PhD THESIS

Synergistic Utilization of Aurantiochytrium limacinum microalgae SR21 and *Aronia melanocarpa* By-Product: Bioproduction and Functional Bar Development

SUMMARY OF THE Ph.D THESIS

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CONTENTS

1. Single-cell Protein: a Potential Substitute in Human and Animal Nutrition

Many microorganisms have been directly utilized as food. SCPs can be lifesaving in less privileged areas where malnutrition is a real and life-threatening problem. A species of alga called Spirulina was grown many years ago in Africa's Lake Chad and was subsequently used as food to compensate for local people's protein shortfall (Junaid *et al.,* 2020). Germans reportedly utilized a certain species of Candida in their meals during World War I, including sausages and soups. Proteins generated from bacterial, fungal and algal cultures were widely used in food and as food from then on. The concept of SCP arose from this method, these proteins are now widely used (Ali *et al.,* 2017).

Researchers and businesses around the world are interested in SCP production. Due to the many promising benefits that these proteins provide, various companies have sprung up that claim to be able to commercialize SCP. However, a key challenge for the industry is sourcing a sustainable, renewable, protein-enriched ingredient. Currently, fishmeal, along with terrestrial plantmeals, constitutes the majority of protein content in diets (Takahashi *et al.,* 2020).

Among others, algae, fungi, yeast and bacteria can be used for SCP production, but each has its own advantages and disadvantages. Bacteria possess higher growth rates, higher protein content, and more sulfur-containing amino acids. From an industrial perspective, methane-oxidizing bacteria are the most advanced and prepared bacteria for SCP production. Yeasts have been used as a source of SCP for a long time. Mushroom SCP has been found to be useful in animal feed, while specific by-products are used in the beverage sector (Bratosin *et al*., 2021; Junaid *et al*., 2020).

3. Research objectives

In order to complete the doctoral thesis, the following objectives were established:

- Optimization of cultivation conditions for *Aurantiochytrium limacinum* SR21 by identifying critical parameters of the cultivation environment, such as sea salt, laboratory glucose, and protein extracts, to maximize biomass production and the content of bioactive compounds, especially docosahexaenoic acid (DHA).
- Maximizing production yield for A. limacinum SR21. Development and implementation of an efficient process to achieve consistent high-protein and DHA content production in dry biomass.
- Evaluation of the antioxidant potential of *Aronia melanocarpa* powder: Analysis and quantification of the powder's antioxidant activity obtained from by-poduct

Aronia melanocarpa peels.

- Development and characterization. This objective focuses on the analyzing and quantifying the antioxidant activity of *Aronia melanocarpa* powder derived from the by-products of *A. melanocarpa* peels, assessing its potential health benefits and applications in various industries.

4. 1st Study. Production of Bioactive Compounds by *Aurantiochytrium limacinum* SR21 into Discontinuous Fermentation

The objective of the current work was to optimize the production process of bioactive compounds and nutraceuticals from the microalga *Aurantiochytrium limacinum* SR21. This will be achieved by identifying and efficiently using carbon and nitrogen sources, as well as optimizing growth parameters within the fermentation process. The ultimate goal is to transform *A. limacinum* SR21 into a significant source of valuable compounds for the food industry, contributing to the efficiency and sustainability.

4.1. Material and Method

Cultivation of *A. limacinum* SR21 (ATCC MYA-1381) used in this study was obtained from the University of Agricultural Sciences and Veterinary Medicine, Food Science department, from Cluj-Napoca. The cells were activated in 10 mL of artificial seawater (ASW) containing 20 g/L glucose (VWR Chemicals, Belgium), 10 g/L yeast extract (Alfa Aesar, Karlsruhe, Germany) and 20 g/L sea salts (Sigma - Aldrich, USA), the composition of ASW being reported by Chin *et al.* (2006).

