

Isolation and Characterization of Hyperthermophilic Nanobacteria from a Hot Spring in Ardabil, Iran

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ABSTRACT

Background and Objective: Nanobacteria are nanometer-scale particles with different shapes, which have been a subject of debate in modern microbiology. They belong to a proposed class of living organisms, specifically cell-walled microorganisms with a size much smaller than the generally accepted lower limit for life. Since some microorganisms are able to continue growth at high temperatures, we aimed to isolate thermophilic bacteria from Gheinarcheh hot spring in Ardabil (Iran) and identify the characteristics of these microorganisms.

Methods: Microbial mats were found in cultures from Gheinarcheh hot spring in North West of Iran. Synthetic media were prepared and used for isolation of protease-producing thermophilic bacteria, and identification of the features of microbial mats. Cultures were incubated at temperature range of 60-120 oC. Scanning electron microscopy, DNA extraction and polymerase chain reaction were used to further identify characteristics of the microbial biofilms.

Results: Microbial biofilms of nanoparticles were detected in our samples. Growth of the bacteria increased at all temperatures tested. Results of scanning electron microscopy showed nano-sized particles in the scale of 60 nm. No band was visible in gel electrophoresis of polymerase chain reaction products.

Conclusion: This study is the first to report the presence of hyperthermophilic nanobacteria in Iran.

Keywords: Nanobacteria, Hyperthermophile, Microbial Mat.

INTRODUCTION

Nanobacteria are nanometer-scale circular and ovoid particles that have received increasing interest in modern microbiology (1). They belong to a main class of living organisms, specifically cell-walled microorganisms with a size much smaller than the usually accepted lower limit for life (2). Nanobacteria are abundant in normal marine limestones and other environments such as continental fresh-water streams, springs, water pipes and caves (3). Their size varies from 50 to 200 nm in diameter (4). They are thought to be involved in formation of kidney stones and arterial plaques (5-9).

A bold theory suggested the existence of nanobacteria as a new life form responsible for a wide range of diseases in humans (3, 10, 11). Nanobacteria are strange particles that have been the subject of debate in contemporary microbiology (1, 12). These particles were first discovered by geologists as 100 nm spherical fragments on mineral surfaces (1, 11), but later identified in human and cow blood, and in several cell culture serums (11).

This study aimed to identify cells present in microbial mat found in Gheinarcheh hot spring in Ardabil, Iran.

MATERIAL AND METHODS

Water and soil sediment samples were collected from different parts of the Gheinarcheh hot spring in Ardabil, Iran (Latitude: 38° 16' 1.3012", Longitude: E 47° 48' 35.1123"). The samples were transferred to laboratory within 2 hours and cultured to isolate possible thermophile species. Several culture media including Muller Hinton agar, nutrient agar, Luria-Bertani broth and six synthetic media that were designed by the authors (media A, B, C, D, E and F) were used in this study.

Synthetic medium A contained 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 0.5 g/l glucose, 0.4 g/l Na₂HPO₄, 0.085 g/l Na₂CO₃, 0.02 g/l ZnSO₄, 0.02 g/l CaCl₂ and 0.02 g/l MgSO₄. Synthetic medium B contained 2% (w/v) ammonium nitrate, 1% (w/v) glucose, 0.4 g/l Na₂HPO₄, 0.085 g/l Na₂CO₃, 0.02 g/l ZnSO₄, 0.02 g/l CaCl₂ and 0.02 g/l MgSO₄. Synthetic medium C contained the same ingredients as medium B except glucose was replaced with sucrose. Synthetic medium D, E and F contained the same ingredients as medium B but D is without ammonium nitrate

or any other nitrogen resource, E is without carbon sources and F included sodium acetate instead of ammonium nitrate.

In order to evaluate microbial mat formation at different temperatures, different media were used and incubated or autoclaved at temperatures ranging from 60 to 120°C. The optimal pH for growth of biofilms was by incubation in synthetic medium B at 95°C. In addition, the pH of media was brought to the initial value (pH range 1.0–13.0, with pH increments of 1.0) at room temperature using HCl or NaOH, and then the media were sterilized by filter papers.

After Gram staining, morphology was examined under light microscope and scanning electronic microscope (SEM). In order to prepare specimens for SEM analysis, the cells were cultured in medium B for 24 hours at 95°C within serum glass bottles. After preparing microscope slides, sticky microbial mats appeared on inner surface of the glass and slides. Inner surface of serum glass bottles was fixed at room temperature for 4 hours using 3% glutaraldehyde in 0.1 M phosphate buffer. After washing with 0.1 M phosphate buffer (pH=7.2 - (3 x 10min), the samples were dehydrated using graded ethanol or acetone solutions with the concentration of 30%, 50%, 70%, 80%, 90%, 96%, 100% for 5-15 min. A chemical drying agent, hexamethyldisilazane (HMDS) was applied instead of critical point drying. Samples were prepared by mixing 100% ethanol into a 1:2 solution of HMDS. Samples were kept in 100% ethanol for 20 minutes, and then transferred to a fresh 1:2 HMDS: ethanol solution for 20 minutes. The glasses were kept in 100% HMDS for 20 minutes, and then the last step was repeated. After metal coating, the samples were analyzed under SEM (TESCAN Co., Czech Republic).

Cells were disrupted by physical and chemical methods. Ultrasonication, freeze/thaw cycles and grinding in liquid nitrogen were carried out as the physical methods. Sodium dodecyl sulfate, lysosome, ethylenediaminetetraacetic acid, ethanol and Triton X-100 were used in the chemical method. The samples were characterized by dsDNA absorption and gel electrophoresis.

