EXTRACELLULAR HYDROLASES PRODUCED BY MICROORGANISMS ISOLATED FROM THE POLLUTED RIVER PASAREA, ROMANIA

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The present work highlights the presence of extracellular hydrolytic enzymes such as amylase, caseinase, cellulase, esterase, gelatinase for some strains belonging to the genera *Aeromonas*, *Bacillus*, *Brachybacterium*, *Enterobacter*, *Exiguobacterium*, *Lysinibacillus*, *Microbacterium*, *Pseudomonas* and *Yersinia* isolated from water samples taken from the putative polluted sector of the Pasarea river, in the area of Tunari, Ilfov county. The 20 investigated strains belong to five families as follow: *Bacillaceae*, *Dermabacteraceae*, *Enterobacteraceae*, *Microbacteriaceae*, *Aeromonadaceae*, *Yersiniaceae* and *Pseudomonadaceae*. The predominant enzymatic activities were the hydrolysis of starch, casein and Tween 80. Strains belonging to the genera *Aeromonas*, *Bacillus* and *Pseudomonas* distinguished themselves by the presence of the five types of enzymatic activities investigated, some of them being combined.

Keywords: extracellular hydrolases, Pasărea river, pollution.

INTRODUCTION

Bacterial extracellular enzymes are widely distributed in several ecosystems (Ruginescu *et. al*, 2020; 2022) and their activity is regulated at this level by environmental factors and at the microenvironment level by enzyme-substrate interactions. Generally, microorganisms have the ability to quickly respond to environmental changes due to their close contact with the environment and relatively rapid rate of growth. As mediators in important biogeochemical processes, i.e. decomposition and transformation of organic matter, release of inorganic nutrients to higher trophic levels and detoxification of xenobiotics, bacterial enzyme activities have the potential to be used as descriptors of biological responses to changing environmental conditions (Cole, 1999).

It is very well known that heterotrophic bacteria which represent the main group in aquatic ecosystems, by their extracellular enzymatic equipment are involved in the process of decomposition and mineralization of organic matter mainly represented by decaying phytoplankton and riverine input (Findlay *et al*.,1998).

The extracellular enzymes of bacteria have an important role in the first stages of the decomposition of organic matter. Enzyme activity can be induced by

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natural saprobic processes or influenced by organic pollution. In the mineralization process, extracellular enzymes break down complex molecules into simple molecules (Hoppe, H.G., 1991) and thus any disruptive factor in enzyme activity or production affects the entire mineralization process (Arnosti, 2003). The main category of extracellular enzymes is represented by hydrolases that can break C-O and C-N that bind monomers but they can also be involved in oxidative reactions by cleaving C-C and C-O bonds (Sinsabaugh, 1994).

The degradation of organic matter and inorganic compounds by heterotrophic bacteria is dependent on the ability of these microorganisms to produce extracellular hydrolytic enzymes (Ojovan *et al*., 2021). Enzyme categories vary depending on the carbon source available in the environment. Generally, bacteria synthesize the constitutive enzymes required to use a simple organic carbon source such as glucose. Although bacteria prefer to use glucose as a substrate, if it becomes unavailable, they have the ability to synthesize the enzymes necessary to process other categories of substrate (Alves *et al*., 2014). Also, thanks to the conjugation phenomenon, bacteria can diversify their enzyme equipment, increasing their survival rate in conditions where the basic substrate for the constitutive enzymes is not available. Microbial enzyme activity is dependent on environmental conditions and is favored by alkaline pH and high temperatures (Chrost, J.R. Siuda, W 2002). Most urban aquatic ecosystems are influenced by human activities, thus organic matter accumulates with the presence of pollutants, a fact that contributes to the decrease in the self-purification capacity of these aquatic environments (Neagu *et al*., 2021). Thus, inorganic nutrient pollution can overtax the hydrolytic capacity of aquatic systems, although this is not a major problem for running waters. In lakes, however, an increase in the enzymatic activity of peptidases and esterase was recorded with increasing trophic status (Burns, R.G., Dick, R.P., 2002). In terrestrial or aquatic environments, the microbial enzymes responsible for oxidation, reduction and methylation release heavy metals from complexes with organic compounds, pass them into an insoluble phase or precipitate them in the aqueous environment, forms in which they have a lower level of toxicity or can be removed easier (Dungan R.S., Frankenberger W.T., 2002). Generally, enzyme activities in aquatic ecosystems can be useful as indicators of pH changes, ofpollution and water quality following the impact of organic effluents from wastewater treatments, as well as of potentially toxic substances such as heavy metals or critical raw materials (CRM) (Burns, R.G., Dick, R.P., 2002).

The aim of this paper was to identify the ability of microbial strains belonging at nine genera isolated from chemically polluted Pasarea River to synthetize extracellular enzymes able to degrade macromolecules into complex chemical composition of aquatic ecosystem, considering the ability of microbial enzyme mixture to degrade xenobiotics and pollutants from the flow water (Alokpa *et al*., 2022). The novelty degree of the study is also supported by seasonal variations in the physico-chemical behavior of the investigated river sector as results of anthropogenic impact which is reflected also in the dynamics of microbial populations and their extracellular enzymatic activities spectrum.

