

**THE INFLUENCE OF COORDINATIVE TARTRATE  
AND MALATOGERMANATE COMPOUNDS ON THE ACTIVITY  
OF  $\alpha$ -L-RHAMNOSIDASE PREPARATIONS FROM *Penicillium tardum*,  
*Eupenicillium erubescens* AND *Cryptococcus albidus***

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**Received:** 26 November 2019; **Accepted:** 15 May 2020

Recently enzyme preparations of microbial origin become increasingly important in different industries. Preparations of  $\alpha$ -L-rhamnosidase are used in the pharmaceutical industry as well as in scientific work as a tool for analytical research. We have obtained purified  $\alpha$ -L-rhamnosidase preparations from *Penicillium tardum*, *Eupenicillium erubescens* and *Cryptococcus albidus* microorganism strains which are effective enzyme producers. The aim of the study was to estimate the ability of germanium coordination compounds to enhance enzyme catalytic activity. The effects of 11 heterometal mixed ligand tartrate (malate-)germanate compounds at 0.01 and 0.1% concentration on the activity of  $\alpha$ -L-rhamnosidase preparations from *Penicillium tardum* IMV F-100074, *Eupenicillium erubescens* and *Cryptococcus albidus* 1001 were studied at 0.5 and 24 h exposition. The inhibitory effect of  $[\text{Ni}(\text{bipy})_3]_4\{\text{Ge}_2(\text{OH})_2(\text{Tart})_3\text{Cl}_2\} \cdot 15\text{H}_2\text{O}$  on *P. tardum*  $\alpha$ -L-rhamnosidase was revealed. All studied compounds except  $[\text{CuCl}(\text{phen})_2][\text{Ge}(\text{OH})(\text{HMal})_2]$  were shown to increase activity of *P. tardum*  $\alpha$ -L-rhamnosidase at a longer term of exposition. Activity of *E. erubescens*  $\alpha$ -L-rhamnosidase was shown to be stimulated by d-metal cation-free compounds. *C. albidus*  $\alpha$ -L-rhamnosidase occurred to be insensitive to all compounds studied.

**Key words:** germanium complexes,  $\alpha$ -L-rhamnosidase, *Penicillium tardum* IMV F-100074, *Eupenicillium erubescens* 248, *Cryptococcus albidus* 1001.

In recent years, enzymes of microbial origin are becoming increasingly important in food, pharmaceutical and chemical industries. Glycosidases, enzymes of the hydrolase family (O-glycoside hydrolases), which catalyze the hydrolysis of O-glycosidic bonds in glycosides, oligo-, polysaccharides, glycolipids and other glycoconjugates are of particular interest to researchers. Among them is  $\alpha$ -L-rhamnosidase ( $\alpha$ -L-rhamnoside-rhamnohydrolase, EC 3.2.1.40), which hydrolytically cleaves the terminal unreduced  $\alpha$ -1,2,  $\alpha$ -1,4 and  $\alpha$ -1,6 bound residues of L-rhamnose in natural products such as naringin, rutin, quercetrin, hesperidin and other rhamnose-containing glycosides [1-3].  $\alpha$ -L-Rhamnosidase has

a wide range of applications: in the food industry, for example, in winemaking to improve the quality and aroma of wines, in the production of citrus juices and drinks to remove bitter components (naringin) that improves the quality and nutritional value of these products; in research as an analytical tool for studying the structure of complex carbohydrate-substituted biopolymers. However, today, preparations of  $\alpha$ -L-rhamnosidase are not available in Ukraine, and the high price of foreign commercial enzyme products (USA) significantly impedes their use in industrial technologies in Ukraine. With this in mind, we searched for effective producers of highly specific  $\alpha$ -L-rhamnosidases among the strains of

microorganisms of various taxonomic groups - bacteria, micromycetes, and yeast from the depositary of IMV, NAS of Ukraine. We selected the most active strains - *Penicillium tardum* [4], *Eupenicillium erubescens* [5] and *Cryptococcus albidus* [6] and obtained  $\alpha$ -L-rhamnosidase preparations from their culture fluids. Then we studied their physiochemical properties, substrate specificity, and functional groups involved in the catalytic process [7-9]. To obtain highly productive microbial enzymes, e.g.  $\alpha$ -L-rhamnosidases, there are several approaches, one of which is the use of substances that enhance their catalytic effect. As  $\alpha$ -L-rhamnosidase modifiers, coordination compounds of germanium, an essential ultratrace element, with various biologically active ligands have received increasing interest recently [10-12]. Due to the peculiarities of electronic configuration, germanium is able to promote tissue oxygenation, enhance blood circulation, strengthen the immune system, promote  $\gamma$ -interferon induction, normalize metabolism, and exhibit antitumor effect [13-16]. A lack of germanium can cause pathological conditions linked to reducing in immunity. Coordination compounds of germanium, which are able to increase the activity of  $\alpha$ -L-rhamnosidases, may be useful in the development of therapeutic compositions based on rhamnose-containing compounds. Earlier [17], we obtained germanium complexes with citric acid and studied their effect on the  $\alpha$ -L-rhamnosidase activity in enzyme preparations from fungi. All complexes were found to exert an activating effect, in contrast to the inorganic salts of  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ , which acted as inhibitors [18, 19].

