PhD THESIS

Recovery of By-products and Solid Organic Waste for the Production of Sweeteners and Flavorings

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(SUMMARY OF THE DOCTORAL THESIS)

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SUMMARY

Introduction

Sugars are the main sources of carbon and energy for most cell types and play important roles in metabolism, growth, resistance to stress, and the development of bacteria, yeasts, fungi, plants, and animals. However, an excess of sugar in the human diet is strongly associated with an increased risk of obesity, heart disease, diabetes, and tooth decay. Considering the strong association between the intake of excess added sugars and the risk of disease, we need to consume lower-calorie, healthy foods with less added sugars. Natural sugars, like those from fruits and vegetables, are generally considered a healthier alternative. Another solution to reduce the amount of added sugars in food would be using low-metabolizable, low-glycemic, natural sweeteners, such as polyols, with great potential in the food industry and medical field. Global policies on adding polyols to foods are supported and recommended to prevent health problems associated with high-sugar foods. In recent years, research efforts have been made to improve the productivity and yield of polyols using simple and inexpensive methods, particularly biotechnological manufacturing methods. Erythritol and mannitol are considered to be safe polyols when added to food. Moreover, they can be biotechnologically produced using food–grade yeasts or lactic acid bacteria (LAB) on different media, including by-products. With the development of new production technologies and alternative resources, erythritol and mannitol will possibly be produced on a larger scale.

Recent advances in food and biotechnology ensure that food processing by-products or waste is perceived as a rich source of bioactive compounds. Food waste and by-products are a severe global problem, especially in many developed countries. Additionally, food demand has increased due to urbanization, population growth, and income growth, and meeting its sustainability remains a major global challenge in the long run. One of the most concerning industries is apple processing, which generates a massive volume of waste, considering the annual processed tonnage of up to 12 million tons (Mt).

Global apple production has amounted to over 87 million tons/year, while 18% are processed, resulting in 20-35% (apple fruit fresh weight) apple pomace (AP). In case apple production and consumption will exhibit the same trend and constant growth, it will increase by 16%, more precisely over 14.17 Mt until 2030. Another major concerning waste is represented by the organic fraction of municipal solid waste (OFMSW). The massive generation of municipal solid waste (MSW) has made its disposal an important challenge to overcome. For example, global MSW production is expected to increase up to 70% by 2050 from 2010 Mt in 2016. In addition to MSW, substantial global industrialization has also contributed to an unprecedented increase in the generation of industrial solid waste. In 2017, the rate of industrial waste generation was approximately 18 times higher than MSW. A large portion of industrial wastes and MSW are organic residues which can be used as substrates in fermentations.

By contrast, the low cost and high abundance of these by-products and waste highlight the economic perspectives of its potentially valuable components to act as important nutritive substrates and precursors for fermentation-derived added-value compounds (sweeteners, flavorings). Innovations range from food production, land use, and emissions all the way to improved diets and waste management. Using modern by-product and waste management approaches via integrated knowledge in innovative fermentation demonstrates opportunities for reducing environmental pollution and integration into a circular economy. With this association in view, integrating AP flour during sourdough fermentation, the nutritional value increases, increase polyols production, highlighting a new approach that could guide innovative fermented foods. Also, using wheat bran as a substrate for enzyme production and followed by a hydrolase can result in bio-vanillin production, the most popular flavor in the world, but with reduced price and natural labeling.

In parallel with the pleasure of sweetness, food consumption is connected with flavoring properties. Vanilla is the main natural flavoring agent used in industries as pharmaceuticals, food, flavoring, and fragrance, in which vanillin is the significant component. The vanillin compound can be produced using the following routes: chemical synthesis, vegetable biosynthesis, and biotechnological processes (the production of bio-vanilla). Bio-vanillin derived from the latter route has significant advantages over the other two, including fulfilling the needs of modern consumer trends by being considered natural and supporting industrial production due to the lack of toxicity and its alternative use of by-products or wastes. Moreover, only natural-labeled vanillin is approved for use in the food industry. Using the knowledge and an innovative process is possible to find a biotechnological route for vanillin production using by-products and waste as a substrate for microorganisms growth after a hydrolyses process. The use of novel approaches involving integrated technologies (microorganisms, enzymes production, hydrolyses process, food technology, industrial and waste valorization) for the production of bio-vanillin is a promising route for a natural and sustainable production with high industrial potential. Solid-state fermentation (SSF) is recognized as a suitable process for enzymes production using by-products or food residues as substrates. However, only a few studies have integrated an evaluation of the feasibility of applying enzymes produced by SSF into subsequent hydrolyses followed by the production of target compounds, e.g., bio-vanillin, lactic acid, through submerged-liquid fermentations.

