DOCTORAL (PhD) THESIS

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ENZYMATIC AND MICROBIAL CONVERSION OF WHEY PERMEATE WITH THE OBJECTIVE OF CREATING VALORIZED PRODUCTS

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1. BACKGROUND AND OBJECTIVES

The valorization of whey - an abundant by-product of cheese manufacturing - has pronounced scientific and industrial significance. The most common route of whey processing is its fractionation, during which the main components of whey (lactose and whey protein) are separated and utilized individually**.** As a result of the high nutritional value of whey proteins, they are usually marketed as dietary supplements, however, the utilization of lactose in the food industry is limited due to its proneness to crystallize and the increasing ratio of lactose intolerant consumers.

In recent years, the use of whey-derived lactose as a precursor of prebiotic galacto-oligosaccharides (GOS) has received increased interest. The enzymatic synthesis of GOS is catalyzed by ß-galactosidase from lactose as a substrate at high concentrations. Therefore, the lactose content of whey needs to be concentrated when used for this purpose, but the possibilities of reaching the high lactose concentration necessary for the reaction have not yet been investigated.

The result of the enzymatic synthesis (so called crude GOS) is a mixture of prebiotic GOS fractions, non-reacted lactose, glucose and galactose as by-products. Lactose and monosaccharides are unwanted components in GOS products, their removal is usually achieved by simulated moving bed (SMB) chromatography. Removal of mono- and disaccharides by selective utilization of various microorganisms could be an alternative to the costly SMB chromatography. Based on the results in connection with this topic, this can be achieved by lactose-positive *Kluyveromyces* strains, but the process requires the use of high initial cell concentrations and the removal of the generated ethanol. Both factors increase costs, and thus lead to the decrease of the competitiveness of selective fermentation.

Another traditional method of deproteinized whey (whey permeate) valorization is lactic acid fermentation, by which value-added fermented beverages can be produced. The central question in connection with the lactic acid fermentation of whey permeate is whether the high nutritional and organic nitrogen source requirements of lactic acid bacteria can be met by this medium. However, we possess limited microbiological and fermentation-kinetics-related knowledge in this field.

The goal my doctoral work was to develop an integrated process for the valorization of whey, the first phase of which consists of membrane separation, and the use of the produced whey permeate as a substrate for GOS synthesis. My next goal was the elaboration of the microbiological foundations for the lactic acid fermentation of whey permeate. Based on the results of the previous steps, my goal was to combine the two processes by using selective lactic acid fermentation to remove mono- and disaccharides from the crude GOS mixture, in order to create a prebiotic fermented beverage-base. Finally, I aimed to investigate the possibilities to moderate the most relevant cost-increasing factors (use of high initial cell concentrations and ethanol produced in large amounts) of yeast-mediated selective fermentation processes, by conducting experiments with *Kluyveromyces* strains.

The objectives of my doctoral work were conceptualized in the following points:

1. Production of concentrated deproteinized whey (whey permeate) by membrane filtration and its utilization for the enzymatic synthesis of galacto-oligosaccharides (GOS)

The main goal here was to produce a substrate suitable for GOS synthesis and the coordination of the necessary ultra- dia- and nanofiltration processes.

2. Elaboration of the lactic acid fermentation of whey permeate

During my work, my goal was to investigate the lactic acid fermentation of whey permeate by using selected lactic acid bacteria (LAB) strains, and various nutrient sources, and finally, to use the results to define an optimal whey permeate-based fermentation media.

3. Selective removal of the mono- and disaccharide content of crude GOS by LAB strains for creating a functional beverage-base

My objective was the screening of LAB strains in an optimized fermentation media based on their selective mono- and disaccharide fermentation abilities. Furthermore, I investigated the selected strains in terms of their fermentation profile and produced metabolites.

4. Selective removal of the mono- and disaccharide content of crude GOS by the application of lactose utilizing yeasts

During my work I investigated the selective mono- and disaccharide utilization of *Kluyveromyces* strains in the purpose of creating high-purity, metabolite-free GOS.

2. MATERIAL AND METHODS

2.1. Production of concentrated deproteinized whey (whey permeate) by membrane filtration and its utilization for the enzymatic synthesis of galacto-oligosaccharides (GOS)

The protein and lactose content of whey was separated by ultra- and diafiltration, then the water content of whey permeate was partially removed by the means of nanofiltration. The substrates of the whey permeate-based GOS synthesis were the fractions collected during membrane filtration. The enzymatic synthesis was carried out in a stirred-tank reactor using 0.5 w/w% Biolacta N5, *Bacillus circulans*-derived ß-galactosidase at 50 °C for 24 hours. The saccharide-composition throughout the reaction was monitored via HPLC.

