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ELASTASE ACTIVITY OF REPRESENTATIVES OF THE GENUS *BACILLUS* ISOLATED FROM THE COASTAL AREA OF THE KINBURN SPLIT

Previously, elastase activities have been found in some microorganisms and presented by metalloproteases, which may play an important role in helping bacterium invasiveness and establishment of infection. The elastases from Bacillus species are serine proteases, however, to date there are few reports on systematic study of these enzymes producers among representatives of Bacillus, as well as studies of their properties. The ***aim*** *of this work was to study some physicochemical characteristics of partially purified elastase enzyme from a number of Bacillus sp. strains.* ***Methods.*** *The objects of the study were strains of Bacillus sp. (L1, L2, L9) isolated from the dry grass of the coastal zone of the Kinburn split (Mykolaiv region). Cultures were grown under submerged cultivation at 28 °C, with a shaking speed of the nutrient medium of 230 rpm for 2 days. Methods of determining elastase activity in the culture liquid supernatant were used.* ***Results.*** *It has been shown that Bacillus sp. L9, L1, and L2 are characterized by high levels of elastase activity (35.80, 28.0, and 33.80 U/mL, respectively). The maximum synthesis of elastase occurs on the second day of cultivation of the producer at 28 °C and shaking at 230 rpm. Maximum hydrolysis of elastin by Bacillus sp. L1 and L2 are carried out at 40 °C but at different pH optima. Thus, for the preparation of Bacillus sp. L1, optimal pH is 8, and for L2 — 10. For the complex enzyme preparation of Bacillus sp. L9, maximum hydrolysis of elastin occurs at pH 9 and temperature 50 °C.* ***Conclusions.*** *Bacillus sp. L9 with elastase activity is promising for continued research on its properties for further practical use.*

Keywords: *Bacillus sp. strains, Kinburn split, elastase activity, pH optimum, thermal optimum.*

Elastases are enzymes that degrade insoluble protein elastin. Elastin is a fundamental component of the extracellular matrix and is responsible for providing tissue with flexibility. This protein is prevalent in numerous tissues, such as the skin, arteries, and lungs. The quantity of elastin present varies across tissue types, forming extensively cross-linked protein fibers in the process. Elastin

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exhibits a high degree of hydrophobicity and is composed of a hydrophobic domain and a cross-linking domain. The hydrophobic domain contains nonpolar amino acids like glycine, proline, valine, and leucine, while the cross-linking domains consist of lysine and alanine and play a role in the cross-linking of elastin (Wis & Weiss, 2009; Schräder et al., 2018; Kielty, Sherratt, & Shuttleworth, 2002). The elasticity and stability of elastin in tissues are influenced by the distinctive alternating hydrophobic and cross-linking domains. Furthermore, these structures help elastin resist proteolysis by most proteases, except for elastases. Some microorganisms have been found to exhibit elastase activities through metalloproteases, which may contribute to bacterium invasiveness and infection establishment (Kielty, Sherratt & Shuttleworth, 2002). The elastases produced by *Bacillus* species are serine proteases (Mitrofanova et al., 2017; AlShaikh-Mubarak et al., 2023). Wang reported that *Bacillus subtilis* produces an alkaline elastase, subtilisin YaB, which has high elastolytic activity and belongs to the subtilisin subfamily (Wang et al., 2006). Elastases from *Bacillus* have commercial applications in the food, cosmetic, and medical industries. Elastases produced by *Bacillus* sp. YaB and *Bacillus* sp. EL31410 have been found to degrade collagen and elastin, which contribute to meat toughness during the process of meat tenderization (Marques, Maróstica & Pastore, 2010). Elastin peptide, an enzymatic breakdown product of elastin, has been noted for its various health and cosmetic benefits, including enhanced skin elasticity, improved blood flow, and activated ligament cells. However, there have been limited reports on the systematic study of elastolytic enzyme producers in *Bacillus* representatives and their properties.

Thus, this paper **aims** to investigate the physicochemical characteristics of partially purified elastolytic enzymes from various *Bacillus* sp. strains.

Materials and Methods. The objects of research were *Bacillus* sp. L1, L2, and L9 isolated from dry grass of the coastal zone of the Kinburn split (Mykolaiv region).