In this work, we continued our previous research and tested as effectors mix-liganded hetero-metal coordination compounds consisting of complex cations and anions [20-22]. Most of them contain central atoms-acceptors "metals of life": Cu, Ni, Zn, Fe, "essential" Ge and ligands of various types: nitrogen-containing heterocyclic bis-chelating antibacterial agents 1,10-phenanthroline, 2,2'-bipyridine and biologically active agents tartaric and malic acids with hydroxyl and carboxyl functional groups. Their composition suggests participating in many processes: electrostatic binding, donor-acceptor interaction and acting as nucleophilic and electrophilic agents. The aim of this work was to study the activity of these complexes as modifiers of  $\alpha$ -L-rhamnosidases from *Penicillium tardum*, *Eupenicillium erubescens* and *Cryptococcus albidus*.

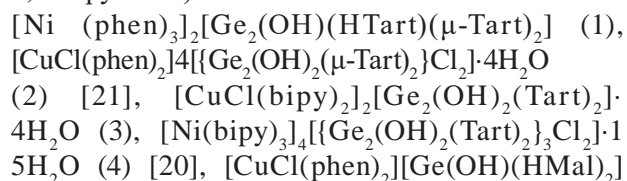
## Materials and Methods

The study was carried out on  $\alpha$ -L-rhamnosidases from *Penicillium tardum* IMV F-100074, *Eupenicillium erubescens* 248 and *Cryptococcus albidus* 1001.

The preparations of  $\alpha$ -L-rhamnosidases were obtained from the culture supernatant of *P. tardum* [4], *E. erubescens* [5] and *C. albidus* [6], by filtration through 4 layers of gauze (for *E. erubescens* and *P. tardum*) or by centrifugation (for *C. albidus*), after separation of biomass and precipitation with ammonium sulfate to 90% saturation. The mixture was kept for 12–16 h at 4 °C and then centrifuged at 5000 g for 30 min. The precipitate was collected, dissolved in 1.5 volumes of 0.01 M phosphate buffer pH 7.0. A sample (about 20–30 mg of protein) was applied to a column (2.5×90 cm) with a neutral TSK-gel Toyopearl HW-60 (Toyo Soda, Japan) equilibrated with 0.01 M phosphate buffer, pH 7.0. Fractions were eluted with the same buffer at a flow rate of 90 ml/h. The protein content was determined using SF-26 at 280 nm. Fractions containing  $\alpha$ -L-rhamnosidase activity were collected, combined and concentrated (~ 5-fold) in vacuo. The obtained samples (4–5 ml, 7–10 mg of protein) were applied to Fractogel DEAE-650-s column (3×35 cm) (Merck, Germany) equilibrated with 0.01 M Tris-HCl buffer pH 7.8. Elution was performed with a linear NaCl gradient (0–1 M, 200 ml) at a flow rate of 24 ml/h.

The  $\alpha$ -L-rhamnosidase activity was determined by the Davis method [23] using naringin as a substrate. The specific  $\alpha$ -L-rhamnosidase activity of the preparations was 120 units/mg of protein for *E. erubescens*, 27 units/mg of protein for *P. tardum* and 12 units/mg of protein for *C. albidus* (protein content - 0.01 mg/ml).

As modifiers of enzyme activity, we used germanium coordination compounds ( $\text{H}_4\text{Tart}$  - tartaric acid, phen - 1,10-phenanthroline, 2,2'-bipyridine). For their synthesis, the original methods were developed [20-22], which involve stepwise interaction of tartrate(malate)-germanate acids and ethanol solutions of complexes of d-metal ions ( $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ) and aromatic amines (1,10-phenanthroline, 2,2'-bipyridine):



(5),  $[\text{Ge}(\text{HMal})_2(\text{phen})] \cdot \text{phen} \cdot 2\text{H}_2\text{O}$  (6) [22],  $[\text{Fe}(\text{bipy})_3]_2\{[\text{Ge}(\text{H}_2\text{O})(\mu\text{-Tart})_2\text{Ge}(\text{H}_2\text{Tart})\{\text{S}_2\text{O}_7\}] \cdot 9\text{H}_2\text{O}$  (7) [18],  $(\text{Hphen})_4[(\mu\text{-Tart})_2(\text{OH})\text{Ge}_2(\mu\text{-O})\text{Ge}_2(\text{OH})(\mu\text{-Tart})_2] \cdot 9\text{H}_2\text{O}$  (8),  $[\text{Zn}(\text{bipy})_3][\text{Ge}_2(\text{OH})_2(\text{Tart})_2] \cdot 5\text{H}_2\text{O}$  (9),  $[\text{Zn}(\text{phen})_3]_2[\text{Ge}_2(\text{OH})(\text{HTart})(\mu\text{-Tart})_2] \cdot 23\text{H}_2\text{O}$  (10),  $[\text{Fe}(\text{phen})_3][\text{Ge}_2(\text{OH})_2(\mu\text{-Tart})_2] \cdot 13\text{H}_2\text{O}$  (11). All compounds were comprehensively characterized by modern physical and chemical methods: elemental analysis, infrared spectroscopy, thermogravimetric analysis, single crystal XRD. Structure data of the synthesized compounds were presented in publications [20-22] and deposited in the Cambridge Crystallographic Database: 1878103 (2), 1883675 (3), 1883676 (4), 1513407 (5), 1513408 (6), 1576554 (7). Representations of the studied compounds are shown in Fig. 1.

When studying the effect of various germanium-containing compounds on the activity of enzymes, we used concentrations of 0.1 and 0.01% and time of exposure 0.5 h and 24 h. The studied compounds were dissolved in 0.1% DMSO.

