Psychrotolerant anaerobes from Lake Podprudnoe, Antarctica and penguin *Spheniscus demersus* colony, South Africa

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ABSTRACT

The study of a sample collected from a wind-made ice sculpture near Lake Podprudnoe, Antarctica led to the isolation of the psychrotolerant strain ISLP-3. Cells of the new isolate are vibrio-shaped that measure 0.5 x 1.0-3.0 µm in size. Growth occurs within the temperature range 5-35°C with the optimum at 22 °C. Salinity range for growth is 0-2 % NaCl with the optimum at 0.25 %. The new isolate grows within a pH range from 6.0 to 9.5 with the optimum at 7.5. Strain ISLP-3 is saccharolytic, growing on the following substrates: *D*-glucose, *D*-ribose, *D*-fructose, *D*-arabinose, maltose, sucrose, *D*-trehalose, *D*-mannose, *D*-cellobiose, lactose, starch, chitin, triethylamine, N-acetylglucosamine, and urea. The best growth occurred on *D*-cellobiose. An environmental sample of pond water near a colony of the endemic species of African penguins, *Spheniscus demersus,* was collected in February 2008 and delivered directly to the Astrobiology laboratory at NSSTC. The microbiological study of this sample led to the isolation of two psychrotolerant strains ARHSd-7G and ARHSd-9G. Both strains are strictly anaerobic bacteria and are able to grow at high pH and low temperatures. The cells of strain ARHSd-7G are motile, vibrio-shaped, spore-forming cells. Optimal growth of this strain occurs at 30 °C, 3 % NaCl, and pH 8.9. The isolate ARHSd-7G combines sugarlytic and proteolytic metabolisms, growing on some proteolysis products including peptone and yeast extract and a number of sugars. The second isolate, ARHSd-9G, exhibits thin, elongated rods that measure 0.4 x 3-5 µm. The cells are motile and spore-forming. Optimal growth of strain ARHSd-9G occurs at 30 °C, 1.75 % NaCl, and pH 8.5. The strain ARHSd-9G is sugarlytic, growing well on substrates such as D-glucose, sucrose, D-cellobiose, maltose, fructose, D-mannose, and trehalose (the only exception is positive growth on yeast extract). In this report, the physiological and morphological characteristics of the novel psychrotolerant, alkaliphilic, and neutrophilic isolates from the Antarctica 2008 expedition will be discussed.

Keywords: Antarctica, *Spheniscus demersus,* psychrotolerant anaerobe, alkaliphile, sugarlytic, Lake Podprudnoe

1. INTRODUCTION

During the past few decades, the investigations of life in extreme environments have provided an ever increasing documentation of the great diversity of microbial life displaying unique physiological characteristics and adaptations to these environments. The term "psychrophile" (cold loving) was coined by Schmidt-Nielsen in 1902 for the description of bacteria capable of growing at 0 °C. Later the term was also used to refer to a number of species of eukaryotic organisms including yeasts, diatoms, algae, lichens, mosses, insects, fish, and mammals. The current definition of psychrophilic microorganisms, as formalized by Morita[1,2], describes the capability to grow at low and subzero temperatures with the optimum growth at 15 °C or lower and the absence of growth and complete lost metabolic activity at the temperature above 20 °C. True psychrophiles undergo cell lysis at room temperature (22 °C), and their proteins (including enzymes) are destabilized resulting in all loss of functionality, if the appropriate cold temperature is not maintained. However the psychrotolerant microorganisms could have high metabolic activity and a relatively prolonged lag-phase for growth at cold temperatures, but they do have growth optima within mesophilic temperature regimes.

Liquid water is essential for metabolic activity to sustain life.[3] The snow algae *Chlamydomonas nivalis* produces the brilliant red spores and alters the albedo of snow to induce a snowmelt to obtain liquid water. Some diatoms and other psychrophiles produce extracellular enzymes and ice-active substances that lead to the pitting of ice and melting the surrounding ice making liquid water available. It is now known that most (if not all) Antarctic Sea-Ice diatoms are able to synthesize these macromolecular ice-active substances that appear to be glycoproteins.[4] Some polar fish produce glycerol to protect against freezing.[5] The synthesis of such cryoprotectants helps these organisms to avoid freezing of the cellular fluids, dehydration, and damage of the cell membrane by ice crystals. Some organisms have developed

mechanisms of freeze tolerance, involving drastic metabolic modifications, resistance to membrane damage, increasing solute concentration, and cell dehydration accompanying ice crystallization.[6]

Cryoprotectants such as glycerol or DMSO are water-miscible liquids, and they penetrate the cell and protect it from freezing by reducing the severity of dehydration effects and preventing the formation of ice crystals. Lyophilization (freeze drying) is a routine method used in microbiology laboratories and cell culture collections to preserve microbial cultures under vacuum at low temperatures (-70 °C to -196 °C). Under specific conditions biological cells are indeed capable of surviving deep freezing, and this feature is possibly a universal characteristic of life.

