CORVINUS UNIVERSITY OF BUDAPEST
PhD School of Food Science

THESES OF DISSERTATION

INVESTIGATION OF GALACTOSIDASE ENZYMES OF COMMERCIALLY AVAILABLE PROBIOTIC BACTERIA

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INTRODUCTION AND OBJECTIVES

In the past decades production of several enzymes became possible on industrial scale due to intensive biotechnological research. Application of these enzymes has great importance for the different industries, in healthcare, analytics and diagnostics. Since enzyme catalysis is not only regioselective but stereoselective, it is possible to make products whose chemical synthesis is too expensive or difficult to implement. Furthermore researchers and developers are now able to elaborate technologies that are effective, energy-saving and environmentally friendly. Introduction and implementation of enzymic technologies are in progress like in other fields of industries. Galactosidase enzymes that catalyse the breakup of galactozyl bonds are being applied in a wide area in the food and pharmaceutical industry.

The α-galactosidase enzyme degrades raffinose and melibiose which inhibit crystallization of saccharose and thus yield may be increased. Furthermore, it degrades galacto-oligosaccharides of soybean origin, enhances gelation properties of galactomannans, it may be used in the treatment of Fabry's disease or in the transformation of blood groups.

The β-galactosidase enzyme is used to degrade lactose in milk, to improve technological and organoleptic properties of lactose containing products, and to produce trans-galacto-oligosaccharides (TrGOS).

Galactosidase enzymes possess transgalactosidase activity beside hydrolytic activity under proper circumstances (high galactose concentration, low water activity, etc.), thus they are capable to synthesis galacto-oligosacchardes with different degree of polymerization. The chain length of the carbohydrate highly depends on the properties of the applied enzyme. These oligosaccharides selectively support growth and activity of probiotic bacteria (*Lactobacillus* and *Bifidobacterium* strains), so they regarded as prebiotics. Commercially available industrial β-galactosidase enzyme products are of *Aspergillus* spp. or *Kluyveromyces lactis* origin.

Although these microorganisms have GRAS status, TrGOS synthesised by them cannot be regarded automatically as prebiotics, because not only the safety but the selectivity or stability are equally important criteria.

It conveys the suggestion that TrGOS should be synthesised by galactosidase enzymes to increase the chance to become prebiotics. Another solution is to synthesize prebiotic TrGOS with free or immobilized probiotic cells.

To produce of fermented probiotic products the *Lactobacillus acidophilus* La-5, the *L. casei* 01 and the *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains are the most often used ones. They have several beneficial physiological effects, i.e. prevention of intestinal infections, alleviation of the symptoms of lactose intolerance, decrease of blood cholesterol and immunomodulation, and have remarkable technological properties.
Although one may find several studies on their technological and physiological properties in the scientific literature, our knowledge about their enzyme system is incomplete.
Such basic information would be great help in the design of prebiotics and synbiotics.

The above mentioned thoughts have motivated me to deepen my knowledge and research the α- and β-galactosidase enzymes of three important probiotic starter cultures: *Lactobacillus acidophilus* La-5, *L. casei* 01 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 as my doctoral work.

Main objectives

- Investigation of the mechanism of synthesis of α- and β-galactosidase enzymes commercially available and applied probiotic strains (*Lactobacillus acidophilus* La-5, *L. casei* 01 and *Bifidobacterium animalis* subsp. *lactis* Bb-12)
    - Study of the effect of the structure of chemically different carbohydrates
    - Exploration of the relationship between growth and enzyme synthesis
    - Determination of their location
    - Determination of the optimal inductor concentration
- Up-scale of enzyme fermentation
- Extraction and purification to homogenity of the α-galactosidase enzyme of *Bifidobacterium animalis* subsp. *lactis* Bb-12 strain
- Characterization of the enzyme protein
    - Estimation of molecular weight
    - Determination of optimal functional conditions
    - Investigation of stability
    - Effect of ions on the activity of the purified enzyme

MATERIALS AND METHODS

In my research work I have used three probiotics available at the Chr. Hansen Company: *Lactobacillus acidophilus* La-5, *Lactobacillus casei* 01 and *Bifidobacterium animalis* subsp. *lactis* Bb-12.

