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Optimization of Hydrolysis and Identification of Bioactive Peptides in Brewery Yeast Residuals

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Industrial waste products are of biotechnological interest due to their abundance and can be utilized as protein sources for the production of bioactive peptides through hydrolysis. The utilization of yeast residues from the brewing industry has been demonstrated to be a viable method for obtaining protein hydrolysates. The degree of hydrolysis and soluble total protein of the hydrolysates were evaluated for optimization purposes to achieve a product with a high proportion of soluble organic nitrogen, increased levels of bioactive peptides and amino acids. Both alkaline and enzymatic hydrolysis processes were tested. The hydrolyses were carried out with the aim of large-scale implementation. The treatments were effective in liberating intracellular content and producing biologically functional hydrolysates, with degree of hydrolysis values ranging from 28 to 63% and total protein from 2.95 to 8.28% at the optimum points confirmed by statistical analysis (analysis of variance (ANOVA)). The hydrolysate produced with Alcalase® exhibited the highest peptide intensity, while the hydrolysate produced with Flavourzyme® showed the highest concentration of free amino acids. The peptides were identified by mass spectrometry and were found to have biological activities according to the Biopep database. This study presents a quick and economically feasible approach for the implementation of a pilot project for the reuse of this waste.

Keywords: bioactive peptides, industrial waste, protein sources, hydrolysis, yeast residues, brewing industry

Introduction

Proteins, peptides, and amino acids are crucial molecular components of living organisms. Unlike amino acids, the notion of proteins and peptides as functional foods aimed at enhancing quality of life is a recent phenomenon.[1] Bioactive peptides obtained from food-derived proteins have been demonstrated to exhibit positive effects on human health, both nutritionally and functionally.[2] After undergoing cleavage, proteins acquire biologically relevant functions and are typically composed of 2 to 20 amino acids.[3]

The consumption of these protein hydrolysates has been shown to improve overall health by reducing the potential for chronic diseases. This is due to various bioactive effects, including inhibition of the angiotensin-converting enzyme (ACE), leading to anti-hypertensive activity, antioxidant properties due to their affinity for reactive oxygen species, antidiabetic effects through inhibition of dipeptidyl peptidase 4 (DPPIV) and dipeptidyl peptidase 3 (DPPIII), chelating abilities towards transition metals, antimicrobial properties, opioid-like effects, immunomodulatory properties, and cytokine-modulating effects.[4]

In the pharmaceutical industry, there is an ongoing effort to develop molecular drug carrier systems using peptides for the chemotherapy treatment of cancers. Peptides

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increase specificity in drug transport and target tumor cells.[5] This effect has also been observed in other diseases through the use of membrane-permeabilizing peptides, based on their specific characteristics.[6]

Studies have focused on the isolation and purification of peptides from hydrolysates of milk, cheese, meat, fish, plants, and marine organisms,[2] which are noble and more expensive. Meanwhile, the residues from industrial protein waste disposal systems are of biotechnological interest due to the abundance of by-products. These hydrolysates can serve as a low-cost, highly available protein source for the production of bioactive peptides through enzymatic hydrolysis,[7] which in addition to providing an alternative to waste disposal, due to the added value, can have the application as functional foods.

Several factors influence the biological activity of functional foods derived from enzymatic hydrolysis, including the source, pre-treatments, and digestion protocols, physical-chemical variables, and the structure of the resulting molecules from these hydrolysates. Literature reports that low molecular mass peptides, such as di- and tripeptides, are more bioactive and have higher antioxidant activity and ACE inhibitory activity.[8]

Some of the important industrial protein waste comes from *Saccharomyces* species, with studies[9] demonstrating that it can be a technically viable protein source. It has been found that even in situations of using yeast residues as industrial by-products, where the cell wall is more resistant, enzymatic treatment is effective in releasing the internal content for hydrolysis.[9]

Despite much technological development aimed at increasing the efficiency of analysis and understanding the enzymatic processes involved and the mechanisms of action of these peptides, there is limited literature on the use of yeast as a raw material in the production of protein derivatives. Furthermore, there are no reports in the literature on the optimization of protocols for the hydrolysis of beer yeast waste based on the evaluation of tri- and di-peptides,[10] which is an innovation, since these two- and three-amino acid molecules can have higher biological activities and can be efficiently evaluated in protein hydrolysates.[11] It is necessary to acquire data from the use of this industrial waste in order to develop better processing conditions and to add value to the waste, as well as to provide faster, safer and more cost-effective approaches.

Thus, the objective of this study was to outline optimal conditions using a full factorial design with central point of the enzymatic and alkaline hydrolysis of yeast from the *Saccharomyces* genus from the industrial beer production process, based on degree of hydrolysis, total protein, and isolation of low molecular weight peptides (di- and tripeptides) and their biological activities from available databases.

Experimental

Yeast residue samples were obtained from a local brewery in Maringá, Paraná, Brazil. Cells of *Saccharomyces pastorianus* strain SaflagerTM W-34/70 (Fermentis), derived from the production of Pilsen beer after a single fermentation process, were used due to the criteria adopted by the company. Alcalase® 2.4 L, Neutrase® 0.8 L, and Flavourzyme® 1000 L were purchased from Novozymes Latin America Ltda (Araucaria, Brazil). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-serine, sodium dodecyl sulfate, tetrahydrated sodium tetraborate, DL-dithiothreitol, formaldehyde, and high-performance liquis chromatography (HPLC)-grade acetonitrile were obtained from Sigma-Aldrich Brasil Ltda (Barueri, Brazil). Deionized water was obtained using a Milli-Q system (Millipore, Billerica, MA). Formic acid was purchased from Acros Organics (New Jersey, USA).