For submerged fermentation, strains were cultivated in Erlenmeyer flasks containing 100 mL of medium of the following compositions, g/L: KH_2PO_4 — 1.0; $MgSO_4 \times 7H_2O$ — 0.75; $ZnSO_4 \times H_2O$ — 0.25; $(NH_4)_2SO_4$— 0.5; maltose — 1.0; gelatin — 10.0; yeast autolysate — 0.15; pH 7.0. Cultures were grown at 28 °C with a rotation speed of 230 rpm for 2 days. At the end of fermentation, the biomass was separated by centrifugation at 5000 g for 30 min. Enzymatic activity was determined in the culture liquid supernatant (CLS). To obtain a partially purified preparation, dry ammonium sulfate salt was added to the culture liquid supernatant to a final concentration of 90%. The mixture was kept for 24 h at 4 °C, centrifuged at 5000 g for 30 min, and the precipitate was collected. The precipitate obtained from the fractionation with ammonium sulfate was dialyzed.

Elastase activity was determined colorimetrically by the color intensity of the solution during the enzymatic hydrolysis of elastin stained with Congo-rot using the Trombridge et al. method (Trowbridge & Moon, 1972). The incubation mixture contained 5 mg of elastin, 2.0 mL of 0.01 M Tris-HCl buffer (pH 7.5) supplemented with 0.005 M $CaCl_2$, and 1 mL of test solution. The mixture was incubated for 5 h at 37 °C. Non-hydrolyzed elastin was separated by centrifugation at 8000 g for 10 min. The color intensity was measured on an SF-26 spectrophotometer at 515 nm. The activity was calculated from a standard curve, which was obtained by measuring the color of the supernatant from the complete enzymatic hydrolysis of known amounts of elastin stained with Congo-rot. An activity unit was taken as the amount of enzyme that catalyzes the hydrolysis of 1 mg of the substrate for 1 min under standard conditions.

The study of the effect of temperature on the enzymatic activity was carried out in the range from 4 to 70 °C and pH from 2.0 to 12.0, the latter was created using 0.01 M phosphate-citrate buffer (PCB).

Protein concentration was determined by the Lowry method (Lowry et al., 1951). The standard

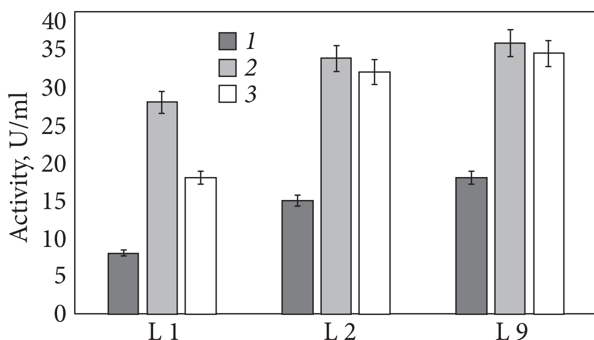


Fig. 1. Dynamics of elastase synthesis by *Bacillus* sp. L1, L2, and L9 during cultivation at 28 °C: 1, 2, 3 — days of cultivation

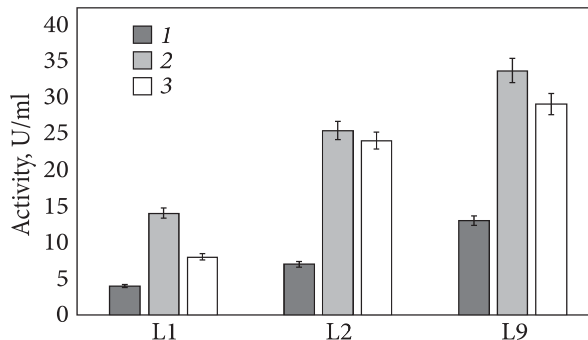


Fig.2. Dynamics of elastase synthesis by *Bacillus* sp. L1, L2, and L9 during cultivation at 42 °C: 1, 2, 3 — days of cultivation

