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SCREENING OF MARINE SPORE-FORMING BACTERIA DEGRADING POLYMER MATERIALS

The development of plastic recycling methods requires the search for new microorganisms capable of their biodegradation. The ***aim*** *of the work was to screen spore-forming bacteria isolated from bottom sediments of the Black Sea for their ability to decompose Impranil and polyethylene terephthalate.* ***Materials and methods.*** *Cultivation of sixty cultures of spore-forming bacteria isolated from the Black Sea was carried out on a solid LB medium supplemented with Impranil (3–4 ml/l) or bis(hydroxyethyl)terephthalate (BHET) (5 mM). The ability of cultures to decompose polymer additives was evaluated by the formation of a transparent zone around the colonies after incubation at 30 ºC and 37 ºC for 14 days.* ***Results.*** *Out of 60 strains, 40 showed a positive result. 22 strains were active towards both plastics, 13 – only towards Impranil, 5 – only BHET. Impranil was decomposed somewhat more actively at 30 °C than at 37 °C. No clear temperature dependence was found in BHET decomposition. The analysis of the species composition showed that 35 of the 40 active strains belonged to seven species of microorganisms. B. subtilis has the highest share of active strains, as this species is represented by 13 strains, each of which was found to be active. The most active strain belonged to the species B. reuszeri.* ***Conclusions****. Spore-forming bacteria isolated from the Black Sea are capable of degrading Impranil and BHET. Enzymes degrading above mentioned polymers are the most widespread among the representatives of the species B. subtilis, B. atrophaeus and B. reuszeri. The highest activity towards Impranil showed B. subtilis, towards BHET– P. megaterium, B. reuszeri and B. licheniformis. Impranil degradation was more active under 30 ºC, but no clear dependence between temperature and BHET degradation was detected.*

Key words: polyethyleneterephthalate, Impranil, biodegradation, spore-forming bacteria, Black Sea.

Due to their remarkable properties, various types of plastic are actively used in many areas of human life. Polyethylene terephthalate or PET is one of the most popular types of plastic, utilized in the production of food [17] and pharmaceutical [9, 22] containers, as well as in the textile industry [13]. However, a significant part of used plastics is not recycled and is discarded into the environment, where it degrades into microplastics which contaminate soils and waters, enter trophic chains, and harm animals and humans [7]. Reducing PET production in the near future is unlikely [11], which creates an urgent need for developing technologies to enhance

the value of used PET waste and combat plastic pollution [23]. Biodegradation can solve the problem from two sides – creating more efficient, energy- and resource-saving methods of PET recycling on the one hand, and theoretically enabling the cleanup of plastic-contaminated ecosystems on the other [4, 12].

PET hydrolases represent the most studied class of enzymes [6]; however, the full extent of PET hydrolase gene distribution remains unknown, particularly among the Firmicutes phylum. PET hydrolytic activity has been demonstrated by p-nitrobenzyl esterase isolated from *Bacillus subtilis* [20]. Consortia from oil-contaminated sites in Texas, comprising mixtures of *Pseudomonas* and *Bacillus* representatives (*B. thuringiensis* C15 and *B. albus* PFYN01), reduced the weight of granular PET [21]. Isolates of *B. cereus* and *B. gottheilii* obtained from coastal mangrove areas exhibited weak PET degradation abilities [3]. A bacterium *B. safensis* YX8, isolated from the soil surface of PET waste, degraded PET nanoparticles [26]. *B. licheniformis* demonstrated the ability to form biofilms on PET surfaces and modify its chemical structure [15].

Given this information, spore-forming bacteria exhibit significant potential for the degradation of synthetic polymers, a field that remains underexplored. Of particular interest are microbial communities in marine environments, as it has been established that marine microorganisms can form biofilms on plastic surfaces [8]. Data on similar properties of Black Sea bacteria are quite limited.

The aim of this study was to screen spore-forming bacteria isolated from Black Sea sediments for their ability to decompose polyethylene terephthalate and Impranil.

Materials and Methods

The study utilized 60 strains of spore-forming bacteria, isolated in previous research from deep-sea sediment samples of the Black Sea and identified (Tables 1, 2) by their morphological, cultural, physiological, and biochemical characteristics, as well as by comparing their fatty acid profiles using the MIDI Sherlock microbial identification system on an Agilent 7890 gas chromatograph equipped with a flame ionization detector [1].

To evaluate the ability of the studied cultures to degrade plastics, nutrient media containing Impranil and BHET were used. Impranil is commonly employed to assess the capability of microorganisms to degrade polymers, particularly polyurethane [5]. BHET consists of one terephthalic acid residue and two ethylene glycol residues, i.e., PET monomers, making it a convenient substrate for detecting PET-hydrolytic activity [25].

