SCIENTIFIC REPORT

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EMERGING STRATEGIES TO IMPROVE THE POTENTIAL OF SPENT BREWER YEAST PROTEINS FOR CREATING NEW FUNCTIONAL FOODS

(Acronym: FunYeast)

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OBJECTIVE: To test the susceptibility to lysis of different spent brewer yeast repitched or non repitched such as to disrupt the cell wall and release the proteins and peptides, whose functionality will be tailored using ultrasound/high pressure.	Degree of achievement 100%

1. Exploring SBY to produce peptides/protein hydrolysates

Activity 1.1. Pre-treatment of spent brewer yeast (SBY) using several yeast lysis procedures such as mechanical/ plasmolysis/ autolysis/enzymatic hydrolysis to break down the cell walls to release the yeast proteins;

This activity was performed using wet spent yeast from the Brewing Pilot Plant of the Faculty of Food Science and Engineering, Galati and dry SBY donated by an industrial beer processor from Ploiesti. The spent yeast was characterized regarding protein content and dry matter content.

Table 1. Protein and dry matter content

Yeast type	Protein content g/100 g sample	Dry matter, g/100 g sample
Wet yeast	13.50±0.35	26.84±0.78
Dry yeast	36.85±0.23	88.41±0.81

The lysis procedures tested for the disruption of cell wall and release of proteins were:

Autolysis: yeast cells were mixed with distilled water at a concentration of 15% (w/v), incubated under shaking at 100 rpm at 55°C, for 48 h. After that, the suspension was inactivated at 80°C, for 10 min, cooled and centrifuged, the resulting supernatant had a protein content of 2.24 g/100 g dry yeast, dry matter content of 7.58% and inhibition rate of 74.10 ± 7.46 %.

Lysis assisted by high pressure: frozen wet SBY was tawed at room temperature and 3 g of biomass (20%) was subjected to several combinations of temperature and pressure using a high-pressure equipment available at the Bioaliment TehnIA Research Center from the Faculty of Food Science and Engineering as follows:

- 300 MPa, for 0, 5, 10, 15,20, 25, 30 minutes;
- 400 MPa, for 0, 5, 10, 15,20, 25, 30 minutes;
- 500 MPa, for 0, 5, 15,20, 25, 30, minutes;

The supernatant was analysed in terms of soluble protein content and antioxidant activity (Table 2).

Table 2. The soluble protein content and antioxidant activity of high-pressure treated yeast samples

Pressure MPa	Holding time (minutes)						
	0	5	10	15	20	25	30
Soluble proteins (% wet yeast)							
300	13.98±0.5	13.20±1.02	13.05±1.61	13.84±3.96	15.78±0.82	10.71±1.65	15.38±0.27
400	5.47±0.25	9.89±0.05	8.75±0.0	10.61±0.01	9.53±0.01	10.18±0.01	9.13±0.02
500	11.04±0.89	9.41±0.04	-	9.30±0.43	10.07±0.31	9.30±0.43	9.80±0.16
Antioxidant activity, DPPH Inhibition rate (%)							
300	81.78±0.0	78.81±0.07	64.39±0.0	74.45±0.14	81.57±0.0	71.36±0.07	76.02±0.00
400	66.53±0.47	66.53±0.47	56.33±4.96	66.20±0.63	66.98±0.31	64.52±6.30	72.95±1.18
500	75.73±2.91	60.17±0.00	-	64.75±2.68	44.84±4.18	68.09±4.10	67.03±8.28

The use of wet SBY created problems in ensuring the homogeneity conditions, as the soluble protein content and antioxidant activity of some control samples was higher than of the samples exposed to high pressure treatment. In tests where the samples were homogenous, the exposure of yeast to high pressure did not lead to cell wall rupture and release of the estimated protein content. The conclusion of this test was that high pressure treatment under the operated conditions is not an effective method of cell wall rupture and the release of intracellular components is not favoured.

Lysis assisted by ultrasound treatment

The ultrasound treatment was performed using a Bandelin, Sonoplus HD 3100 that operates at 200 W and 20 kHz. In this experiment, pulsation mode, amplitude and time were varied (Table 3). After ultrasound treatment, the suspension was centrifuged and the resulting supernatant was analysed for dry matter, soluble protein content, Z-disintegration index and antioxidant activity (ABTS and DPPH method).

Table 3. The conditions applied for performing ultrasound treatment

Amplitude, %	Time min	Pulsation mode
20	2.5	Off
20	5	Off
20	7.5	Off
50	2.5	Off
50	5	Off
50	7.5	Off
70	2.5	Off
70	5	Off
70	7.5	Off
20	2.5	On
20	5	On
20	7.5	On
50	2.5	On
50	5	On
50	7.5	On
70	2.5	On
70	5	On
70	7.5	On

The disintegration index Z showed that higher amplitudes and longer holding times do not exert a positive effect on yeast cell disintegration. At low amplitudes there were no significant differences in the degree of Z score between continuous and pulsed ultrasound treatment. Ultrasonication performed under pulsation mode, and high amplitudes caused lower disintegration of yeast cells.

Dry matter content of the native sample was about 29.9 g/100 g dry yeast. The dry matter content increased during ultrasound treatment, with increasing amplitude and exposure time. The highest dry matter content was obtained when ultrasound treatment was conducted under pulsation mode at 70% amplitude for 7.5 minutes of exposure. (40,02±0,13 g/100 g dry yeast). To obtain a high dry matter content, the ultrasound treatment should be performed at high amplitudes and long exposure time under pulsation mode.

The soluble protein content was obtained for samples exposed to ultrasound under pulsation mode, at 70% amplitude, for 5 minutes and the lowest at 50% amplitude.

