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GERMANIUM (IV) COMPLEXES WITH GLUCONIC ACID AS EFFECTORS OF *PENICILLIUM TARDUM* AND *EUPENICILLIUM ERUBESCENS* α -L-RHAMNOSIDASES

α -L-Rhamnosidase (α -L-rhamnoside-rhamnhydrolase EC 3.2.1.40) showing specificity for terminal α -1,2-, α -1,4- and α -1,6-linked rhamnose residues, which often present in glycoconjugates and synthetic glycosides, can be successfully used in biotechnology for the hydrolysis of rhamnopyranoside residues present in some bioflavonoids, glycoproteins, glycolipids, and other glycoconjugates. Previously, we have shown that a significant part of the coordination compounds of various metals act as effectors of the activity of α -L-rhamnosidases. **The aim** of this investigation was to study the effect of a number of newly synthesized coordination compounds of Ge(IV) and Ba(II), (Co(II), Ni(II), Cu(II), Zn(II) with gluconic acid on the activity of *Penicillium tardum* and *Eupenicillium erubescens* α -L-rhamnosidases. **Methods.** The objects of the study were *Penicillium tardum* and *Eupenicillium erubescens* α -L-rhamnosidases. α -L-Rhamnosidase activity was determined by the Davis method using naringin as a substrate. Coordination compounds Ge(IV) and Ba(II), Co(II), Ni(II), Cu(II), and Zn(II) with gluconic acid were used as enzyme activity modifiers. The synthesized complexes correspond to the formulas $[M(H_2O)_6][Ge_2(OH)_2(C_6H_8O_7)_2] \cdot nH_2O$ ($M = Ba(1), n = 2; Co(2), n = 4; Ni(3), n = 4; Cu(4), n = 4; Zn(5), n = 3$). **Results.** The effect of coordination compounds 1–(5) on the activity of α -L-rhamnosidase in two strains of *Penicillium tardum* and *Eupenicillium erubescens* was studied depending on the exposure time and concentration of the effector. It was shown that compound (3) at a concentration of 0.01% (1 h incubation) led to a slight (by 5%) increase in the activity of *P. tardum* α -L-rhamnosidase. Compound 1 at a concentration of 0.1% led to a decrease in the activity of *P. tardum* α -L-rhamnosidase by 29% during the first hour, and after 24 h of incubation, a decrease in the inhibitory effect to 15% was noted. Compounds 2 and (4) activated the enzyme by 9–39% at 1h exposure. At a concentration of 0.1% and

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exposure time of 1 h, compound 1 increased the activity of *E. erubescens* α -L-rhamnosidase by 80%, while at a decrease in concentration to 0.01%, the activity increased only by 29%. In general, it should be noted that in most cases, an increase in the duration of incubation up to 24 h led to a decrease in the level of activation (or inhibition) and a return to the control values of enzyme activity. **Conclusions.** The variety of effects of metal coordination compounds on the activity of enzymes, depending on the nature of the cation and the origin of the enzyme, has been established. The involvement of Ba(II) had the greatest activating effect on the activity of *E. erubescens* α -L-rhamnosidase compared to other metals.

Keywords: α -L-rhamnosidase, *Eupenicillium erubescens*, *Penicillium tardum*, coordination compounds, gluconic acid.

Currently, hydrolytic enzymes are acquiring an important role in biotechnology for the production of biologically active substances of various natures and actions. The main advantage of the use of them is the high specificity, which allows one to obtain a purer product with fewer purification steps. Microbial hydrolases have great potential for industry due to the abundance of their sources and wide opportunities for selection and mutagenesis. α -L-Rhamnosidase (α -L-rhamnoside rhamnohydrolase EC 3.2.1.40) showing specificity for terminal α -1,2-, α -1,4-, and α -1,6-linked rhamnose residues, which often present in glycoconjugates and synthetic glycosides, can be successfully used in biotechnology for the hydrolysis of flavonoids, glycoproteins, glycolipids, and other glycoconjugates. The scope of the use α -L-rhamnosidase is quite wide: in the food industry, in particular in winemaking to improve the quality and aroma of wine, in the production of citrus juices and the production of drinks from them, to remove bitter components (naringin), thereby improving the quality and nutritional value of these products, as well as in scientific research as an analytical tool for studying the structure of complex carbohydrate-containing biopolymers [1–4].

