

I. ECOSYSTEMS AND BIODIVERSITY

ISOLATION, IDENTIFICATION, MORPHOLOGICAL AND ADHESION PROPERTIES OF MICROORGANISMS FROM ANTARCTIC SOILS AND LICHENS

M. Dimitrova, S. Etem, K. Jordanova, N. Chipev, Ts. Ignatova-Ivanova

Abstract. In the present study we present the results from the isolation and study of microorganisms from Antarctic soils and lichens. The soil and lichen samples were gathered from the Livingston Island, South Shetland Islands. For the isolation of the different microorganism, different nutritious media were used. The isolated microorganisms were macro-morphologically and micro-morphologically studied. We also investigated the adhesion properties of defined microorganisms by using the SEM system JSM 5510. Our results demonstrated that the Antarctic continent is a habitat for a diversity of species of microorganisms despite the harsh living conditions. Some of the investigated species showed very good adhesion properties. Isolation of DNA was carried out by the company Progen LTD–Sofia using the Thermo Scientific Gene JET Genomic DNA Purification Kit. The DNA samples will be used for the exact species identification of the microorganisms. A databank including full inventory of the isolated species will be created.

Keywords: Antarctica, Livingston Island, microorganisms, adhesion properties, SEM JSM 5510.

INTRODUCTION

Antarctica is the most remote continent on Earth. It is particularly well suited for studies involving the aerial transport and survival of microorganisms, as it is isolated from the rest of the world by the Southern Ocean and Antarctic circumpolar current. Where this meets the other world oceans (the Antarctic convergence), the differential temperature of the air masses above warmer low-latitude water and colder high-latitude water discourages mixing.

Antarctic microbial diversity is the result of a balance between evolution, extinction and colonization. In addition, it is important to know whether the microorganisms already present are transient or resident – this is particularly true for the Antarctic environment, as selective pressures for survival in the air are similar to those that make microorganisms suitable for Antarctic colonization. The source of potential airborne colonists is widespread, as they may have originated from plant surfaces, animals, water surfaces or soils and even from bacteria replicating within the clouds [5].

In Antarctica itself, the greatest terrestrial biodiversity is in the maritime regions, where numerous moss and lichen species are found together with Antarctica's only two flowering plant species (*Deschampsia antarctica* and *Colobanthus quitensis*) [11]. The biodiversity of larger organisms generally declines with increasing latitude as conditions get colder and drier and the length of the growing season decreases [4]. Indeed, continental Antarctica is occupied by only the most durable species, such

as lichens and the microorganisms. On the basis of culture-dependent studies, it was concluded that bacteria from Antarctic soils are probably cosmopolitan, similar to those found in soils elsewhere in the world; however, more recent molecular (DNA)-based investigations indicate that there are novel species and genera in Antarctica [1].

The aim of the present paper was to isolate microorganisms from Antarctic soils and lichens and to characterize the adhesion capacity of some of the species. DNA of the studied microorganisms was isolated using the Thermo Scientific Gene JET Genomic DNA Purification Kit (Progen LTD–Sofia) and the exact species identification is in progress.

MATERIALS AND METHODS

1. Soil Samples

1.1. Sampling Sites

The soil samples were collected during the austral summer of 2015 from ice-free areas on Livingston Island at a depth of 0–5 cm. The sites were chosen to represent different habitat characteristics and noted as follows: **S1** (soil from the cliff west from the Base); **S2** (soil from the slope above the beach east from the Bulgarian Base) and **S3** (soil from Hesperides Point); **P1** - lichens (lichens from the cliff west from the Bulgarian Base); **P2** (lichens from the slope above the beach east from the Bulgarian Base) and **P3** (lichens from Hesperides Point).

1.2. Soil Analysis

Water and humus content as well as pH of soil samples was determined [1].

1.3. Cell Suspensions

Cell suspensions were prepared from 5 g soil samples suspended in 45 ml sterile 0.9% NaCl (Sigma-Aldrich)[6].