The base for the plastic-containing media was solid LB medium (lysogeny broth), which was sterilized by autoclaving, cooled to 50–60 °C, and supplemented with the polymers separately under constant stirring using a magnetic stirrer: 3–4 mL of Impranil emulsion per 1 liter of LB or a previously prepared BHET solution to a final concentration of 5 mM [25]. Experiments with each type of plastic were conducted independently. The BHET solution was prepared as follows: a 1 M stock solution of BHET was made in dimethyl sulfoxide, heated to 60 °C, and stirred until the substrate was completely dissolved. The prepared media were poured into Petri dishes: approximately 20 mL per dish with a diameter of 92 mm.

Table 1

The species composition of the studied cultures

Cypher	Species name	Cypher	Species name
B1	*Priestia megaterium* 03	B31	*Bacillus atrophaeus* 200
B2	*Unknown* B	B32	Unknown 222
B3	*Bacillus pumilus* 049	B33	*Bacillus subtilis* 203
B4	*Priestia megaterium* 052	B34	Unknown A05
B5	*Bacillus licheniformis* 048	B35	*Bacillus subtilis* 1223
B6	*Shouchella clausii* 046	B36	Unknown 045
B7	*Shouchella clausii* 050	B37	Unknown 79.2
B8	*Brevibacillus reuszeri* 031	B38	*Lysinibacillus sphaericus* 66
B9	*Bacillus pumilus* 229	B39	Unknown 801
B10	*Brevibacillus reuszeri* 039	B40	*Sutcliffiella halmapala* 9
B11	*Bacillus subtilis* 247	B41	*Bacillus subtilis* 231
B12	*Bacillus licheniformis* 012	B42	*Brevibacillus reuszeri* 44
B13	*Priestia megaterium* 036	B43	*Metabacillus idriensis* 2
B14	*Priestia megaterium* 42	B44	*Bacillus licheniformis* 240
B15	*Bacillus licheniformis* 1	B45	*Bacillus licheniformis* A
B16	*Priestia megaterium* 116	B46	Unknown 043
B17	*Priestia megaterium* 98	B47	Unknown 043.1
B18	*Bacillus licheniformis* 026	B48	Unknown 117
B19	*Bacillus licheniformis* 014	B49	*Shouchella clausii* 020
B20	*Priestia megaterium* 55	B50	*Priestia megaterium* 122
B21	*Bacillus subtilis* 053	B51	*Shouchella clausii* 07
B22	*Bacillus subtilis* 211	B52	*Bacillus subtilis* 231
B23	*Unknown* 040	B53	*Bacillus subtilis* 217
B24	*Priestia megaterium* 054	B54	*Bacillus subtilis* 021
B25	*Priestia megaterium* 233	B55	*Priestia megaterium* 051
B26	*Bacillus subtilis* 204	B56	*Bacillus atrophaeus* 200
B27	*Bacillus subtilis* 212	B57	*Alkalihalobacillus clausii* 07
B28	Unknown 239	B58	*Robertmurraya siralis* 57
B29	*Bacillus subtilis* 1	B59	*Bacillus pumilus* A
B30	*Bacillus subtilis* B	B60	*Bacillus* sp. 1222

The test microorganisms were streaked on meat-peptone agar (MPA) to obtain 24-hour cultures. The cultures were cultivated at 30 °C for 24 hours. The following day, each culture was inoculated onto the plastic-containing media using inoculation loop. The cultures were incubated at 30 °C and 37 °C for 14 days. All inoculations were performed in duplicate for each culture and temperature condition.

The results were assessed by observing the formation of transparent zones in the media surrounding active colonies, and their widths were measured. This analysis provided information about the potential of microorganisms to degrade the selected polymers [18]. Cultures forming transparent zones larger than 3 mm in at least one experiment were considered highly active, those with zones between 2 and 3 mm were considered moderately active, and those with zones of 1.5 mm or less were deemed low-activity cultures. This grading scale was chosen arbitrarily, as the study aimed to conduct a preliminary evaluation of Black Sea bacteria's ability to degrade synthetic polymers and to identify the most active cultures.

Results and Discussion

Degradation of Impranil and BHET by the studied microorganisms

Out of the 60 strains tested for their ability to hydrolyze Impranil and BHET, a total of 40 showed positive results. The list of active cultures is presented in Table 2. Among these, 22 strains were active against both types of plastics, 13 strains exhibited activity only towards Impranil, and 5 strains showed activity exclusively against BHET.