Previously [5, 6], as a result of screening, we have selected producers of α -L-rhamnosidases, in particular, *Penicillium tardum* and *Eupenicillium erubescens*, which showed higher activity compared to other studied enzyme producers. α -L-Rhamnosidases were isolated and purified from the culture liquid, and their properties and catalytic activity were studied. It is known that various synthetic effectors are able to increase

the activity of enzymes and the efficiency of hydrolysis of substrates. Previously [7–9], we have shown that a significant part of the coordination compounds of various metals act as effectors of the activity of α -L-rhamnosidases. Therefore, the purpose of this study was to investigate the effect of a number of newly synthesized coordination compounds of Ge(IV) with Ba(II) (Co(II), Ni(II), Cu(II), Zn(II)) and gluconic acid on the activity of α -L-rhamnosidases from *Penicillium tardum* and *Eupenicillium erubescens*. Interest in compounds of this structure is based on the results of experimental studies of their biological properties [10], which indicate that salts of gluconic acid normalize the activity of the main enzymes in myocardial metabolism such as lactate dehydrogenase, creatine phosphokinase, and succinate dehydrogenase as well as increase the activity of oxide-hydride enzymes, Na⁺/K⁺-ATPase. Also, the effect of calcium gluconate on the activity of enzymes in the scutal lining of the intestines of animals has been shown in [11].

Materials and methods. The objects of research were α -L-rhamnosidase *Penicillium tardum* IMV F-100074 and *Eupenicillium erubescens* 248 from the collection of live cultures of the Zabolotny IMV NAS of Ukraine. The isolation and purification of α -L-rhamnosidase has been described by us earlier [5, 6].

The activity of α -L-rhamnosidase was determined using Davis' method [12]. The assay mixture contained 0.2 mL of 0.1% naringin (Sigma, USA) solution in 0.1 M PCB pH 5.2 and 0.2 mL l enzyme solution. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 5 mL diethylene glycol (90%) and 0.1 mL of

4 N NaOH. The residual naringin was measured at 420 nm. One unit of the α -L-rhamnosidase activity was defined as the amount of enzyme that releases 1 μ mol of naringin per min in the solution. The specific α -L-rhamnosidase activity of the preparations was 27 units/mg of protein for *P. tardum* and 110 units/mg of protein for *E. erubescens* (protein content — 0.01 mg/mL).

As modifiers of enzyme activity, coordination compounds of Ge(IV) with Ba(II), Co(II), Ni(II), Cu(II) or Zn(II) and gluconic acid have been used.

Chemicals for synthesis of new complexes 1—(5) were readily available from commercial sources and used as received without further purification: germanium(IV) oxide (GeO_2 , 99.99%), gluconic acid (50% solution in water, CAS 527-07-1), BaCO_3 , $\text{Co}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, $\text{Ni}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, $\text{Cu}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$, and $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ (all ©Sigma Aldrich, 99%).

For the synthesis of compounds, GeO_2 (0.65 g) was dissolved in 200 mL of hot water (90 °C), added with 2.05 mL gluconic acid (50% solution in water), stirred, and then slowly evaporated at 80 °C to a 20 mL volume. After cooling to 25 °C, 0.0031 mol of barium carbonate (or the acetate of a 3d metal) was added and stirred until the reagent was completely dissolved. In the solid state, the compounds were isolated by salting out with ethanol. The precipitates were separated on a Schott glass filter and dried in the air at 20—25 °C. Yield: 62—70%.