1.4. Bacterial Enumeration

The cultural method was chosen to plate decimal dilution series (10^{-1} – 10^{-5}) of soil suspensions and to enumerate copiotrophic bacteria and actinomycetes. The inoculated agar plates were incubated in dark at 4°C for 8 days.

2. Media

Media with yeast and malt extracts (M 2) in content g/l: glucose - 4; yeast extract - 4; malt extract - 10; agar-agar – 20, pH 7,3.

Media oat agar (M 3) in content g/l: oat meal – 20; agar-agar – 18; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 0,1; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ – 0,1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ – 0,1, pH – 7,2.

Media starch agar (M 4) in content g/l: starch – 10; K_2HPO_4 – 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 1; NaCl – 1; $(\text{NH}_4)_2\text{SO}_4$ – 2; CaCO_3 – 2, agar-agar – 20, pH – 7,0 – 7,4.

Media glycerol-asparagine agar (M 5) in content g/l: L asparagines– 1; glycerol – 10; K_2HPO_4 – 1; agar-agar – 20, pH 7,0 – 7,4.

Media Gauze agar in content: starch – 20; KNO_3 – 1; K_2HPO_4 – 0,5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0,5; NaCl – 0,5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 0,01; agar-agar – 30, pH 7,2 – 7,4.

Media starch-ammonia agar SSA in content g/l: starch – 10; $(\text{NH}_4)_2\text{SO}_4$ – 2; K_2HPO_4 – 1;

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 1; NaCl – 1; CaCO_3 – 3, agar-agar – 20, pH – 7,0.

The media were sterilized by autoclaving at 121°C for 20 min.

3. DNA isolation

The isolation of DNA was carried out by the company Progen LTD–Sofia using the Thermo Scientific Gene JET Genomic DNA Purification Kit. The DNA samples will be used for the exact species identification of the microorganisms.

4. Analysis by SEM JSM 5510.

The following experiment was carried out in order to analyze adhesion ability: a microorganism species from sample P2 (*Streptomyces* sp.) was cultivated on M2 media. When an amount of 10^{-8} CFU was reached, a steel plate was put in the middle. Before use the steel panels (10x4x0,2 mm) were treated with 70% $\text{C}_2\text{H}_5\text{OH}$ and washed with water. After 120 h the steel plate was weighed again, so that the thickness of the biofilm could be assessed. The structure of the layer formed on the steel plates was analyzed by Scanning Electron Microscopy (SEM) JSM 5510.

RESULTS AND DISCUSSION

The soil samples were analyzed for pH and moisture content (Table 1). The moisture content of the soils was high at 79,5% – 96,5%, and even higher for plot S1 – 96,5%. The pH of the soils was close to neutral and varied in a relatively narrow range.

Table 1.Characteristics of the studied soils

Soil sample	S1	S2	S3
Moisture	96,5%	88,9%	79,5%
pH	6,91	6,99	6,75

After 10 days of culture on the different culture media various microbial colonies were obtained (Table 2). The data showed that the slowest growth among all the samples was recorded on medium M2, regardless of the fact that the medium was rich in organic compounds. It is interesting to note that in the samples from lichens higher number of microorganisms were recorded on medium M2. The reason for this probably lies in the symbiotic interactions between the two organisms, inhabiting extreme habitats. Growth on this medium was recorded only for samples S2 from soil, and P2 and P3 from lichens.

The Antarctic is a habitat characterized with very severe living conditions even for the well adapted species. The limiting factors for microbial growth in this seemingly hostile environment are manifold. For example, low pH could severely impede bacterial metabolism, but thanks to the buffering of ammonia and alkaline dusts, these values are kept between pH 5.4 and 6.8, and dissolved organic carbon concentrations can reach several mg l^{-1} , typical for lake water conditions.