Highly active cultures include 11 strains (B1, B6, B8, B10, B11, B19, B27, B52, B53, B54, B56), with moderate activity – 17 strains (B2, B3, B5, B12, B13, B16, B17, B18, B21, B22, B26, B28, B29, B30, B31, B33, B35), while low-activity cultures include 12 strains (B7, B9, B20, B23, B25, B39, B40, B41, B44, B55, B59, B60). The ratio of active to inactive strains is illustrated in Figure 1.

Formation of transparent zones around colonies of active cultures is shown in Fig. 2.

The species composition of bacteria capable of degrading Impranil and BHET

The species composition of active cultures was analyzed: 35 out of 40 active cultures belonged to seven types of microorganisms: *Priestia megaterium* (B1, B11, B13, B16, B17, B20, B25, B55); *Shouchella clausii* (B6, B7); *Brevibacillus reuszeri* (B8); *Bacillus licheniformis* (B5, B12, B18, B19, B44); *Bacillus pumilus* (B3, B9, B59); *Bacillus subtilis* (B21, B22, B26, B27, B29, B30, B33, B35, B41, B52, B53, B54); *Bacillus atrophaeus* (B31, B56); Bacillus sp. (B60); *Sutcliffiella halmapala* (B40). The species of five more strains is unknown.

The number of active cultures among representatives of each species is presented in Table 3. All studied *B. atrophaeus* cultures exhibited the ability to degrade both types of polymer compounds. However, since only two strains of this species were included in the study, the sample size does not allow these results to be considered reliable. It can be assumed that the ability to degrade polymers was most common among *B. subtilis* isolates. All cultures of this species were capable of degrading Impranil, and 77% could degrade BHET. Additionally, 77%

Table 2

The size of the transparent zones around colonies of active strains, mm

№	Species affiliation	Cypher	Degradation of Impranil		Degradation of BHET	
			30 °C	37°C	30 °C	37°C
1	***Priestia megaterium* 03***	**B1**	**2.5**	**-**	**4**	**1**
2	*Unknown* B	B2	1.5	2.0	-	-
3	*Bacillus pumilus* 049	B3	2.0	2.0	-	-
4	*Bacillus licheniformis* 048	B5	2.5	1.5	-	-
5	***Shouchella clausii* 046**	**B6**	**3.5**	**2.5**	**-**	**-**
6	*Shouchella clausii* 050	B7	1.0	1.5	1,5	-
7	***Brevibacillus reuszeri* 031**	**B8**	**2.5**	**1.0**	**1**	**4.5**
8	*Bacillus pumilus* 229	B9	1.0	-	-	-
9	***Brevibacillus reuszeri*** 039	**B10**	**2.0**	**2.5**	**3.0**	**4.5**
10	***Bacillus subtilis* 247**	**B11**	**4.5**	**1.5**	**-**	**-**
11	*Bacillus licheniformis* 012	B12	-	-	2.0	-
12	*Priestia megaterium* 036	B13	2.0	2.5	-	-
13	*Priestia megaterium* 116	B16	2.0	-	0.5	2.5
14	*Priestia megaterium* 98	B17	-	2.0	-	-
15	*Bacillus licheniformis* 026	B18	2.5	-	1	1
16	*Bacillus licheniformis* 014	**B19**	**4.0**	**-**	**3.5**	**0.5**
17	*Priestia megaterium* 55	B20	1	1.5	-	-
18	*Bacillus subtilis* 053	B21	1	2	1	1
19	*Bacillus subtilis* 211	B22	2	2.5	1	1
20	*Unknown* 040	B23	1	-	-	0,5
21	*Priestia megaterium* 233	B25	-	-	-	1
22	*Bacillus subtilis* 204	B26	2.5	2.5	1.5	1.5
23	***Bacillus subtilis* 212**	**B27**	**3.5**	**1.5**	**-**	**1**
24	Unknown 239	B28	-	2	-	-
25	*Bacillus subtilis* 1	B29	2.5	2	1	-
26	*Bacillus subtilis* B	B30	1	2.5	1	1
27	*Bacillus atrophaeus* 200	B31	2	2.5	1	1.5
28	*Bacillus subtilis* 203	B33	2	2	1	1.5
29	*Bacillus subtilis* 1223	B35	2	1	1	0.5
30	Unknown 801	B39	-	-	-	1
31	*Sutcliffiella halmapala* 9	B40	-	-	-	1
32	*Bacillus subtilis* 231	B41	-	1.5	-	-
33	*Bacillus licheniformis* 240	B44	1	1	-	-
34	***Bacillus subtilis* 231**	**B52**	**7**	**-**	**1**	**1**
35	***Bacillus subtilis* 217**	**B53**	**4**	**-**	**-**	**-**
36	***Bacillus subtilis* 021**	**B54**	**5**	**-**	**1**	**1**
37	*Priestia megaterium* 051	B55	0.5	1	1	-
38	***Bacillus atrophaeus* 200**	**B56**	**4**	**-**	**1**	**1**
39	*Bacillus pumilus* A	B59	1	-	-	-
40	*Bacillus* sp. 1222	B60	-	-	1	-

Note: * – the most active strains are shown in bold.

