ROMANIAN ACADEMY
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SUMMARY OF THE PhD THESIS

IDENTIFICATION OF NOVEL HALOPHILIC AND HALOTOLERANT MICROBIAL STRAINS ABLE TO PRODUCE BIOTECHNOLOGICALLY IMPORTANT HYDROLYTIC ENZYMES

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INTRODUCTION

Enzymes are ubiquitous components of living organisms that catalyze metabolic reactions at rates high enough to sustain life. Besides their vital biological functions, enzymes have multiple commercial uses: biocatalysts in industrial processes (e.g., conversion of starch to glucose in the food industry), therapeutic agents (e.g., removal of fibrin clots from bloodstream), analytic reagents (e.g., detection of glucose in blood) and manipulative tools (e.g., lysozyme, restriction enzymes).

The global enzymes market, valued at more than USD 11 billion in 2021, is dominated by hydrolases, particularly proteases, amylases, lipases and cellulases. Microorganisms are the main sources from which these enzymes are obtained due to the economic, technical and ethical advantages they present in comparison to animal and plant organisms.

Among the various microbial sources of hydrolytic enzymes, microorganisms that survive in extreme environmental conditions have gained increasing attention in the scientific community over the last decades. Their enzymes, often called extremozymes, perform the same catalytic functions as their mesophilic counterparts, but additionally they operate under severe physicochemical conditions that generally inactivate enzymes produced by non-extremophiles. Due to their robustness, extremozymes have proven to be useful in numerous industrial and environmental applications that require harsh conditions, such as high/low temperatures, acidic/alkaline pH, or high salinity.

A group of extremophilic microorganisms that produce biotechnologically valuable hydrolases is represented by halophiles. They are adapted to survive in highly saline environments and are typically categorized on the basis of their salt requirement/tolerance as extreme halophiles (preferring media with 2.5–5.2 M NaCl), moderate halophiles (optimal growth at 0.5–2.5 M NaCl) or halotolerant microorganisms (non-halophiles that tolerate relatively high salt concentrations, often up to 2.5 M NaCl). Extracellular enzymes produced by both halophilic and halotolerant microorganisms have evolved to retain structural stability and catalytic activity over a wide range of salinities. These salt-adapted macromolecules, compared to their mesophilic counterparts, show an excess of acidic amino acids at the protein surface and a general decrease in hydrophobic amino acid frequency.

The major applications in which such extremozymes have been proposed to be used are biofuel production, food processing, bioremediation of polluted hypersaline environments and bio-cleaning of mural paintings affected by efflorescence. Despite their

practical utility, salt-adapted enzymes are not widely produced for commercialization currently.

Studies that aim to explore the diversity of microorganisms in hostile/extreme environments have a key role in both identifying new enzymes with special properties (such as activity and stability in multiple extreme conditions) and discovering novel and more efficient producers of such biocatalysts. Numerous saline environments, both in Romania and in other parts of the world, have not yet been investigated from this perspective. As a consequence, in order to identify novel strains of microorganisms able of synthesizing enzymes with unique structural and functional adaptations, it is necessary to direct microbiological research to under-/un- investigated ecosystems.

In this context, **the aim of the present thesis** was to investigate the diversity of microorganisms in various salt ecosystems to create a collection of novel halophilic and halotolerant strains of bacteria, archaea and microfungi able of producing hydrolases with properties useful in biotechnological processes involving high salinities.

To achieve the proposed goal, the research was focused on five objectives: 1) to collect water, sediments and halite samples from seven environments with different salinities (five salt lakes in Romania, two areas of the Black Sea and an extreme habitat in the Atacama desert), 2) to cultivate, isolate and identify halophilic and halotolerant strains of bacteria, archaea and microfungi, 3) to test the potential of strains to produce various extracellular hydrolytic enzymes (glycosidases, esterases and proteases), 4) to quantify the activity of hydrolases produced by the selected strains and determine the catalytic properties of some of them, 5) preliminary evaluate the efficacy of halotolerant esterases, produced by a selected strain, in the bio-cleaning of mural paintings.

The present thesis consists of five chapters, the first two being devoted to the current state of knowledge in the field of halophiles. In *Chapter 1*, emphasis is placed on taxonomic and metabolic diversity of halophilic microorganisms, as well as on structural and physiological adaptations they have acquired in order to survive in environments characterized by high salt concentrations. In *Chapter 2*, the biotechnological importance of halophilic microorganisms and halotolerant enzymes is highlighted by presenting both the technological applications in which they are currently used and the potential applications.

The next three chapters present the experimental part of the thesis. *Chapter 3* describes the study of taxonomic and physiological diversity of halophilic and halotolerant strains isolated from the seven investigated environments. *Chapter 4* describes the study of the activity of hydrolases produced by some halophilic and halotolerant strains selected from

the laboratory collection. *Chapter 5* presents the preliminary assessment of the efficacy of halotolerant esterases in a practical application involving saline conditions, i.e., the bio-cleaning of murals. Finally, the general conclusions, the list of published articles and the bibliographic references are presented.

I. THEORETICAL CONSIDERATIONS

CHAPTER 1

THE BIOLOGY OF HALOPHILIC MICROORGANISMS: OVERVIEW

Halophiles are a heterogeneous group of extremophilic organisms able to survive and even thrive in highly saline environments, unfavorable for the existence of most life forms known to us (Oren, 2002).

The world of halophiles is highly diverse, representatives of this group of organisms being found in all three domains of life: *Archaea*, *Bacteria* and *Eukarya*. The great diversity of halophiles is expressed not only at the phylogenetic level but also at the physiological level. In this regard, the metabolic diversity of halophilic microorganisms includes oxygenic and anoxygenic phototrophs, aerobic chemo-organoheterotrophs and chemolithoautotrophs, fermenters, denitrifiers, sulfate reducers, methanogens and acetogens. Moreover, considerable diversity also exists in the mechanisms these microorganisms use to cope with the osmotic pressure exerted by the high salt concentration of the surrounding medium (Oren, 2011).

The extreme halophiles *par excellence* are the archaeal representatives of the class *Halobacteria* (Phylum *Euryarchaeota*). Currently, the class is divided into three orders (*Halobacteriales*, *Haloferacales* and *Natrialbales*) that comprise 60 genera and 267 species.

Halophilic bacteria are spread over a large number of phylogenetic branches. Currently, of the 35 phyla in the domain *Bacteria*, 12 (*Actinobacteria*, *Bacteroidetes*, *Balneolaeota*, *Cyanobacteria*, *Fibrobacteres*, *Firmicutes*, *Proteobacteria*, *Rhodothermaeota*, *Spirochaetes*, *Tenericutes*, *Thermotogae*, *Verrucomicrobia*) comprise moderately- or extremely-halophilic representatives and many more include halotolerant taxa that have the ability to grow both in the presence and in the absence of salts (Parte, 2018).

Within the domain *Eukarya*, halophily is less widespread, the best salt-adapted eukaryotes being represented by a few species of green algae, yeasts, filamentous fungi and protozoa (McGenity and Oren, 2012).

CHAPTER 2

THE ROLE OF HALOPHILIC MICROORGANISMS IN BIOTECHNOLOGY

In comparison to other groups of extremophiles, halophilic microorganisms are currently used in a relatively small number of biotechnological applications. Some technological processes involving halophilic microorganisms are centuries-old and existed long before their underlying microbiological aspects were understood. Two examples of such technologies are the production of salt by evaporation of seawater and the production of certain types of Asian fermented foods. Furthermore, the two most successful industrial processes involving halophilic microorganisms are the production of β-carotene by the unicellular alga *Dunaliella* and the production of ectoine using various species of moderately halophilic bacteria (Oren, 2010).

In addition to the biotechnological processes in which halophilic microorganisms are currently used, many products synthesized by halophiles (e.g., bacteriorhodopsin, enzymes, polysaccharides, polyhydroxyalkanoates, biosurfactants, antimicrobials) or processes performed by them (e.g., degradation of toxic compounds) have found different potential uses (Oren, 2002).

Halophilic microorganisms represent promising sources of biotechnologically valuable hydrolases, such as proteases, lipases, esterases, amylases, cellulases, xylanases, pectinases, inulinases and nucleases. These salt-adapted macromolecules, compared to those produced by thermophilic and alkaliphilic microorganisms, are not widely produced for commercialization currently. However, numerous studies carried out in recent years show that these biocatalysts have many potential applications and can be used to streamline those biotechnological processes in which hypersaline conditions cause the rapid inactivation of common enzymes (Amoozegar et al., 2017).

II. ORIGINAL CONTRIBUTION

Identification of novel halophilic and halotolerant microbial strains able to produce biotechnologically important hydrolytic enzymes

THE AIM OF THE STUDY

Considering the increasing demand for enzymes with special catalytic properties (e.g., stability at extreme temperatures, pH values, salinities, etc.), numerous studies have been directed towards the bioprospecting of microorganisms from environments dominated by extreme physicochemical conditions. Among the extremophilic microorganisms, halophiles represent promising sources of salt-tolerant enzymes that could be used in various biotechnological processes where high salt concentrations would otherwise inhibit enzymatic transformations.

In this context, the **aim** of the present thesis was to investigate the diversity of microorganisms in various salt ecosystems to create a collection of novel halophilic and halotolerant strains of bacteria, archaea and fungi able of producing hydrolases with properties useful in biotechnological processes involving high salinities.

The research was focused on five **objectives**:

1. To collect water, sediments and halite samples from seven environments with different salinities (five salt lakes in Romania, two areas of the Black Sea and an extreme habitat in the Atacama Desert);
2. To cultivate, isolate and identify halophilic and halotolerant strains of bacteria, archaea and fungi;
3. To test the potential of strains to produce various extracellular hydrolytic enzymes (glycosidases, esterases and proteases);
4. To quantify the activity of hydrolases produced by the selected strains and determine the catalytic properties of some of them;
5. Preliminary evaluate the efficacy of halotolerant esterases, produced by a selected strain, in the bio-cleaning of mural paintings.