Hydrolysis

Before the hydrolysis, the dry weight of the liquid samples was determined for calculation purposes and referred to as the "Substrate." This was accomplished by drying the sample in an oven at 105 °C until its mass stabilized. The amount of reagents and dilutions were calculated in order to carry out the different treatments according to the statistical design presented in Table 1. For each level, the enzyme and NaOH were added in mass proportion to the substrate mass.

For the enzymatic hydrolyses, the *in natura* samples were weighed considering their total weight, in 250 mL Erlenmeyers and the pH was adjusted to 8, 7.5, and 7 for treatments with Alcalase®, Neutrase® and Flavourzyme®, respectively. After adjustment, the samples were heated in a water bath to 65 °C and then the enzymes were added. The containers were transferred to an agitated oven at 110 rpm for 1 h at 60, 60, and 50 °C, respectively for Alcalase®, Neutrase® and Flavourzyme®. After the hydrolysis process, the inactivation of the enzymes was carried out in a water bath at 90 °C for 15 min (Figure 1). Both temperatures and pH were defined based on the recommendations of the manufacturer for best enzyme conditions.[12]

In the experimental treatment with alkaline hydrolysis using NaOH, the samples were prepared in 50 mL Becker flasks. The containers were transferred to a water bath at

Table 1. Complete factorial design with central point (CP); concentration of the crude sample and its dilution in distilled water (m/v), and reagents in relation to yeast dry matter, for enzymatic and alkaline hydrolyses

Experiment	Crude sample		Enzyme/Substrate		NaOH/Substrate	
	Level	Concentration / %	Level	Concentration / %	Level	Concentration / %
A	+1	100	+1	10	+1	100
B	+1	100	−1	1	−1	50
C	−1	50	+1	10	+1	100
D	−1	50	−1	1	−1	50
CP	0	75	0	5.5	0	75

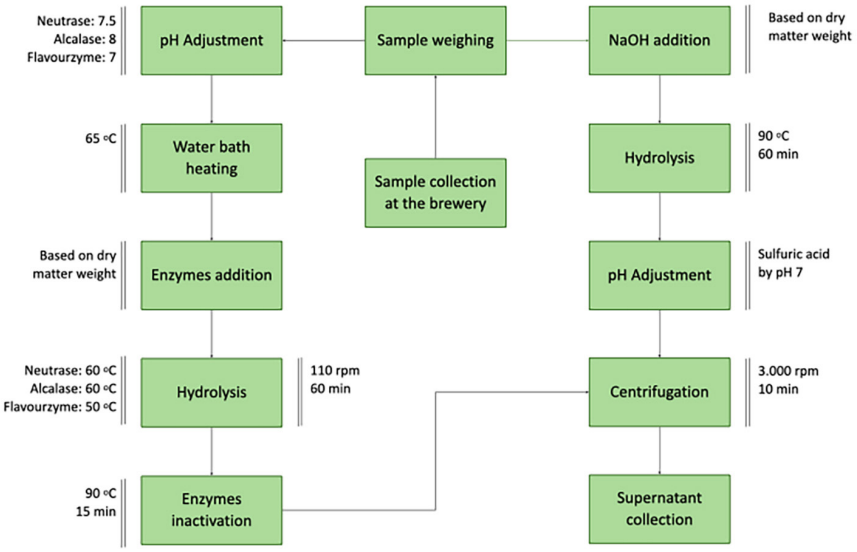

Figure 1. Flowcharts demonstrating the steps of hydrolysis.

90 °C for 1 h. Subsequently, the pH was adjusted with 50% sulfuric acid to 7 ± 0.1. All experiments involved centrifuging the samples for 10 min at 3,000 rpm in a Falcon tube, and collecting the supernatant for subsequent analysis of total protein and degree of hydrolysis, which were stored in a refrigerator at 4 °C (Figure 1).

Statistical analysis

The experimental design is crucial to ensure the robustness of the data, in order to provide the necessary information to generate a response surface that can establish the best conditions to maximize the results.[13] Experiments were designed based on a full factorial analysis at two levels to verify the response surface with a central point, with the sample concentration and reagent (enzyme or NaOH) as input variables and degree of hydrolysis and nitrogen percentage as output variables. Table 1 presents how the experiments were designed.

The treatments, defined by the design, were carried out in triplicate. Each of the samples was later used for protein (Pt) and degree of hydrolysis (GH) analysis, whose results fed the calculation and statistical analysis spreadsheet. The data obtained from degree of hydrolysis and total protein were subjected to analysis of variance (ANOVA) with $p < 0.05$ (significance level greater than 5%) through Sisvar 5.7 software.[14]

Total protein

The total protein content (Pt), expressed as a percentage, was determined using the Kjeldahl method. 0.2 g of sample was weighed and 2 g of catalytic mixture (containing copper sulfate) and 5 mL of PA sulfuric acid was added. The tubes containing the sample and reagents were digested by increasing the temperature to 150 °C for 1 h, and then to 250 °C for 1 h and finally to 350 °C until complete digestion, when the sample becomes light green. The sample was then allowed to cool to room temperature overnight.