The fatty acid content of flask and bioreactor fermentations was determined using Gas Chromatography-Mass Spectrometry. Transesterification with 1% SO_4 in methanol was used to obtain the fatty acid profile from the total lipids (Dulf *et al.*, 2017; Mitrea *et al.*, 2019). A PerkinElmer Clarus 600 T gas chromatograph coupled with a mass spectrometer (PerkinElmer, Inc., Shelton, CT, USA) was used to measure the methylated fatty acids in the sample. A Supelcowax 10 capillary column (Supelco Inc., Darmstadt, Germany) with a 60 m × 0.25 mm i.d. and a 0.25 μm film thickness received a volume of 0.5 μL of sample.

4.2. Results and discussions

The content of fatty acids in the flask and bioreactor is presented in Table 3.5 and Fig. 3.5. As can be observed, lauric acid (+15.15%), palmitic acid (+3.30%), palmitoleic acid (+5.25%), α–linolenic acid (+31.25%), eicosapentaenoic acid (+29.23%), adrenic acid (+17.13%) and docosahexaenoic acid (+43.13%) were in a

higher value in the flask fermentation. In the bioreactor, a higher quantity of fatty acids was observed for tridecanoic acid (+200%), myristic acid (+14.70%), 11–tetradecenoic acid (+44.44%), pentadecanoic acid (+270.47%), cis–7 hexadecenoic acids (+71.42%), margaric acid (+294.66%), stearic acid (+30%), oleic acid (+74.07%), linoleic acid (+53.03%), γ–linolenic acid (+141.17%). Also, total lipids were almost double in flask 8.93 ± 0.25 (g/100g dry biomass) compared to the bioreactor 4.89 ± 0.35 (g/100g dry biomass).