All experiments were performed in 7 replicates. Student's *t*-test was used to perform statistical analysis. The data are presented as mean  $\pm$  standard error ( $M \pm m$ ) and are considered significant at  $P < 0.05$ . The results presented in graphs were processed using Microsoft Excel 2007.

## Results and Discussion

Previously, based on inhibitory and kinetic analysis with Dixon and Lineweaver-Burk plots and using group-specific reagents, we revealed some active functional groups in  $\alpha$ -L-rhamnosidases from *P. tardum*, *E. erubescens* and *C. albidus* [7-9]. A significant inhibition (more than 50%) of the activity of all the studied enzymes by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide may indicate an important role of carboxyl groups of aspartic or glutamine amino acids in the catalytic activity of the enzymes. However, partial inhibition may indicate the presence on the protein surface of other amino acid residues, which are also an important for the enzymatic reaction. A sulfhydryl group of cysteine is known to play important role in the activity of many proteins. Since p-chloromercuribenzoate, sodium arsenite, and N-ethylmaleimide inhibit the activity only of *E. erubescens*  $\alpha$ -L-rhamnosidase, we suggest the presence of sulfhydryl groups in its active site. Moreover, this inhibition was not abolished by dithiothreitol, mercaptoethanol and cysteine. Involvement of the histidine's imidazole group in the

activity of  $\alpha$ -L-rhamnosidases of all three producers is suggested by a decrease in the enzymes activity by more than 90% when this group is modified by photooxidation in the presence of methylene blue and diethyl pyrocarbonate, which specifically reacts with histidine residues at pH 5.5-7.5. It was found that metal-containing groups do not involve in the catalysis by  $\alpha$ -L-rhamnosidases of *P. tardum* and *C. albidus*, i.e., these enzymes are metal-independent enzymes, while the activity of the  $\alpha$ -L-rhamnosidase of *E. erubescens* depends on the presence of metal ions (inhibition by EDTA, o-phenanthroline). Identification of catalytically important groups in the enzyme active site allows predicting its behavior in reaction media and control catalysis in order to optimize enzymatic processes in biotechnology.

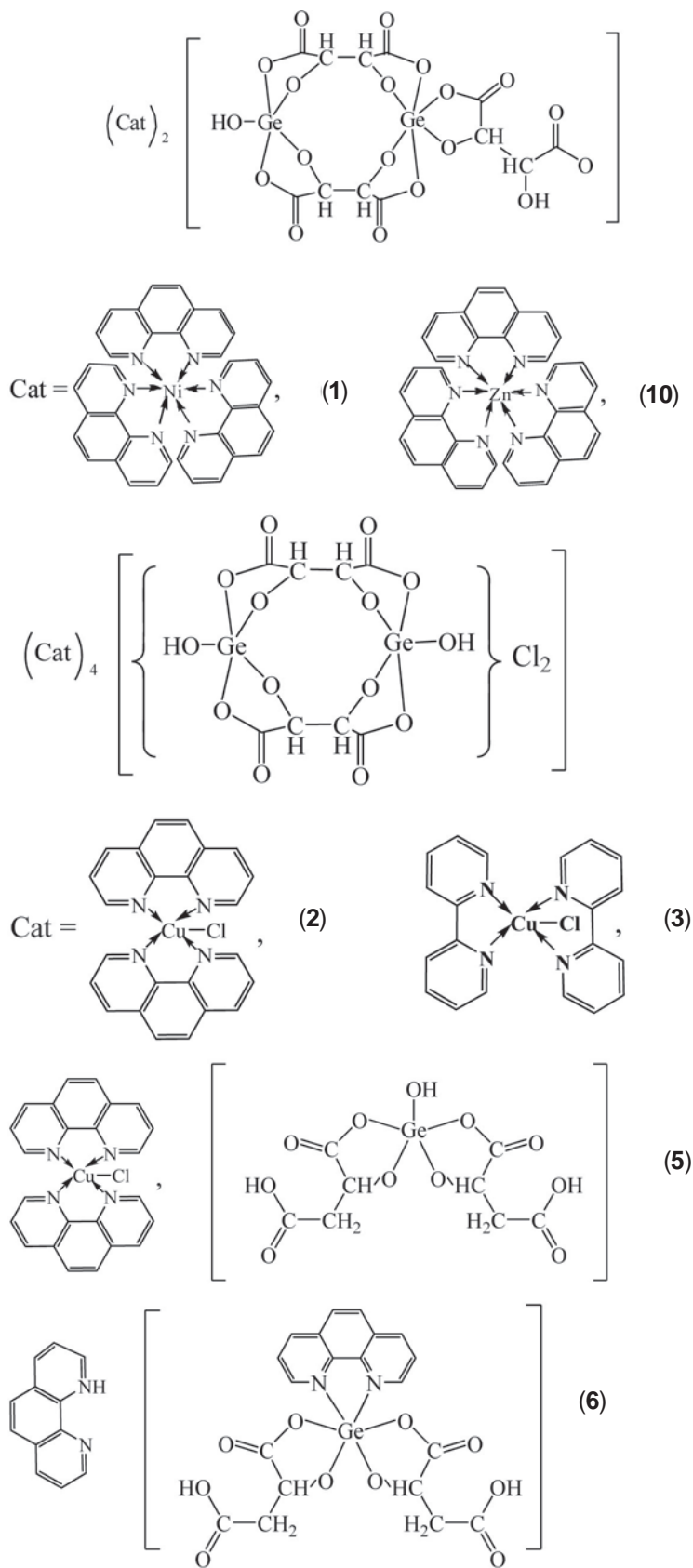
It is known [1-3] that  $\alpha$ -L-rhamnosidases belong to the enzymes, which do not require the presence of metals for their activity, however metal ions can change the conformation of the molecule and make active sites more accessible to the substrate, at that, the orientation of the distant groups can also be changed. It is not possible to predict precisely what effect each metal will have on enzyme activity.