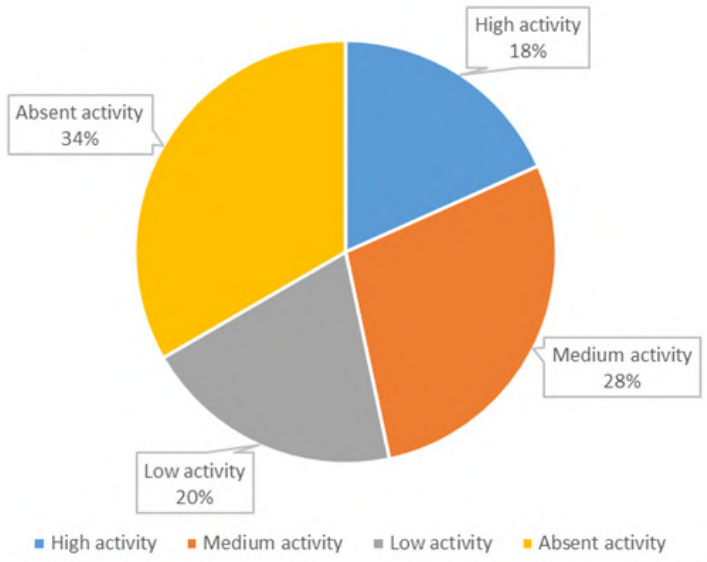

Fig. 1. The ratio of strains with high, medium, low and absent activity among 60 studied strains

of these cultures degraded both types of plastics. In general, representatives of six species (*P. megaterium, S. clausii, B. reuszeri, B. licheniformis, B. subtilis,* and *B. atrophaeus*) were able to degrade both Impranil and BHET. Cultures of *Bacillus pumilus* degraded only Impranil, while *S. halmapala* and *Bacillus sp.* degraded only BHET, but due to the small number of strains representing these species, these data cannot be considered reliable. Among the 11 cultures whose species affiliation could not be established, 3 were active against Impranil, and 2 against BHET. One culture exhibited activity against both polymers. However, according to the data of Table 2, this activity was low.

Various studies [3, 13, 20] describe the ability of *Bacillus* species to degrade polyethylene terephthalate (PET), which is confirmed by the results obtained in this study. Previous researches [3, 15, 20, 21, 26] indicate that the *Bacillus* genus is among the most common carriers of PET-degrading enzymes among the Firmicutes phylum. This is also confirmed by our data, as members of the *Bacillus* genus accounted for the largest proportion (34%) of the 40 active cultures. The *p*-nitrobenzyl esterase BsEstB produced by *B. subtilis* has been described [20]. It is possible that this enzyme is responsible for the degradation of both Impranil and BHET.

The ability of *P. megaterium* to biodegrade polystyrene, which, like PET and BHET, contains a benzene ring, has been demonstrated [16]. Additionally, *P. megaterium* was identified in consortium "No. 46," which exhibited PET-degrading activity [24]. While *P. megaterium* is known to form biofilms on plastic surfaces, according to the obtained results, it may also participate directly in biodegradation by breaking down PET degradation products, such as BHET.

B. licheniformis has previously been studied for its ability to degrade polylactic acid [2]. However, based on our data, it can be assumed that this species might also have the potential to degrade PET.

