BULGARIAN ACADEMY OF SCIENCES
INSTITUTE OF MICROBIOLOGY "STEPHAN ANGELOFF“

NIKOLINA ATANASOVA ATANASOVA

DEGRADATION OF PLASTICS BY THERMOPHILIC AND HALOPHILIC BACTERIA ISOLATED FROM BULGARIAN EXTREME NICHES

SUMMARY

On a dissertation for the award of the educational and scientific degree "Doctor"
In the field of higher education 4. Natural sciences, mathematics and informatics,
Professional direction: 4.3 Biological sciences (specialty Microbiology)

Supervisor: Prof. Margarita Kambourova, DSc

Members of the scientific juri:
Prof. Penka Petrova, DSc
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The dissertation contains in 180 pages, 38 figures and 23 tables. 187 literary sources are included in the bibliographic list. The experimental work was carried out in the laboratory "Extremophilic Microorganisms", "Stephan Angeloff" Institute of Microbiology, BAS.

The dissertation work was discussed and directed for defense on 28.03.2023 at an extended session of a National seminar on "General Microbiology" at “Stephan Angeloff” Institute of Microbiology, BAS.

The defense of the dissertation work will take place at an open meeting in the presence of the scientific jury on ..... from..... in the meeting hall of “Stephan Angeloff” Institute of Microbiology ", BAS.

The defense materials are available in the office of the scientific secretary of “Stephan Angeloff” Institute of Microbiology, BAS.

I express my gratitude to my scientific supervisor, Prof. Margarita Kamburova, for her support, patience, guidance and advice in carrying out the research and shaping the dissertation work.

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Abbreviations used:

AZCL - azurin crosslinked

C1-K – halophilic community from sample 1, cultivated in the absence of plastic

C1-PCL – halophilic community from sample 1 cultivated in the presence of polycaprolactone

C1-PVA – halophilic community from sample 1, cultivated in the presence of polyvinyl alcohol

C2-K – halophilic community from sample 2, cultivated in the absence of plastic

C2-PCL – halophilic community from sample 2 cultivated in the presence of polycaprolactone

C2-PVA – halophilic community from sample 2 cultivated in the presence of polyvinyl alcohol

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

GPC – gel permeation chromatography

HDPE - high density polyethylene

LDPE - low density polyethylene

MK-K – a community from Marikostinovo hot spring, cultivated in the absence of plastic

MK-PCL – a community from Marikostinovo hot spring, cultivated in the presence of polycaprolactone

MK-PS – a community from Marikostinovo hot spring, cultivated in the presence of polystyrene

NaLS – sodium lauryl sulfate

NBS – N-bromosuccinimide

PCB – polyhydroxybutyrate

PCL – polycaprolactone

PE – polyethylene

PES – polyethersulfone

PMSF – phenylmethylsulfonide fluoride

pNPB - para-nitrophenyl butyrate

pNPP – para-nitrophenyl palmitate

PP - polypropylene

PS – polystyrene

PVA – polyvinyl alcohol

PVC – polyvinyl chloride

SDS – sodium dodecyl sulfate

SEM/SEM – scanning electron microscope

Introduction

The massive production of plastics gradually leads to their entry into almost all areas of human life, making it easier, safer and more colorful. The amount of plastics produced increased more than twenty-fold between 1964 and 2020, it is expected to double by 2035 compared to 2020 and almost quadruple by 2050. The advantages of plastics such as lightness, low cost, high durability and inertness against many chemicals, physical agents and microbial attacks determine their diverse applications and contribute to economic growth. On the other hand, however, the inertness of the materials from which plastics are made allows them to remain intact for hundreds of years. Their difficult degradation and their rapid accumulation in the environment have led to public concern about their pollution. Plastics are one of the main reasons for the accumulation of ecologically polluting waste, the deterioration of the environment and climate change, the depletion of natural resources, the reduction of biodiversity, the increase of carcinogenic diseases in humans. The excessive use of plastics and the increasing pressure on landfills to dispose of plastic waste has determined the huge interest in the past few years in the possibility of their biodegradability. Many hopes for their recycling are placed on microorganisms. The extraordinary properties of extremozymes and the metabolic characteristics of their producers offer a new approach for the bioremediation of polluted extreme habitats or the development of new composting processes to contribute to solving one of the biggest societal problems, namely plastic pollution.

Purpose and tasks

A purpose of this dissertation is to study the ability of extremophilic bacteria isolated from Bulgarian extreme niches to effectively degrade different types of plastics.

Tasks:

1. Enrichment of microbial communities from hot springs and salt flats in environments with different types of plastic as the sole carbon source.

2. Screening of the extremophilic strains available in the laboratory collections (thermophiles and halophiles) for growth in nutrient media with different types of plastics as a carbon source.

3. Phylogenetic and molecular analysis of microbial communities.

4. Isolation of pure strains from the microbial communities.

5. Screening of microbial communities and isolates for esterase enzyme activity and selection of promising degrading strain for further research.

8. Optimization of the physical and chemical parameters for optimal microbial degradation of the selected plastics.

9. Determination of kinetic characteristics and efficiency of the degradation process in pure microbial culture and microbial community.

10. Evaluation of plastic surface changes and biofilm formation by scanning electron microscopy.

11. Purification and characterization of the properties of a degrading enzyme isolated from a selected plastic-active strain.

12. Determination of the mechanism of action of the degrading enzyme on the substrate.

Methods

1. Collection of samples from Bulgarian extreme niches

1.1. Hot spring sampling and community enrichment

Samples containing plastic residues (coffee cups, bottles, plates, children's toys, medicine packages) and waters near the plastic waste were collected from five Bulgarian hot springs located in southwestern Bulgaria: Rupi, Levunovo, Marikostinovo, Simitli and Dolno Osenovo.

1.2. Saline habitat sampling and culture enrichment

Two types of samples, lye and mud containing plastic residues, were collected from Atanasovsko Lake (Burgas Salterns) (33% salinity) and Pomorie Salterns (34% salinity) in sterile glass bottles and stored in a thermostat bags at 4ºC during transportation to the laboratory.

2. Determination of esterase activity

Enzyme activity was determined by measuring esterase activity. The hydrolysis of p-nitrophenyl palmitate (pNPP) as a substrate was determined spectrophotometrically. One unit of esterase activity was defined as the amount of enzyme required to release 1 μM p-nitrophenol in one minute under the conditions described. The molar extinction coefficient for p-nitrophenol at pH 7.5 was 3.39×10^3/M.

3. Determination of protein concentration

Protein content was determined by Lowry's method.

4. Metagenomic analysis of microbial communities

Metagenomic analyzes were performed at Eurofins Genomics Europe, Ebersberg, Germany. DNA was extracted with a commercial kit according to the manufacturer's instructions. 16S rRNA genes were PCR amplified from the extracted DNA using specific primers and analyzed by Amplicon sequencing on the Illumina MiSeq platform.

5. Scanning Electron Microscopy (SEM)

6. Isolation of pure strains from the thermophilic community and screening of the isolates

To isolate pure strains, each colony was spread in triplicate and single colonies with different appearance were selected for further work. A screening procedure for PCL-degrading bacteria was performed. Biodegradation activity was evaluated by the formation of clear zones around the growth spots.

7. Phylogenetic analysis of the isolated bacteria

Petri dishes containing pure strains were sent for phylogenetic analysis to Macrogen Europe BV, Amsterdam, Netherland, where the complete 16S rRNA gene sequences were

sequenced using universal bacterial primers 27F and 1492R. Isolates were sequenced and identified in Macrogen.

8. Optimization of PCL degradation parameters for thermophiles

8.1. Temperature optimum – it was measured in the region of 50-65°C.

8.2. The pH optimum – it was measured in the range of 6 to 9.

8.3. Influence of PCL concentration – it was determined at a substrate concentration of 0.1 to 0.7%.

9. Measurement of bacterial biomass

Measurement of optical density or microscopic counting in MK-PCL was hindered due to the formation of visible biofilms on the plastic surface although optical density was used in the initial stages of our work to evaluate the approximate growth. During the characterization of the biodegradation process, however, microbial growth was assessed by measuring the protein concentration in the culture fluid and in cells removed from the PCL surface after SDS treatment.

10. Measurement of gravimetric weight

The biodegradation of PCL was determined by a gravimetric method over 4 weeks. The weight loss of PCL pearls in one day (degradation rate) was calculated based on the decrease in their gravimetric weight according to the formula: $DR = (W_0 - W)/7$, where DR is the degradation rate, W_0 is the weight of pearls PCL at the end of the previous week (mg), W is the weight of the pearls at the end of the current week.

11. Determination of carbohydrate-degrading activity of B. thermoruber strain 7 on AZCL-substrates.

12. Gel-permeation chromatography

The products of PCL (Mw 14,000) degradation at different stages of the degradation process were analyzed by Gel Permeation Chromatography (GPC) analysis. Assays were performed at 45°C with an elution rate of 1.0 ml/min.

13. Enzyme purification

14. Characterization of the properties of the purified lipase

14.1. pH optimum – it was determined in the range of 6 to 9.

14.2. Temperature optimum – it was determined in the interval from 45 to 65°C.

14.3. Thermostability - thermostability was determined by the lipase pre-incubation for various times at 60°C in the absence or presence of 5 mM $CaCl_2$ and the residual activity was determined at 55°C.

14.4. Effect of some metal ions and inhibitors on the enzyme activity.

The effect of metal ions, EDTA, surfactants and inhibitors on the enzyme activity was investigated by incubating the samples for 30 min at room temperature with the selected chemicals (5 mM): $ZnSO_4.7H_2O$, KCl, $CoCl_2.6H_2O$, NaCl, $CaCl_2$, $FeCl_3.6H_2O$, $MnCl_2.4H_2O$, $MgCl_2.6H_2O$, $HgCl_2$, $CuSO_4.5H_2O$, sodium dodecyl sulfate (SDS), Tween 20, ethylenediamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Na- lauryl sulfate (NaLS) and N-bromosuccinimide (NBS). Residual lipase activity was at optimal conditions against a control containing no added chemicals.

15. Determination of the maximum velocity of the enzyme reaction (Vmax)

Results and discussion

1. Degradation of plastics by thermophilic microorganisms

1.1. Cultivation of the strains from the laboratory collection of thermophiles using different plastics as a carbon source

The strains available in the collection were initially grouped based on their phylogenetic proximity into eleven groups (Table 1).

Table 1. Thermophilic strains from the laboratory collection tested for growth and esterase activity in minimal medium with plastic as a sole carbon source

Group	Species, Number of strains	Control		PS		PP		PCL		PVA	
		OD	U/ml	OD	U/ml	OD	U/ml	OD	U/ml	OD	U/ml
1	*Bacillus licheniformis* – 5	0.4	0	0.6	208.3	0.4	291.6	0.4	129	0.5	137.5
2	*Geobacillus stearothermophilus* – 5	0.11	0	0.16	250	0.6	208	0.39	104.2	0.3	83.3
3	*Geobacillus thermodenitrificans* - 2	0.19	0	0.6	375	0.29	125	0.36	208	0.4	208
4	*Geobacillus* sp. – 3	0.22	0	0.7	246	0.21	83.3	0.33	166	0.4	125
5	*Brevibacillus thermoruber* – 22	0.16	0	0.14	0	0.11	0	0.3	125	0.1	0
6	*Anoxybacillus flavithermus* – 19	0.23	0	0.22	0	0.09	0	0.2	0	0.3	87.5
7	*Anoxybacillus gonensis* – 14	0.11	0	0.01	125	0.45	158.3	0.1	0	0.1	0
8	*Aeribacillus pallidus* – 6	0.22	0	0.21	0	0.11	0	0.11	0	0.12	41.7
9	*Desulfurococcus* - 2	0.26	0	0.23	0	0.24	0	0.09	0	0.09	0
10	*Hydrogenobacter* - 1	0.10	0	0.09	0	0.05	0	0.31	166	0.13	175
11	*Thermus* - 1	0.11	0	0.11	0	0.07	0	0.26	83.3	0.27	125

The determined enzyme activities for each type of plastic revealed highest values for PS of 375 U/ml, for PP –291.6, for PCL – 208, for PVA – 208. The highest activities were observed in the three groups strains belonging to the genus *Geobacillus*. Of particular interest was the high activity of the enzyme in groups 2 and 3 belonging to the species *Geobacillus thermodenitrificans* and *Geobacillus stearothermophilus* when bio-undegradable plastics PS and PP were used as substrates. The formation of a biofilm, which contributes to a close contact between the substrate and the esterase-synthesizing microorganism, is an important condition for the degradation of slowly degradable substrates such as plastics. No good film-forming ability was observed for the esterase-synthesizing thermophilic strains from the laboratory collection. The degradation of the plastic surface by them was carried out by the chance encounters with esterase molecules freely diffusing in the culture liquid, that determined the slow rate of degradation despite the recorded high enzyme activities.