CHAPTER 3

TAXONOMIC AND PHYSIOLOGICAL DIVERSITY OF HALOPHILIC AND HALOTOLERANT MICROORGANISMS ISOLATED FROM BRACKISH, SALINE AND HYPERSALINE ENVIRONMENTS

3.1 MATERIALS AND METHODS

3.1.1 Sampling and measurement of physicochemical parameters

3.1.1.1 Water and sediments from salt lakes located in Romania

Water and sediment samples were collected from five salt lakes located in the Romanian Plain: Lake Amara (AM), Lake Balta Alba (BA), Lake Caineni Bai (CB), Movila Miresii Salt Lake (MM), and Braila Salt Lake (BSL), during a two-day field trip in August 2019 (Figure 3.1). Physicochemical parameters (temperature, pH, dissolved oxygen, oxidation-reduction potential, salinity, electrical conductivity) were measured *in situ* with a portable multiparameter instrument for water analysis (Hanna HI98194).

Figure 3.1. Geographic locations and overview photos of the five studied lakes (Ruginescu et al., 2020).

3.1.1.2 Water from the Black Sea

Water samples were collected in April 2021 from two locations of the Black Sea seashore: Eforie Nord and Cap Aurora (Figure 3.2).

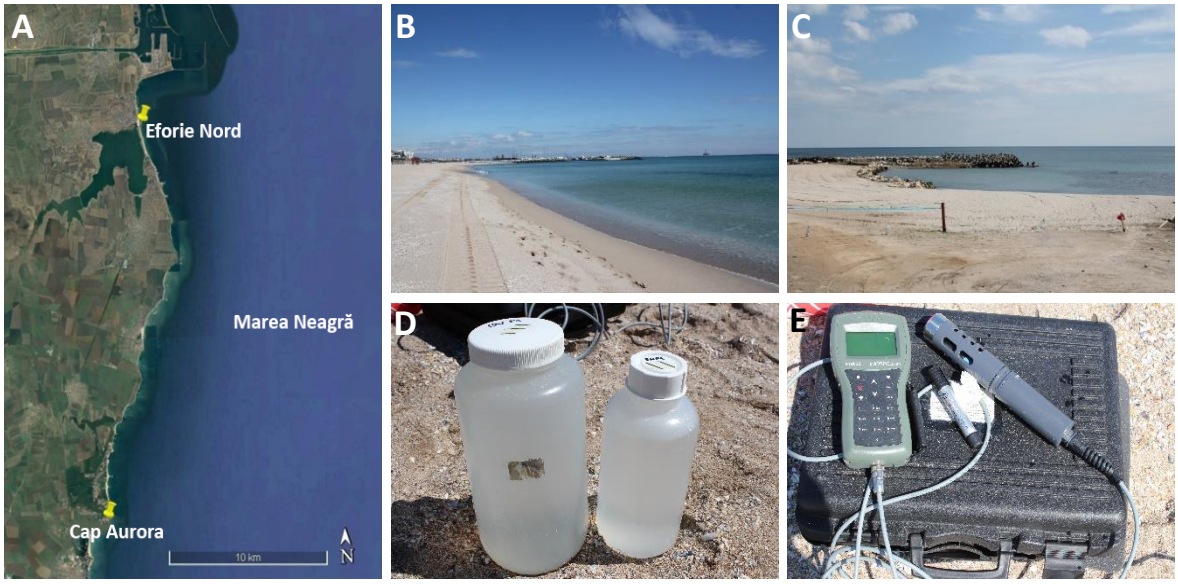

Figure 3.2. Collection of water samples from the Black Sea. (**A**) Geographic locations of the sampling sites; (**B**) Eforie Nord sampling site; (**C**) Cap Aurora sampling site; (**D**) Water samples; (**E**) Multiparameter instrument for water analysis.

3.1.1.3 Halites from the Atacama Desert

Halite samples were collected in November 2018 from the Salar de Atacama, the largest salt flat in Chile. The sampling site area, located about 60 km from San Pedro de Atacama, was distinguished by rugged salt crusts composed almost exclusively of NaCl that covered the soil surface. Halite crystals were collected in sterile plastic containers. Samples were kept at ambient temperature during transportation to the laboratory in Romania and further refrigerated until processing.

3.1.2 Cultivation and abundance estimation of halophilic/halotolerant microorganisms

In order to isolate halophilic and halotolerant microorganisms and estimate their cultivable fraction, water and sediment samples were serially diluted and plated onto four different growth media (MH, JCM-168, MA and ES) with salinities ranging from 3,4% to about 22% (w/v).

3.1.4 Screening for extracellular hydrolase activities

The capability of halotolerant, slightly-halophilic and moderately-halophilic bacteria and fungi to produce hydrolytic enzymes (protease, lipase, amylase, cellulase, xylanase and pectinase) was qualitatively assayed on modified MH or ES media containing one of the

following substrates of interest (g/L): casein (1), Tween-80 (1), starch (1), carboxymethyl cellulose (CMC) (0.5), xylan (1) or pectin (1). Glucose and proteose-peptone were removed from the composition of these assay media.

Extremely halophilic archaea and bacteria were screened for hydrolytic enzyme production on modified JCM-168 media containing the same substrates mentioned above. It is important to note that casamino acids were removed from the composition of these assay media.

3.1.5 Genotypic identification and phylogenetic analysis of the selected strains

Bacterial, archaeal and fungal isolates with distinct phenotypic characteristics (colony morphology, salt tolerance/requirement, extracellular hydrolytic enzyme profiles) were selected for molecular identification. To this end, the following steps were followed: 1) genomic DNA extraction, 2) PCR amplification of the 16S rRNA gene (in the case of bacterial and archaeal strains) or sequence ITS1-5.8S-ITS2 (in the case of fungal strains), 3) sequencing of amplicons, and 4) comparison of sequences obtained with those in *GenBank* to identify the degree of similarity between them. The phylogenetic relations between the strains investigated in this work and the type species were established by drawing phylogenetic trees.

3.2 RESULTS AND DISCUSSION

3.2.1 Sampling sites description

3.2.1.1 The salt lakes in Romania

The five sampled lakes are highly dynamic ecosystems whose water chemistry and temperature fluctuate seasonally depending on climate conditions (i.e., rainfall, solar radiation intensity and water evaporation rate). Based on water salinity, the investigated environments were of three types: brackish (AM and BA), saline (CB) and hypersaline (MM and BSL) (Gâștescu, 1971).

The physicochemical parameters measured at the time of sampling (August 2019) were presented in Table 13.

Table 13. Physicochemical properties of the sampled lakes[1,2].

Lake	pH	T (°C)	DO (mg/L)	ORP (mV)	EC (mS/cm)	Salinity (g/L)	Chloride (g/L)
AM	8.81 ± 0.3	25.49 ± 0.03	11.54 ± 1.29	12.67 ± 2.27	19.05 ± 0.25	11.31 ± 0.15	3.54 ± 0.07
BA	10.16 ± 0.25	24.4 ± 0.0	3.86 ± 0.19	-158 ± 17.9	20.78 ± 0.18	12.36 ± 0.11	5.52 ± 0.07
CB	9.03 ± 0.23	29.96 ± 0.88	2.53 ± 1.81	-220 ± 58.1	52.8 ± 8.98	35.67 ± 5.85	17.91 ± 0.87
MM	10.15 ± 0.26	22.87 ± 0.09	2.12 ± 0.51	-94.7 ± 16.7	107.8 ± 0.2	>70*	38.52 ± 0.54
BSL	8.05 ± 0.09	36.67 ± 2.75	0.71 ± 0.57	-339 ± 43.9	168.1 ± 4.9	>70*	150.5 ± 5.9

[1]Data were presented as mean ± standard deviation (n = 3). [2]Abbreviations: T, Temperature; DO, Dissolved Oxygen; ORP, Oxidation Reduction Potential; EC, Electrical Conductivity. *Values exceeded the detection limit of the measuring instrument.

3.2.1.2 The Black Sea

The physicochemical parameters measured at the time of sampling (April 2021) were presented in Table 14.

Table 14. Physicochemical properties of the Black Sea.

Location	T (°C)	pH	DO (mg/L)	EC (mS/cm)	Salinity (g/kg)
Eforie Nord	9,3 ± 0,2	9,4 ± 0,2	4,8 ± 1	29,1 ± 0,1	18,01 ± 0,08
Cap Aurora	11,3 ± 0,4	9,2 ± 0,3	9,9 ± 0,9	28,9 ± 0,2	17,87 ± 0,14

[1]Data were presented as mean ± standard deviation (n = 3). [2]Abbreviations: T, Temperature; DO, Dissolved Oxygen; EC, Electrical Conductivity.

3.2.1.3 The Atacama Desert

The Atacama Desert, stretching for over 1000 km along the Pacific Coast of Northern Chile, is considered as the driest and oldest extant desert, becoming one of the most extreme environments on Earth. Extensive areas in the hyper-arid core of the desert (22–26 °S) receive an average annual rainfall of only 2 mm, representing about 100-fold and 10–50-fold less than other arid deserts such as the Gobi and the Sahara, respectively (Azua-Bustos et al., 2012; Drees et al., 2006).

The halite agglomerate collected from the northern area of the Salar de Atacama presented an irregular shape with rough surfaces, due to wind erosion. In addition to a white surface crust, the presence of pink-green nodules layers suggested the colonization by pigments-producing microbial communities (Kampf et al., 2005; Finstad et al., 2016).

3.2.2 Abundance of cultured halophilic and halotolerant microorganisms

3.2.2.1 Abundance of cultured microorganisms in water and sediment samples from salt lakes

The cultured fractions of the microbial communities inhabiting the five investigated environments varied considerably in abundance not only from one ecosystem to another but also between the different sites of the same lake (Figure 3.6).