MATERIAL AND METHODS

Water column samples were collected from two sites, marked as Dimieni Bridge (DB – $44^0$55′24.9″N, $27^0$14′34.4″E) and Tunari Dam (TD – $44^0$54′73.7″N, $26^0$16′23.0″E), during summer (July – D1), autumn (November – D2), winter (February – D3) in 2020 and spring (April – D4) in 2021 from the Pasarea River. The samples were collected in sterile, glass containers and subsequently stored at a temperature of 4^0C, until investigation in the laboratory. The water salinity, total dissolved solids, conductivity and pH were recorded in situ by using a multiparameter HI 9828 Hanna Instrument.

For the *isolation of the bacterial strains* serial dilution were prepared using solidified nutrient broth culture media. The Petri dishes were incubated at 28^0C for 24–48 hours and after these intervals, some bacterial strains were randomly selected based on their morphology and color. Selected strains further were characterized for Gram staining (Helebian *et al*., 1981; Suslow *et al*., 1982; Moledj, 1986) and catalase/oxidase activity (Steel, 1961). To confirm Gram staining results, 3% KOH test was used. Cultures becoming viscous and forming a mucoid string in 15 sec. in contact with this solution were assessed as Gram–negative. Catalase was elicited by adding a 3% H_2O_2 solution over a fresh bacterial culture and appearance of gas bubbles was considered positive reaction (Azhar *et al*., 2014). The oxidase test is based on phenylenediamine oxidized to indophenol and dark purple color is considered a positive reaction (Shields *et al*., 2010).

Critical Raw Materials (European Commission Classification) presence in the water samples has been determined by XRF analysis using XRF Rigaku ZSX100e, Supermini model (Catana *et al*., 2023; Neagu *et al*., 2021).

Identification of the strains by 16S rDNA analysis – the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) has been used for total genomic DNA extraction following the standard protocol for bacteria. Purity and concentration were measured using a BioDrop DUO UV/VIS spectrophotometer. The 16 S rRNA gene has been amplified by PCR in a 50 µL final reaction volume containing 1× Mango Master Mix (Bioline), 0.2 µM of each primer (27F and 1492R), 50–250 ng DNA template and bidistilled water (Fredriksson *et al*., 2013). The PCR reactions were performed following the protocol: 3 min denaturation at 95^0C, 35 cycles of 1 min denaturation at 95^0C, 1 min annealing at 57^0C, 90 s extensions at 72^0C and a final extension step of 5 min at 72^0C. The obtained amplicons were checked on agarose gel (1%, w/v) and then purified with QIAquick PCR Purification Kit (Qiagen). The gene sequencing was performed by a commercial sequencing service provider (Macrogen Europe B.V.) using primer 27F. The sequences were further compared to known sequences available in the NCBI public database using the BLASTN algorithm. The phylogenetic trees were reconstructed in MEGA X from the resulting alignments (CLUSTALW algorithm) with 16S rRNA gene sequences of related reference strains, using the Neighbor-joining method and the Tamura-Nei model (Tamura and Nei, 1993).

Extracellular hydrolytic enzymes – The ability of selected bacteria to produce extracellular hydrolytic enzymes (amylase, protease, lipase/esterase, cellulase and gelatinase) was qualitatively assayed on modified solidified culture medium (meat extract 3g L^{-1}, agar 15g L^{-1}, NaCl 5g L^{-1}) containing 1 g/L of one of the interest substrates as follow: Tween 80, starch, casein, gelatin. For the cellulase assay the amount of substrate carboxymethyl cellulose (CMC) added in culture media was 0.5 g/L and for gelatinase assay, the agar has been removed from the medium composition (Enache *et al*., 2007; Ruginescu *et al*., 2020). Tested microbial isolates were inoculated onto the surface of agar plates using fresh solid inoculum and incubated at 28^0C for 48 hours. The hydrolytic activities towards casein, starch and CMC were considered positive by appearance of a clear zone around the colonies after flooding the plates with certain solutions: 1N HCl (casein), 0.3% I_2–0.6% KI solution (starch) and 0.1% Congo red (CMC). Esterase/lipase activity was judged as positive by appearance of an opaque halo around the colonies (Rohban *et al*., 2009; Menasria *et al*., 2018). The presence of gelatinases liquefies the medium (Azhar *et al*., 2014) after incubation at 30^0C for 24h. The experiments were conducted in triplicate, and results were expressed qualitatively as levels of enzyme activities (LEA) using the diameter of the hydrolysis zone (in millimeters) from which it was subtracted the culture spot diameter. The microbial strains were classified as having high (LEA > 10), medium (LEA 5–10), low (LEA < 5) or no hydrolytic activities (Ruginescu *et al*., 2020; Enache *et al*., 1999; 2004).