Therefore, we hypothesized the possibility of isolating more efficient degraders from natural habitats containing plastic waste.

1.2. Characteristics of the sampling sites

Five springs in Southwest Bulgaria with water temperature from 46 to 72°C were selected for sampling (Table 2). This temperature interval coincides with the growth region of obligate thermophilic bacilli, growing between 50 and 70°C with an optimal temperature of 55-65°C (Bonch-Osmolovskaya and Atomi, 2015). Thermophilic microorganisms are most preferred for biotechnological use because of their faster growth, easier cultivation, and developed enzyme gene expression systems. The measured pH of the waters was neutral-slightly alkaline.

Table 2. Hot springs in Bulgaria from which samples were taken

Sample №	Hot springs for sampling	T°C	pH
1	Rupi	72	8.8
2	Levunovo	71	8.0
3	Marikostinovo	57	8.3
4	Simitli	46	7.1
5	Dolno Osenovo	55	7.2

1.3. Determination of growth and esterase activity in thermophilic communities from different springs

Microorganisms in the samples were enriched for 120 h of cultivation in 20 ml of minimal medium with the appropriate plastic. Growth (OD_{660}) and esterase activity were measured every 24 h. For undegradable plastics (PP and PS), the observed OD_{660} was not significantly different from that of the control (up to two-fold), due to the slow release of degradation products. In the case of biodegradable PCL and PVA, OD_{660} exceeded that of the controls up to 6 times (sample from Marikostinovo, growing on PCL) and 2.5 times (sample from Rupite, growing on PVA). Additionally, in some of the samples with plastics, dense growth on the surface of the plastic particles (Marikostinovo sample growing on PCL) or aggregate formation (Rupi sample growing on PVA) was observed (Table 3).

The esterase group and especially the lipase subgroup have been reported as major enzymes involved in the degradation of a number of plastics in which the main linkage in the molecule

is the ester (Pathak, 2017; Yu et al., 2009; Singh et al., 2014). Determination of the esterase activity of the samples was used to characterize the enzyme present in the culture fluids. Esterase activity was recorded in one of the samples for each type of plastic, except PCL, where esterase activity was reported in two of the samples. The sample from Marikostinovo was active in the degradation of PP, PS and PKL. The highest esterase activity was found in the Marikostinovo sample of PKL, which determined our further interest in this sample.

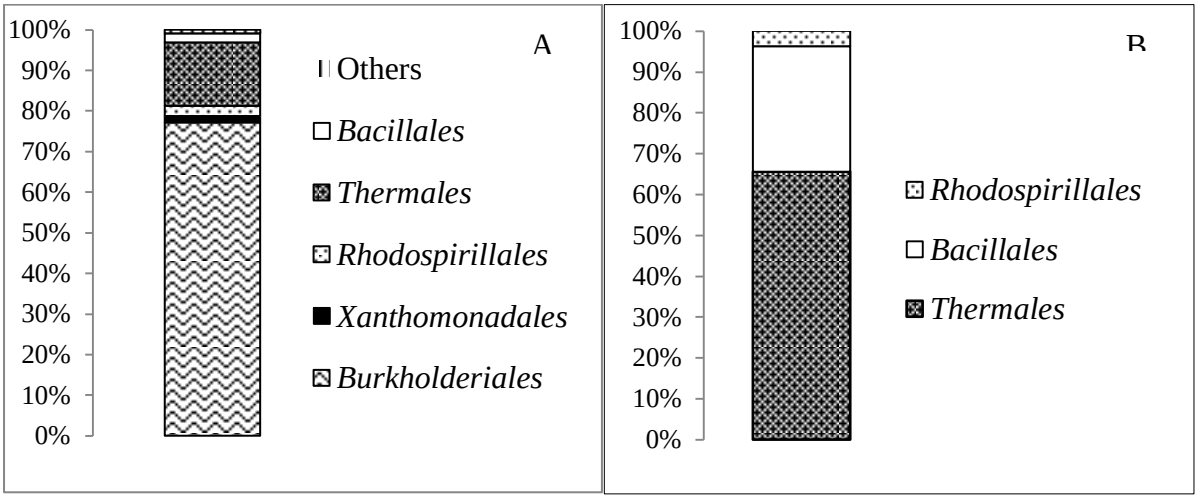
Table 3. Growth and esterase activity of thermophilic microbial communities (72nd hour of cultivation)

Sample	**Control**		**Polypropilene (PP)**			**Polystirene (PS)**			**Polycaprolactone (PCL)**			**Polyvinyl alcohol (PVA)**		
	OD	U/ml	OD	U/ml	Number of isolates	OD	U/ml	Number of isolates	OD	U/ml	Number of isolates	OD	U/ml	Number of isolates
1	0.12	0	0.13	0		0.18	0		0.13	0		0.30	54.83	1
2	0.09	0	0.22	0		0.13	0		0.32	102.1	2	0.18	0	1
3	0.11	0	0.15	46.3	1	0.22	69.75	1	0.70	375	12	0.15	0	
4	0.08	0	0.23	0		0.12	0		0.15	0		0.16	0	
5	0.10	0	0.12	0		0.15	0		0.12	0		0.19	0	

1.4. Phylogenetic diversity in the Marikostinovo community cultivated without plastic and in the presence of PCL and PS

The metagenomic analysis showed that the community composition in the plastic-free control (MK-K) (Table 4 and Figure 1, A) was characterized by a high diversity (11 phylogenetic groups, 5 orders) and a strong dominance of the phylum *Proteobacteria* (81.2%), mostly *Betaproteobacteria* (77.2%). The presence of *Proteobacteria* is universal for environmental samples. Other represented phyla were *Deinococcus-Thermus* (15.5%) and *Firmicutes* (2.3%). Community composition in the presence of PCL (MK-PCL) (Table 4 and Figure 1, B) was more restricted (7 phylogenetic groups, 3 orders). The significant decrease of *Proteobacteria* to 3.7%, represented only by the species *Elioraea tepidiphila* in MK-PCL led to the flourishing of the types *Deinococcus-Thermus* (65.6%) and *Firmicutes*, Class *Bacilli* (30.7%). The increase of *Bacillales* was more than 13-fold and of *Deinococcus-Thermus* more than 4-fold. The predominance of the genera *Meiothermus* (58.1%) and *Brevibacillus* (18.5%)

suggested their active involvement in PCL degradation. A similar dominance of plastic-active taxa has been reported for some marine communities isolated from samples containing plastic debris (Dussud et al., 2018; Tourova et al., 2020). Unfortunately, a comparison between the composition of thermophilic communities with and without plastic was not possible due to the lack of such information in the literature. 16S rRNA sequences identified in both communities were deposited in NCBI with BioProject accession number PRJNA766622 (https://www.ncbi.nlm.nih.gov/sra/PRJNA766622 , Accessed on 29 September 2021).



Фиг.1. Bacterial orders represented in: A, MK-K; B, MK-PCL.

Table 4. Taxonomic groups identified in MK-K and MK-PCL

Identified group	**Phylogenetic affiliation**	**Sequences (%)**	
		MK-C	**MK-PCL**
Caldimonas sp.	Phylum *Proteobacteria*, Order *Burkholderiales*	42.2	-
Tepidimonas ignava	Phylum *Proteobacteria*, Order *Burkholderiales*	21.0	-
Meiothermus sp.	Phylum *Deinococcus-Thermus*, Order *Thermales*	14.5	58.1
Tepidimonas sp.	Phylum *Proteobacteria* Order *Burkholderiales*	13.7	-
Bacillales	Phylum *Firmicutes*, Order *Bacillales*	2.3	11.6
Thermomonas sp.	Phylum *Proteobacteria*, Order *Xanthomonadales*	1.5	-
Elioraea tepidiphila	Phylum *Proteobacteria* Order *Rhodospirillales*	1.1	3.7
Thermaceae	Phylum *Deinococcus-Thermus*, Order *Thermales*	1.1	7.5
Roseomonas	Order *Rhodospirillales* Family *Acetobacteraceae*	0.9	-
Acetobacteraceae	Class *Alphaproteobacteria* Order: *Rhodospirillales*	0.5	-
Burkholderiales	Phylum *Proteobacteria* Order: *Burkholderiales*	0.3	-
Brevibacillus	Phylum *Firmicutes* Order *Bacillales*	-	18.5
Paenibacillus	Phylum *Firmicutes* Order *Bacillales*	-	0.4
Bacillaceae	Phylum *Firmicutes*	-	0.2

The number of taxonomic groups represented in the MK-PS sample cultured in polystyrene containing medium was similar to that in the control - 11 in the control and 10 in the PS sample (Table 5), which is explained by the lower selective pressure of the substrate in the presence of the difficult-to-degrade PS.

Table 5. Characterization of the biodiversity of a community from Marikostinovo in an environment with polystyrene (MK-PS)

Identified group	**Phylogenetic affiliation**	**Sequences (%)**	
		МК-К	**МК-ПС**
Caldimonas sp.	Phylum *Proteobacteria*, Order *Burkholderiales*	42.2	19.2
Tepidimonas ignava	Phylum *Proteobacteria*, Order *Burkholderiales*	21.0	-
Meiothermus sp.	Phylum *Deinococcus-Thermus*, Order *Thermales*	14.5	53.6
Tepidimonas sp.	Phylum *Proteobacteria* Order *Burkholderiales*	13.7	-
Bacillales	Phylum *Firmicutes*, Order *Bacillales*	2.3	7.6
Thermomonas sp.	Phylum *Proteobacteria*, Order *Xanthomonadales*	1.5	-
Elioraea tepidiphila	Phylum *Proteobacteria* Order *Rhodospirillales*	1.1	1.3
Thermaceae	Phylum *Deinococcus-Thermus*, Order *Thermales*	1.1	4.3
Roseomonas	Order *Rhodospirillales* Family *Acetobacteraceae*	0.9	1.9
Acetobacteraceae	Class *Alphaproteobacteria* Order: *Rhodospirillales*	0.5	0.6
Burkholderiales	Phylum *Proteobacteria* Order: *Burkholderiales*	0.3	-
Brevibacillus sp.	Phylum *Firmicutes* Order *Bacillales*	-	10.4
Rubrobacter sp.	Phylum *Actinobacteria*, Order *Rubrobacterales*	-	0.9
Paenibacillus sp.	Phylum *Firmicutes* Order *Bacillales*	-	0.6

As with PCL, the relative proportion of different groups was different. There was a significant decrease in the proportion of *Proteobacteria* from 80.3% to 21.1%, accompanied by an increase in the proportion of *Bacillales* from 2.3% to 18.8%, and the genus *Brevibacillus* was not identified in the control (taxa represented by >0.1% of the total sequences present were identified) reached 10.4% in PS containing medium, which suggested the active participation of representatives of this genus in the degradation of this type of

plastic. Another recurring feature of the MK community is the significant increase in the proportion of representatives of the genus *Meiothermus* in both types of plastic.

1.5. Identification of thermophilic bacteria degrading different types of plastics

Eighteen strains in total were isolated from samples from the different springs incubated with different types of plastics. As a result of the amplification with the PCR primers and the subsequent sequencing with the sequencing primers, the nucleotide sequences of the entire 16S rRNA genes were identified. 16S rRNA genes of the isolates were deposited in the Gene Bank on 29/01/2021 and 15/04/2021 under the numbers MW541894 to MW541896 and MW927322 to MW927332. Sequences were compared to the bank-deposited data using the Basic Local Alignment Search Tool (BLAST).

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=%20BlastSearch&LINK_LOC=blasthome) .

The eighteen isolates were assigned to eight thermophilic species from three families, two phyla, *Firmicutes* and *Deinococcus-Thermus* (Table 6). Among the isolates, the phylum *Firmicutes*, Class *Bacilli*, Order *Bacillales* dominated with 17 isolates - nine strains of *Brevibacillus thermoruber* (*Paenibacillaceae*), two strains of *Aneurinibacillus thermoaerophilus* (*Paenibacillaceae*), two strains of *Geobacillus thermodenitrificans* (*Bacillaceae*) and one strain of *Meiothermus cateniformans* (*Thermaceae*). The dominant number of bacilli is consistent with the common belief that they are essential microorganisms in degradation and biotransformation processes (Mahdi et al., 2016). The representatives of *Brevibacillus thermoruber* (*Paenibacillaceae*) were the most numerous, which suggested the active participation of these microorganisms in the degradation of plastics. *Deinococcus-Thermus* phylum is represented by only one isolate assigned to the species *Meiothermus cateniformans*. Esterase activity during growth in PCL medium was recorded in two of the samples, from Levunovo (54.83 U/ml) and from Marikostinovo (375 U/ml) (Table 3). Among 14 strains isolated from these two samples, twelve visibly different colonies originated from the Marikostinovo community (MK-PCL). They were purified by triplicate plating on rich medium and identified.

