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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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PHASE 2

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:

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) Mixing the protein and carbohydrate solutions resulted at step 1 and 2, 1:1 (v/v);
5) Heat treatment at 70°C, for 100 minutes;
6) 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 1**) are responsible for the antioxidant activity of the mixtures.

Table 1. 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 1**.

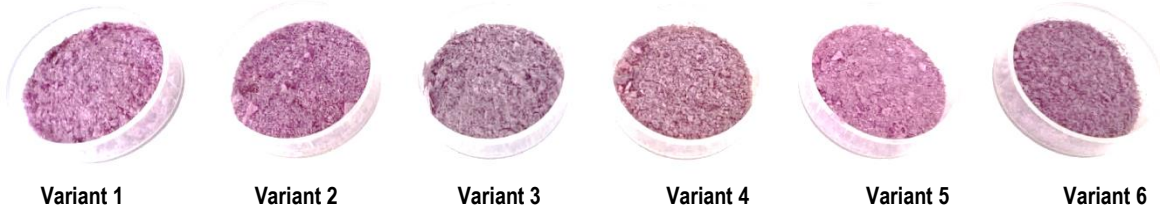


Figure 1. The freeze-dried powders resulted after testing different encapsulation variants

The physico-chemical characterization revealed that all the tested powders had a high stability as the water activity value was lower than 0.3. As shown in **Figure 1**, 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.

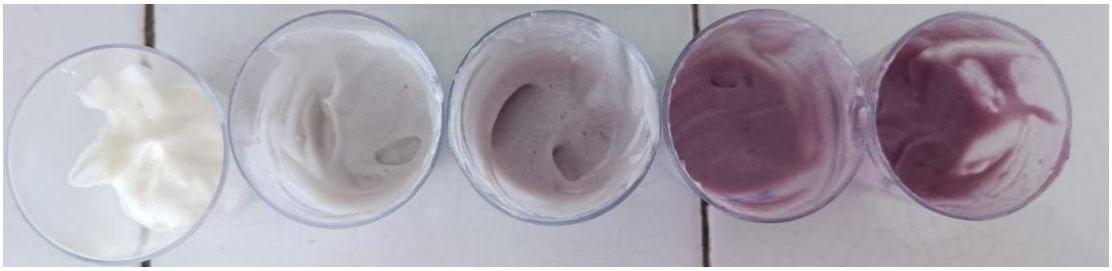
2. Foam powder for deserts

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 2**).



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

Figure 2. 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 2**. The anthocyanins and polyphenols content increased with increasing the powder content.

Table 2. Physico-chemical characteristics of the desert foam with microencapsulated anthocyanins powder

Sample	pH	aw	L*	a*	b*	Total anthocyanins mg C3G/g d.w.	Total polyphenols mg GAE d.w
Control	7.41	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.

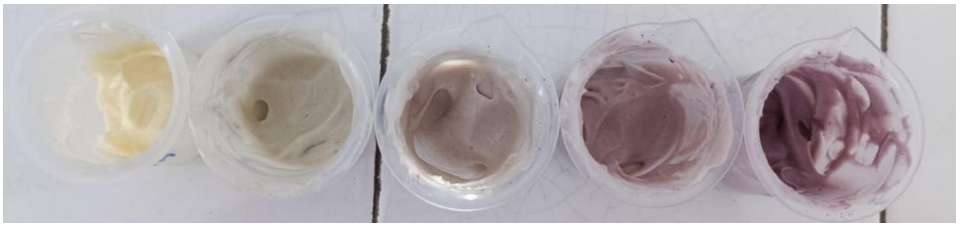
2. Mix for a cream for cookies

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 2**). The physico-chemical characterization of the cream with anthocyanins addition is presented in **Table 3**.



Control **1,2%** **2,4%** **6%** **10%**

Figure 2. Cream for cookies with microencapsulated anthocyanins based on yeast protein conjugates

Table 3. 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 coming months, the tests will be continued by designing a new food product with microencapsulated anthocyanins.

Estimated result indicators	Obtained result indicators	Degree of achievement
- Two conjugates with increased functionality	- Two conjugates with increased functionality in terms of antioxidant activity.	**100%**
- One microcapsules powder	- One microcapsules powder	
- One presentation to international conferences	- Three participations international conferences: The 11th International Symposium "Insights of Future Foods - from Concepts and Challenges to Technological Innovations, 3rd Food Chemistry Conference: Shaping a Healthy and Sustainable Food Chain through Knowledge, 37th EFFoST International Conference - Sustainable Food and Industry 4.0: Towards the 2030 Agenda - One participation to a national conference: MED-FARM LAB 2023 - Conferința Națională de Analize Medico-Farmaceutice de Laborator: De la Concepte Teoretice la Aplicaţii Practice	
- 3 articles submitted to ISI/BDI journals	- Three articles accepted for publication in ISI journal ranked according to Web of Science in Q1 and Q2: 1. 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**	

2. Dumitrașcu 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**
3. Banu I., Patrascu L., Vasilean I., Dumitrașcu 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**