Elemental analyses for germanium, barium, cobalt, nickel, copper, and zinc were performed using inductively coupled plasma atomic emission spectroscopy with an Optima 2000 DV instrument (PerkinElmer); analyses for C, H, and N were performed in an Elemental Analyzer CE-440. The elemental analysis resulted in obtaining the following gross formulas with the contents (in %) of individual elements and the color.

1 — $\text{C}_{12}\text{H}_{34}\text{O}_{24}\text{Ge}_2\text{Ba}$: C 17.06/18.40; H 4.03/3.95; Ge 17.18/17.37; Ba 16.23/16.10 (white);

2 — $\text{C}_{12}\text{H}_{38}\text{O}_{26}\text{Ge}_2\text{Co}$: C 18.05/18.21; H 4.76/4.55; Ge 18.17/18.33; Co 7.39/7.23 (pink);

3 — $\text{C}_{12}\text{H}_{38}\text{O}_{26}\text{Ge}_2\text{Ni}$: C 18.05/18.17; H 4.76/4.62; Ge 18.17/18.41; Ni 7.39/7.19 (green);

4 — $\text{C}_{12}\text{H}_{38}\text{O}_{26}\text{Ge}_2\text{Cu}$: C 17.93/18.14; H 4.73/4.59; Ge 18.06/18.27; Cu 7.97/7.69 (blue);

5 — $\text{C}_{12}\text{H}_{36}\text{O}_{25}\text{Ge}_2\text{Zn}$: C 18.22/18.11; H 4.56/4.38; Ge 18.35/18.20; Zn 8.23/8.57 (white).

Thermogravimetric analysis was carried out using Q-1500D with a heating rate of 10 °C/min in the air in the temperature range 20—1000 °C. The IR absorption spectra of the complexes were collected on a Frontier spectrophotometer (PerkinElmer) using KBr pellets in the 400—4000 cm^{-1} range. The most important absorption bands in the IR spectra of complexes 1—(5) were assigned in compliance with the literature data, including data for the germanium(IV) coordination compounds [13].

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 1 h and 24 h. The studied compounds were dissolved in 0.1% DMSO. All experiments were performed in 5—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. The elemental analysis of complexes 1—5 has demonstrated that compounds with the composition M (Ba, Co, Ni, Cu, Zn): Ge: ligand = 1:2:2 are formed in all the cases.

IR spectra of complexes 1—5 contain the absorption bands $\nu_{\text{as}}(\text{COO}^-)$ and $\nu_{\text{s}}(\text{COO}^-)$, which indicates the presence of coordinated carboxylates groups in these compounds (Table 1). Compared with the IR spectrum of gluconic acid, a band of C=O valence vibrations of the carboxyl group lacks in the spectra of the complexes. The deprotonation of the alcoholic OH groups of the ligands and their coordination are shown by the absorption bands $\nu(\text{C—O})$ and $\nu(\text{Ge—O})$. There are also deformational vibrations of free C—OH groups. The IR spectra contain deformational

$\delta(\text{H}_2\text{O})$ vibrations typical for hexaaqua complexes of metals.

The IR spectra of 1–(5) contain absorption band deformational vibrations $\delta(\text{Ge}-\text{OH})$. Therefore, the molecules of the new compounds contain hydroxyl and carboxyl groups bound to the complexing agent, and germanium is part of the complexes in a hydrolyzed form.

The occurrence of intense $\nu(\text{OH})$ stretching vibration bands in the IR spectra of all the complexes evidences the presence of crystal water molecules in their compositions.

When studying the thermal stability of the synthesized complexes, we have established that their thermolysis proceeds in a similar way. At the first stage, an endotherm is observed within the range of 60–250 °C. The wide temperature range, high process temperature, and corresponding weight loss curves allow us to conclude that the complexes incorporate both crystallized and coordinated water molecules joined by hydrogen bonds (8, 10, 10, 10, and 9 molecules of water in complexes 1, 2, 3, 4, and 5, respectively).