Table 6. Phylogenetic affiliation of the strains isolated from the different samples in the presence of any of the four types of plastics

Strain No	**Plastic type**	**Sequence number**	**Closest relatives, %**	**Phylum, Class, Order, Family**
1	ПВА	MW541891	*Anoxybacillus kamchatkensis* G10, 99.73	*Firmicutes*; *Bacilli*; *Bacillales; Bacillaceae*
2	ПВА	MW541892	*Geobacillus vulcani* strain 2C, 99.80	*Firmicutes;Bacilli*; *Bacillales; Bacillaceae*
3	ПС	MW541893	*Fictibacillus gelatini* strain LMG 21880, 99.86	*Firmicutes;*, *Bacilli*; *Bacillales; Bacillaceae*
4	ПКЛ	MW541894	*Brevibacillus thermoruber* strain B4M, 99.86	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
5	ПКЛ	MW541895	*Brevibacillus thermoruber* strain B4M, 99.73	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
6	ПКЛ	MW541896	*Brevibacillus thermoruber* strain BT2, 99.46	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
7	ППП	MW541897	*Brevibacillus borstelensis* strain Gp-1, 100	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
8	ПКЛ	MW927322	*Aneurinibacillus thermoaerophilus*, 99.12	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
9	ПКЛ	MW927323	*Aneurinibacillus thermoaerophilus*, 99	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
10	ПКЛ	MW927324	*Geobacillus thermodenitrificans*, 99.42	*Firmicutes*; *Bacilli*; *Bacillales; Bacillaceae*
11	ПКЛ	MW927325	*Geobacillus thermodenitrificans*, 99.89	*Firmicutes*; *Bacilli*; *Bacillales; Bacillaceae*
12	ПКЛ	MW927326	*Brevibacillus thermoruber*, 99.36	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
13	ПКЛ	MW927327	*Brevibacillus thermoruber*, 99.80	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
14	ПКЛ	MW927328	*Brevibacillus thermoruber*, 99.84	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
15	ПКЛ	MW927329	*Brevibacillus thermoruber*, 99.32	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
16	ПКЛ	MW927330	*Brevibacillus thermoruber*, 99.71	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*
17	ПКЛ	MW927331	*Brevibacillus thermoruber*, 99.77	*Firmicutes*; *Bacilli*; *Bacillales; Paenibacillaceae*;
18	ПКЛ	MW927332	*Meiothermus cateniformans*, 99.14	*Deinococcus-Thermus*; *Deinococci*; *Thermales*; *Thermaceae*

1.6. PCL-degrading thermophilic strains isolated from Marikostinovo community

The high number of isolates and the highest established esterase activity of the MK-PCL sample determined our further interest in these strains and their biodegradation activities.

Table 7. Growth and esterase activity of the pure strains and their combination with strain 7 after 48 h of cultivation in PCL medium

Strain №	**Species, 16S rRNA gene sequence number**	**Pure strain**		**Pure strain+strain 7**	
		OD_{660}	**Esterase activity (U/ml)**	**OD_{660}**	**Esterase activity (U/ml)**
1	*Aneurinibacillus thermoaerophilus*, MW927322	0.08	0	0.22	97
2	*Aneurinibacillus thermoaerophilus*, MW927323	0.10	115	0.19	325
3	*Geobacillus thermodenitrificans*, MW927324	0.13	65	0.16	62
4	*Geobacillus thermodenitrificans*, MW927325	0.14	63	0.15	0
5	*Brevibacillus thermoruber*, MW927326	0.16	32	0.17	62
6	*Brevibacillus thermoruber*, MW927327	0.16	20	0.25	32
7	*Brevibacillus thermoruber*, MW541894	0.2	290	-	-
8	*Brevibacillus thermoruber*, MW541895	0.14	0	0.30	32
9	*Brevibacillus thermoruber*, MW927328	0.14	42	0.30	135
10	*Brevibacillus thermoruber*, MW927329	0.11	25	0.11	50
11	*Brevibacillus thermoruber*, MW927330	0.27	20	0.11	27
12	*Meiothermus cateniformans*, MW927331	0.2	42	0.10	70
	MK community, PRJNA766622	0.2	375	-	-

They were assigned to four species: *Aneurinibacillus thermoaerophilus, Geobacillus thermodenitrificans, Brevibacillus thermoruber* and *Meiothermus cateniformans*. The highest enzyme synthesis was observed in the community, 375 U/ml. Among the pure strains, the highest esterase activity of 290 U/ml was measured for *Brevibacillus thermoruber* strain 7 (Table 7). Relatively high activity was also reported for *Aneurinibacillus thermoaerophilus* strain 2 (115 U/ml) and *Meiothermus cateniformans* strain 12 (42 U/ml). Co-cultivation of strain 2 and strain 7 resulted in higher activity, while the esterase activity in the cultivation of

strain 7 and strain 12 was lower than that of strain 7 alone, therefore strain 12 was not used for further experiments.

The pure strains isolated from MK were tested for the formation of clear zones around colonies on agar minimal medium with PCL (Mw 14,000) as the sole carbon source (Fig. 2). Good growth and halos were observed in *B. thermoruber* strain 7, *A. thermoaerophilus* strain 2 and *M. cateniformans* strain 12. These results are consistent with the occurrence of prominent fractions of *Brevibacillus* and *Meiothermus* established by the metagenomic analysis.

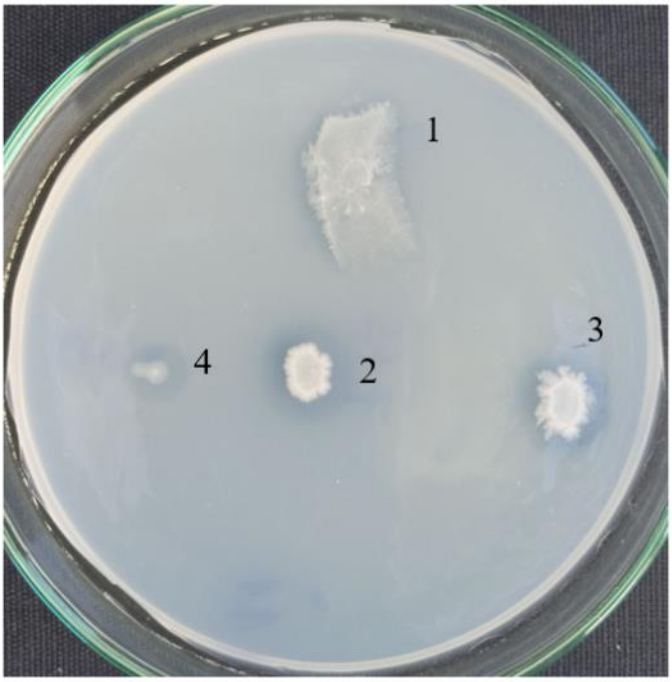


Fig. 2. Clear halos of PCL-degrading bacteria isolated from Marikostinovo. 1, MK-PCL; 2, *Brevibacillus thermoruber* strain 7; 3, *Aneurinibacillus thermoaerophilus* strain 2; 4, *Meiothermus cateniformans* strain 12.

The thermophilic PCL-degrading bacteria reported so far belong to the genus *Streptomyces*. They were active at 40-50°C and were isolated from mesophilic niches. *Streptomyces thermonitrificans* PDS-1 was isolated from fish processing industry compost (Nakasaki et al., 2006), and *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* 76T-2 was isolated from soil in Taiwan (Chua et al., 2013). In our experiments, strain 7, which showed the highest activity, was isolated from hot spring MK, an ecological niche typical for thermophiles. It was identified as belonging to the species *Brevibacillus thermoruber* and was designated as the primary degrading microorganism. It represents not only the first reported thermophilic *Bacillus* capable of degrading PCL, but PCL-degrading bacilli have no been reported even in the mesophilic group. The genus *Brevibacillus* is known to be of great

biotechnological interest (Panda et al., 2014), but so far only its activity towards low density polyethylene (LDPE) has been reported.

1.7. Optimization of the physico-chemical parameters for PCL degradation

In the pH optimization process for degradation, the highest esterase activity of MK-PCL and *B. thermoruber* strain 7 was found at pH 7.5, while for the co-culture of strain 7 and strain 2 it was at pH 8.0 (Fig. 3). This result corresponds to the slightly alkaline pH of the spring water. Enzyme activity increased with temperature, reaching a maximum at 55°C for all variants (Fig. 4), which is close to the melting temperature of PCL (55-60°C), at which the amorphous regions become soft and flexible. The established optimal temperature is superior to the two thermophilic producers described in the literature, which belong to *Streptomyces* and are active at temperatures of 40-50ºC (Nakasaki et al., 2006; Chua et al., 2013). Increasing the temperature above 55ºC resulted in melting of the plastic, particularly well demonstrated at 65ºC where no activity was recorded.

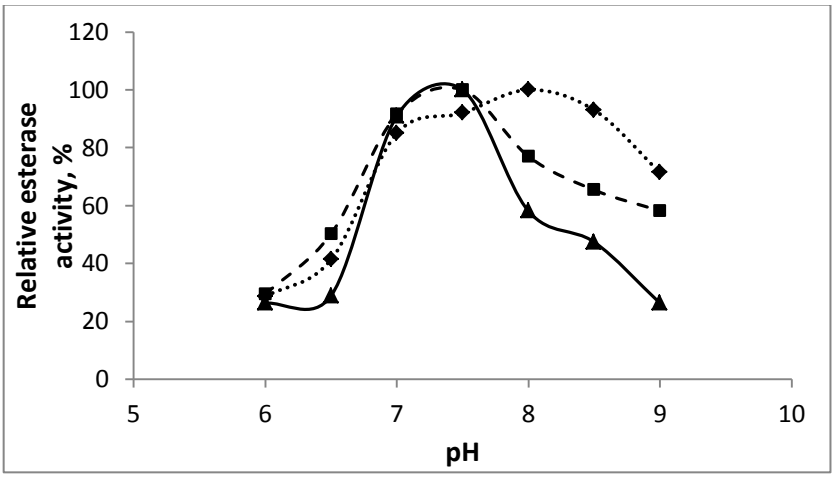


Fig. 3. pH optimum of degradation at a temperature of 55ºC. --■--, MK-PKL; ▲ , strain 7; ···♦···, strains 7+2

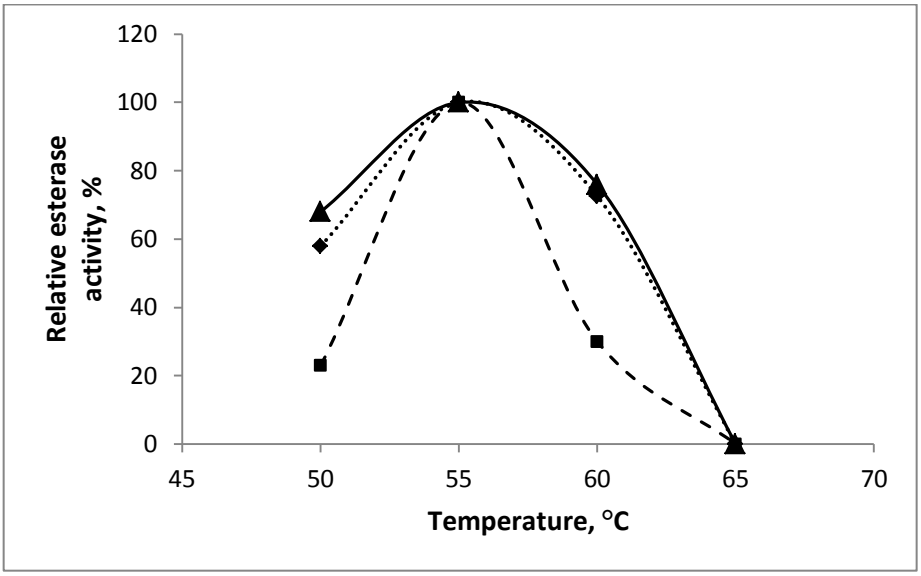


Fig. 4. Temperature optimum of degradation at pH 7.5. --■--, MK-PKL; ▲ , strain 7; ···♦···, strains 7+2

The influence of substrate concentration in the medium is presented in Fig. 5. The highest enzyme activity was measured at a PCL concentration of 0.3%, 375 U/ml for MK-PCL, 290 U/ml for strain 7 and 325 U/ml for the co-culture of the two pure strains.

