Thermal stable α-galactosidase from *Penicillium restrictum* for biodegradation of galactooligosaccharides

Nataliia Borzova

D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kyiv

Keywords:

A-galactosidase
Penicillium restrictum
Stability
Specificity
Galacto-oligosaccharides

Article history:

Received 4.01.2024
Received in revised form 23.04.2024
Accepted 2.07.2024

Corresponding author:

Nataliia Borzova
E-mail: nvb.imv@gmail.com

DOI: 10.24263/2304-974X-2024-13-2-9

Abstract

Introduction. Studying the functional properties of practically important enzymes, in particular α-galactosidases, is a necessary step towards obtaining stable preparations with high selectivity of action to be used in the food industry for biodegradation and modification of raw materials.

Materials and methods. Gel filtration and ion exchange chromatography on TSK-gels were used to purify the microbial α-galactosidase. To determine the activity and specificity of the enzymatic action, *p*-nitrophenyl-α-D-galactopyranoside, raffinose, stachyose, and galactomannan were used as substrates.

Results and discussion. α-Galactosidase with a specific activity of 49.1 IU/mg was isolated from the culture liquid of *Penicillium restrictum* and purified to a homogeneous state. The molecular weight of the enzyme was 17 kDa according to the data of gel filtration on Sepharose 6B. The optimum pH for the enzyme action was 4.0, and the thermal optimum was 60 °C. Enzyme showed high stability in the temperature ranged from 40 to 60 °C. The half-life of α-galactosidase from *Penicillium restrictum* at 70°C increased in 2-fold in the presence of 20–40% glycerol and 2.3-fold in the presence of 4 M sorbitol. K_m for the hydrolysis of *p*-nitrophenyl-α-galactopiranoside, raffinose, stachyose, and galactomannan were 0.9 mM, 2.2 mM, 2.4 mM, and 3.8 mM, respectively. It was shown that the rate and efficiency of hydrolysis of galactose-containing natural substrates by *Penicillium restrictum* α-galactosidase decreased with increasing their molecular weight. The enzyme was completely inhibited in the presence of 10^{-3} M Ag^{+}, Hg^{2+}, Ca^{2+}, and Cd^{2+}. α-Galactosidase from *Penicillium restrictum* was resistant to action of proteinase K, pronase E, and trypsin.

Conclusions. The high thermal stability and efficiency of α-galactosidase from *Penicillium restrictum* for the modification of galactooligosaccharides suggests its broad-range applicability for food and animal feed processing.

Introduction

Currently, industry for the production of food and feed increasingly involves biotechnological processes. Among them, enzymatic treatment is a convenient and effective way for obtaining food products of improved quality (Meszaros et al., 2021). Compared to inorganic catalysts, enzymes have such valuable properties as high specificity of action, selectivity for certain substrates, exceptional efficiency under conditions of moderate temperatures, normal pressure and neutral pH values, as well as environmental friendliness and safety (Bilal and Iqbal, 2020).

Commercial enzymes are produced by microorganisms, mainly by bacteria or fungi. The diversity of microorganisms and their adaptation systems, which ensure the existence of microbes in extreme conditions, allow us to find enzyme producers with the most diverse properties among them (Anisha et al., 2009; Huang et al., 2018; Lee et al., 2012). In addition, modern methods of genetic and molecular engineering make it possible to successfully modify them to improve technological properties (Boukid et al., 2023; Cao et al., 2009).

Today the market of microbial enzymes is extremely wide and a significant share of it is occupied by glycosidases, which are characterized by the ability to cleave the glycosidic bond in oligo-, polysaccharides and glycoconjugates (Singh et al., 2016; Yi et al., 2021). Most of such enzymes are used in technologies for processing plant raw materials of various compositions (Choi et al., 2015).

Microbial α-galactosidases (EC 3.2.1.22) are enzymes that play an important role in the cleavage of alpha-galactosidic bonds in carbohydrates, such as raffinose, melibiose, stachyose, galactomannans and galacto-glucomannans, which are present in food products and are important components of the diet of the general population (Ivanov et al., 2021). Enzyme α-galactosidase can be used in food industry to hydrolyze galactooligosaccharides in soy-based food production (soybean milk, soy protein, and others) and to treat sugar beet molasses promoting sugar crystallization process (Alvarez-Cao et al., 2019; Bhatia et al., 2020). Cleavage of alpha-galactosidic bonds in the difficult-to-digest galactooligosaccharides of the raffinose family helps maintain gut health, ensure efficient digestion, and reduce discomfort associated with soy milk and flour consumption. This enzyme is often used as a food additive to improve digestion in combination with amylolytic and lipolytic enzymes ("Beano Ultra", "Doctor's Best Digestive Enzymes", "Vitacost Gas Enzyme Alpha-galactosidase").

Wide ranges of microorganisms are involved in the industrial production of α-galactosidase: *Aspergillus niger, Bacillus subtilis, Trichoderma reesei, Saccharomyces cerevisiae, Mortierella vinacea,* and others (Katrolia et al., 2014). However, the search for new producers of α-galactosidases, which have special functional properties and substrate specificity, remains an urgent task. Enzymes that exhibit activity in a wide range of pH and temperature values attract the special attention of researchers, as these indicators are an important characteristic of biocatalyst stability.

Earlier, in the culture fluid of the soil micromycete *Penicillium restrictum*, high levels of α-galactosidase activity was detected. The purpose of the present study was to isolate and characterize α-galactosidase from *P. restrictum*, to evaluate its substrate specificity and efficiency to hydrolyse various galactooligosaccharides.

Materials and methods

Strain, medium, and growth conditions

The object of the study was α-galactosidase from *Penicillium restrictum* Gilman & Abbott, a strain registered in the Depository of the D.K. Zabolotny Institute of Microbiology and Virology of National Academy of the Sciences of Ukraine under the number of IMV F-100139. Cultivation of *P. restrictum* to obtain the enzyme was carried out in a liquid medium of the following composition, g/l: KH_2PO_4 – 1.6; $MgSO_4 \times 7H_2O$ – 0.75; $CaCl_2$ – 0.3; $(NH_4)_2SO_4$ – 0.8; yeast autolysate – 0.15, soy flour – 10, pH 6.0 in Erlenmeyer flasks at a temperature of 25 °C under shaking of 220 rpm for 5 days.

