of stable ace-inhibitory peptides. LWT - Food Sci. Technol. 111, 77–84. https://doi.org/10.1016/j.lwt.2019.05.011.

Marson, G.V., Lacour, S., Hubinger, M.D., Belleville, M.P., 2022. Serial fractionation of spent brewer's yeast protein hydrolysate by ultrafiltration: a peptide-rich product with low RNA content. J. Food Eng. 312, 110737 https://doi.org/10.1016/j.jfoodeng.2021.110737.

Xie, J., Cui, C., Ren, J., Zhao, M., Zhao, L., Wang, W., 2017. High solid concentrations facilitate enzymatic hydrolysis of yeast cells. Food Bioprod. Process. 103, 114–121. https://doi.org/10.1016/j.fbp.2017.03.004.

Powers, J.-P.S., Hancock, R.E., 2003. The relationship between peptide structure and antibacterial activity. Peptides 24, 1681–1691. https://doi.org/10.1016/j.peptides.2003.08.023.

Jenssen, H., Hamill, P., Hancock, R.E.W., 2006. Peptide antimicrobial agents. Clin. Microbiol. Rev. 19, 491–511. https://doi.org/10.1128/CMR.00056-05.

Mookherjee, N., Anderson, M.A., Haagsman, H.P., Davidson, D.J., 2020. Antimicrobial host defence peptides: functions and clinical potential. Nat. Rev. Drug Discov. 19, 311–332. https://doi.org/10.1038/s41573-019-0058-8.

Chan, D.I., Prenner, E.J., Vogel, H.J., 2006. Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. Biochim. Biophys. Acta - Biomembr. 1758, 1184–1202. https://doi.org/10.1016/j.bbamem.2006.04.006.

DeBose-Boyd, R.A., 2008. Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. Cell Res. 18, 609–621. https://doi.org/10.1038/cr.2008.61.

Zhao, Y., 2008. Structure and function of angiotensin converting enzyme and its inhibitors. Chin. J. Biotechnol. 24, 171–176. https://doi.org/10.1016/S1872-2075(08)60007-2.

Shields, H.J., Traa, A., Van Raamsdonk, J.M., 2021. Beneficial and Detrimental effects of reactive oxygen species on lifespan: a comprehensive review of comparative and experimental studies. Front. Cell Dev. Biol. 9 https://doi.org/10.3389/fcell.2021.628157.

Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., Jacob, R., 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. J. Agric. Food Chem. 51, 3273–3279. https://doi.org/10.1021/jf0262256.

Costa, E.M., Oliveira, A.S., Silva, S., Ribeiro, A.B., Pereira, C.F., Ferreira, C., Casanova, F., Pereira, J.O., Freixo, R., Pintado, M.E., Carvalho, A.P., Ramos, Ó.L., 2023. Spent yeast waste streams as a sustainable source of bioactive peptides for skin applications. Int. J. Mol. Sci. 24, 2253. https://doi.org/10.3390/ijms24032253.

Schaich, K.M., Tian, X., Xie, J., 2015. Hurdles and pitfalls in measuring antioxidant efficacy: a critical evaluation of ABTS, DPPH, and ORAC assays. J. Funct. Foods 14, 111–125. https://doi.org/10.1016/j.jff.2015.01.043.