Chromosomal DNA was segregated from 4g of frozen cells using the Seigo SHIMA

Method(13). Amplification was done using polymerase chain reaction (PCR) with five universal primers (three primers for 16 srDNA bacteria, one primer for archaeal 16S rDNA and one primer for haloarchaeal community.(Considering the extremely small size of bacteria, serial dilution was prepared to obtain reasonable counts. A thirty-fold serial dilution (1 M, 0.1 M, 0.01 M, 0.001 M...) was prepared to create accurate highly diluted solutions. Effect of ethidium bromide on the microbial mats and E.coli was evaluated and compared.

RESULTS

Morphological and molecular examination of microbial biofilms present in the media was performed. Reproduction of microbial mats (containing microbial cells) in each medium was studied. Formation of biofilm was only observed in the synthetic medium B.

Gram staining and light microscopy
Light microscopy displayed very delicate, superfine and transparent biofilms. Results of Gram staining showed the biofilms resemble the properties of Gram-negative bacteria (Figure 1).

Figure 1- Result of Gram staining of nanobacteria within biofilms under light microscope (100X magnification)

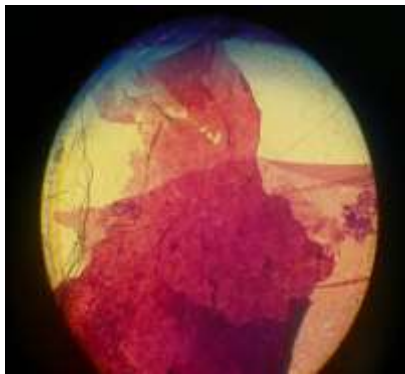
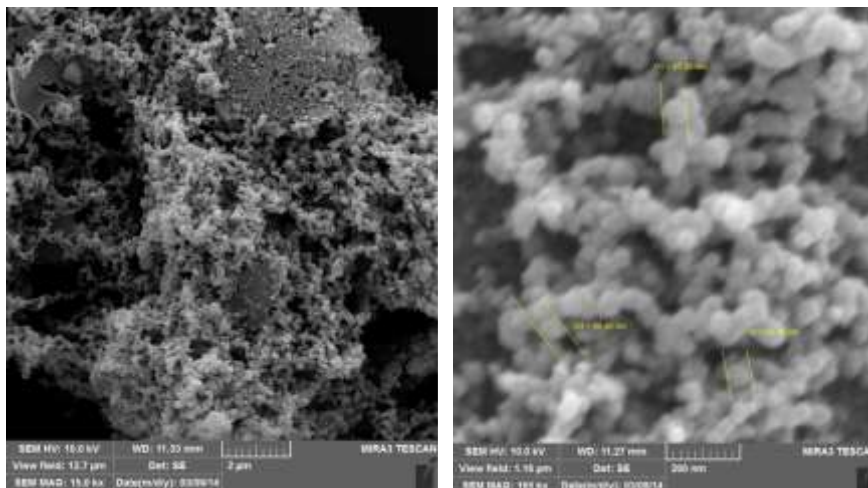


Figure 2- SEM micrograph of nanobacteria with a small size (scale bar 60 nm) and cocci-shaped morphology



SEM analysis showed the presence of coccoid nanobacteria. Size of the cells was determined to be about 60 nm (Figure2).

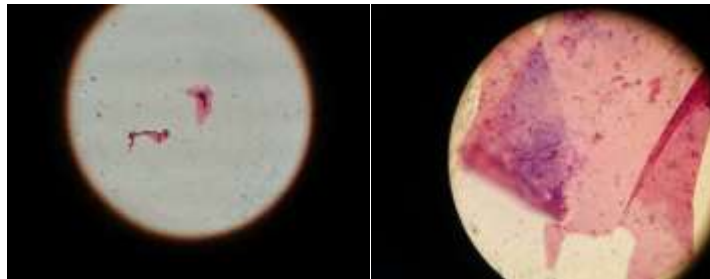
Growth under different temperatures

The rate and velocity of growth correlated to the temperature increments. The temperature increment increased from 60°C to 95 °C, with

10 °C increase in autoclave. Increasing the temperature increased the growth rate so that the microbial mat mass increased significantly after 4 hours of autoclaving (Figure3).

The microbial cells were active at various pH values (1.0 to 13.0). The optimal pH for the growth and cellular activity was in the 7.0-13.0 range (Figure4).

Figure 3- Effect of incubation of samples in pH 1 (a) (10X magnification) and in pH 13 (b) (100X magnification). Signs of microbial mat growth were evident after Gram staining and in light microscopy.



DISCUSSION

Nanobacteria are present in almost every environment ranging from freshwater springs to thermal springs. Although there has been increasing interest in finding Nano-sized microorganisms in the past few years, only a limited number of studies are available on thermophilic nanobacteria. A study in 1985, reported the presence of very small microorganisms in microbial mat of an alpine thermal spring(3, 14). More recent studies confirmed the presence of nanobacteria with different shapes in biofilms from a hot spring in Austria with temperature of 45.6 °C (15, 16). The nanobacteria found in the mentioned studies were spherical, filamentous and rod-shaped, and in different sizes (17). No study has yet reported the presence of hyperthermophilic nanobacteria. In this study, we found a spherical Nano-sized bacterium, which was able to survive autoclaving, and form microbial mat even at dilution of 10-24. However, more studies are required to

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evaluate the characteristics of these microorganisms.

CONCLUSION

Nano bacterial cells (size range 30-300 nm) are abundant in a wide spectrum of carbonate precipitating environments ranging from hypersaline environments to normal marine, subaerial caliches, speleothems, birdbath scum and pipe scale. They can be isolated from seawater, fresh water, well water, and even human body.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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