Production and characterization of α-galactosidase from *Thermomyces lanuginosus*

PhD thesis

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1. The background and the aims of the research

The real meaning of the word “nutrition” has changed due to the results of economic and social development. Today nutrition does not only mean that nutrients and energy are provided to our body, but it is an essential aspect of the production and consumption of such foodstuff, which play a role in the maintenance of health.
Those foods which have positive effects on health beside their nutritive power, are called functional food. There are several ways for food to become functional: if a component with negative physiological effect is removed or replaced, if the concentration of a component with positive physiological effect is increased or if a component which is beneficial to the health is added to it. The provision of the population having special nutritional needs means an additional task and challenge for the specialists in food science and the food industry. The demand continuously increases for food which can be consumed by people suffering from deficiency diseases and allergy.
Nowadays the design and production of functional food is a dynamically developing segment of food industry. Key research priorities of the industry targeting the next decades include:

- the increase of processing efficiency with a reduction of environmental impact;
- the expansion of the development of value-added technologies;
- the understanding and the exploitage of the interactions of components in formulated food;
- the development and the promotion of strategies to control food related illnesses;
- creation of functional foods that promote health and well-being.

The enzyme technologies take an important part in the accomplishment of these tasks. The enzyme reaction is not only regioselective, but stereoselective. The enzyme reaction proceeds under mild circumstances and is practically harmless for the environment.

The most frequently utilized enzyme in food processing belongs to the class of hydrolases. The amylolytic, the proteolytic, the pectolytic, the lactase, the invertase and the lipase enzymes have been utilised on industrial scale for many decades. At present α-galactosidase (EC 3.2.1.22) is applied in the beet sugar industry for the enhancement of sucrose yield by the hydrolysis of raffinose.

α-Galactosidases could be expansively applied in other fields of food production. The functionality of food can be improved by their application. The enzyme is capable to hydrolyze the flatulence causing galactooligosaccharides, which belong to raffinose family. By its utilization the antinutritive oligosaccharides of legumes can be eliminated therefore they become functional and their nutritional value is improved. The locust bean gum is a food additive with an outstanding gelling feature, but it is available only in a limited amount. This product is successfully replaceable by modified galactomannan from guar which is produced by means of α-galctosidase enzyme. After a formation of the favourable mannose:galactose rate the modified galactomannan techno-functional character is better.

Several α-galactosidases are known to possess transferase activity. They can be exploited to produce transgalacto-oligosaccharides with various chemical structures, which are applicable as prebiotic in functional food. Such special α-galactosidases were reported which are capable to transfer galactose to cyclodextrines both as a side chain and built in their rings. These heteropolymers are applicable for microencapsulating of bioactive materials. The industrial exploitation of α-galactosidase is expected in the near future.

The great majority of industrial enzymes are produced by fermentation applying microbes. The natural habitat of filamentous fungi is the decayed part of plants which contain polymers with diverse compositions. They have to synthesize and secrete various enzymes to ensure the nutrients for their survival and propagation. These characteristics of the fungi are very attractive to utilize them in the production of enzymes. The enzymes produced by thermophilic fungi are generally more thermostable, than their counterpartners from mesophilic fungi. So their biotechnological exploitation has numerous advantages such as shorter technological time, better utilization of capacities and longer lifetime. At the same

time its inactivation is not so problematic, as the enzymes from extreme thermophilic bacteria.

Among the thermophilic fungi *Thermomyces lanuginosus* has the widest temperature range of growth. This fungus is not pathogen and does not produce toxins. It can be propagated easily and it is a rich source of numerous extracellular enzymes such as the hemicellulases, the amylolytic, the pectolytic, the lipase and the phytase enzymes. From among their hemicellulases most information is known about the xylanase enzyme. Its sequence and crystal structure are defined. The researches on xylanase enzyme were driven by that *T. lanuginosus* is non-cellulolytic; therefore it produces cellulase free thermostable xylanase. Data on the other glycosylhydrolases from *T. lanuginosus* i.e. the α-galactosidases, which participate in the degradation of the hemicelluloses, are very limited. There is only one report about the α-galactosidase enzyme from *T. lanuginosus*, but it does not deal with the improvement of enzyme production. In order to get a detail information about α-galactosidase enzyme from *T. lanuginosus,* it was aimed to study its enzyme production, to recover and characterize the enzyme, and to investigate possible utilizations.