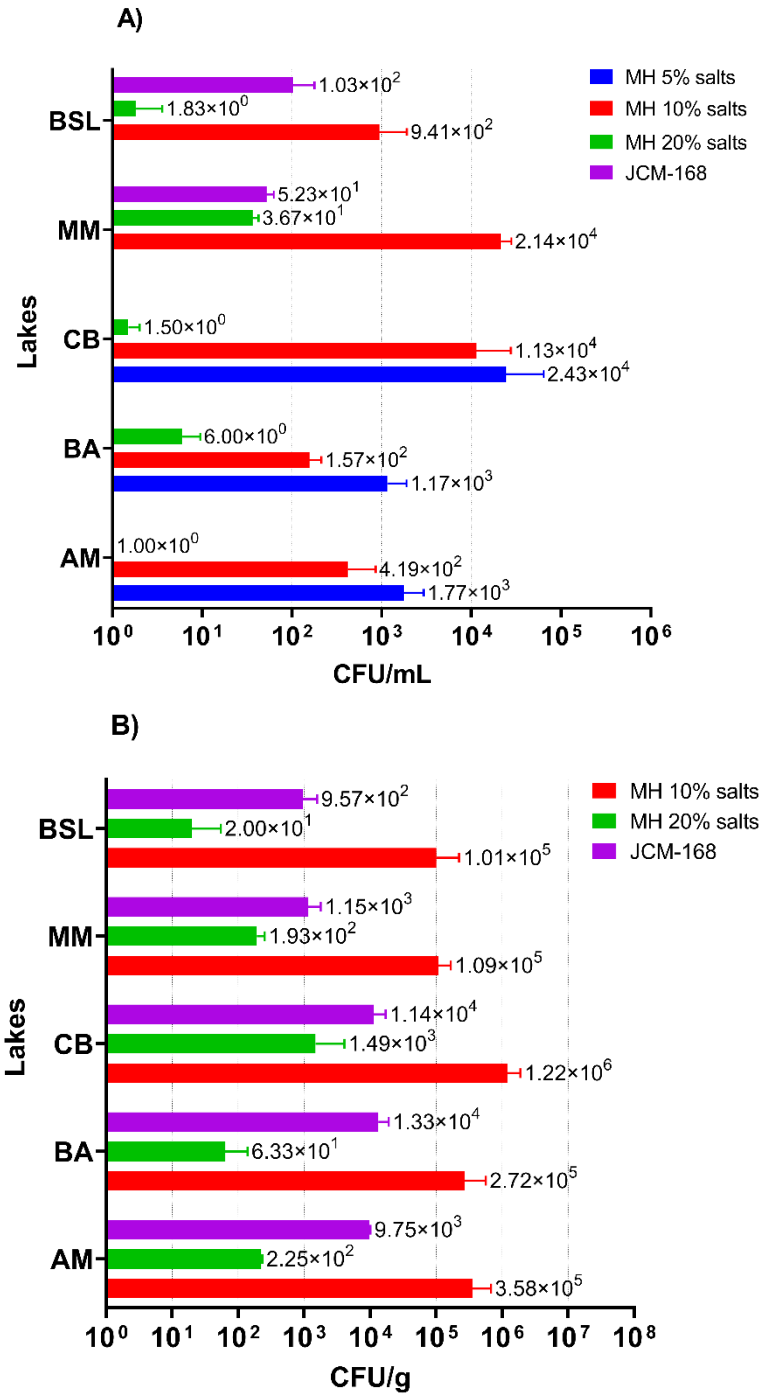

Figure 3.6. The abundance, expressed as colony-forming units (CFU) per 1 mL of water (**A**) or 1 g of wet sediment (**B**), of the cultured fractions of halophilic/halotolerant microbial communities inhabiting the five investigated lakes. Bars show the differences between the three sampling sites.

3.2.2.2 Abundance of cultured microorganisms in water samples from the Black Sea

The density of heterotrophic bacteria was higher in the water samples collected from Cap Aurora (2.2×10^3–1.32×10^4 CFU/mL) than in those collected from Eforie Nord (2.8×10–9.3×10^2 CFU/mL). Comparing the two media used, it was found that the number of CFU/mL was higher on the enriched seawater medium (ES) than on MA medium. Considering that MA medium has a salt content of 3.42%, representing a value almost double that of the Black Sea water (1.8%), it is possible that its relatively high salinity is responsible for inhibiting the development of some halosensitive species.

3.2.2.3 Abundance of cultured microorganisms in halite samples from the Atacama Desert

Microbial cultures obtained from halite crusts of the Salar de Atacama under various growth conditions led to the highest abundance ($1.6\times10^5 \pm 2.1\times10^4$ CFU/g) when using MH media containing 10% NaCl. Higher salt concentrations gave lower isolation rates and recovered colonies with less diverse morphologies. In the presence of 20% NaCl in MH and JCM-168 growth media, the cell density decreased to $5.8\times10^4 \pm 3.4\times10^3$ and $1.2\times10^4 \pm 3.1\times10^3$ CFU/g, respectively.

3.2.3 Phenotypic characteristics of microbial isolates

3.2.3.1 Bacterial, archaeal and fungal isolates from salt lakes

- *Halophily and halotolerance of the isolates*

A total of 244 microbial isolates (182 bacteria, 22 archaea and 40 fungi) from the five sampled lakes were obtained in pure cultures and tested for the ability to grow at different salt concentrations. The majority (141 isolates, 57.8%) grew between 0 and 2 M NaCl (optimally at 0–1 M) and thus they were categorized as halotolerant. However, some of these isolates, particularly those recovered from the hypersaline lake BSL, were able to grow slowly (>10 days of incubation) up to 3–3.5 M NaCl and therefore they were considered extremely halotolerant. A significantly smaller fraction of isolates was constituted by moderately (75 isolates, 30.7%) and extremely halophilic (28 isolates, 11.5%) microorganisms, whose growth has shown to be dependent on relatively high salt concentrations. While most of the former grew in the NaCl range of 0.5–3 M (optimally at 0.5–1 M), the majority of the latter grew between 2 and 4.5 M NaCl (optimally at 2–3.5 M) (Figure 3.10).

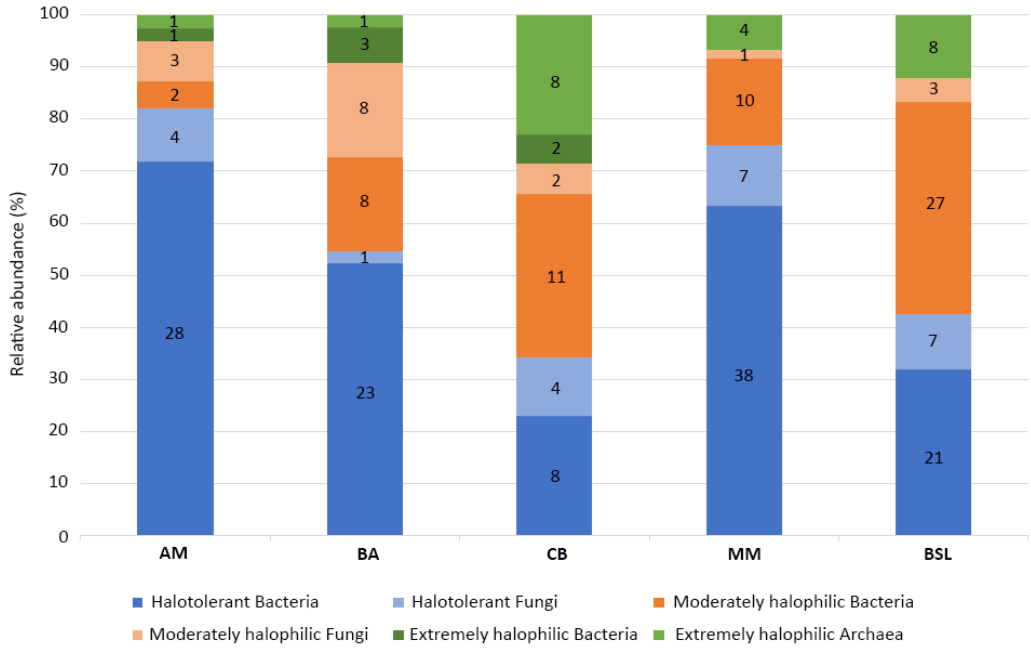

Figure 3.10. Relative abundances of halotolerant and halophilic microorganisms isolated from the five investigated lakes. The numbers in the bar graphs indicate the number of isolates.

3.2.3.2 Bacterial isolates from the Black Sea

- *Halophily and halotolerance of the isolates*

A total of 64 bacterial isolates from the Black Sea were obtained in pure cultures and tested for the ability to grow at different salt concentrations. The majority (47 isolates; 73%) required salts to grow and thus they were categorized as halophilic strains. Among them, the majority (38 strains; 59%) were classified as slightly-halophilic (optimal ≤3.4% salts) and only 9 strains (14%) were classified as moderately-halophilic (optimal> 3.4% salts). The other 17 strains (27%) were able to grow up to 7-15% (w/v) salts and thus they were categorized as halotolerant.

3.2.3.3 Bacterial isolates from the Atacama Desert

Despite the relatively high density of bacteria detected in halite samples, the morphological diversity of colonies was limited, the majority being round, cream-colored, shiny and smooth in appearance. In this regard, only 23 apparently distinct colonies were chosen for subsequent isolation. All these isolates were moderately halophilic. Most of them grew in the NaCl range of 0.5–4 M and showed optimal growth at 0.5–2 M NaCl.

3.2.4 Production of extracellular hydrolytic enzymes

3.2.4.1 The hydrolytic potential of microbial isolates obtained from salt lakes

All 244 microbial isolates were screened for the ability to synthesize hydrolytic enzymes capable of degrading various types of substrates, such as proteins (i.e., casein), lipids (i.e., Tween-80) and polysaccharides (i.e., starch, CMC, xylan and pectin). While 87 isolates (36%) produced none of the six hydrolases tested, the majority (157 isolates, 64%) showed single or combined hydrolytic activities. The enzymes most frequently produced by the microorganisms cultured from each of the five salty lakes were protease and lipase. However, major differences in the type and number of extracellular hydrolytic activities were observed between the various bacterial, archaeal and fungal isolates.

From a total of 182 bacterial isolates, 72 were subjected to taxonomic identification. Comparative analysis of partial 16S rRNA gene sequences revealed that bacterial isolates fell within three classes: *Bacilli* (58.3%), *Gammaproteobacteria* (40.3%) and *Actinobacteria* (1.4%). The hydrolytic potential of the representatives of the *Bacilli* class (genera *Bacillus*, *Virgibacillus*, *Salinicoccus*, *Marinococcus*, *Halobacillus*, *Planococcus*, *Thalassobacillus* and *Salimicrobium*) was much higher than that of the representatives of the *Gammaproteobacteria* (genera *Halomonas*, *Salinivibrio*, *Vibrio*, *Idiomarina* și *Psychrobacter*). In this regard, most strains of the *Bacilli* class produced proteases (78%) and lipases (66%), followed by xylanases (52%), amylases (47%), cellulases (40%) and pectinases (19%). On the other hand, the strains included in the *Gammaproteobacteria* class produced particularly lipases (31%) and proteases (20%) (Table 18).

