IMPROVED LYSOZYME METHOD TO OBTAIN CYANOBACTERIA IN AXENIC CULTURES

I. SARCHIZIAN [1], I.I. ARDELEAN [2]

Isolation of cyanobacteria from natural environments is needed to obtain axenic cultures of cyanobacteria which can be mainly used for studies in fundamental and applicative researches. The aim of this study is to present the results concerning the purification from sulphurous mesothermal spring of cyanobacteria in axenic culture by an improved lysozyme method which uses a second antibiotic, tienam, added after organic nutrients.

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

INTRODUCTION

Cyanobacteria have been found to inhabit a wide variety of places with a high degree of success; they possess many physiological characteristics that are able to tolerate some of the most extreme conditions (Van Den Hoek *et al.*, 1995). They are a large and morphologically diverse group of phototrophic prokaryotes, which occur in almost every habitat on earth. The cosmopolitan distribution of cyanobacteria indicates that they can cope with a wide spectrum of global environmental stresses such as heat, cold, desiccation, salinity, etc. (Bhadauriya *et al*., 2008). Although several methods to obtain axenic cultures of cyanobacteria have been suggested (Ferris and Hirsch., 1991, Bolch and Blackburn, 1996; Connell and Cattolico, 1996; Watanabe *et al.*, 1998; Kim *et al.*, 1999; Choi *et al*., 2007), heavily contaminated material, such as field-collected samples, are still very difficult to detach from contaminants. 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, 1988; Choi *et al*., 2007), and other germicidal chemicals (Bolch and Blackburn, 1996; Connell and Cattolico, 1996; Watanabe *et al.*, 1998; Kim *et al*., 1999; Vázquez-Martínez *et al*., 2004).

This study deals with the isolation and purification cyanobacteria from mesothermal sulphurous spring in axenic culture using classical lysozyme treatment (Kim *et al*., 1999) improved by addition of organic nutrients to promote

[1] Ovidius University, Constanța 900470
[2] Institute of Biology, 060031 Bucharest

the growth of heterotrophic contaminants before the addition of tienam. The purity of these two cultures was checked both by culture-dependent and culture-independent classical methods.

MATERIAL AND METHODS

Study area and sampling. Cyanobacterial samples with contaminants were collected in May 2009 from sulphurous mesothermal spring (Obanul Mare) placed near Mangalia City (43˚49'53.6''N; 28˚34'05.3''E) (Fig. 1). The samples collected in sterile bottles were used to isolate cyanobacteria, by dilution and inoculation into conical flasks with either BG_{11} media with different value of pH (Rippka *et al*., 1979) or nitrate-free BG_{11} media (BG_0) to isolate nitrogen fixing cyanobacteria, heterocystous or not.

Fig. 1. The satelitary map of mesothermal sulphurous spring Obanul Mare (43˚49'53.6''N; 28˚34'05.3''E), placed near Mangalia Marsh – collection of cyanobacterial samples (www.GoogleEarth.com).

Culture conditions. Natural samples, inoculated in either BG_{11} or BG_0 liquid media, were incubated in culture room for 10 weeks at 25 ± 2ºC and illuminated with fluorescent tubes having the photon rate of 50 µmol $m^{-2}s^{-1}$ at the surface of culture vessels. Enrichment cultures were obtained using either BG_0 liquid or solidified media, BG_{11} with higher pH (pH = 9,6 and pH = 8,6) or supplemented with natrium sulphide to eliminate aerobic bacteria and enrich facultative anoxigenic cyanobacteria.

Purification was done using the classical lysozyme treatment to eliminate heterotrophic bacteria from cyanobacterial cultures (Kim *et al*., 1999) taking the advantage of other antibiotic treatment (Carmichael and Gorham, 1974; Bolch and Blackburn, 1996; Vazquez-Martınez *et al.*, 2004; Choi *et al*., 2007; Sarchizian and Ardelean, 2010), with special emphasis on the fact that organic nutrients have been added before the second antibiotic (tienam). The antibiotics used in the growing

vessels for our study are: lysozyme – 20 μg/ml final concentration; tienam IV (imipenem 500 mg/cilastatin 500 mg) – 100 μg/ml final concentration. The greater capacity of imipenem within 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 to generate axenic cultures of cyanobacteria (Choi *et al*., 2007; Sarchizian and Ardelean, 2010).