The main objects of the research are the following:

- Elaboration of enzyme fermentation technology for a selected strain
  - Optimization of the composition of medium
  - Standardization of the cultivation of inoculum
  - Elaboration of the fermentation technology
- Development of a procedure for the recovery and purification of the α-galactosidase enzyme
- Characterization of the enzyme protein
  - Molecular mass, isoelectric point, carbohydrate content
  - Quantification of optimum parameters for the activity
  - Determination of catalytic features of the relevant enzyme to elucidate their use in possible application

2. Materials and methods

The applied chemicals were analytical grade and purchased from the firms of Sigma, Reanal, Merck and Pharmacia.

The *Thermomyces lanuginosus* strains used in the experiments originated from different culture collections. Fermentation was carried out in shaken flasks cultivation.

For the determination of the α-galactosidase activity 15 mM p-nitro-phenyl-α-D-galactopyranoside was used as substrate. After five-minute reaction time the enzyme reaction was terminated by adding 0.1 M sodium carbonate solution. The released amount of p-nitrophenol was determined by spectrophotometry at 405 nm. One unit (U) of α-galactosidase activity was expressed as the amount of enzyme that releases 1 μmol p-nitrophenol in 1 minute under the relevant condition.

For the purification of the α-galactosidase enzyme low pressure liquid chromatography was used applying various resins for the protein separations.

The columns were connected to FPLC and GradiFrac systems controlled by FPLCAssistant and FPLCDirector softwares (Pharmacia).

For the determination of the protein concentration Biuret, Lowry, modified Lowry methods and light-absorption at 280 nm were applied. To determine reducing sugars Somogyi-Nelson and cooper-bicinchoninate methods were used. The quantities and the qualities of the carbohydrates were determined by HPLC method. The quantification of galactose was carried out by test kit manufactured by Roche firm (Roche Diagnostics GmbH, Mannheim, Germany).

The determination of protein molecular mass was done using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For staining of protein Coomassie Brillant Blue reagent was applied. The isoelectric point was estimated by electric focusing on agarose gel.

3. Results

Seventeen *T. lanuginosus* strains cultivated on raffinose substrate were ranked based on their α-galactosidase activities. *T. lanuginosus* CBS 395.62b strain with the best activity was selected for the elaboration of enzyme fermentation technology. The activity of this strain was 15.7 U/mL defined at 58 °C which is two and half times higher than the average productivity of all other investigated strains.

From the investigated growth substrates only sucrose, raffinose, lactosucrose®, L-arabinose and galactomannans induced the synthesis of extracellular α-galactosidase enzyme. Some agricultural products and by-products (pea flour, wheat bran extracts) also efficiently induce the synthesis of α-galactosidases. Among the investigated inducers, sucrose proved to be the best one for enzyme production. The majority of the tested strains showed better or near identical activities on sucrose than on raffinose growth substrate. The *T. lanuginosus* strain selected for enzyme production exhibited 23 % higher activity in sucrose than in raffinose containing media. The industrial feasibility of the elaboration of fermentation technology, which applies sucrose as carbon source is promising, since it is a more available and cheaper nutrient than raffinose. To maximize the production of the α-galactosidase of *T. lanuginosus* CBS 395.62b strain, the optimum concentrations of sucrose and ammonium acetate as well as the preparing of inoculum and fermentation culture were determined. The optimal composition of the medium is the following: sucrose 30 g, ammonium acetate 9 g, KH_2PO_4 3 g, K_2HPO_4 2 g, $MgSO_4*7H_2O$ 1 g, Vogel's mineral solution 1mL. The above mentioned components are dissolved in McIlvaine buffer (pH=7.5) and its volume is adjusted to 1000 mL. For the initiation of enzyme fermentation two-day old inoculum culture is advised.

After seven- or eight-day fermentation using the elaborated technology about 100 U/mL enzyme activity was achieved. The optimization of fermentation medium enhanced the enzyme yield six times. This achievement is outstanding comparing to the data presented so far in the literature, while the majority of publications communicate only α-galactosidase activities of 1-5 IU/mL

The α-galactosidase was isolated by precipitation with ammonium sulphate from the filtrate of the ferment broth. For the enzyme purification a four-step chromatographic process was elaborated, in which the separations were carried out by combinations of ion-changing and gel filtration chromatography. Applying this procedure a 114-fold purification and a yield of 59 % was achieved, and an enzyme preparation with 1813 U/mg was gained. The homogeneity of the enzyme protein was proved by SDS-PAGE. The molecular mass was calculated to be 94 kDa and the isoelectric point of the enzyme was 3.9-4.1. The α-galactosidase enzyme seems to have glycoprotein character and its carbohydrate content was estimated to be 5.3 (w/w) %. The distribution of carbohydrates linked to the enzyme protein was the following: 56 % of D-mannose, 36 % of glucosamine, 8 % of D-galactose and the D-glucose content was less than 1 %.