Table 18. Distribution in classes of bacterial strains producing hydrolases from salt lakes.

Class (No. of strains)	**Protease** No. of strains (%)	**Lipase** No. of strains (%)	**Amylase** No. of strains (%)	**Cellulase** No. of strains (%)	**Xylanase** No. of strains (%)	**Pectinase** No. of strains (%)
Gammaproteobacteria (29)	6 (20.7)	9 (31)	2 (6.9)	1 (3.4)	3 (10.3)	2 (6.9)
Bacilli (42)	33 (78.6)	28 (66.7)	20 (47.6)	17 (40.5)	22 (52.4)	8 (19)
Actinobacteria (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0

The *Bacillus* isolates were generally halotolerant and exhibited higher hydrolytic activities compared to the other cultured taxa. In this regard, they produced combinations of three or more enzymes, mainly protease (19 isolates), lipase (17 isolates), xylanase (17 isolates), cellulase (16 isolates) and amylase (14 isolates). Moreover, six isolates (BSL P1.8,

MM P1.8A, AM P2.6, AM N P1.17, BA N P2.7, CB N P1.6) were able to degrade all six substrates tested (Figure 3.15).

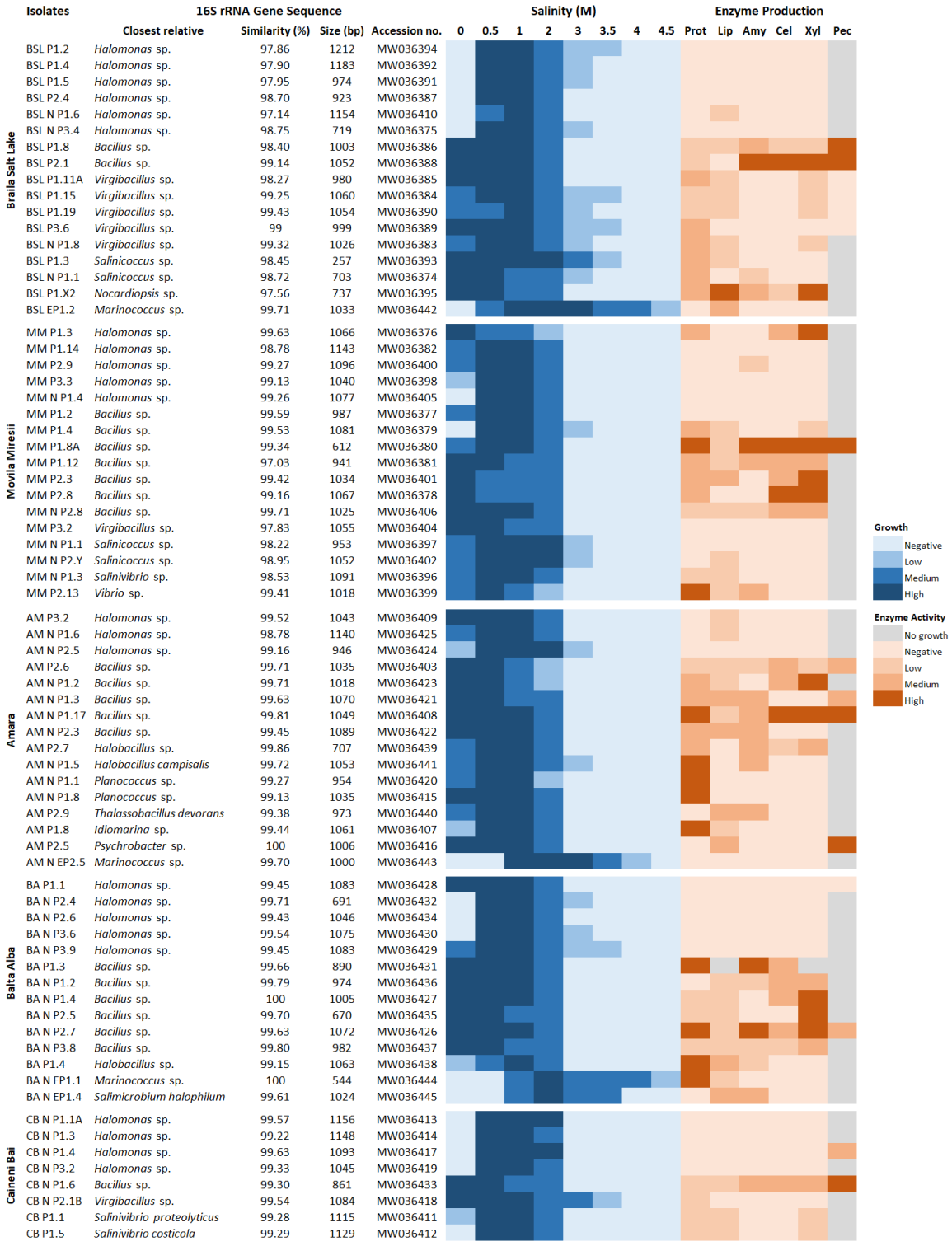

Figure 3.15. Heat maps showing the ability of the Bacterial isolates – taxonomically identified on the basis of the 16S rRNA gene sequence analysis – to grow at different salt concentrations and produce extracellular hydrolytic enzymes.

In contrast to bacterial isolates, the potential of the 22 archaeal representatives to produce extracellular hydrolytic enzymes was quite limited. In this regard, the only two hydrolases produced by only a few isolates were amylase (four isolates, 18%) and cellulase (two isolates, 9%). The most promising strain in terms of extremozymes production was related to *Haloterrigena turkmenica* (Figure 3.16).

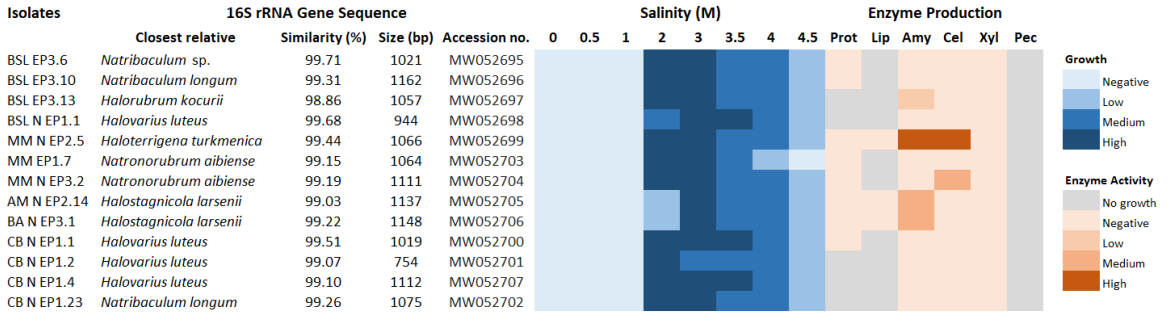

Figure 3.16. Heat maps showing the ability of the Archaeal isolates – taxonomically identified on the basis of the 16S rRNA gene sequence analysis – to grow at different salt concentrations and produce extracellular hydrolytic enzymes.

Among the 40 fungal isolates, 30 (75%) were pectinase producers, 17 (42.5%) cellulase producers, 15 (37.5%) protease producers, 14 (35%) xylanase producers, 13 (32.5%) lipase producers, and only 6 (15%) isolates produced amylase. The most promising fungal strains in terms of hydrolase production were related to *Aspergillus*, *Penicillium* and *Emericellopsis*.

3.2.4.2 The hydrolytic potential of microbial isolates obtained from the Black Sea

Among the 64 bacterial strains isolated from the Black Sea, 53 (82.8%) showed the ability to degrade at least one of the substrates tested. The enzymes most frequently produced were represented by lipases (60.9%), followed by amylases (43.7%), proteases (42.2%), cellulases (31.2%), pectinases (21.9%) and xylanases (17.2%). The origin of samples from which the isolates were recovered did not influence their extracellular enzymatic profiles.

Comparative analysis of partial 16S rRNA gene sequences revealed that bacterial isolates fell within six classes: *Gammaproteobacteria* (42.2%), *Alphaproteobacteria* (9.4%), *Betaproteobacteria* (3.1%), *Flavobacteriia* (23.5%), *Bacilli* (15.6%) and *Actinobacteria* (6.2%) (Table 19).

The most promising strains in terms of hydrolase production were related to representatives of the following genera: *Pseudoalteromonas*, *Paraglaciecola*, *Polaribacter*, *Aquimarina*, *Cellulophaga*, *Bacillus*, *Metabacillus*, *Isoptericola* and *Streptomyces*.

Table 19. Distribution in classes of bacterial strains producing hydrolases from the Black Sea.

Class (No. of strains)	**Protease** No. of strains (%)	**Lipase** No. of strains (%)	**Amylase** No. of strains (%)	**Cellulase** No. of strains (%)	**Xylanase** No. of strains (%)	**Pectinase** No. of strains (%)
Gammaproteobacteria (27)	8 (29.6)	22 (81.5)	10 (37)	7 (25.9)	4 (14.8)	4 (14.8)
Alphaproteobacteria (6)	0	3 (50)	0	0	0	0
Betaproteobacteria (2)	0	0	0	0	0	0
Flavobacteriia (15)	8 (53.3)	9 (60)	7 (46.6)	9 (60)	4 (26.6)	1 (6.6)
Bacilli (10)	9 (90)	3 (30)	9 (90)	2 (20)	1 (10)	6 (60)
Actinobacteria (4)	2 (50)	2 (50)	2 (50)	2 (50)	2 (50)	3 (75)

3.2.4.3 The hydrolytic potential of microbial isolates obtained from the Atacama Desert

From a total of 23 bacterial isolates, most (19 strains) showed lipolytic activity, followed by pectinolytic (11 strains), caseinolytic (one strain) and weakly cellulolytic (one strain) activities. None of the cultured strains was able to degrade starch, gelatin and inulin. The 16S rRNA gene sequence analysis showed that seven isolates (AD-2, AD-8, AD-9, AD-18, AD-20 G, AD-23 and AD-L1) were related to members of the *Halomonas* genus and AD-6 was homologous to *Idiomarina* species.