The sample was distilled in a Lucadema still (Luca-74) with the addition of 20 mL of 50% NaOH solution, and

the evolved sample was collected in a 150 mL Erlenmeyer flask containing 20 mL of a mixture of 4% boric acid and indicator (alcoholic solution of methyl red and bromocresol green), until 50 mL was reached. The solution was then titrated with 0.1 mol L^{-1} hydrochloric acid until the indicator turned pink. The volume consumed in the titration served as the basis for calculation.[15] The conversion factor of 5.8 was used for yeasts.[16]

Degree of hydrolysis

To determine the DH, the *o*-phthalaldehyde (OPA) methodology,[17] was conducted, where serine was used as a standard solution and distilled water as a blank. Absorbance readings were taken in a Kasvi UV-Vis spectrophotometer (K37-UVVIS) at 340 nm, from cuvettes containing 3 mL of OPA solution and 400 µL of sample solution in distilled water (1:100, m/v), after exactly 2 min of reaction. The value of constants α and β were taken as 1 and 0.4, respectively. Total hydrogen was used as 7.5.[16] The degree of hydrolysis was expressed as a percentage.

Solid-phase extraction

SPE was performed on 3 mL columns (C18 500 mg; Millipore-Sigma) following the column conditioning and equilibration steps, sample filtration, aqueous and organic elution, using the methodology described by Huang *et al.*[18] with modifications. Acetonitrile solution with 0.1% formic acid was first used to activate the column, with a total of three volumes of the solution, followed by three volumes of Milli-Q water with 0.1% formic acid. The supernatant sample was again centrifuged for 10 min at 3,000 rpm and mixed with formic acid at a ratio of 0.1% for application to the SPE column. The aqueous elution consisted of three volumes of Milli-Q water in 0.1% formic acid solution. The sample was collected by organic elution, with three volumes of the column, using a solution of acetonitrile and Milli-Q water (70:30, v/v) and 0.1% formic acid.

Sample preparation and liquid chromatography

Following the rapid loss-neutral mass spectrometry (MS/MS) analysis methodology,[10] 100 mg of the samples were weighed and conditioned with the addition of 1.0 mL of ammonium bicarbonate solution (0.1939 g in 50 mL of Milli-Q water), mixed in a vortex for 1 min. The second dilution was made with 100 µL of this solution and 900 µL of the mobile phase, a solution of acetonitrile in Milli-Q water (70:30, v/v) and 0.1% formic acid, mixed in a vortex for 1 min, followed by centrifugation for 10 min at 3,000 rpm and conditioning in a refrigerator for 1 h. For the liquid chromatography mass spectrometry (LC-MS/MS) injection and reading process, the final dilution was performed with 100 µL of the supernatant and 900 µL of the mobile phase. The samples that underwent solid-phase extraction (SPE) were directly diluted in the mobile phase in a 6:4 (v/v) ratio.

The mobile phase was pumped at an isocratic flow rate of 300 µL min^{-1} through a column with a C18 stationary phase with particle size of 3.5 µm (4.6 × 50 mm). The samples were injected in a volume of 5 µL, running for 5 min at room temperature on a PremierXE Triple-Quadruple Mass Spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray ionization (ESI) source and positive ionization mode at 4.0 kV, with the desolvation gas temperature and source block temperature at 350 and 100 °C, respectively. Additionally, the cone voltage was set at 20 V, collision energy was 15 V, and collision gas pressure of argon was set at 3.0×10^{-3} Torr.

Mass spectra interpretation

The rapid LC-MS/MS method based on 46 Da neutral loss was used to identify potential di- and tripeptides resulting from yeast hydrolyses. The potential dipeptides (Tables S1-S2) and tripeptides (Tables S3-S28) are presented in Supplementary Information (SI) section, considering the 20 most important amino acids. This method is based on the rapid (5 min) tracking of protonated molecules where there is a selective 46 Da neutral loss, referring to the carboxylic acid portion. After searching for possible peptides, collision-induced dissociation (CID) fragmentation is conducted, and a search for fragments is performed through de novo sequencing.[10]

The fragmentation spectra of the neutral loss scans peaks were interpreted,[19] starting from the identification of y1 ions (referring to possible protonated amino acids), y2 and b2 ions (for tripeptides), confirming amino acid residues, Table S29 presented the protonated amino acids (y1), immonium ions, and the related ions to each amino acid.

Results and Discussion

Degree of hydrolysis and total protein

The main challenge in using residual yeast from industries is the release of intracellular compounds through the breaking of the cell wall, which becomes larger and more rigid due to reuse in the fermentation process. The focus of research using this material is to

find optimal conditions for breaking the cell wall and transforming the proteins into bioactive peptides. It is of utmost importance to have a low cost to make it viable for large-scale use by the industry, while presenting the highest possible amount of molecules with biological activity.

The raw, untreated samples exhibited a Pt content of 3.1 ± 0.1% in the supernatant and 10.2 ± 0.1% in the homogenized sample (i.e., with the yeast suspended). Table 2 shows the highest gain in total soluble protein in the Alcalase® (+1, +1) treatment as compared to the raw homogenized sample, with a rise of 8.28% from the initial 10.16%, resulting in a mass balance of 81.5%. This indicates that the experiments yielded the maximum percentage of protein that could be extracted from the raw homogenized sample. The experiment utilizing a low amount of Alcalase® (+1, −1) still showed a satisfactory amount of soluble protein, yielding a value of 75.2%. These values pertain to soluble proteins, peptides, and amino acids.