2.2. Elaboration of the lactic acid fermentation of whey permeate

I worked with seven *Lactobacillus* and two *Lactococcus* strains. The LAB strains were isolated from probiotic products and dairy starters or originated from various culture collections. Vitamin and amino acid requirements of the strains were investigated by agar- and disk diffusion methods. During fermentation experiments, whey permeate with 20 g/L lactose content was supplemented with a mixture of mineral salts, inorganic (ammonium-sulphate) or organic (whey protein, soy and casein peptones) nitrogen sources. The experiments were carried out in 15-50 mL centrifuge tubes, with 10^7 cell/mL initial cell concentration, at 30 °C, anaerobically, for 48 hours. Growth (OD_{600}, spread plate technique), acidification (pH meter), amino acid utilization (OPA-method) and carbohydrate profiles (HPLC) were measured during experiments.

2.3. S Selective removal of the mono- and disaccharide content of crude GOS by LAB strains for creating a functional beverage-base

Selective fermentative abilities of nine LAB strains were screened in a crude Vivinal GOS syrup-based fermentation media which was diluted to 15 g/L total carbohydrate content and supplemented with 0.25-10 g/L soy or casein peptone. Three strains *(L. acidophilus N2, L. paracasei PB9* and *L. plantarum* 2108) were selected based on the results, and were used in further experiments with 2.5 g/L soy or casein peptone supplementation. As previous results suggested that the rapid drop of pH hindered carbohydrate utilization of the strains, the acidification of the fermentation media was controlled by 0.05-0.15 M phosphate buffer. The experiments were carried out in 15-50 mL centrifuge tubes, with 10^7 cell/mL initial cell concentration, at 30 °C, anaerobically, for 72 hours. Growth (OD_{600}, spread plate technique), organic acid (HPLC) and D-lactate production (enzymatic kit), carbohydrate profiles (HPLC) and the release of ß-galactosidase into the fermentation broth (ONPG-method) were monitored during experiments.

2.4. Selective removal of the mono- and disaccharide content of crude GOS by yeasts

Three *Kluyveromyces* strains: *K. marxianus* DMB Km-RK, *K. lactis* DMB Kl-RK and *K. nonfermentans* NCAIM Y.01443 were used.

Vivinal GOS syrup was diluted to 100 g/L and supplemented with 5 g/L yeast extract or with a mixture of minimal salts. The experiments were carried out in 100 mL fermentation media, with $5*10^6$ cell/mL initial cell concentration, at 30 °C-on, and 220 rpm shaking for 72 hours. Growth (OD_{600}), acidification (pH meter) and carbohydrate composition (HPLC) were monitored.

3. RESULTS

3.1 Production of concentrated deproteinized whey (whey permeate) by membrane filtration and its utilization for the enzymatic synthesis of galacto-oligosaccharides (GOS)

By the UF/NF (ultrafiltration/nanofiltration) mediated fractionation of demineralized whey I produced a substrate suitable for GOS synthesis. The reaction shifted towards hydrolysis in whey permeate fractions collected during UF, and Biolacta ß-galactosidase N5-catalyzed GOS synthesis dominated in the concentrated, 200 g/L lactose content whey permeate, collected during NF. During GOS synthesis, the yield of oligosaccharide fractions (34 w/w %) was close to the maximum values characteristic to Biolacta N5 (36 w/w %), and the degradation of GOS fractions only occurred slowly. Production of lactose concentrations above 200 g/L with NF resulted in lactose loss. Furthermore, the native pH of the concentrated whey permeate (pH=7.5) was proven to be appropriate for GOS synthesis, thus modification of the pH is unnecessary.

3.2. Elaboration of the lactic acid fermentation of whey permeate

Addition of organic nitrogen sources to the whey permeate-based fermentation media was necessary to stimulate growth and carbohydrate utilization of the investigated LAB strains. *Lactococcus* strains were unable to utilize large molecular weight whey protein, due to their low proteolytic activities. Although *Lactobacillus* strains were able to grow in the presence of whey protein (5-8*10^7 cell/mL final cell concentration), they did not decrease lactose levels of whey permeate in the exponential growth phase, they did, however, utilize 11-51%. of its amino acid content.

Therefore, it can be concluded that the investigated strains used amino acids in whey protein as a sole nutrient source to satisfy their nitrogen and carbon requirements alike. This phenomenon could not be observed in the case of smaller molecular weight peptones, as growth ($8*10^7$-10^8 cell/mL final cell concentration), amino acid (29-73%) and lactose (14-25%) utilization were all pronounced in their presence throughout the entirely of the fermentation. The observed differences are most likely connected to the induction of the protease enzyme required for the hydrolysis of whey proteins.