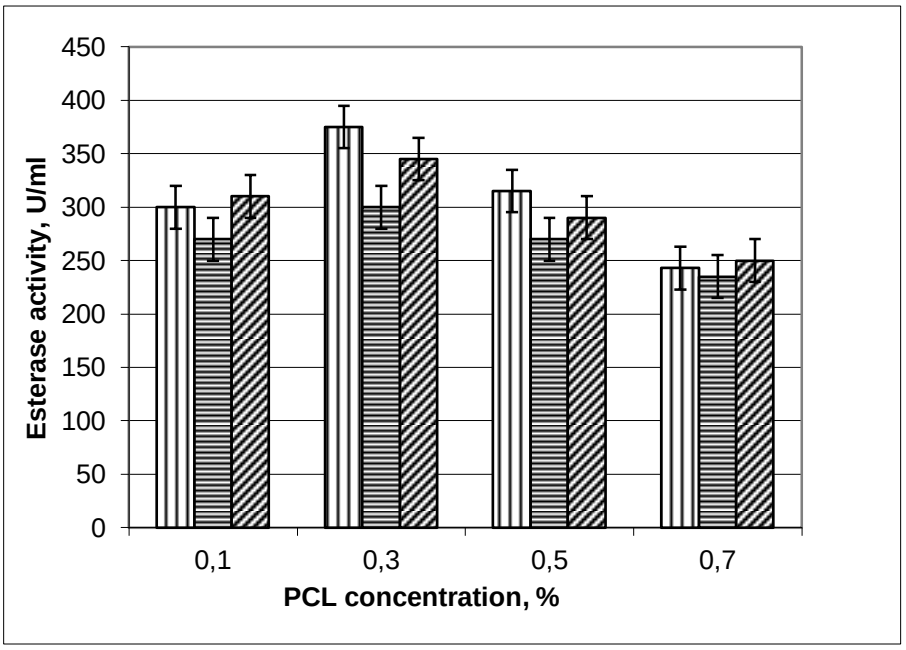


Fig. 5. Esterase activity (48 h) at different concentrations of PCL in the medium: ▥, MK-PCL; ▤, *B. thermoruber* strain 7; ▨, co-culture of *B. thermoruber* strain 7 and *A. thermoaerophilus* strain 2.

1.8. Carbohydrate degrading activity of B. thermoruber strain 7 using AZCL-substrates

When investigating the ability of the strain to degrade polysaccharides, it was found that it degraded two of the total 10 AZCL-substrates investigated: arabinan and amylose, which

together with its ability to hydrolyze PCL and fats makes the strain very promising for biotechnological exploitation.

1.9. Characteristics of the PCL biodegradation process

The biodegradation process of PCL from community MK, strain 7 and co-culture of strain 7 and strain 2 was followed for 4 weeks in a liquid medium with 0.3% PCL (Mw 80,000) at 55ºC, pH 7.5, shaker speed 80 rpm (Table 8).

Table 8. Efficiency of PCL degradation by *B. thermoruber* strain 7, and *B. thermoruber* strain 7 + *A. thermoaerophilus* strain 2

Microorganism	**Week**	**Final weight**	**Weight loss in 7 days (mg)**	**PCL degradation rate (mg/day)**	**Weight loss in 7 days (%)**	**Protein content (mg/ml)**			**Activity (U/ml)**
						Protein in free cells (mg)	**Biofilm protein (mg)**	**Total Protein (mg)**	
Control	4	150 ± 9	0	0	0	0	-	0	0
Community	1	88.2	61.8	8.83	41.2	0.23	1.31	1.54	375
	2	48.8	39.4	5.63	26.3	0.45	1.56	2.01	375
	3	20.1	28.7	4.1	19.1	0.63	1.39	2.02	333.8
	4	0	20.1	2.87	13.4	0.17	0.84	1.01	291.6
B. thermoruber strain 7	1	134.7	15.3	2.18	10.2	0.75	0.05	0.80	290
	2	115.2	19.5	2.78	13	0.78	0.15	0.93	290
	3	84.2	31.0	4.42	20.6	0.84	0.19	1.03	258
	4	54.5	29.7	4.24	19.8	0.11	0.14	0.25	125
B. thermoruber strain 7 + *A. thermoaerophilus* strain 2	1	125	25	3.57	16.7	0.82	0.13	0.95	325
	2	96.8	28.2	4.02	18.8	1.06	0.19	1.25	308
	3	67.6	29.2	4.17	19.5	1.18	0.19	1.37	280
	4	37.7	29.9	4.27	19.9	0.72	0.17	0.89	248

Complete degradation of PCL and no trace of the pearls was observed after four weeks in the presence of the MK-PCL community (Fig. 6), while the gravimetric weight loss was 63.6% after the action of strain 7 alone and 74.9% in the culture of strain 7 with strain 2 for the same time period.

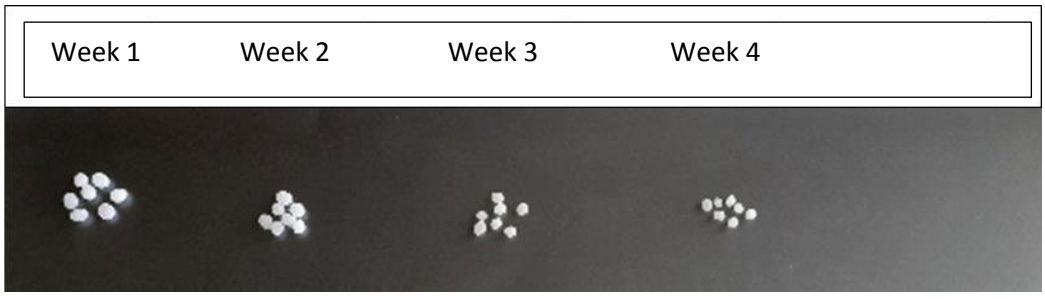


Fig. 6. Change in appearance of pearls incubated in the presence of MK-PCL community

The constant weight of PCL in the control flasks showed no self-degradation during the experiment. A high rate of degradation was observed at the beginning of the process with a

maximum of 8.83 mg/day in MK, while using the pure strains the highest levels of~4 mg/day were measured in the second part of the process. The gravimetric weight loss of PCL was 41.2% of MK and 10.2% of strain 7 after a seven-day process.

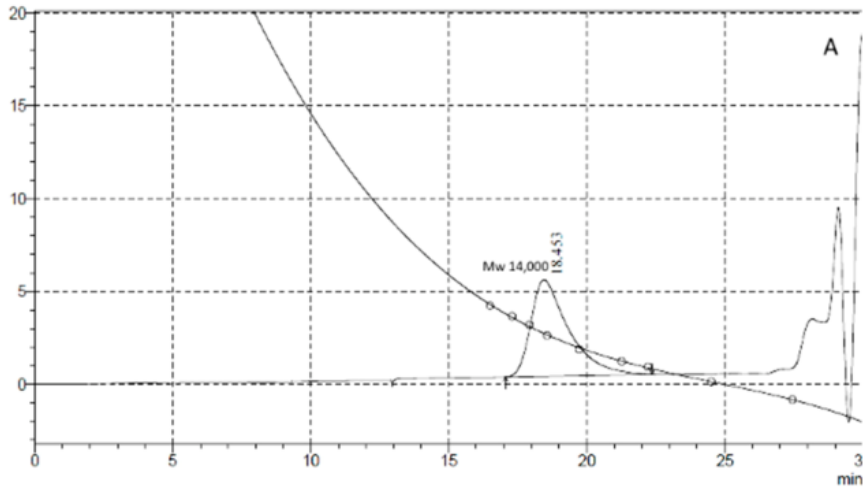
Co-cultivation of *B. thermoruber* strain 7 and *A. thermoaerophilus* strain 2 in our experiment resulted in only 10% increase in degraded polymer compared to strain 7, possibly as a result of the simultaneous action of enzymes from both organisms, but not as a synergistic effect from degradation. The predominance of *Meiothermus* and its low esterase activity suggest its active involvement mainly in biofilm formation and/or in the utilization of degradation intermediates. At the end of the first week, the highest activity of 375 U/ml was measured in the culture fluid of MK, for strain 7 it was 290 U/ml and for strain 7 + strain 2 - 325 U/ml and over time they all showed a weak trend to decrease. The enzyme was not detected in the sonicated cells, indicating its extracellular accumulation.

The difference in enzyme activity recorded in the MK-PCL samples, strain 7 or the co-culture of strain 7 and strain 2 was not as significant as the rate of degradation. The greater efficiency of the degradation process by MK-PCL is explained by the formation of a biofilm, which puts the microorganisms and the substrate in direct contact and hence more effective action of the enzyme. The concentration of cells defined as protein in the biofilm reached 7.3 times higher levels in MK-PCL, compared to the other two samples. The low protein concentration in the plastic-bound cells of strain 7 and its co-culture with strain 2 suggested that these two strains were not the main contributors to community biofilm formation.

1.10. Identification of the degradation products of polycaprolactone

The results from the analysis of PCL degradation products were obtained using gel permeation chromatography. The characteristic peaks in the elution profiles of the three samples were very similar for the samples from 24, 48, 72, 96, 120 and 168 h of cultivation. A different elution profile was observed using MK-PCL, strain 7 and the co-culture of strain 7 and strain 2, which was particularly well demonstrated at the 48th hour of cultivation (Fig. 7). A peak corresponding to a molecular weight of 14,000 of the PCL substrate appeared at an elution volume of 18.45 ml in the samples of strain 7 and strain 7 + strain 2, but with a different proportion – 83.74% for strain 7 and 54.99% for the co-culture. The only additional peak in the supernatant of strain 7 was at an elution volume of 27.06 ml, which corresponded to the monomer ε-caprolactone (14.99%). The registration of the monomer in the supernatant of strain 7 suggests that this microorganism is unable to assimilate ε-caprolactone or its assimilation is slower than its release from the PCL molecule. The presence of only the monomer as a degradation product indicates that the enzyme from this strain is an exo-

enzyme that hydrolyzes ester bonds at the ends of the polymer chain. In the mixed culture of strain 7 and strain 2, three other peaks with elution volumes of 24.4, 25.1 and 26.9 ml were observed, corresponding to intermediates with molecular weights of hexamer (22.78%), trimer (3.67%) and monomer (4.25%). The appearance of various low molecular weight intermediates in the supernatant of the co-culture of strain 7 and strain 2 supports the suggestion that strain 2 synthesizes an enzyme(s) different from that of strain 7 that acts in the interior of the polymer chain. In contrast to our results, *Streptomyces thermonitrificans* PDS-1 was reported to be solely able to degrade PCL, but its co-cultivation with *Bacillus licheniformis* HA1 resulted in accelerated degradation and an increased amount of degraded polymer because HA1 promoted the degradation of intermediates (Nakasaki et al., 2006). In the supernatant from the community, the PCL peak was almost absent, indicating that MK-PCL is the most effective in the degradation of PCL, since various microorganisms are involved in the assimilation process. The recorded peaks with elution volumes of 23.82 ml and 25.79 ml corresponded to 12-mer (8.2%) and dimer (91.7%), Monomer was also not recorded, suggesting the presence of microorganisms in the community that carry out the complete mineralization to H_2O and CO_2.



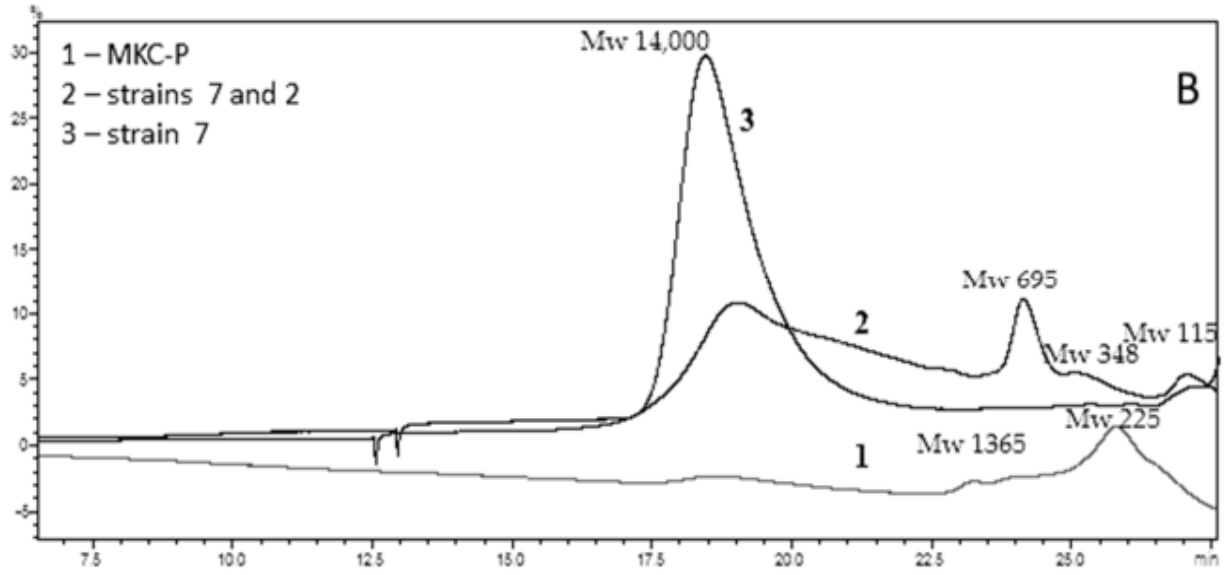


Fig. 7. Elution profile of the degradation products of PCL at 48 h of cultivation. (A), control – pure PCL; (B), 1, MK-PCL; 2, co-culture of *B. thermoruber* strain 7 and *A. thermoaerophilus* strain 2; 3, *B. thermoruber* strain 7.