It can be noted that yeast dilution generally increases hydrolysis efficiency, as the Pt values of the treatments with NaOH, Neutrase®, and Alcalase®, with a dilution of 50%, showed a value higher than half the Pt value of the undiluted samples. Thus, the efficiency is 46, 69, and 90% (experiment C), compared to 28, 59, and 81.5% (experiment A), respectively. For Flavourzyme®, the yield was 37% for the most concentrated and 36% for the most diluted, not showing that dilution made a difference in practice. It is observed that experiments B and D (with lower limits of enzymes and NaOH) do not show much difference from experiments A and C (with higher limits of enzymes and NaOH).

In a scientific study by Mirzaei *et al.*,[20] it was discovered that the correlation between the degree of hydrolysis in autolysis and biological activity (both in terms of ACE inhibition and antioxidant activity) is not proportional. This is in contrast to the results obtained from hydrolysates produced through enzymatic treatment. The findings suggest that these biological properties may be related to the hydrophobic amino acids present in the hydrolysates. However, it is important to note that the degree of hydrolysis is a combined measurement of both amino acid and peptide content. A high degree of hydrolysis could indicate a large amount of amino acids and a small amount of peptides, and it is well established that peptides have a positive effect on biological activities.

The degree of hydrolysis of a sample is commonly used as an indicator to compare the efficiency of hydrolysis among different products and processes. The degree of hydrolysis refers to the extent of hydrolytic degradation of the proteins contained in the sample. During hydrolysis, various peptides of varying sizes are generated, depending on the specificity of the enzyme.[21] With the aim of optimizing the process for the production of stable ACE inhibitors, Amorim *et al*.[22] used extracts of *Cynara cardunculus* and measured the degree of hydrolysis of the samples. The best results (40%) were achieved for this variable after 8 h of treatment with 8% enzyme, on material after four fermentative beats. The degree of hydrolysis results obtained after analysis for the treatments are represented in Table 3.

Table 2. Values of total protein (Pt) after treatment

Experiment	Crude sample	Chemical/S	Pt / %			
			NaOH	Neutrase®	Alcalase®	Flavourzyme®
A	+1	+1	2.86 ± 0.1	5.97 ± 0.3	8.28 ± 0.4	3.80 ± 0.1
B	+1	−1	2.93 ± 0.1	4.50 ± 0.2	7.64 ± 0.2	3.25 ± 0.1
C	−1	+1	2.36 ± 0.1	3.52 ± 0.1	4.55 ± 0.5	1.82 ± 0.2
E	−1	−1	1.82 ± 0.2	2.89 ± 0.3	4.29 ± 0.2	1.28 ± 0.1
CP	0	0	2.60 ± 0.1	4.55 ± 0.6	6.58 ± 0.2	2.70 ± 0.11

Table 3. Degree of hydrolysis (DH) values, in percentage, after treatment

Experiment	Crude sample	Chemical/S	DH / %			
			NaOH	Neutrase®	Alcalase®	Flavourzyme®
A	+1	+1	32.68 ± 2.4	24.41 ± 0.6	33.35 ± 0.1	49.48 ± 1.3
B	+1	−1	28.16 ± 4.2	27.88 ± 1.2	31.22 ± 0.3	45.35 ± 0.6
C	−1	+1	18.24 ± 1.1	19.54 ± 1.2	30.40 ± 0.7	61.80 ± 0.1
D	−1	−1	16.81 ± 2.2	21.31 ± 0.4	28.90 ± 0.2	59.83 ± 0.7
CP	0	0	30.51 ± 3.8	23.67 ± 0.6	34.78 ± 0.1	60.11 ± 1.7

Satisfactory degrees of hydrolysis were obtained for all tested enzymes, with Flavourzyme® having the highest efficiency. However, the total mass content of hydrolyzed material is lower due to its low protein content. In the best experiment with Flavourzyme®, we have approximately 49% hydrolysis of 3.8% protein, which corresponds to a total percentage of 1.9% in mass of peptides and amino acids. For the treatments performed with Alcalase®, when the same calculation is made, the total percentage in mass is approximately 2.7% of peptides and amino acids, or 42% more hydrolyzed content. It is worth noting that a high degree of hydrolysis may indicate a higher amount of amino acids than peptides.

Another important aspect from an industrial viewpoint is that even with low percentages of enzymes or NaOH, good hydrolysis results are still obtained. Additionally, the hydrolyses performed with Neutrase® showed better hydrolysis degree results when lower enzyme concentrations were used, demonstrating higher efficiency at lower concentrations, probably due to the reduced interference of molecules that may bind to the active site of the enzyme, reducing its activity. On the other hand, diluted samples, using the same level of enzymes and NaOH, had reduced hydrolysis degree, very likely due to the fact that the latter stage of the reaction involves only the protein, and dilution ends up reducing the reaction rate.

It was also observed that dilution of yeast residue increased the degree of hydrolysis with Flavourzyme®, indicating that it is more efficient in diluted solutions or that dilution may be reducing some inhibitory effect contained in the yeast residue. This does not occur in the other studied processes.