The optimal pH of the enzyme activity was between pH=5.0-5.5 and in this pH range the optimum temperature was 65 °C determined at presence of p-nitro-phenyl-α-D-galactopyranoside substrate. The enzyme preparation was stable at 55°C between pH=6.4 and 8.3 for at least one day. It was determined that the α-galactosidase enzyme at 60°C under pH=4.0 and pH=4.5 was inactivated in one hour and 7.5 hour, respectively. Residual activities measured at pH=5.0, pH=5.5, pH=6.0, pH=6.5, pH=7.0, pH=7.5, pH=8.0, pH=8.5 and pH=9.0 were 52, 58, 68, 75, 79, 45, 51, 59 and 19 %, respectively. During the treatment at 65 °C the enzyme was inactivated at pH=4.0 in 20 minutes, at pH=4.5 in 40 minutes, at pH=7.5, pH=8.5 and pH=9.0 in 3 hours, at pH=5.0 in 4 hours at pH=5.5 and pH=7.0 in 5 hours, however at pH=6.0 and pH=6.5 it kept more than 40 % of its activity and at

pH=8.0 10 % residual activity was measured after 6 hours. When incubated at 70 °C or higher, the enzyme preparation lost its activity very quickly.

The relevant α-galactosidase enzyme is active on p-nitro-phenyl-α-D-galactopyranoside, melibiose, raffinose and stachyose, but it was not capable to liberate galactose from intact galactomannans.

The kinetic parameters of the enzyme were determined, and following values were found: K_m=1.13 mM, V_{max}=2498 μmol*min^{-1}*mg^{-1} for p-nitro-phenyl-α-D-galactopyranoside; K_m=1.61 mM, V_{max}=4434 μmol*min^{-1}*mg^{-1} for raffinose and K_m=1.17 mM, V_{max}=4889 μmol*min^{-1}*mg^{-1} for stachyose. α-Galactosidase showed the highest affinity to raffinose and had similar affinity to p-nitro-phenyl-α-D-galactopyranoside and stachyose substrates.

The manganese ions serve as activators, while the calcium, the zinc and the mercury ions act as inhibitors of the enzyme.

The hydrolysis of melibiose and raffinose happened at 55 °C and pH=5.5 (McIlvaine buffer) applying 50 U α-galactosidase for 1 g substrate, when the concentration of the substrates was between 1 and 8 (w/w) % in 3 hours. In case of raffinose hydrolysis, trimer and tetramer oligosaccharides as intermedier products were also formed. In case of the hydrolysis of stachyose only 60 % conversion was observed after 24 hours.

The synthesis of the α-galactosidase enzyme of *T. lanuginosus* CBS 395.62b strain was different when various growth substrates were applied. In the presence of sucrose and galactomannan the enzyme was secreted into the medium, while in melibiose containing medium the enzyme was synthesized in intracellular form. In the production of extracellular α-galactosidase enzyme the quality of growth substrate was also decisive. Under optimal circumstances one order of magnitude less activity was reached on trimer than on sucrose. One of the possible explanations for this is that the concentration of galactomannan cannot be

increased above 1 (w/v) % because of its gel forming capacity. When galactomannan concentration was applied in 1 (w/v) %, it caused problems during the isolation of enzyme. The difference in the synthesized enzymes was proved, while the molecular masses of galactomannan and sucrose induced enzymes are 54 kDa and 94 kDa, respectively.

4. Aspects of biotechnological exploitation and further works

- Industrial production of α-galactosidase is feasible by scaling up the technology that was elaborated on laboratory scale. For the development and the realization of the enzyme production molasses is suggested as raw material because it is cheap and contains raffinose and sucrose. Both of them induce the synthesis of enzyme. Further advantage of the utilization of molasses is that it is rich in easily available amino acids.
- To map the extracellular α-galactosidases of the fungus *Thermomyces lanuginosus* it would be worth to isolate the α-galactosidase enzymes from ferment broth containing wheat bran extract and to characterize them.
- The determination of the sequence of amino acids gives possibility to classify the relevant enzyme into the family of glycohydrolases.
- The identification of the catalytic mechanism of the enzyme by molecular methods.
- The produced enzyme is capable for the degradation of the antinutritive galactooligosaccharides; therefore it can be utilized for reduction or elimination of galactooligosaccharides known as flatulence factors that are found in legumes. In this way these raw materials become functional, moreover their nutritional values are improved.
- Development of the production of transgalactooligosaccharides, which can be used as prebiotic by the optimization of the transfer reaction.