This study investigates the biodiversity of prokaryotic microorganisms of some Antarctic ecosystems, the samples of which were collected during the *2008 Tawani International Antarctica Expeditions* by Richard B. Hoover. Currently we have described a number of new obligately/facultatively anaerobic and aerobic bacterial strains that are tolerant to low temperatures.

1.1 Ecosystems and diversity of microbial psychrophiles

The ecosystems of Earth with constant low temperature include the regions of the Arctic and Antarctic with polar ice sheets, glaciers and permafrost, the snow caps and glaciers of high mountains, and the deep sea of world Ocean. The Polar Regions contain large areas of frozen ground or permafrost. In these regions, the soils and sediments remain at or below 0 °C for two or more years in succession.[7] Permafrost underlies more than 20 % of the world's land area and is primarily controlled by climatic factors and characterized by extreme terrain conditions and landforms. On Earth the permafrost can be several hundreds of meters thick. It is 600 to 800 meters thick in Central Yakutia of East Siberia. During a relatively short period of the polar summer, a thin surface zone (active region) of the permafrost sediments undergoes thawing.

Another vast low-temperature ecosystem is the deep sea floor (oceans cover three quarters of the surface of our planet). The psychrophiles that inhabit this global-scale ecosystem (with a constant temperature of 4 °C below a depth of 1,000 m) are true extremophiles as they are adapted not only to low temperatures, but also to other environmental constraints.[8] In the ocean's depths and sediments these microorganisms are faced with extremely high pressures, and therefore must be piezo-psychrophiles or baro-psychrophiles.[9]

The microbial communities that are found on sea ice, which comprise bacteria, algae, fungi and protozoa[10] are exposed to salt concentrations of several molar in brine veins at −20 °C, and are therefore halo-psychrophiles. On the snow surface of glaciers and the polar ice caps, psychrophiles are exposed to strong ultraviolet radiation.[11] The endolithic microbial communities found in rocks of the Antarctic dry deserts and oases (e.g., lichens, yeasts, cyanobacteria and heterotrophic bacteria) survive desiccation and luck of nutrient availability.[12]

In alpine and arctic caves and cracks, psychrophilic and psychrotolerant bacteria also inhabit a cold environment in the total absence of light (troglo-psychrophiles). In caves with temperatures around 10 °C, most of the bacteria were psychrotolerant. However, in colder caves, such as the Larshullet cave of northern Norway, most of the strains isolated were true psychrophiles, growing better at 4 °C than 20 °C.[13]

The physiological and phylogenetic diversity of psychrophilic and psychrotolerant microorganisms that were detected and isolated from cold ecosystems is very broad. This includes almost all main physiological groups of bacteria and archaea. Fungi and yeast are also common in cold environments. The producers or synthetics of organic matter in cold ecosystems include not only photosynthetics (cyanobacteria, diatoms, green and brown algae), but also chemolithoautotrophic bacteria, such as homoacetogens and methanogens. Active anaerobes that have a truly psychrophilic nature occurred in the following physiological groups: fermentative bacteria, methanogens, acetogens, sulfate-reducers, iron-reducers, and nitrate-reducers. In the last decade attention has been paid to the search for psychrophilic strains of methanogens. Also methane-oxidizing aerobic bacteria were found in the cold peat bogs of the Arctic regions.[14] The first Gram-positive sulfate-reducing bacterium from Antarctica *Desulfotomaculum antarcticum* strain No.64 was isolated in 1968.[15] However, truly psychrophilic Gram-negative sulfate-reducers capable of growth at − 1.8 °C and with growth optima at 7-10 °C were only isolated in 1999 from the permanently cold marine sediments off the coast of Svalbard.[16]

1.2 The adaptation to low temperatures

To optimal functioning every microorganism has to be adjusted to the natural environments, and this should be reflected in its physiological characteristics such as range and optima of temperature, pH, salinity, etc. Any extreme changes in the environmental conditions inflict a stress on an organism.[17] The intensivity and time duration of the changes usually determines whether the organism is killed, ceases growth, or has an increased lag- phase of growth and reduced biomass yield.[18,19] Most bacteria are able to tolerate small changes in the environmental parameters and can adapt over the time

scale of minutes, hours, or days.[20] Microorganisms could perform this by yielding to the stress conditions and making suitable provisions for survival, or by attempting to resist the stress.[21] For most microorganisms, this tolerance can be pushed to maximum limits if the cell is provided with a sufficient opportunity to sense and adapt to the deteriorating environment. Entire groups of microorganisms, such as psychrophiles, have adapted their lifestyles to prefer (and sometimes to require) these extreme environments. Changes in environmental conditions away from the optimal value can cause the induction of many elaborate stress responses. These strategies are generally directed at survival rather than to growth. According to recent reviews, the mechanisms of adaptation to cold temperatures could be connected to the changing of capacities of proteins (more flexible structure and conformation changes), increasing the fluidity of membranes by changing the unsaturation degree of fatty acids, modifications to *ante-iso-/iso-* branching patterns, and by shortening the fatty acid chain length. Also, the synthesis of antifreeze glycoproteins and peptides can further depress the freezing point of water within the cells.