The antioxidant activity was measured using DPPH and ABTS method. The highest inhibition rates were obtained by using ABTS method and the lowest when using DPPH method. The ultrasound treatment applied for 2.5 minutes gave the highest antioxidant activity at 50% amplitude.

The proteins for yeast suspension exposed to ultrasound treatment have been analysed also by using fluorescence spectroscopy method to assess changes of the Trp and Tyr residues. Shifts in maximum emission have been observed during ultrasound treatment indicating that amino acids are becoming less exposed.

Among the three cell lysis methods, ultrasound treatment resulted in the highest release of protein content.

Activity 1.2. Testing the combination between conventional and nonthermal technologies to obtain highly functional SBY protein hydrolysates/peptides

Three variants have been tested to obtain peptides/ hydrolysates from yeasts

Variant 1

Steps:

1) Preparation of 12 % yeast suspension;
2) Homogenization with Ultraturax at 15.000 rpm, for 10 minutes;
3) Autolysis at 70°C, for 1 hour;
4) Adjustment at pH 7,0;
5) Enzymatic hydrolysis using bromeline, neutrase and trypsin at the following hydrolysis conditions: temperature 50°C, time between 0 - 67 h;
6) Cooling to room temperature and pH adjustment to 7.0;
7) Enzyme inactivation;
8) Centrifugation at 9000 rpm, 10 min;
9) Hudrolysate characterisation.

Variant 2

Steps:

1) Preparation of 12 % yeast suspension;
2) Homogenization with ultrasounds at 70% amplitude, under continuous mode, for 30 minutes;
3) Ajustare suspensie la pH 7,0;
4) Adjustment at pH 7,0;
5) Enzymatic hydrolysis using bromeline, neutrase and trypsin at the following hydrolysis conditions: temperature 50°C, time between 0 - 67 h;
6) Cooling to room temperature and pH adjustment to 7.0;
7) Enzyme inactivation;
8) Centrifugation at 9000 rpm, 10 min;
9) Hudrolysate characterisation.

Variant 3

Steps:

1) Preparation of 12 % yeast suspension;
2) Pretreatment with EnzymeBrew, for 60 minutes, at 55°C;
3) Adjustment at pH 7,0;
4) Enzymatic hydrolysis using bromeline, neutrase and trypsin at the following hydrolysis conditions: temperature 50°C, time between 0 - 67 h;

5) Cooling to room temperature and pH adjustment to 7.0;
6) Enzyme inactivation;
7) Centrifugation at 9000 rpm, 10 min;
8) Hydrolysate characterisation.

The hydrolysates have been characterized for:

- Hydrolysis degree using OPA method
- Colour using L^*, a^*, b^* coefficients
- Dry matter content using reference method
- Soluble protein content using Lowry method
- Antioxidant activity by using DPPH and ABTS method

The type of variant, and the enzyme used for hydrolysis influenced all the parameters considered for characterisation of the yeast samples. The degree of hydrolysis was influenced by the time and type of enzyme, the highest degree of hydrolysis being obtained after 67 hours of hydrolysis using neutrase. The dry matter content, soluble proteins and antioxidant activity increased with increasing time, the highest values were obtained when neutrase was used in enzymatic hydrolysis. The results have shown that there were no significant differences between variant 1 and variant 2, whereas variant 3 under parameters operated did not conduct to the protein hydrolysis (Table 4) and did not cause any changes in the antioxidant activity (Table 5 a, b).

Table 4. The influence of time and enzyme type on the degree of hydrolysis (%) of yeast samples

Sample type	Hydrolysis time (hours)		
	2	4	8
Control	8.22±0.43	8.78±0.07	8.43±0.46
Control with Enzyme brew	8.91±0.65	8.32±0.35	8.17±0.13
Hydrolysate with bromeline	6.9±0.05	9.02±1.04	9.06±0.09
Hydrolysate with neutrase	9.58±0.82	9.5±0.65	9.75±1.03
Hydrolysate with trypsin	9.23±0.03	9.25±1.33	8.69±0.44

Table 5. The influence of time and enzyme type on the antioxidant activity of yeast samples using DPPH (a) and ABTS method (b) (µM Trolox/ g dried yeast)

a)

Sample type	Hydrolysis time (hours)		
	2	4	8
Control	48.82±0.01	34.42±0.01	44.51±0.01
Control with Enzyme brew	55.23±0.01	57.12±0.01	31.9±0.01
Hydrolysate with bromeline	44.20±0.01	36.94±0.01	24.61±0.01
Hydrolysate with neutrase	49.53±0.01	39.46±0.01	35.54±0.01
Hydrolysate with trypsin	53.09±0.01	45.63±0.01	36.10±0.0

b)

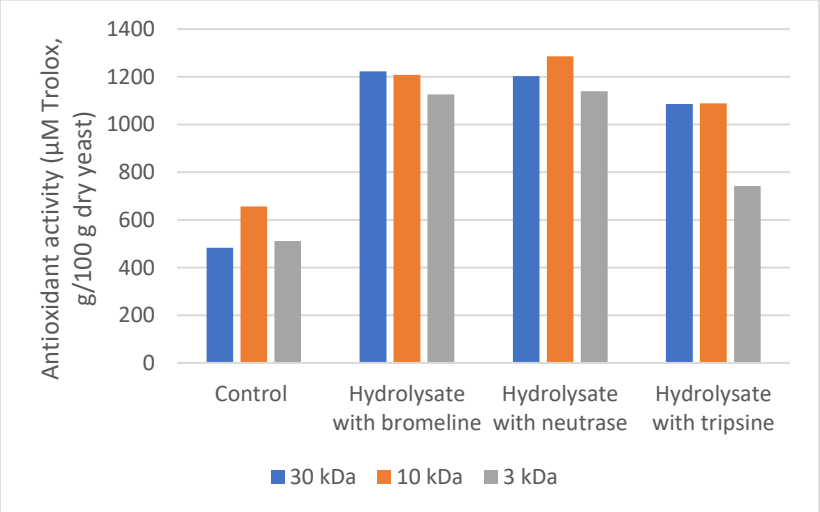
Sample type	Hydrolysis time (hours)		
	2	4	8
Control	661±17	619±2	685±62
Control with Enzyme brew	659±1	711±26	662±18
Hydrolysate with bromeline	645±22	713±9	627±67
Hydrolysate with neutrase	707±57	686±18	724±30
Hydrolysate with trypsin	659±10	690±22	692±25