Statistical analysis

The ANOVA showed that the model was significant, considering a significance level of 5%, for the results obtained in all treatments.

The results for both output variables showed that the sample concentration had the greatest effects on all treatments. Although the enzyme/substrate ratio demonstrated effects on the results, the interactions between the variables were not significant at the 95% confidence level in any of the enzyme treatments. The concentration of NaOH also did not show a significant effect on the results. Tables S21-S28 in SI section present the analysis of variance for all treatments and dependent variables.

Figures 2 and 3 show the response surface for the degree of hydrolysis (%) and total protein (%) of the treatments.

Figure 2 depicts the response surface for the degree of hydrolysis of the treatments, considering the variation of enzyme concentration for Alcalase® and NaOH results as quadratic. Meanwhile, the results for Neutrase® and Flavorzyme® exhibit a linear behavior.

Figure 3 depicts the response surface for total protein of the treatments, showing all of them a linear behavior in the selected range. After statistical analysis, the optimum points with the highest DH and Pt were selected for each of the four treatments. Tables 4 and 5 show the input variables (sample and reagent concentration) and the output variables (DH and Pt) with their calculated values and actual experimental results, which were experimentally tested for validation. Detailed information about the Pareto chart test is in the SI section.

Comparison of the optimal points of the treatments showed that the results varied from 27.88 to 61.80% in DH and from 2.93 to 8.28% in total protein (Pt). However, the two variables did not show a direct proportionality, meaning that a higher DH does not necessarily mean a higher Pt based on the data obtained in this study. This means that when seeking to obtain a product with a higher content of the mixture of amino acids and peptides, the conditions of Table 5 should be used, and for higher protein content, the conditions of Table 4 should be used. We can observe that the theoretical optimal points for each treatment were very close to the experimental optimal points in both protein total and degree of hydrolysis, supporting the validity of the mathematical model.

Mass spectrometry

The inherent properties of the sample necessitated the use of SPE to reduce interferents and enhance ionization sensitivity in the electrospray ionization source compared to the preparation described by Poliseli *et al.*[10] Neutral loss scans of 46 Da (NL46) were performed on the samples within the mass-to-charge ratio (*m/z*) range of *m/z* 50 to 210 and *m/z* 210 to 400 to observe amino acids and di- and tripeptides. The mass spectra from *m/z* 50 to 210 are presented in Figure S1, in SI section for the results of DH and Pt for NaOH, Neutrase®, Alcalase®, and Flavourzyme®.

In the mass spectra, we can observe various amino acids and some peptides in the treatments presented, demonstrating the clearness of the methodology in presenting the results. The spectra in the range of *m/z* 210 to 400 are shown in the Figure S2 (SI section).

In the interpretation work, the ion signals with an average intensity of at least two readings from the triplicate, above 500 units after processing the spectrum were considered. Thus, the intensities of the ions corresponding to the amino acids in the samples are contained in Table 6.