Table 2. Number of obtained colonies on the different media

Media/Sample	S1	S2	S3	P1	P2	P3
SSA	$28 \cdot 10^6$	$13 \cdot 10^5$	$42 \cdot 10^6$	$38 \cdot 10^6$	$10 \cdot 10^3$	$72 \cdot 10^7$
Gauze	$50 \cdot 10^5$	$32 \cdot 10^6$	$27 \cdot 10^5$	$24 \cdot 10^5$	-	$32 \cdot 10^4$
M5	$21 \cdot 10^5$	$38 \cdot 10^6$	$19 \cdot 10^5$	$6 \cdot 10^5$	$32 \cdot 10^3$	$83 \cdot 10^7$
M2	-	$4 \cdot 10^5$	-	-	$13 \cdot 10^5$	$70 \cdot 10^7$
M4	$24 \cdot 10^5$	$49 \cdot 10^7$	$64 \cdot 10^6$	$12 \cdot 10^4$	$21 \cdot 10^6$	$43 \cdot 10^5$
M3	$33 \cdot 10^5$	$61 \cdot 10^7$	$29 \cdot 10^6$	$58 \cdot 10^6$	$15 \cdot 10^6$	$31 \cdot 10^4$

The isolated species of microorganisms were subjected to micro-morphological and macro-morphological studies. The results are presented in Figures 1 and 2. The micro-morphological characteristic suggested that among the isolated species dominated representatives of *Streptomyces sp.* and *Actinomyces sp.* These results confirmed previous studies on microbial diversity in Antarctica [7]. The microscopic pictures also confirmed the presence of high microbial species diversity in some of the samples. It could be clearly seen the presence of diverse structure of the aerial mycelium in the isolated species (Fig. 1 A,B and C), and the cells of the microorganisms refer to the Gram positive bacteria. The Antarctic habitat is characterized by harsh living conditions. The limiting factors for the microbial growth in these hostile conditions may be manifold. For example, low pH could severely impede bacterial metabolism, but thanks to the buffering of ammonia and alkaline dusts, these values are kept between pH 5.4 and 6.8, and dissolved organic carbon concentrations can reach several mg l^{-1} , typical for lake water conditions. Natural solar radiation at high altitudes may strongly inhibit microbial activity, but clouds themselves can provide shading from damaging and bactericidal UV radiation [5]. There are a number of ways in which different microorganisms are adapted to survival, through tolerance of cold, pH and UV radiation, using spores, mini cells and other small resistant cell forms. One particular adaptation is the ability to withstand long-term natural cryopreservation – samples of permafrost sediments with ages from 5–10 thousand to 2–3 million years have been found to contain viable micromycete and bacterial cells

[8]. Another potential response might be cells to remain viable but not divide in transit.

Microbes in Antarctica adapt to aridity the same way as microbes in hot deserts do: when water becomes scarce, the organisms simply dry up, shut down metabolic activity, and wait in a "cryptobiotic" state until water again becomes available [3]. Microbes can also become dormant in a cryptobiotic state known as "anhydrobiosis" when they become dehydrated due to low water availability.

Our results confirmed the high diversity of Antarctic microorganisms despite the harsh environment. This may be due to the ability of the microorganisms to remain vital for a long time at low temperature –natural cryopreservation. Another possibility for survival at low temperatures could be some kind of relationships (i.e. symbiosis) between lichens and *Streptomyces sp.* and *Actinomyces sp.* microorganisms. There are no data in the literature on such relationships but our result (Fig. 1. A,B) suggest their presence. The possible role of such relationships in the survival of microorganisms remains open for further research.

In previous studies [7] we demonstrated that one of the isolated from Antarctic soils species (*Streptomyces sp.*) showed good adhesion capacity and could be used as a corrosion inhibitor. This finding motivated us to test another species for adhesion properties. In order to analyze the adhesion capacity of one of the isolated microorganisms (sample P2 on M2) an experiment *in vitro* was carried out. The increase in the weight of the metal plate of area 2 cm^2 after the adhesion process was 1.9 mg or 0.95 mg/cm^2 . The results were visualized using

CEM JSM 5510 and are presented in Fig. 3. In general, it could be concluded from the obtained

results that the studied microorganism species demonstrated good adhesion capacity.