3.3. Selective removal of the mono- and disaccharide content of crude GOS by LAB strains for creating a functional beverage-base

During screening of the selective fermentative abilities of the LAB strains, *Lactobacillus acidophilus* N2, *Lactobacillus paracasei* PB9 and *Lactobacillus plantarum* 2108 showed promising results in terms of glucose removal, but mono- and disaccharide-free GOS was not achieved in either case.

Based my assumptions, the low level of carbohydrate utilization was caused by the rapid acidification of the fermentation media, therefore, I examined the effect of pH control in experiments with the three selected strains.

Buffering stimulated mono- and disaccharide consumption of the LAB strains, increased the cell-mass generation, overall acid production, diversity of produced organic acids, and the release of ß-galactosidase enzyme into the fermentation broth through cell lysis as well. Although high ß-galactosidase activities were measured in the fermentation broth, GOS fractions were not hydrolyzed at all, which indicated the selectivity of the harbored ß-galactosidases for the DP2-sized fractions.

L paracasei PB9 and *L. plantarum* 2108 were able to remove the non-lactose disaccharides of the DP2 fraction, most likely due to the probiotic characteristics of these two strains. Fermentation by *L. paracasei* PB9 and *L. plantarum* 2108 resulted in high purity GOS products (Yield: 100%, Final Purity: 94-97%). This required 2.5 g/L soy or casein peptone supplementation and the application of 0.10 M, pH=7 phosphate buffer. The result of the process was a pH≤5 fermented product, considered safe, that contained approximately 5 g/L of prebiotic GOS fractions along with considerable amounts of L-lactate (6.2-6.5 g/L) and citrate (approx. 1.6 g/L) as well as smaller quantities of D-lactate (approx. 0.3 g/L), tartaric acid (0.4-0.8 g/L) and propionic acid (0-0.3 g/L) produced by the LAB strains.

In order to achieve the therapeutic effects of GOS consumption, approximately 500 mL of the fermentation product needs to be ingested. Consumption of such amounts does not surpass international recommendations for phosphate and D-lactate intake, hence the fermented GOS produced here is appropriate as a base for prebiotic beverages, which also contain the health-promoting effects (anti-microbial, immune-stimulating etc.) of lactic acid produced by the strains.

3.4. Selective removal of the mono- and disaccharide content of crude GOS by yeasts

Based on selective GOS fermentation experiments conducted with *Kluyceromyces* strains, the selective fermentation process carried out by *K. marxianus* DMB Km-RK with low initial cell concentrations ($5*10^6$ cell/mL) is applicable for the production of high-purity, mono- and disaccharide free GOS products (97% yield, 100% purity). For this, the supplementation of crude GOS with 5 g/L yeast extract was necessary. During fermentation, *K. marxianus* Km-RK produced

considerable amounts (40 g/L) of ethanol, removal of which requires further purification steps.

Although *Kluyveromyces nonfermentans* Y.01443 was unable to remove the DP2 fraction of crude GOS it removed the complete glucose and galactose content of the fermentation media, in the case of 5 g/L yeast extract supplementation. As *Kluyveromyces nonfermentans* Y.01443 did not produce ethanol, monosaccharide and ethanol free GOS could be achieved by the application of this strain. Such, partially purified GOS products are highly favorable for application in infant formulas.

4. CONCLUSION AND PROPOSALS

1) Whey can be processed for GOS synthesis, without considerable lactose loss by the developed UF/NF process. With this method, a concentrated whey permeate with 200 g/L lactose content can be produced, which then can be used for a Biolacta N5 ß-galactosidase-catalyzed GOS synthesis without further pH adjustment.

2) The supplementation of whey permeate with soy or casein peptone is necessary to promote the growth and carbohydrate utilization of the investigated LAB strains. Addition of large molecular weight whey protein, however, is not recommended, as *Lactococcus* strains are unable to utilize it and *Lactobacillus* strains use its amino acid content as a sole nutrient- and energy source, instead of lactose.

3) *L. paracasei* PB9 and *L. plantarum* 2108 strains are suitable for the selective removal of mono- and disaccharide content of crude GOS in the presence of 15 g/L initial carbohydrate concentration, 2.5 g/L soy or casein peptone supplementation and buffering with 0.1-15 M phosphate buffer at pH=7. Under the above conditions, products are free from mono- and disaccharides-, possess favorable organic acid profiles (contain mainly lactic and citric acid), have pH below 5, and thus can be considered safe.