In the atempt to obtain axenic cultures of cyanobacteria and to totally eliminate eukaryotes from the cultures we also used cycloheximide – 20 μg/ml (Rippka, 1988).

Testing the purity of the culture. For cultivation-dependent methods cyanobacterial cultures were diluted appropriately, transferred in sterile Petri dishes, mixed with solid Luria-Bertani medium (0.1 ml of diluted solution per 10 ml of medium) and then allowed to harden (pour plates). Alternatively, the diluted cultures (30 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 (spread plates). These plates were sealed with Parafilm and incubated under white fluorescent illumination of 50 μmol $m^{-2}s^{-1}$ at the surface of culture vessels, in an inverted position at 30 °C for 7 days.

For cultivation-independent methods cyanobacterial cultures were treated with acridine orange and inspected with an epifluorescence microscope, following the previously shown protocol (Ardelean *et al.*, 2009; Sarchizian and Ardelean, 2010) 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 heterotrophic bacteria with CellC software and assess the validity of CellC enumeration by comparison with manual counting results. 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

Different morphological types of microorganisms are presented in natural samples of sulphurous water, as shown by light microscopy (Fig. 2A,B) or by epifluorescence (Fig. 2C).

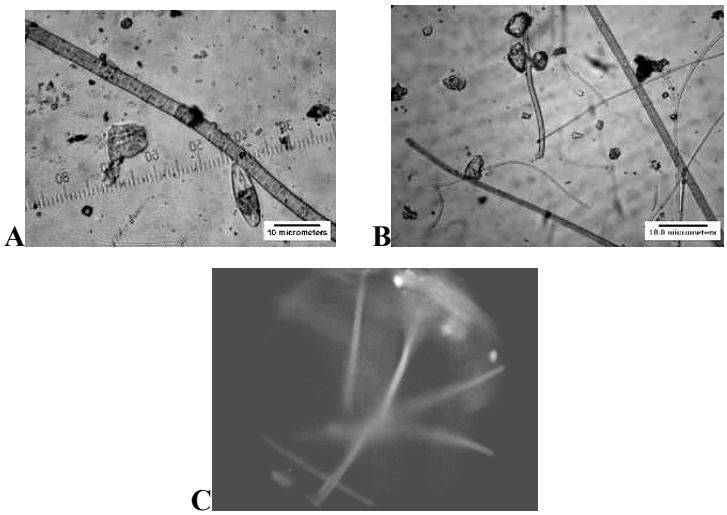

Fig. 2. Microorganisms in a sample from sulphurous mesothermal spring Obanul Mare (Mangalia) as viewed by light microscopy (A, B) and natural chlorophyll fluorescence (C).

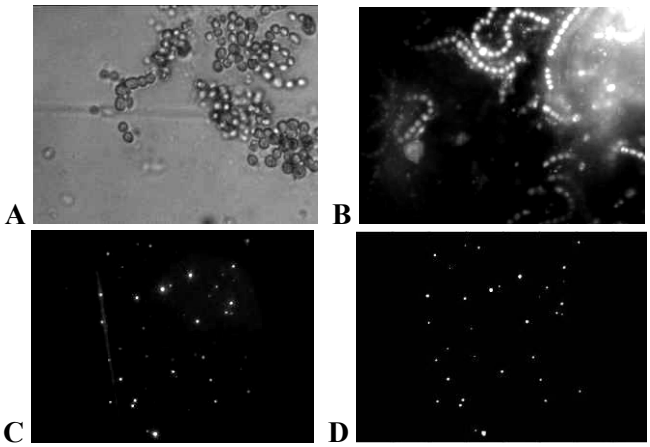

Fig. 3. The abundance of heterotrophic contaminants present in cyanobacterial culture visualized by light microscopy (A) and by epifluorescence microscopy using AO stain (B); digital image analysis of panel C (heterotrophic bacteria labeled with DAPI stain) using CellC software (D).

Enriched cultures of nitrogen fixing cyanobacteria were obtained by selective cultivation and passages on BG_0 medium either liquid or solid. Investigation of cyanobacterial culture by epifluorescence microscopy using acridine orange or DAPI revealed the presence of heterotrophic contaminants before antibiotic treatment (Fig. 3).