1.11. SEM analysis of changes in the surface of plastics

In scanning electron microscopy (SEM) analysis, it was observed that the surface of the PCL pearls of the control samples was not completely smooth (Figure 8, A), but its appearance did not change after 2 weeks of incubation. Cultivation of *B. thermoruber* strain 7 for 72 h resulted in the appearance of bubble-like small bumps on the surface of the pearls (Figure 8, B) and surface deformations (Figure 8, C and D) that were not significantly different from the deformations of the controls pearls (Figure 8, A). Bacterial cells measuring 1.1 × 0.6 μm were rarely observed on the pearl surfaces (Figure 8, D). Three-week cultivation in the presence of strain 7 resulted in significant changes in PCL surface area. The formation of shallower (Fig. 8, E and F) or deeper (Fig. 8, G and H) folds was characteristic. The presence of attached cells was still rare (Fig. 8, H), suggesting that changes in the PCL surface were primarily due to the release of enzymes into the culture medium and not so much to biofilm formation**.**

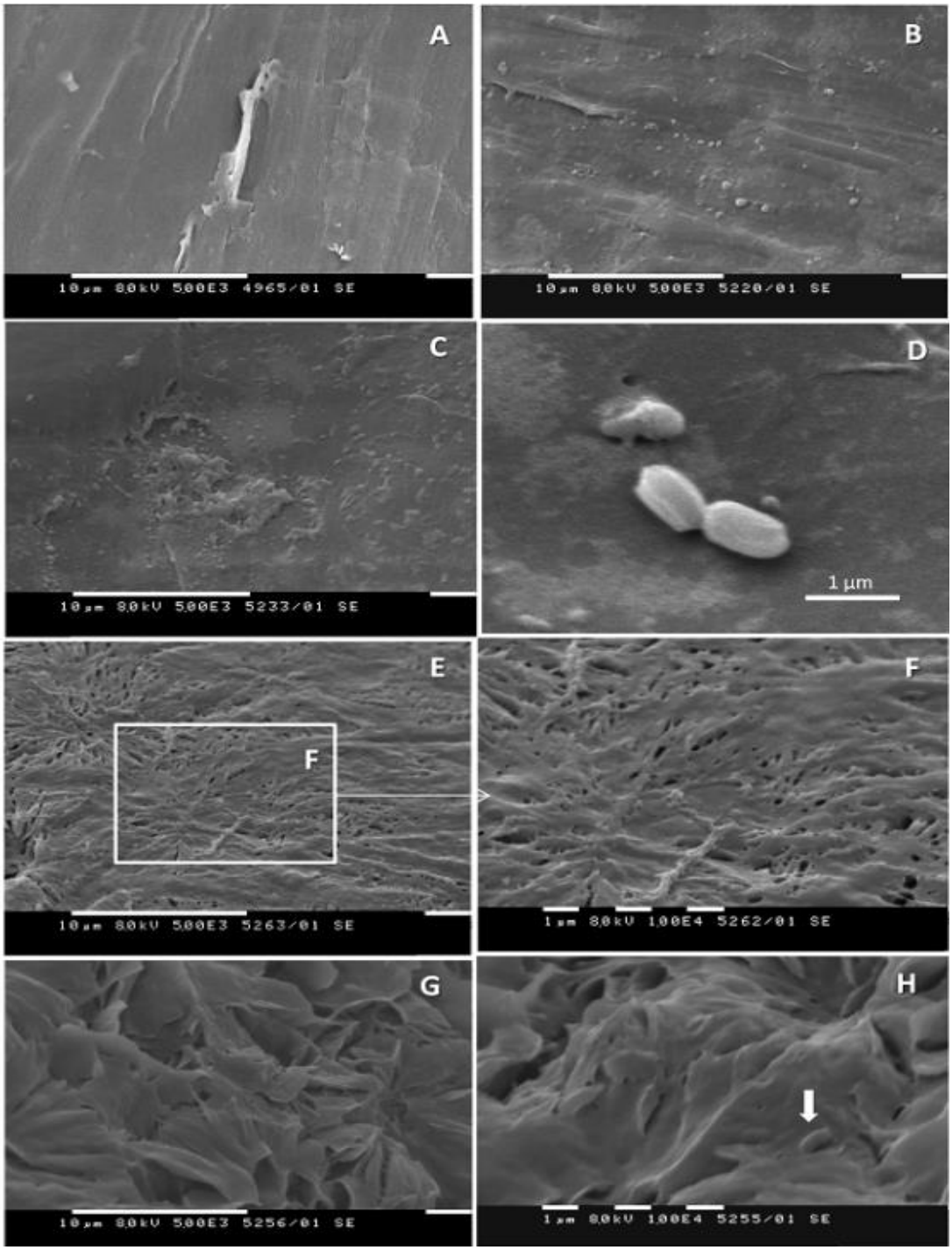


Fig. 8. Surface changes of PCL pearls cultured in the presence of *Brevibacillus thermoruber* strain 7. A, control. B - D, PCL pearls in the presence of the strain for 72 h. PCL surface deformations include bubble-like protrusions (B and C), single attached bacteria are occasionally observed (D, arrow). E–H, culture in the presence of the strain for 3 weeks. The PCL surface is characterized by more superficial (E, F) or deeper (G, H) folds with a low frequency of attached bacteria (H, arrow).

The SEM images of the PCL surface when the bacterial community was cultivated were significantly different from those of the single strain. At 72 hours, clumps of attached cells (Fig. 9, A and B) and biofilm development of microcolonies were observed. They were

different in length, width and length to width ratio. The first type were cells with a size of 1.05 ± 0.13 × 0.63 ± 0.10 μm, a length/width ratio of 1.6 (Figure 9, G) and were morphologically similar to those of strain 7 (Figure 9, D). They represented 25% of all cells measured. The predominant cell type (68%) measured 1.45 ± 0.29 × 0.45 ± 0.05 μm, length/width ratio 3.22 (Fig. 9, G–I). Of the third morphological type, only a few bacteria were observed and represented 7% of the measured cells. They were pear-shaped with dimensions of 1.07 ± 0.12 × 0.45 ± 0.02 μm, length/width ratio 2.4 (Fig. 9, I). At the third week, the frequency of significantly deformed areas did not increase significantly compared to the 72-hour interval, but there was degradation of the plastic in depth, with a large proportion of the bacteria observed below the surface of the plastic pearls. Granular structures were established, and parts of the bacterial cells were visible beneath them (Fig. 9, J and K). This granular material was probably composed of extracellular polymers synthesized by the biofilm, with undegraded plastic fragments incorporated into them.

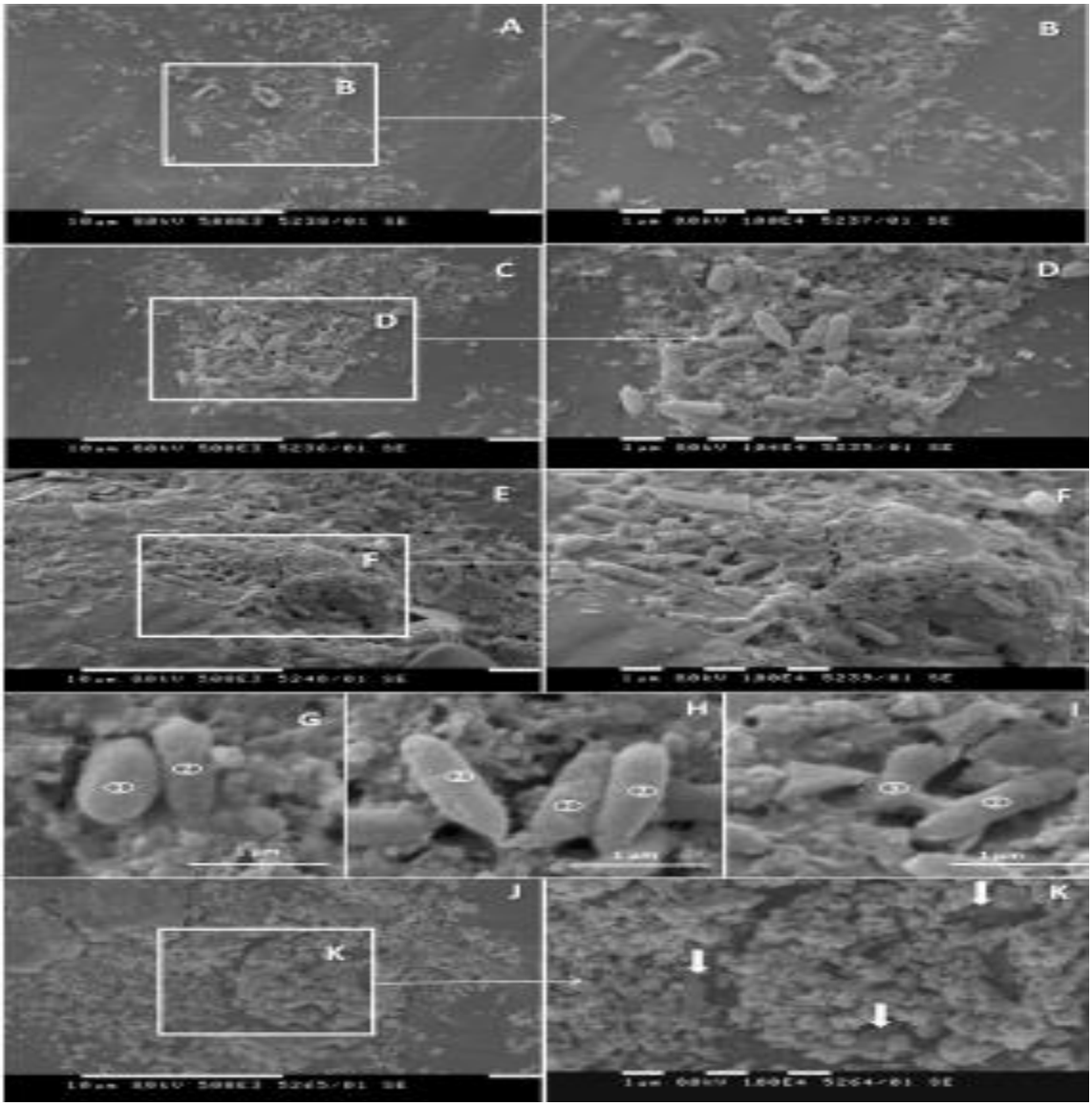


Fig. 9. Biofilm formation on the surface of PCL pearls in the presence of the bacterial community. A - F, 72 h culture. Bacterial cell attachment (A, B), microcolony formation (C, D) and bacterial penetration into the plastic (E, F). Biofilm bacteria on PCL pearls are of three morphological types. (G, H, I): (1) oval cells morphologically similar to cells of B. thermoruber 7 (5, D); (2) rod cells; (3) pear-shaped cells. After three weeks of culture (J, K), areas of granular material predominated and bacterial cells were beneath this material (K, arrow).

The effective degradation of PCL by the community at the beginning of the process can be explained by the presence of a microorganism that attaches to the plastic surface and contributes to biofilm formation. SEM results suggest that this biofilm contains at least three different strains of bacteria. One of them was morphologically similar to the few cells adherent to PCL in the sample inoculated with *B. thermoruber* strain 7. Apparently, strain 7 does not have good biofilm-forming properties and the presence of other strains in the community apparently increases its adhesion abilities. The relatively better attachment of the

cells of "morphotype strain 7" at 72 hours indicates that there may be a synergistic interaction between the community strains in the colonization of the plastic surface.

Close contact between plastic and microorganisms through biofilm formation favors the biodegradation process (Ganesh et al., 2020). The slowdown in the rate of degradation in the later stages observed here could be explained by the inhibition of the metabolic flux by the accumulated biofilm. The increase in the late-stage degradation rate in the presence of pure strains confirmed the observation that strain 7 and strain 2 did not form a good biofilm, and the increased degradation was associated with an increase in the number of lipase-producing cells in the culture fluid. Such an assumption is also confirmed by the higher concentration of protein in the supernatant in these variants compared to MK-PCL. At the same time, the protein in the biofilm removed from the plastic is several times higher. SEM studies confirmed damage to the plastic surface in all variants, but biofilm formation only in MK-PCL.

2. Lipase from B. thermoruber strain 7

The efficient degradation of PCL by strain 7 determined our interest in isolating the enzyme responsible for the biodegradation.