Microbiological examinations

For the quantification of bifidobacteria, and MRS agar for that of lactic acid bacteria TPY and MRS agar was used, respectively.

Realization of enzyme fermentation experiments

The enzyme fermentation experiments were performed in Erlenmeyer flasks of different size. All fermentations were started with one-day inoculum (inoculation with 1 (v/v)%). Inoculums were made in TPYG and MRSG medium that were inoculated with 5 v/v% cell suspension. Fermentations lasted 1-2 days at 37°C. Samples were taken at determined intervals; fermentations were followed by measuring pH and the activity of galactosidase enzyme.

The propagation of *B. animalis* subsp. *lactis* Bb-12 strain was up-scaled in a Biostat B (B. BRAUN) benchtop fermentor with net 2-liter volume. The fermentation lasted 20 hours at 37°C with 150 rpm agitation. Anaerobic circumstances were created by a gas mixture (10% carbon-dioxide in nitrogen gas) supplied through the aerating system.

Cell disruption

Galactosidase enzymes of bifidobacteria and lactic acid bacteria are intracellular ones, so disruption of cells are necessary for their detection. A mechanical method was applied for cell disruption with high pressure French press disruptor. Prior to disruption cells were separated by centrifuging (12000 rpm, 10 minutes, 4°C), then washed three times with a buffer (0.2 M McIlvaine pH 6.6). Finally, cells were collected and used in the French press. The cell disruption was performed in three consecutive cycles under 800 Psi pressure.

Measuring the activity of galactosidase enzyme

To measure the activity of galactosidase enzyme McIlvaine buffer was used (pH 6.6). The mixture made up of 0.5 ml substrate (in case of α-galactosidase: 15mM p-nitrophenyl-α-D-galactopyranoside; in case of β-galactosidase 15 mM p-nitrophenyl-β-D-galactopyranoside) and 0.3 ml McIlvaine buffer (pH 6.6) was pre-incubated for 5 minutes at 37°C, then the 5 minute reaction was started with properly diluted sample. Reaction was stopped after 5 minutes with 5 ml 0.1 M Na_2CO_3 solution, then absorbance was measured at 405 nm with spectrophotometer.

One unit of galactosidase enzyme is the amount that releases 1μM p-nitrophenol in one minute under reaction conditions.

Determination of protein content

To determine the protein content the Bradford method, and another method based on absorption of light at 280 nm were used.

Determination of enzyme location

B. animalis subsp. *lactis* Bb-12 cells from 20-hour culture broth containing raffinose were collected, and after three consecutive cell disruptions the suspension was centrifuged. Pellets containing cell debris were suspended in 1 ml 200 mM McIlvaine buffer (pH 6.6) and this fraction was regarded as the part that is connected to the cell membrane. Enzymes located in the cytoplasm were in the supernatant.

Enzyme purification procedures

Extraction and purification of α-galactosidase enzyme was performed with the combination of cell disruption, ion exchange chromatography (DEAE Sepharose Fast Flow, Q Sepharose), hydrophobic interaction (Phenyl Sepharose) and gelfiltration (Sephadex G200). Chromatographic steps of enzyme purification were performed with the help of columns connected to FPLC (Fast Performance Liquid Chromatography) equipment (Pharmacia, Uppsala Sweden) at 4°C. Supernatant collected after centrifugation was applied on the columns in several steps. Ultrafiltration was used as concentration and desalinization between chromatographic steps of protein purification. Protein solution was concentrated with Amicon ultrafilter having 10 kDa cut-off value. Samples were applied on the membrane with 3 bar overpressure from above. The ultrafiltration was performed on ice.

Determination of molecular weight

Estimation of the molecular weight of the purified enzyme was performed with SDS-PAGE gel electrophoresis. Ten percent Mini-PROTEAN TGX precast gel was applied.

Determination of the optimal pH and temperature of the enzyme

To determine the temperature optimum of the enzyme McIlvaine buffer (pH 6.6) was used and activity of α-galactosidase was measured in the range of 25 to 60°C. To determine pH optimum two kinds of buffers were used: 200 mM McIlvaine buffer (pH 3.0 to 7.0) and TRIS/HCl buffer (pH 7.2 to 9.0), and activity measurements were performed at 37°C.