RESULTS

Isolation and preliminary characterization of strains

From the investigated column water samples taken from the polluted river Pasarea along of four seasons, 20 strains were randomly selected to be successively passed on solidified culture media until their purification. Their morphological aspects revealed transparent, matte white, glossy white cream-pink, yellow, orange, red colonies with a flat, umbonate or convex profile, with a well-defined margin, regular, radial, round, with a mucous appearance. According with the results obtained after test with 3% KOH, 11 isolates were considered Gram positive (55%) and 9 Gram negative (45%). Tests for metabolic activities indicated 8 strains (40%) positive for oxidase and 14 (70%) for catalase. On the other hand, 12 strains (60%) were judged as negative for oxidase and six (30%) for catalase. Three isolates (1, 4N and 5N) showed positive reaction to both tests for oxidase and catalase, two of them being isolated in the winter and one in the spring of the year 2021, respectively.

Physico-chemical parameters of area

The sampled river Pasarea appears to be a dynamic ecosystem whose physico-chemical parameters (Table 1) fluctuate seasonally depending on

atmospheric conditions like rainfall, solar radiation intensity and human impact (Ojovan *et al*., 2021). The physico-chemical parameters recorded at the sampling time are showed in Table 1. Their variable pH values from neutral to slow alkaline was relatively similar with values reported for other flow water (Iticescu *et al*., 2019) and during the four seasons, the pH values were between 6.82 (winter-DB) and 9.55 (winter-TD). The increase in the amount of suspended solids (TDS) led to the increase in electrical conductivity; the lowest values of TDS were recorded in the summer season, 345 at the DB and 354 at the TD. The maximum values were reached in the autumn season at the DB (510) and in the winter, at the TD (454). The electrical conductivity also varied in the same ratio with minimum values in the summer season in the two sampling points, the maximum values being reached in the autumn season at the DB, respectively the winter season at the TD. The water salinity in the area did not undergo major changes, the values being between 0.33-0.51 (DB), the maximum value recorded in the autumn and 0.34-0.45 (TD) with maximum in the winter. Thus, the variation is correlated with the values of TDS that equally influenced also the electrical conductivity values. The recorded temperature was relatively similar between the sampling points and have seasonal variation from around $4/6^0$C during were the winter until to 28^0C in the summer (Table 1) when selected the majority of the investigated strains.

Table 1
The seasonal variation of some physical-chemical parameters in situ recorded at BD (Dimieni Bridge) and TD (Tunari Dam) sampling point in river Pasarea

Season (year)	Summer (2019)		Autumn (2019)		Winter (2020)		Spring (2020)	
Sampling site	DB	TD	DB	TD	DB	TD	DB	TD
pH	8.44	8.27	8.27	9.16	6.82	9.55	7.45	8.95
Total dissolved solids (ppm)	345	354	510	385	468	454	396	445
Conductivity 1 (µS/cm)	691	707	1023	771	936	909	792	890
Salinity (PSU)	0.33	0.34	0.51	0.38	0.46	0.45	0.39	0.44
Temperature (^{0}C)	28.7	28.7	11.1	11.6	6.7	3.8	10.4	12.5

Extracellular hydrolytic enzymes

All isolates were tested for their capacity to produce extracellular enzymes such as esterases, cellulases, amylases, gelatinases and caseinases. The results from Fig. 1 showed that the most common hydrolases were those of the esterase type (8 strains – 29%) with a hydrolysis diameter between 2 mm and 30 mm, respectively and caseinases (8 strains – 29%) with a smaller hydrolysis diameter, between 3 mm and 9 mm. The presence of gelatinases was recorded for six strains (21%) out of the 20 isolates, the medium supplemented with starch was hydrolyzed by only four strains (14%) with a hydrolysis diameter between 2 mm and 8 mm and only two (7%) strains degraded the medium supplemented with carboxymethylcellulose, with a hydrolysis diameter of 10 mm and 20 mm, respectively.

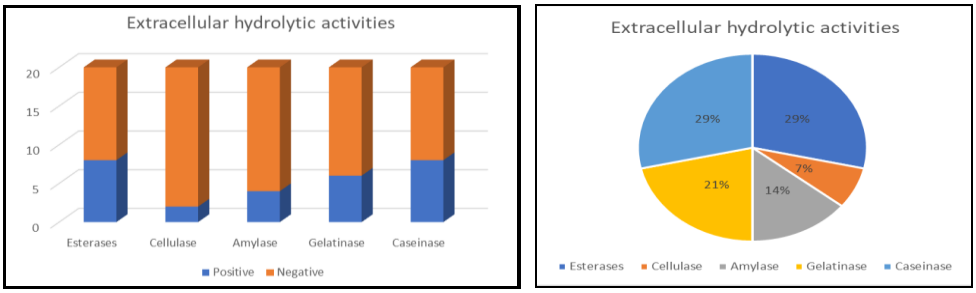

Figure 1. The capacity of the investigated isolates to produce extracellular enzymes - esterases, cellulases, amylases, gelatinases and caseinases