Our research showed that the effect of germanium compounds on the activity of the studied  $\alpha$ -L-rhamnosidases varied with different exposure times and concentrations. The most different effect was observed on the activity of  $\alpha$ -L-rhamnosidase of *P. tardum* (Fig. 2, A, B). Thus, an increase in the activity of  $\alpha$ -L-rhamnosidase of *P. tardum* up to 18% was observed at the action of compound (3) at a concentration 0.01% and exposure time 0.5 h (Fig. 2, A), whereas an increase in exposure time to 24 h at the same concentration of compound (3) led to an increase in activity by 31% and at a concentration 0.1% - by 50%.

Compounds (7) and (9) at both concentrations and exposure time 0.5 h did not affect the activity of the studied  $\alpha$ -L-rhamnosidase (activity was at the control level). All other compounds inhibited the activity of  $\alpha$ -L-rhamnosidase from *P. tardum*. The most significant decrease (by 50-70%) in the activity was registered under the action of compound (4) at both concentrations.

The activity of the effectors decreased with increasing exposure time. Compound (5) was the only exception. Its effect (degree of inhibition) did not depend on exposure time and remained constant.

The maximum increase in the activity of  $\alpha$ -L-rhamnosidase of *E. erubescens* occurred under the



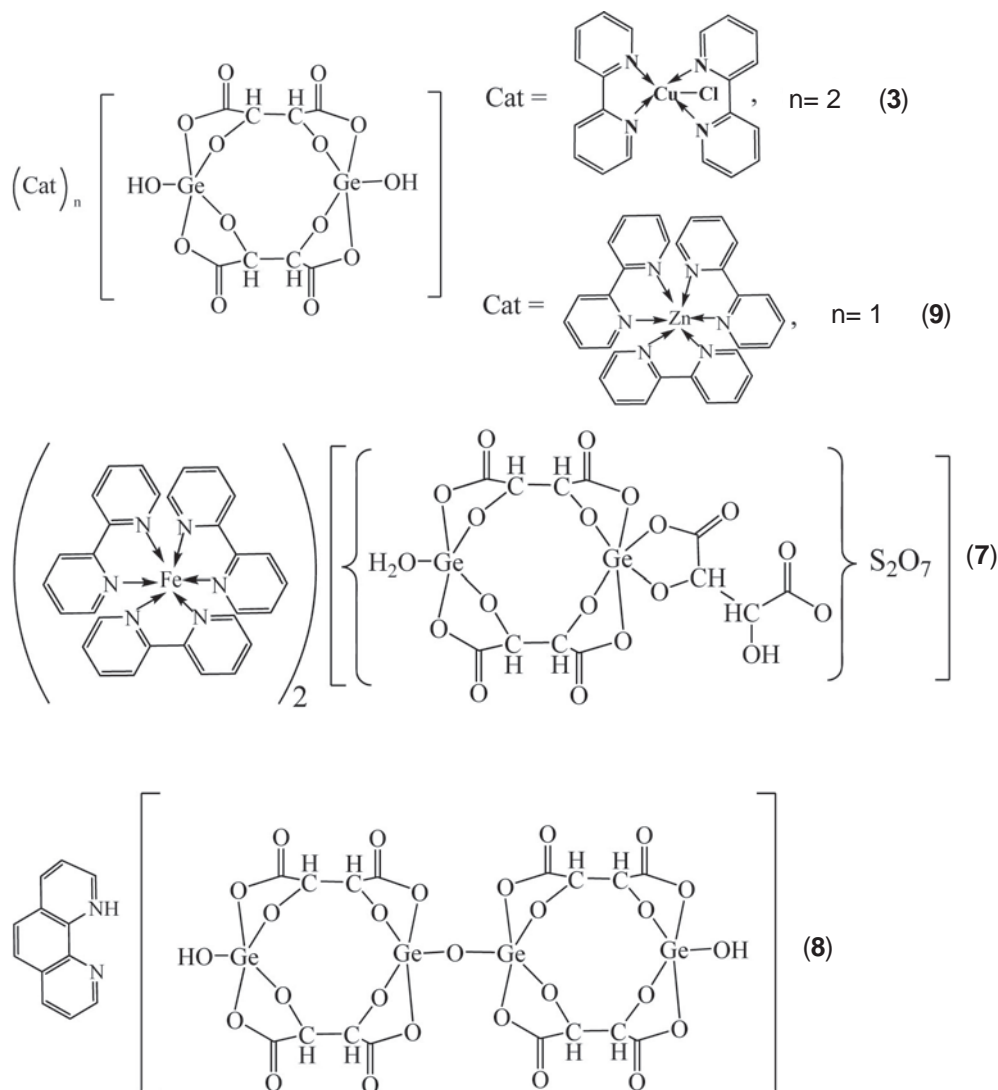


Fig. 1. Representations of compounds (1)-(11). Compounds (1)-(11) are stable toward hydrolysis in an aqueous solution, dilution does not change their composition. These compounds can dissociate into complex cations and anions. For example,  $[\text{CuCl}(\text{bipy})_2]_2[\text{Ge}_2(\text{OH})_2(\text{Tart})_2] \leftrightarrow 2[\text{CuCl}(\text{bipy})_2]^+ + [\text{Ge}_2(\text{OH})_2(\text{Tart})_2]^{2-}$  (3)  $(\text{Hphen})_4[(\mu\text{-Tart})_2(\text{OH})\text{Ge}_2(\mu\text{-O})\text{Ge}_2(\text{OH})(\mu\text{-Tart})_2] \leftrightarrow 4(\text{Hphen})^+ + [(\mu\text{-Tart})_2(\text{OH})\text{Ge}_2(\mu\text{-O})\text{Ge}_2(\text{OH})(\mu\text{-Tart})_2]^{4-}$

action of compound (3) at a concentration 0.01% and compounds (6), (8) at a concentration 0.1%, exposure time 0.5 h (Fig. 3, A). With an increase in exposure time to 24 h (Fig. 2, B), the activating effect of compounds (1), (6) and (8) was observed at a concentration 0.1%. It should be noted that the effect of compounds (9)-(11) on the  $\alpha$ -L-rhamnosidase from *E. erubescens* was minimal. The activity was similar to the control values at various time of exposure. The inhibitory effect of compound (1) and (2), (4), (5), (7) was also observed at concentration 0.01%. A decrease in the inhibitory effect with an increase in

the exposure time was also observed, similar to  $\alpha$ -L-rhamnosidase from *P. tardum*.

Compounds (1)-(11) exerted the least pronounced effect on  $\alpha$ -L-rhamnosidase from *C. albidus* compared to the enzymes from *P. tardum* and *E. erubescens* (Fig. 4, A, B). Activity did not change at different exposure times and was predominantly at the control level. There was only slight increase in the activity (from 5 to 25%) h at various concentrations of compounds (3), (4), (7), (8), (9)-(11) and exposure time 0.5 h. Such increase was eliminated with an increase in exposure time to 24 h. The ex-