5. New scientific results

1. Seventeen *Thermomyces lanuginosus* strains cultivated in shaken flasks were ranked based on their extracellular activities. Among the productivities of the investigated strains considerable differences were determined. For the production of α-galactosidase the *T. lanuginosus* CBS 395.62/b was selected. The spectrum of growth substrates, which induce synthesis of α-galactosidase enzyme of *T. lanuginosus* was determined. Sucrose proved to be the best inductor. The synthesis of the enzyme was induced by oligo- and polysaccharides containing α-galactoside linkage (raffinose and galactomannans), L-arabinose, as well as the biosynthetic lactosucrose used as prebiotic.

2. It was determined that intracellular α-galactosidase was synthetised by *T. lanuginosus* CBS 395.62b strain.

3. Fermentation technology on a laboratory scale was elaborated for the production of α-galactosidase by the *T. lanuginosus* CBS 395.62b strain. For the initiation of the fermentation two-day old inoculum culture was defined as optimum. The productivity of the enzyme fermentation was enhanced six times by the optimization of the composition of the medium.

4. A procedure was developed for the recovery of α-galactosidase enzyme. The molecular mass of the electrophoretic homogenous α-galactosidase enzyme produced on sucrose was 94 kDa and and its electric point was pI=3.9-4.1. The enzyme is a glycoprotein with 5.3 % carbohydrate content in which the patterns of the main components are 56 % mannose, 36 % glucosamine, 8 % galactose. The pH optimum of the enzyme is in the range of pH=5.0-5.5 and its optimum temperature is 65 °C on p-nitro-phenyl-a-D-galactopyranoside. Manganese ions affect as weak activators, while calcium, zinc and mercury ions act as strong inhibitors for the activity. In the presence of silver ions the enzyme is inactivated.

5. Concerning the catalytic features of the enzyme it was concluded that it hydrolyzes the melibiose, the raffinose and the stachyose beside the p-nitro-phenyl-α-D-galactopyranoside, and it was not active on galactomannans. Under specific circumstances transferase activity was observed.
   The kinetic parameters of the enzyme preparation were the followings K_m=1.13 mM, V_{max}=2498 μmol*min^{-1}*mg^{-1} on p-nitro-phenyl-α-D-galacto-pyranoside; K_m=1.61 mM, V_{max}=4434 μmol*min^{-1}*mg^{-1} on raffinose and K_m=1.17 mM, V_{max}=4889 μmol*min^{-1}*mg^{-1} on stachyose substrates.

6. It was proved that the *T. lanuginosus* CBS 395.62b strain produces at least two α-galactosidase enzymes with various molecular masses depending on the applied growth substrate. The molecular mass of the enzyme synthesized on sucrose was 94 kDa, while the enzyme produced on locust bean galactomannan was 54 kDa.

6. List of publications related to the subject of the dissertation

Scientific papers

1. Rónaszéki G, Nguyen DQ, **Rezessy-Szabó JM**, Hoschke Á, Bhat MK (2000) Screening the strains of thermophilic fungus *Thermomyces lanuginosus* for amylolytic activities. Acta Aliment **29**:71-79
2. **Rezessy-Szabó JM**, Bujna E, Hoschke Á (2002) Effect of different carbon and nitrogen sources on α-galactosidase activity originated from *Thermomyces lanuginosus* CBS 395.62/b. Acta Aliment **31**: 73-82
3. **Rezessy-Szabó JM**, Nguyen DQ, Bujna E, Takács K, Kovács M, Hoschke Á (2003) *Thermomyces lanuginosus* CBS 395.62/b strain as rich source of α-galactosidase enzyme. Food Technol Biotechnol **41**: 55–59