Regarding the biotechnological route, SSF is gaining popularity due to its positive economic and environmental impacts. The process of fermentation by microorganisms has been used for centuries to increase the nutritional character of foods, and many studies employ different wastes as substrates for the growth of microorganisms. The enzymes produced by microorganisms under SSF conditions can be used to reduce the price for sweeteners and flavors production. These enzymes are able to increase sugars, also increasing bio-accessibility and biological activity. SSF is superior to submerged fermentation due to higher productivity, lower water and energy requirement, easy aeration, lower demand of sterility, resemblance to the natural habitat of microorganisms, easier downstream processing, utilization of cheaper agro-industrial residues as solid substrates, and environmental friendliness.

Research objectives

The thesis aimed to identify a suitable solution for food by-products and waste materials via biotechnological and food processing approaches. Additionally, increasing consumer interest in fermented products has driven the emergence of several novel foods, including by-products that enrich sourdough fermentation. Considering these aspects, the present study aimed to formulate and evaluate an attractive sourdough system enriched with AP flour and fermented using a selective culture of *S. cerevisiae* (baker's yeast) and *Fructilactobacillus florum* DSM 22689 (known to produce polyols) as a GRAS (Generally Recognized as Safe) and cheap microorganisms, with the final goal of reducing sugars and increase of polyols productions. Sourdough fermentation was monitored and analyzed for 72 hours. Lactic acid bacteria (LAB) typically dominate sourdough cultures in symbiotic combinations with yeasts. Enriching sourdough fermentation with other by-products increases protein digestibility, total soluble/insoluble fiber content, reduces food's glycemic index and improves the bioavailability of minerals. The most significant contribution in addition of AP to the sourdough is the fermentable sugars (in wheat flour doughs, these sugars are limited). These sugars extend the fermentation time by favoring mainly lactic fermentation in co-cultures and polyols production. Also, AP by-products are certainly an important source of dietary fiber. Dietary fiber slows down many processes associated with the digestion of glycaemic carbohydrates, such as gastric emptying, small intestinal transit, and transport from the lumen to the mucosal surface.

The final part of the thesis presents the production of enzymes by SSF, considering the economic efficiency of the biotechnological process of obtaining flavors such as vanillin using enzymes. In order to make the process more eco-efficient, the substrate used was wheat bran without supplement of nutrients. The fungi used were evaluated during 10 days of SSF. After this step, the enzymes obtained were used to hydrolase OFMSW to increase sugars content. The sugars obtained after hydrolysis can be a source of cheap substrate for obtaining flavors. In the end, the strains, enzymes, and flavoring constituents obtained from by-products and waste may be encapsulated. Therefore the microencapsulation strategy was investigated via a comprehensive literature study. In order to make the process more efficient, the encapsulation of the strain with different polymers as chitosan, alginate, and pectin is proposed. Additionally, biocompatibility, bioadhesiveness, and biodegradability for the most popular polymers (chitosan, alginate, and pectin) were evaluated.

In order to achieve the aim of the thesis, the following aspects were investigated and materialized into four objectives:

O.1. Integrating apple pomace flour in sourdough fermentation using a selective culture of microorganisms to increase the nutritional value and polyols production.

O.2. Development of methods for vanillin production from agro-industrial by-products via strain metabolism.

O.3. Solid-state fermentation of wheat bran for enzymes production to obtain a cheap enzyme enrich substrate for OFMSW hydrolysis followed by submerged fermentation for lactic acid production, and for vanillin production.

O.4. The efficiency of the biotechnological process via encapsulation of the strain, enzymes, and bioactive compounds from waste and by-products.

The results of this thesis were published in three review articles (one ISI indexed journal with IF 8.429 – in *Critical Reviews in Biotechnology* Journal;

the second ISI indexed journal with IF 12.563 – in *Trends in Food Science & Technology* Journal; the third ISI indexed journal with IF 4.329– in *Polymers* Journal). In addition, two original articles (one ISI indexed journal with IF 5.64 – in *Frontiers in Microbiology* Journal; the second ISI indexed journal with IF 5.816 – in *Journal of Fungi*) were published.