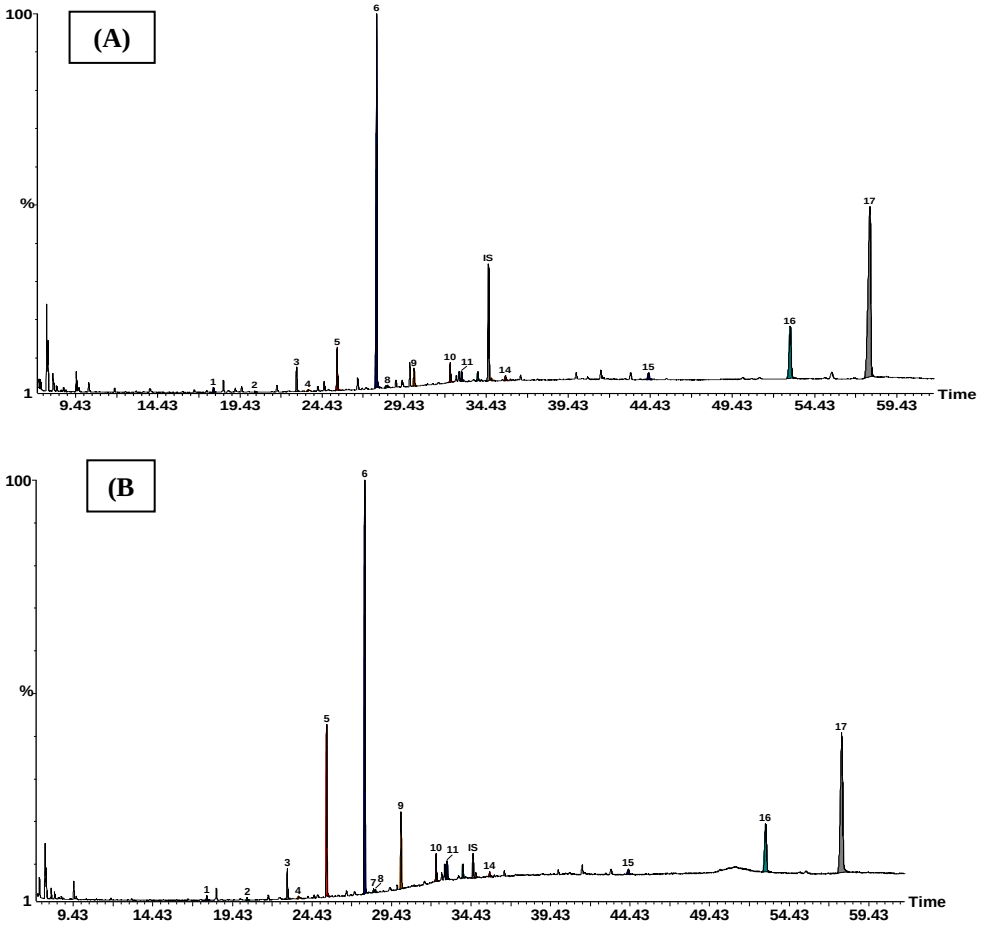

Figure 3.5. GC–MS analysis of fatty acids from the flask (A) and bioreactor fermentation (B). Peaks: (1) lauric acid; (2) tridecanoic acid; (3) myristic acid, (4) 11-tetradecenoic acid; (5) pentadecanoic acid; (6) palmitic acid; (7) cis-7 hexadecenoic acid; (8) palmitoleic acid; (9) margaric acid; (10) stearic acid, (11) oleic acid, (12) linoleic acid; (13) γ–linolenic acid; (14) α–linolenic acid; (15) eicosapentaenoic acid, (16) adrenic acid; (17) docosahexaenoic acid (DHA)

5. 2nd Study. Nutritional and Physico-Chemical Characteristics Of Innovative Bars Enriched with *Aronia melanocarpa* By-product Powder

The primary objective is to explore the nutritional contribution and functional properties of *A. melanocarpa* by-products, elucidating their role in enhancing the health profile of food products. The study delves into various applications within the food industry, including the development of innovative bars, emphasizing the valuable role of these by-products as sources of antioxidants and other bioactive compounds.

5.1. Material and Method

Dynamic rheological measurements of the protein bars were conducted using an Anton Paar MCR 72 rheometer (Anton Paar, Graz, Austria) equipped with a Peltier plate system (P-PTD 200/Air) and a temperature controller (set at T = 4 °C and room temperature). The rheometer had a smooth parallel plate with a diameter of 50 mm (PP-50-67300). Initially, in the center of the lower plate of the Peltier system was added 3 g of the sample, with a 1.5 mm gap between the plates, and allowed to rest for 5 minutes (Teleky *et al.,* 2022). After providing the sample, any excess was removed, and silicon oil was added to prevent drying. Oscillatory frequency sweep tests were then performed at angular frequencies ranging from 0.628 to 628 rad/s to determine the dynamic storage modulus (elastic) (G′, Pa) and loss modulus (viscous) (G″, Pa). G′ and G″ represent the material's ability to store elastic deformation energy and the viscous portion of the material, respectively. The results from three experiments, each with replicates, were presented as the mean value ± standard deviation (SD), where $n = 3$. With Graph Prism Version 8.0.1 was performed statistical analysis (GraphPad Software Inc., San Diego, CA, USA).

5.2. Results and discussions

In the context of our study, the application of dynamic rheological measurements on nutritional bars prior to manufacturing holds significant importance for several reasons (Fig. 4.2). Dynamic rheology, which involves the study of a material's flow and deformation behaviour under various conditions, provides crucial insights into the structural and textural characteristics of food products. Here, we discuss the relevance of applying dynamic rheological measurements in the pre-manufacturing phase of aronia-enriched bars in comparison with control bars. To select the best composition variant for the bars, the measurements were performed on all batches at room temperature and 4 °C (Fig. 4.2).Comparing nutritional values of the control bar and the aronia bar: the results of the nutritional analysis for the two bar variants, the control bar and the aronia bar, are presented in the Table 4.5.

