Algal Research 23 (2017) 43–50

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Establishment of axenic cultures from cyanobacterium *Aphanizomenon flos-aquae* akinetes by micromanipulation and chemical treatment

Sigitas Šulčius *, Kristina Slavuckytė, Milda Januškaitė, Ričardas Paškauskas

Laboratory of Algology and Microbial Ecology, Nature Research Centre, Akademijos str. 2, LT-08412 Vilnius, Lithuania

ARTICLE INFO

Article history:
Received 7 September 2016
Received in revised form 16 January 2017
Accepted 18 January 2017
Available online xxxx

Keywords:
Nostocales
Isolation
Purification
Pure cultures
Bacterial contamination

ABSTRACT

Filamentous cyanobacteria are an environmentally and biotechnologically important species. However, the isolation and purification techniques for these organisms remain poorly developed and rarely used in studies of their biology, ecology and growth requirements, mainly due to the need of species- or strain-specific approaches and labour-intensive work. Here we propose a simple protocol for the establishment of an axenic (pure) culture of filamentous cyanobacterium *Aphanizomenon flos-aquae* from its akinetes. We tested the effect of different physical and chemical treatments on akinetes viability and germination time, removal of epiphytic and contaminating bacteria and growth of the recovered cultures. The protocol consists of three steps: 1) capturing the *A. flos-aquae* akinetes using a micromanipulator, 2) akinete treatment with a TESC buffer, containing 1% of CTAB and 3) transferring the akinetes to the growth medium. We further demonstrate the increased growth of axenic *A. flos-aquae* compared to their bactericized counterparts, which provides insights into cyanobacteria-bacteria interactions.

1. Introduction

The establishment and maintenance of axenic (pure) cultures of different microorganisms, including cyanobacteria, is one of the greatest challenges in the field of fundamental and applied microbiology [1,2,3,4]. The difficulties associated with the isolation and cultivation of axenic cyanobacterial strains limits their exploitation in biotechnological processes and uses for the production of various biologically active compounds, in particular, those of pharmaceutical and therapeutical value [5]. It also circumvents the definitive characterization of cultured strains for taxonomic classification and nomenclature [6,7]. Unlike in PCR-based studies, non-axenic strains may lead to a pronounced bias in the analysis of the genome sequencing data of cultured organisms. The isolation of pure cultures is also an obligate necessity to understand the biology and function of cultivated microorganisms as well as their ecological interactions with other species [8,9]. Hence, numerous studies over the last several decades attempted to develop more efficient methods for cell isolation, primarily aiming to increase precision, throughput and cell recovery rates [3,10,11,12,13].

Filamentous cyanobacteria are globally distributed photosynthetic microorganisms of great ecological and economic importance. In many natural environments they constitute a major component of the aquatic biomass playing a significant role in the food web dynamics and biogeochemical cycles [14]. Due to their capability to synthesize an array of natural bioactive compounds, cyanobacteria have great potential for multiple industrial applications. Commercially and biotechnologically, the most important filamentous species are *Arthrospira platensis*, *Aphanizomenon flos-aquae* and several members belonging to genera *Nostoc* and *Anabaena* [15,16,17]. However, the establishment of axenic cultures of these cyanobacteria remains rather difficult, and is considered to be time consuming and labour intensive work that requires strain-specific approaches [18].

Cyanobacteria are very morphologically diverse microorganisms ranging from simple unicellular to complex filamentous forms. The recent "polyphasic" approach subdivides all cyanobacteria into five subsections, where subsections IV and V include all cyanobacteria exhibiting cell differentiation. The prevalent types of differentiated cells are heterocytes, capable of atmospheric nitrogen fixation by the oxygen-sensitive nitrogenase enzyme complex, and akinetes. The later are the dormant stage cells produced exclusively by cyanobacteria belonging to the order Nostocales [19]. Akinetes develop from one or several neighbouring vegetative cells and possess a thick multi-layered cell wall, which makes akinetes more resistant to physical and chemical treatment, and enables the organism to survive in an unfavourable environment until the onset of suitable conditions for vegetative growth.