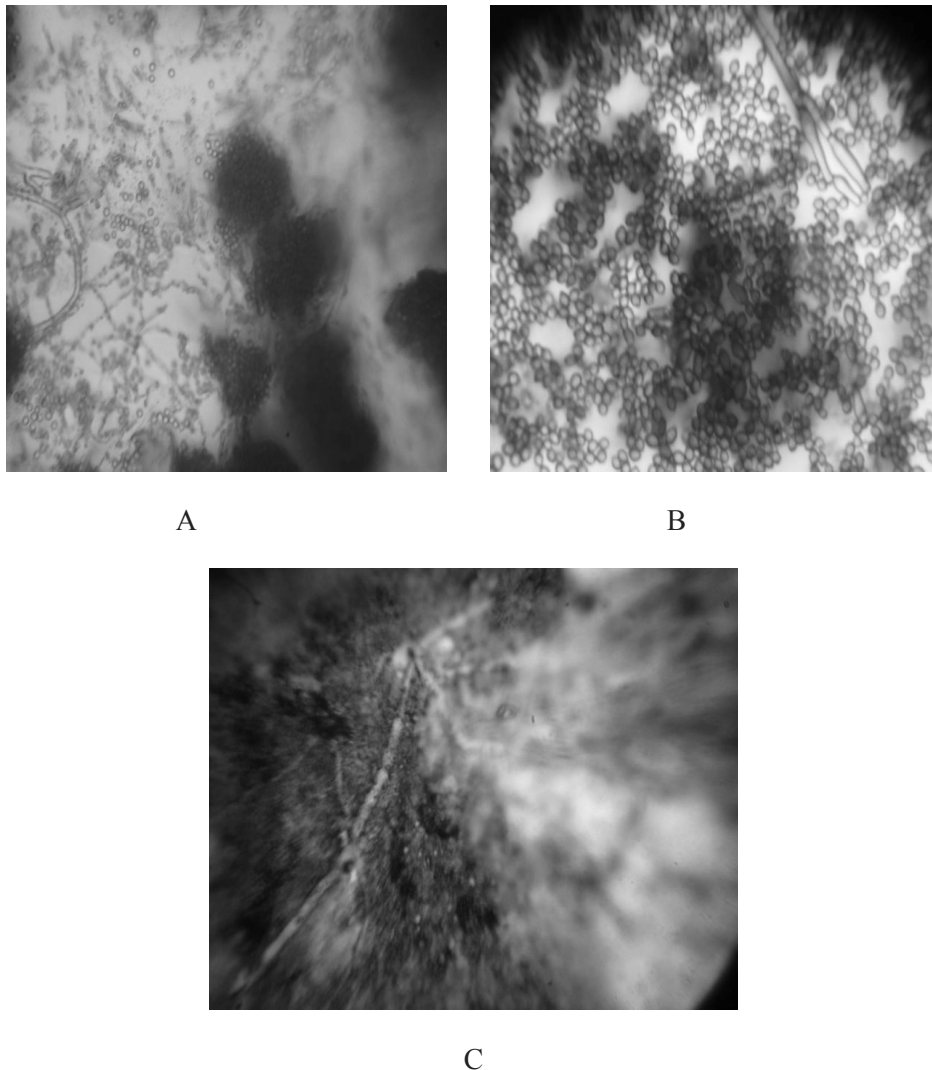


Fig. 1. Light microscopic visualizations of preparation imprint (A, B) and Gram stained (C) of isolated strains: A) sample P2 on M2; B) sample P1 on M3; C) sample P3 on Gause medium. (pictures were taken using microscope OPTIKA (Italy) at magnification 1000 by immersion).

The adhesion capacity of the studied microorganism species may have connection with the life style in the harsh environment. Microorganisms were found to play an active role in ice nucleation and in creating precipitation in some types of clouds [10]. Super cooled water needs a surface on which to freeze and this could be provided by mineral particles, bio-aerosols or microbial cells. Schnell (1976) stated that microorganisms attached to mineral particles were responsible for ice nucleation rather than the mineral particles themselves. Nowadays, the

microbial ability to act as an ice nucleus is crucial for agriculture where ice nucleation can cause frost injuries to plants even at relatively warm temperatures close to the freezing point of water [9]. The most prominent bacterium with the ice nucleation gene is *Pseudomonas spp.*, although ice nucleation-negative species have been detected in cloud samples collected from the Northern Hebrides [2]. The protein is located in the surface of the outer membrane and serves as a template for the alignment of water molecules for ice crystallization.