The studies and experiments described in this thesis were performed within the Fermentative Biotechnology Laboratory at “Life Science Institute King Michael I of Romania” from The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca and in the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) in Potsdam, Germany under the guidance of the supervisor Prof. Dan Cristian Vodnar.

The PhD thesis is structured in two main parts: state-of-the art containing one literature review article (Chapter 1) and original research containing the working hypothesis/objectives (Chapter 1 from the second part) and the general methodologies (Chapter 2), followed by the own research articles (Chapters 3-6), general conclusions and recommendations (Chapter 7), respectively the originality and innovative contributions of the thesis (Chapter 8).

In what concerns the first part (literature review articles), studies were identified by conducting PubMed, Web of Science Core Collection, Scopus, and Google Scholar electronic searches. After the literature screening presented within the state of the art, anaerobic conditions and a source of fructose is recommended for heterolactic fermentation to increase polyols production. AP is rich in fermentable sugars, like fructose (19.2%), creating a great condition for LAB. Also, AP, wheat bran, and OFMSW are among the cheapest and highly available. The strategies investigated, like bioprocesses, enzymes production, hydrolyses, encapsulation techniques, support the concept of waste and by-products management for zero waste.

In what concern the second part, the experiments, which involved the cultivation of microorganisms, were conducted in submerged and solid-state batch fermentation under controlled and sterile conditions. All the process was conducted in accordance with literature screening and with the help of members' expertise from Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) in Potsdam, Germany, especially for enzymes production and hydrolyses process. The cultivation conditions for the investigated strain were established and optimized by literature screening. The analyses and determinations were performed in duplicates and triplicates for every individual study and analysis.

The statistical analyses were performed using GraphPad Prism Version 8.0.1 software (Graph Pad Software Inc., San Diego, CA, USA) and IBM SPSS Statistics 19. The results were expressed as the means±standard deviation (SD). Data normality was studied using the Shapiro-Wilk test. One-way ANOVA with posthoc Tukey HSD was used to determine if there were significant differences between two samples for each acid, polyols, and sugars identified. In the case of F value, a $p<0.05$ was obtained, the calculations were continued, and the significance of the differences was obtained for groups of two by two samples. Scheffé, Bonferroni, and Holm posthoc tests were also applied to consolidate the results. In most cases, the same meaning was obtained as in Tuckey test. At each time interval, mean ± standard deviation (SD) (n=3) was passed because the data were parametric (normal). At each time interval was passed Tukey HSD p-value and Tukey HSD inference. The symbols were used as follows ** $p<0.01$, * $p<0.05$, NS $p>0.05$.

Chapter 3 capitalizes food by-products (apple pomace) and integration into a continuous flow of food biotechnological processes. This paper integrates the performance of traditional sourdough enriched with apple pomace and fermented by a selective LAB and yeast (mono- and co-cultures). The selective culture is represented by *Fructilactobacillus florum* to create a hetelactic fermentation to obtain polyols as erythritol and mannitol.

Chapter 4 presents the biotechnological routes towards sustainable industrial production of vanillin. This chapter demonstrates that the use of novel approaches involving integrated technologies (microorganisms, enzymes production, substrate hydrolyses, food technology, genetic engineering, industrial waste valorization) for the production of bio-vanillin is a promising route for a natural and sustainable production with high industrial potential.

Chapter 5 presents the economic efficiency of the biotechnological process for enzymes production and enzyme activity obtained via SSF. Following optimization, cellulase and glucoamylase have maximum activities after 7 and 5 days of SSF on cereal by-products. Enzymes were then used for the hydrolysis of the OFMSW. During hydrolysis, glucose increased considerably, which may be used to produce target compounds (e.g., vanillin or lactic acid) through submerged-liquid fermentations.

Chapter 6 presents chitosan, alginate, and pectin-based microencapsulation as an efficient method for encapsulating strains, enzymes, and bioactive compounds. Using polymers, as mentioned above, for the strains used could be better exploited for an extended period of time, and also an integrated system of SSF and submerged fermentation can be created.

The general conclusions were:

1. Enriching the sourdough fermentation with different by-products increases protein digestibility, total soluble/insoluble fiber content, reduces the glycemic index of food, and improves bioavailability of minerals.

2. Integrating apple pomace flour during sourdough fermentation, the nutritional value increased (organic acids and fermentable sugars), highlighting a new approach that could guide innovative fermented foods.