Unsaturation of fatty acid chains is the change that is most commonly found to occur when the temperature is reduced. This increases the fluidity of the membrane because unsaturated fatty acid groups create more disturbances to the membrane than saturated chains, and is achieved by desaturases situated in the membrane itself and thus are able to react quickly.[17] Also, the average fatty acid chain length may be shortened, which would have the effect of increasing the fluidity of the cell membrane. After a drop of temperature, an increase in the amount and/or kind of branched fatty acids may occur. Sometimes, there may be a reduction in the proportion of cyclic fatty acids and thus an increase in mono-unsaturated straight chain fatty acids.[22]

Cold shock response can involve the expression of up to 50 different cold shock proteins depending on the species, as well as the rate and extent of temperature drop.[23] There are two groups of proteins produced at the gene expression during the cold shock response. Cold shock proteins (Csps) are synthesized at low temperature and the larger the severity of the shock, the more Csps produced.[21,24] A second group of cold-induced proteins, the cold acclimation proteins (Caps) has also been described that are comparable to Csps and are continuously synthesized during prolonged growth at low temperatures, and differentiate psychrophiles from mesophiles.[25] Cold shock proteins can stabilize mRNA and re-initiate protein production. Others are also linked to maintaining the fluidity of the membrane such as inducible desaturases.
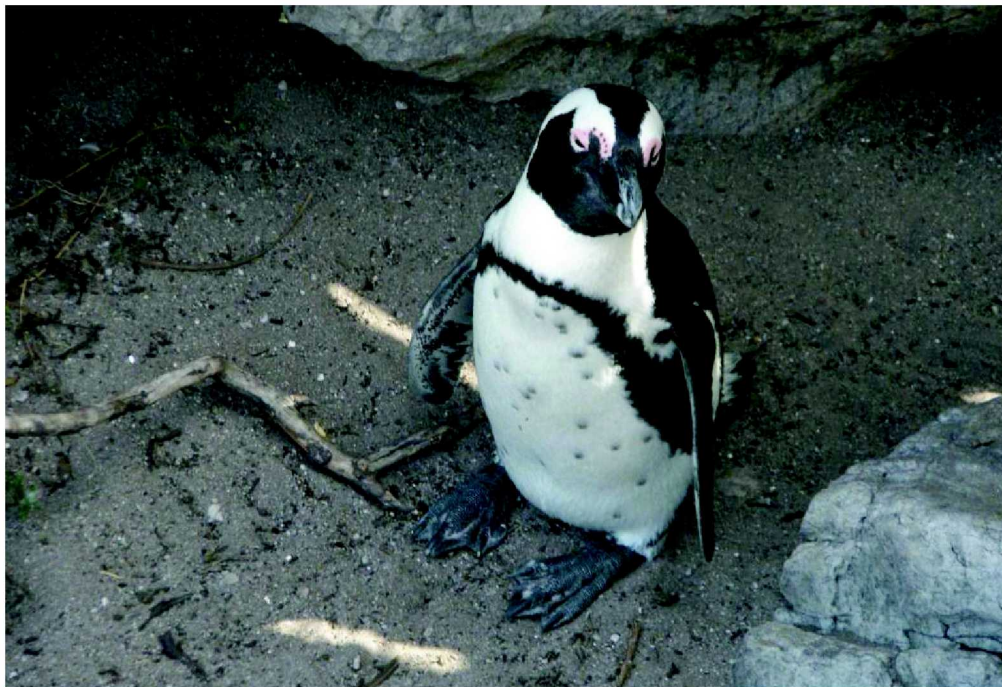

Fig. 1. African penguin *Spheniscus dimersus* at Stony Point, Betty's Bay, South Africa.

Fig. 2. "Ice sculpture" near Lake Podprudnoye in Schirmacher Oasis, Antarctica.