4) The selective fermentation of crude GOS by *L. paracasei* PB9 and *L. plantarum* 2108 results in a product that contains GOS fractions, L-lactate and citrate, which can be used as a base for functional beverages. Considering the therapeutic dose of GOS and the recommendations for phosphate-intake, the use of 0.1 M phosphate buffer in the fermentation process and 500 mL daily consumption is recommended. Hereinafter, it is advised to look

for food-grade buffering agents, other than phosphate and test supplementation of the fermentation products with various flavorings (fruit aromas or concentrates), as well as sweetening agents. Conduction of organoleptic tests in order to develop marketable functional beverages is also necessary during the product development process.

6) If the goal was to develop metabolite-free high purity GOS products, *Kluyveromyces marxianus* DMB Km-RK proved to be a suitable strain for this task. When crude GOS was diluted to 100 g/L total carbohydrate and supplemented with 5 g/L yeast extract, the fermentation process resulted mono- and disaccharide-free GOS products. Following the fermentation step, removal of ethanol and the concentration of GOS is required.

6) A glucose, galactose and ethanol-free GOS product was achieved by *Kluyveromyces nonfermentans* Y.01443, when the GOS syrup was diluted to 100 g/L total carbohydrate and supplemented with 5 g/L yeast extract during fermentation. Product of this fermentation process can play a significant role in lactose-containing prebiotic products, especially among infant formulas.

5. NEW SCIENTIFIC RESULTS

1) Based on the results connected to the whey permeate-based GOS synthesis, I conclude the following: (i) A lactose concentration of above 200 g/L lactose needs to be achieved during concentration of the UF permeate, in order to avoid unwanted hydrolysis; (ii) the GOS yields obtained with concentrated whey permeate is comparable to those obtained with buffered, purified lactose solutions; (iii) and the native pH (pH=7.5) of the substrate produced by the proposed membrane separation processes is favorable in terms of the GOS yield of the enzymatic catalysis.

2) The investigated LAB strains require organic nitrogen supplementation for significant fermentation of whey permeate. Out of the applied organic nitrogen sources, whey protein proved to be unsuitable, while soy and casein peptones, containing small molecular weight peptides stimulated growth and lactose utilization considerably. In fermentation media containing whey protein and lactose, *Lactobacillus* strains utilized the amino acid content of whey protein as a sole carbon and nitrogen source. This phenomenon could not be observed in the presence of peptones. Based on these results, the induction of the proteolytic system by whey proteins modified the metabolic pathways of the investigated *Lactobacillus* strains in a way that resulted in the preference of amino acid side chains as a carbon source instead of lactose.

3) ß-galactosidase enzymes of the investigated *L. acidophilus* N2, *L. paracasei* PB9 and *L. plantarum* 2108 strains seem to be specific for the degradation of the <3 DP fraction of GOS, as the released ß-galactosidase from the lysed cells into the fermentation media did not decrease the amount of DP3-6 components.

4) *Lactobacillus paracasei* PB9 and *Lactobacillus plantarum* 2108 strains are suitable for the selective fermentation of mono- and disaccharides from GOS syrup diluted to 15 g/L and supplemented with 2.5 g/L soy or casein peptone. For the appropriate saccharide utilization, setting the initial pH to 7 with 0.1 M phosphate buffer is necessary.

5) Buffering influences the amount and spectra of the organic acids produced by *L. acidophilus* N2, *L. paracasei* PB9 and *L. plantarum* 2108 strains. While without buffering, only lactic acid is produced in considerable amounts, buffering causes the production of citrate of increased amounts and smaller concentrations of tartaric and propionic acids though unknown metabolic pathways.

6) *Kluyveromyces marxianus* Km-RK strain is suitable for the production of mono- and disaccharide-free GOS products, when crude GOS is diluted to 100 g/L and supplemented with 5 g/L yeast extract. This process can be suitable for the industrial production of mono- and disaccharide-free GOS due the applied moderate dilution factor and low initial cell concentrations.

7) *Kluyveromyces nonfermentans* NCAIM Y.0144 strain is able to remove glucose and galactose from crude GOS diluted to 100 g/L and supplemented with 5 g/L yeast extract, without ethanol production.

6. PUBLICATIONS RELATED TO THE PhD THESIS

6.1 Scientific Journal Articles

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PÁZMÁNDI, M., KOVÁCS, Z., MARÁZ, A., (2020): Potential of *Lactobacillus* strains for the production of fermented functional beverages enriched in galacto-oligosaccharides. Sumbitted for publication

6.2 International Conferences

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6.3 Domestic Conferences

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