Nine isolates were tested positive for a single type of hydrolases, of which two for gelatinase, two isolates produced esterases (diameter of hydrolysis was 25 mm and 3 mm, respectively), for caseinase four isolates had a smaller diameter of hydrolysis, of only 3 mm, 5 mm, and 9 mm, respectively, and a cellulase-producing isolate (diameter of hydrolysis 10 mm) (Table 2). Four isolates had the ability to degrade media supplemented with two types of substrates, two of which showed esterase and amylase activity, one isolate hydrolyzed media supplemented with gelatin and casein, and another isolate hydrolyzed media supplemented with esterase and casein. Two isolates had the ability to produce three types of hydrolases.

Table 2
The capacity of investigated strains to produce extracellular hydrolases. The numbers from the brackets representing hydrolysis zone excluding colony diameter.

Strains	BLAST correspondence genus	Esterase (TW 80) (mm)	CMC (mm)	Amylase (mm)	Gelatinase	Caseinase (mm)
5n (D1)	*Microbacterium*	negative	negative	negative	negative	positive (5)
P1-2 (D.3)	*Bacillus*	positive (5)	negative	positive (8)	negative	negative
P1-4 (D.3)	*Brachybacterium*	negative	negative	negative	positive	negative
P1a-5 (D3)	*Pseudomonas*	positive (20)	negative	negative	negative	negative
P1-5 (D.3)	*Pseudomonas*	positive (25)	negative	negative	negative	negative
P1-1 (D.3)	*Pseudomonas*	positive (30)	negative	negative	positive	positive (3)
P2-5(D.4)	*Bacillus*	negative	negative	negative	negative	positive (5)
P2-2 (D.4)	*Aeromonas*	negative	negative	negative	positive	positive (4)
P2-7 (D.4)	*Aeromonas*	positive (10)	negative	negative	negative	positive (5)
P1-1 (D.4)	*Aeromonas*	positive (15)	negative	positive (5)	positive	negative
P2-8 (D.4)	*Yersinia*	negative	negative	negative	negative	negative
P2-3 (D.4)	*Bacillus*	negative	negative	negative	negative	positive (9)
P2 -1 a (D.4)	*Aeromonas*	positive (2)	negative	positive (2)	negative	negative
P1-2 (D.4)	*Aeromonas*	positive (2)	positive (20)	positive (5)	positive	positive (4)

Strains	BLAST correspondence genus	Esterase (TW 80) (mm)	CMC (mm)	Amylase (mm)	Gelatinase	Caseinase (mm)
P2-4 (D.4)	*Bacillus*	negative	positive (10)	negative	negative	negative
P2-1b (D4)	*Lysinibacillus*	negative	negative	negative	negative	negative
P1-3 (D4)	*Pseudomonas*	negative	negative	negative	negative	negative
P2-6 (D.4)	*Bacillus*	negative	negative	negative	negative	negative
4N (D.1)	*Exiguobacterium*	negative	negative	negative	negative	positive (3)
R 1 (D1)	*Enterobacter*	positive (3)	negative	negative	negative	negative

They were isolated in the winter and spring seasons respectively and produced hydrolases such as esterase with a hydrolysis diameter of 30 mm, gelatinase and caseinase (smaller diameter, only 3 mm), and in the other isolate esterase, amylase and gelatinase. The strain P1-2 (D4) isolated in the spring season stood out for its ability to degrade media supplemented with all five types of substrates for which it was tested, being producers of esterase (2 mm), cellulase (20 mm), amylase, gelatinase (5mm) and caseinase (4mm). Four isolates out of 20 were not able to degrade any media supplemented with the five substrate types (TW80, CMC, starch, gelatin or casein) for which they were tested. Considering the level of enzymatic activity (LEA) the strains are assigned as having high (LEA > 10) – seven strains, medium (LEA 5–10) – seven strains and low (LEA < 5) – eight strains, hydrolytic activities. There were recorded four strains without extracellular enzymatic activities (LEA – 0).