Purification of α-galactosidase from *P. restrictum*

Isolation of the enzyme was carried out by fractionation with ammonium sulfate: dry salt was added to the supernatant of the culture liquid up to 90 percent saturation under pH control. The mixture was kept for 24 h at 4 °C and centrifuged at 2.5×g for 30 min. The precipitate was collected, dissolved in three times the volume of 3 M ammonium sulfate, and 0.01 M sodium azide was added for preservation. The methods of ion exchange and gel permeation chromatography were used to purify the enzyme. The sediment obtained as a result of fractionation with ammonium sulfate was centrifuged, dissolved in 1.5 volumes of the appropriate buffer, pH 6.0 – 7.5. The samples were subjected to gel filtration on a column (1.8×50 cm) with neutral TSK gel — Toyopearl HW-55 (Toson, Japan), equilibrated with 0.01 M phosphate buffer, pH 6.0. Elution was carried out at a rate of 20 ml/h. Ion exchange chromatography was performed on a column (2.5×40 cm) with Toyopearl DEAE-650 (M) (Toson, Japan), equilibrated with 0.01 M Tris-HCl buffer pH 7.5. The sample (about 100 mg of protein) was applied to the column, elution was carried out with the same buffer in a linear NaCl gradient (0-1 M, 200 ml each) at a rate of 30 ml/h. Fractions showing enzymatic activity were collected, combined and concentrated under vacuum approximately 5 times.

Rechromatography was performed on a column (1.3×52 cm) with Sepharose 6B, equilibrated with 0.01 M phosphate buffer, pH 6.0. Elution was carried out with the same buffer with 0.1 M NaCl at a rate of 60 ml/h. 1 ml of sample (20 mg of protein) was applied to the column. Protein content was recorded on a DeNovix DS-11 spectrophotometer/fluorimeter, fractions containing enzymatic activity were collected, combined and concentrated (10 times) by evaporation under vacuum.

Molecular weight determination

Determining of the enzymes molecular weight (MW) in the native system was performed by the gel-filtration on the column (1.3×50 cm) with Sepharose 6B. Elution was done by the 0.01 M phosphate buffer (pH 5.0) with 0.1 M NaCl. Standard curve for MW calculations was plotted using high-molecular protein-markers (Pharmacia, Sweden): ribonuclease (13.7 kDa), proteinase K (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa). The calibration curve was plotted using the gel-phase distribution coefficient (K_{av}) versus logarithm of the molecular weight (Log Mw).

$$K_{av} = (Ve - Vo)/(Vc - Vo),$$

where Ve is elution volume, Vo is column void volume, Vc is geometric column volume.

Enzyme activity assays

α-Galactosidase activity was determined using as a substrate *p*-nitrophenyl-α-D-galactopyranoside (*p*-NPGal) ("Sigma-Aldrich", USA). To determine activity 0.1 ml of the enzyme solution was mixed with 0.2 ml 0.1 M phosphate-citrate buffer (PCB) pH 5.0 and 0.1 ml 0.01 M substrate solution in PCB. Reaction mixture was incubated for 10 min at 40 ºC. The reaction was stopped by adding 2 ml of 1 M sodium bicarbonate. The amount of released nitrophenol as a result of hydrolysis was determined colorimetrically by the absorption at 400 nm. One international unit of enzyme activity (IU) was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min at 40 °C in 0.1 M PCB, pH 5.0.

Determination of the rate of splitting of raffinose, stachyose and guar galactomannan was carried out in the supernatant of *P. restrictum* by estimating the amount of galactose formed because of the hydrolysis of the corresponding substrate. The amount of galactose was determined by the dinitrosalicylic method (Miller, 1959). The reaction mixture containing 0.25 ml of culture liquid and 0.25 ml of substrate (1% galactomannan or 50 mM solutions of raffinose and stachyose in 0.1 M PCB pH 5.2 was incubated for 30 min at 40 ºC, then 0.25 ml of dinitrosalicylic reagent was added and boiled for 10 min. The color intensity was estimated spectrophotometrically at 500 nm. Galactose was used as a standard.

The content of total protein in the supernatant of the culture liquid was determined by the Lowry method (Lowry et al., 1951). The calibration curve was constructed using bovine serum albumin as a standard.

Characteristics of enzyme

The optimum pH for the activities of α-galactosidase was determined by incubating the enzyme preparation with *p*-NPGal in 0.1 M citrate, PCB and 0.01 M Tris-HCl buffers at the pH range from 2 to 9. Activities were measured at 24 h using the standard protocol. The optimum temperature of α-galactosidase was determined by incubating the assay mixture for 10 min at temperature ranging from 5 to 80 ºC. Thermal stability was measured by preincubation of the enzymes at the optimum pH at different temperatures (30, 40, 50, 60, 65, 70 and 75 °C) with the exposition time of 180 min.

Thermal inactivation of α-galactosidase was studied at 55–75 °C, pH 4.0–6.0 (0.1 M PCB). The enzyme samples (5 IU/ml) were kept at given temperature for 15-180 min; the aliquots in 0.1 ml were collected in definite intervals (15 min) for measurement of residual activity. Enzyme treatment with glycerol and sorbitol was carried out as follows: to 5-40 % glycerol or 0.1-5.0 M sorbitol in 0.1 M PCB was added enzyme solution (5 IU/ml) and the mixture was incubated at room temperature for 60 min. Thermal inactivation was carried out as described above.

Kinetic experiments were carried out at 37 °C at the pH 5.0. The maximum reaction rate (V_{max}) and Michaelis constant (K_m) were determined according to Lineweaver-Burk from curves defining dependence of the enzyme reaction rate on the substrate (from 0.1 to 10 mg/ml). Inhibition studies were performed using D-galactose at concentrations ranging from 1 to 10 mM.

The efficiency of hydrolysis was evaluated by the ratio of V_{max} to $K_{m.}$

The half-life period (in min) was calculated from the first-order inactivation rate constants, which were obtained from linear regression in logarithmic coordinates by equation:

$$t_{1/2} = \ln 2/k,$$

where k is the rate constant and ln2 is the natural log of 2 = 0.693.

To determine the resistance of galactosidase to the action of proteolytic enzymes, commercial and laboratory protease preparations were used: proteinase K, pronase E *(Streptomyces griseus),* trypsin ("Merk", Germany), and a laboratory sample of protease from *Bacillus sp.* The reaction mixture contained equal amounts of galactosidase and protease (≈1 mg). Enzyme solutions in 0.1 M PCB pH 5.0 were mixed and kept under stirring for 1–24 hours. Glycosidase activity was measured as described above. Thermal inactivation of enzyme in the presence of proteases was performed according to the thermal inactivation method.

