ROMANIAN ACADEMY

INSTITUTE OF BIOLOGY BUCHAREST

Ph.D. THESIS

SUMMARY

MODERATELY HALOPHILIC AND HALOTOLERANT BACTERIA FROM SALINE HABITATS: PHYSIOLOGY, BIOCHEMISTRY, BIONANOTECHNOLOGY

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KEY WORDS:

EXTREME CONDITIONS
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SALINE HABITATS
HALITE
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MINE UNIREA
BAIA BACIULUI LAKE
BIODETERIORATION
PINK PIGMENTATION
HUMOR MONASTERY
BIOTECHNOLOGICAL APPLICABILITY
SILVER NANOPARTICLES
CAROTENOID PIGMENTS

INTRODUCTION

Although some habitats such as hot springs, deserts and hypersaline lakes, glacial caps, and ocean depths have been considered to be sterile in the past, more recent research (after the years 2000) has shown that they are populated by a wide variety of "extremophilic" microorganisms, capable of maintaining vital processes and even developing under extreme environmental conditions. There are also microorganisms adapted to several types of environmental stress, commonly referred to as "polyextremophiles".

Studying these cases not only demonstrates the principles of living in hostile environments but also provides arguments for the nature of life itself and the properties of the first organisms to colonize Terra at a time when conditions were much more hostile than those tolerated by most of the life forms that populate the planet today.

Some of these extremophilic microorganisms cannot survive under moderate conditions, preferred by most living organisms and are poorly adaptable to variable environmental conditions, while others present a higher flexibility. In this latter category, *moderately halophilic bacteria*, which grow optimally at 0.5-2.5M NaCl, are also included. A particular group consists of *halotolerant bacteria,* capable of growth at salt concentrations exceeding the limit of 0,5M, but whose optimum salinity value is much lower (less than 0,2M NaCl). Many microorganisms initially described as halotolerant are moderately halophilic and vice versa.

Even if less studied, compared to extreme halophilic microorganisms, moderately halophilic and halotolerant bacteria populate a wide range of aquatic and terrestrial habitats and also some salt preserved foods.

These microorganisms have a remarkable applicative potential in the biotechnological field, not only by producing compounds of industrial interest (enzymes, polymers), but also due to some physiological properties that can contribute to facilitating their exploitation in industrial interest (resistance to high NaCl concentrations, which minimizes the contamination risk, simple nutritional requirements).

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This PhD thesis **contributes** to the knowledge of the ***adaptive potential*** of moderately halophilic and halotolerant bacteria in extreme environmental conditions (osmotic stress) and their ***applicative importance*** resulting from the capacity to synthesize some biomolecules such as halophilic extracellular enzymes, exopolymers, carotenoid pigments. Acquiring certain

information related to the physiological characteristics of these microorganisms contributes to understanding the nature of halophilism and halotolerance.

The **source of isolation** of halophilic and halotolerant bacterial strains was represented by different types of saline media from Romania, of natural or anthropic origin, as follows: salt wall of Mina Unirea (Slănic Prahova), infilling mortar from the wall of the Humor Monastery (Suceava) and the salt lakes of Baia Baciului (Slănic Prahova), Balta Albă (Buzău County), Amara (Ialomița County), Movila Miresei (Brăila County) and Ocnele Mari (Vâlcea County). Except for the investigated historical monument, most sampling sites are located in the south-eastern part of Romania, in the sub-Carpathian area of the Southern Carpathians.

The **main scientific approaches** addressed the *isolation and selection* of halophilic and halotolerant bacterial strains from various saline habitats, *cellular physiology aspects* (testing of anaerobic growth capacity, determination of optimum salinity value and salinity range for growth, determination of the temperature and pH ranges for growth, the ability to synthesize exopolysaccharides and to produce biofilms), as well as aspects related to their *metabolic peculiarities* (synthesis of halophilic hydrolytic enzymes: amylases, lipases, esterases, proteases). At the same time, it was followed the *selection of moderately halophilic and halotolerant bacterial strains* able to synthesize *extracellular hydrolytic enzymes and exopolysaccharides* with applicative potential.

In the case of Humor Monastery, the aim was to establish the origin of pink biopigmentation on mural painting and to test colloidal solutions of gold and silver nanoparticles in order to establish a strategy for the decontamination of areas affected by biodegradation and a treatment to prevent future colonisations.