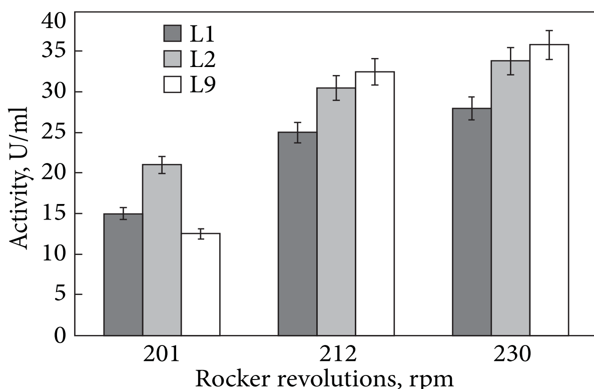


Fig. 3. Effect of the rotation speed on the elastase activity of *Bacillus* sp. L1, L2, and L9

Table 1. **Effect of the volume of medium in the flask on the elastase activity of the supernatant of the culture liquid of *Bacillus* sp. L1, L2, and L9**

Volume of medium in the flask, mL	Elastase activity, U/mL		
	Bacillus sp. L1	*Bacillus* sp. L2	*Bacillus* sp. L9
50	27.0 ± 0.09	32.9 ± 0.10	33.1 ± 0.09
100	28.0 ± 0.09	33.80 ± 0.09	35.0 ± 0.09
150	24.3 ± 0.50	27.7 ± 0.09	28.8 ± 0.09
200	17.3 ± 0.90	21.1 ± 0.09	23.5 ± 0.09
250	10.1 ± 0.09	12.3 ± 0.09	17.4 ± 0.09

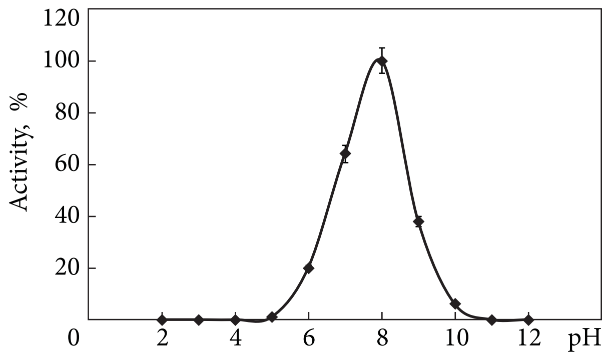
curve of bovine serum albumin (BSA) (1 mg/mL) was constructed.

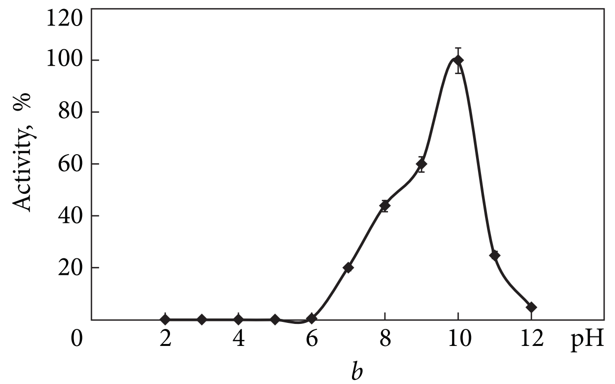
All experiments were performed in no less than 3—5 replications. Statistical processing of the results of the experimental series was carried out by standard methods using Student's t-test. The value of the null hypothesis $p < 0.05$ was taken as the critical level of reliability.

Results. Previously (Gudzenko & Varbanets, 2024), a screening of 15 bacterial strains revealed that several strains of *Bacillus* sp. L9, L2, and L1 obtained from dry grass in the coastal zone of the Kinburn Spit showed promise in further research on the production of specific proteolytic enzymes associated with elastase activity. The strains' catalytic activity was the basis for this determination. Since the biosynthesis of enzymes depends on the cultivation conditions of the producer, we studied some of their parameters, in particular, temperature, level of aeration of the nutrient medium, and rotation speed of the shaker.

When studying the influence of cultivation temperature, it was shown (Fig. 1) that the maximum synthesis of elastase activity by strains of *Bacillus* sp. L9, L2, and L1 are observed at 28 °C on the second day of cultivation of all studied strains.