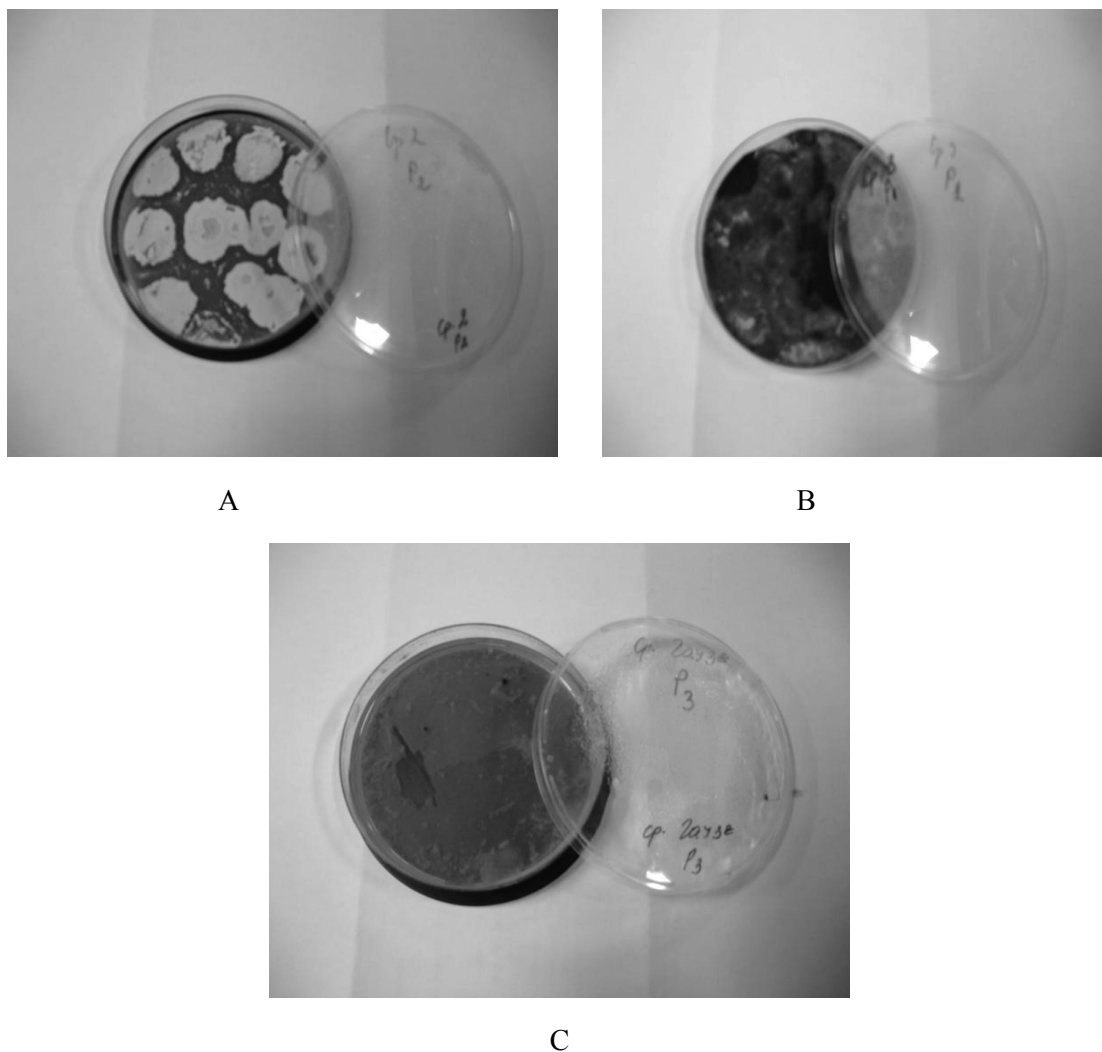


Fig 2. Pictures of the colonies of isolated species: A –sample P2 on M2; B - sample P1 on M3; C -sample P3 on Gause (pictures were taken using stereomicroscope OPTIKA (Italy) at magnification 1000)