In order to isolate and characterize the halophilic and halotolerant bacterial strains, there were used *classical microbiology* **techniques** (estimation of the number of colony forming units, strain isolation on artificial culture media and their purification, selection of halophilic and halotolerant bacteria on media with specific inhibitors), *microscopy* (optical and SEM), *biochemistry* (assessing the extracellular enzyme synthesis by qualitative and quantitative methods), *spectroscopy* (UV-VIS, FT-IR - Fourier transform coupled infrared spectroscopy, XRF - X-ray fluorescence), *thermal analysis* (DSC), *molecular biology* (genomic DNA extraction, DNA concentration determination, PCR amplification of 16S rRNA gene, agarose gel electrophoresis, 16S rRNA sequence analysis and phylogenetic tree construction).

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The present paper is structured in two parts and contains 75 figures, out of which 72 in the original contributions part and 28 tables, of which 26 in the second part.

The first part (**chapters I-VII**) presents the current state of the art, including general information on the physiological and biochemical characteristics of moderately halophilic and halotolerant bacteria, as well as their applicative potential.

The second part (**chapters VIII-XI**) presents the original contributions, constituted by the results obtained in the study of moderately halophilic and halotolerant bacteria isolated from different types of saline habitats in Romania, representing the subject of the PhD thesis. Each chapter containing personal results is organized in several subchapters, each of those presenting the materials and methods used, the original results and the discussions, as well as the conclusions. The paper ends with a chapter of general conclusions. The bibliography contains 304 titles of papers published in national and foreign journals and is preceded by the list of papers published by the author on the subject of the PhD thesis.

The first chapter contains a short history of the research on halophilic and halotolerant bacteria from saline habitats in Romania. The first concerns about the study of microbial communities in hypersaline habitats referred to the salt lake Techirghiol (Ţuculescu et al., 1965). At the Institute of Biology Bucharest of the Romanian Academy, these studies, initiated under the leadership of Acad. Prof. G. Zarnea, have been continued and developed over the last 20 years.

In **the second chapter** are presented the ecological particularities of halophilic and halotolerant bacteria from saline habitats. There are two types of biologically important media in which the salinity factor interacts with microbial populations, namely: the soil and water. Several less common habitats, such as some desert plant and animal species, some salt preserved foods, or even some mural paintings, can also be mentioned.

Chapter III contains information about the cellular structure of halophilic and halotolerant bacteria, highlighting only the special characteristics of the cellular constituents, because halophilic bacteria have many common features with the non-halophilic related members.

Chapter IV contains considerations on the taxonomy and nomenclature of halophilic bacteria. They are present in 8 phylums: *Actinobacteria, Bacterioidetes, Cyanobacteria, Firmicutes, Proteobacteria, Spirochaetes, Tenericutes* and *Thermotogae* and, excepting the order *Halanaerobiales* (composed only by halophilic bacteria) and the family *Halomonadaceae* (almost exclusively halophilic) are found in a close phylogenetic relationship with the non-halophilic relatives.

Chapter V includes aspects related to the physiological and biochemical diversity of halophilic and halotolerant bacteria. Most halophilic bacteria have complex nutritional requirements at high salinities. They can use both common nutrient substrates and other compounds that are difficult to degrade (hydrocarbons, halogenated organic compounds) as a source of carbon and energy. The variety of metabolic types of halophilic bacteria decreases as salinity increases. Among the halophilic bacteria there are some representatives that constitute heterotrophic aerobic and anaerobic life models, as well as models of oxygenic and anoxygenic phototrophy, with autotrophic, photoheterotrophic and chemolithotrophic nutrition.

Chapter VI describes the two adaptation strategies used by halophilic bacteria to survive the osmotic stress generated by the presence of high concentrations of salt in the environment, namely the ”salt-in” strategy and the "compatible solution" strategy.

Chapter VII presents the bionanotechnological potential of moderately halophilic and halotolerant bacteria. Many of these produce various compounds of interest (osmoprotectors, enzymes, polymers and pigments), which are applicable in fields such as medicine, food, paper, pharmaceutical, cosmetics, agrochemical, textile industries and also in the bioremediation of waste water and polluted environments. Combining biomolecules synthesized by halophilic and halotolerant bacteria with different nanomaterials is of high interest for diagnosis, cellular marking and biosensing.