When analyzing the results of research on complexes 1–5, it was established that the obtained compounds belong to the cation-anion type. In the complex anion, the ligand is coordinated to germanium with hydroxyl and carboxyl oxygen atoms and has a dimeric structure. In the cation, the coordination polyhedron of Ba, Co, Ni, Cu, or Zn is formed by the oxygen atoms of six water molecules. The synthesized complexes correspond to the formulas $[\text{M}(\text{H}_2\text{O})_6] [\text{Ge}_2(\text{OH})_2(\text{C}_6\text{H}_8\text{O}_7)_2] \cdot n\text{H}_2\text{O}$ (M = Ba(1), n=2; Co 2, n=4; Ni(3), n=4; Cu(4), n=4; Zn(5), n=3) and the scheme of structure presented in Fig. 1.

The study of the effect of coordination compounds 1–5 on the activity of two strains *Penicillium tardum* and *Eupenicillium erubescens* of α -L-rhamnosidase showed that only complex 3 at a concentration of 0.01% after 1 h of incubation led to a slight (by 5%) increase in the activity of *P. tardum* α -L-rhamnosidase (Fig. 2, a). The

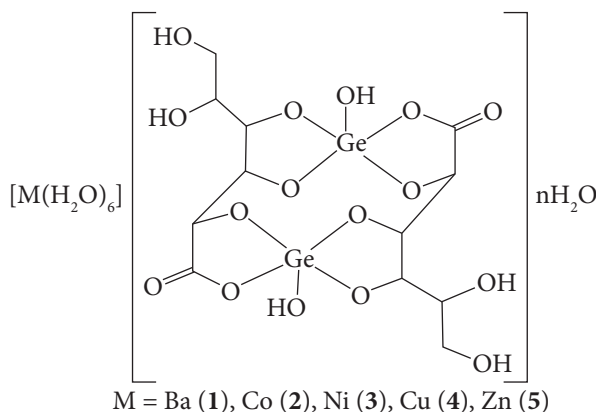


Fig. 1. Scheme of the structure of complexes 1–5

effect of complexes 1–5 at a concentration of 0.1% turned out to be more diverse. It was shown that after 1 h of incubation, complex 1 reduced the activity of *P. tardum* α -L-rhamnosidase by 29% (Fig. 2, a). With an increase in the exposure time to 24 h (Fig. 2, b), a decrease in its inhibitory effect by up to 15% was noted. All the other complexes, depending on the incubation time, either had no effect on the enzyme (3 and 5), or increased activity by 9–39%. Thus, the greatest activation (by 39%) was noted under the action of complex 2 after 1 h of incubation. With an

Table 1. Selected data for the IR spectra of complexes 1–(5)

Vibrations of the functional groups / $\nu(\text{cm}^{-1})$	Complex				
	1	2	3	4	5
OH stretching	3300	3413	3375	3400	3420
Asymmetric COO^- stretching	1678	1684	1690	1682	1694
H_2O deformational	1604	1616	1601	1600	1604
Symmetric COO^- stretching	1353	1350	1340	1355	1359
C—OH deformational	1245	1220	1221	1220	1244
C—O stretching	1069	1091	1070	1067	1089
Ge—OH deformational	833	847	813	825	865
Ge—O stretching	636	631	635	636	624