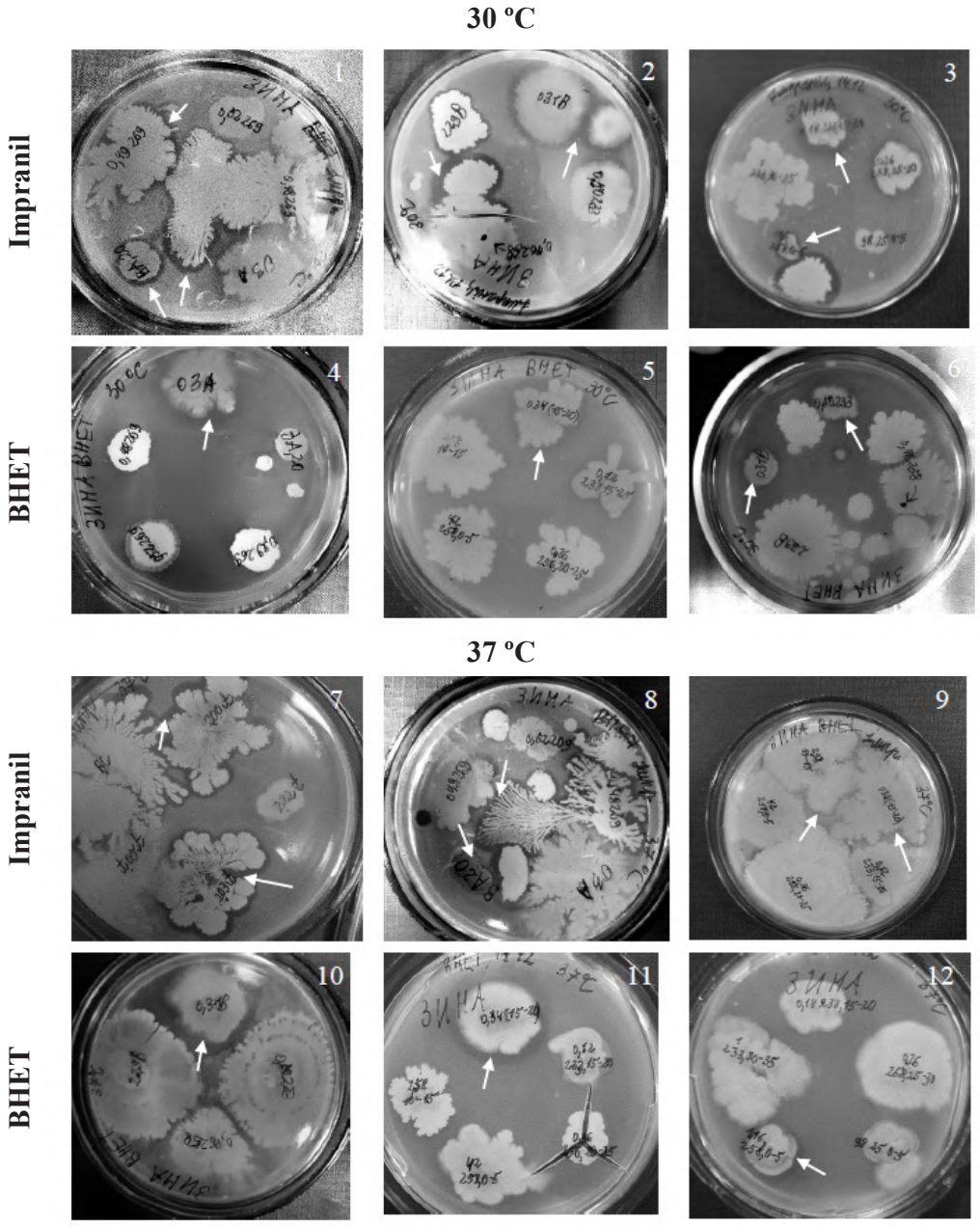

Fig. 2. Decomposition of Impranil and BHET by the studied cultures

Note: arrows indicate transparent areas around active cultures:
1 – B2 (BA: 1.5 mm), B3 (049D: 2 mm), B5 (048D: 2.5 mm);
2 – B6 (046C: 3.5 mm), B8 (031B: 2.5 mm); 3 – B19 (014A: 4 mm), B16 (116C: 2 mm);
4 – B1 (03A: 4 mm); 5 – B10 (039: 3.5 mm); 6 – B7 (050A: 1.5 mm), B8 (031B: 1 mm);
7 – B31 (200A: 2.5 mm), B33 (203A: 2 mm); 8 – B2 (BA: 2 mm), B5 (048D: 1.5 mm);
9 – B10 (039: 2.5 mm), B11 (247: 1.5 mm); 10 – B8 (031B: 4.5 mm);
11 – B10 (034: 4.5 mm); 12 – B16 (116C: 2.5 mm)

Table 3

The share of active cultures among representatives of different species

Species	Cultures that were studied	The number of active against Impranil	The number of active against BHET	The number of active against BHET and Impranil
Priestia megaterium	11	6	4	3
Shouchella clausii	4	2	1	1
Brevibacillus reuszeri	3	2	2	2
Bacillus licheniformis	7	4	3	2
Bacillus pumilus	3	3	-	-
Bacillus subtilis	13	13	10	10
Bacillus atrophaeus	2	2	2	2
Bacillus sp.	1	-	1	-
Sutcliffiella halmapala	1	-	1	-
Alkalihalobacillus clausii	1	-	-	-
Robertmurraya siralis	1	-	-	-
Lysinibacillus sphaericus	1	-	-	-
Metabacillus idriensis	1	-	-	-
Unknown	11	3	2	1

B. reuszeri is known for its wide enzymatic activity spectrum and its ability to degrade polyethylene and polyurethane [10]. Polyurethane, like PET, contains a benzene ring. Given that *B. reuszeri* can utilize BHET, it is plausible that it may also be capable of degrading PET.