The establishment of pure bacterial cultures relies on two fundamental aspects. First, a pure culture has to be derived from the progeny of a single cell [10]. Second, it has to be free of all other free or cell surface attached contaminants, including eukaryotes, prokaryotes and viruses. Both conditions are hardly, if at all, achievable for many isolated

* Corresponding author.
E-mail address: sigitas.sulcius@gmail.com (S. Šulčius).

http://dx.doi.org/10.1016/j.algal.2017.01.006
2211-9264/

filamentous cyanobacteria to date. Cells of filamentous cyanobacterial are interconnected and organized into chains (filaments and trichomes), and thus, in most cases, filaments (or trichomes) rather than single cells are invoked in the isolation of axenic cultures [20,21,22,23]. Moreover, most of the filamentous cyanobacteria are colonized by epiphytic (cell surface attached) bacteria and form intimate symbiotic associations [24,25,26,27,28]. To overcome this problem and remove epiphytic bacteria, purification steps are required, often involving multiple physical (centrifugation, sonication, UV, etc.) and chemical (antibiotics, detergents, etc.) treatments of the targeted cells, which in turn, impair their survival [21,22,26,29]. For example, during the isolation of the filamentous cyanobacteria *Phormidium animalis*, Vázquez-Martínez with collaborators [30] used centrifugation, differential filtration, washing, sonication of the filaments to disrupt them into smaller fragments and, finally, treating them with a mixture of antibiotics. A combination of different techniques was also used by Katoh et al. [22] to isolate the filamentous cyanobacteria *Nostoc commune*, which included serial dilutions, repeated re-isolation on solid and liquid media, micromanipulation and treatment with different antibiotics and cycloheximide. In the study of Fujishiro and colleagues [26] who reported successful isolation of an axenic culture of the freshwater unicellular cyanobacterium *Aphanothece sacrum*, which is widely used in the food industry of Japan, similar multiple extensive manipulations resulted in only producing 3 pure cultures out of 3000 selected cells. All together, these examples illustrate that intensive handling not only prolongs the time of the isolation procedure, but also significantly affects the viability of the targeted cells. Consequently, this reduces the throughput and the overall success rate of the cyanobacteria establishment into the pure cultures [2].

The aim of this study was to develop a simple protocol for the isolation of pure cultures from single cell of the filamentous akinete-forming cyanobacterium *Aphanizomenon flos-aquae*. The proposed protocol involves three steps: 1) capturing the akinetes using a micromanipulator, 2) chemical treatment of the cyanobacterial akinetes and 3) akinete transfer to the growth medium. We assessed the effect of different physical and chemical treatments on akinete viability, removal of contaminating bacteria and growth of recovered cultures.

2. Material and methods

2.1. Description of the cyanobacterial strain and culture conditions

The cyanobacterial strain used in the present study, *Aphanizomenon flos-aquae* 2012/KM1/D3, was isolated from surface water collected in the Curonian Lagoon (N 55°30′, E 21°15′), Lithuania [31]. The strain was maintained as a unicyanobacterial yet non-axenic culture in a modified AF-6 medium [21] without the addition of nitrogen source under a 14/10-h light-dark cycle of approximately 120 μmol of photons m^{-2} s^{-1} using cool white fluorescent illumination at 20 °C. The strain is available at the Collection of pure cultures of algae and cyanobacteria at the Nature Research Centre (Vilnius, Lithuania) [32].

Filaments of the *A. flos-aquae* strain 2012/KM1/D3 exists as straight single filaments of some 118.9 μm (±70.5 μm) long and 3.6 μm (±0.6 μm) in width, while they tending to agglomerate into irregular forms of bundles, then reaching the stationary growth phase. Akinetes of the *A. flos-aquae* strain are elongated and cylindrical with an average size of 28.8 μm (±8.5 μm) × 5.0 μm (±0.3 μm).