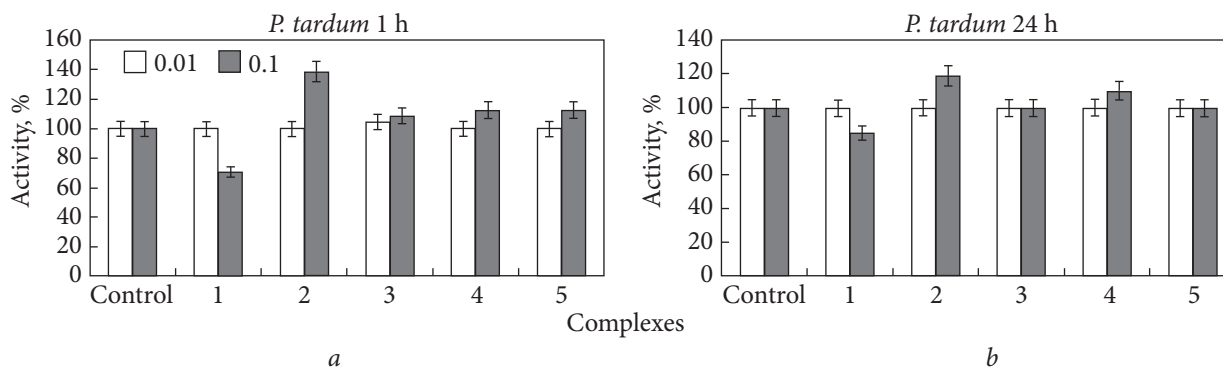


Fig. 2. The effect of germanium-containing compounds on the activity of *P. tardum* α -L-rhamnosidase: *a* — exposure time 1 h, *b* — exposure time 24 h

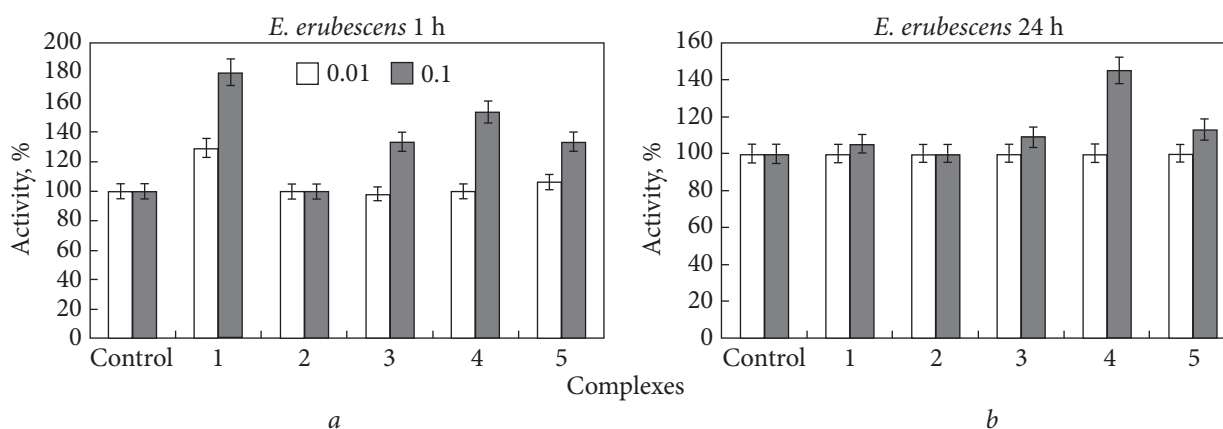


Fig. 3. Influence of germanium-containing compounds on the activity of *E. erubescens* α -L-rhamnosidase: *a* — exposure time 1 h, *b* — exposure time 24 h

increase in exposure time, the activating effect decreased to 19%. A similar effect is also shown for complexes 3–5. After 1 h of incubation, the activity increased by 9–13%. An increase in the duration of incubation up to 24 h contributed to a decrease in activation from 13 to 10% with complex 4 and a return to control values with complexes 3 and 5.