Regarding *S. clausii*, *B. atrophaeus*, and *B. pumilus*, data on their polymer degradation capabilities are limited. However, based on the obtained results, it can be inferred that these three species are more inclined to degrade Impranil than BHET. As for *S. halmapala*, only low activity against BHET was observed.

Temperature dependence of culture activity

In general, Impranil was more actively degraded at 30 °C than at 37 °C, although the data are insufficient to establish a clear correlation (Fig. 3). Specifically, the activity of eleven cultures increased with a rise in cultivation temperature, while for sixteen cultures, activity was higher at 30 °C. For three strains, activity was independent of temperature. A total of twenty-one cultures degraded Impranil at both temperatures, 11 strains were active only at 30 °C, and three strains only at 37 °C.

No clear dependence of BHET degradation efficiency on temperature was observed (Fig. 4). For ten cultures, activity increased with a rise in temperature, while for seven, activity was higher at 30 °C. For another seven cultures, activity was independent of temperature. Sixteen cultures degraded BHET at both temperatures, five only at 30 °C, and five exclusively at 37 °C.

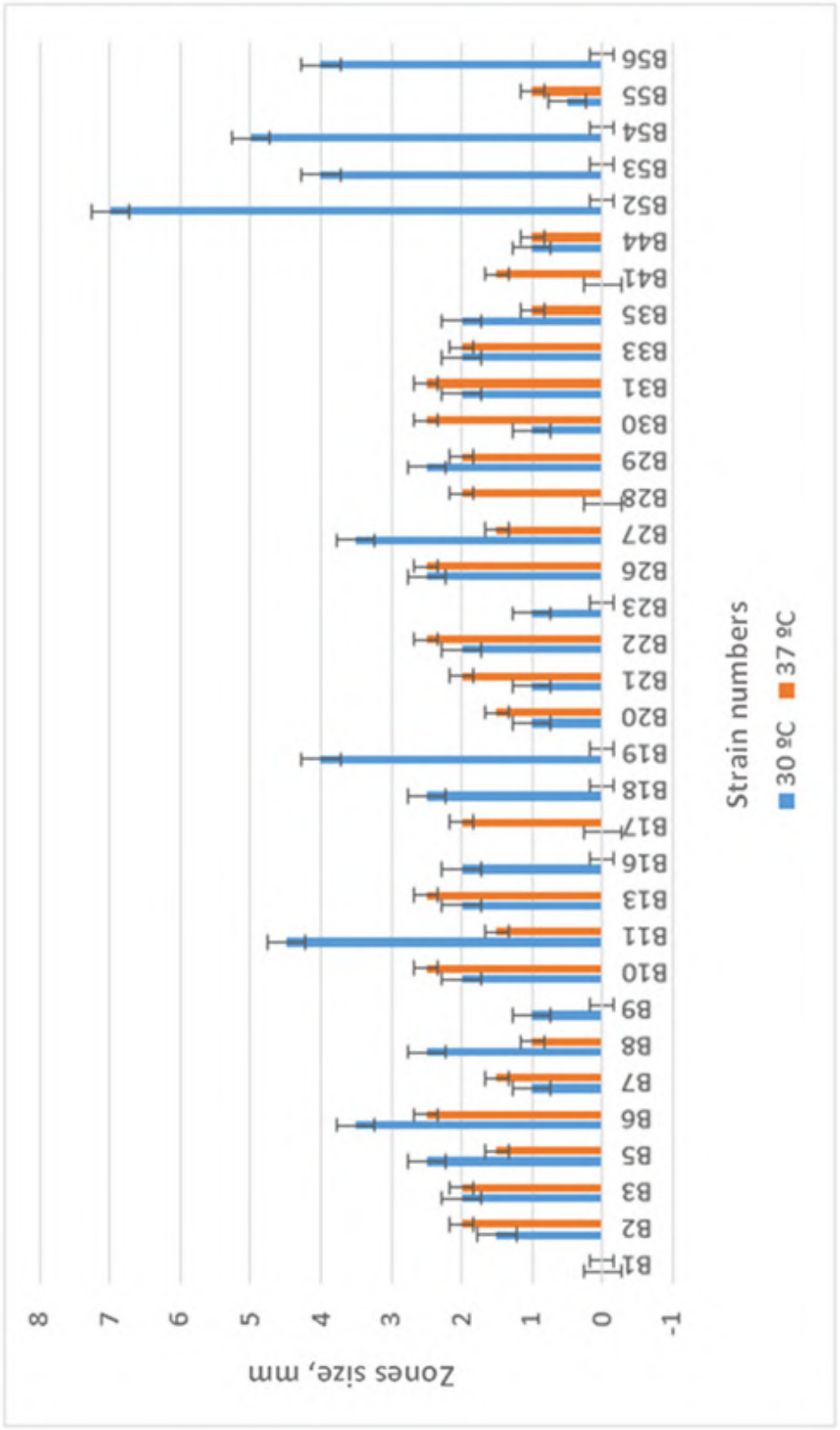

Fig. 3. Dependence of Impranil decomposition on temperature

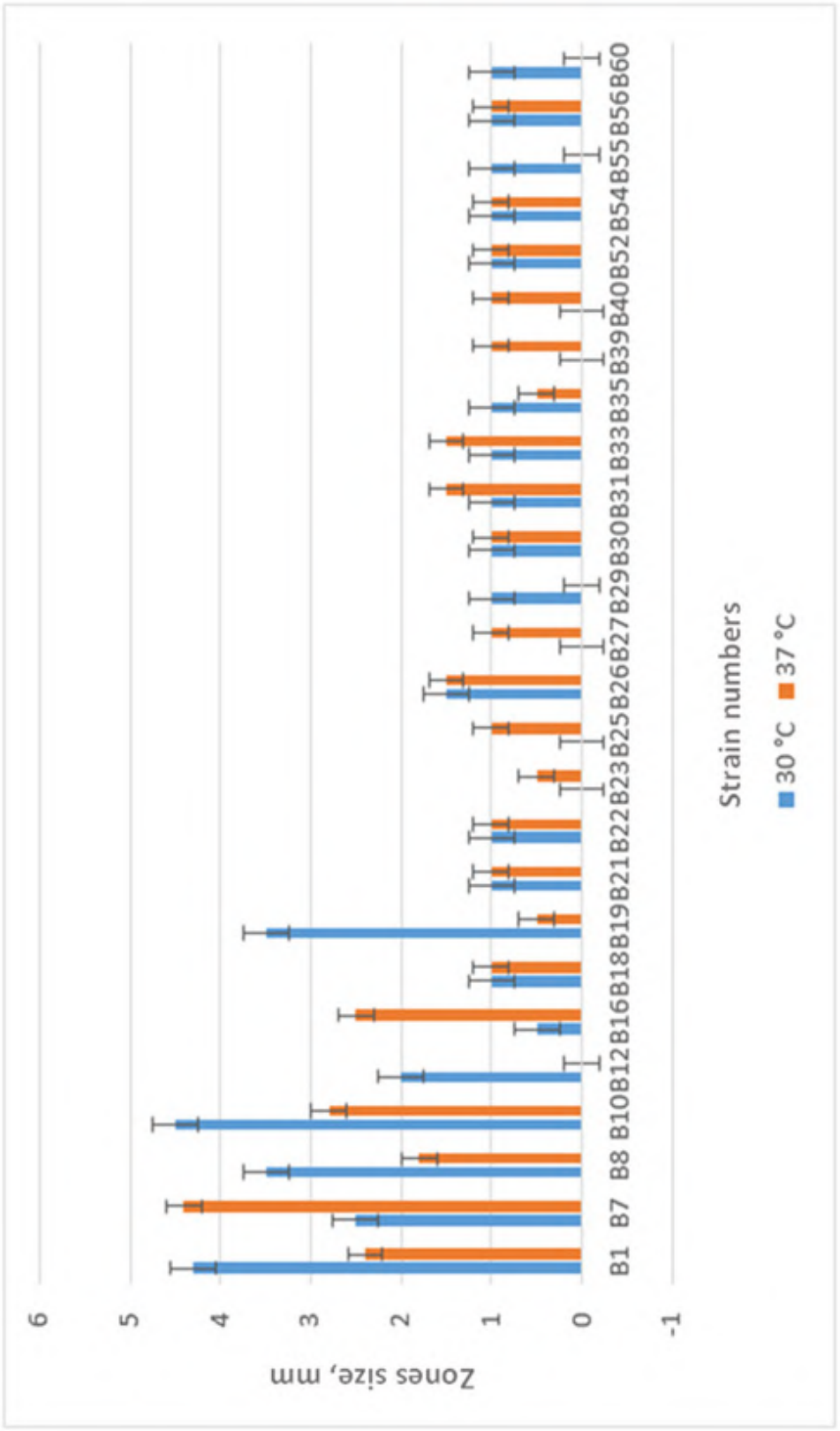

Fig. 4. Dependence of BHET decomposition on temperature

Thus, spore-forming bacteria isolated from the Black Sea sediments exhibit potential in the biodegradation of polymeric compounds, particularly PET and Impranil. Enzymes responsible for the degradation of these polymers are most common in representatives of *B. subtilis*, *B. atrophaeus*, and *B. reuszeri*. The highest activity against Impranil was observed in strains of *B. subtilis*, while *P. megaterium*, *B. reuszeri*, and *B. licheniformis* demonstrated the most