2. MATERIALS AND METHODS

2.1 Bacterial strains, isolation, and growth conditions

Strains ARHSd-7G and ARHSd-9G were isolated from the guano of the African penguin *Spheniscus demersus*. Environmental samples of the penguin guano were collected from a small tidal pool with the assistance of Park Rangers at the Stony Point Nature Reserve, Betty's Bay, South Africa (S 34 ° 21'25"; E 18 ° 28'16"). The samples collected measured a temperature of 15 °C, pH 6.8, and salinity 3 %. The sample was directly examined by visible light and dark field microscopy and then returned to the Astrobiology Laboratory of NSSTC in Huntsville.[28] Phase-contrast microscopy of the sample at NSSTC revealed the presence of diverse motile microbial cells ($\sim 10^{13}$ cells per ml). Enrichment cultures of ARHSd- 9G were grown by injecting 0.3 ml portions of the samples into alkaline (pH 9) anaerobic media with 1 % NaCl or 3% NaCl, and enrichment cultures of ARHSd-7G were grown by injecting 0.3 ml samples into marine anaerobic media with pH 7 and 3 % NaCl. All tubes were incubated at 3 °C for 2 weeks. Pure cultures were obtained by the dilution method on a medium with D-glucose, and colony growth was achieved through the roll-tube method on a 3 % agar medium. For the cultivation of strain ARHSd-7G, a medium for neutrophilic bacteria was used and it contained (1^{-1}): NaCl, 5 g,; KCl, 0.3g,; KH_2PO_4, 0.3g,; $MgSO_4*7H_2O$, 0.1g,; NH_4Cl, 1.0g,; $CaSO_4*7H_2O$, 0.0125g,; $NaHCO_3$, 0.4g, (added after cooling); Na_2S*9H_2O, 0.4g, (added after cooling); resazurin, 0.0001g,; yeast extract, 0.1g, (added after cooling); D-glucose, 5.0g, (added after cooling); vitamin solution[26], 2ml,; and trace mineral solution[27], 1ml. Enrichment cultures of strain ARHSd-9G were obtained with a medium containing (1^{-1}): NaCl, 30 g,; Na_2CO_3, 2.76 g, (added after cooling); $NaHCO_3$, 24 g, (added after cooling); NH_4Cl, 1.0 g,; KCl, 0.2 g,; KH_2PO_4, 0.2 g,; $MgCl_2*6H_2O$, 0.1 g,; Na_2S*9H_2O, 0.4 g, (added after cooling); resazurin, 0.0001g,; yeast extract, 0.1g, (added after cooling); D-glucose, 5.0g, (added after cooling); vitamin solution[26], 2ml,; and trace mineral solution[27], 1ml. High-purity nitrogen was used as the gas phase. Cultures were incubated at 5-35°C, and substrates were added at a concentration of 3 g 1^{-1}.

Strain ISLP-3 was isolated from an ice sculpture in the vicinity of Lake Podprudnoye, Antarctica (also known as Proglacial Lake). A large field of "ice sculptures" formed by the summer melt and wind erosion processes was

discovered by the expedition vehicle, and these "ice sculptures" contained a large number of dark rocks and dust grains. Sunlight was able to penetrate the ice and results in localized melting that forms liquid water films around each of the ice enclosed rocks. These water films provide an ideal environment for the growth of photoautotrophic cyanobacteria, bacteria, diatoms, other algae, and organotrophic bacteria. Strain ISLP-3 was isolated from one of the ice sculptures, and the frozen sample was slowly melted in a sterile flask under a pure nitrogen atmosphere. The melted liquid (0.5 ml) was injected into a Hungate tube with anaerobic medium and incubated at 3 °C for 2-3 weeks. Pure cultures were obtained by the dilution method on a medium with D-glucose, and colony growth was achieved through the roll-tube method on a 3 % agar medium. The medium for the cultivation of the isolate contained (1^{-1}): NaCl, 10 g,; KCl, 0.3g,; KH_2PO_4, 0.3g,; $MgSO4*7H_2O$, 0.1g,; NH4Cl, 1.0g,; CaSO4*7H2O, 0.0125g,; NaHCO3, 0.4g, (added after cooling); Na2S*9H2O, 0.4g, (added after cooling); resazurin, 0.0001g,; yeast extract, 0.1g, (added after cooling); D-glucose, 5.0g, (added after cooling); vitamin solution[26], 2ml,; and trace mineral solution[27], 1ml. High-purity nitrogen was used as the gas phase, and cultures were incubated at 5-22°C. Substrates were added at a concentration of $3 \text{ g } 1^{-1}$.

The culture growth of all studied strains was estimated with a phase-contrast microscope (Fisher Micromaster) by direct cell counting, and with measurements of optical density at 510 nm on a spectrophotometer (Genesis 5; Spectronic Instruments, USA).

2.2 Morphological studies

The cell morphologies of the three strains were examined under a phase-contrast microscope (Fisher Micromaster), and the microstructures of the cells' surfaces were examined using a Hitachi S-4000 field-emission scanning electron microscope (not shown).

2.3 Metabolic properties

Dependence on Na+ ions for each strain was checked by replacing sodium salts with potassium salts. Each strain was exposed to differing amounts of NaCl in order to determine the optimal percentage of salinity for growth. The isolates were also grown on medium with varying pH values and incubated at differing temperatures to determine the optimal pH and temperature for cell growth. Lastly, the isolates were tested for their ability to grow on a variety of substrates, or donors of electrons, including sugars, proteolysis products, organic acids, proteins, and alcohols.

2.4 Antibiotic susceptibility

Antibiotic inhibition was determined at concentrations of 250 $\mu g \text{ ml}^{-1}$ for ampicillin, kanamycin, gentamycin, rifampicin, tetracycline; and 100 $\mu g \text{ ml}^{-1}$ for chloramphenicol.

3. RESULTS

3.1 Colonies and cell morphology

The colonies of strains ARHSd-7G and ARHSd9G are cream in color with dense centers. Both strains produce raised, circular colonies with ARHSd-7G colonies ranging from 1-1.5 mm in diameter and ARHSd-9G colonies ranging from 3–5 μm in diameter. There is also a clearing around the colonies. The isolate ISLP-3 possess cream colored, smooth, and glossy colonies circular in shape and 1-3 mm in diameter. These colonies also are encompassed by a clearing and possess a convex, raised quality.