Determination of enzyme stability

Stability of the enzyme was measured in the function of time at different temperature and pH values. The McIlvaine buffer was used in the range of pH 5.0 to pH 7.5, and the purified enzyme protein was incubated in these buffers between 35 and 50 °C, and sample was taken at certain intervals. Enzyme activity was measured according to the standard procedure.

To evaluate enzyme stability the surface response method was chosen, that takes into account the interaction of pH and temperature.

Effect of ions on enzyme activity

Effect of different ions was examined at 37 °C in the presence of 50 mM McIlvaine buffer (pH 6.5) by enzyme activity measurement.

The following compounds were tested, and were used in 10 mM concentration in McIlvaine buffer (pH 6.5): $AgNO_3$, $CaCl_2$, $CoCl_2*6H_2O$, $CuSO_4$, $HgCl_2$, KCl, $MgCl_2*6H_2O$, $MnSO_4$, $NiCl_2$ and $ZnSO_4$.

RESULTS

Extraction of intracellular galactosidase enzymes was accomplished by cell disruption with the French Press homogenizator that uses high pressure. I have determined that in order to extract maximal protein and proper galactosidase activities three consecutive disruption cycles have to be performed.

Lactobacillus casei 01 strain

The evaluation of growth properties of *L. casei* 01 strain revealed proper growth in nutrient broth containing both glucose, and glucose and lactose. During the 24-hour of fermentation the cell concentration increased from 10^7 to 10^9 CFU/mL. The highest cell concentration ($1.78 \cdot 10^9$ CFU/mL) was detected, if MRSG nutrient broth supplemented with 0.5 (w/v)% lactose was applied. In this system the lowest β-galactosidase activity (0.03 U/100 mL) was detectable, if only glucose was presented, and the highest (0.08 U/100 mL), if the nutrient broth contained MRSG with 0.5% lactose. The low enzyme activity values, similar as in case of *L. acidophilus* La-5, are probably due to the glucose content (2 (w/v)% of the medium, which provides enough carbon source for the microbial growth, and also causes catabolic repression on the synthesis of β-galactosidase enzyme. The optimal lactose concentration for the highest β-galactosidase activity (0.38 U/100 mL) was 1 (w/v)%. I found the inducer effect of lactose on enzyme synthesis in case of *L. casei* 01 strain.

Lactobacillus acidophilus La-5 strain

The growth properties of this strain were evaluated on four different carbohydrates (glucose, lactose, raffinose, melibiose). All the examined substrates supported growth properly. In glucose containing broth the culture reached a stationery growth phase at the 9^{th} hour of fermentation. There are differences in utilization of oligosaccharides at the initial phase of fermentation, which differences are supposedly derive from the different mechanism of the uptake transport systems and the biosynthesis of the necessary glycosylic hydrolase enzymes. However, at the end of the fermentation on both lactose and raffinose the cell number were approximately $2.88 \cdot 10^9$, which is similar as in case of control medium (glucose substrate). It is interesting that in melibiose containing broth La-5 strain produced only 10^8 order of magnitude cell number by the end of the fermentation. Beside the evaluation of growth properties the process of galactosidase synthesis also were studied. According to the result of α-galactosidase enzyme activity in the presence of 2 (w/v)% raffinose 13.51 U/100 mL

activity was detectable, while in case of glucose as sole carbon source *L. acidophilus* La-5 α-galactosidase was not synthesized. This can be explained: that the broth with 2 (w/v)% glucose, which can easily be taken up, and serve enough energy for La-5 strain. The synthesis of α-galactosidase can be induced and its production is growth-associated. For the enzyme induction the optimal raffinose concentration is 1.5 (w/v)% which ensures 23.5 U/100 mL α-galactosidase activity. Further increase of raffinose concentration causes the decrease of α-galactosidase activity.