The following conclusions were drawn from the testing of the 3 variants:

- For the first two variants tested, the degree of hydrolysis, dry matter, soluble protein content and antioxidant activity varied according to the type of enzyme used and the time of hydrolysis;
- There were no significant differences between variant 1 and 2 in terms of the characteristics tested, therefore, it was decided to continue the experiment using variant 1 for obtaining the hydrolysates;
- The enzymatic hydrolysis performed directly on the supernatant did not show significant changes compared to control sample, therefore it was no longer considered for performing the next experiments.

The next step consisted in checking if the antioxidant activity from hydrolysates obtained after 67 h of hydrolysis with neutrase is associated to the formation of peptides. The supernatant was separated using membranes with 30 kDa, 10 kDa and 3 kDa. Gel electrophoresis indicated the presence of peptides, whereas, the antioxidant activity measurements (Figure 1) highlighted that the peptides present in the sample possess antioxidant activity.

a)



b)

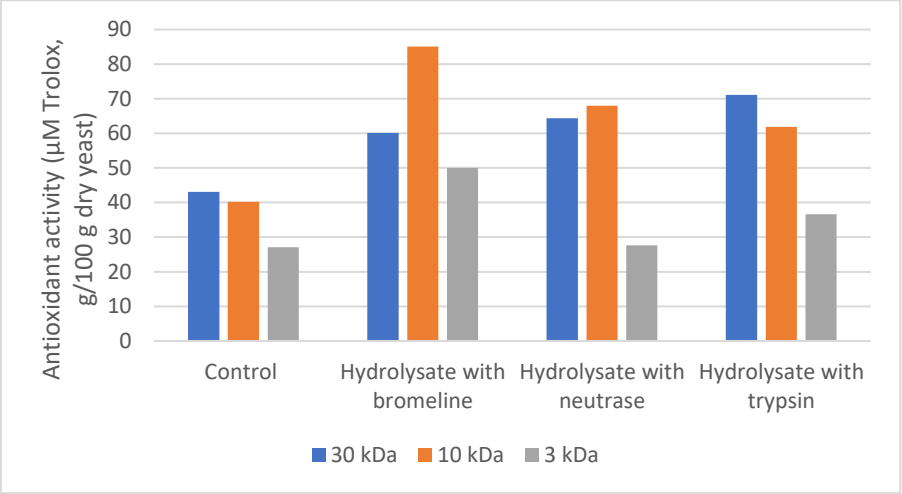


Figure 1. Antioxidant activity of hydrolysates separated with different cut-off membranes using ABTS method (a) and DPPH method (b)

The technological functionality of protein hydrolysates resulted after 67 hours of hydrolysis under the conditions applied for variant 1, have been checked by evaluating the foaming capacity, emulsifying properties and solubility of lyophilised samples.

The assessment of the foaming properties of hydrolysates was performed by using dispersions with a concentration of 6%. The incorporation of air into these dispersions was performed at different homogenization speeds (5000, 7000 and 9000 rpm) for 2 min using the Ultraturax shaker (IKA T18 basic). The foaming capacity was appreciated as the increase in volume of the foam (%) at the end of the homogenization, and the stability of the foam (%) and the collapse of the foam was determined after 15 min and 60 min of keeping at room temperature. Compared to the unhydrolyzed yeast extract that showed an increase in volume of 15% under the conditions of shaking at 5000 rpm, it was noticed that the hydrolysis of yeast proteins with their own enzymes, but especially with exogenous enzymes, led to a significant improvement in foaming properties. For all the analyzed samples it was observed that volume increased with increasing the stirring rate. The protein hydrolysates obtained with trypsin showed the highest values of the increase in volume as well as a very good stability for 15 min of the obtained foams, being followed by the protein hydrolysates obtained with bromeline. The stability of the foams showed values between 62% and 96% even after 60 min of storage at room temperature, but the foams were much less dense compared to the moment of completion of the shaking.

In order to assess the emulsifying properties of yeast protein hydrolysates, emulsions were prepared from mixtures consisting of protein suspension of 6% concentration and sunflower oil (ratio 1:1). The soluble protein content of the hydrolysates subjected to emulsification varied between 2.67% for the hydrolyzed control sample using endogenous enzymes and 6.62% for the protein hydrolysis obtained with neutrase. The protein suspension-oil mixtures were subjected to emulsification in stirring conditions at 5000 rpm for 2 min with the help of the Ultraturax stirrer (IKA T18 basic). The emulsions thus obtained were characterized from a rheological point of view, using the AR 2000ex rheometer, TA Instruments Ltd. Emulsions were subjected to dynamic oscillatory tests using a geometric system of plate-plate type. The emulsions based on protein hydrolysates obtained with neutrase and trypsin presented higher values of G" compared to G' throughout the scanned deformation range, indicating that the more advanced hydrolysis of proteins affects the property of forming firm and stable emulsions.