Effect of various reagents on the enzyme activities

The effects of various chemical reagents and cations (L-cysteine, β-Mercaptoethanol, Glutation, Dithiothreitol (DTT), *p*-Chlormercuribenzoate (*p*-CMB), Diethyl pyrocarbonate (DEPC), N-ethylmaleimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC methiodide), Succinic anhydride, Hydrogen peroxide, Guanidine chloride, Potassium ferricyanide, Sodium azide, *o*-Phenanthroline, NH^{4+}, K^{+}, Na^{+}, Li^{+}, Ag^{+}, Hg^{2+}, Cd^{2+}, Ca^{2+}, Co^{2}, Mg^{2+}, Ni^{2+}) on enzyme activity were investigated by their incorporation at a concentration of 0.01 M in standard assay. Reactions were carried out for 60 min at 40 °C and pH 5.0 (0.1 M PCB) in the presence or absence of the compounds examined.

All experiments were performed in 3–5 repetitions. The analysis of the obtained results was carried out by their statistical processing using the Student's t-test. The results presented graphically were obtained using the Microsoft Excel 2007 program. Values at P 0.05 were considered reliable.

Results and discussions

Purification and characterization of α-galactosidase from *P. restrictum*

α-Galactosidase with a specific activity of 49.1 IU/mg of protein was obtained from the culture fluid of *P. restrictum* as a result of precipitation with ammonium sulfate and 3-step purification on TSK gels and Sepharose 6B (Table 1).

Table 1

Steps for purification of α-galactosidase from *P. restrictum*

Steps	Total protein, mg	Total activity, IU	Specific activity, IU/mg	Yield, %	Fold purification
Culture liquid supernatant (0.5 l)	450	3000	6.7	100	1.0
90 % ammonium sulfate	390	2700	6.9	86	1.1
TSK-gel Toyopearl HW-55	120	2123	17.7	26	2.6
Toyopearl DEAE-650M	52	1444	27.8	12	4.1
Sepharose 6B	20	1045	49.1	4.4	7.3

According to gel filtration, the MW of α-galactosidase was 17 kDa (Figure 1).

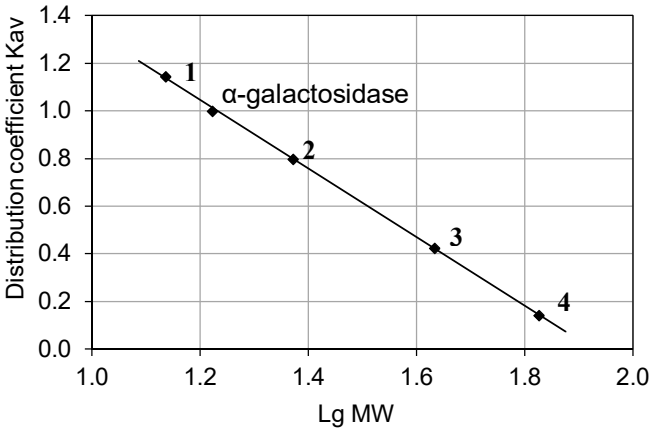

Figure 1. Molecular weight (MV) determination of *P. restrictum* α-galactosidase in the native system.

Molecular markers:
ribonuclease (13.7 kDa) (1), proteinase K (25 kDa) (2), chicken ovalbumin (43 kDa) (3), bovine serum albumin (67 kDa)(4), α-galactosidase – purified enzyme.

A similar low molecular weight α-galactosidase (18 kDa) from *Aspergillus awamori* was also described (Vidya et al., 2020). However, molecular weight in the range from 100 to 400 kDa are more typical for fungal α-galactosidases: 210 kDa in *Rhizopus* sp. (Cao et al., 2009), 118 kDa in *A. awamori* (El-Gindy et al., 2008), 106 kDa in *A. foetidus* (Liu et al., 2012), 99 and 430 kDa in *A. niger* (Borzova and Varbanets, 2007; Othman et al., 2023).

The enzyme had optimum activity at 60 °C and pH 4.0. In the pH range of 4.0-6.0, the half-life period of α-galactosidase of *P. restrictum* at 60 °C was 180-210 min, and at 65 °C – 20 min (Figure 2).

The thermal stability of the enzyme is higher than that of the previously described α-galactosidases of the micromycetes *Penicillium canescens* and *A. oryzae* (Borzova and Varbanets, 2010; Wang et al., 2020). In addition, *P. restrictum* α-galactosidase retained 100 % activity at pH 4.0-5.0 in the temperature range of 20-50 °C during 4 hours of incubation and during storage at 4 °C. Other researchers (Anisha et al., 2009; Farzadi et al., 2011; Yakimova et al., 2017) showed that the pH value of the optimum for the action of α-L-rhamnosidase of fungal and bacterial producers varies in a wide range from 2.0 to 11.0. However, unlike bacterial enzymes that operate in a region close to neutral or alkaline, fungal α-galactosidase is more active at slightly acidic values.

The resistance of the enzyme to proteolysis was also investigated. The activity of α-galactosidase of *P. restrictum* was evaluated after treatment with protease of *S. griseus*, proteinase K, protease of *Bacillus* sp. and trypsin. It was shown that *P. restrictum* α-galactosidase retained more than 90 % of its activity after 24 hours of incubation with proteases. High resistance to the action of proteolytic enzymes is also noted for other α-galactosidases, in particular, α-galactosidase of *A. oryzae* (Wang et al., 2020), tetrameric α-galactosidase from *Gibberella* sp. and α-galactosidase of *Rhizopus* sp. from the GH36 family (Cao et al., 2009). The enzyme from *Pleurotus djamor* also showed resistance to acid protease and varying degrees of tolerance to other proteases:

trypsin > collagenase type I > α-chymotrypsin-neutral protease > proteinase K

(Hu et al., 2017).