significant activity against BHET, which was used as an indicator of PET degradation enzymes. Impranil was generally degraded more effectively at 30 °C, whereas no clear temperature-dependent correlation was observed for BHET. These results provide a foundation for further studies and suggest the potential of Black Sea sediment bacteria as polymer degraders, as their enzymatic systems remain active at relatively low temperatures, offering the possibility of developing energy-efficient plastic biodegradation technologies.

The studied bacteria are facultative anaerobes; however, their polymer-degrading ability was tested only under aerobic conditions. Conducting similar experiments under anaerobic conditions could provide more data into the mechanisms of plastic biodegradation under natural conditions.

The experiment duration was compared with other studies. In experiments focused on PET biodegradation, strains of *Pseudomonas* and *Bacillus* were cultivated on LB media with Rhodamine B to test lipolytic activity, with cultivation lasting 24 hours. For general hydrolytic and polyesterase activity, agar plates with tributyrin were used, with cultivation lasting five days [19]. Impranil degradation using purified enzymes lasted for 24 hours [5], while an esterase from *Enterobacter sp.* degraded approximately 80% of BHET in 120 hours [19]. Thus, 14 days is sufficient for the enzymes of active strains to diffuse through the agar and react with Impranil and BHET.

The obtained data indicate a significant potential of the Black Sea bacteria in the decomposition of polymer compounds. Future research should focus on screening a broader range of spore-forming bacteria and other microbial groups from the Black Sea and investigating the nature of the degradation products.

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СКРИНІНГ ЧОРНОМОРСЬКИХ СПОРОУТВОРЮВАЛЬНИХ БАКТЕРІЙ ЩОДО ЗДАТНОСТІ ДО БІОДЕГРАДАЦІЇ ПОЛІМЕРНИХ МАТЕРІАЛІВ