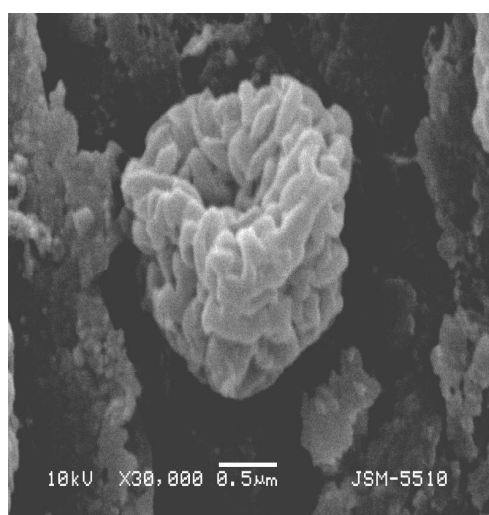


Fig 3. Adhesion ability of the microorganisms from sample P2 on M2

In general, the adhesion properties of microorganisms are undesirable both in agriculture and industry, as for example they can plug-up pipes, contribute to the wearing-up of metal constructions, etc. However, in our previous studies we have demonstrated that adhesion of Antarctic microorganisms on metal surfaces can inhibit corrosion [7] and further experiments are underway to confirm the positive role of microorganisms in corrosion protection.

To date, studies of life in remote icy environments have generated a huge range of estimates of microbial numbers, and increasingly, estimates of activity and impact are starting to be generated. These microorganisms represent a significant carbon pool. The implication for future studies of such icy environments is that it is essential to know how quickly they are accumulated (growth rate vs. input rate), i.e. whether the microorganisms identified are merely transient organisms, established but not dividing, established and dividing slowly or established and reproducing effectively.

ACKNOWLEDGMENTS: *This study was partly funded by project RD-08-266/10.03.2015 of Shumen University.*

REFERENCES

- [1] Adams BJ, Bardgett RD, Ayres E. Diversity and distribution of Victoria Land biota. *Soil Biol Biochem*, vol. 38, 2006, 3003–3018.
- [2] Ahern HE, Walsh KA, Hill TCJ & Moffett BF. Ice-nucleation negative fluorescent pseudomonads isolated from Hebridean cloud and rain water produce biosurfactants. *Biogeosciences*, vol 3, 2006, 1561–1586.
- [3] Aislabie JM, Chour K-L, Saul DJ, Miyauchi S, Ayton J, Paetzold RF & Balks MR. Dominant bacteria in soils of marble point and Wright Valley Victoria L and Antarctica. *Soil Biol. Biochem.*, vol. 38, 2006, 3041–3056.
- [4] Convey P. Terrestrial ecosystem responses to climate change in the Antarctic. 'Fingerprints' of Climate Change – Adapted Behaviour and Shifting Species Ranges (Walther G-R, Burga CA & Edwards PJ, eds), Kluwer, New York, 2001, 17–42.
- [5] David A. Pearce, Paul D. Bridge, Kevin A. Hughes, Birgit Sattler, Roland Psenner & Nick J. Russel. Microorganisms in the atmosphere over Antarctica. *FEMS, Microbiol Ecol.*, vol. 69, 2009, 143–157.
- [6] Encheva, M., N. Zaharieva, A. Kenarova, N. Chipev, V. Chipeva, P. Hristova, I. Ivanova, P. Moncheva. Abundance and activity of soil actinomycetes from Livingston Island, Antarctica. *Bulgarian Journal of Agricultural Science*, vol.19 (2), 2013, 68–71.
- [7] Ignatova-Ivanova Ts., R. Ivanov, N. Chipev. 2015. Isolation of microorganisms from Antarctic soils and their use as possible corrosion inhibitors. *International Journal of Research Studies in Bio Sciences (IJRSB)*, vol. 3 (1), 2015, 164–168.
- [8] Kochkina GA, Ivanushkina NE, Karasev SG, Gavrish EY, Gurina LV, Evtushenko LI, Spirina EV, Vorob'eva EA, Gilichinskii DA & Ozerskaya SM. Survival of micromycetes and actinobacteria under conditions of long-term natural cryopreservation. *Microbiology*, vol. 70, 2001, 356–364.
- [9] Morris CE, Georgakopoulos DG & Sands D. Ice nucleation active bacteria and their potential role in precipitation. *J Phys. IV*, vol. 121, 2004, 87–103.
- [10] Morris CE, Sands DC, Bardin M, Jaenicke R, Vogel B, Leyronas C, Ariya PA & Psenner R. Microbiology and atmospheric processes: an upcoming era of research on bio meteorology. *Biogeosciences Discuss.*, vol. 5, 2008, 191–212.
- [11] Smith RIL. Vascular plants as indicators of regional warming in Antarctica. *Oecologia*, vol. 99, 1994, 322–328.