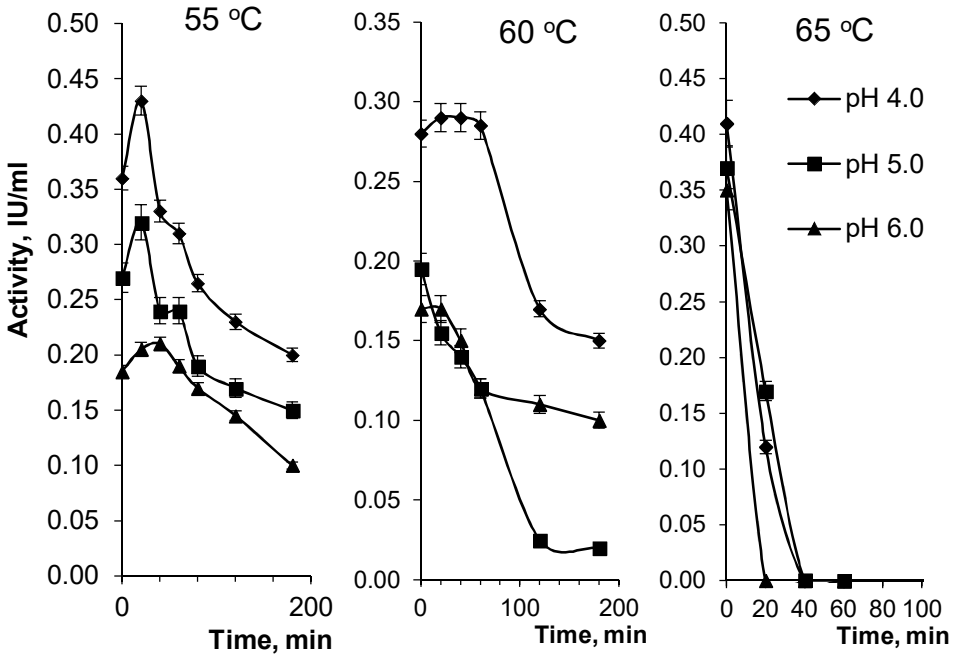

Figure 2. Thermal stability of α-galactosidase *P. restrictum* at different temperature and pH.
The mean values and standard errors are shown (n=5)

Therefore, α-galactosidase of *P. restrictum* may be an interesting object of research in terms of its activity and stability, especially in the context of use in industrial processes.

Kinetic parameters of α-galactosidase from *P. restrictum*

Currently, there is data in the literature that synthetic *p*-nitrophenyl substrates inhibit many of the studied α-galactosidases at high concentrations (Bhatia et al., 2020; Katrolia et al., 2014). The curve of dependence of *P.restrictum* α-galactosidase activity on time had the usual form, where there is a gradual increase in the activity of the enzyme, but after a certain period of time the reaction rate becomes constant. The rate of p-NPG hydrolysis by *P.restrictum* α-galactosidase plateaued at substrate concentrations of 4–5 mg/ml or 1.5 mM at different pH (Figure 3). Inhibition of the reaction was observed at substrate concentrations > 10 mg/ml.

α-Galactosidase of *P. restrictum* cleaved α-1,6-linked galactose from oligo- and polygalactosides at a high rate. It was shown that the rate and efficiency of hydrolysis of galactose-containing natural substrates by *P. restrictum* α-galactosidase decreased with increasing their molecular weight (Table 2). In addition, the speed of splitting galactose from the main chain of oligosaccharides (raffinose and stachyose) was significantly higher than when splitting the galactosidic bond of mannan side chains.

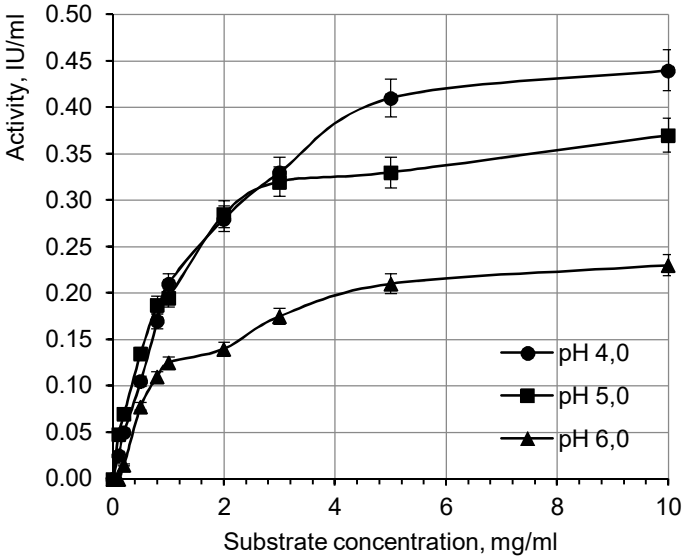

Figure 3. Dependence of α-galactosidase reaction rate on substrate concentration at different pH (0.1 M PCB, 37 ºC).
The mean values and standard errors are shown (n=3)

Table 2

Kinetic parameters of α-galactosidase *P. restrictum*

Substrate	Type of bond	K_m, mM	V_{max}, μmol/min/mg	V_{max}/ K_m
p-NPGal	α-1	0.9±0,05	90.1±2.5	100.1±4.1
Raffinose	α-1,6	2.2±0.08	134.6±3.8	61.3±1.7
Stachyose	α-1,6	2.4±0.08	116.5±4.5	48.1±2.2
Galactomannan	α-1,6	3.8±0.11	27.5±1.4	7.1±0.2

The high rate of hydrolysis of oligosaccharides indicates the suitability of this enzyme for use as a dietary supplement to improve digestion when using various products, especially legumes, and to improve the quality of products containing soy milk or flour (Boukid et al., 2023; Hu et al., 2017). The capacity of the enzyme to degrade the polysaccharide galactomannan of guar is also a valuable property for its use in the food industry. Enzymatic hydrolysis of guar galactomannan improves its ability to gel, which makes it possible to reduce the cost of production processes of ice cream and frozen desserts, sauces, cream cheeses, in comparison with the use of carob gum (Katrolia et al., 2012). In addition, the glycosidic modification of galactomannans, which are present in a large number of tree biomass, can provide bioprocessing plants with a sufficient number of raw materials for the production of ethanol (Choi et al., 2015).

Effects of metal ions and reagents

Nowadays, wide ranges of specific reagents, which modify the side chains of amino acids in protein molecules with varying efficiency, are known. Most of them are used to identify functionally active groups of proteins and study their structure, but often this approach helps to obtain more stable and active forms of enzymes.

It was shown that the activity of α-galactosidase of *P. restrictum* is completely inhibited in the presence of cations Ag^{+}, Cd^{2+}, Ca^{2+}, Hg^{2+} (Figure 4).