The rheological tests of forced flow, during which the values of the shear tension (Pa) and viscosity (Pa·s) of the samples were recorded in the conditions of the gradual increase of the shear rate (s-1) in the range of 0.1-100, revealed significant differences between the emulsion-forming properties of the protein hydrolysates of yeast. For all the analyzed samples, the shear tension increased over the entire range of shear speeds tested.

OBJECTIVE: To use nonthermal technologies to promote glycation through Maillard reaction to obtain a new class of bioactive ingredients based SBY peptide/protein conjugates	**Degree of achievement 100%**

2. Tuning the techno-functional characteristics of SBY peptides/ proteins/ through controlled glycation assisted by nonthermal technologies

T 2.1. Optimization of glycation procedure of SBY peptides/proteins by combining conventional glycation method (dry or wet heating) with ultrasounds and/or high pressure to obtain SBY conjugates with increased functionality;
T 2.2. Characterization of SBY peptide/protein-polysaccharide conjugates;

The improvement of the techno-functional characteristics of SBY proteins/peptides was conducted on the sample hydrolysed with bromelain (B) or neutrase (N) obtained in the stage 1. Three methods for obtaining conjugates were tested, namely:

a) Conventional wet glycation;
b) Ultrasound (US) - assisted wet glycation;
c) Combination of US followed by conventional glycation;
The carbohydrates used in the conjugation reaction were: glucose (G), maltodextrin (MD) and dextran (D).

Variant A

Steps:

10) Preparation of 2 % protein hydrolysate with neutrase (N) or bromelain (B) in 0.1 M phosphate buffer, pH 7.0;
11) Preparation of 2% carbohydrate (G, D or MD) in 0.1 M phosphate buffer, pH 7.0;
12) Storage under refrigeration for 24 h;
13) Mixing the protein and carbohydrate solutions resulted at step 1 and 2, 1:1 (v/v);
14) Heat treatment at 70°C, for 100 minutes;
15) Cooling to room temperature.

Variant B

Steps:

1) Preparation of 2 % protein hydrolysate with neutrase (N) or bromelain (B) in 0.1 M phosphate buffer, pH 7.0;
2) Preparation of 2% carbohydrate (G, D or MD) in 0.1 M phosphate buffer, pH 7.0;
3) Storage under refrigeration for 24 h;
4) Exposure to US treatment (50% amplitude, 5 on, 5 of), at 70 C, for 13 minutes of the protein hydrolysate;
5) Mixing the resulted sample with the carbohydrate solution prepared at step 2, 1:1 (v/v);
6) Heat treatment at 70°C, for 100 minutes;

7) Cooling to room temperature.

Variant C

Steps:

1) Preparation of 2 % protein hydrolysate with neutrase (N) or bromelain (B) in 0.1 M phosphate buffer, pH 7.0;
2) Preparation of 2% carbohydrate in 0.1 M phosphate buffer; pH 7.0;
3) Storage under refrigeration for 24 h;
4) Mixing the protein and carbohydrate solutions resulted at step 1 and 2, 1:1 (v/v);
5) Exposure to US treatment, 50% amplitude, 5 on, 5 off, at 70 C, between 5 to 23 minutes of the protein-carbohydrate mixture;
6) Cooling to room temperature.

The Maillard conjugates prepared using one of the three variants presented above, were characterized for the following parameters: pH, glycation degree (GD) using OPA method, antioxidant activity using ABTS assay, early stage, intermediate and advanced Maillard components by measuring the absorbance at 284 nm, 304 nm and 420 nm, whiteness and chroma parameters calculated based on the L*, a*, b* coefficients. All tested parameters were dependent on the type of hydrolysate, type of carbohydrate and glycation method.

When performing conventional glycation on the hydrolysate resulted from the action of N (75°C, for 100 min), the pH value resulted in conjugates prepared with G, D and MD were: 6.68±0.01, 6.8±0.01, 6.79±0.01, respectively. The exposure to US of protein sample before glycation had no major influence on glycation of the samples prepared with D and MD, where the measured pH values were similar. The exposure to US of the protein:carbohydrate mixture, generated lower pH values (compared to conventional glycation) in conjugates prepared with G and D, whereas for conjugates obtained with dextran, the pH increased. The increased pH was attributed to US-heat-driven exposure of inherently hidden domains that increased the number of the free amino groups content.

The GD was affected by the glycation method, the use of US generated lower GD values and increased antioxidant activity, the highest antioxidant activity being measured for the N:D-US conjugate (6067 ± 108 μM/ TEAC dry sample).

The conjugates prepared with hydrolysate resulted from the action of B, generated lower pH values, higher GD values and lower antioxidant activity than conjugates prepared with hydrolysate prepared with N, regardless of the carbohydrate type and glycation method.

The US exposure between 5 – 23 minutes of protein carbohydrate mixture, increased the antioxidant activity of the conjugates prepared with N, the highest values being recorded in N:D and N:MD conjugates. These two variants were selected further for developing an encapsulation matrix for anthocyanins.

The functional properties of the Maillard conjugates were assessed in terms of foaming capacity, foam stability, emulsifying activity, emulsions stability and rheological behavior. The samples obtained through variant C of processing, using yeast protein hydrolysates prepared with neutrase and bromelain, different types of carbohydrates and subjected to ultrasounds for various periods of time, were foamed and emulsified with sunflower oil (0.25 oil fraction) by means of UltraTurax (IKA T18 basic). The US treatment, the enzymes used for preparing the protein hydrolysates and the carbohydrate used as partner in the Maillard reaction influenced both the foaming capacity and the stability of the foams. The highest overrun of 109-125% was noticed in case of the samples prepared with peptide mixtures released by neutrase and maltodextrin, subjected to US treatment for 23 min. Regarding the emulsifying activity, the experimental results indicated that samples based on Maillard conjugates exhibited higher emulsifying activity compared to the corresponding controls, consisting of protein hydrolysates

prepared with neutrase or bromelain. The only exception concerned the conjugate based on protein hydrolysate obtained with bromelain and glucose. The rheological behavior of the emulsions was tested by means of AR 2000ex rheometer (TA Instruments Ltd). In case of all investigated samples, the stepped flow test indicated the increase of the shear stress values and the decrease of the apparent viscosity while gradually raining the shear rate. The highest apparent viscosity of 0.27 Pa·s at shear rate of 1 s^{-1} was registered for the emulsion prepared with peptide mixture released by bromelain and glucose, US treated for 23 min.