L. acidophilus La-5 strain showed β-galactosidase activity only on lactose and raffinose (7.11 and 0.62 U/100 mL, respectively) among the four applied substrates. On glucose and melibiose no activity was detected. On lactose substrate, which contains β-galactosidic bond, more than 10-fold higher β-galactosidase activity was detected compared to raffinose substrate. The inducer concentration was optimized, which resulted 0.5 (w/v)% lactose concentration. Further increase of lactose concentration (1 to 2 w/v %) did not significantly affect the enzyme activity (7.56 and 8.51 U/mL, respectively). I have verified the repressing effect of glucose on β-galactosidase enzyme synthesis. If both lactose and glucose are present in the culture medium β-galactosidase activity was only 0.75U/ 100 mL, while in absence of glucose the enzyme activity was 7.5 U/ 100 mL.

***Bifidobacterium animalis* subsp. *lactis* Bb-12 strain**

To reveal the mechanism of β-galactosidase enzyme activity TPYG nutrient broth supplemented with lactose (0.1 and 0.5 w/v% concentration) was used. Bb-12 strain grew well in the nutrient medium, and after 24-hour fermentation the cell concentrations reached a level of $5\text{-}7 \cdot 10^8$ CFU/mL. β-galactosidase activity varied in range of 1.5 to 2.5 U/mL depending on the applied medium and fermentation time. Beta-galactosidase activity was also detectable if glucose was the sole carbohydrate. Supplementation with lactose did not result relevant β-galactosidase activity increase, however lactose influenced the dynamics of enzyme synthesis. Change of β-galactosidase activity was evaluated in TPY medium supplemented with 2% of carbohydrates with different chemical structure (glucose, lactose, and raffinose). Beta-galactosidase was detectable also in media, which did not contain molecules with β-galactosidic linkage. This phenomenon indicates the constitutive enzyme synthesis. Application of lactose substrate resulted 5-8-fold higher enzyme activity referring to the values of other carbohydrates. The optimal lactose concentration is between 1.0 (w/v) % and 1.5 (w/v) %. In almost all cases 1.5 (w/v)% lactose concentration was enough

to maximize the enzyme production. In these cases the productivity of β-galactosidase varied between 27-29 U/10^{10}·CFU × h. Lactose in 2.5 (w/v)% or higher concentration inhibited the enzyme synthesis.

In the presence of all examined carbohydrate (glucose, lactose, raffinose) the activity of α-galactosidase was detectable. Thus I determined that Bb-12 strain constitutively synthesizes α-galactosidase enzyme. The highest activity (9.46 U/100 mL) was measured at the 15th hour of fermentation in presence of 2(w/v)% raffinose. According to the results 1% raffinose is enough to maximize the enzyme productivity (13 U/10^{10}·CFU·× h), which was observable at between 15th and 20th hour of fermentation. In the evaluation of α-galactosidase location *B. animalis* subsp. *lactis* Bb-12 strain revealed that 91% of activity is located in the cytoplasm and only 9% is located linked to the cell wall. A four-step method including chromatography was used to extract and purify β-galactosidase enzyme from *B. animalis* subsp. *lactis* Bb-12 strain for the purpose of characterizing the enzyme. The homogeneity of the enzyme protein was examined by SDS-PAGE method. According to results the molecular weight of β-galactosidase produced by *B. animalis* subsp. *lactis* Bb-12 strain is approximately 50 kDa. Removed from the environment and purified to homogeneity the enzyme lost its stability, and rapidly lost the activity.

To characterize the physical and chemical properties of α-galactosidase from *B. animalis* subsp. *lactis* Bb-12 strain the optimal environmental conditions were evaluated. The range of optimal pH is 5.5 to 7.0, while the range of temperature optimum is 35 to 45 °C. Temperature over 45°C resulted in 35% activity loss.

The highest stability was detected at 35 °C and pH 6.5. Under these circumstances the half-time was 50 hours. At this temperature on pH 5.0-5.5 the enzyme lost its 50% of activity after 4.2 hours of incubation. Half-time of the enzyme have decreased rapidly (to 40 minutes) when it was incubated at 40 °C on pH 5.0-5.5. These values on pH range of 6.0-7 are 15.33 and 24 hours. An interesting observation was that in mildly alkali milieu the enzyme kept 60% of its activity for 30 hours. At higher temperature (45°C) the enzyme lost half of its activity after 8 minutes on pH 5-5.5. This time length doubled if the pH was 6-6.5, however on pH 7.5 the half-time was approximately 5.5 hours. At 50°C incubation temperature in the whole analyzed pH range the enzyme inactivated rapidly.