CHAPTER 4

CHARACTERIZATION OF SOME SALT-TOLERANT HYDROLASES PRODUCED BY HALOPHILIC AND HALOTOLERANT STRAINS

4.1 MATERIALS AND METHODS

4.1.1 Quantitative assays of hydrolytic activities

4.1.1.1 Protease assay

Principle of the method: Enzymatic degradation of proteins leads to the release of peptides and amino acids, including tyrosine. The latter interacts with the Folin-Ciocalteu reagent and generates blue chromatophores that can be quantified by measuring the optical density (Cupp-Enyard, 2008).

4.1.1.2 Esterase assay

Principle of the method: Enzymatic degradation of *p*-nitrophenyl butyrate leads to the release of *p*-nitrophenol that forms the chromophore *p*-nitrophenolate by deprotonation. The latter can be quantified by measuring the optical density (Pliego et al., 2015).

4.1.1.3 Lipase assay

Principle of the method: Enzymatic hydrolysis of oil leads to the release of fatty acids which can be quantified by titration with an alkaline solution (NaOH) in the presence of a pH indicator (phenolphthalein) (Gupta et al., 2003).

4.1.1.4 Xylanase assay

Principle of the method: Xylose released by enzymatic hydrolysis of xylan reduce 3,5-Dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The latter, due to its reddish color, can be quantified by measuring the optical density at 540 nm (Miller, 1959).

4.1.1.5 Cellulase assay

Principle of the method: Enzymatic hydrolysis of carboxymethylcellulose leads to the release of glucose which reduce 3,5-Dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The quantification of the latter is performed by measuring the optical density at 540 nm (Miller, 1959).

4.2 RESULTS AND DISCUSSION

4.2.1 Quantitative evaluation of hydrolase activities

Bacterial and fungal strains that showed both promising hydrolytic activities (estimated semi-quantitatively in section 3.2.4) and biotechnologically valuable physiological characteristics (i.e., fast growth rate, the ability to tolerate a wide range of salt concentrations, the ability to produce multiple hydrolases) were selected and subjected to quantitative evaluation of enzyme activities.

4.2.1.1 Protease activity

Among the microbial strains tested, the highest proteolytic activities were detected in the culture supernatant of *Bacillus* sp. BA N P2.7 (61 U/mL), *Bacillus* sp. MM P1.8A (55 U/mL), *Bacillus* sp. AM N P1.17 (47 U/mL) and *Salinivibrio* sp. MM N P1.3 (45 U/mL) (Table 20).

Table 20. Results of quantitative determinations of protease activity of selected strains.

Strain	**Growth conditions**				**Protease assay conditions**			**Protease activity (U/mL)**
	Medium	Temp. (°C)	Shaking (rpm)	Time (days)	Temp. (°C)	Time (min)	pH	
Bacillus sp. MM P1.8A	MH with 0.5 M NaCl and 1% casein, pH 7	30	140	3	37	20	7.5	55.7 ± 6.4
Bacillus sp. AM N P1.17				3				47.5 ± 4.4
Bacillus sp. BA N P2.7				3				61.2 ± 2.7
Salinicoccus sp. BSL N P1.1				3				1.2 ± 0.3
Salinivibrio sp. MM N P1.3				3				45 ± 0.7
Virgibacillus sp. BSL N P1.8				2				1.9 ± 0.2
Marinococcus sp. BA N EP1.1				6			7	1.3
Nocardiopsis sp. BSL P1.X2				7				4.3 ± 0.6
Penicillium sp. BSL FP3.2				5				7.6 ± 0.1
Aspergillus sp. BSL FP1.2				5				1 ± 0.1
Idiomarina sp. AD-6	MH with 1 M NaCl and 1% casein, pH 7.2	28		4			7.5	1.8 ± 0.76

4.2.1.2 Esterase activity

The highest esterolytic activity, among the microbial strains tested, was produced by *Bacillus* sp. BA N P3.3 (0.175 U/mL) (Table 21).

Table 21. Results of quantitative determinations of esterase activity of selected strains.

Strain	**Growth conditions**				**Esterase assay conditions**			**Esterase activity (mU/mL)**
	Medium	Temp. (°C)	Shaking (rpm)	Time (days)	Temp. (°C)	Time (min)	pH	
Bacillus sp. BA N P2.7	MH with 0.5 M NaCl and 1% Tween80, pH 7	30	140	4	37	15	7.5	38.4 ± 0.8
Bacillus sp. BA N P1.2				4				23.9 ± 0.9
Bacillus sp. BA N P3.3				4				175 ± 7.1
Bacillus sp. BA N P3.8				4				17 ± 0.1
Bacillus sp. AM N P1.17				4				44 ± 0.8
Bacillus sp. CB N P1.6				4				31.4 ± 0.6
Bacillus sp. MM P1.8A				4				25.7 ± 0.2
Salinivibrio sp. MM N P1.3				4				17.4 ± 0.1
Salinivibrio sp. CB P1.1				4				15.4 ± 0.2
Psychrobacter sp. AM P2.5				4				2.8 ± 0.1
Penicillium sp. BSL FP3.2				5				30.4 ± 1
Penicillium sp. MM FP1.4				5				63.7 ± 3.6

4.2.1.3 Lipase activity

Lipase activity was determined in the culture supernatant of 12 microbial strains previously selected for their ability to decompose Tween 80 (Table 21). The results of titrimetric determinations showed that only two strains produced extracellular lipolytic enzymes able to hydrolyze olive oil. These strains were represented by *Bacillus* sp. AM N P1.17 and *Penicillium* sp. MM FP1.4 (Table 22).

Table 22. Results of quantitative determinations of lipase activity of selected strains.

Strain	Growth conditions				Lipase assay conditions			Lipase activity (U/mL)
	Medium	Temp. (°C)	Shaking (rpm)	Time (days)	Temp. (°C)	Time (min)	pH	
Bacillus sp. AM N P1.17	MH with 0.5 M NaCl, 0.5% Tween 80 and 1% olive oil, pH 7	30	140	3	37	30	7.5	2.83 ± 0.17
Penicillium sp. MM FP1.4								2.66 ± 0.16

4.2.1.4 Xylanase activity

Among the microbial strains tested, *Bacillus* sp. BA N P1.4 exhibited the highest xylanolytic activity (29.3 U/mL) (Table 23).

Table 23. Results of quantitative determinations of xylanase activity of selected strains.

Strain	Growth conditions				Xylanase assay conditions			Xylanase activity (U/mL)
	Medium	Temp. (°C)	Shaking (rpm)	Time (days)	Temp. (°C)	Time (min)	pH	
Bacillus sp. BSL P2.1	MH with 0.5 M NaCl and 1% xylan, pH 7	30	140	3	37	10	7.5	1.8
Bacillus sp. MM P1.8A				3				2.3 ± 0.1
Bacillus sp. MM P2.8				3				16.2 ± 0.5
Bacillus sp. BA N P1.4				3				29.2 ± 0.2
Bacillus sp. AM N P1.17				3				9 ± 0.4
Nocardiopsis sp. BSL P1.X2				4				2.4 ± 0.1
Penicillium sp. BSL FP3.2				5				4
Emericellopsis sp. MM FP1.2				5				5.1 ± 0.5
Emericellopsis sp. SED MM2				5		15		0.85
Emericellopsis sp. SED A1				5		15		1.1

4.2.1.5 Cellulase activity

Six bacterial strains belonging to the genus *Bacillus* and two fungal strains belonging to *Penicillium* and *Aspergillus* genera were quantitatively assayed for extracellular cellulolytic activities. Among them, *Bacillus* sp. AM N P1.17 produced the highest cellulase activity (0.076 U/mL), followed by *Penicillium* sp. BSL FP3.2 (0.062 U/mL) (Table 24).

Table 24. Results of quantitative determinations of cellulase activity of selected strains.

Strain	Growth conditions				Cellulase assay conditions			Cellulase activity (mU/mL)
	Medium	Temp. (°C)	Shaking (rpm)	Time (days)	Temp. (°C)	Time (min)	pH	
Bacillus sp. BSL P2.1	MH with 0.5 M NaCl and 0.5% CMC, pH 7	30	140	3	37	30	7	55.1 ± 0.8
Bacillus sp. MM P2.8				3				46.7 ± 0.1
Bacillus sp. AM N P1.17				3				75.6 ± 3.1
Bacillus sp. BA N P1.4				3				57.9 ± 0.8
Bacillus sp. BA N P1.2				3				37.7 ± 0.6
Bacillus sp. CB N P1.6				3				53.3 ± 1.2
Penicillium sp. BSL FP3.2				5				62.4 ± 0.4
Aspergillus sp. MM FP1.1				5				47.1 ± 0.7

4.2.2 Characterization of proteases produced by *Bacillus* sp. AM N P1.17

4.2.2.2 Optimal conditions for protease production

Protease production by *Bacillus* sp. AM N P1.17 was affected by culture conditions, such as temperature and pH. The highest protease activity was achieved when the strain was cultured for 72 h at 30 °C, in a medium whose initial pH was 8. Also, protease production was significantly affected by the incubation time. In this regard, the protease activity increased from 18.6 U/mL after 24 h to 44.9 U/mL after 48 h and to 50.4 U/mL after 72 h. Comparing these results with the growth kinetics (Figure 4.6), it was found that protease activity reached the maximal level in the stationary phase of the growth curve.

