AXENIC CULTURE OF A DIAZOTROPHIC FILAMENTOUS *CYANOBACTERIUM* ISOLATED FROM MESOTHERMAL SULPHUROUS SPRINGS (OBANUL MARE – MANGALIA)

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Isolation and purification of cyanobacteria from ecosystems is needed to obtain cultures of cyanobacteria which can be of use for laboratory studies in fundamental and applicative research, and to improve the knowledge concerning microbiota of the given ecosystem. This study presents the results concerning the purification in axenic culture of a diazotrophic filamentous cyanobacterium isolated from sulphurous mesothermal spring. The purification of cyanobacteria has been done by the use of antibiotic treatment, with special emphasis on the timing between organic nutrients addition and antibiotic addition.

Key words: cyanobacteria, axenic culture, antibiotics, epifluorescence.

INTRODUCTION

Axenic (bacteria-free) cultures of cyanobacteria are usually obtained by single-cell isolation, density gradient centrifugation and rinsing (Vaara *et al*., 1979; Bolch and Blackburn, 1996), UV irradiation, filtration, or treatment with antibiotics (Rippka, 1988; Castenholz, 1989; Choi *et al*., 2007), and other germicidal chemicals (Kim *et al*., 1999, Bolch and Blackburn, 1996; Connell and Cattolico, 1996; Watanabe *et al*., 1998; Kim *et al*., 1999; Vázquez-Martínez *et al*., 2004).

The aim of this paper is to isolate a heterocystous cyanobacterium from mesothermal sulphurous spring (Obanul Mare-Mangalia) in axenic culture and to check the purity both by culture dependent and culture-independent classical methods.

MATERIALS AND METHODS

Study area and sampling. Samples were collected in October 2008 and May 2009 from sulphurous mesothermal spring (Obanul Mare) placed near Mangalia City (43˚49’53.6’’N; 28˚34’05.3’’E). The samples collected in sterile bottles were divided into sub-samples, one being immediately fixed with buffered formaldehyde (2% final concentration) and the second one used to isolate cyanobacteria by inoculation into conical flasks with either BG_{11} medium or nitrate-free BG_{11} medium (BG_0) (Rippka *et al*., 1979).

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Culture conditions. Natural samples inoculated in either BG_{11} or BG_0 media, either solid or liquid, were incubated in culture room at 25 ± 1º C and illuminated with fluorescent tubes having the photon rate of 50 μmol $m^{-2}s^{-1}$ at surface of the culture vessels.

Methods to eliminate heterotrophic bacteria and eukaryote microorganisms from diazotropic cultures of cyanobacteria. We used the classical antibiotic treatment to eliminate heterotrophic bacteria from cyanobacteria cultures (Carmichael and Gorham, 1974; Bolch and Blackburn, 1996; Kim *et al*., 1999; Choi *et al*., 2007; Vázquez – Martínez *et al*., 2004) with special emphasis on the timing between organic nutrients addition and antibiotic addition (see results). The antibiotics are the following, together with their final concentrations in the growing vessels: tienam IV (imipenem 500 mg/cilastatin 500 mg) – 100 μg/mL; cefalexin – 10 μg/mL and nalidixic acid – 10 μg/mL. Up to our best knowledge, for the first time we used augmentin (875 mg amoxiciline, 125 mg clavulanic acid) – 10 μg/mL, in the atempt to obtain axenic cultures of cyanobacteria. In order to eliminate eukaryotes we used cycloheximide – 20 μg/mL (Rippka, 1988).

Test for purity of filamentous cyanobacteria. Bacterial contamination of treated samples of cyanobacterial cultures was tested by cultivation-dependent and – independent methods. For cultivation-dependent methods the antibiotic-treated samples of cyanobacterial culture were diluted appropriately, pipetted onto sterile Petri dishes, mixed with molten but cool solid Luria-Bertani (LB) medium (0.1 mL of diluted solution per 10 mL of medium) and then allowed to harden. Alternatively, the diluted cultures (10 μL were placed on the surface of the hardened, solid medium in the Petri dishes and spread over the medium with a bent glass rod. These plates were sealed with Parafilm and incubated at 30°C for 24 hours (or more).

For cultivation-independent methods the antibiotic-treated samples of cyanobacterial cultures were centrifugated (10 minutes at 1,000 g) in order to pellet cyanobacterial filaments and to obtain the heterotrophic, nonfilamentous contaminants in the supernatant. The supernatant was treated with the fluorochrome acridine orange, filtered through Millipore filters (0.22 μm) and inspected with an epifluorescence microscope, following the previously shown protocol (Ardelean *et al*., 2009) adapted from literature (Sherr *et al*., 2001).