Cells of strain ARHSd-7G are motile vibrions with sizes 0.6x1.2-2.4 μm (Figure 3b). They can be observed as single cells, as pairs, and as long, irregular chains. Gram-stained cells exhibit fuchsia and pink shading indicating that the organism is gram-variable. Strain ARHSd-7G is a sporulating organism, as it forms spores when nutrients are underprovided. Cells of strain ARHSd-9G exhibit elongated straight or slightly curved rods that are motile by peretrichous flagella (Figure 3a). ARHSd-9G is spore-forming, and the spores are located terminally. Cells of strain ISLP-3 exhibit a "baseball" shape with abnormal configurations due to a tendency to form clumps of cells through the excretion of a mucopolysaccharide (Figure 4). Cells occur in pairs and are slightly angled, and at times, Y-shape cells may be observed.
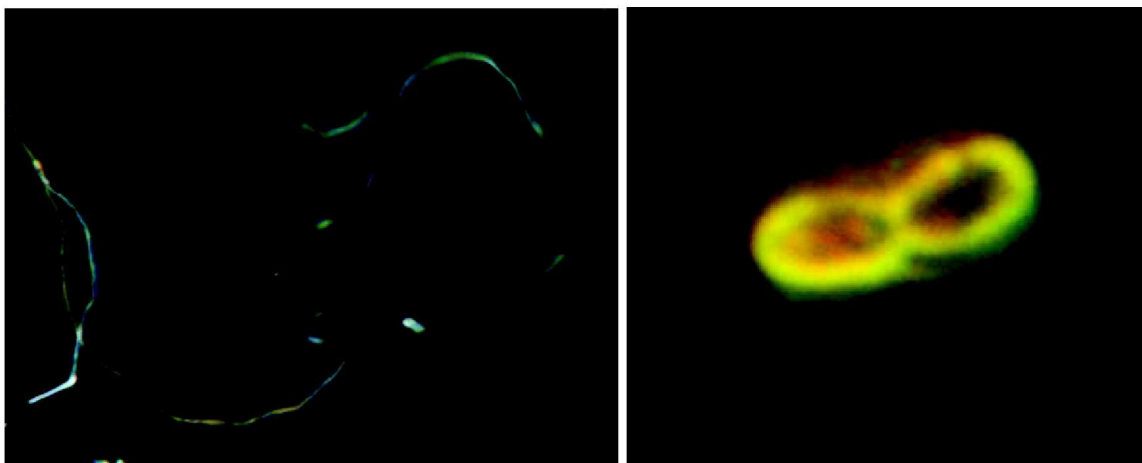
Fig. 3a,b: a. Cell morphology of strain ARHSd-9G; b. Dividing cells of strain ARHSd-7G.

3.2   Metabolic properties

The isolate ARHSd-7G is an obligate anaerobe that grows exclusively under anaerobic conditions. The strain is catalase negative and dependent on Na+ ions for growth. Optimum growth is observed at 3 % (w/v) NaCl, and the organism grows at a range of 0.5-24.0 % (w/v) NaCl (Figure 7). No growth is observed at 0.1% NaCl. Strain ARHSd-7G grows at a temperature range of 3-40 °C with an optimum growth temperature of 30°C (Figure 5). The range of pH upon which the strain is able to grow is 5.2 – 10, with an optimum at pH 8.9 (Figure 6). The organism is capable of growth on the following substrates: D-glucose, maltose, D-ribose, D-fructose, D-trehalose, D-cellobiose, sucrose, D-mannitol, yeast extract, peptone, casamino acids, pyruvate, and triethylamine. No growth is found on D-mannose, D-arabinose, L-arabinose, lactose, starch, acetate, lactic acid, butyrate, propionate, citrate, Na+ formate, trimethylamine, betamine, methanol, ethanol, glycerine, or acetone. The strain metabolized monomeric and bimeric sugars and products of proteolysis, thus it was determined to be a sugarlytic and proteolytic organism. ARHSd-7G has a strictly fermentative metabolism determined as the addition of any acceptors of electrons (Fe^{3+}, NO_3^{2-}, SO_4^{2-}, SO_3^{2-}, S^o, $S_2O_4^{2-}$) did not stimulate growth. Also, the isolate is unable to reduce sulfur compounds to H_2S.
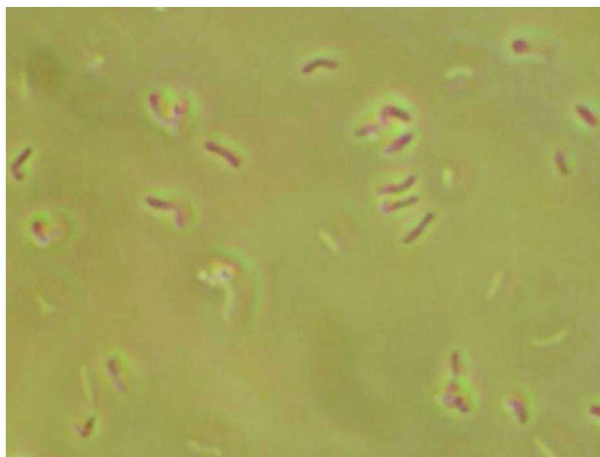
Fig. 4. Cell morphology of strain ISLP-3.