When the cultivation temperature increases to 42 °C (Fig. 2), a decrease in the synthesis of elastase activity by the studied cultures is observed. Hence, after the first day of incubation





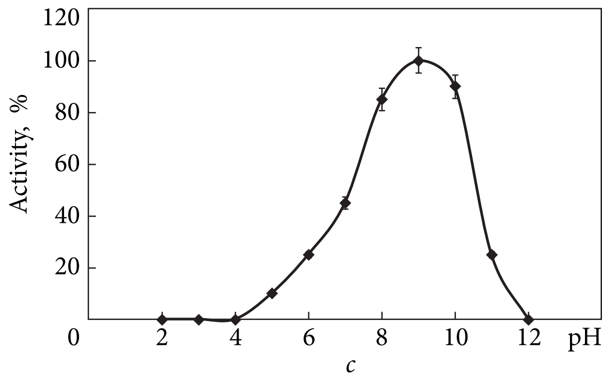
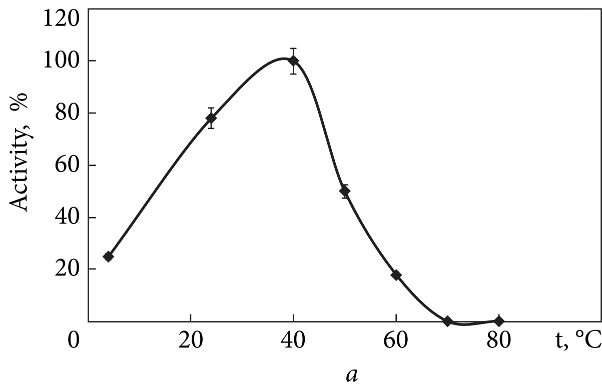
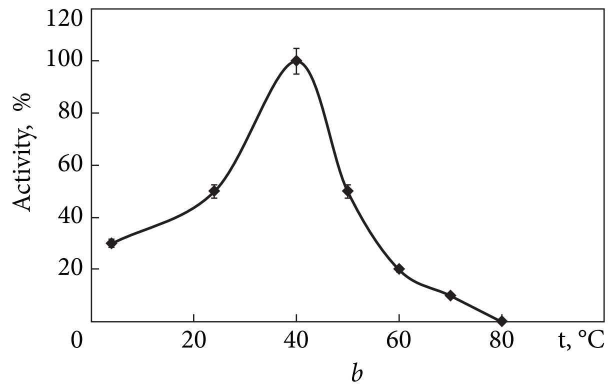


Fig. 4. pH-optimum action of elastase preparations from *Bacillus* sp.: *a* — L1, *b* — L2, *c* — L 9. Activity is expressed as % of the maximum activity





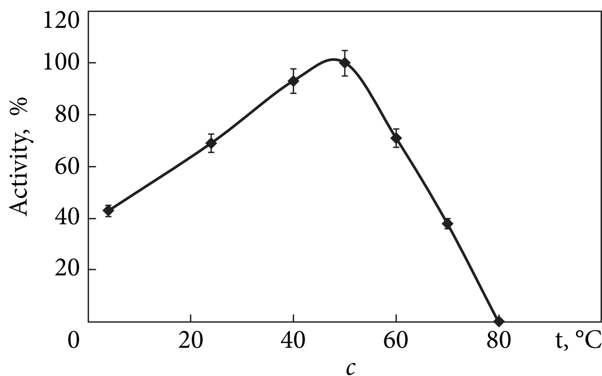


Fig. 5. Thermal optimum of action of elastase preparations from *Bacillus* sp. L1 (*a*), L2 (*b*), L 9 (*c*). Activity is expressed as % of the maximum activity

at 28 °C, the enzyme activity of *Bacillus* sp. L1 and L2 culture liquid supernatants was twice that of the activity measured during cultivation at 42 °C, whereas the enzyme activity of *Bacillus* sp. L9 culture liquid supernatant was 1.4 times higher than that measured during cultivation at 42 °C. On the second day of cultivation (Figs. 1 and 2), similar dynamics was observed. The elastase activity in the supernatant of the culture liquid of *Bacillus* sp. L1 doubled and for *Bacillus* sp. L2 and L9 increased by 1.33 and 1.06 times, respectively, when cultivated at lower temperatures. On the third day of cultivation, the same pattern remained: elastase activity in the supernatant of the culture liquid of *Bacillus* sp. L1, L2, and L9 was higher by 2.25, 1.33, and 1.19 times, respectively, when grown at 28 °C than at 42 °C.