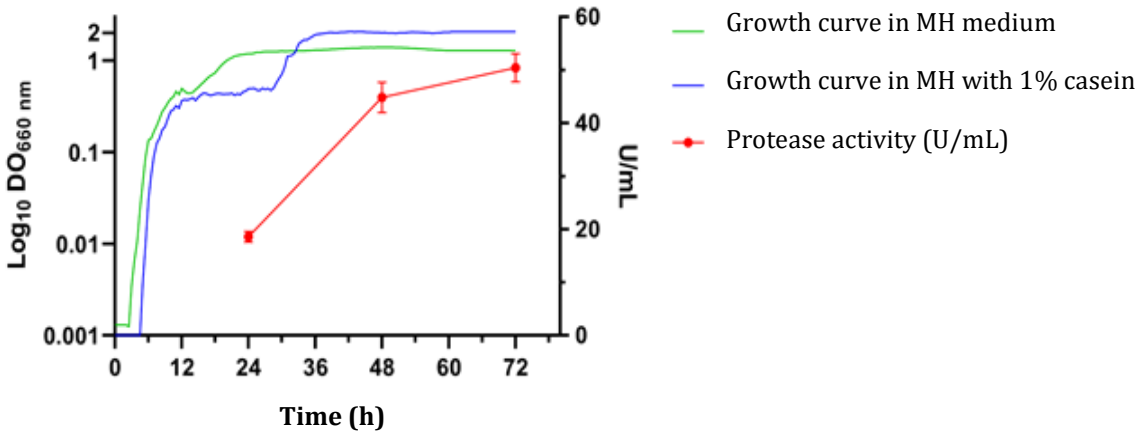

Figure 4.6. The growth curve of *Bacillus* sp. AM N P1.17, correlated with protease activity in the culture supernatant.

4.2.2.3 Catalytic properties of proteases

Under standard assay conditions (i.e., 37 °C, pH 8), the specific protease activity of the culture supernatant was 1.75 U/mg. After concentrating proteins by acetone precipitation, the proteolytic activity was enhanced to 11.6 U/mg (Table 25).

Table 25. Summary of extracellular protease precipitation.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	47.5	27.1	1.75	100	1
Precipitated proteins	22	1.9	11.6	46.3	6.6

Concentrated proteases were active over a wide range of temperatures (20–80 °C) and pH values (6–10). The highest protease activity was detected at temperatures between 50 and 70 °C, at pH 8 (Figures 4.7 A,B). Although proteases showed optimal activity at high temperatures, they were not stable over time at temperatures above 20 °C (Figure 4.7 D). The enzymes were active at salinities between 0 and 1 M NaCl (Figure 4.7 C).

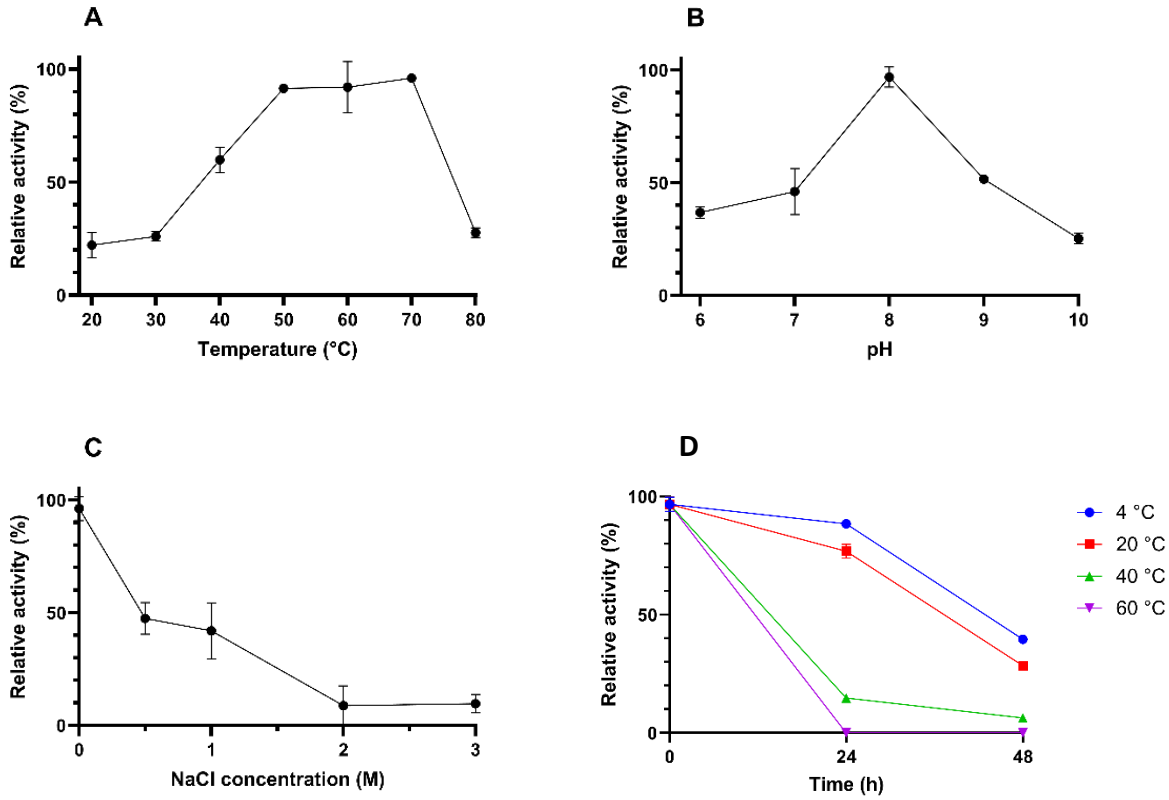

Figure 4.7. The effects of temperature (**A**), pH (**B**) and NaCl concentration (**C**) on the activity of concentrated proteases obtained from *Bacillus* sp. AM N P1.17; (**D**) The effect of temperature on the stability of proteases.

4.2.3 Characterization of esterases produced by *Bacillus* sp. BA N P3.3

4.2.3.2 Catalytic properties of esterases

The specific esterase activity of the culture supernatant was 0.016 U/mg under standard assay conditions (i.e., 37 °C, pH 7.5). After concentrating proteins by acetone precipitation, the esterolytic activity was enhanced to 0.124 U/mg (Table 26).

Table 26. Summary of extracellular esterase precipitation.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	0.175	10.75	0.016	100	1
Precipitated proteins	0.06	0.485	0.124	34	7.75

Concentrated esterases were active over a wide range of temperatures (20–80 °C) and salt concentrations (0–4 M NaCl), at neutral and alkaline pH. Esterases were stable at temperatures between 4 and 40 °C for at least 48 h. At higher temperatures, the enzymes lost their activity after 2 h of incubation (Figure 4.11).

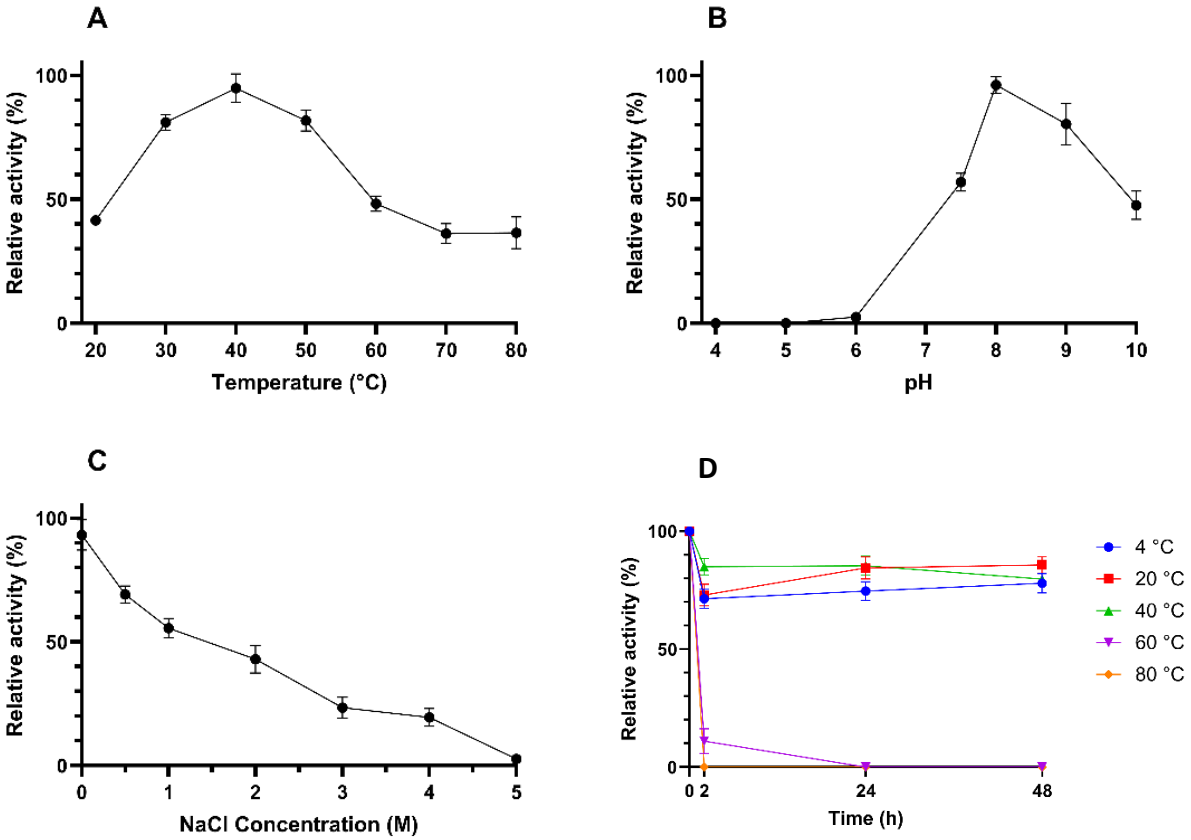

Figure 4.11. The effects of temperature (**A**), pH (**B**) and NaCl concentration (**C**) on the activity of concentrated esterases obtained from *Bacillus* sp. BA N P3.3; (**D**) The effect of temperature on the stability of esterases.

4.2.4 Characterization of xylanases produced by *Bacillus* sp. BA N P1.4

4.2.4.2 Catalytic properties of xylanases

Under standard assay conditions (i.e., 37 °C, pH 7), the specific xylanase activity of the culture supernatant was 2.43 U/mg. Following purification by acetone precipitation, the xylanolytic activity was enhanced to 11.3 U/mg (Table 27).

Table 27. Summary of extracellular xylanase precipitation.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	29.2	12	2.43	100	1
Precipitated proteins	21	1.86	11.3	72	4.65

Concentrated xylanases were active at different temperatures (20–80 °C), pH values (5–10), and salt concentrations (0–5 M NaCl). Enzymes were stable at temperatures between 4 and 40 °C for at least 42 h (Figure 4.13).