Lysozyme treatment and testing the purity of cyanobacteria culture. In order to obtain axenic culture, a lysozyme stock solution (20 g/L) was freshly prepared by dissolving lysozyme in distilled water (Kim *et al.*, 1999). The solution was filtered through a 0.25 μm Millipore filtre and then diluted with sterile distilled water over the range of test concentration. Cyanobacteria selectively sub-cultivated on BG_0 were incubated 24 hours in darkness at 4 ºC in liquid BG_0 medium with 3 mL Tris HCl 10 mM 7,6 and 20 mM EDTA, and lysozyme 20 μg/mL. Following this dark incubation, the cultures was washed with steriled water, centrifugated 4 minutes at 4 ºC and 8500 g, followed with addition of cycloheximide 20 μg/mL and the resultant pellets were further re-suspended in sterile distilled water and rinsed four times, then incubated 240 hours in light at 25 ºC in BG_0 medium. A test to confirm that axenic cultures of cyanobacteria was performed using BG_{11} and another test was carried out with LB agar medium (1 % tryptone, 0.5 % NaCl, and 0.5 % yeast extract). The plates were incubated at 30 ºC in the dark for 4 days. After 24 hours of dark incubation no bacterial colony was shown on plates, but after 96 hours of dark incubation 5 bacterial colonies (corresponding to 165 bacterial cells/mL) were counted.

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 a 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 strict autotrophic cyanobacteria in darkness). In light the growth of cyanobacteria is promoted, thus the antibiotic should be absent. After one cycle of treatment 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 30 minutes in the presence of the organic substrates (1 % or 10 % LB in BG_{11}), tienam been added afterwards. The rationale of this attempt is based on recent findings who 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 (Graumann, 2006; Francia *et al.*, 2007), changes which should protect the population against that antibiotic (Dubnau and Losick, 2006). According to our working hypothesis raised on these findings, it is expected that adding the second antibiotic (tienam) after the organic substances would reduce the

chances of growing cells within heterotrophic bacterial populations to communicate within the same species (population) to develop in due time the protective mechanisms (Graumann, 2006; Dubnau and Losick, 2006; Francia *et al.*, 2007) against antibiotic presence.

In Figure 4 there is shown one cyanobacterial strain isolated in axenic culture.

Fig. 4. Axenic filamentous, nitrogen fixing but notheterocystous, alkalo-tolerant (pH = 9,6) cyanobacterium (natural epifluorescence analysed with ImageJ software) isolated and purified by the improved lysosyme method.

CONCLUSIONS

Axenic culture of a filamentous, nitrogen fixing but no theterocystous, alkalo-tolerant (pH = 7.3–9.6) cyanobacterium was isolated by the use of the improved lysozyme method presented in this paper. The improvement consists in using tienam as second antibiotic whose action occurred when the growth of heterotrophic bacteria was sustained by the addition of organic substrates before tienam treatment.

Acknowledgments. We thank Dr. Tech. Jyrki Selinummi (Department of Signal Processing, Tampere University of Technology, Finland) for kind advice and helpful discussions concerning the use of software CellC (http://www.cs.tut.fi/sgn/csb/cellc/) for automated image analysis to count heterotrophic bacteria labeled with acridine orange and Professor dr. Maria Teodora Onciu (*Ovidius* University, Constantza, Romania) and dr. Aurelia Brezeanu (Institute of Biology, Romania) for kind advice and support. Also we want to thank Dr. Hee Mock-OHH for his kindness to send us reprints of his work.