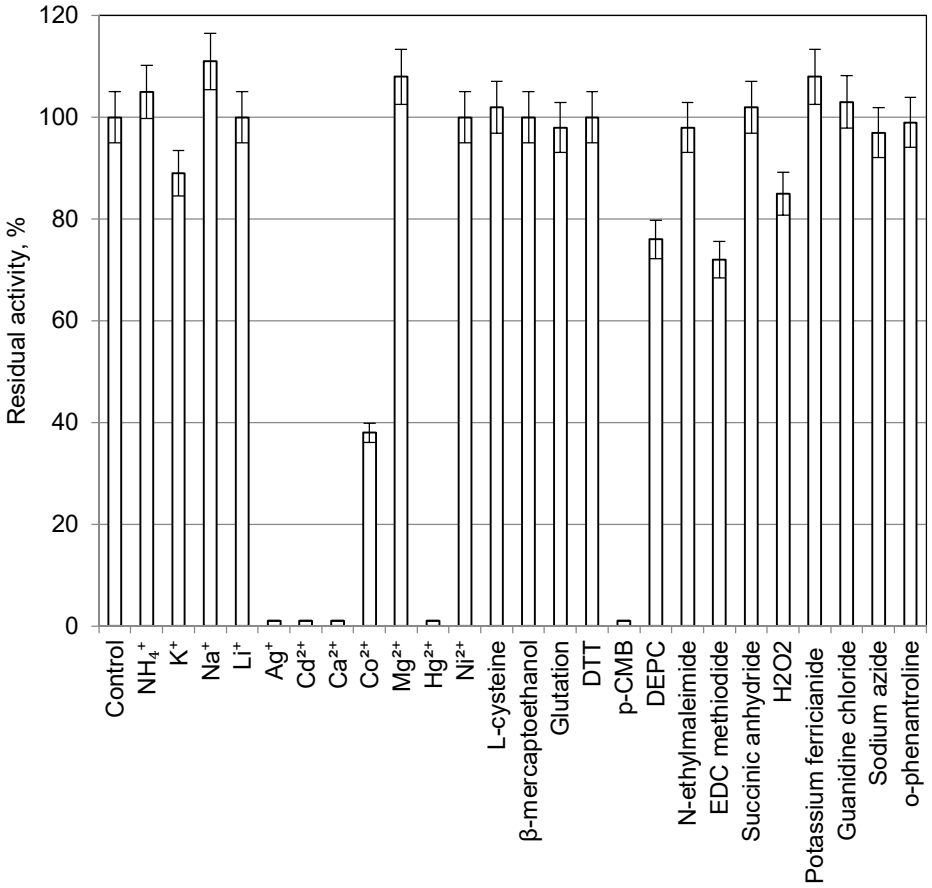

Figure 4. The influence of various chemical reagents on the activity of α-galactosidase. Concentration of additives 0.01M, pH 5.0, 37 ºC, time exposition 60 min.
The mean values and standard errors are shown (n=5)

In addition, the reversible nature of activity inhibition in the presence of Hg^{2+} and Ag^{+} was established, which is characteristic of most of the described glycosidases. Inactivation by Hg^{2+} ions was noted in enzymes from *P. djamor* (Hu et al., 2017), *A. niger* (Othman et al., 2023), *Pseudobalsamia microspora* (Yang et al., 2015). Inhibition of enzyme activity in the presence of carboxyl modifying reagent EDC metiodide and DEPC was noted, which indicates the important role of carboxyl groups of dicarboxylic acids and the imidazole group

of histidine for the activity of α-galactosidase of *P. restrictum*. Today it is known that α-galactosidases from the GH4, GH27, GH36, and GH57 families can use glutamic or aspartic acids as a catalytic nucleophile (Chen et al., 2020; Okuyama et al., 2009) and NH^{+}(His) acts as an electrophile in sweet almond α-galactosidase (Dey and Pridham, 1972).

Sulfhydryl groups of cysteine residues often play an important role in the manifestation of the functional activity of many proteins. Under conditions close to physiological, they are effective nucleophilic agents with high reactivity. Their modification can be carried out with the help of iodoacetamide, aminoethylation with ethyleneimine, reaction with *p*-CMB, oxidation of H_2O_2 to derivatives of cysteine acid, oxidation under mild conditions with the formation of inter- and intramolecular disulfide bonds under the action of ferricyanide, condensation with N-ethylmaleimide according to pH 7.0.

Modification of the SH-groups of the studied α-galactosidase p-CHMB led to a complete loss of its activity, and hydrogen peroxide inhibited the *P. restrictum* enzyme by 13 %. At the same time, ferricyanide and N-ethylmaleimide had almost no effect on the activity of α-galactosidase of *P. restrictum*. Thus, a wide variability of effects among modifiers of SH groups within the studied enzyme molecule was noted, which may be a consequence of different availability of sulfhydryl groups for these reagents. The enzyme was insensitive to the influence of chelating agents (o-phenanthroline) and reagents of thiosulfide exchange. Stability in the presence of the denaturing agent guanidine chloride may indicate the monomeric structure of the studied glycosidase molecule.

Therefore, the obtained data suggest that functionally active and reactive groups in the studied glycosidase are the carboxyl groups of dicarboxylic amino acids, the imidazole group of histidine, and the sulfhydryl groups of cysteine. By modifying these groups, it is possible to influence the conformational stability of the *P. restrictum* enzyme and develop a strategy for obtaining a thermostable enzyme preparation based on it.

Thermal stabilization of α-galactosidase from *P. restrictum*

Increasing the stability of industrially important proteins, which allows effective use of biocatalysts for a long time with minimal loss of their activity, is definitely a priority task of modern applied enzymology. The lack of data on all essential aspects of the mechanism of protein denaturation does not allow us to offer universal ways of their stabilization today and requires an individual approach to solving these problems in accordance with the structural and functional features of the protein (Lopina, 2017).

It is known that enzymes can be inactivated by the aggregation mechanism. In this case, one of the ways to stabilize the protein is to increase the viscosity of the solution, which allows reducing the frequency of collisions between enzyme molecules and slowing down inactivation.

Studies of the effect of high concentrations of glycerol and sorbitol on the thermostabilization of the enzyme showed that glycerol at a concentration of 25–40% had no protective effect on the α-galactosidase of *P. restrictum*. The use of sorbitol solutions in concentrations of 1.6 and 2.5 M at a temperature of 65 ºC (pH 4.0–5.0) made it possible to increase the half-life of *P. restrictum* α-galactosidase to 60–180 min (Figure 5). In the presence of 5 M sorbitol, 50–70% of the enzyme activity was preserved for 120–180 min at 65 and 70 ºC (Figure 6).