Phylogenetic analysis

The phylogenetic tree reconstructed from 16S rDNA sequences grouped investigated strains in two major clades from ancestor. The first clad is represented by the members of family *Enterobacteriaceae* and *Pseudomonadaceae* with strains belonging to the genera *Aeromonas*, *Enterobacter*, *Yersinia* and *Pseudomonas* (Figure 2). Between members of this genera grouped 11 strains from our investigated isolates, from which five has been isolated from TD point (P 2-2, P2-1a, P2-7, 1 and P2-8) and six from DB (P1-1, P1-2, P1-3, P1-1/D3, P1-5 and P1a-5) generally in warm seasons. From these isolates five grouped with *Aeromonas* (P 2-2, P2-1a, P2-7, P1-1, P1-2), four with *Pseudomonas* (P1-3, P1-1/D3, P1-5 and P1a-5) and one each with *Enterobacter* (1) and *Yersinia* (P2-8) strains. The second clade is constituted by members of families *Bacillaceae*, *Dermabacteraceae* and *Microbacteriaceae* with strains grouping with members of *Microbacterium* (5N), *Brachybacterium* (P1-4), *Exiguobacterium* (4N), *Lysinibacillus* (P2-1b) and *Bacillus* (P2-3, P2-4, P2-5, P2-6). Four of the strains grouped in this clad (4N, 5N, P1-2 and P1-4) were isolated from DB and the five remaining were isolated from TD. According to the blast analysis (table 3) the investigated strains revealed a high degree of similarity (>97%) with the members of genera with which they grouped in the phylogenetic tree. There are some

exceptions for the strain P 1-3 which has 95,41% similarity with *Pseudomonas psychrophila* and strain 4N which showed 94,52% similarity with *Exiguobacterium* species. Based on this preliminary data, this isolate should be analyzed as a possible new strain for this genus from *Bacillaceae* family.

Generally, most of the investigated strains belonging to the five families previously mentioned have been isolated in the warm seasons, excepting strains 1, 4N and 5N, considering the randomly criteria applied in this way and should be noticed that in this season, the chemical composition of the river water indicated a putative pollution in this way mainly due to the presence of some critical raw materials (Bunker *at al*., 2016; European Comission – 2020) like cerium, germanium, holmium, terbium and neodymium compounds. These elements have been detected sporadically either to DB or TD, seasonally, with a content from investigated sample (10 mL of water) varying from 2,6mass% (germanium in autumn at DB) until to 26.2mass% (neodymium in autumn at TD). The other elements have different concentration like 6.1mass% holmium, 8.1mass% terbium (increasing in warm seasons until to 14mass%) and 30mass% for cerium in the spring sample.

Table 3
The BLAST correspondence between investigated strains and similar 16S rRNA sequences already deposited in data bank

Strains	**BLAST correspondence strain**	**Similarity degree %**
P1-2 (D.3)	*Bacillus sp.*	99,7
P1-5 (D.3)	*Pseudomonas alcaligenes*	99,67
P1a-5 (D3)	*Pseudomonas sp.*	99,7
P2-5(D.4)	*Bacillus sp.*	93,31
P2-2 (D..4)	*Aeromonas popoffii*	99,45
4N (D.1)	*Exiguobacterium*	**94,52**
R1 (D1)	*Enterobacter kobei*	98,22
P2-7 (D.4)	*Aeromonas salmonicida*	98,44
P1-1 (D.3)	*Pseudomonas aeruginosa*	98,73
P1-1 (D.4)	*Aeromonas hydrophila*	97,86
P2-8 (D.4	*Yersinia intermedia strain*	98,07
P2-3 (D.4)	*Bacillus sp.*	97,31
P2 -1 a (D.4)	*Aeromonas popoffii*	97,28
5n (D1)	*Microbacterium marytipicum*	98,37
P1-2 (D.4)	*Aeromonas allosaccharophila*	97,04
P1-4 (D.3)	*Brachybacterium muris/zhongshanense*	97,93
P2-4 (D.4)	*Bacillus sp.*	99,65
P21b(D4)	*Lysinibacillus macroides*	97,73
P1-3(D4)	*Pseudomonas psychrophila*	**95,41**
P2-6(D.4)	*Bacillus sp.*	97,04

CONCLUSIONS

The investigated strains from this paper have been assigned to nine genera of bacteria belonging to five families: *Bacilaceae*, *Dermabacteraceae*, *Enterobacteraceae*, *Microbacteriaceae* and *Pseudomonadaceae* respectively. Most of them are known as infectious agents and having ressistance to a wide spectrum of antibiotics (Parcalabioru *et al*., 2021; Weist *et al*., 2013). The strains belonging to some genera like *Aeromonas, Enterobacter* and *Yersinia* are well known as agents for various illnesses. Thus, their presence in Pasarea river indicates an athropogenic impact and a high degree of pollution in accordance with the presence of CRMs too. From these elements the cerium appears to have adverse effects towards environments and aquatic ecosystems (NCBI 2021) and his presence in flow waters could be a consequence of its usage as oxidizing/reducing plating agent, surface treating agent, environmental catalyst paint being necessary to be quantified (Wang *et al*., 2018; Catana *et al*., 2023). In order to cope with the chemical pollution, the microbial communities from the flow waters synthetize extracellular hydrolases which can contribute to decresing degree of contamination by xenobiotics (Alokpa *et al*., 2022). In our cases a number of five types of hydrolases such as amylase, esterase/lipase, caseinase, gelatinase and cellulase was detected for the investigated strains which demonstrates the ability to degrade macromolecules currently present or accidentally deposited in the ecosystem. Some of the strains, namely 4N belonging to genera *Exiguobacterium* which is know from the literature (Yang *et al*., 2015) as plastics degrading argued for pollution with plastic and microplastics matter in Pasarea river and adiacent plain areas. The presence of the strain in the flow water could be considered also a consequence of plastic pollution resulting from human activities. The combined extracellular hydrolitic activities for some strains (P1-2, P2-1a, P2-7, P2-2) belonging to *Aeromonas* genera could be considered for their use in the treatment of some polluted flow water sectors or other water bodies. Thus, the ability of extracellular hydrolases mixture (Alokpa *et al*., 2022) to transform low quantity of CRM in environmental ecosystem conditions could be exploited for further development of several systems (Lulea *et al*., 2022) for polluted area monitoring, management, treatment and recovering for reutilisation.