2.1. Purification of lipase

A three-step scheme was developed to purify the enzyme synthesized by *B. thermoruber* strain 7 (Table 9), resulting in a purification factor of 23.1 fold and a final yield of 51.4%, which is a very good yield for a lipophilic enzyme

Table 9. A scheme of lipase purification

Purification stage	Volume (ml)	Protein		Activity		Specific activity (U/mg)	Purification fold	Yield, %
		mg	%	U/ml	%			
Supernatant	1470	292	100	138181	100	473.2	1	100
Ultraconcentrate	5	110.1	37.7	79040	57.2	717.9	1.52	57.2
DEAE-Sepharose	28	6.51	2.23	71025	51.4	10910	23.1	51.4

The molecular weight of the purified lipase, determined by SDS-PAGE, was 28 kDa.

2.2. Qualitative test for an enzyme activity

In a qualitative test to identify enzyme activity, equally large halos were found for the preliminary equilibrated in activity of ultraconcentrate and pure enzyme, while no such clearing was observed in the buffer-containing control (Fig. 10).

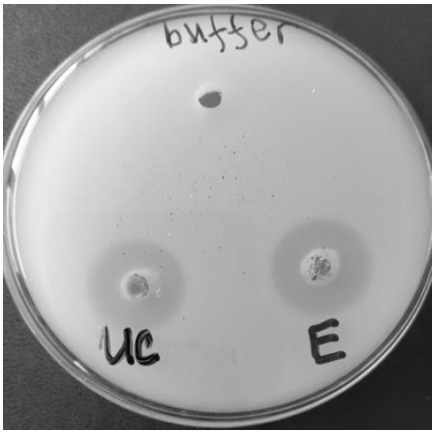


Fig. 10. Clear halos were observed in agar medium containing 0.1% PCL (Mw 14000) when 50 µL UC (ultraconcentrate) or E (pure enzyme) containing 145 U each was incubated in the wells at 55°C for 24 hours, 0.05 M sodium phosphate buffer, pH 7.5, was used as a control.

2.3. Characterization of the properties of the purified enzyme

The results of the study of the temperature optimum for the action of the pure lipase showed the highest activity at 55°C, a temperature typical for thermophilic enzymes (Fig. 11).

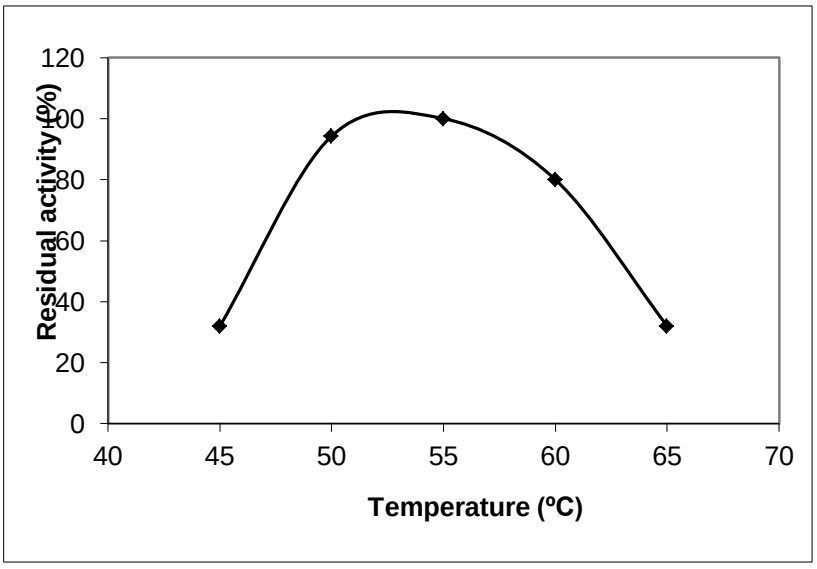
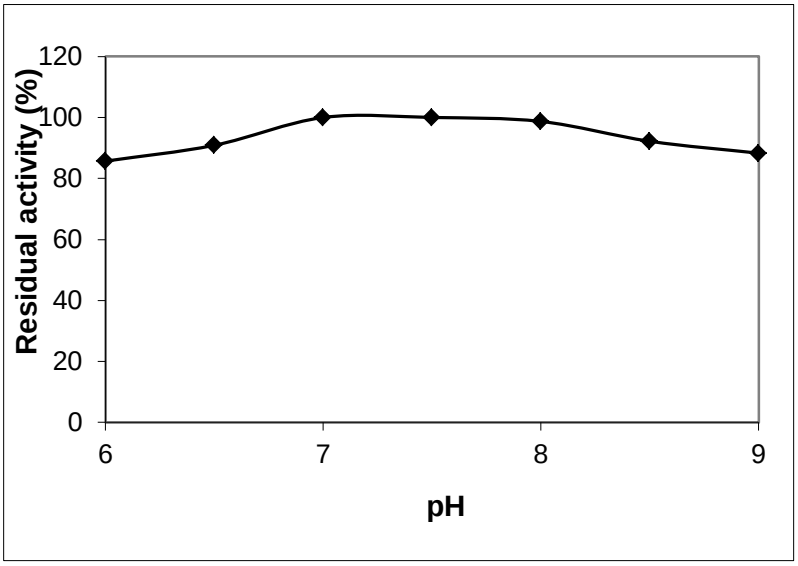


Fig. 11. Effect of temperature on the lipase activity of the purified enzyme from strain 7, pH 7.5.

The temperature optimum of this enzyme (55°C) coincides with the melting temperature of PCL 55-60°C (Gajanand et al., 2014), which makes the thermophilic enzyme particularly suitable for the efficient degradation of PCL. The chains in the amorphous polymer domains can gain sufficient mobility to access the active sites of the enzyme when the enzymatic

hydrolytic reactions proceed at a temperature close to the melting temperature (Kawai et al., 2019).

The lipase from strain 7 showed an optimum at pH 7.0-8.0, being active in a very wide pH range – the recorded units remained above 80% at pH 6.0 to 9.0 (Fig. 12).



Фиг. 12. pH optimum of the purified lipase from strain 7

Investigation of the effect of temperature on the stability of the enzyme showed that it was among the most thermostable with a half-life of 5 h at 60°C, which increased up to 6 h in the presence of 5 mM Ca^{2+} (Fig. 13).

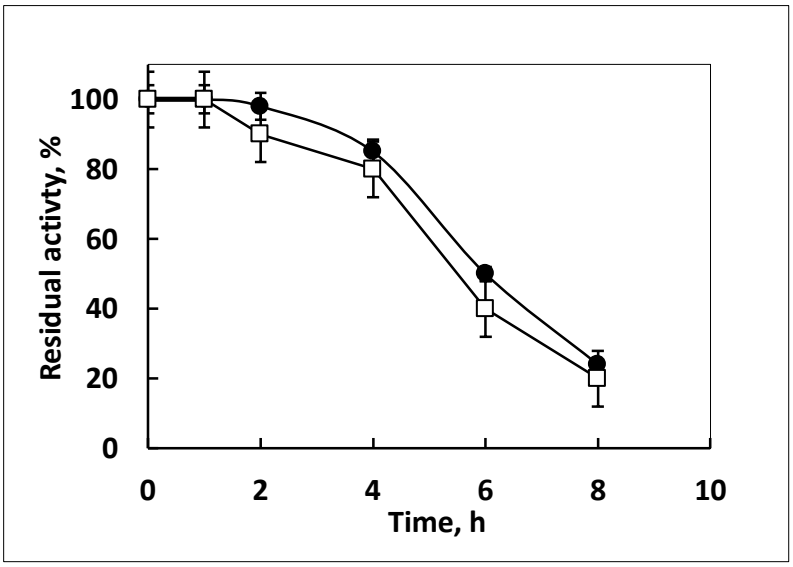


Fig. 13. Thermostability of the purified lipase after pre-incubation at 60°C for different time (hours). □, pure enzyme; •, pure enzyme + 5mM $CaCl_2$. Residual activity was determined at 55°C.

Most of the metal ions slightly decreased the enzyme activity, while Fe^{3+} strongly inhibited it (Table 10). The only metal ion with a positive effect on activity was Ca^{2+}. The enzyme was sensitive to detergents, inhibitors and EDTA. The observed negative effect of the chelating agent EDTA confirms that enzyme activity depends on the presence of Ca^{2+} in the active site. Inhibition of enzyme activity by transition and heavy metal ions may result from the formation of complexes with the reactive groups of the enzyme, thereby denaturing it (Cassia Pereira et al., 2017; Yeboah et al., 2021). The inhibitory effect of detergents such as SDS, Tween 20 and DTT is probably the result of lowering the surface tension of the aqueous system and possible modification of the distribution of the enzyme between the lipid surface and the aqueous phase (Cassia Pereira et al., 2017, Gargouri et al., 1983). The strong inhibition by the tryptophan inhibitor NBS, the thiol inhibitor PCMB and the serine inhibitor PMSF indicates the important role of these amino acids in the catalytic mechanism and the possible modification of the active site under the influence of the inhibitors. The effect of NaLS is related to its ability to form hydrogen bonds with the enzyme surface (Wang et al., 2018).

Table 10. Influence of some metal ions, inhibitors, and surfactants on lipase activity

Metal ions (5 mM)	**Relative activity (%)**	**Inhibitors, surfactants (5 mM)**	**Relative activity (%)**
Control	100	Control	100
Ca^{2+}	106.7	NaLS	4.7
Mg^{2+}	93.3	SDS	4.5
Co^{2+}	93.3	DTT	3.3
K^{+}	89.6	Tween 20	2.9
Na^{+}	80.0	NBS	2.8
Cu^{2+}	80.0	PMSF	2.5
Mn^{2+}	80.0	EDTA disodium salt	0
Hg^{2+}	66.7		
Zn^{2+}	66.7		
Fe^{3+}	33.3		

Determination of the substrate specificity of the enzyme using as a substrate para-nitrophenyl palmitate (pNPP) containing 16 carbon atoms in the palmitic residue or para-nitrophenyl butyrate (pNPB) containing 4 carbon atoms in the butyrate chain clearly demonstrated its belonging to the group of lipases (EC 3.1.1.3) characterized by a preference for hydrolysis of long-chain fatty acid residues rather than true esterases (EC 3.1.1.1). The

enzyme activity determined with pNPP was higher compared to pNPB at all temperatures tested (Table 11).

Table 11. Enzyme activity using p-nitrophenyl palmitate and p-nitrophenyl butyrate as substrates

Substrate	**30°C**	**35°C**	**40°C**	**45°C**	**50°C**	**55°C**	**60°C**
	U/ml	**U/ml**	**U/ml**	**U/ml**	**U/ml**	**U/ml**	**U/ml**
p-nitrophenyl palmitate	87.5	97	170.8	209	267	280	224
p-nitrophenyl butyrate	37.5	56	79.2	102	116	-	

2.4. PCL degradation products by purified lipase isolated from B. thermoruber strain 7

GPC analysis of PCL degradation products revealed that the enzyme from strain 7 attacks the main chain of PCL. Comparison of the intermediates revealed an almost identical elution profile when PCL was degraded by the ultraconcentrate or the pure enzyme, suggesting that the isolated lipase was the only enzyme synthesized by *B. thermoruber* strain 7 and active on PCL (Fig. 14). The elution volume of the main peak in both samples was very similar, 28.005 ml for the pure enzyme sample and 28.438 ml for the ultraconcentrate. The peak content was 65.034% and 66.909%, and the calculated molecular weight of 115 and 125 for the pure enzyme and the ultraconcentrate, respectively, is similar to the molecular weight of the monomer ε-caprolactone (114). A peak (13.657%) with an elution volume of 26.515 mL and a Mw of 252 corresponding to a dimer was observed only in the pure enzyme sample. The second largest peak with an elution volume of 25.632 mL and 25.701 mL and a peak area of 14.72% and 23.651% and a Mw of 454 for the pure enzyme and the ultraconcentrate, respectively, represents a tetramer. The elution volumes of the two smallest peaks in the two samples correspond to octamer and 12-mer molecular weight intermediates. As the only peak observed in the supernatant after 48 h of cultivation of strain 7 was the monomer, a reverse reaction can be assumed after a longer time of enzyme action.