The activity of *E. erubescens* α -L-rhamnosidase (Fig. 3, *a, b*) was affected differently by coordination compounds 1–(5). Thus, at a concentration of 0.1% and an exposure time of 1 h, complex 1 increased the activity of the enzyme by 80% (Fig. 3, *a*), while at a decrease in concentration to 0.01%, the activity increased only by 29%. An increase in the action time of the test compound

to 24 h (Fig. 3, *b*) resulted in a decrease in activity to the control level (at a concentration of 0.01%), or to 5% activation (at a concentration of 0.1%). Complex 2 did not affect the activity of *E. erubescens* α -L-rhamnosidase regardless of the incubation time and concentration (Fig. 3, *a, b*). We also showed (Fig. 3, *b*) that at the 0.01% concentration of complexes 1–5 and exposure time of 24 h, the activity was at the control level. The same results were noted for complex 4 at a concentration of 0.01% and an exposure time of 1 h (Fig. 3, *a*).

Discussion. The use of biologically active substances of microbial origin for practical and theoretical purposes is one of the main strategies of modern biotechnological processes. This

is primarily due to the unlimited sources, in particular microbial producers, the high rate of reproduction of which and the controllability of the conditions for the synthesis of biopolymers by them create the conditions for obtaining substances with predetermined properties and specificity of action. Today, microorganisms are the most technological and economic source of obtaining such enzymes as hydrolases, which have useful properties for the use of them in the food, pharmaceutical, and chemical industries, as well as medicine. To increase the activity of enzymes, a number of approaches can be used, in particular, the optimization of cultivation conditions and the use of synthesis inducers of both synthetic and natural origin. But in recent years, special attention of researchers has been directed to the search for substances that can directly affect the activity of enzymes, in particular, coordination compounds of transition metals. Coordination compounds of germanium, cobalt, and nickel with organic ligands have already proven themselves as promising enzyme activators [14–16]. The low toxicity and wide spectrum of biological activity encourage researchers to synthesize new complex compounds [13]. We investigated the influence of a wide range of coordination compounds on the activity of α -L-rhamnosidases and α -galactosidases of microorganisms and established various effects, both qualitatively and quantitatively, depending on the nature of the cation and anion of the complex, metal, and ligand. Analysis of the nature of the interaction of complexes with enzymes allows us to conclude that promising effectors among coordination compounds of biologically active metals and ligands are compounds whose structural organization ensures the synergism of the action of all components. Also, we have shown [7–9] that various germanium complexes with bioligands can be recommended for a targeted search of α -L-rhamnosidase effectors. It was established that the most effective of the 20 tested

compounds was the compound tris(bipyridine) nickel(II) with μ -dihydroxylarato-germanate (IV), which increased the activity of *E. erubescens* α -L-rhamnosidase by 9.7 times and *P. tardum* by 8 times (exposure time 24 h, effector concentration 0.1%), as well as tris(bipyridine) nickel(II) bis(citrate)germanate hydrate (IV), the maximum activating effect of which at the same concentration increased the activity of *E. erubescens* α -L-rhamnosidase by 2.5 times and *P. tardum* by 5 times.

As for the compounds of germanium with various metals and gluconic acid studied in this work, they turned out to be less effective than the coordination compounds we studied earlier. Thus, the complex $[M(H_2O)_6][Ge_2(OH)_2(C_6H_8O_7)_2] \cdot nH_2O$ ($M=Ba$, $n=2$) stimulates the activity of *E. erubescens* α -L-rhamnosidase by 80%, while the complex $[M(H_2O)_6][Ge_2(OH)_2(C_6H_8O_7)_2] \cdot nH_2O$ ($M=Co$, $n=4$) stimulates the activity of *P. tardum* α -L-rhamnosidase by only 39%. The observed effects are the sum of the effects of all components of the complex and are unlikely to be related to the effect of the metal cation. Thus, previous studies showed that *E. erubescens* α -L-rhamnosidase is a metal-dependent enzyme, and its activity was inhibited by Ba(II) ions by 36%, and Ni(II), Zn(II), Co(II), and Cu(II) — by 10–18% [6]. At the same time, Co(II) ions suppressed the activity of *P. tardum* α -L-rhamnosidase by more than 60% [8], however, the coordination compound, which includes this metal, has a stimulating effect on the activity of the enzyme. The noted features may be related to the location of functional groups and donor centers in the molecules of enzymes and effectors. Although the studied compounds are inferior to previously studied activators and inhibitors of a similar nature in terms of effectiveness, they also provide new information and can help in solving the important issue of the mechanism of interaction between enzymes and complex chemical molecules.