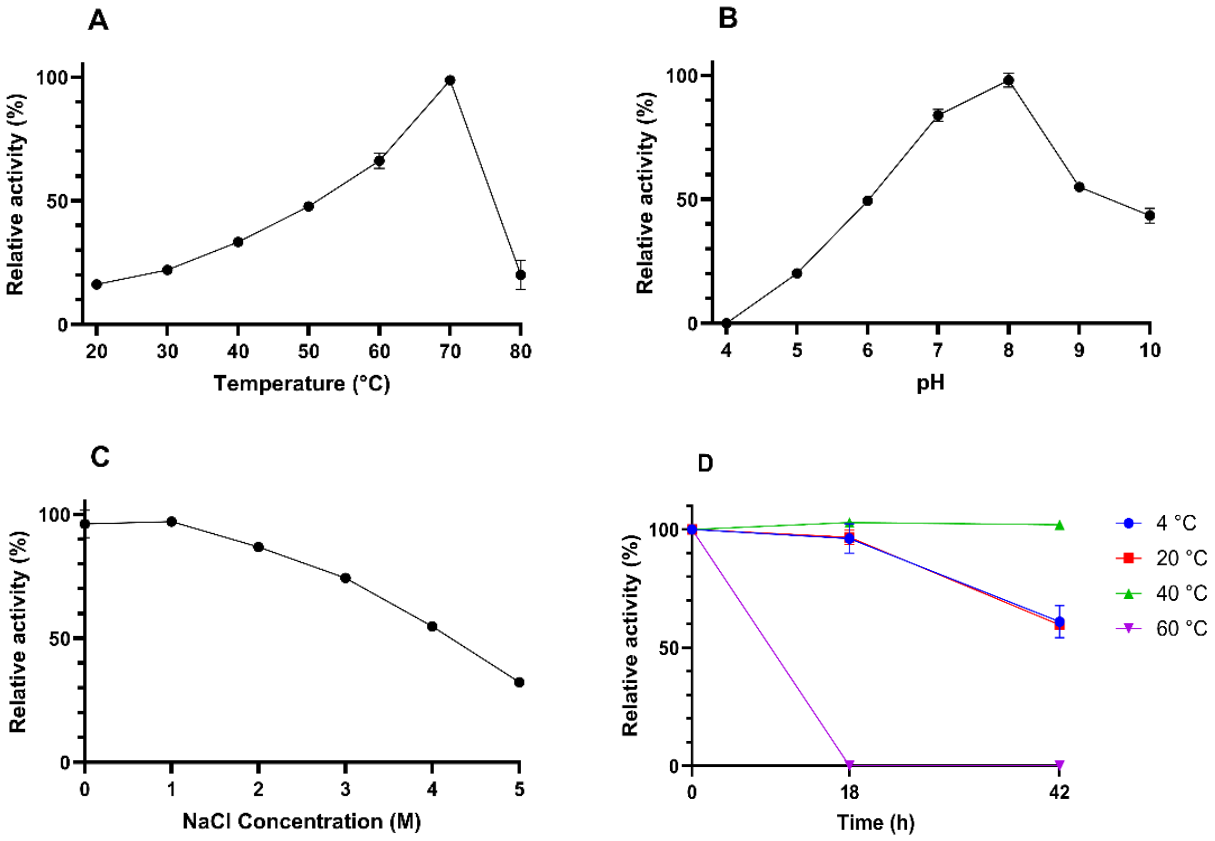

Figure 4.13. The effects of temperature (**A**), pH (**B**) and NaCl concentration (**C**) on the activity of concentrated xylanases obtained from *Bacillus* sp. BA N P1.4; (**D**) The effect of temperature on the stability of xylanases.

4.2.5 Characterization of cellulases produced by *Bacillus* sp. AM N P1.17

4.2.5.2 Catalytic properties of cellulases

The specific cellulase activity of the culture supernatant was 0.008 U/mg under standard assay conditions (i.e., 37 °C, pH 7). After concentrating proteins by acetone precipitation, the cellulolytic activity was enhanced to 0.032 U/mg (Table 28).

Table 28. Summary of extracellular cellulase precipitation.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	0.071	8.66	0.008	100	1
Precipitated proteins	0.043	1.33	0.032	60.5	4

Concentrated cellulases were active at 20–80 °C (optimally at 70 °C), at pH 4–10 (optimally pH 7) and at 0–5 M NaCl (optimally at 0 M). The enzymes were stable at temperatures between 4 and 40 °C for at least 48 h. At 60 °C, cellulases retained 73% of the maximal activity after 2 h of incubation, but they completely lost their activity after 24 h of incubation (Figure 4.14).

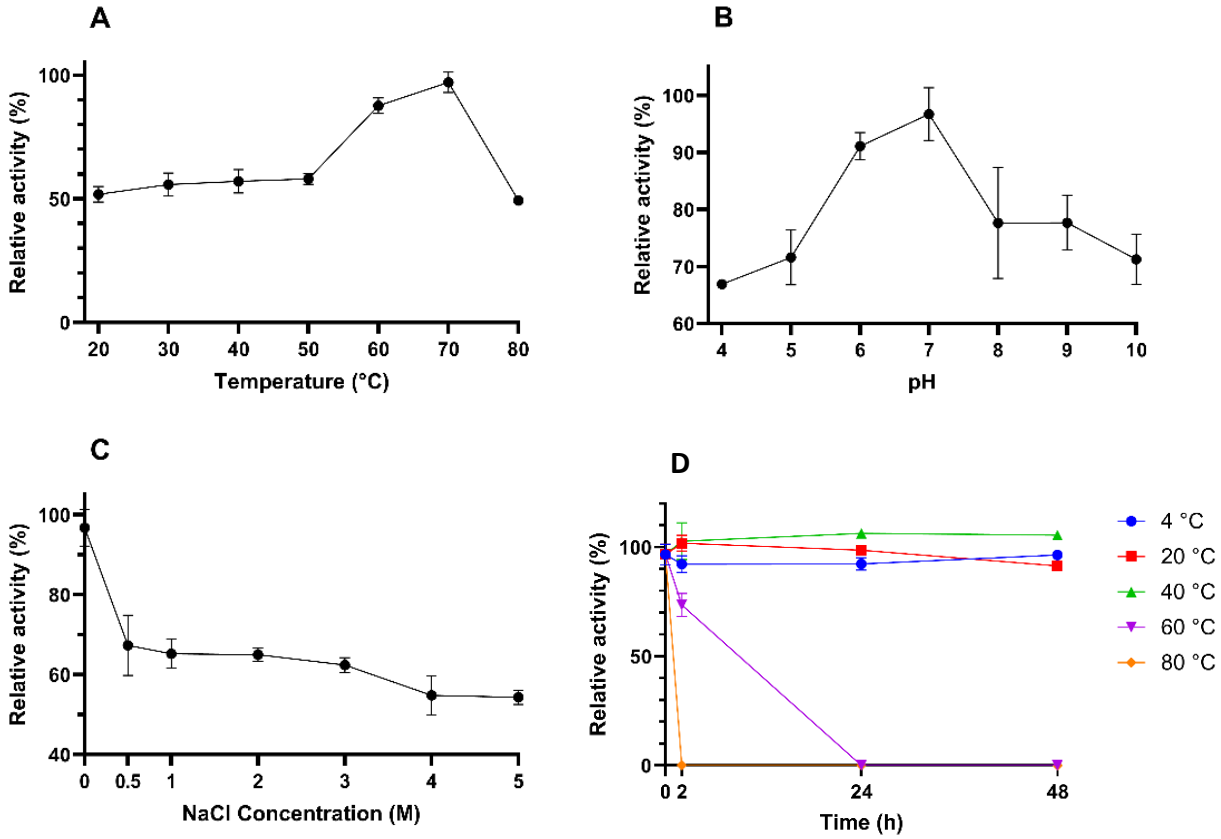

Figure 4.14. The effects of temperature (**A**), pH (**B**) and NaCl concentration (**C**) on the activity of concentrated cellulases obtained from *Bacillus* sp. AM N P1.17; (**D**) The effect of temperature on the stability of cellulases.

CHAPTER 5

PRELIMINARY EVALUATION OF THE EFFICACY OF HALOTOLERANT ESTERASES IN BIO-CLEANING APPLICATIONS

5.1 MATERIALS AND METHODS

5.1.1 Preparation of laboratory models

The efficacy of utilizing halotolerant esterases produced by *Bacillus* sp. BA N P3.3 in the cleaning of wall paintings was qualitatively evaluated on laboratory models represented by painted bricks that simulated the mural surface. Briefly, the following steps were performed to obtain the laboratory models: (a) wetting the bricks, (b) inserting the lime and the tow in special trays, (c) incorporating the sand, (d) placing the mortar on the wet bricks, (e) applying the pigments (red, yellow, blue, green), and (f) slow drying for 30 days. Then, three layers of acrylic resin Paraloid B-72, sunflower oil or wax were applied on the painted surfaces using a brush (Figure 5.1).

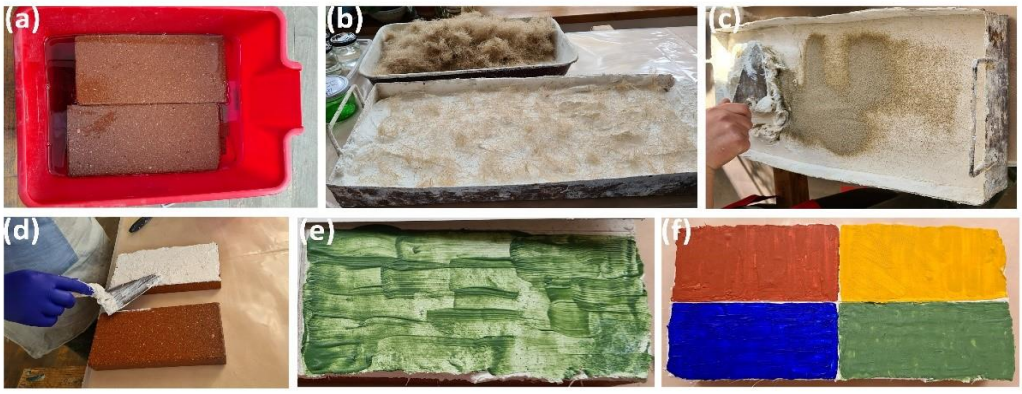

Figure 5.1. Preparation of the painted laboratory models.