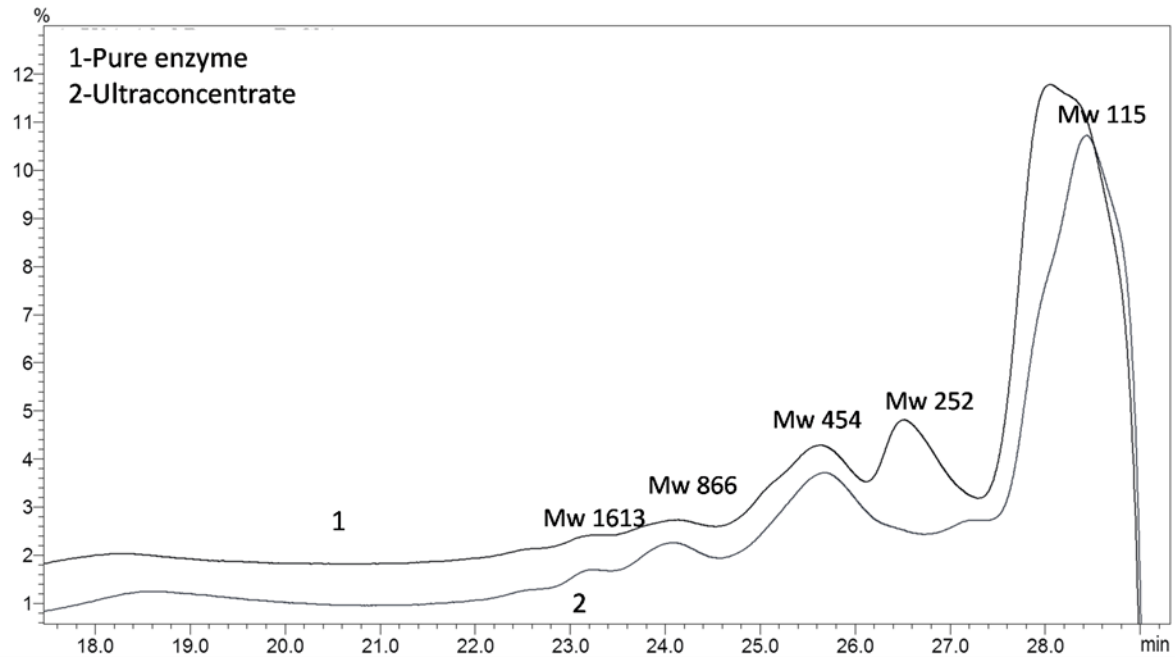


Fig. 14. Gel-permeation elution profile of PCL degradation intermediates after one week of cultivation of *B. thermoruber* strain 7. (1), pure enzyme; (2), ultraconcentrate.

SEM analysis of the surface of PCL pearls after their one-week incubation in a buffer solution of ultraconcentrate or pure enzyme (3000 U/ml) revealed profound morphological changes (Fig. 15). Microscopic images of the PCL control cultured in the absence of enzyme show relief without characteristic surface defects after the incubation period (A). Observations of the plastic surface after incubation with ultraconcentrate (B) and pure enzyme (C, D) confirmed degradation of the pearls. The very similar indication of degradation effects with clearly distinguishable plastic holes (marked by asterisks) and grooves (marked by arrows) observed in both samples again confirmed that the only PCL active enzyme synthesized by this strain is the lipase under investigation. The SEM micrograph after higher magnification (D) revealed the biodegradation ability of the enzymes even in the bottom layers of PCL pearls. In both samples, material with a granular structure was observed, probably containing plastic fragments in the initial stage of the enzyme action.

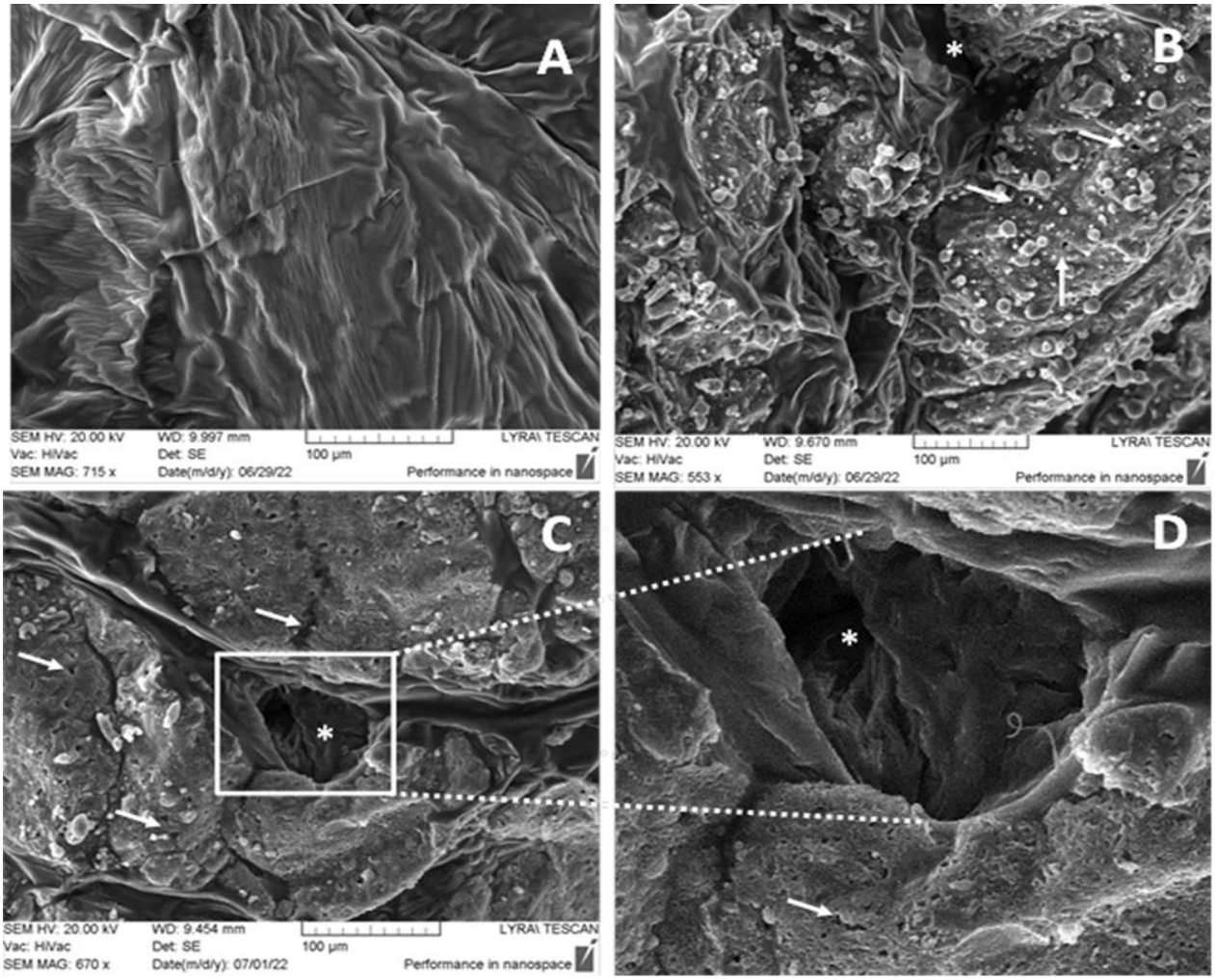


Fig. 15. Changes in the surface of PCL pearls, confirming degradation process. (A) control; (B) PCL pearls incubated in the presence of the ultraconcentrate; (C, D) PCL pearls incubated in the presence of the pure enzyme.

To the best of our knowledge, the isolated and characterized lipase from *B. thermoruber* strain 7 is the first reported thermostable enzyme capable of degrading PCL and among the small number of enzymes capable of degrading plastics. The lack of information on enzyme characteristics, biochemical mechanisms, and degradation products (Ru et al., 2020) precludes comparison of the properties of lipase from strain 7 and enzymes from other producers.

3. Degradation of plastics by halophilic bacteria

3.1. Screening of halophiles from the laboratory collection

The halophilic isolates stored in the laboratory were grouped into three groups depending on their phylogenetic affiliation:

Group 1: phylum *Firmicutes*, Class *Bacilli*, genera *Virgibacillus* and *Salinicoccus*

Group 2: phylum *Proteobacteria*, Class *Gammaproteobacteria*, genus *Halomonas*

Group 3: phylum *Proteobacteria*, Classes *Alphaproteobacteria* and *Gammaproteobacteria*, genera *Nesiotobacter*, *Chromohalobacter*, *Salinivibrio* and *Cobetia*.

Growth (OD_{660}) and esterase activity in each flask were determined after culturing each group in saline medium with a different type of plastic as the sole carbon source for two weeks at 30°C, 15% NaCl (Table 12).

Table 12. Halophilic strains from the laboratory collection tested for growth and esterase activity

Group	Species, number of strains	Control		PS		PP		PVA		PCL	
		OD	U/ml	OD	U/ml	OD	U/ml	OD	U/ml	OD	U/ml
1	*Virgibacillus salarius* – 2 *Virgibacillus marismortui* – 1 *Virgibacillus olivae* – 1 *Salinicoccus roseus*– 1	0.60	0	0.60	0	0.55	0	0.40	0	0.20	0
2	*Halomonas venusta* – 2 *Halomonas variabilis* – 3 *Halomonas smyrnensis* – 1 *Halomonas eurhalina* – 1	0.70	0	0.60	0	0.90	0	0.60	0	0.32	0
3	*Nesiotobacter exalbescens* – 1 *Chromohalobacter canadensis* - 1 *Salinivibrio costicola* – 1 щам *Cobetia marina* – 1 щам	0.52	0	0.70	0	0.84	0	0.60	0	1.0	0

Results, presented in table 12 clearly revealed that none of the halophilic strains from the laboratory collection were effective in degrading any of the four types of plastics used.

Table 13. Halophilic niches from which samples were collected

Sample №	**Origin of the sample**	**pH**
C1	Lye from Atanasovsko lake	8.0
C2	Water from Atanasovsko lake	8.7
C3	Mud from Atanasovsko lake	8.2
C4	Water from the beginning of Atanasovsko lake	8.1
C5	Mud from Pomorie salterns, cell PS18	8.1
C6	Saturated salt solution, cell PS18	8.0
C7	Diaper over the salt water, cell PS18	8.0

To study the degrading ability of halophilic microorganisms, samples of lye, water and mud were collected from the Burgas salterns and Pomorie salterns (Table 13).

3.3. Determination of growth and esterase activity in halophilic communities

Microorganisms represented in the samples were enriched by culturing of two g of mud or two ml of water/lye in 20 ml of minimal medium with the appropriate plastic for two weeks, after which 200 µl were transferred to 20 ml of fresh medium and cultured for another two weeks.

Table 14. Growth, esterase activity and number of isolates for the enriched communities of the different samples after 14 days of cultivation

Sample	**Control**		**ПП**			**ПС**			**ПКЛ**			**ПВА**		
	OD_{660}	U/ml	**OD_{660}**	**Е/мл**	**Number of isolates**	**OD_{660}**	**Е/мл**	**Number of isolates**	**OD_{660}**	**Е/мл**	**Number of isolates**	**OD_{660}**	**Е/мл**	**Number of isolates**
C1	0.30	0	0.51	0		0.49	0		0.51	52.8		0.14	29.0	4
C2	0.26	0	0.10	0		0.37	0		0.09	60.3	4	0.11	51.3	2
C3	0.15	0	0.16	17.0	5	0.26	0		0.16	0		0.17	18.4	
C4	0.38	0	0.33	31.0	3	0.25	0		0.27	27.9	3	0.24	0	
C5	0.39	0	0.16	0		0.21	26.3	4	0.45	0		0.43	0	
C6	0.18	0	0.20	0		0.26	0		0.43	0		0.25	0	
C7	0.28	0	0.46	5		0.48	0		0.46	0		0.22	0	

The results of the measured optical densities and esterase activity are reflected in Table 14. Comparison of microbial growth with and without plastic showed a higher optical density for C1 in the presence of PP, PS and PCL, while C2 did not show an increase in this indicator with any of the plastics used compared to K, despite the observed visible changes in the color of plastics used. Obviously, the possibility for biofilm formation affects the accuracy of using this indicator for growth assessment also in halophiles. The highest levels of esterase activity were found in C2-PCL, followed by C1-PCL and C2-PVA. To further characterize the ability of halophiles to degrade plastics, C1 and C2 growing in PCL medium were selected.

3.4. Phylogenetic diversity in the C2 halophilic community cultivated without plastic and in the presence of PCL

The phylogenetic diversity of the community with the highest activity was selected for a metagenomic analysis. Its structure was revealed after cultivation in the absence (C2-K) and presence of PCL (C2-PCL) (Table 15).

All identified groups from both communities belonged to *Alpha-* and *Gamma-Proteobacteria*. Seventeen phylogenetic groups assigned to seven orders were represented in the control (C2-K), while in C2-PCL twelve groups were assigned to five orders, testifying to the selective pressure of PCL on microorganisms unable to digest it (Table 16). There was no significant difference in the taxa represented, especially at higher phylogenetic levels. In contrast to C2-K, in C2-PCL three orders from the classes *Alpha-* and *Gammaproteobacteria* were represented, while the orders *Rhodobacterales* and *Nevskiales* were not present. However, the relative proportion of the two classes *Alpha-* and *Gamma-Proteobacetria* differed significantly in the two samples. *Alphaproteobacteria* dominated in C2-K (61.3%), while in C2-PCL it was present with only 6.9%. At the same time, a significant dominance of *Gammaproteobacteria* was observed for C2-PCL (93.1%). Under the selective pressure of PCL as the only carbon source, the portion of the family *Halomonadaceae*, order *Oceanospirillales* was sharply changed - from 24.1% in C2-C to 89.9% suggesting the main role of its representatives in the process of degradation of PCL and the possible activity of the genus *Halomonas*, whose 16S rRNA sequences increased almost threefold.