Chapter VIII describes the methodology we used for the isolation, selection and characterization of bacterial strains from salt crystals harvested from the wall of Mine Unirea (Slănic Prahova), as well as the obtained results. Of a total of 13 isolated halotolerant bacterial strains, most of them developed at low temperatures (12-15°C) and under dark conditions, proving a good adaptation to the microclimate conditions offered by the habitat of origin. BLAST analysis of 16S rRNA sequences indicated a 100% similarity of the 1/9 halotolerant strain with *Bacillus subtilis* AJ276351 and of 1/12 strain with *Virgibacillus halodenitrificans* AY543168. The strains 1/2 and 1/6 phylogenetically group with *Bacillus licheniformis*, respectively *Paraliobacillus quinghaiensis* species. It is the first mention of the presence of these halotolerant bacterial species, determined by molecular methods, in saline habitats from Romania.

Bacillus subtilis strain 1/9 showed the ability to hydrolyse all 6 tested enzymatic substrates, and the strains 1/1, *Bacillus licheniformis* 1/2 and *Virgibacillus halodenitrificans* 1/12 synthesized halophilic lipases, active at high salt concentrations (3M NaCl) with biotechnological potential in the pharmaceutical, agrochemical, oleochemical industries, etc.

Chapter IX deals with the isolation, selection and characterization of some strains of halophilic and halotolerant bacteria from the hypersaline lake Baia Baciului (Slănic Prahova), of

anthropic origin. Two strains of moderately halophilic bacteria and one halotolerant strain were isolated and it was found that the salinity interval in which the growth occurs is narrower and the optimum salinity value is lower in the case of the moderately halophilic strains, compared to those recorded for the halotolerant strain.

Using molecular methods there were identified species of *Idiomarina loihiensis* (halotolerant) and *Marinobacter lipolyticus* (moderately halophilic), present in the hypersaline lake Baia Baciului.

The isolated strains exhibit at least one of the tested extracellular hydrolytic activities (hydrolysis of starch, gelatin, casein, Tween 80), while *Idiomarina loihiensis* BB6 and *Marinobacter lipolyticus* BB49 presented combined enzymatic activities. At the same time, the bacterial strain *Idiomarina loihiensis* BB6 also showed the ability to synthesize a thermostable exopolysaccharide, having the melting point at 207ºC and a rich content of amino and sulfate groups, characteristics that represent an advantage for its use in biotechnological applications. The property of synthesizing extracellular exopolysaccharides is correlated, in the case of the strain *Idiomarina loihiensis* BB6 with its ability to form biofilms.

In **chapter X** are presented the isolation, selection and characterization of moderately halophilic and halotolerant bacterial strains from the natural salt lakes: Balta Albă (Buzău County), Amara (Ialomiţa), Movila Miresei (Brăila) and Ocnele Mari (Vâlcea). It has been found that the ratio established between the number of isolated moderately halophilic bacterial strains and that of halotolerant strains is dependent on the salinity of the environment from which they were isolated. In the case of Balta Albă lake, the relatively large number of strains with amylolytic activity can be correlated with the polyextremophilic character of this lake, being known that the pH 8-9 and the temperature values between 30ºC and 37ºC favor the amylase synthesis in halophilic microorganisms, in general and in the halophilic bacteria, in particular.

Chapter XI summarizes the results of the research carried out for the first time in Romania, concerning the origin of pink biopigmentation on the mural painting in the prenave of Humor Monastery (Suceava County). The isolation, selection and characterization of some halophilic bacterial strains from infilling mortar taken from the biodeteriorated area were performed, as well as the testing of the inhibitory activity of colloidal silver and gold nanoparticles, with the purpose of their use in strategies for biodegradation prevention.

The SEM examination of the infilling mortar sample revealed the presence of a microbial consortium consisting of bacillary-shaped cells and long, filamentous bacterial cells characteristic to actinomycetes, that form a "hyphae" network on the surface of the mortar. Under laboratory conditions there were isolated a halotolerant bacterial strain, producing carotenoid pigments and

capable of forming biofilms and a borderline extreme halophilic strain of actinomycetes. The biological features of the isolated bacterial strains explain their involvement in the biopigmentation process present in the Humor monastery. The obtained data demonstrate that the 5 Hum strain (identified by 16S rRNA sequence analysis as grouping with *Halobacillus* genus) grows and multiplies throughout the year, while the growth of the Act H strain occurs only when the temperature exceeds 20°C, corresponding to late spring, summer and early autumn periods.