REFERENCES

1. Ardelean I., S. Ghiță, I. Sarchizian, 2009, Epifluorescent method for quantification of planktonic marine prokaryotes, In: Proc. *2nd International Symposium on New Research in Biotechnology*, Series F (Special volume), ISSN 1224-7774, pp. 288-296.
2. Bhadauriya P., R. Gupta, S. Singh, P. Singh Bisen, 2008, n-Alkanes variability in the diazotrophic cyanobacterium *Anabaena cylindrica* in response to NaCl stress, *World J Microbiol Biotechnol*, **24**, pp. 139-141.
3. Bolch C.J.S. and S.I. Blackburn, 1996, Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa* Kutz, *J Appl Phycol*, **8**, pp. 5-13.
4. Carmichael W.W. and P.R. Gorham, 1974, An improved method for obtaining axenic clones of planktonic blue-green algae, *J. Phycol.*, **10**, pp. 238-240.
5. Castenholz R.W., 1988, Culturing methods for cyanobacteria, *Methods Enzymol*, **167**, pp. 68-93.
6. Choi G-G., M-S. Bae, C.-Y. Ahn, H.-M. Oh, 2007, Induction of axenic culture of *Arthrospira* (*Spirulina*) *platensis* based on antibiotic sensitivity of contaminating bacteria, *Springer Science + Business Media B.V.*, 2007, **30**, pp. 87-92.
7. Clewell D.B. and K.E. Weaver, 1989, Sex pheromones and plasmid transfer in *Enterococcus faecalis*, *Plasmid*, **21**, pp. 175-184.
8. Connell L. and R.A. Cattolico, 1996, Fragile algae: axenic culture of field-collected samples of *Heterosigma carterae*, *Mar Biol*, **125**, pp. 421-426.
9. Dubnau D. and R. Losick, 2006, Bistability in bacteria, *Mol Microbiol*, **61**, pp. 564-572.
10. Dunny G.M., B.L. Brown, D.B. Clewell, 1978, Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone, *Proc Natl Aca Sci USA*, **75**, pp. 3479-3483.
11. Ferris M.J. and Hirsch C.F., 1991, Method for isolation and purification of cyanobacteria, *Appl Environ Microbiol*, **57**, pp. 1448-1452.
12. Francia M.V., K.E. Weaver, P. Goicoechea, P. Tille and D.B. Clewell, 2007, Characterization of an active partition system for the **Enterococcus faecalis** pheromone-responding plasmid pAD1**, J Bacteriol**, **189**, pp. 8546-8555.
13. Graumann P.L., 2006, Different genetic programmes within identical bacteria under identical conditions: the phenomenon of bistability greatly modifies our view on bacterial populations, *Molecular Microbiology*, **61(3)**, pp. 560-563.
14. Kim J.-S., Y-H Park, B.-D. Yoon, H.-M. Oh, 1999, Establishment of axenic cultures of *Anabeana flos-aquae* and *Aphanothece nidulans* (cyanobacteria) by lysozyme treatment, *J Phycol*, **35**, pp. 865-869.
15. Rippka R., J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, 1979, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *Journal of Geneneral Microbiology,* **111**, pp. 1-61.
16. Rippka R., 1988, Isolation and purification of cyanobacteria, *Methods Enzymol*, **167**, pp. 3-27.
17. Sarchizian I. and I.I. Ardelean, 2010, Axenic culture of a diazotrophic filamentous *Cyanobacterium* isolated from mesothermal sulphurous spring (Obanul Mare – Mangalia), *Rom J. Bio. – Plant Biol*, **55**, pp. 47-53.
18. Selinummi J., J. Seppälä, O. Yli-Harja, J.A. Puhakka, 2005, Software for quantification of labeled bacteria from digital microscope images by automated image analysis, *BioTechniques*, **39**, pp. 859-863.
19. Vaara T., M. Vaara, S. Niemela, 1979, Two improved methods for obtaining axenic cultures of cyanobacteria, *Appl Environ Microbiol*, **38**, pp. 1011-1014.

20. Van Den Hoek C., N.H. Mann, H.M. Jahns, 1995, *Algae*, *An introduction to phycology*, Cambridge University Press.
21. Vázquez-Martínez G., M.H. Rodriguez, F. Hernández-Hernández, J.E. Ibarra, 2004, Strategy to obtain axenic cultures from field-collected samples of the cyanobacterium *Phormidium animalis*, *Journal of Microbiological Methods*, **57**, pp. 115-121.
22. Watanabe M.M., M. Nakagawa, M. Katagiri, K. Aizawa, M. Hiroki, H. Nozaki, 1998, Purification of freshwater picoplanktonic cyanobacteria by pour-plating in "ultra-lowelling-temperature agarose", *Phycol Rev*, **42**, pp. 71-75.