T 2.3. Molecular modeling investigations on SBY peptides/proteins in different environmental conditions;T 2.4. Affinity evaluation between SBY peptides/proteins and polysaccharides with different molecular weight.

For the *in silico* investigations the primary structure of the main proteins from *Saccharomyces cerevisiae* were taken from UniProt database (https://www.uniprot.org/). The complete yeast proteins digestion, with the enzymes used in the experimental study, was simulated using dedicated *in silico* tools, namely BIOPEP-UWM and Peptide Cutter. The biological activity of the released peptides was checked against the content of the BIOPEP-UWM database. The bioactive peptides from yeast proteins typically consist of 2 to 16 amino acids, and might ensure health promoting effects, because of different physiological functions, such as the antioxidative, antihypertensive, antidiabetic, antimicrobial properties etc. Mainly di- and tripeptides (**Table 6**) are responsible for the antioxidant activity of the mixtures.

Table 6. Peptides with antioxidant activity released through hydrolysis with bromelain, neutrase and trypsin from *Saccharomyces cerevisiae* proteins

Uniprot ID	Bioactive peptides: amino acids sequence [position in the initial protein]
P36010	IKL [39-41], IR [105-106] – bromelain LW [133-134] - neutrase
P38894	EL [236-237], IR [312-313, 357-358, 402-403, 447-448, 492-493, 537-538, 582-583, 627/628], WG [228-229], YA [64-65, 1050-1051], YF [143-144] – bromelain
P26263	IR [153-154, 533-534], WG [412-413, 460-461], KYL [8-10], YA [56-57, 61-62, 89-90] - bromelain IR [533-534], LPK [462-463], LK [334-335, 538-539] - neutrase LK [14-15] - trypsin
P32768	EL [338-339, 383-384, 428-429, 473-474], IR [312-313, 357-358, 402-403, 447-448, 492-493, 537-538, 582-583, 627-628, 672-673, 717-718, 762-763, 807-808, 852-853, 897-898, 942-943, 987-988, 1023-1024], WG [228-229], YA [64-65, 1512-1513], YF [143-144] – bromelain
P16467	WG [412-413, 460-461], KYL [8-10], YA [56-57, 61-62, 89-90, 405-406] – bromelain LH [96-97], LPK [462-464] - neutrase IR [316-317, 533-534] - trypsin
P06169	EL [78-79], WG [412-413, 460-461], KYL [8-10], YA [56-57, 61-62, 89-90] – bromelain LH [96-97], LPK [462-464] - neutrase IR [316-317, 533-534], LK [14-15] - trypsin
B0FGR2	EL [149-150, 391-392], IR [188-189] – bromelain LK [276-277], VW [249-250], ISW [78-80] - neutrase

The models of the peptides resulting from the enzymes assisted hydrolysis were generated using Hyperchem 8.0 molecular modeling software (Hyperchem®, Hypercube, Canada, 2002). The geometry of the models was optimized using Amber 3 force field and the sequence of Steepest Descent and Fletcher-Reeves algorithms. In order to estimate the impact of the thermal treatment applied during the conjugation reaction, the optimized models were further subjected to molecular dynamics simulations for heating and equilibration at 22 and 70°C. Although the temperature increase from 22 to 70°C led to the different spatial orientation of the amino acid side chains, due to the small molecular size and the lack of three-dimensional structure, no important changes were observed in the degree of exposure of the functional groups of the equilibrated peptides at different temperatures.

In order to appreciate the affinity between the model molecules used as partners in the Maillard reactions at laboratory scale experiments, the molecular docking method was further applied. The peptides acted as receptor, while the carbohydrate acted as ligand in the docking carried out using the Blind Docking Server (available at http://bio-hpc.eu/software /blind-docking-server/). The binding energy of the two molecules within the complex was used to estimate the molecular affinity. Regardless of the carbohydrate used as a ligand, due to the larger molecular size, a better affinity was observed between the molecules of the complexes formed with tripeptides compared to those with dipeptides. Increasing the temperature in the molecular dynamics stage up to 70°C (according to the treatment applied in variant C for obtaining the conjugates) led to the different orientation of the side chains of the amino acids, affecting the atomic contact with the carbohydrates, and consequently the value of the binding energy. The molecular mass of the carbohydrate used as a ligand significantly influences both the contact surface with the peptides and the binding energy. Higher affinity was observed for the tripeptide-maltodextrin complexes, when binding energy varied between -3.00 and -2,60 kcal/mol.