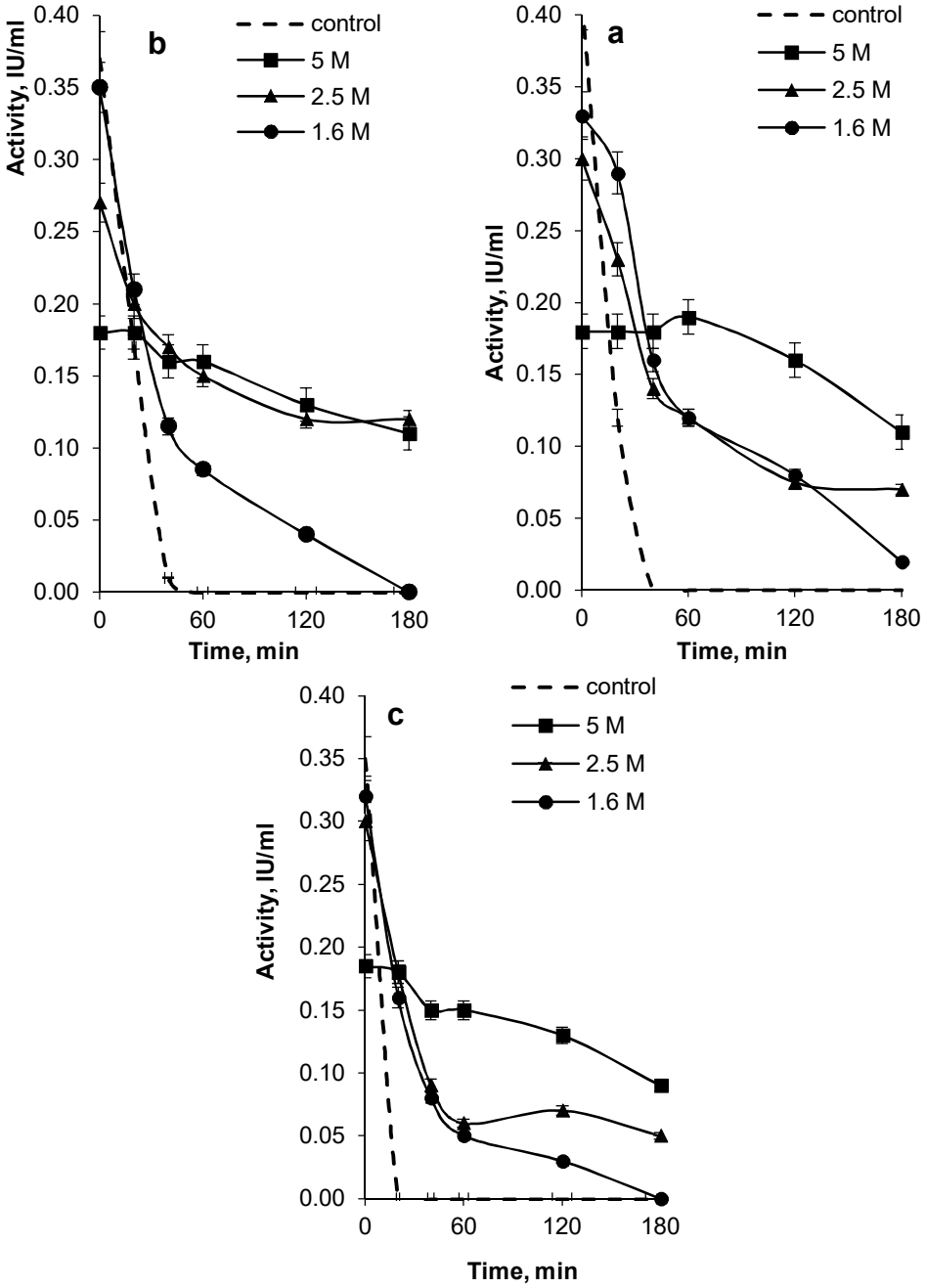

Figure 5. Thermoinactivation of *P. restrictum* α-galactosidase at 65 °C in the presence of different concentrations of sorbitol:
a, pH 4.0; b, pH 5.0; c, pH 6.0.