Project reports

1. Hoschke Á, **Rezessy-Szabó JM**, Rónaszéki G, Nguyen DQ (1998) Production and evaluation of industrial potential of the thermostable amylolytic and xylanolytic enzymes from the thermophilic fungus, *Thermomyces lanuginosus*. COPERNICUS CIPA-CT 94-0232 project final report
2. Bhat MK, Macris BJ, Claeyssens M, Kolev DN, Hoschke Á, Biely P, Bennett NA, Ryan J, Yusuf I, Kekos D, Christakopolous P, Katapodis P, Nerinckx W, Ntuma P, Petrova S, Atev A, Bakalova NG, Benadova RS, **Rezessy-Szabó JM**, Rónaszeki G, Nguyen DQ *et al.* (1998) Production and evaluation of industrial potential of the thermostable amylolytic and xylanolytic enzymes from the thermophilic fungus, *Thermomyces lanuginosus*. Final edited and exploitation technical progress report, IFR, Reading Laboratory
3. Hoschke Á, **Rezessy-Szabó JM**, Bujna E, Mayer Á, Bognár CS, Barna Zs (2000) Production and evaluation of functional food ingredients in improving the nutritional quality of food and human health. Final Report of IC-CT 96-1000 Project

Full text proceedings

1. Bhat MK, Bennet NA, Hoschke Á, **Rezessy-Szabó JM**, Rónaszéki G, Macris BJ, Katapodis P, Biely P, Vranska M (1997) Thermostable glucoamylase and xylanase for food applications. 8th European Congress on Biotechnology, Budapest
2. Hoschke Á, **Rezessy-Szabó J**, Nguyen DQ, Bujna E, Czukor B (1999) Enzymatic degradation of antinutritive oligosaccharides of legumes, Proceeding of Euro Food Chem., Budapest. **In**: Proceedings of Euro Food Chem X: Functional Food – A new challenge for the food chemists (eds: Lásztity R, Pfannhauser W, Simon-Sarkadi L, Tömösközi S) 778-783
3. **Rezessy-Szabó JM**, Nguyen DQ, Hoschke Á (2000) Formation of α-galactosidase enzyme by *Thermomyces lanuginosus*. Proceeding of 14th Forum for Applied Biotechnology 2000 **65/3a:**319-322
4. Hoschke Á, Nguyen DQ, **Rezessy-Szabó JM** (2000) Termofil gomba *Thermomyces lanuginosus* extracelluláris enzimek vizsgálata. Acta Microbiol Debrecina 142-146

Lectures at scientific conference

1. Bhat MK, Bennet NA, Hoschke Á, **Rezessy-Szabó JM**, Rónaszéki G, Macris BJ, Katapodis P, Biely P, Vranska M (1997) Thermostable glucoamylase and xylanase for food applications. 8th European Congress on Biotechnology, Budapest
2. **Rezessy-Szabó JM**, Nguyen DQ, Bujna E, Rónaszéki G, Hoschke Á (1998) Glükoamiláz enzim előállítása *Thermomyces lanuginosus* fonalas gombával. Lippay János – Vas Károly Tudományos Ülésszak, Budapes
3. **Rezessy-Szabó JM**, Bujna E, Hoschke Á (2000) Különböző szén- és nitrogénforrások hatása *Thermomyces lanuginosus* eredetű α-galaktozidáz enzim termelésére. Lippay-Vas Tudományos Ülésszak, Budapest

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1. Hoschke Á, Nguyen DQ, **Rezessy-Szabó JM** (2000) Termofil gomba *Thermomyces lanuginosus* extracelluláris enzimek vizsgálata. Fermentációs Kollokvium, Debrecen
2. **Rezessy-Szabó JM**, Nguyen DQ, Bujna E, Hoschke Á (1999) Production of α-galactosidase by *Thermomyces lanuginosus*. First Hungarian Conference of Micology, Budapest
   Abstract in Acta Microbiologica et Immunologica Hungarica **46:**345
3. **Rezessy-Szabó JM**, Nguyen DQ, Hoschke Á (2000) Formation of α-galactosidase enzyme by *Thermomyces lanuginosus*. 14th Forum for Applied Biotechnology Gent, Belgium
4. **Rezessy-Szabó JM**, Sinkó I, Bujna E, Nguyen DQ (2000) Enhancement of productivity of alpha-galactosidase by optimisation of medium composition. First Joint Meeting of the Slovenian Society for Microbiology and the Hungarian Society for Microbiology, Keszthely
5. **Rezessy-Szabó JM**, Nguyen DQ**,** Bujna E, Takács K, Kovács M, Hoschke Á (2002) *Thermomyces lanuginosus* CBS 395.62/B strain as rich source of α-galactosidase enzyme. Power of Microbes, Opatjia, Croatia
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