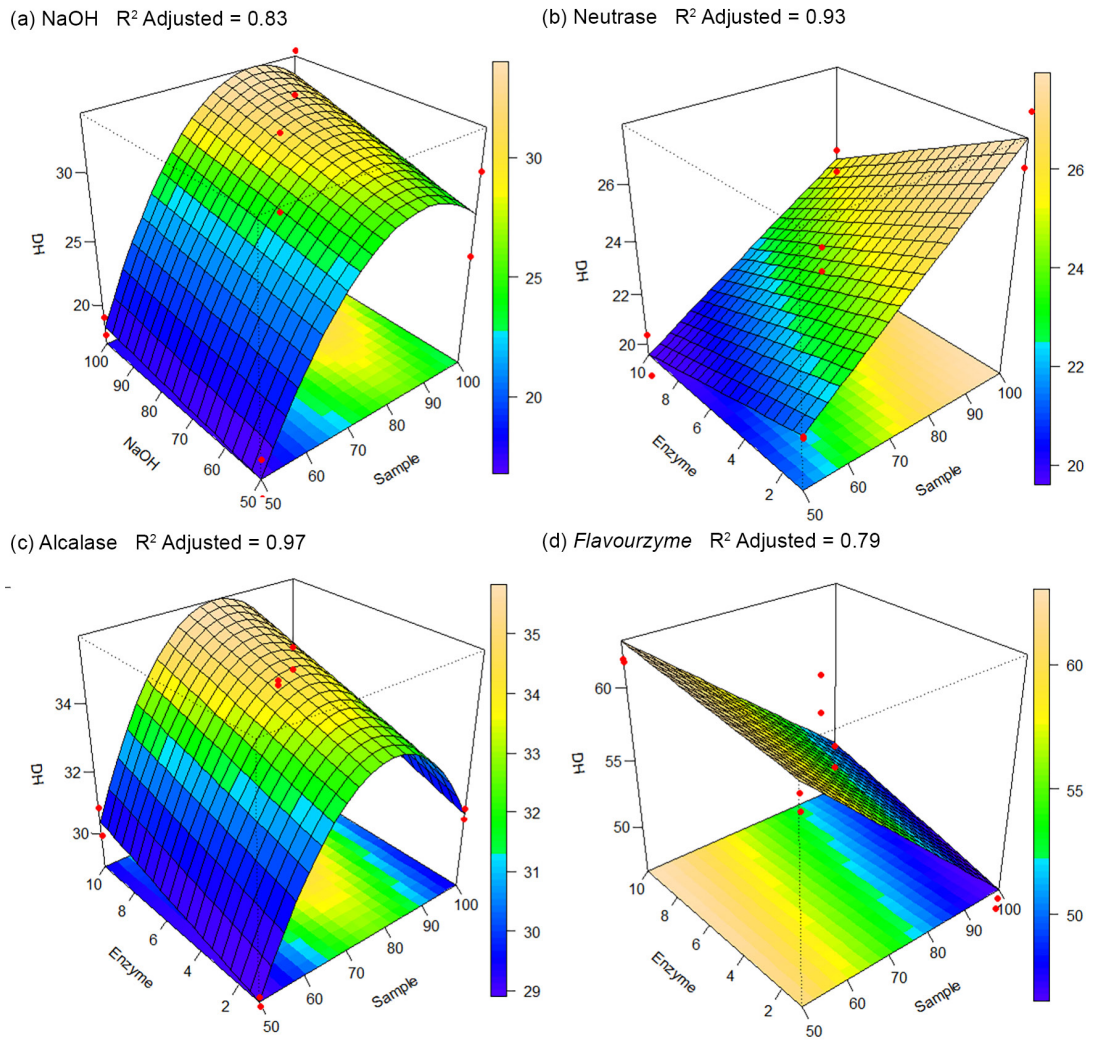

Figure 2. Response surface for hydrolysis degree results of treatments (a) NaOH; (b) Neutrase®; (c) Alcalase®; (d) Flavourzyme®.

The values of the intensities with their respective standard deviations are shown in Table S29, SI section.

Table 6 demonstrates that the intensity of amino acids consistently appeared higher in Pt treatments compared to DH treatments, most likely due to the greater efficiency of Alcalase® in producing amino acids under conditions of higher concentrations of yeast and reagents. The presented results were highly favorable for the Flavourzyme® enzyme process in obtaining free amino acids, suggesting a greater efficiency of this enzyme due to the observation of more intense peaks. When compared to alkaline hydrolysis, enzymatic hydrolysis showed superiority in terms of ion intensities related to amino acids. It is possible to observe the presence of the amino acids proline, valine, leucine/isoleucine, and phenylalanine in all samples, which have nonpolar properties.

The mass spectra from *m/z* 210 to 400 are presented in Figure S2 for improved results of DH and Pt for NaOH, Neutrase®, Alcalase®, and Flavourzyme®. In the mass spectra presented in Figure S2, we can observe, in the range of *m/z* 210 to 400, a higher number of peptides as expected, and apparently, the peptides are more intense in the treatments optimized for a higher level of Pt. To verify whether these changes between DH and Pt treatments are significant and whether the change of the catalyst significantly interferes with the peptide intensities, we conducted a Tukey's test with significance of $p < 0.05$. The results are presented in Table 7.

Significance level $p < 0.05$ by Tukey's test

The data from Table 7 shows that the peptides obtained after treatment with NaOH and enzymes present signals with greater intensity for the treatment with higher protein, which corroborates the hypothesis that the high protein condition favors peptides, while conditions with lower protein concentrations favor a higher degree of hydrolysis, possibly due to an increase in free amino acids.