Реферат

Розвиток методів утилізації пластиків потребує пошуку нових мікроорганізмів, здатних до їх біодеградації. ***Метою роботи*** *був скринінг спороутворювальних бактерій, виділених з донних осадів Чорного моря, щодо здатності до розкладання поліетилентерефталату та Імпранілу.* ***Матеріали і методи.*** *Культивування шістдесяти чорноморських спороутворювальних бактерій здійснювали на агаризованому живильному середовищі LB, у яке додатково вносили Імпраніл (3–4 мл/л) або біс(гідроксиетил)терефталат (БГЕТ) (5 мМ). Здатність культур до розкладання полімерних добавок оцінювали за утворенням навколо колоній прозорої зони після інкубації при 30 ºС та 37 ºС упродовж 14 діб.* ***Результати.*** *З 60 штамів 40 показали позитивний результат. 22 штами були активні щодо обох видів пластику, 13 – тільки щодо Імпранілу, 5 – виключно щодо БГЕТ. 35 штамів з 40 активних належали до семи видів мікроорганізмів. Найбільшу кількість активних штамів виявлено серед культур Bacillus subtilis. Найактивніша культура належала до виду Bacillus reuszeri. Імпраніл дещо активніше розкладався при 30 °С, ніж при 37 °С. Не знайдено чіткої залежності у розкладанні БГЕТ від температури.* ***Висновки.*** *Спороутворювальні бактерії, виділені з Чорного моря, здатні до деградації Імпранілу та БГЕТ. Найбільше ферменти, що викликають деградацію вказаних полімерів, поширені у представників видів Bacillus subtilis, Bacillus atrophaeus та Bacillus reuszeri. Найвищою активністю щодо Імпранілу характеризуються представники виду Bacillus subtilis, щодо БГЕТ– Priestia megaterium, Bacillus reuszeri та Bacillus licheniformis. Імпраніл ефективніше розкладається при 30 ºС, для БГЕТ чіткої залежності швидкості деградації від температури не знайдено.*

Ключові слова: морські спороутворювальні бактерії, Чорне море, полімерні матеріали, біодеградація.

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