CONCLUSIONS

1. Halotolerant bacterial strains isolated from salt crystals from the wall of **Mine Unirea (Slănic Prahova)** develop in wide salinity ranges (0-3M or 0-4M NaCl), and most have the ability to grow at low temperatures (12- 15°C) and in dark conditions, demonstrating a good adaptation to the microclimate conditions offered by the habitat of origin, namely Mine Unirea (Slănic Prahova).

2. Halotolerant bacterial strains isolated from salt crystals, phylogenetically grouping with *Bacillus licheniformis* and *Bacillus subtilis*, show the ability to grow anaerobically and have a psychrotolerant character.

3. The obtained results show that in the salt Mina Unirea (Slănic Prahova) wall structure there are halotolerant microorganisms with a rich extracellular enzymatic activity, which can contribute in time to the geochemical transformations of the salt deposit.

4. The ability of most salt crystals isolates to synthesize esterases recommends these strains for further studies concerning the lipid degradation or the bioremediation of waste waters in saline conditions.

5. The amylase activity present in the case of some investigated strains varies inversely with salinity. The synthesized halophilic lipases are active at salt concentrations exceeding the values for optimal growth of the producing halotolerant strains and have a biotechnological potential in the pharmaceutical, agrochemical and oleochemical industries.

6. Gram-negative, aerobic and facultatively anaerobic strains, phylogenetically grouping with *Idiomarina loihiensis* (halotolerant) and *Marinobacter lipolyticus* (moderately halophilic), having the ability to grow in a wide range of temperature (4 - 45 ° C) and pH (6-9), were identified from **Baia Baciului lake** using molecular techniques.

7. *Idiomarina loihiensis* BB6 halotolerant strain was found to develop in a much wider salinity range than the moderately halophilic *Marinobacter lipolyticus* BB49 strain. Optimal growth occurs at a higher NaCl concentration in the case of BB6 strain compared to BB49 strain.

8. The bacterial strain *Idiomarina loihiensis* BB6 has the ability to synthesize a thermostable exopolysaccharide with the melting point at 207ºC and a rich content of amino and sulfate groups, which is an advantage for its use in the bioremediation of pollutants and waste waters.

9. The researches carried out on samples taken from **Balta Albă (Buzău), Amara (Ialomiţa), Movila Miresei (Braila County) and Ocnele Mari (Vâlcea County)** confirmed the role of salinity as a limiting factor of the diversity of bacterial species. The ratio established between the number of isolated moderately halophilic bacterial strains and that of the halotolerant strains is dependent on the salinity of the medium from which they were isolated.

10. The polyextremophilic character of Balta Albă lake (intermediate salinity and alkaline pH) favors the development of a relatively large number of halophilic bacteria with amylolytic activity.

11. This PhD thesis approaches, for the first time in Romania, the study of pink biopigmentation on mural painting, highlighting the bacterial origin of the biodegradation process, by assigning the role of biodeteriogen to the halotolerant strain 5 Hum, that groups phylogenetically with *Halobacillus naozhouensis*. It is responsible for the production of pink biopigmentation in the monastery prenave, due to its ability to synthesize carotenoid pigments and to produce biofilms.

12. On the basis of the carried-out researches, it was found that in the **Humor Monastery**, the microclimate conditions (temperature, relative humidity) and the presence of efflorescences favor the growth and multiplication of the halophilic and halotolerant bacteria, respectively the colonization of the painting layer and mortars.

13. The efficiency of silver nanoparticles obtained from white strawberry callus extract in inhibiting the growth of the 5 Hum halotolerant strain recommends them for the incorporation into structural or infilling mortars in order to prevent the biodegradation process.

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Investigating the physiological and biochemical particularities of moderately halophilic and halotolerant bacterial strains isolated from different types of saline habitats has contributed to increasing the knowledge about their ability to survive and multiply under osmotic stress conditions and understanding the nature of halophilism and halotolerance.

The results obtained within this doctoral thesis confirm the metabolic versatility and the ability to synthesize biomolecules (halophilic extracellular enzymes, exopolysaccharides, carotenoid pigments) of moderately halophilic and halotolerant bacteria isolated from saline

habitats in Romania, with applicability in medicine, food industry, pharmaceutical, agrochemical industry and in the bioremediation of waste waters and polluted media.

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