REFERENCES

1. Alokpa, K., Lafortune, P., Cabana, H., 2022, Application of laccase and hydrolases for trace organic contaminants removal from contaminated water, Environmental Advances, 100243.
2. Alves, P.D.D., Siqueira, F.d.F., Facchin, S., Horta, C.C.R., Victória, J.M.N., Kalapothakis, E., 2014, Survey of Microbial Enzymes in Soil, Water, and Plant Microenvironments, *TOMICROJ*, **8**, pp. 25–31.
3. Arnosti, C., 2003, Microbial Extracellular Enzymes and their Role in Dissolved Organic Matter Cycling, *Academic Press*, pp. 315–342.

4. Azhar, M., Uniyal, V., Chauhan N., Rawat, S.D., 2014, Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India, *International Journal of Current Microbiology and Applied Sciences*, **3**, **12**, pp. 753–760
5. Basic Local Alignment Search Tool. Avai. online: https://blast.ncbi.nlm.nih.gov/Blast.cgi.
6. Bunker, B.C., Casey, W.H., 2016, The Impact of Oxides on Environmental Chemistry, in *The Aqueous Chemistry of Oxides*, Bunker, B.C., Casey, W.H., Eds.; Oxford University Press: Oxford, UK, pp. 537–582.
7. Burns, R.G., Dick, R.P. (Eds.), 2002, Enzymes in the Environment, Activity, Ecology, and Applications; Books in soils, plants, and the environment; Marcel Dekker, New York, NY, USA, ISBN 978-0-8247-0614-2.
8. Catana, D.R., Podosu, A., Florescu, I.L., Mihai, A.A., Enache, M., Cojoc, R., Moldoveanu, M., 2023, Quantitative Analyses of Chemical Elements in *Phragmites australis* as *Bioindication of Anthropization in Urban Lakes*, *Sustinability*, **15**, p. 553.
9. Cole, J., 1999, Aquatic Microbiology for Ecosystem Scientists: New and Recycled Paradigms in Ecological Microbiology, *Ecosystems*, **2**, pp. 215–225
10. Chrost, J. R., Siuda, W., 2002, Ecology of Microbial Enzymes in Lake Ecosystems, in *Enzymes in the environment*, p. 52
11. Dungan, R.S., Frankenberger, W.T., 2002, Enzyme-Mediated Transformations of heavy metals/metalloids. Agricultural research service, U.S. Department of Agriculture.
12. Enache, M.; Itoh, T., Kamekura, M., Teodosiu, G., Dumitru, L., 2007, *Haloferax prahovense* Sp. Nov., an Extremely Halophilic Archaeon Isolated from a Romanian Salt Lake, *Int. J. Syst. Evol. Microbiol*, **57**, pp. 393–397.
13. Enache, M., Dumitru, L., Faghi, A.M., 1999, Occurrence of halocins in mixed archaebacteria culture, *Proc. Inst. Biol*., II, pp. 151–154.
14. Enache, M., Faghi, A.M., Dumitru, L., Teodosiu, G., Zarnea, G., 2004, Halocin Hf1 a bacteriocin produced by *Haloferax* sp. GR1, *Proc. Rom. Acad. Series* B, **6**, pp. 27–32.
15. European Commission. Study on the EU's List of Critical Raw Materials–Final Report; European Commission: Brussels, Belgium, 2020.
16. S. Findlay, RL, Sinsabaugh, DT, Fischer, P, Franchini, 1998, Sources of dissolved organic carbon supporting planctonic bacterial production in the tidal freshwater Hudson River, *Ecosys 1*: pp. 227–239, **16**. K, Suberkropp, A. Michelis, H-J, Lorch, JCG, Ottow, 1988, Effect of sewage treatment plant effluents on the distribution of aquatic hyphomycetes in the river Erms, Schwa¨bische Alb, *F.R.G. Aquat Bot*, **32**, pp. 141–153.
17. Fredriksson, N.J., Hermansson, M., Wilén, B.-M, 2013, The Choice of PCR Primers Has Great Impact on Assessments of Bacterial Community Diversity and Dynamics in a Wastewater Treatment Plant. PLoS ONE, **8**, e76431.
18. Halebian, S., Harris, B., Finegold, S. M., Rolfe R.D. 1981, Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria, *J. Clin. Microbiol*., **13**, **3**, pp. 444–448.
19. Hoppe, Hans-Georg., 1991, Microbial Extracellular Enzyme Activity: A New Key Parameter in Aquatic Ecology, 10.1007/978-1-4612-3090-8_4.
20. Iticescu, C., Georgescu, L.P., Murariu, G., Topa, C., Timofti, M., Pintilie, V., Arseni, M., 2019, Lower Danube Water Quality Quantified through WQI and Multivariate Analysis, *Water*, **11**(6), p. 1305.
21. Latorre, J.D., Hernandez-Velasco, X., Wolfenden, R.E., Vicente, J.L., Wolfenden, A.D., Menconi, A., Bielke, L.R., Hargis, B.M., Tellez, G., 2016, Evaluation and Selection of *Bacillus* Species Based on Enzyme Production, Antimicrobial Activity, and Biofilm Synthesis as Direct-Fed Microbial Candidates for Poultry, *Front. Vet. Sci*., **3**, 95.
22. Lulea, A.C., Ruginescu, R., Banciu, R.M., Pantazi, C., Brinduse, E., Ion, M., Quintela, S., Elejalde, E., Fernandez-de-Castro, L., Villaran, M.C., Ruiz-de-Vergara, Z., Ruiz, C., Epure,