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КОМПЛЕКСИ ГЕРМАНІЮ(IV) З ГЛЮКОНОВОЮ КИСЛОТОЮ — ЕФЕКТОРИ α -L-РАМНОЗИДАЗ *PENICILLIUM TARDUM* ТА *EUPENICILLIUM ERUBESCENS*

α -L-Рамнозидаза (α -L-рамнозид-рамногідролаза ЕС 3.2.1.40), що проявляє високу специфічність щодо кінцевих α -1,2-, α -1,4- і α -1,6-зв'язаних залишків рамнози, які часто присутні у глікокон'югатах та синтетичних глікозидах, може бути успішно використана в біотехнології для гідролізу рамнопіранозидних залишків, присутніх у деяких біофлавоноїдах, глікопротеїнах, гліколіпідах та інших глікокон'югатах. Раніше нами було показано, що значна частина досліджених координаційних сполук різних металів поводить як ефектори активності α -L-рамнозидаз. **Метою** даного дослідження було вивчити вплив низки ново-синтезованих координаційних сполук Ge(IV) з Ba(II), Co(II), Ni(II), Cu(II), Zn(II) та глюконовою кислотою на активність α -L-рамнозидази *Penicillium tardum* та *Eupenicillium erubescens*. **Методи.** Об'єктом дослідження були α -L-рамнозидази *Penicillium tardum* та *Eupenicillium erubescens*. Активність α -L-рамнозидази визначали за методом Девіса з використанням нарингину як субстрату. Як модифікатори активності ферментів використані координаційні сполуки Ge(IV) з Ba (II), Co(II), Ni(II), Cu(II), Zn(II) і глюконовою кислотою. Синтезовані комплекси відповідають формулам $[M(H_2O)_6][Ge_2(OH)_2(C_6H_8O_7)_2] \cdot nH_2O$ (M = Ba(1), n = 2; Co 2, n = 4; Ni(3), n = 4; Cu(4), n = 4; Zn(5), n = 3). **Результати.** Досліджено вплив координаційних сполук 1—5 на активність α -L-рамнозидази двох штамів *Penicillium tardum* та *Eupenicillium erubescens* залежно від часу експозиції та концентрації ефектора. Показано, що сполука 3 в концентрації 0,01 % (1 год інкубації) приводила до незначного (на 5 %) підвищення активності α -L-рамнозидази *P. tardum*. Сполука 1 в концентрації 0,1 % знижувала активність α -L-рамнозидази *P. tardum* на 29 % протягом першої години, а після 24 год інкубації відзначали зниження інгібувальної дії до 15 %. Сполуки 2 та (4) активували ензим на 9—39 % за експозиції 1 год. При концентрації 0.1 % та часі експозиції 1 год сполука 1 на 80 % підвищувала активність α -L-рамнозидази *E. erubescens*, тоді як при зниженні концентрації до 0.01 % активність підвищувалася лише на 29 %. В цілому можна відзначити, що в більшості випадків збільшення тривалості інкубації до 24 год призводило до зниження рівня активації (або інгібування) та повернення до контрольних значень активності ензимів. **Висновки.** Встановлено різноманітність впливів координаційних сполук металів на активність ензимів в залежності від природи катіону та походження ензиму. Залучення Ba(II) мало найбільший активуючий ефект на активність α -L-рамнозидази *E. erubescens* у порівнянні з іншими металами.

Ключові слова: *Penicillium tardum*, *Eupenicillium erubescens*, α -L-рамнозидаза, координаційні сполуки Ge(IV) з Ba(II), Co(II), Ni(II), Cu(II), Zn(II) та глюконовою кислотою.