OBJECTIVE: To design and develop different encapsulation methods based on SBY conjugates for the sustained release of anthocyanins that can be used as a novel formulation for food applications	**Degree of achievement 100%**

3. Development of a new entrapping matrix based on SBY peptide/protein-carbohydrate conjugates for anthocyanins

T 3.1. Testing the affinity between SBY conjugates and anthocyanins

Protein conjugates with D or MD were tested for their affinity for anthocyanins. Aronia pomace was used as a source of anthocyanins. Aronia extract was obtained using the following protocol: the dried aronia pomace with a dry weight of 93% was grounded and mixed with an 80% ethanol solution, at a ratio of 1:10 (weight/volume) ratio, that was further exposed for 30 min to ultrasound treatment (50% amplitude, 5 on, 5 off, 20°C). Then, the mixture was centrifuged, and the resulting supernatant was concentrated. The composition of the extract was analysed using HPLC, four main anthocyanins being identified as follows: cyanidine-3-O-galactoside (24.72 mg/g), cyanidine-3-O-glucoside (1.71 mg/g), cyanidine-3-O-arabinoside (8,90 mg/g), cyanidine-3-O-xyloside (1.82 mg/g). The affinity assessment between conjugates obtained according to the protocol described above and anthocyanins from aronia pomace was performed by using spectrofluorimetric measurements, which involved measuring the fluorescence emitted by tryptophan residues from conjugates prepared with MD or D in the presence of different concentrations of aronia extract.

The fluorescence of protein solutions decreased with increasing anthocyanins concentration, suggesting the interaction of anthocyanins with SBY proteins/peptides. Based on the Stern Volmer constants, the application of US treatment led to a higher affinity of anthocyanins for N:MD conjugate.

T 3.2. Testing several encapsulation techniques based on SBY conjugates for the protection of anthocyanins; T 3.3. Optimization of the encapsulation technique for anthocyanins release on target; T 3.4. Advanced characterization of the microcapsules.

The N:MD and N:D conjugates prepared by using US were further tested for their ability to encapsulate anthocyanins. The following encapsulation variants were tested:

Variant 1

Steps:

1) Preparation of an aqueous solution of 5 % protein hydrolysate with neutrase (according to the protocol developed in the stage 1/2022), pH 7.0;
2) Preparation of an aqueous solution of 5 % maltodextrin concentration, pH 7.0;

3) Mixing the protein and carbohydrate solutions in a ratio of 1:1 (v/v);
4) Exposure of the resulted mixture to US treatment at 35% amplitude for 23 minutes, followed by exposure for 1 hour, at 70°C;
5) Cooling to room temperature;
6) pH adjustment to 7.0;
7) The addition of the aronia extract (according to the protocol provided previously) in the conjugated sample, using a weight ratio of 1:1;
8) Freeze-drying.

Variant 2

Steps:

1) Preparation of an aqueous solution of 5% protein hydrolysate with neutrase (according to the protocol developed in the stage 1/2022), pH 7.0;
2) Preparation of an aqueous solution of 5% maltodextrin concentration, pH 7.0;
3) Preparation of an aqueous solution of 5% yeast cellular wall (resulted in the hydrolysis process, after the separation by centrifugation of hydrolysed extract), pH 7.0;
4) Mixing the protein and carbohydrate solutions, in a ratio of 1:1 (v/v);
5) Exposure of the resulted mixture to US treatment at 35% amplitude for 23 minutes, followed by exposure for 1 hour, at 70°C;
6) Cooling to room temperature;
7) pH adjustment to 7.0;
8) Mixing the conjugate resulted above with the cellular wall solution, at a volume ratio of 3:1;
9) The addition of the aronia extract (according to the protocol provided previously) in the conjugated sample, using a weight ratio of 1:1;
10) Freeze-drying.

Variant 3

1) Preparation of an aqueous solution of 5% yeast cellular wall (resulted in the hydrolysis process, after the separation by centrifugation of hydrolysed extract), pH 7.0;
2) The addition of the aronia extract (according to the protocol provided previously) in the yeast cellular wall sample, using a weight ratio of 1:1;
3) Freeze-drying.

Variant 4

1) Preparation of an aqueous solution of 5% protein hydrolysate with neutrase (according to the protocol developed in the stage 1/2022), pH 7.0;
2) The addition of aronia extract (according to the protocol provided previously) in the protein sample, using a weight ratio of 1:1;
3) Freeze-drying.

Variant 5

Steps:

1) Preparation of an aqueous solution of 5 % protein hydrolysate with neutrase (according to the protocol developed in the stage 1/2022), pH 7.0;
2) Preparation of an aqueous solution of 5 % dextran concentration, pH 7.0;
3) Mixing the protein and carbohydrate solutions, in a ratio of 1:1 (v/v);
4) Exposure of the resulted mixture to ultrasonic treatment at 35% amplitude for 23 minutes, followed by exposure for 1 hour, at 70°C;

5) Cooling to room temperature;
6) pH adjustment to 7.0;
7) The addition of aronia extract (according to the protocol provided previously) in the conjugated sample, using a weight ratio of 1:1;
8) Freeze-drying.

Variant 6

Steps:

1) Preparation of an aqueous solution of 5% protein hydrolysate with neutrase (according to the protocol developed in the stage 1/2022), pH 7.0;
2) Preparation of an aqueous solution of 5% dextran concentration, pH 7.0;
3) Preparation of an aqueous solution of 5% yeast cellular wall (resulted in the hydrolysis process, after the separation by centrifugation of hydrolysed extract), pH 7.0;
4) Mixing the protein and carbohydrate solutions, in a ratio of 1:1 (v/v);
5) Exposure of the resulted mixture to ultrasonic treatment at 35% amplitude for 23 minutes, followed by exposure for 1 hour, at 70°C;
6) Cooling to room temperature;
7) pH adjustment to 7.0;
8) Mixing the conjugate resulted above with the cellular wall solution, at a volume ratio of 3:1;
9) The addition of aronia extract (according to the protocol provided previously) in the conjugated sample, using a weight ratio of 1:1;
10) Freeze-drying.

The images of the samples after freeze-drying are presented in **Figure 2**.