Beside the half-time values the rate of inactivation were also examined. To evaluate the data response surface method was applied. The half-time and rate of inactivation values were

evaluated together, and the temperature range of 35-37°C and pH range of 6.5-7 can be proposed to bioconversion experiments. Several metal ions were examined to reveal the effect on enzyme activity in 10 mM concentration. In this concentration none of the examined metals increased the enzyme activity. However Co^{2+}, Ag^{2+} Hg^{2+} ions have inhibited the enzyme functions. Hg^{2+} ion by 75%, Ag^{2+} by 72% and Co^{2+} by 64% decreased the enzyme activity of α-galactosidase.

The results of my PhD research contain numerous novelties, and contribute to research to reveal and understand the enzyme systems of the applied probiotic strains, and to design and develop functional foods (prebiotics, probiotics and synbiotics).

Possible developments of results

- All-round characterization of the purified enzyme, and determination of kinetic parameters, substrate specificity, exploration of catalytic mechanism.
- Bioconversion researches that include detection of hydrolysis and transfer reactions.
- Optimization of the conditions of bioconversion studies.

NEW SCIENTIFIC ACHIEVEMENTS

1. I have proved that the *Lactobacillus acidophilus* La-5 probiotic strain grows well on glucose, lactose, raffinose and melibiose substrates. At the end of fermentation $5{,}0x10^8$ and $5{,}0x10^9$ CFU/mL cell concentration may be achieved. Furthermore, α-galactosidase enzyme may be induced with raffinose, the β-galactosidase with lactose. Synthesis of enzymes is related to growth. I have shown that glucose have repressive effect during the synthesis of galactosidase enzymes. The optimal lactose concentration is in the range of 1 to 2%, where the activity in case of *L. acidophilus* strain is 7.56 and 8.51 U/ 100mL. Higher lactose concentration has inhibitory effect on β-galactosidase activity. The α-galactosidase enzyme is synthesized inductively, and raffinose proved to be the best inductor in 1.5 (w/v)%.

2. The *B. animalis* subsp. *lactis* Bb-12 synthesizes β-galactosidase enzyme in medium containing glucose and lactose, but I have determined that with the application of lactose substrate 5 to 8-times higher enzyme activity may be achieved than with the other tested carbohydrates. The optimal lactose concentration is between 1.0 and 1.5 (w/v)%. Presumptively, the β-galactosidase enzyme is synthesized in constitutive way by the bacterium.

3. I have determined that the *B. animalis* subsp. *lactis* Bb-12 strain constitutively synthesizes the α-galactosidase enzyme. I have found that even 1% raffinose is sufficient to reach the maximal α-galactosidase productivity (13 $U/10^{10}$ CFU × h). This maximum value may be measured at the 15^{th}-20^{th} hour of fermentation. The most significant portion (91% of the activity) of intracellular α-galactosidase enzyme is located in the cytoplasm, and only 9% is located connected to the cell wall.

4. I have purified the α-galactosidase enzyme of the *B. animalis* subsp. *lactis* Bb-12 strain in several chromatographic steps. Molecular weight of the α-galactosidase enzyme purified to homogenity was estimated to be 50 kDa. Optimal temperature of the enzyme is between 35-45 °C. Furthermore I have found that the enzyme have wide pH optimum (pH 5.5 to 7.0). The Co^{2+}, Ag^{+} and Hg^{2+} ions inhibit activity of α-galactosidase enzyme.

5. I have examined the stability of α-galactosidase enzyme of *B. animalis* subsp. *lactis* Bb-12 strain with the surface response method. I have determined that the longest half-life time (50 hours) may be achieved if the enzyme is incubated at 35°C and pH 6.5. Based on the common evaluation of half-life time and inactivating rate I suggest that bioconversion experiments should be conducted in the pH range of 6.5 to 7.0 and at temperature range of 35 to 37 °C.

PUBLICATIONS IN THE FIELD OF THE DISSERTATION

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