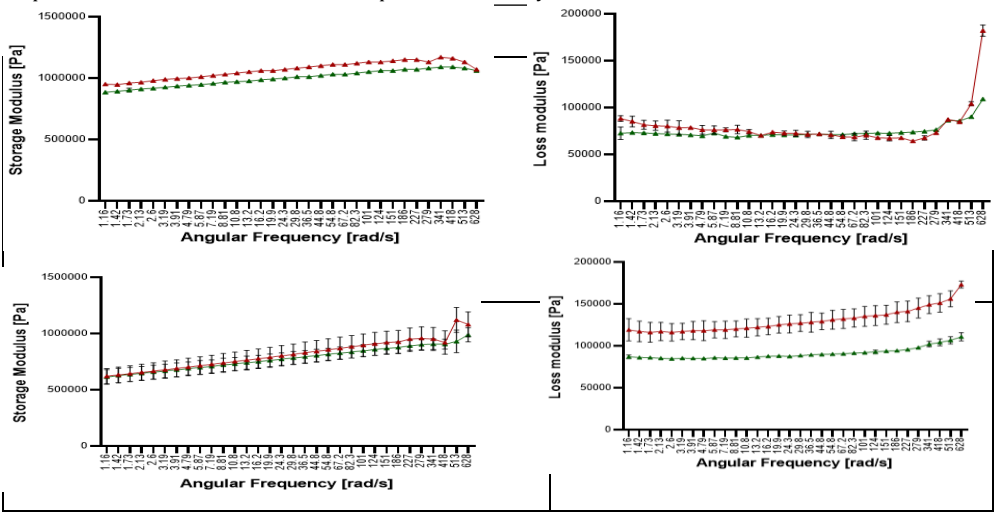

Figure 4.2. Properties related to the flow and deformation behaviour of the bars before and after enrichment with aronia powder at room temperature (a, b) and at 4xC (c, d), where red is the control bar and green colour is supplemented with aronia by-product bar

Table 4.5.

The results of the nutritional analysis for the two bar variants

Sample	Control bar	Bar with aronia
Dry matter (%)	90.52	90.43
Humidity (%)	9.47	9.56
Ash (%)	1.82	1.87
Protein (%)	12.20	12.28
Fat (%)	20.51	20.46
Carbohydrates (%)	45.41	45.47
Fiber (%)	10.58	10.35
Kcal	429.87	430.01
Kj	1798.59	1799.16

The descriptive statistics table provides the mean ± SD (*n = 3*). A two-way ANOVA was conducted to explore significant differences between the control bar and bar with aronia, followed by Sidak's multiple comparisons tests. In this analysis, the second column was compared to the first column. The following symbols indicate Different levels of significance: N.S. (not significant).

These characteristics are essential for maintaining the quality of the product over the long term. Regarding ash content, a minor difference was observed between the two variants, with the aronia bar having a slightly higher value of 0.05%. However, the discrepancy does not significantly influence the product's quality or organoleptic characteristics.

3rd Study. Consumers Perception on Nutritional Bars. A Case Study: Nutritional Bars Based on Aronia By-Products

The aim of this study is to investigate and analyze consumer perceptions and preferences regarding nutritional bars, with a specific focus on those made from aronia by-products. This research seeks to understand the factors that influence consumer choices, the credibility of information sources, and the preferred product variants.

5.2. Material and method

A quantitative survey with 24 items with different possibilities of responses (yes, no, multiple choices) was conducted during February – May 2024. Questionnaires were designed and distributed with the aim of identifying the consumer perceptions and preferences regarding nutritional bars, with a specific focus on those made from aronia by-products. The questionnaires are structured in four parts, concerning: demographic profile, the knowledge about protein and aronia bars, the perceptions and expectations from protein and aronia bars, and general perceptions concerning aronia bars. They were assigned to 384 inhabitants of Cluj-Napoca municipality, which have residence in Cluj-Napoca, and villages located in vicinity of the town.

5.3. Results and Discussions

The aronia-based protein bars are the most preferred variant among respondents, reflecting a strong consumer interest and potential market for these products. Integrated functional foods also have significant appeal, suggesting that products combining aronia with other functional ingredients are well-received. Powders, while less popular than bars and integrated foods, still hold a notable share of preferences. Capsules and other product forms are less favoured, indicating limited consumer interest. The minimal percentage of non-specific preferences suggests that most consumers have a clear idea of their preferred aronia-based product type.