Variant 1 **Variant 2** **Variant 3** **Variant 4** **Variant 5** **Variant 6**

Figure 2. The freeze-dried powders resulted after testing different encapsulation variants

The physico-chemical characterization revealed that all the tested powders had a high stability as the water activity value was lower than 0.3. The colour parameters measured in terms of L*, a*, b* coefficient varied according to the tested variant. The encapsulation efficiency of anthocyanins varied between 67.09% and 88.72%, the highest value being measured in variant 2, and the lowest in variant 3. The results have shown that the encapsulation efficiency of polyphenols was lower compared to that of polyphenols. The retention efficiency of anthocyanins varied between 58.25% to 66% and of polyphenols between 46.87% and 69.95%. The stability evaluation of anthocyanins and polyphenols during the digestion in simulated gastric and intestinal juice showed that the best protection of anthocyanins and polyphenols was provided by variant 6.

The stability of the samples in terms of encapsulation retention and efficiency, water activity, colour parameters was tested for 60 days at 4°C and 20°C. The stability of the samples was strongly dependent on the storage temperature, the powders stored under refrigeration conditions presented a better stability compared with those kept at room temperature. Among all the tested variants, variant 6 was further selected for the development of a new product with increased functionality.

OBJECTIVE: To use the powder ingredient containing microcapsules to develop innovative value-added food products	Degree of achievement 100%

4. Development of a new food product with increased functionality

T 4.1. Formulation of a new value-added food product by incorporation of the microcapsules powder

Initially, the functional evaluation of the powder was tested by adding it into two commercial products: foam for deserts, and cream for cookies.

The following ingredients were used to prepare the foam for deserts:

1. Plants based beverage

Composition: water, soy, rice, almonds, calcium carbonate, sea salt, natural flavour, vitamin D, vitamin B2, vitamin B12.

2. Foam powder for deserts

Composition: sugar, glucose syrup, starch, hydrogenated oils, emulsifier: esters of acetic acid with mono and diglycerides of fatty acids, milk fat, flavours.

3. Anthocyanins microcapsules powder based on peptides conjugates from brewers yeasts

Composition: brewers yeast hydrolysate, dextran, aronia pomace extract, yeast cellular wall.

The foam was obtained by mixing the plants based beverage and powder for desert using a weight/volume ratio of 4:1. Then, it was added the microcapsules powder for up to 10%. The resulted mixture was homogenized with a mixer at maximum speed for 5 minutes (**Figure 3**).

Control **1%** **2%** **5%** **10%**

Figure 3. Foam for deserts with microencapsulated anthocyanins based on yeast protein conjugates

The main physico-chemical characteristics of the foam with microencapsulated anthocyanins are presented in **Table 7**. The anthocyanins and polyphenols content increased with increasing the powder content.

Table 7. Physico-chemical characteristics of the desert foam with microencapsulated anthocyanins powder

Sample	pH	a_w	L*	a*	b*	Total anthocyanins mg C3G/g d.w.	Total polyphenols mg GAE d.w
Control	7.41	0.935	66.08 ± 0.01	-1.13 ± 0.01	5.54 ± 0.01	-	17.82 ± 0.59
1%	7.00	0.933	59.56 ± 0.29	-0.02 ± 0.01	3.23 ± 0.01	0.12 ± 0.01	28.63 ± 4.11
2%	6.73	0.952	56.40 ± 0.04	1.09 ± 0.02	2.66 ± 0.01	0.25 ± 0.05	28.20 ± 1.57
5%	6.11	0.952	51.30 ± 0.01	4.85 ± 0.02	1.61 ± 0.00	0.53 ± 0.04	32.91 ± 1.81
10%	5.88	0.952	47.93 ± 0.01	6.74 ± 0.01	0.99 ± 0.01	0.77 ± 0.01	39.85 ± 4.58

The second commercial product on which it was tested the powder with anthocyanins, was a cream for cookies that was obtained with the following ingredients:

1. Plants based beverage

Composition: water, soy, rice, almonds, calcium carbonate, sea salt, natural flavour, vitamin D, vitamin B2, vitamin B12.

2. Mix for a cream for cookies

Composition: glucose syrup, vegetal palm fat, modified starch, sugar, emulsifier, aromas, food colouring.

3. Anthocyanins microcapsules powder based on peptides conjugates from brewers yeasts

Composition: brewers yeast hydrolysate, dextran, aronia pomace extract, yeast cellular wall.

The cream was prepared by mixing the plants based beverage with the cream mix using a weight/volume ratio of 5:1, followed by the addition of microcapsules powder in a concentration that ranged between 0 – 10%. The resulted mixture was homogenized with a mixer at maximum speed for up to 5 minutes (**Figure 4**). The physico-chemical characterization of the cream with anthocyanins addition is presented in **Table 8**.

Control **1.2%** **2.4%** **6%** **10%**

Figure 4. Cream for cookies with microencapsulated anthocyanins based on yeast protein conjugates

Table 8. Physico-chemical characteristics of the cream for cookies with microencapsulated anthocyanins powder

Sample	pH	aw	L*	a*	b*	Total anthocyanins mg C3G/g d.w.	Total polyphenols mg GAE/d.w.
Control	7.13	0.967	60.57 ± 0.04	-2.47 ± 0.02	17.24 ± 0.10	-	-
1,2%	6.90	0.962	67.64 ± 0.05	-2.18 ± 0.01	12.19 ± 0.02	-	19.65 ± 0.01
2,4%	6.55	0.971	56.40 ± 0.04	1.09 ± 0.02	2.66 ± 0.01	0.19 ± 0.01	22.42 ± 0.55
6%	6.16	0.971	51.30 ± 0.01	4.85 ± 0.02	1.61 ± 0.00	0.25 ± 0.00	26.80 ± 2.12
10%	5.93	0.963	47.93 ± 0.01	6.74 ± 0.01	0.99 ± 0.01	0.72 ± 0.03	37.38 ± 0.99

The pH of the samples decreased with increasing the microencapsulated anthocyanins powder, whereas the water activity did not change significantly. The functionality evaluated in terms of total anthocyanins and total polyphenols increased reaching a maximum at 10% concentration of anthocyanins powder.