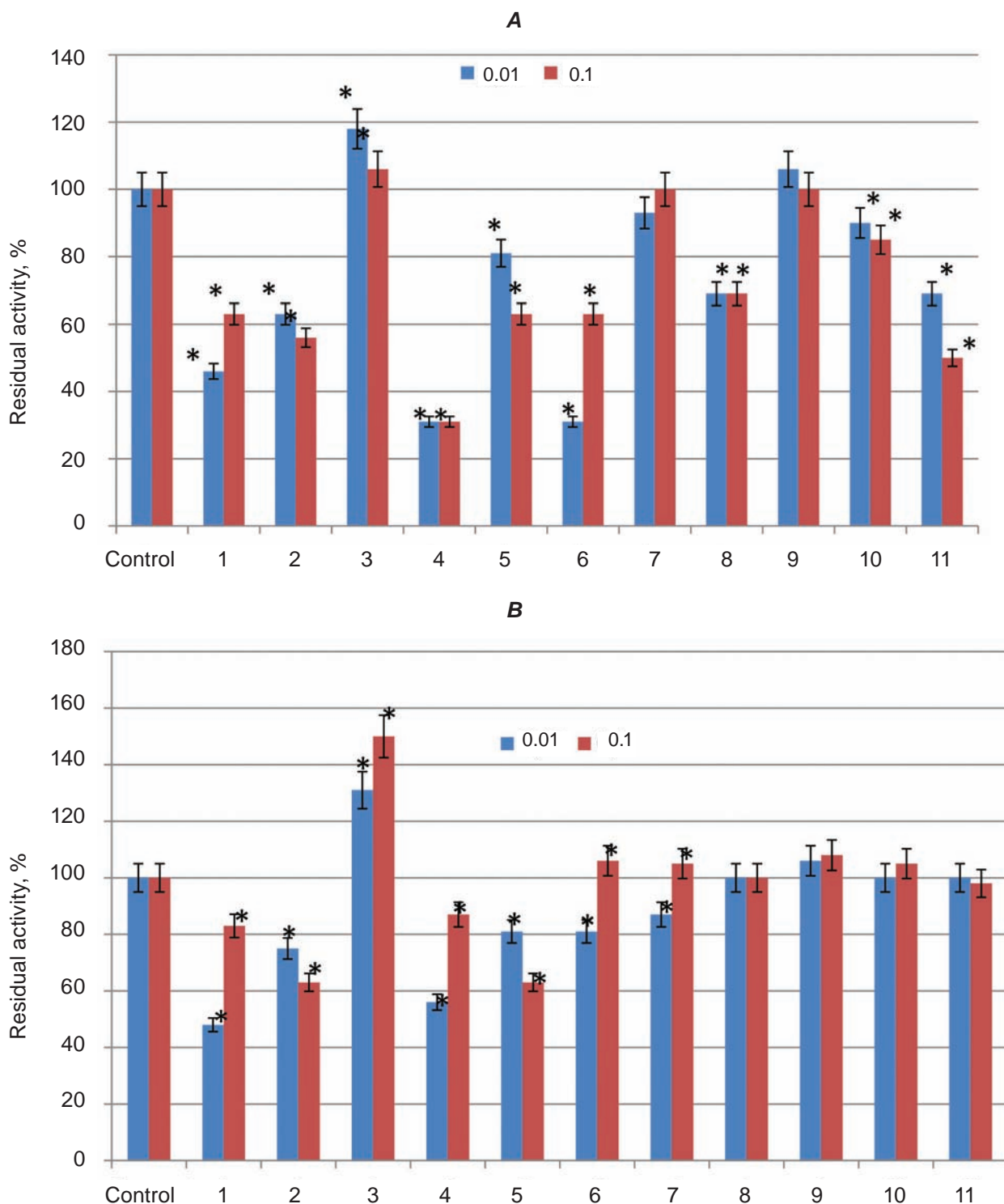


Fig. 2. The effect of germanium-containing compounds on the activity of  $\alpha$ -L-rhamnosidase from *P. tardum*. **A** – exposure time 0.5 h, **B** – exposure time 24 h. \*Significantly different to control

ception was compound (6), which at a concentration 0.01% increased enzyme activity by 10%.

The results obtained showed that the effects of various germanium-containing compounds on the

activity of  $\alpha$ -L-rhamnosidases from various producers have peculiar features. The changes in nature and intensity of the impact of compounds (1)-(11) as effectors with the concentration and exposure time