P., Purcarea, C., Vasilescu, A., 2022, Fast Electrochemical Measurement of Laccase Activity for Monitoring Grapes' Infection with *Botrytis cinerea*, *Processes*, **10**, 3.

23. Menasria, T., Aguilera, M., Hocine, H., Benammar, L., Ayachi, A., Si Bachir, A., Dekak, A., Monteoliva-Sánchez, M., 2018, Diversity and Bioprospecting of Extremely Halophilic Archaea Isolated from Algerian Arid and Semi-Arid Wetland Ecosystems for Halophilic-Active Hydrolytic Enzymes, *Microbiol. Res*., **207**, pp. 289–298.
24. Moaledj, K., 1986, Comparison of Gram-staining and alternate methods, KOH test and aminopeptidase activity in aquatic bacteria: their application to numerical taxonomy, *J. Microbiol. Methods*, **5**, 5–6, pp. 303–310.
25. National Center for Biotechnology Information. PubChem Compound Summary for CID 73963, Cerium Dioxide; National Center for Biotechnology Information: Bethesda, MD, USA, 2021.
26. Neagu, S., Enache, M., Cojoc, R., Ruginescu, R., Moldoveanu, M., Florescu, L., Lucaci, I., 2021, Seasonal variation of the water color from the IOR lake – Bucharest. *Oltenia. Studii şi comunicări. Ştiinţele Naturii Tom.*, **37**(1), pp. 205–210.
27. Ojovan, B., Catana, R., Neagu, S., Cojoc, R., Lucaci, A.I., Marutescu, L., Florescu, L., Ruginescu, R., Enache, M., Moldoveanu, M., 2021, Metabolic Potential of Some Functional Groups of Bacteria in Aquatic Urban Systems, *Fermentation*, **7**, pp. 242.
28. Pircalabioru, G.G., Popa, L.I., Marutescu, L., Gheorghe, I., Popa, M., Czobor Barbu, I., Cristescu, R., Chifiriuc, M.C., 2021, Bacteriocins in the Era of Antibiotic Resistance: Rising to the Challenge, *Pharmaceutics*, **13**, p. 196.
29. Ruginescu, R., Lavin, P., Iancu, L., Menabit, S., Purcarea, C., 2022, Bioprospecting for Novel Bacterial Sources of Hydrolytic Enzymes and Antimicrobials in the Romanian Littoral Zone of the Black Sea. *Microorganisms*, **10**, 12.
30. Ruginescu, R., Gomoiu, I., Popescu, O., Cojoc, R., Neagu, S., Lucaci, I., Batrinescu-Moteau, C., Enache, M., 2020, Bioprospecting for novel halophilic and halotolerant sources of hydrolytic enzymes in brackish, saline and hypersaline lakes of Romania, *Microorganisms*, **8**, 12.
31. Rohban, R., Amoozegar, M.A., Ventosa, A., 2009, Screening and Isolation of Halophilic Bacteria Producing Extracellular Hydrolyses from Howz Soltan Lake, *Iran. J. Ind. Microbiol. Biotechnol*., **36**, pp. 333–340.
32. Sinsabaugh, R., 1994, Enzymic analysis of microbial pattern and process. *Biology and Fertility of Soils*, **17**, pp. 69–74, 10.1007/BF00418675.
33. Steel, K. J., 1961, The Oxidase Reaction as a Taxonomic Tool., *J. Gen. MicroMol*, **25**, pp. 297–806.
34. Suslow, T. V., M. N Schroth, and M. Isaka, 1982, Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining, *Phytopathology*, **72**, pp. 917–918.
35. Tamura, K., Nei, M., 1993, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, *Mol. Biol. Evol*, **10**, pp. 512–526.
36. Wang, X., Zhu, M., Li, N., Du, S., Yang, J., Li, Y., 2018, Effects of CeO_2 nanoparticles on bacterial community and molecular ecological network in activated sludge system, *Environmental Pollution*, **238**, 516e523.
37. Weist, K., Diaz Hogberg, L., 2014, ECDC Publishes 2013 Surveillance Data on Antimicrobial Resistance and Antimicrobial Consumption in Europe, *Eurosurveillance*, **19**, p. 20962.
38. Yang, Y., Yang, J, Wu, WM, Zhao, J., Song, Y., Gao, L., Yang, R., Jiang, L., 2015, "Biodegradation and Mineralization of Polystyrene by Plastic-Eating Mealworms: Part 2. Role of Gut Microorganisms", *Environ. Sci. Technol*, **49**, 20, pp. 12087–12093.