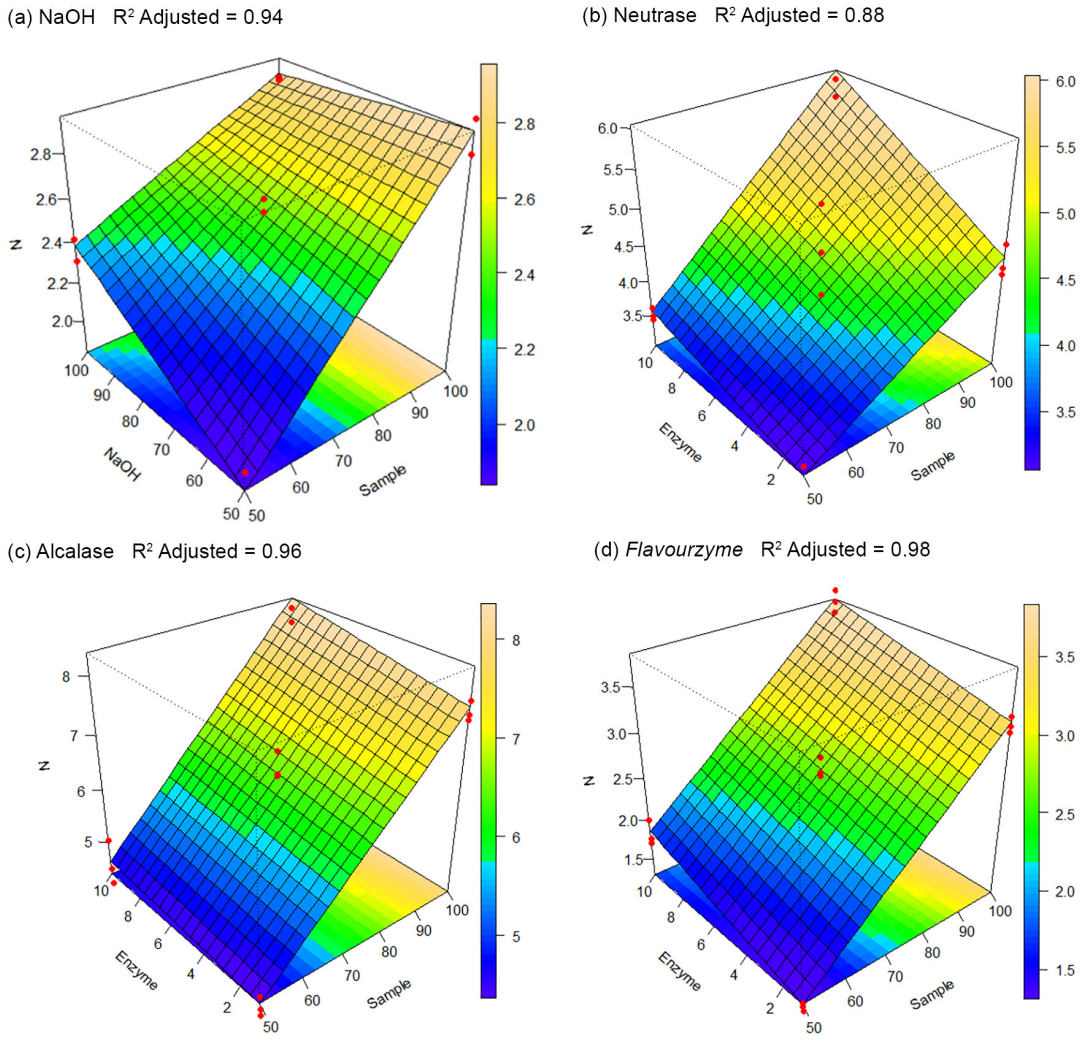

Figure 3. Response surface for total protein results of treatments (a) NaOH; (b) Neutrase®; (c) Alcalase®; (d) Flavourzyme®.

Table 4. Optimal degree of hydrolysis results

Treatment	NaOH / %	Neutrase® / %	Alcalase® / %	Flavourzyme® / %
Crude sample	89	100	80	50
Chemical/S	100	1	6	10
Theoretical result	33.93	27.95	35.83	63.00
Experimental result	32.68 ± 2.4	27.88 ± 1.2	33.64 ± 1.6	61.80 ± 0.6

Table 5. Optimal points for total protein

Treatment	NaOH / %	Neutrase® / %	Alcalase® / %	Flavourzyme® / %
Crude sample	100	100	100	100
Chemical/S	50	10	10	10
Theoretical result	2.95	6.03	8.28	3.83
Experimental result	2.93 ± 0.1	5.97 ± 0.3	8.28 ± 0.4	3.80 ± 0.1

Among the treatments, Alcalase® showed higher intensity for the treatments with higher protein for SH, (I/L)(I/L), VGT, SG(I/L), F(I/L), (I/L)F, and FF. The treatment with Flavourzyme® GH has a high degree of SV, and NaOH has a higher intensity of the peptide GMT.

The ions of *m/z* 177, 205, 243, 245, 279, and 313

Table 6. Average peak intensity (in triplicates) in the 46 Da (NL46) neutral loss scan in the range of *m/z* 50 to 210, corresponding to amino acids

Treatment		Optimal samples							
		NaOH		Neutrase®		Alcalase®		Flavourzyme®	
Amino acid	*m/z*	DH	Pt	DH	Pt	DH	Pt	DH	Pt
Serine	106	–	501^{a}	557^{a}	907^{b}	393^{a}	380^{a}	432^{a}	564^{a}
Proline	116	1319^{a}	1589^{a}	707^{a}	1806^{a}	2070^{a}	657^{a}	7551^{a,b}	17480^{b}
Valine	118	233^{a}	911^{a,b,c}	639^{a,b}	1958^{d}	1638^{c,d}	616^{a,b}	1324^{b,c,d}	1743^{d}
Isoleucine/Leucine	132	739^{a}	3890^{a}	1487^{a}	14437^{b}	10956^{b}	1947^{a}	11530^{b}	10942^{b}
Histidine	156	239^{a}	435^{a,b,c}	449 ^{a,b,c}	801^{c}	387^{a,b}	372^{a,b}	515 ^{a,b,c}	665^{b,c}
Fenilalanine	166	5578^{a}	7625^{a,b}	2060^{a}	23375^{c}	21921^{b,c}	5185^{a}	36007^{c}	27568^{c}
Tirosine	182	251^{a}	62^{a,b}	249^{a}	803^{b}	708^{a,b}	–	607^{a,b}	631^{a,b}

Superscript letters: significance level $p < 0.05$ by Tukey's test.

Table 7. Average intensity of peaks (triplicate) in the neutral loss scan of 46 Da (NL46) in the range of *m/z* 210 to 400, corresponding to peptides

Ions	Peptides	Optimal samples							
		NaOH		Neutrase®		Alcalase®		Flavourzyme®	
m/z		DH	Pt	DH	Pt	DH	Pt	DH	Pt
177	GT	322^{a}	271^{a}	368^{a}	1.057^{c}	279^{a}	236^{a}	798^{b,c}	524^{b}
205	SV	761^{a}	1658^{a}	836^{a}	1181^{a}	1388^{a}	540^{a}	8999^{b}	7048^{b}
243	SH	112^{a}	273^{a}	293^{a}	1101^{c}	206^{a}	2088^{d}	709^{b}	1555^{c}
245	(I/L)(I/L)	238^{a}	–	152^{a}	1054^{b}	375^{a}	1790^{b}	–	286^{a}
277	VGT; SG(I/L)	180^{a}	167^{a}	126^{a}	704^{a,b}	143^{a}	1262^{b}	189^{a}	409^{a}
279	F(I/L); (I/L)F	708^{a,b}	442^{a,b}	292^{a}	1464^{b,c}	387^{a,b}	2240^{c}	242^{a}	328^{b}
309	GMT	453^{a}	618^{a}	393^{a}	593^{a}	242^{a}	614^{a}	299^{a}	548^{a}
313	FF	300^{a}	249^{a}	132^{a}	779^{b}	347^{a}	877^{c}	220^{a}	240^{a}