In the second stage, a vegetable sauce was obtained that can be paired with salads, steaks or cheeses. The ingredients used to obtain the vegetable sauce were: mustard, tomato paste, sesame paste (tahini), pomegranate concentrate and powder containing conjugates based on yeast peptides and dextran, and yeast cell wall as encapsulation material with encapsulated anthocyanins form aronia. The patent documentation associated with this product was sent to the state office for inventions and trademarks.

Estimated result indicators	Obatined result indicators	Degree of achievement
- Project web page	- www.funyeast.ugal.ro	**100%**
- 5 papers submitted to publication in ISI/BDI journals	- **1 paper** in ISI journal. Dumitrascu L., Lanciu (Dorofte) A., Aprodu I. A preliminary study on using ultrasounds for the valorization of spent brewer's yeast. The Annals of the University Dunarea de Jos of Galati – Food Technology, 46(2), 1-15. https://www.gup.ugal.ro/ugaljournals/index.php/food - **4 papers** in ISI journals ranked in first (**Q1**) and second quartile (**Q2**) (according to Web of Science), as follows: ✓ Dumitrascu, L., Brumă(Călin) M., Turturică M., Enachi E. Ceoromila A., Aprodu I. Ultrasound assisted Maillard conjugation of yeast protein hydrolysate with polysaccharides for encapsulating the anthocyanins from aronia, Antioxidants 2024, 13, 570, https://doi.org/10.3390/antiox13050570. **Q1** ✓ Dumitrașcu L., Borda D., Aprodu I. 2023. Alternative processing options for improving the proteins functionality by Maillard conjugation. Foods, 12(19), 3588. https://www.mdpi.com/2304-8158/12/19/3588. **Q1** ✓ Dumitrascu L., Lanciu Dorofte A., Grigore-Gurgu L., Aprodu I. 2023. Proteases as tools for modulating the antioxidant activity and functionality of the spent brewer's yeast proteins. Molecules, 28(9), 3763. https://www.mdpi.com/1420-3049/28/9/3763. **Q2** ✓ Banu I., Patrascu L., Vasilean I., Dumitrascu L., Aprodu I. 2023. Influence of the protein-based emulsions on the rheological, thermo-mechanical and baking performance of muffin formulations. Applied Sciences, 13(5), 3316. https://www.mdpi.com/2076-3417/13/5/3316. **Q2**	**100%**

	- **Position paper** Dumitrascu L. (2022). Non-animal protein sources and resources- new strategies of valorization into value-added ingredients. Food, nutrition and environment: Position papers in Central European space, pp. 129-138. Publisher: Croatian Academy of Engineering, Zagreb, ISBN: 978-953-7076-30-6.	
- One presentation to conferences	**International conferences** ✓ Dumitrascu L., Aprodu I. Enachi E., Chitescu C. Spectroscopy-based investigations on the interaction between spent brewer's yeast peptides and anthocyanins from Aronia pomace. 37th EFFoST International Conference - Sustainable Food and Industry 4.0: Towards the 2030 Agenda, 6-8 November 2023, Valencia, Spain (poster presentation).https://effostconference.com/ ✓ Dumitrascu L., Brumă (Călin) M, Aprodu I. In silico investigations on the biological activity of the peptides encrypted in various proteins from brewer's yeast. 3rd Food Chemistry Conference: Shaping a Healthy and Sustainable Food Chain through Knowledge, 10 - 12 October 2023, Dresden, Germany (poster presentation). https://www.elsevier.com/events/conferences/food-chemistry-conference ✓ Bleoanca I., Nigrou R., Lanciu Dorofte A., Dumitrașcu L., Aprodu I., Borda D., Stan F., Fetecau C. Development and characterization of yeast protein films. Euro-Aliment 2023 - The 11th International Symposium "Insights of Future Foods - from Concepts and Challenges to Technological Innovations", 19-20 October 2023, Galati, Romania (poster presentation). https://euroaliment.ugal.ro/index.php/en/ ✓ Dumitrascu L. Non-animal protein sources and resources- new strategies of valorization into value-added ingredients. 11th Central European Congress on Food and Nutrition, 27-30 September 2022, Čatež ob Savi, Slovenia (oral presentation).https://cefood2022.si/ ✓ Aprodu I., Enachi E., Borda D., Dumitrascu L. Saccharomyces cerevisiae as source of carrier material for bioactive compounds encapsulation. 11th Central European Congress on Food and Nutrition, 27-30 September 2022, Čatež ob Savi, Slovenia (poster presentation). https://cefood2022.si/ **National conference** ✓ Aprodu I., Brumă (Călin) M., Vasilean I., Dumitrascu L. In vitro and in silico evidences on the biological activity of the peptides encrypted in various food proteins. MED-FARM LAB 2023 - Conferința Națională de Analize Medico-Farmaceutice de Laborator: De la Concepte Teoretice la Aplicaţii Practice, 6-8 Octombrie 2023, Galaţi, România (oral presentation).https://www.medfarmlab.ugal.ro/index.php/ro/	**100%**
Initiation of patent documentation	- Patent application no. OSIM A/00155/3.04.2024 Dumitrașcu L., Aprodu I., Călin, M., Turturică, M. Sos vegetal cu peptide din drojdia de bere și antociani din fructe de aronia și procedeu de obținere al acestuia.	**100%**