Table 15. Taxonomic groups identified in C2-K and C2-PCL

Identified groups	**Phylogenetic level**	**Relative portion of sequences, %**	
		C2-C	**C2-PCL**
Halomonadaceae	*Phylum Proteobacteria, Class Gammaproteobacteria Order Oceanospirillales*	24.1	49.7
Hyphomonadaceae	*Phylum Proteobacteria, Class Alphaproteobacteria Order Caulobacterales*	19.5	3.8
Halomonas sp.	*Phylum Proteobacteria, Class Gammaproteobacteria Order Oceanospirillales*	15.7	40.2
Alcanivorax sp.	*Phylum Proteobacteria, Class Gammaproteobacteria Order Oceanospirillales*	13.0	0.2
Phyllobacteriaceae	*Phylum Proteobacteria, Class Alphaproteobacteria Order Hyphomicrobiales*	4.9	0.8
Martelella sp.	*Phylum Proteobacteria, Class Alphaproteobacteria Order Hyphomicrobiales*	3.1	1.0
Chromohalobacter sp.	*Phylum Proteobacteria, Class Gammaproteobacteria Order Oceanospirillales*	2.9	2.4
Hyphomicrobiaceae	*Phylum Proteobacteria, Class Alphaproteobacteria Order Hyphomicrobiales*	2.3	-
Pelagibacterium sp.	*Phylum Proteobacteria, Class Alphaproteobacteria Order Hyphomicrobiales*	2.3	-
Methylophaga sp.	*Phylum Proteobacteria, Class Gammaproteobacteria Order Thiotrichales*	1.8	0.6
Marinicauda sp.	*Phylum Proteobacteria, Class Alphaproteobacteria Order Caulobacterales*	1.6	0.2
Algiphilus aromaticivorans	*Phylum Proteobacteria, Class Gammaproteobacteria Order Nevskiales*	1.1	-
Proteobacteria	*Phylum Proteobacteria*	0.9	0.4
Rhizobiales	*Phylum Proteobacteria, Class Alphaproteobacteria Order Rhizobiales*	0.3	0.1
Rhodobacterales	*Phylum Proteobacteria, Class Alphaproteobacteria, Order Rhodobacterales*	0.1	-
Rhodobiaceae	*Phylum Proteobacteria, Class Alphaproteobacteria Order Hyphomicrobiales*	0.1	-

3.5. SEM analysis of changes in the surface of plastics

SEM analyzes confirmed the resistance of non-biodegradable PP and PS to the action of bacterial communities, in contrast to the activity to biodegradable plastics. The SEM data were consistent with the enzymatic assay results, with no visible changes on the surfaces of PS and PCL incubated with community C1 (only salt crystals were observed) and changes on the surface of PP were negligible, while the surface of PVA was significantly altered (Fig. 16). Cultivation of C2 was accompanied by biofilm formation on PCL surface and its damage. Regarding PVA, both communities cause visible changes on the surface of the plastic.

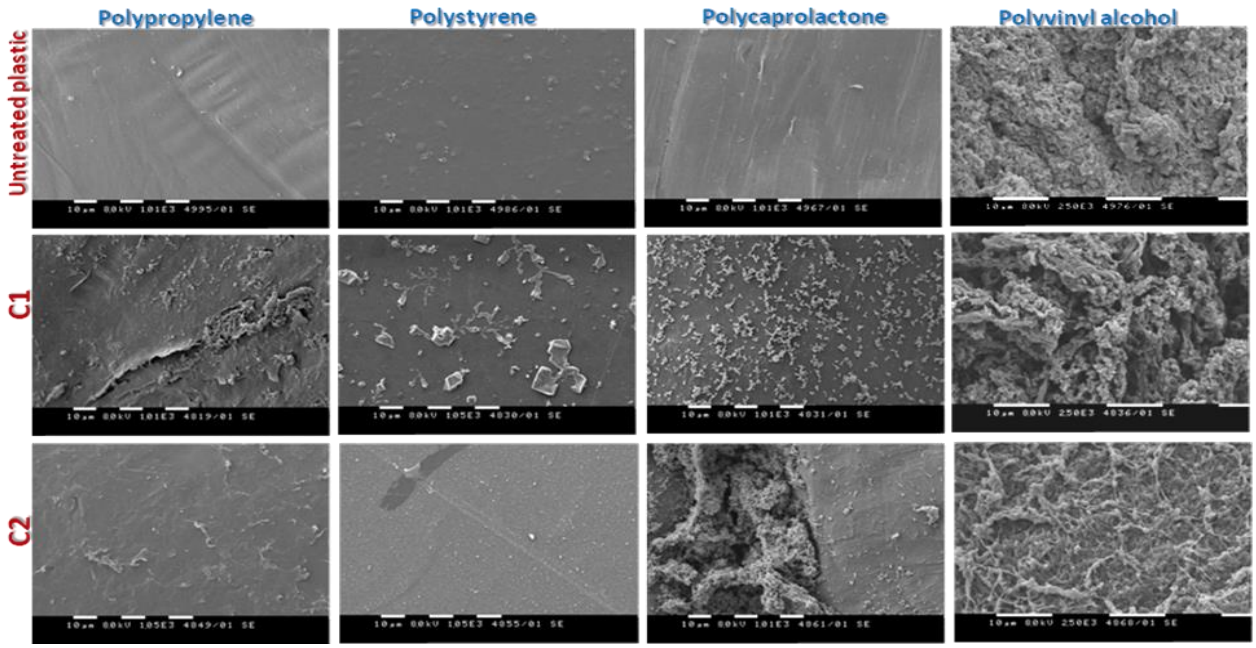


Fig. 16. Changes in the surface relief of the four investigated types of plastics after 14 days of incubation in the presence of bacterial communities C1 and C2.

3.6. PCL-degrading halophilic strains isolated from community C2

Twenty-five strains were isolated from the culture fluid of the second passage of sample C2-PCL. Six of them showed esterase activity, three in PVA cultivation and one each in PKL, PP and PS cultivation (Table 16).

Table 16. Phylogenetic affiliation and esterase activity after 14 days of cultivation

Strain No	**Phylogenetic affiliation**	**Isolated from sample:**	**Used plastic**	**Growth (OD_{660})**	**Activity (U/ml)**
1	*Virgibacillus marismortui*	1	ПВА	0.13	7.7
2	*Oceanobacillus picturae*	2	ПВА	0.35	4.4
3	*Virgibacillus salarius*	1	ПВА	0.20	13.2
4	*Virgibacillus salarius*	2	ПКЛ	0.20	2.75
5	*Virgibacillus salarius*	3	ПП	0.11	2.75
6	*Oceanobacillus picturae*	5	ПС	0.2 + agregates	2.75

Like the thermophiles, all halophilic isolates were assigned to the family *Bacillaceae*. They belonged to two species of the genus *Virgibacillus*, *V. salarius* (3 strains) and *V. marismortui* (1 strain) and the genus *Oceanobacillus*, *O. picturae* (2 strains). The degree of 16S rRNA gene similarity to the phylogenetic neighbors in all isolates was > 97%. Despite the significant increase in the number of sequences assigned to the genus *Halomonas*, established by metagenomic analysis, a pure strain from this genus was not isolated, suggesting its active participation in the degradation of the products obtained from the action of the primary degrader, but also an inability to attack the molecule of PCL.

Conclusions

1. Thermophilic communities from five hot springs located in Southwest Bulgaria demonstrated an ability to degrade some of the four tested plastics, polycaprolactone, polyvinyl alcohol, polystyrene, and polypropylene as the sole carbon source. The highest degrading activity was observed against polycaprolactone in the sample from Marikostinovo spring.
2. Metagenomic analysis revealed a reduction in natural phylogenetic diversity in the community cultivated in the presence of ε-polycaprolactone (7 taxonomic groups) compared to the community without plastic (11 taxonomic groups).
3. A strong dominance of the phylum *Proteobacteria* was observed in a mineral nutrient medium without plastic, while in its presence representatives of *Deinococcus -Thermus* and *Firmicutes* were dominant phyla.
4. Esterase activity against polycaprolactone was found in 12 isolates, the highest being *Brevibacillus thermoruber* strain 7.
5. When following the biodegradation process for four weeks, a 100% loss of the physical weight of the polycaprolactone pearls was found, while they lost 63.6% of their weight in the presence of a strain *B. thermoruber* 7.
6. Different products of the polycaprolactone degradation after the action of the community and the pure strain were identified by gel-permeation analysis, confirming the participation of a larger number of microorganisms in the degradation process in the community.
7. Electron microscopic analysis demonstrated the significant deformation of the plastic surface during the degradation process involving the microbial community and *B. thermoruber* strain 7.
8. The pure enzyme showed a molecular weight of 28 kDa, a temperature optimum of 55°C, a pH optimum of 7.0-8.0 and high thermostability – its half-life after pretreatment at 60°C was more than five hours.
9. By scanning electron microscopy (SEM analysis), significant changes such as shallower or deeper folds on the surface of PCL pearls incubated with ultraconcentrate or pure enzyme were observed.

10. Samples collected from the lye and water mud of Lake Atanasovsko, Burgas salterns and Pomorie salterns showed esterase activity after cultivation with the plastics polycaprolactone, polyvinyl alcohol, polystyrene and polypropylene. It was accompanied by significant changes of the plastic surface as determined by scanning electron microscopy.

11. Metagenomic analysis of the community from the water of Lake Atanasovo with PCL revealed a selective pressure from the plastic leading to a flourishing of the family *Halomonadaceae*, *Gamma-Proteobacteria* at the expense of *Alpha-Proteobacteria* in the control.

12. Among the 25 strains isolated from water from Atanasowsko lake and phylogenetically determined, six showed esterase activity, three after cultivation with PVA and one for each, PCL, PP and PS culture.

Contributions

1. For the first time, a bacterial community, degrading the ε-polycaprolactone plastic at 55°C was isolated - the highest temperature among those reported so far, in which changes in the plastic properties of PCL favoring microbial action were observed.
2. For the first time in thermophilic and halophilic communities, a strong selective pressure on biodiversity was observed from the used PCL plastic as the only carbon source.
3. The first able to degrade polycaprolactone thermophilic bacillus *Brevibacillus thermoruber* strain 7 was isolated from the thermophilic community from Marikostinovo spring.
4. As a result of the established high degradation efficiency of 0.3% PCL in the medium, the PCL pearls were completely degraded in four weeks. The degradation rate achieved is among the highest reported for a plastic substrate.
5. The mechanism of more efficient degradation of PCL by the thermophilic community compared to strain 7 is explained with the parallel presence of microorganisms that digest the oligomers obtained from the activity of strain 7, as well as those that favor the formation of biofilm on the PCL surface.
6. Purified lipase is the first reported thermostable enzyme capable of degrading PCL. The coincidence between the temperature optimum of strain 7 lipase (55°C) and the melting point of PCL 55–60°C determines the possibility for an efficient enzymatic process with the participation of ultraconcentrate or pure enzyme. An additional contribution to an efficient enzyme process is the wide pH range in which the enzyme is active – over 80% at pH 6.0 to 9.0.
7. The obtained results suggest an effective application of the isolated community, *B. thermoruber* strain 7, and the enzyme synthesized by it to solve one of the main problems of our society, namely the treatment of plastic waste.
8. The isolated and phylogenetically defined thermophilic and halophilic strains capable of degrading to varying degrees polycaprolactone, polyvinyl alcohol, polystyrene and polypropylene are a basis for future research on the degradation of plastics.

Publications related to the dissertation:

1. Atanasova, N., Stoitsova, S., Paunova-Krasteva, T., Kambourova, M. Plastic degradation by extremophilic bacteria. International Journal of Molecular Sciences, 22, 11, 2021, no 56. Q1, JCR IF (Scopus): 5.923

2. Atanasova, N., Paunova-Krasteva, T., Stoitsova, S., Radchenkova, N., Boyazdhieva, I., Petrov, K., Kambourova, M. Degradation of poly(ε-caprolactone) by a thermophilic community and Brevibacillus thermoruber strain 7 isolated from Bulgarian hot spring. Biomolecules, 11, 10, MDPI, 2021, 1488. Q2, SJR (Scopus): 4.879

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Citations of the publications related to the dissertation

Cited publication:

Atanasova, N., Stoitsova, S., Paunova-Krasteva, T., Kambourova, M. (2021). Plastic degradation by extremophilic bacteria. International Journal of Molecular Sciences, 22, 11, no 56. Q1, SJR IF (Scopus): 5.923

Citing publication:

1. Borthakur, D., Rani, M., Das, K., Shah, M. P., B. K. Sharma (2021). Bioremediation: an alternative approach for detoxification of polymers from the contaminated environment. Letters in Applied Microbiology

2. Chattopadhyay, I. (2021). Role of microbiome and biofilm in environmental plastic degradation. Biocatalysis and Agricultural Biotechnology, DOI:10.1016/j.bcab.2021.102263
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