ИЗОЛИРАНЕ, ИДЕНТИФИКАЦИЯ, МОРФОЛОГИЯ И АДХЕЗИОННИ СВОЙСТВА НА МИКРООРГАНИЗМИ ОТ АНТАРКТИЧЕСКИ ПОЧВИ И ЛИШЕИ

М. Димитрова, С. Етем, К. Йорданова, Н. Чипев, Ц. Игнатова-Иванова

Резюме. В настоящата статия представяме резултати, получени от изолиране на микроорганизми от Антарктически почви и лишей. Почвените и лишейните проби са взети от остров Ливингстън. За изолиране на различни групи микроорганизми бяха използвани различни хранителни среди. Изолираните микроорганизми бяха подложени на макроморфологичен и микроморфологичен анализ. В допълнение изследвахме адхезионните свойства на микроорганизмите чрез СЕМ система JSM 5510. Резултатите показват, че независимо от суровите условия за живот Антарктида притежава едно голямо природно богатство от различни видове микроорганизми, част от

които живеят във взаимоотношения с лишеите, които се срещат там. Някои от изолираните микроорганизми проявяват добра адхезионна способност. Предстои да бъде направена и видова идентификация на намерените видове микроорганизми по изолирана от нас ДНК чрез Thermo Scientific Gene JET Genomic DNA Purification Kit от фирма Pirogen LTD–София.

Ключови думи: Антарктида, остров Ливингстон, микроорганизми, адхезионни свойства, СЕМ система JSM 5510

Assoc. Prof. Tsvetoslava Ignatova-Ivanova, PhD

Shumen University “Konstantin Preslavski”
Department of Biology
115 Universitetska Str., Shumen, Bulgaria
E-mail: radi_cvet@abv.bg*

Marina Dimitrova

Shumen University “Konstantin Preslavski”
Department of Biology
115 Universitetska Str., Shumen
Bulgaria

Sevgi Etem

Shumen University “Konstantin Preslavski”
Department of Biology
115 Universitetska Str., Shumen
Bulgaria

Prof. Nesho Chipev, PhD

Shumen University “Konstantin Preslavski”
Department of Biology
115 Universitetska Str., Shumen, Bulgaria

Kristina Jordanova

Shumen University “Konstantin Preslavski”
Department of Biology
115 Universitetska Str., Shumen, Bulgaria

Доц. д-р Цветеслава Игнатова-Иванова

Шуменски Университет „Епископ Константин Преславски”
Катедра Биология
ул. „Университетска” 115
Шумен - 9712, България

Марина Димитрова

Шуменски Университет „Епископ Константин Преславски”
Катедра Биология
ул. „Университетска” 115
Шумен - 9712, България

Севги Етем

Шуменски Университет „Епископ Константин Преславски”
Катедра Биология
ул. „Университетска” 115
Шумен - 9712, България

Проф. д-р Нешо Чипев

Шуменски Университет „Епископ Константин Преславски”
Катедра Биология
ул. „Университетска” 115
Шумен - 9712, България

Кристина Йорданова

Шуменски Университет „Епископ Константин Преславски”
Катедра Биология
ул. „Университетска” 115
Шумен - 9712, България