The level of aeration of the nutrient medium plays a significant role in the biosynthesis of elastase by the studied cultures of *Bacillus* sp. (Table 1). Thus, the highest elastase activity of 35.80, 33.80, and 28.00 U/mL for *Bacillus* sp. L9, L2, and L1, respectively, appeared when the three strains were grown in 100 mL of culture medium.

It was shown (Fig. 3) that for maximum elastase synthesis, effective aeration is also necessary: at higher rotation speeds of the shaker, the activity was significantly higher. Thus, at a speed of 230 rpm, the activity was 3 times higher than at 201 rpm for the *Bacillus* sp. L9 strain, 1.86 times for the L1 strain, and 1.6 times for the L2 strain.

An important characteristic of enzyme preparations is the optimal conditions for their action such as pH and temperature. To study these parameters, we obtained partially purified complex enzyme preparations of *Bacillus* sp. L1, L2, and L9 and showed (Fig. 4) that they differ in pH optimum and pH range. The enzyme preparation of *Bacillus* sp. L1 actively degrades elastin in the neutral and alkaline pH zone with an optimum effect at pH 8.0 (Fig. 4, *a*). *Bacillus* sp. L2 is active in the pH range from 7.0 to 11.0 with a pH optimum of 10.0 (Fig. 4, *b*), and the complex enzyme preparation of *Bacillus* sp. L9 (Fig. 4, *c*) has a pH optimum of 9.0, but at pH 8.0 and 10.0, it exhibited 85 and 90% activity, respectively.

A study of optimal temperatures for elastin hydrolysis showed that enzyme preparations of *Bacillus* sp. L1 and L2 (Fig. 5, *a, b*) are active in the range from 4 to 60 °C, and the preparation of *Bacillus* sp. L9 (Fig. 5, *c*) — from 4 to 70 °C. In this case, the temperature optimum for elastin hydrolysis with preparations L1 and L2 is at 40 °C (Fig. 5, *a, b*) and with preparation L9 — at 50 °C (Fig. 5, *c*).

Thus, it was shown that the maximum synthesis of elastase occurs on the second day of cultivation of the producer at 28 °C and rotation of the shaker at 230 rpm. Maximum hydrolysis of elastin by *Bacillus* sp. L1 and L2 takes place at a temperature of 40 °C but at different pH optima. Thus, for the preparation of *Bacillus* sp. L1, optimal pH is 8, and for L2 — 10. As for the complex enzyme preparation of *Bacillus* sp. L9, the maximum hydrolysis of elastin occurs at pH 9 and temperature 50 °C.

Discussion. In biotechnological processes involving enzymes, some parameters are important, in particular their synthesis in a shorter cultivation period, as well as a lower temperature for growing the producer. The results obtained demonstrate that, according to these two indicators, all three studied strains are promising: they synthesize the enzyme already on the second day of cultivation, and it is more active when grown at a lower (28 °C) rather than at a higher temperature (42 °C). It is known (Zhou et al., 2013) that the optimal growing temperature varies among different elastase-producing strains. The authors (He et al., 2004), having studied the influence of different growing temperatures of *Bacillus* sp. EL31410 from 39 °C to 28 °C, showed that the maximum elastase activity was obtained when the cells were cultivated at 30 °C.

Partially purified enzyme preparations differ somewhat in the temperature optimum for elastin hydrolysis: 40 °C — for *Bacillus* sp. L1 and L2, and 50 °C — for *Bacillus* sp. L 9. This difference in the optimal operation conditions is pos-

sibly due to the difference in the mechanisms of binding of the enzyme to the substrate, as well as changes under the influence of temperature in the conformation of both the enzyme protein molecule and the substrate.