The microphotographs taken with a Canon digital camera (PowerShot A420, 4.0 megapixels) were used for automated image analysis and heterotrophic cells enumerations (Selinummi *et al*., 2005), CellC being an easy-to-use automated image analysis software, which allows the analysis of multiple digital microscope images (http://www.cs.tut.fi/sgn/csb/cellc/). Samples labelled with AO (0.01%) were visualized with epifluorescence microscopy for counting the number of heterotrophic bacteria with CellC software and assess the validity of CellC enumeration by comparison with manual counting results (Ardelean *et al*., 2009 and Selinummi *et al*., 2005). During this procedure, CellC can do the segmentation

and the analysis of the Total Count image, and then, the segmentation of cells from the background and extraction of individual cell clusters (Selinummi *et al.*, 2005). At first, the brightness variation from the image background was corrected because of uneven illumination and for background autofluorescence; second, the cell pixels are separated from background pixels by global thresholding, producing a binarized image with white cells on a black background. Clustered cells are then separated from each other by marker-controlled watershed segmentation that is based on cell shape.

RESULTS AND DISCUSSION

Microorganisms in the samples collected from sulphurous mesothermal spring. As one can see in Figure 1 there are different morphological types of microorganisms presented in natural samples, some of them being photosynthetic ones as shown by transmission light microscopy (Fig. 1A, B and C) or by epifluorescence (Fig. 1D).

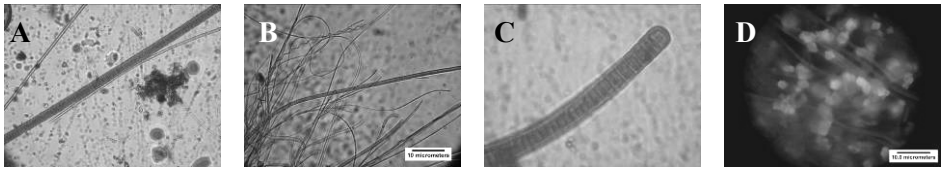

Fig. 1. Microorganisms in sample from sulphurous mesothermal spring as viewed by transmission light microscopy (A, B), basic fuxine coloration (C) and natural chlorophyll fluorescence (D).

Isolation of a heterocystous cyanobacterium by selective cultivation and passages on BG_0 media. In Figure 2 we showed the macropscopic aspect of culture on Petri dishes after 2 months of cultivation at 25ºC on continuous illumination (Fig. 2A) and also the microscopic aspects of heterocystous cyanobacteria (Fig. 2B, C, D) isolated by selective cultivation on BG_0, either liquid or solidified.

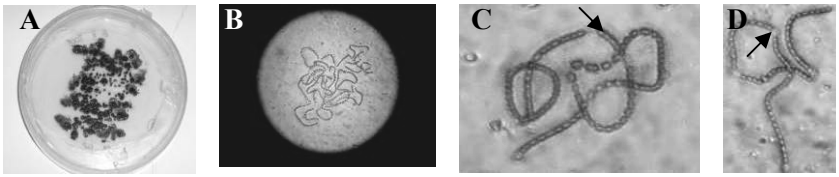

Fig. 2. The macroscopic aspect of cyanobacterium culture on BG_0 media after 2 months of cultivation (A) and the microscopic aspects of heterocystous cyanobacteria isolated from culture (B, C, D – fuxine basic coloration); the arrows indicate the presence of heterocysts.

Visualisation of heterotrophic contaminants present in the cyanobacterial culture before antibiotic treatment. We investrigated the cyanobacterial culture by epifluorescence microscopy using acridine orange to find the heterotrophic contaminants occurring before antibiotic treatment. The presence of a great number of heterotrophs through the filamentous cyanobacteria is illustrated in Figure 3.

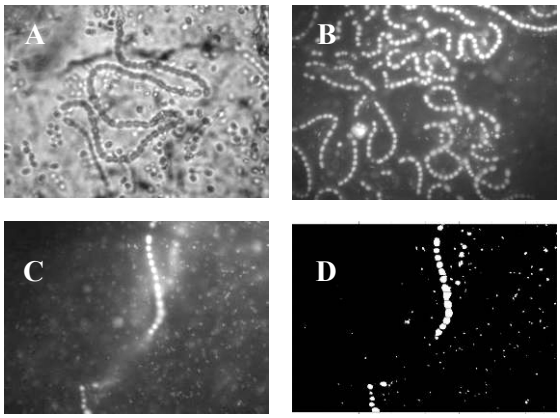

Fig. 3. The abundance of heterotrophic contaminants present in cyanobacterial culture visualized by light microscopy (A) and by epifluorescence microscopy using AO stain (B, C); digital image analysis of panel C (both cyanobacterial cells and heterotrophic bacteria) using CellC software (D).