2. Apple pomace flour addition in wheat flour had a positive effect on cell viability, with constant growth, especially in fermentations with 95% wheat flour and 5% apple pomace, mainly in co-culture fermentation.

3. In the fermentation process with *S. cerevisiae*, apple pomace induced mannitol production, and in co-culture, increased mannitol production by approximately 5%.

4. With the development of new production technologies, erythritol and mannitol will possibly be produced on a larger scale; genetically engineered microorganisms might offer new exciting possibilities for the future, yielding very good results with enormous potential for the food and pharmaceutical industries.

5. Using a sterile wheat four, the properties of sourdough can be controlled by the added microorganism mix; all of the strains showed high fermentation activity on fructose, glucose, and maltose, the main soluble carbohydrates of sourdough.

6. The future perspective needs to be focused on fruit by-products that can delay the drying of bread.

6. Wheat bran can be used by *A. awamori* to grow and produce enzymes without requiring nutrients supplementation.

7. Future work should target the optimization of enzyme production in SSF with tests of different temperatures, moisture contents, or even with different mixtures of nutrients (glucose + yeast extract + minerals, etc.) to enhance the hydrolysis step.

8. Studies using SSF bioreactors that could allow for larger volumes of wheat bran and mixing would definitely provide useful results for the scale-up of the system.

9. Further work could be carried out to investigate wheat bran and organic fraction of municipal solid waste as important substrates for the fermentation process targeting other valuable compounds.

10. Fermentation products using thermophilic and extremophilic organisms could benefit from such a SSF-submerged integrated approach.

11. A challenge for by-products and waste materials may be the drying or autoclaving of substrates, recommended especially for by-products and organic fraction of municipal waste, in order to stop spontaneous fermentation with microorganisms already existing in the substrate, which can significantly depreciate its carbohydrate content.

Originality and personal contributions

The results presented in this thesis might be considered helpful for the scientific community from the biotechnology, food, and nutrition fields. This research can be considered a comprehensive study for by-products and waste materials valorization via biotechnological processes with the final aim to integrate into a circular economy.

Identifying and integrating the performance of traditional food products enriched with by-products and fermented with selective cultures of LAB and yeast (mono- and co-cultures) can increase the nutritional food value. The selective cultures represent a possibility of creating a specific fermentation mix to obtain target compounds such as erythritol and mannitol that contribute to future applications. Also, this thesis represents a comprehensive report related to the analysis of apple pomace before and after drying processing.

The investigation related to the biotechnological route aimed to build and evaluate an attractive sourdough system using a selective culture of *S. cerevisiae* (baker's yeast) and *Fructilactobacillus florum* (known to produce polyols) as a GRAS (Generally Recognized as Safe) and cheap microorganisms, with the final goal of reducing sugars and increase of polyols productions. This study is a step forward for future research projects related to the by-products valorization via their integration in fermented products. According to the most recent comprehensive review study on this topic, there are no scientific articles investigating a sourdough fermentation using a selective culture to produce in situ polyols.

Investigating sweetening and flavoring compounds using by-products and waste matrices is the future approach. Regarding the production of enzymes by SSF has the best economic efficiency and is highly recommended to obtain flavors such as vanillin, or added-value compounds such as organic acid-lactic acid. In addition, to make the process more efficient, the substrate used was wheat bran without nutrients supplementation. Following optimization, cellulase and glucoamylase have maximum activities after 7 and 5 days of SSF. Enzymes were then used for the hydrolysis of the organic fraction of municipal solid waste. During hydrolysis, glucose increased considerably, which may be used to produce target compounds (e.g., vanillin or lactic acid) through submerged-liquid fermentations.

The economic efficiency of the biotechnological process via encapsulation of the strains, enzymes, and bioactive compounds with biopolymers such as chitosan, alginate, and pectin-based represents a strong strategy in the bioeconomy field, increasing the industries' interest in by-products and waste materials re-valorization. The encapsulation of microorganisms can be an integrated and connected use between SSF and submerged fermentation for one system approach (SSF – enzymes production –> Hydrolyses –> followed by submerged fermentation with microencapsulated strains in the same reactor).

Finally, this thesis brings new data regarding the use of specific by-products deriving from the apple processing industry and organic fraction waste provided from municipal waste and integrate into the biotechnological process to obtain sweeteners and flavors as erythritol or mannitol and vanillin. This thesis integrates the concept of bioeconomy within the field of biotechnology by valorizing industrial by-products and waste materials via biotechnological and food processes.