The isolate ARHSd-9G is catalase negative and dependent on Na+ ions for growth. It grows at a range of 0.25-15.0 % NaCl with the optimal growth observed at 1.75 % NaCl (Figure 8). Strain ARHSd-9G grows at an optimal temperature of 30°C and at a range from 5 - 40°C (Figure 10). The range of pH upon which the organism is able to grow is from 6.5-10.0, with an optimum at pH 8.5 (Figure 9). The strain grows well on the following substrates: D-glucose, maltose, D-ribose, D-fructose, D-trehalose, D-cellobiose, sucrose, D-mannitol, D-mannose, D-arabinose, lactose, and N-

acetylglucosamine. No growth was observed on formate, acetate, pyruvate, lactate, propionate, butyrate, citrate, oxylate, methanol, ethanol, glycerol, acetone, betaine, trimethylamine, triethylamine, peptone, yeast extract, casamino acids, starch, or urea.

Strain ISLP-3 is a catalase negative, obligately anaerobic microorganism. This strain is psychrotolerant and grows from a range of 5 - 35°C with an optimum of 22 °C (Figure 13). The organism does not need NaCl in its medium for growth. It does have an optimum of 0.25 % NaCl in the presence of NaCl with a range of growth from 0–2 % (w/v) (Figure 11). The range of pH for growth occurs between 6.0 and 9.5, with the optimum at 7.5 (Figure 12). ISLP-3 is able to grow on the subsequent compounds: triethylamine, chitin, N-acetylglucosamine, urea, *D*- glucose, *D*-arabinose, *D*-trehalose, maltose, sucrose, *D*-ribose, *D*-mannose, lactose, starch, and *D*-cellobiose. The best growth is observed on *D*-cellobiose. Growth was never observed on the following substrates: formate, acetate, pyruvate, lactate, propionate, butyrate, citrate, oxalate, methanol, ethanol, glycerol, *D*-mannitol, acetone, betaine, trimethylamine, peptone, yeast extract, casamino acids, pectin, and *D*-fructose.

3.3 Antibiotic susceptibility

Strains ARHSd-7G and ISLP-3 was sensitive to all checked antibiotics. Strain ARHSd-9G was resistant to ampicillin, kanamycin, gentamycin, rifampicin, and chloramphenicol, but sensitive to tetracycline.
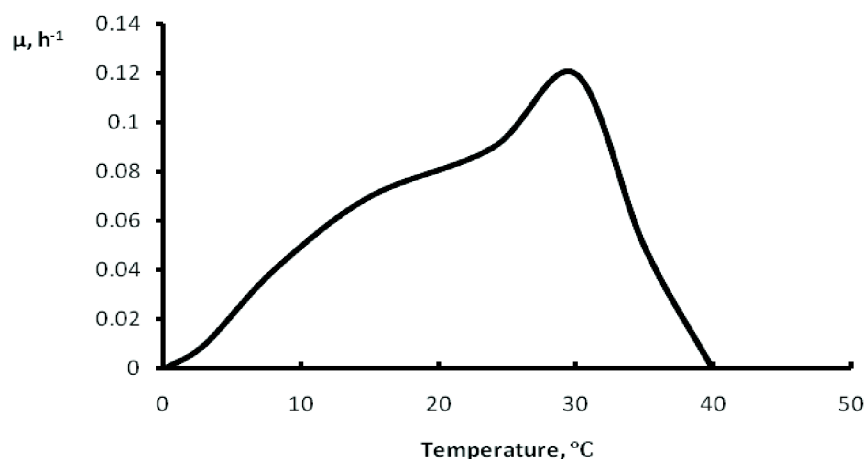
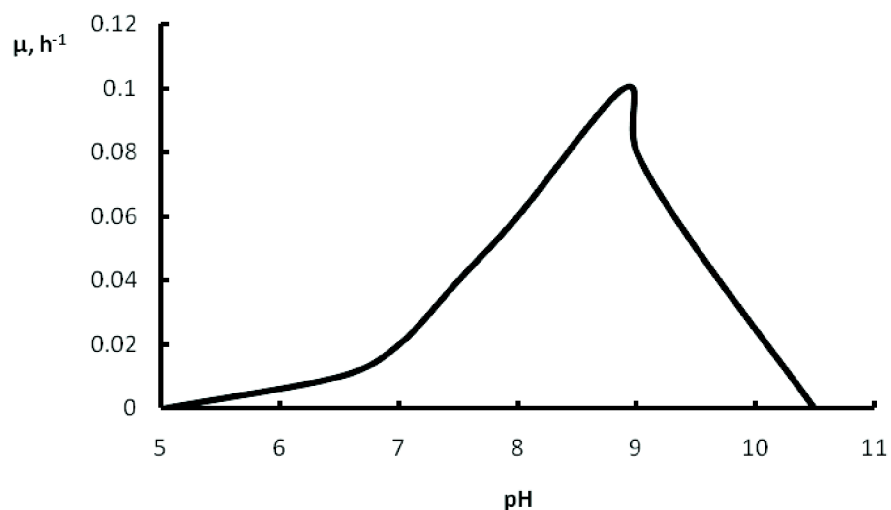Fig. 5. The growth of strain ARHSd-7G in dependence upon temperature.