Antibiotic treatment and testing the purity of cyanobacteria culture. In order to obtain axenic culture of diazotrophic cyanobacteria, the culture (20 mL) was incubated 24 hours in darkness at 30 ºC in liquid BG_0 supplemented with antibiotic and LB. Following this dark incubation the culture was washed with sterile BG_0 media and further incubated 24 hours in light at 25 ºC in BG_0. This overall treatment is one cycle of purification.

The rationale of antibiotic treatment (Carmichael and Gorham, 1974; Bolch and Blackburn, 1996; Kim *et al*., 1999; Vázquez-Martínez *et al*., 2004; Choi *et al*., 2007) is that in darkness, in the presence of (even limited amount of) organic carbon and nitrogen sources, as well as vitamins, the cellular growth of heterotrophic bacteria is promoted, these bacteria being more sensitive against different types of anitbiotics than bacteria which are not during cellular growth (as should be the case of photosynthetically-grown cyanobacteria in darkness). In light, in the absence of the antibiotic and organic resources, the growth of cyanobacteria is promoted. After one cycle of treatment with the four antibiotics, the heterotrophs still have a significant presence (results not shown).

In order to try to completely eliminate the heterotrophic contaminants, the antibiotic method was modified simply by incubating the culture for 60 minutes in the presence of the organic substrates (1% or 10% LB in BG_0), antibiotic being

added afterwards. The rationale of this attempt is based on recent findings which show that some bacteria are able to communicate within the same species (population), (Dunny *et al*., 1978; Clewell and Weaver, 1989) and can react to the presence of an antibiotic in its growing medium by significant changes at cellular and molecular level (Prudhomme *et al*., 2006; Graumann, 2006; Francia *et al*., 2007), changes which should protect the population against that antibiotic (Dubnau and Losick, 2006). According to our working hypothesis, it is expected that adding the antibiotic after the organic substances would reduce the chances of growing cells within bacterial populations to communicate within the same species (population) in order to develop those mechanisms which should protect the population against that antibiotic.

Following the above treatment, with 60 minutes incubation in darkness in the presence of organic compounds (1% or 10% LB in BG_0) before the addition of antibiotics, the cyanobacterial samples were tested for purity by culture dependent method. The supernatants collected from the culture treated with antibiotics were inoculated into solid test media (LB) and inspected to distinguish macroscopic colonies of heterotrophs. With this improved method, after three cycles, axenic cultures were obtained only using tienam or augmentin, no heterotrophic colony being apparent after 24 hour of incubation on LB in both cases (Table 1).

The decrease in number of heterotrophic bacteria per milliliter in cultures of the isolated heterocystous cyanobacterium, during three cycles of antibiotic treatment (cell densities after 1st and 2nd cycles were measured by growth independent method (epifluorescence) and the last one (3rd cycle) by growing dependent method-colony counting after 24 hours of incubation on LB media).

Table 1

Antibiotic	**Number of heterotrophs (per mL) after 1st cycle of purification**	**Number of heterotrophs (per mL) after 2nd cycle of purification**	**Number of heterotrophs (per mL) after 3rd cycle of purification**
Tienam	15.340	5.120	0
Augmentin	12.600	7.250	0

The use of improved method with the other two antibiotics (cefalexin and nalidixic acid) reduced the number of heterotrophs in different amounts (results not shown), but no true axenic cyanobacterial culture was obtained.

The greater capacity of imipenem from tienam composition to reduce the number of contaminant bacteria and the ability of cyanobacteria to tolerate incubation with it in the dark appear to make imipenem superior to other β-lactam antibiotics which have been used in an attempt to generate axenic cultures of cyanobacteria. The very broad antibacterial spectrum of imipenem may allow it to be more generally useful in eliminating a wide range of different heterotrophic

bacteria which may be encountered in efforts to produce axenic cyanobacteria (Ferris and Hirsch, 1999).

CONCLUSIONS

A heterocyst-forming cyanobacterium was isolated in axenic culture by the improved antibiotic method using tienam and augmentin, the last one being used, to our best knowledge, to obtain axenic culture of cyanobacteria for the first time. The main contribution of this paper is based on the fact that the classical antibiotic method has been improved by promoting the growth of heterotrophic bacteria in the absence of antibiotic, which was added after one hour.

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