5.1.2 Preparation of hydrogels

The enzymatic solution used in the bio-cleaning experiments was represented by extracellular proteins obtained by acetone precipitation from the culture supernatant of *Bacillus* sp. BA N P3.3.

The bio-cleaning process involved the incorporation of the enzyme solution into hydrogels and the application of this mixture on the surfaces of artificially aged laboratory models. Four types of polysaccharide gels represented by Vanzan NF-C (Vanderbilt Minerals), Gellano Kelcogel (C.T.S.), Agarart (C.T.S.) and Klucel (Ashland) were tested.

5.1.3 Bio-cleaning treatment

The cleaning treatments involved the application of hydrogels containing the esterase solution on the surface of the painted laboratory models (1 mL enzyme/6.25 cm^2) and their incubation for 5 h and 10 h at room temperature (Figure 5.2). Then, the hydrogels were removed from the treated surfaces, and traces of enzyme solution were removed using cotton swabs moistened in distilled water.

Negative controls were treated in the same manner as the samples, with the only exception that the enzyme solution was replaced by the enzyme buffer.

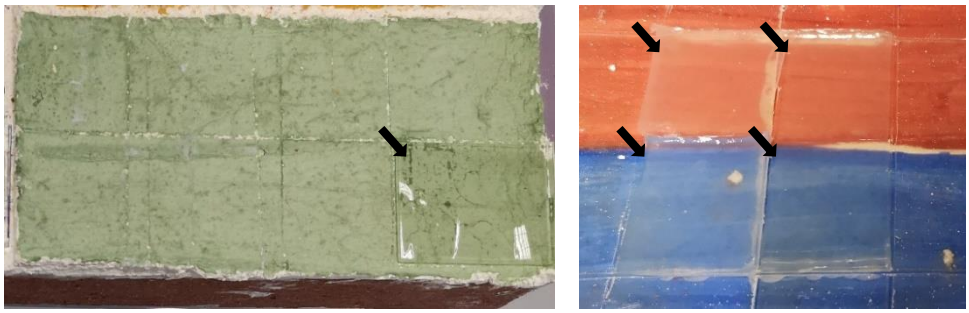

Figure 5.2. The application of hydrogels (Agarart and Gellano Kelcogel) on the laboratory models.

5.1.4 Qualitative methods to evaluate the efficacy of bio-cleaning

Morphological changes due to the hydrolysis of Paraloid B-72, sunflower oil or wax were examined by optical and scanning electron microscopy (SEM).

5.2 RESULTS

5.2.1 Characteristics of hydrogels

- Vanzan NF-C does not require high temperatures to dissolve, so there is no risk of thermal inactivation of the enzymes. It is relatively easy to apply and remove, but it is partially absorbed into the pores of the substrate.
- Gellano Kelcogel requires high temperatures (>80 °C) to dissolve, and the enzymes should be added to the mixture only when the temperature has dropped to about 50 °C. Incorporation of the enzymes into the gel is difficult because the latter solidifies relatively quickly at temperatures below 60 °C.
- Agarart also requires high temperatures to dissolve, but solidification is slower and takes place at a lower temperature than Gellano Kelcogel. This feature allows the enzymes

to be incorporated more easily, without the risk of thermal inactivation. Application and removal from wall surfaces are easy.

- Klucel has the advantage that dissolution is carried out at low temperatures and thus there is no risk of enzyme degradation. However, there are the following disadvantages: a) it takes a long time (~24 h) to dissolve, b) large amounts must be used to obtain the semi-solid consistency, c) the removal requires repeated washing with cotton wool soaked in water because it penetrates the pores of the substrate.

5.2.2 Qualitative evaluation of the efficacy of bio-cleaning treatments

The esterase-treated surfaces of the painted laboratory models showed morphological changes that indicated the partial removal of acrylic resin Paraloid B-72, sunflower oil and wax. In comparison to the untreated control, the bio-cleaned surfaces appeared to be brighter and less reflective. Moreover, SEM analyses showed textural differences between treated and untreated areas. The partial removal of targeted substrates could be attributed to the uneven distribution of enzymes on the treated surfaces.

In the case of oil-enriched laboratory models, the efficacy of bio-cleaning treatments could not be correctly evaluated on red and yellow pigmented areas due to the detachment of the painted layers after enzyme's application. However, samples enriched with Paraloid B-72, which served as a consolidant, were not affected by this phenomenon. In this case, it was observed that the efficacy of the bio-cleaning treatments was not significantly affected by the type of pigment.

The contact time of enzymes with the treated surfaces was an important parameter of the bio-cleaning process. In this regard, it was found that after 10 h of exposure, the deposits were hydrolyzed over larger areas compared to the 5 h treatments.

The ability of halotolerant esterase produced by *Bacillus* sp. BA N P3.3 to remove a widely utilized consolidant, i.e., Paraloid B-72, oil and wax from the painted surfaces of laboratory models represented a promising result in the field of bio-cleaning of mural paintings, stone artworks, as well as icons. To the best of our knowledge, this is the first report that utilized a salt-tolerant enzyme in a bio-cleaning process.

GENERAL CONCLUSIONS

Considering the biotechnological importance of salt-tolerant enzymes, the growing demand for these molecules on the global market and the current need for more efficient producers of such biocatalysts, the main aim of the present study was to isolate and identify novel strains of halophilic and halotolerant microorganisms able of synthesizing hydrolases with practical applicability. In order to achieve this goal, seven environments whose microbial communities have been poorly studied in terms of hydrolytic activities (five salt lakes in Romania, the Black Sea ecosystem and an extreme habitat in the Atacama Desert) were sampled and subjected to bioprospecting studies.

From the present study, the following conclusions were drawn:

1. A total of 331 halophilic and halotolerant isolates were obtained from water, sediment and halite samples collected from seven brackish, saline, and hypersaline environments. Among them, 269 (81.3%) were bacterial isolates, 40 (12.1%) were fungal isolates, and 22 (6.6%) were archaeal isolates.

2. The screening for hydrolytic enzymes showed that 230 (69.5%) isolates were able to produce single or combined hydrolytic activities (protease, lipase, amylase, cellulase, xylanase, pectinase). Based on the semi-quantitative estimations of hydrolytic activities, a total of 133 strains (112 bacteria, 16 fungi and 5 archaea) with the highest catalytic activities were selected. These strains constituted the laboratory collection.

3. The taxonomic identification of bacterial strains from the laboratory collection led to their classification into 39 genera distributed in six classes: *Bacilli* (50 strains), *Gammaproteobacteria* (40 strains), *Flavobacteria* (15 strains), *Actinobacteria* (4 strains), and *Alphaproteobacteria* (3 strains). The fungal strains were classified into three genera of the classes *Eurotiomycetes* (12 strains) and *Sordariomycetes* (4 strains), and the archaeal strains were classified into four genera of the class *Halobacteria*.

4. The quantitative evaluation of proteolytic, esterolytic/lipolytic, cellulolytic and xylanolytic activities of 25 selected bacterial and fungal strains showed that halotolerant strains belonging to the genus *Bacillus* produced the most intense hydrolytic activities.

5. Proteases, esterases, cellulases and xylanases produced by three selected strains (i.e., *Bacillus* sp. AM N P1.17, BA N P3.3, and BA N P1.4) were active over a wide range of salt concentrations, temperatures and pH values. Due to such functional properties, these

hydrolases could be suitable in various industrial and environmental applications that require harsh physicochemical conditions.

6. The ability of halotolerant esterases produced by *Bacillus* sp. BA N P3.3 to remove Paraloid B-72, oil, and wax from the painted surfaces of laboratory models represented a promising result in the field of bio-cleaning of mural paintings, stone artworks, as well as icons. However, further studies must be performed to optimize the bio-cleaning process and ensure the uniform and complete removal of these deposits, which often affect the aesthetic quality of murals.

The results of the present study encourage the continuation and extension of research on the novel halophilic and halotolerant strains from the laboratory collection. On the one hand, halotolerant strains of the genus *Bacillus* that showed rapid growth rates and intense hydrolytic activities at the laboratory level may be tested at pilot station level. On the other hand, halophilic strains that showed slow growth rates may be subjected to genetic engineering techniques, i.e., cloning and overexpressing the genes encoding the enzymes of interest.

Also, the collection of novel bacterial, archaeal and fungal strains offers the opportunity to conduct numerous other bioprospecting studies. In this regard, the strains may be screened for both the ability to produce various compounds of biotechnological interest (polyhydroxyalkanoates, polysaccharides, bacteriorhodopsin, antibiotics) and the potential to decompose various toxic substances (hydrocarbons, organic solvents, organic compounds) in order to establish their effectiveness in bioremediation of hypersaline environments or treatment of saline industrial effluents.

LIST OF PUBLISHED ARTICLES

Articles published from the PhD thesis

- **Ruginescu R.**, Enache M., Popescu O., Gomoiu I., Cojoc R., Batrinescu-Moteau C., Maria G., Dumbravician M., Neagu S., **2022**. Characterization of some salt-tolerant bacterial hydrolases with potential utility in cultural heritage bio-cleaning. *Microorganisms* 10, 644; https://doi.org/10.3390/microorganisms10030644; **IF 4,128**
- **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, 1903; doi:10.3390/microorganisms8121903; **IF 4,128**
- **Ruginescu R.**, Purcarea C., Dorador C., Lavin P., Cojoc R., Neagu S., Lucaci I., Enache M., **2019**. Exploring the hydrolytic potential of cultured halophilic bacteria isolated from the Atacama Desert. *FEMS Microbiol. Lett.* 366, fnz224; https://doi.org/10.1093/femsle/fnz224; **IF 2,742**

Other articles on related topics

- 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(4):242. DOI: 10.3390/fermentation7040242; **IF 3,975**
- Cojoc L.R., Enache M.I., Neagu S.E., Lungulescu M., Setnescu R., **Ruginescu R.**, Gomoiu I., 2019. Carotenoids produced by halophilic bacterial strains on mural paintings and laboratory conditions, *FEMS Microbiol. Lett.* 366, fnz243, https://doi.org/10.1093/femsle/fnz243; **IF 2,742**
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