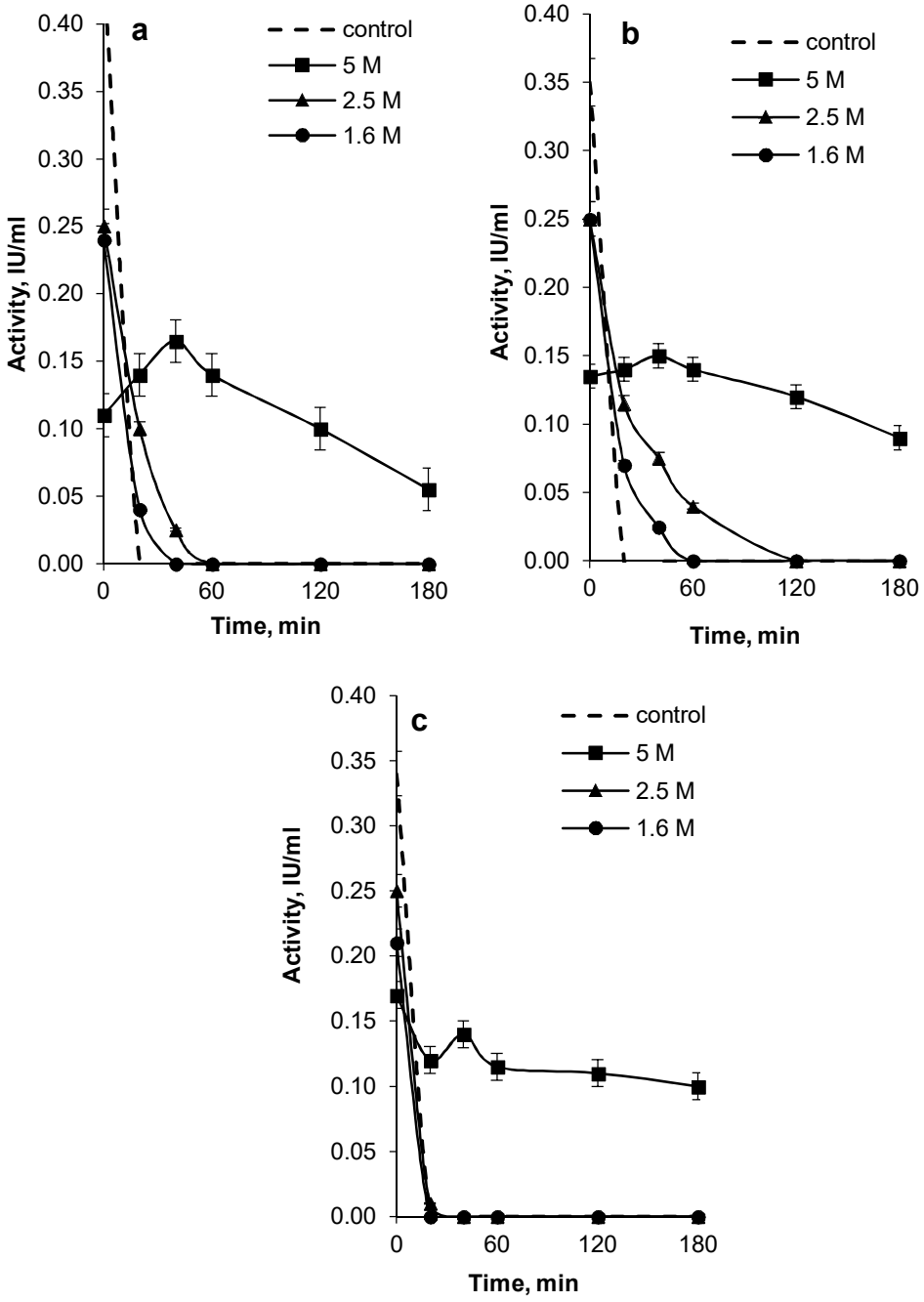

Figure 6. Thermoinactivation of *P. restrictum* α-galactosidase at 70 °C in the presence of different concentrations of sorbitol:
a, pH 4.0; b, pH 5.0; c, pH 6.0.

Conclusions

1. The results of the study of the functional and thermal stability of the practically important microbial α-galactosidase, which was isolated from the culture fluid of the micromycete *P. restrictum*, are presented. The enzyme showed high activity and stability in the pH range from 4.0 to 6.0 and at temperatures from 40 to 60 °C. It was established that the use of high concentrations of sorbitol allows for a significant increase in the half-life of the enzyme under conditions of thermal denaturation at 70-75 °C.
2. The functional properties and specificity of action on α-1,6-linked galactosyl residues in the main and side chains of galactoglycosides indicate the potential of *P. restrictum* α-galactosidase for use in industrial processes where rapid and efficient conversion of carbohydrate substrates is required. The use of α-galactosidase for the biodegradation of galactooligosaccharides of the raffinose family (raffinose and stachyose) allows for to improvement of the quality of soy products, which are the basis of dietary nutrition of various segments of the population. As a result of processing soy milk with α-galactosidase, difficult-to-digest oligosaccharides are transformed into simple sugars — galactose, glucose, sucrose, or fructose, which increases its nutritional value and allows the consumer to be spared from negative effects, including flatulence, diarrhea and other inefficient food digestion. In addition, the use of α-galactosidase in combination with other glycosidases for the modification of fruit pectins makes it possible to obtain functional drinks and purees with a high content of prebiotic oligosaccharides. With the help of α-galactosidase also can to improve the gelling ability of guar galactomannan. This will make it possible to replace more expensive raw materials in the food industry, such as locust bean gum, with more affordable.
3. Thus, the obtained active enzyme preparation is of considerable value not only for understanding and supplementing the theoretical aspects of protein chemistry and engineering but also can compete with imported enzyme compositions and significantly expand the use of enzymatic biotransformation in technological processes of the food industry.

References

Alvarez-Cao M.E., Cerdán M.E., Gonzalez-Siso M.I., Becerra M. (2019), Bioconversion of beet molasses to alpha-galactosidase and ethanol, *Frontiers in Microbiology*, 10, 405, https://doi.org/10.3389/fmicb.2019.00405

Anisha G.S., John R.P., Prema P. (2009), Biochemical and hydrolytic properties of multiple thermostable α-galactosidases from *Streptomyces griseoloalbus*: obvious existence of a novel galactose tolerant enzyme, *Process Biochemistry,* 44(3), pp. 327−333, https://doi.org/10.1016/j.procbio.2008.11.009

Bhatia S., Singh A., Batra N., Singh J. (2020), Microbial production and biotechnological applications of α−galactosidase, *International Journal of Biological Macromolecules,* 150, pp. 1294−1313, https://doi.org/10.1016/j.ijbiomac.2019.10.140

Bilal M., Iqbal H.M.N. (2020), State-of-the-art strategies and applied perspectives of enzyme biocatalysis in food sector − current status and future trends, *Critical Reviews in Food Science and Nutrition*, 60(12), pp. 2052−2066, https://doi.org/10.1080/10408398.2019.1627284

Borzova N.V., Varbanets L.D. (2007), α-Galactosidase of *Aspergillus niger*: purification and properties, *Studia Biologica,* 1(1), pp. 53−64.

Borzova N.V., Varbanets L.D. (2010), Termoinaktivaciya α-galaktozidazy *Penicillium canescens, Ukrainskii Biokhimicheskii Zhurnal*, 82(3), pp. 24−30.

Boukid F., Ganeshan S., Wang Y., Tülbek M.Ç., Nickerson M.T. (2023), Bioengineered enzymes and precision fermentation in the food industry, *International Journal of Molecular Sciences,* 24(12), 10156, https://doi.org/10.3390/ijms241210156

Cao Y., Wang Y., Luo H., Shi P., Meng K., Zhou Z., Zhang Z., Yao B. (2009), Molecular cloning and expression of a novel protease-resistant GH-36 alpha-galactosidase from *Rhizopus* sp. F78 ACCC 30795, *Journal of Microbiology and Biotechnology*, 19(11), pp. 1295−1300, https://doi.org/10.4014/jmb.0904.4003

Chen S.C., Wu S.P., Chang Y.Y., Hwang T.S., Lee T.H., Hsu C.H. (2020), Crystal structure of α-galactosidase from *Thermus thermophilus*: insight into hexamer assembly and substrate specificity, *Journal of Agricultural and Food Chemistry,* 68(22), pp. 6161−6169, https://doi.org/10.1021/acs.jafc.0c00871

Choi J.M., Han S.S., Kim H.S. (2015), Industrial applications of enzyme biocatalysis: Current status and future aspects, *Biotechnology Advances,* 33(7), pp. 1443−1454, https://doi.org/10.1016/j.biotechadv.2015.02.014

Dey P.M., Pridham J.B. (1972), Biochemistry of α-galactosidases. *Advances in Enzymology and Related Areas of Molecular Biology,* 36, pp. 91−130, https://doi.org/10.1002/9780470122815.ch3

El-Gindy A.A., Ali U.F, Ibrahim Z.M., Isaac G.S. (2008), A cost-effective medium for enhanced production of extracellular α-galactosidase in solid substrate cultures of *Aspergillus awamori* and *A. carbonarius*, *Australian Journal of Basic and Applied Sciences,* 2(4), pp. 880−899.