Analysis of the data obtained gives us a possibility to believe that of the three studied strains, the most promising for further research is *Bacillus* sp. L9, whose elastase activity is maximum on the second day of cultivation and is 35.80 U/mL. The maximum hydrolysis of elastin occurs at pH 9 and temperature 50 °C. According to some parameters, a partially purified enzyme preparation with elastase activity of the *Bacillus* sp. L9 is similar to those described in the literature for the strains of *Bacillus subtilis* AKAL7 and *Exiguobacterium indicum* AKAL1 (Emon et al., 2020). The optimal temperature, initial pH of the media, and shaking speed for protease production by these strains were 30 °C, pH 9.0, and 120 rpm, respectively. The optimum temperature and pH of the partially purified protease from both strains was 40 °C and pH 9.0, respectively. Protease from these strains was stable at pH 7.0—12.0 and temperatures up to 50 °C (Hakim et al., 2018; AlShaikh-Mubarak et al., 2023). The best reaction pH for gasm32 elastase was determined to be 8.0, which is the same as for *P. aeruginosa* and the *B. megaterium*-TK1 strain isolated from saltwater. This optimal pH was different from those obtained for other elastases in other studies, such as the alkaline elastase from *Micrococcus luteus* (pH 9.3), *B. megaterium* (pH 7.5), *P. aeruginosa* ZuhP13 (pH 7.5), and the alkalophilic *Bacillus* strain Ya-B (pH 11.75) (AlShaikh-Mubarak et al., 2023).

37 °C was the optimal reaction temperature for the elastase of *C. indologenes*, 40 °C — for the serine elastase of *P. aeruginosa* ZuhP13, 60 °C — for *Bacillus* Ya-B elastase, and the temperature range of 57—59 °C is optimal for the elastase of *Micrococcus luteus* (AlShaikh-Mubarak et al., 2023).

Thus, the results of our research and analysis of literature data indicate that elastases of different strains of microorganisms differ in such parameters as pH and thermal optimum.

Our data on the elastolytic activity of *Bacillus* sp. L9 are promising for continued research into its properties for further practical use.

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ЕЛАСТАЗНА АКТИВНІСТЬ ПРЕДСТАВНИКІВ РОДУ *BACILLUS*,
ІЗОЛЬОВАНИХ З ПРИБЕРЕЖНОЇ ЗОНИ КІНБУРНСЬКОЇ КОСИ

Раніше еластазна активність була виявлена у деяких мікроорганізмів і представлена металопротеазами, які можуть відігравати важливу роль у сприянні інвазивності бактерій та встановленні інфекції. Еластази видів *Bacillus* є сериновими протеазами. Проте на сьогодні не багато повідомлень щодо систематичного вивчення продуцентів еластолітичних ензимів у представників *Bacillus,* а також досліджень їх властивостей. **Метою** даної роботи було вивчення деяких фізико-хімічних характеристик частково очищених ферментів з еластазною активністю ряду штамів *Bacillus* sp. **Методи.** Об'єктами дослідження були штами *Bacillus* sp. (L1, L2, L9), виділені із сухої трави прибережної зони Кінбурнської коси (Миколаївська обл.). Культури вирощували в умовах глибокого культивування при 28 °С, зі швидкістю перемішування живильного середовища 230 об/хв протягом 2 діб. Використовували методи визначення еластолітичної активності в супернатанті культуральної рідини. **Результати.** Показано, що *Bacillus* sp. L9, L1 та L2 характеризуються високим рівнем еластазної активності (відповідно 35,80, 28,0 та 33,80 од/мл). Максимальний синтез еластази відбувається на другий день культивування продуцента при 28 °С та за обертання шейкера 230 об/хв. Максимальний гідроліз еластину *Bacillus* sp. L1 і L2 відбувається за температури 40 °С, але при різних оптимумах pH. Так, для ензимного препарату *Bacillus* sp. L1 оптимальним є pH 8, а для L2 — pH 10. Максимальний гідроліз еластину комплексним ферментним препаратом *Bacillus* sp. L9 відбувається при pH 9 і температурі 50 °С. **Висновки.** Штам *Bacillus* sp. L9 з еластазною активністю є перспективним для продовження досліджень його властивостей щодо подальшого практичного використання.

Ключові слова: *Bacillus sp., Кінбурнська коса, еластазна активність, оптимум pH, термооптимум.*