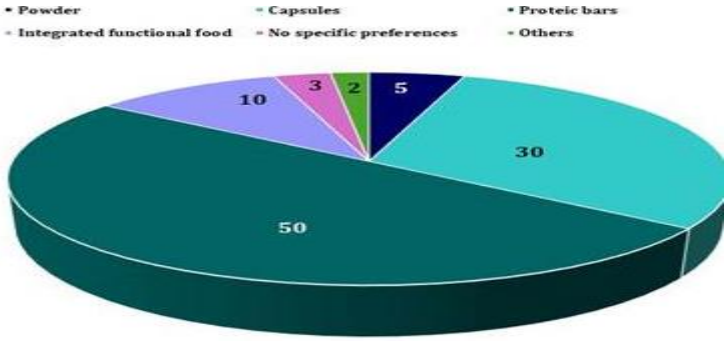

Figura 5.8. Variantele preferate de produse pe bază de aronia

6. Conclusions and recommendations

The results obtained in the studies lead to the following general conclusions, which are presented. *Aurantiochytrium limacinum* SR21 is a promising resource for the food and nutraceutical industry, providing proteins, polyunsaturated fatty acids, especially omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as valuable bioactive compounds like organic acids, including monounsaturated fatty acids such as oleic acid, and saturated fatty acids such as palmitic acid. Investing in research and development to optimize cultivation, extraction, and processing techniques could maximize the yield and bioavailability of these beneficial compounds, thereby enhancing their application in functional foods, dietary supplements, and other health-promoting products. The bars and freeze-dried powder from the by-product of Aronia melanocarpa retained their nutritional and antioxidant properties, specifically, 12% protein, 20% fat, and a caloric content of 430 kcal. Additionally, they exhibited a significant increase in phenolic compounds by 755.74% after the digestion phase. Further research into optimizing processing methods and exploring additional applications in food and beverage industries could enhance the utilization of these valuable by-products. The freeze-drying process of the by-product of Aronia melanocarpa maintained and improved the antioxidant properties of the powder. Additionally, it demonstrated a significant increase in antioxidant activity, with a 61% improvement compared to the initial by-product, highlighting the antioxidant potential of the powder. Belief in the benefits of protein bars and chokeberry, although less significant, still contributes to the overall understanding of consumer perceptions. This information could be valuable in targeting educational or marketing efforts to improve familiarity and trust in these products.

Based on the conclusions, the recommendations are formulated, as follows. Optimization of the cultivation process of Aurantiochytrium limacinum SR21. The primary recommendation is to continue research efforts to optimize the cultivation conditions of Aurantiochytrium limacinum SR21. By scaling up the production process to an industrial level, valuable compounds can be produced more efficiently and sustainably from microalgae on a larger scale, which could have a significant impact on the food industry and health food supplements. Development of nutritional bar production technology. Considering the significant increase in protein and docosahexaenoic acid (DHA) content in Aurantiochytrium limacinum SR21, it is recommended to research and develop an efficient technology for producing bars enriched with this micro-algae. This could provide an innovative way to incorporate these beneficial nutritional compounds into daily dietary consumption. Optimization of the Freeze-Drying Process form Aronia Melanocarpa By-Product. Considering the

significant improvement in antioxidant activity through the freeze-drying process of *Aronia melanocarpia* by-product, exploring and further developing of freeze-drying technology, with a focus on freese-drying materials, is suggested. This effort could lead to the refinement of the process and the attainment of improved results regarding the quality and effectiveness of Aronia melanocarpa by-product freeze-drying. Our research data suggested that the combination of Aurantiochytrium limacinum SR21 and *Aronia melanocarpia* provide synergistic benefits. It is recommended to assess the synergy between these two sources of bioactive compounds for the development of innovative food products or nutritional supplements.

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