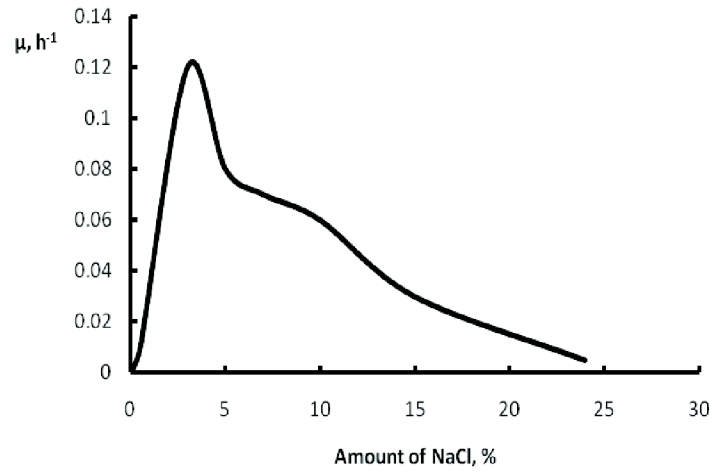Fig. 6. The growth of strain ARHSd-7G in dependence upon pH.

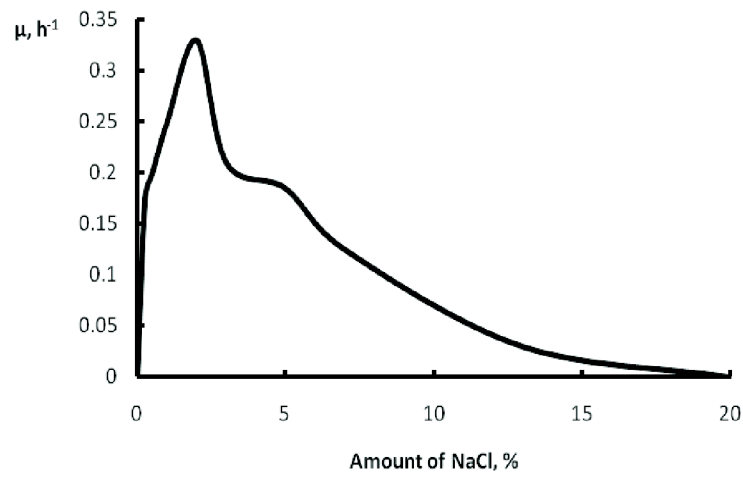Fig. 7. The growth of strain ARHSd-7G in dependence upon NaCl (w/v).

Fig. 8. The growth of strain ARHSd-9G in dependence upon NaCl.
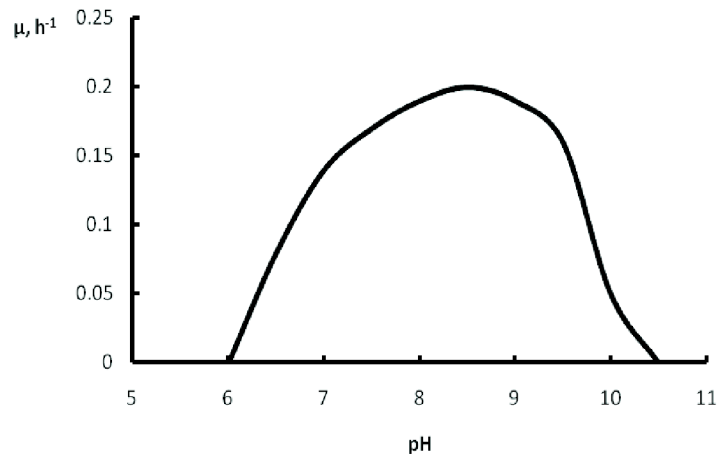