Farzadi M., Khatami S., Mousavi M., Amirmozafari N. (2011), Purification and characterization of α-galactosidase from *Lactobacillus acidophilus*, *African Journal of Biotechnology*, 10(10), pp. 1873−1879, https://doi.org/10:1873–1879. 10.5897/AJB10.357

Hu Y., Tian G., Zhao L., Wang H., Ng T.B. (2017), A protease-resistant α-galactosidase from *Pleurotus djamor* with broad pH stability and good hydrolytic activity toward raffinose family oligosaccharides, *International Journal of Biological Macromolecules,* 94(Pt A), pp. 122−130, https://doi.org/10.1016/j.ijbiomac.2016.10.005

Huang Y., Zhang H., Ben P., Duan Y., Lu M., Li Z., Cui Z. (2018), Characterization of a novel GH36 α-galactosidase from *Bacillus megaterium* and its application in degradation of raffinose family oligosaccharides, *International Journal of Biological Macromolecules,* 108, pp. 98−104, https://doi.org/10.1016/j.ijbiomac.2017.11.154

Ivanov V., Shevchenko O., Marynin A., Stabnikov V., Gubenia O., Stabnikova O., Shevchenko A., Gavva O., Saliuk A. (2021),Trends and expected benefits of the breaking edge food technologies in 2021–2030, *Ukrainian Food Journal*, 10(1), pp. 7–36, https://doi.org/10.24263/2304-974X-2021-10-1-3

Katrolia P., Rajashekhara E., Yan Q., Jiang Z. (2014), Biotechnological potential of microbial α-galactosidases, *Critical Reviews in Biotechnology,* 34(3), pp. 307−317, https://doi.org/10.3109/07388551.2013.794124

Lee J., Park I., Cho J. (2012), Production and partial characterization of a-galactosidase activity from an Antarctic bacterial isolate, *Bacillus* sp. LX-1, *African Journal of Biotechnology,* 11(60), pp. 12396−12405, https://doi.org/10.5897/AJB12.1219

Liu C.Q., He G.Q. (2012), Multiple α-galactosidases from *Aspergillus foetidus* ZU-G1: purification, characterization and application insoybean milk hydrolysis, *European Food Research and Technology* 234(5), pp. 743−751. https://doi.org/10.1007/s00217-012-1679-x

Lopina O.D. (2017), Enzyme inhibitors and activators. In: Senturk M. (Ed.), *Enzyme Inhibitors and Activators*. InTech. Available at: http://dx.doi.org/10.5772/67248

Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. (1951), Protein measurement with folinphenol reagent, *Journal of Biological Chemistry,* 193, pp. 265−275.

Meszaros Z., Nekvasilova P., Bojarová P., Křen V., Slamova K. (2021), Advanced glycosidases as ingenious biosynthetic instruments, *Biotechnology Advances,* 49, 107733, https://doi.org/10.1016/j.biotechadv.2021

Miller G.L. (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugars, *Analytical Chemistry,* 31(3), pp. 426−428, https://doi.org/10.1021/ac60147a030

Okuyama M., Matsunaga K., Watanabe K.I., Yamashita K., Tagami T., Kikuchi A., Ma M., Klahan P., Mori H., Yao M., Kimura A. (2017), Efficient synthesis of α-galactosyl

oligosaccharides using a mutant *Bacteroides thetaiotaomicron* retaining α-galactosidase (BtGH97b), *The FEBS Journal,* 284(5), pp. 766−783, https://doi.org/10.1111/febs.14018

Othman A.M., Elshafei A.M., Elsayed M.A., Ibrahim G.E., Hassan M.M., Mehanna N.S. (2023), Biochemical characterization and insights into the potency of the acidic *Aspergillus niger* NRC114 purified α-galactosidase in removing raffinose family oligosaccharides from soymilk yogurt, *BMC Biotechnology*, 23(1), 3, https://doi.org/10.1186/s12896-023-00773-x

Singh R., Kumar M., Mittal A., Mehta P.K. (2016), Microbial enzymes: industrial progress in 21st century, *3 Biotech*, 6(2), 174, https://doi.org/10.1007/s13205-016-0485-8

Vidya C.H., Gnanesh Kumar B.S., Chinmayee C.V., Singh S.A. (2020), Purification, characterization and specificity of a new GH family 35 galactosidase from *Aspergillus awamori*, *International Journal of Biological Macromolecules,* 156, pp. 885−895, https://doi.org/10.1016/j.ijbiomac.2020.04.013

Wang J., Yang X., Yang Y., Liu Y., Piao X., Cao Y. (2020), Characterization of a protease-resistant α-galactosidase from *Aspergillus oryzae* YZ1 and its application in hydrolysis of raffinose family oligosaccharides from soymilk, *International Journal of Biological Macromolecules,* 158, pp. 708−720, https://doi.org/10.1016/j.ijbiomac.2020.04.256

Yakimova B., Tchorbanov B., Stoineva I. (2017), Alpha-galactosidase and invertase from *Penicillium chrysogenum* sp.23. Purification, characteristics and hydrolysis of raffinose, *Bulgarian Chemical Communications,* 49(2), pp. 101−106

Yang D., Tian G., Du F., Zhao Y., Zhao L., Wang H., Ng T.B. (2015), A fungal alpha-galactosidase from *Pseudobalsamia microspora* capable of degrading raffinose family oligosaccharides, *Applied Biochemistry and Biotechnology*, 176(8), pp. 2157−2169, https://doi.org/10.1007/s12010-015-1705-0

Yi D., Bayer T., Badenhorst C.P.S., Wu S., Doerr M., Höhne M., Bornscheuer U.T. (2021), Recent trends in biocatalysis, *Chemical Society Reviews,* 50(14), pp. 8003−8049, https://doi.org/10.1039/d0cs01575j

Cite:

UFJ Style

Borzova N. (2024), Thermal stable α-galactosidase from *Penicillium restrictum* for biodegradation of galactooligosaccharides, *Ukrainian Food Journal*, 13(2), pp. 316–330, https://doi.org/10.24263/2304-974X-2024-13-2-9

APA Style

Borzova, N. (2024). Thermal stable α-galactosidase from *Penicillium restrictum* for biodegradation of galactooligosaccharides. *Ukrainian Food Journal, 13*(2), 316–330. https://doi.org/10.24263/2304-974X-2024-13-2-9