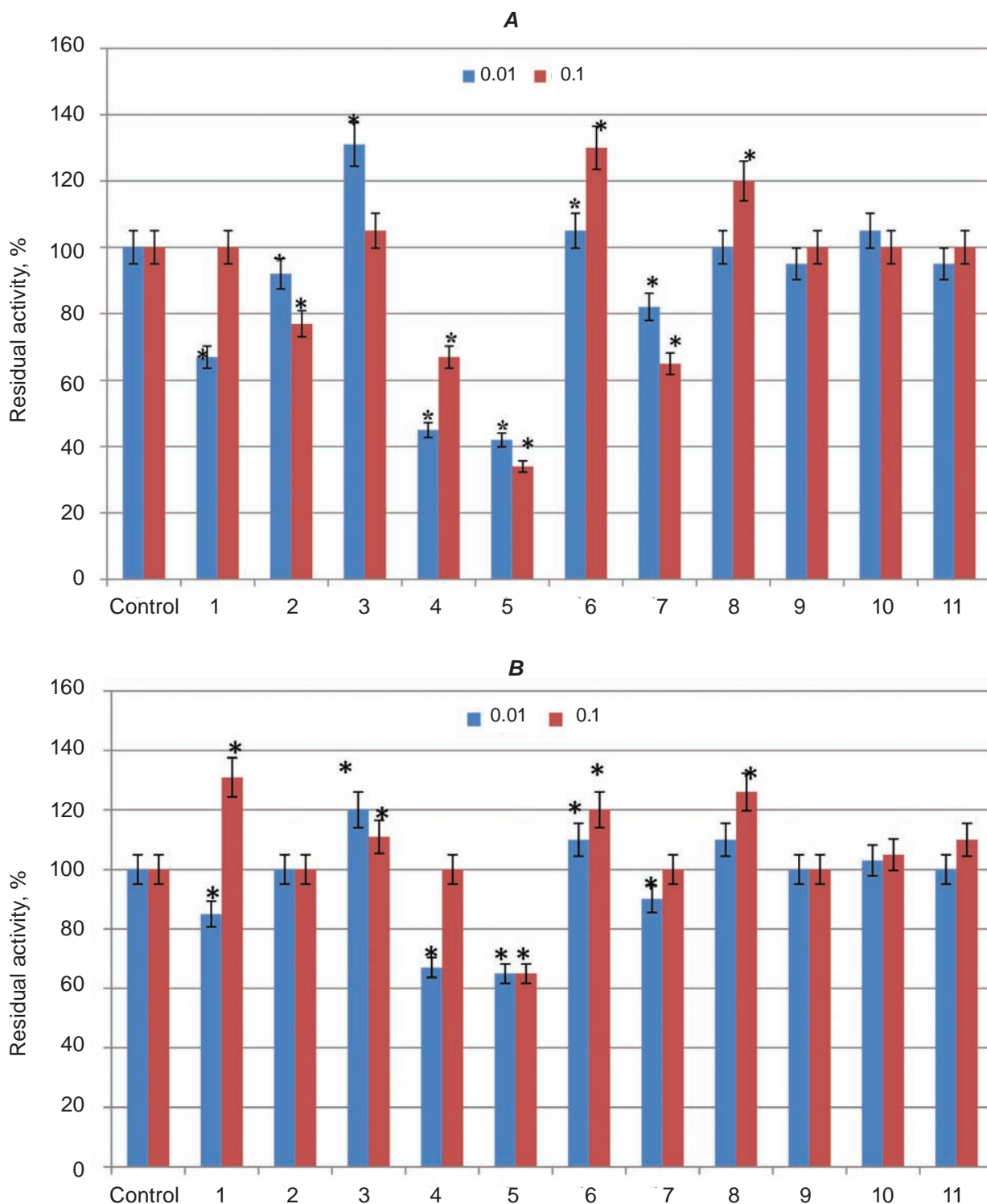


Fig. 3. The effect of germanium-containing compounds on the activity of  $\alpha$ -L-rhamnosidase from *E. erubescens* **A** – exposure time 0.5 h, **B** – exposure time 24 h. \*Significantly different to control

can be accounted for by dissociation of these compounds in aqueous solution. The presence of cations and anions allows us to consider the compounds as both nucleophilic and electrophilic activators. Thus,

the compound (3) at both studied concentrations affected the enzyme from *P. tardum* first as an inhibitor, and then as an activator (Fig. 2). Whereas, in the experiment with *E. erubescens*, the picture