Superscript letters: significance level $p < 0.05$ by Tukey's test.

presented dipeptides with reported biological activity in the literature. The ions of *m/z* 277 and 309 were identified as tripeptides, not previously reported in the database, and observed for the first time. Table 8 presents the interpretation of the fragmentation spectrum performed by de novo sequencing, the sample identification results, as well as the biological activity found in the literature.

Peptides with ACE and DPPIV inhibitory activity can be observed from all tested treatments, however, glucose stimulation was only found in the peptide from *m/z* 245 ion present in the samples hydrolyzed with Neutrase® and Alcalase®. Different peptides can be obtained depending on the defined approach. An example is the high intensity of the *m/z* 205 ion in the treatment with Flavourzyme® which presents the SV peptide, a DPPIV inhibitor.

Dipeptides with important functions such as ACE inhibition for GT, (I/L)(I/L), (I/L)F, and FF; DPP III and IV inhibition for SV, SH, (I/L)(I/L), F(I/L), and FF; and glucose metabolism stimulation in (I/L)(I/L) were observed. There were no records in the database for the biological activity of the tripeptides found. Figures containing the fragmentation spectra of the peaks and identification of the peptides are in Figures S3-S10, SI section.

The methodology adopted for peptide identification has proven to be efficient as in other studies. Meurer and co-workers[11,32] used the same protocol to analyze different substrates in order to verify bioactive peptides and bio-stimulating activity, and the treatment with Alcalase® as being the most efficient in obtaining more intense di- and tripeptides with bioactivities already demonstrated in the literature.[11] The next step will be the feasibility study of the product for testing in animal feed and human food.

Conclusions

Several hydrolysis processes were optimized to obtain bioactive peptides. The degree of hydrolysis and total protein were evaluated in order to obtain a product with the highest proportion of soluble organic nitrogen and higher levels of peptides and amino acids. The amino acids obtained were mainly proline, valine, leucine/isoleucine, and phenylalanine, both with hydrophobic characteristics. The hydrolysate

Table 8. Amino acid sequence of peptides identified by LC-MS/MS

Ions							Sequence	Biological activity	Reference
$[M + H]^+$	y1	Residue	Imonium	Related	b2	y2			
177	120(T)	57(G)	–	–	159	–	GT	ACE inhibitor	23
205	118(V)	87(S)	–	–	187	–	SV	DPPIV inhibitor	24
243	156(H)	87(S)	–	–	225	–	SH	DPPIV inhibitor	24
245	132(I/L)	113(I/L)	86	–	227	–	(I/L)(I/L)	DPPIV inhibitor; glucose stimulant and ACE inhibitor	24-27
277	120(T)	157(VG)	72	–	157	177	VGT	–	–
	132(I/L)	145(SG)	86	72	145	189	SG(I/L)	–	–
279	132(I/L)	147(F)	120	–	261	–	F(I/L)	DPPIII and DPPIV inhibitor	28,29
	166(F)	113(I/L)	120	–	261	–	(I/L)F	ACE inhibitor	23,30
309	120(T)	189(MG)	–	–	189	251	GMT	–	–
313	166(F)	147(F)	120	–	295	–	FF	ACE inhibitor; DPPIV inhibitor	31

ACE: angiotensin-converting enzyme; DPPIV: dipeptidyl peptidase 4; DPPIII: dipeptidyl peptidase 3.

resulting from the treatment with Flavourzyme® was the one that showed the highest intensity of amino acids, therefore, it is the process that can be used industrially to obtain these nutrients. The dipeptides obtained were GT, SV, SH, (I/L)(I/L), F(I/L), (I/L)F, FF, and the tripeptides VGT, SG(I/L), and GMT, which have important functions such as ACE inhibition for GT, (I/L)(I/L), (I/L)F and FF; DPP III and IV inhibition for SV, SH, (I/L)(I/L), F(I/L), and FF; and stimulation of glucose metabolism in (I/L)(I/L). There were no records in the database of biological activity for the tripeptides found. The hydrolysate obtained in the treatment with Alcalase® was the one that showed the highest intensity of peptides, therefore, it is the process that can be used industrially to obtain these bio-stimulants. This work presented a rapid approach that may be feasible for implementing a pilot project for the reuse of this waste.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Fernanda Della Rosa was responsible for investigation and writing de original draft; Angelica P. Tonin for data analysis; Beatriz Rocha for analysis; Marco A. R. Santos for experimental design and data analysis; Fabiane Cocari for data analysis; Lucio Cardoso Filho for conceptualization and experimental design; Valquíria M. S. Ribeiro for validation and manuscript review; Eduardo C. Meurer for funding acquisition, validation, manuscript writing, review and editing, experimental design, and supervision.

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