Fig. 9. The growth of strain ARHSd-9G in dependence upon pH.
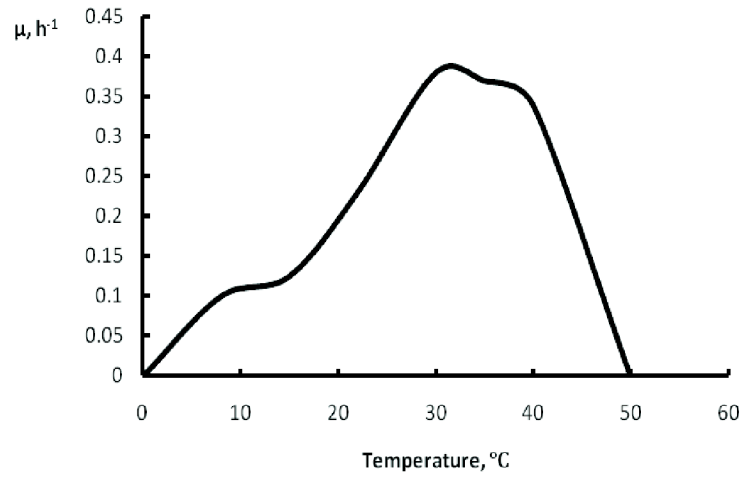
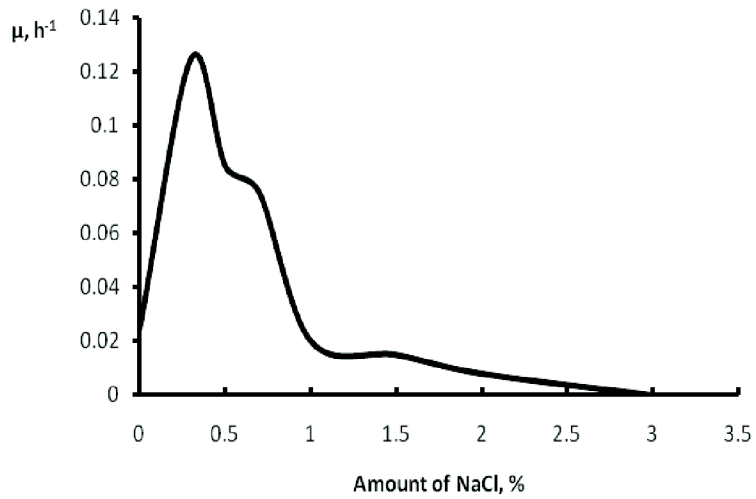Fig. 10. The growth of strain ARHSd-9G in dependence upon temperature.

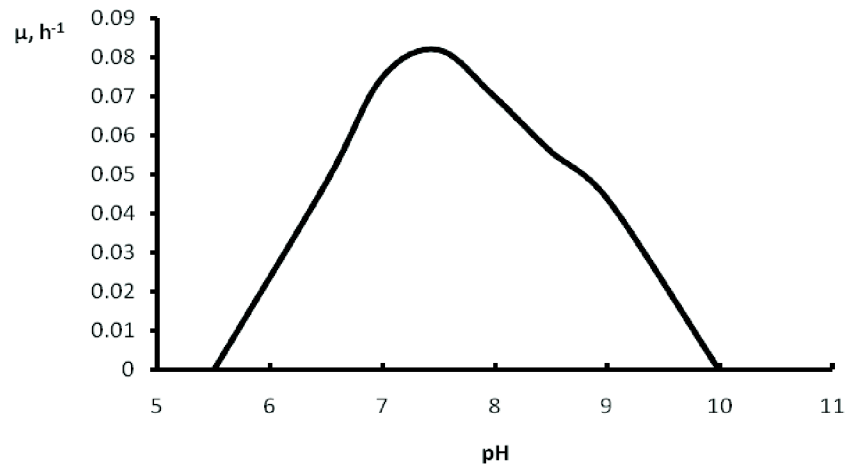Fig. 11. The growth of strain ISLP-3 in dependence upon NaCl.

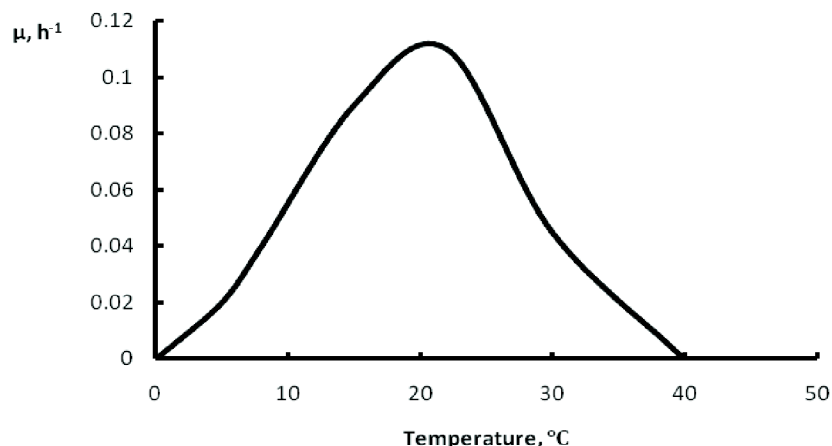Fig. 12. The growth of strain ISLP-3 in dependence upon pH.

Fig. 13. The growth of strain ISLP-3 in dependence upon temperature.

4. CONCLUSIONS

The conducted research showed presence of many diverse anaerobic microorganisms in the samples of studied the ecosystem. New isolated strains showed psychrotolerant nature and tolerant to high pH on anaerobic media. All of these strains are sugarlytic that are able to grow on other substrates except for the strain ISLP-3 which is strictly sugarlytic. Interesting results of the antibiotic susceptibility experiment need to be studied in detail in the future due to the isolation of plasmids that could be responsible for this feature. Also, the high salinity tolerance of strains ARHSd-7G and ARHSd-9G need to be studied in detail in accordance to better understand the role of these strains within *in-situ* ecology.

New isolates represent obvious interest in biotechnology since they are carriers of new biomolecules with unique properties. This research has impact on fundamental science such as biodiversity of Antarctica and other extreme ecosystems.

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