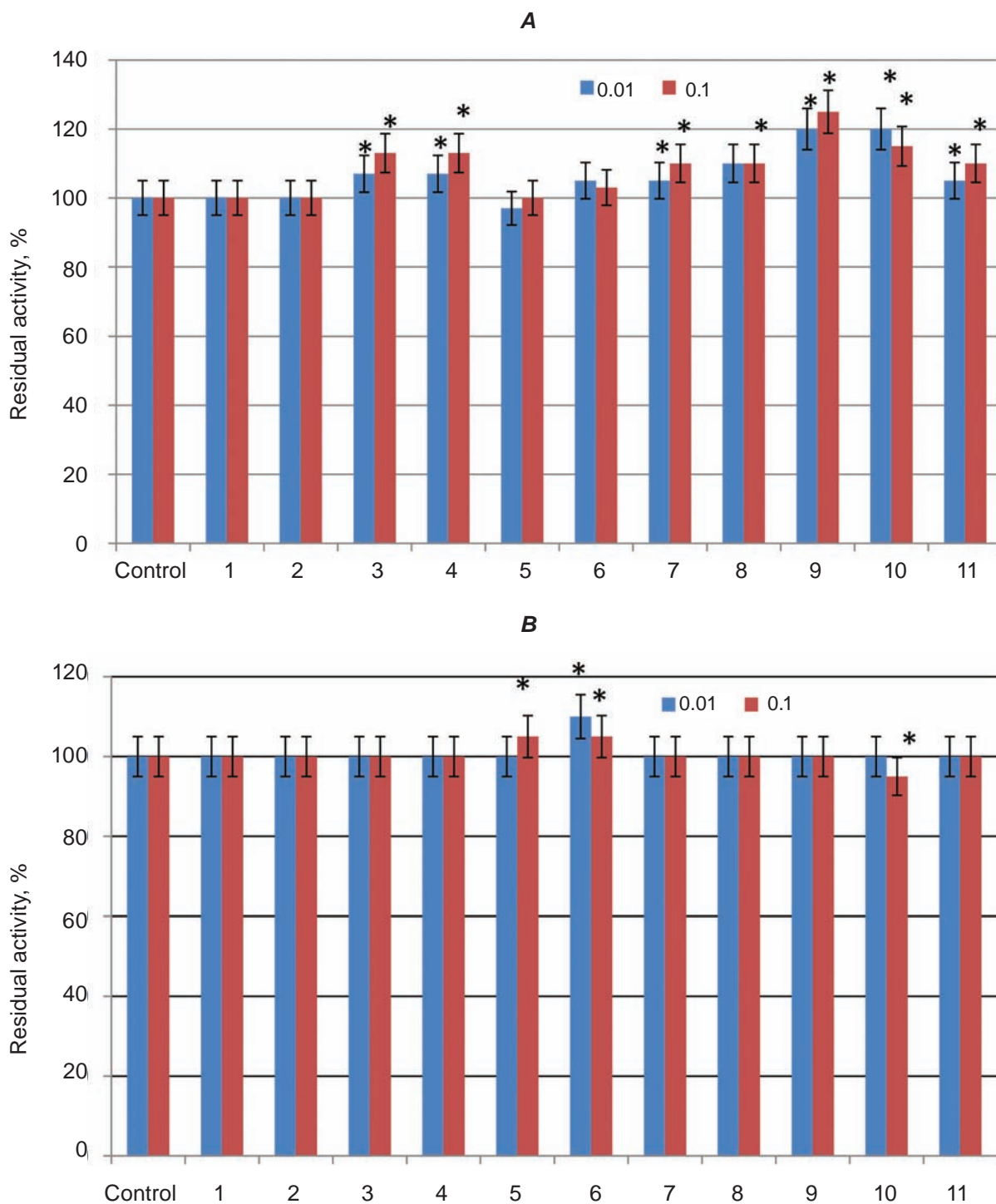


Fig. 4. The effect of germanium-containing compounds on the activity of  $\alpha$ -L-rhamnosidase from *Cryptococcus albidus*. **A** – exposure time 0.5 h, **B** – exposure time 24 h. \*Significantly different to control

changed: the same compound was shown to act as activator and its effect did not depend on the concentration and increased with time. Something similar was observed for compounds (1) and (4). They inhibited the enzyme activity from *P. tardum*, did not

affect *C. albidus*, while compound (1) increased and compound (4) inhibited the activity of the enzyme from *E. erubescens*. The inhibitory effect of compounds (1) and (4) on *P. tardum* can be accounted for by the preventing substrate binding to the enzyme



functional groups. The activity of  $\alpha$ -L-rhamnosidase from *E. erubescens* increased in the presence of compound (1), since it does not compete with zinc-containing enzyme for the substrate binding sites. On the contrary, it facilitates accessibility of the reactive sites. Unlike compound (1), compound (4) is an inhibitor. It contains a chloride ion, which blocks zinc in the enzyme active site and thus, prevents the donor/acceptor enzyme-substrate interaction.