The genomic analysis of *A. flos-aquae* sequence data revealed at least 7 different epiphytic bacteria associated with the *A. flos-aqua* filaments (unpublished data). The bacterial colonization pattern was visualized by epifluorescence microscopy and showed that most of epiphytic bacteria are specifically attached to heterocytes (Fig. 1). Some other bacteria are also present during the maintenance of the *A. flos-aquae* culture and colonize filaments, including akinetes (Fig. 7a) with no clear pattern (personal observation). However, in contrast to contaminating or exopolysaccharide associated bacteria, the epiphytic bacteria were not removed by filtration, centrifugation, shaking and/or sonication of the culture suspension.

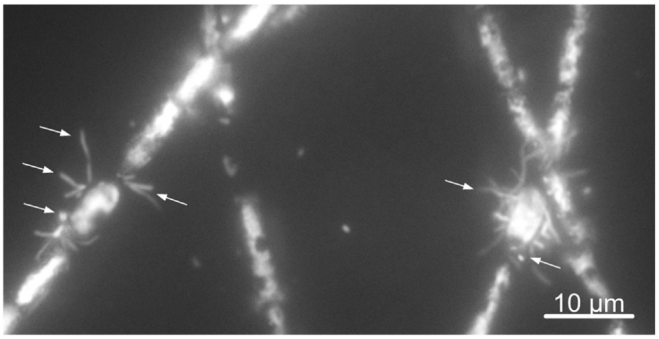

Fig. 1. Epifluorescence microscopy image of *Aphanizomenon flos-aquae* filaments and epiphytic bacteria (arrows) attached to heterocytes.

2.2. Isolation of cyanobacterial akinetes by micromanipulation

Aphanizomenon flos-aquae culture, of late exponential growth phase, was directly subjected to micromanipulation, with no prior promotion of akinete differentiation or homogenization. An inverted microscope Nikon Eclipse Ti*S* (Nikon Corporation, Tokyo, Japan) equipped with an Integra 3™ micromanipulator (Research Instruments Ltd., Falmouth, Cornwall, England, United Kingdom) with sterile glass micro-capillaries (ID 15 μm) was used to isolate single *A. flos-aquae* akinetes from a culture suspension to 200 μl of buffer solutions or a sterile growth medium according to experimental treatments (see below).

A schematic representation of the cyanobacteria's akinete isolation and purification procedure developed in this study is given in Fig. 3. A culture suspension of 500 μl in volume was transferred and placed on a sterile glass microscope slide in the centre of the microscope viewfield. The micromanipulator was operated according to the instructions given by the manufacturer. Targeted *A. flos-aquae* akinetes were captured by a microcapillary (Fig. 2) filled with either a buffer solution or sterile growth medium. After capturing the akinete from the culture suspension within the microcapillary, it was transferred to 200 μl of a buffer solution. The akinete was resuspended in the buffer solution and then transferred into a 96-well plate containing 200 μl of a sterile growth medium (Fig. 3). For the control, freezing and UV treatments captured *A. flos-aquae* akinetes were directly ejected into a 96-well plate with a growth medium and then treated as described below (see *Experimental manipulations*). After the corresponding treatment, the isolated akinetes were subsequently incubated at the conditions as described above for the *A. flos-aquae* strain 2012/KM1/D3.

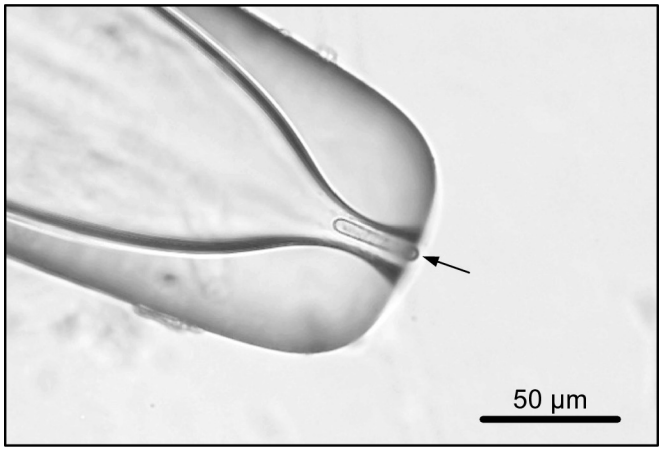

Fig. 2. Isolation of *Aphanizomenon flos-aquae* akinetes (arrow) by microcapillary.