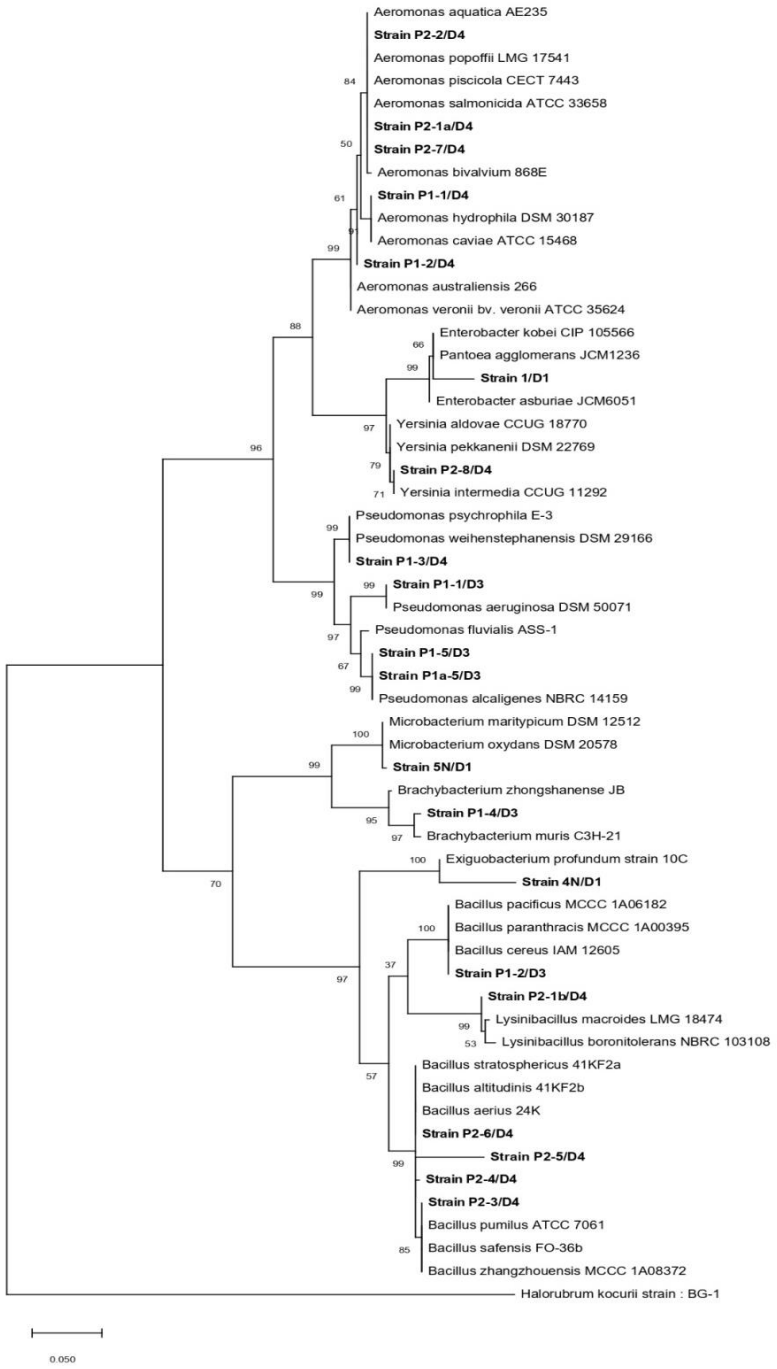

Figure 2. Phylogenetic tree derived from the 16S rRNA gene sequences showing the position of the investigated strains between memebres of genera *Aeromonas*, *Bacillus*, *Brachybacterium*, *Enterobacter*, *Exiguobacterium*, *Lysinibacillus*, *Microbacterium*, *Pseudomonas* and *Yersinia*. The tree was reconstructed by the neighbour-joining method. Bootstrap values >50 % (100 replicates) are shown. Bar, 0.05 substitutions per nucleotide position.