In the experiments with the enzyme from *E. erubescens*, along with compound (3), significant activity was also observed for compounds (6) and (8), which do not contain d-metal cations, but are able to bind to the active site of a metal-containing  $\alpha$ -L-rhamnosidase. Differences in the influence of (1)-(11) on the activity of the enzymes from *P. tardum*, *E. erubescens* and *C. albidus* indicate that activation and inhibition occur with the involvement of different functional groups of these enzymes. Most likely, no bonds with such groups were formed in the case of *C. albidus*, hence most of the compounds were inactive towards it. The data presented differ from the previously reported results on citrate-germanate complexes as  $\alpha$ -L-rhamnosidase modifiers [16] and indicate the essential role of the composition of complex germanium-containing anion, as the complex cations were the same.

It should be mentioned that when we have analyzed the publications on  $\alpha$ -L-rhamnosidases, no other examples of metal-containing compounds capable of activating the enzyme were found, except for the ones we studied earlier [17-19]. The cations  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$  in inorganic salts act as inhibitors of catalytic activity. That is, the unique combination of the biological properties of Ge, Cu, Ni, tartrate acid and 2,2'-bipyridine is a key factor. Therefore, the compounds  $[\text{CuCl}(\text{bipy})_2]_2[\text{Ge}_2(\text{OH})_2(\text{Tart})_2] \cdot 4\text{H}_2\text{O}$  (3) and  $[\text{Ni}(\text{bipy})_3]_4\{[\text{Ge}_2(\text{OH})_2(\text{Tart})_2]_3\text{Cl}_2\} \cdot 15\text{H}_2\text{O}$  (4) could be considered as the most promising effectors of  $\alpha$ -L-rhamnosidases from various producers and require further studies. Our findings that the compound  $[\text{CuCl}(\text{bipy})_2]_2[\text{Ge}_2(\text{OH})_2(\text{Tart})_2] \cdot 4\text{H}_2\text{O}$  (3) increases  $\alpha$ -L-rhamnosidases activity are important, since these enzymes are used in the food industry to improve product quality.

Thus, coordination compounds with tartrate-germanate anions and Cu (II) and Ni (II) 2,2'-bipyridine complex cations are currently of particular scientific and practical interest. The selected metal ions are essential elements and bonded to the biologically active ligands [10-12]. Related complexes

demonstrate the ability to induce interferon and have immunostimulating effect, low toxicity, cardio-protective, antihypertensive, antiarrhythmic properties, and exert a beneficial effect in cardiovascular and chronic respiratory diseases, pneumonia, neuropsychiatric and metabolic disorders [21, 22]. The compounds that can act as effectors on *Penicillium tardum* and *Eupenicillium erubescens*  $\alpha$ -L-rhamnosidases activity will have a wide range of practical applications.

*Conflict of interest.* Authors have completed the Unified Conflicts of Interest form at [http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi\\_disclosure.pdf](http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf) and declare no conflict of interest.

The research was carried out within the framework of the research project "Functional, biological and physico-chemical properties of membrane and extracellular molecules of microorganisms and relationship from peculiarities of primary structure". State Registration number 0118U000214.

## ВПЛИВ КООРДИНАЦІЙНИХ ТАРТРАТО-І МАЛАТОГЕРМАНАТНИХ СПОЛУК НА АКТИВНІСТЬ $\alpha$ -L-РАМНОЗИДАЗ *Penicillium tardum*, *Eupenicillium erubescens* ТА *Cryptococcus albidus*

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Останнім часом ензими мікробного походження набувають все більшого значення в різних галузях промисловості. Препарати  $\alpha$ -L-рамнозидази використовуються у фармацевтичній промисловості, а також у науковій роботі як інструмент для аналітичних досліджень. Нами одержано очищені препарати  $\alpha$ -L-рамнозидази із штамів мікроорганізмів *Penicillium tardum*, *Eupenicillium erubescens* і *Cryptococcus albidus* – ефективних продуцентів ензиму. Мета роботи – оцінити здатність координаційних сполук германію підвищувати каталітичну активність ензимів. Вивчено вплив 11 гетерометальних змішанолігандних тартрато-(малато-)герма-

натів у концентрації 0,01 і 0,1% на активність препаратів  $\alpha$ -L-рамнозидази *Penicillium tardum* IMV F-100074, *Eupenicillium erubescens* 248 та *Cryptococcus albidus* 1001 за експозиції 0,5 і 24 год. Виявлено інгібувальний ефект сполуки  $[\text{Ni}(\text{bipy})_3]_4[\{\text{Ge}_2(\text{OH})_2(\text{Tart})_2\}_3\text{Cl}_2] \cdot 15\text{H}_2\text{O}$  на  $\alpha$ -L-рамнозидазу *P. tardum*. Встановлено, що всі досліджувані сполуки, крім  $[\text{CuCl}(\text{phen})_2][\text{Ge}(\text{OH})(\text{HMal})_2]$ , підвищували активність  $\alpha$ -L-рамнозидази *P. tardum* за тривалішого часу експозиції. Показано, що активність  $\alpha$ -L-рамнозидази *E. erubescens* стимулювалась сполуками, які не містять у своєму складі катіонів d-металів.  $\alpha$ -L-Рамнозидаза *C. albidus* виявилася нечутливою до всіх досліджуваних сполук.

**Ключові слова:** *Penicillium tardum* IMV F-100074, *Eupenicillium erubescens* 248, *Cryptococcus albidus* 1001,  $\alpha$ -L-рамнозидаза, германійвмісні сполуки.

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