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An Efficient Protocol for Long-Term Preservation of Cyanobacteria

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Abstract: A simple modification in preparation of nutrient agar meant for agar slants resulted in a convenient and efficient matrix for long-term cyanobacterial preservation. In the modified protocol, agar concentration was increased and cyanobacterial cells were mixed rapidly in the molten agar before solidification. Solidified mixture was cut into cubes and air dried. The resulting agar flakes were stored in the dark. Periodically some flakes were inoculated in fresh medium to study regeneration of viable filaments in order to access the efficiency of the method in preserving cyanobacteria in dehydrated form. Possible outer contamination could be removed by washing the agar flakes in 1% sodium hypochlorite for one minute prior to their inoculation in fresh medium. The percentage of agar used and amount of cyanobacterial cells entrapped were the factors that influenced the period of preservation. A comparison of some biochemical and physiological characters in six regenerated cyanobacterial strains to their free-living counterparts showed that the dried agar flakes were completely reliable as preserving material for at least a period of three years. During this period the entrapped cyanobacterial cells did not need further maintenance. This process of maintaining cyanobacteria is extremely convenient as it reduces (1) input of chemicals and manpower required for maintaining cyanobacteria in batches (2) possible cross-contamination among various cyanobacteria maintained in liquid batch cultures (3) use of glassware and (4) storage space. In addition, the cells could be maintained in a near dormant state, and that minimized the chances of alterations in their native characters.

1. Introduction

Cyanobacteria are an ancient group of photosynthetic, gram negative, photoautotrophic prokaryotes which are cosmopolitan in nature. They inhabit every conceivable habitat on earth ranging from terrestrial to aquatic from frigid regions to tropical areas (Carr and Whitton, 1982; Stal, 1995). Cyanobacteria are also known to exist in deserts where they remain dormant for most of the time (Hu *et al*., 2003). They occur in soil, on rocks, in fresh as well as in salt water (Hoffmann, 1989; Kulasooriya, 1998; Nayak and Prasanna, 2007). They exist as free-living and are also capable of symbiotic associations with a wide variety of organisms, ranging from protists, plants, animals and fungi (Rai, 1990; Adams, 2000; Whitton, 2000; Rai *et al.,* 2000; 2002).

Cyanobacteria are now regarded to have enormous potential in serving humanity in more ways than one. Their diazotrophic nature, wide distribution pattern and capability to enter into N_2–fixing symbiosis make them attractive research interest. Cyanobacteria have a long history of usage in agriculture as bio-fertilizer (De, 1939; Watanabe *et al.,* 1951; Singh, 1961; Whitton, 2000). Other potential applications of cyanobacteria in diverse fields of human welfare have also gained a lot of attention in recent years. This includes their use as food and animal feed, in bioremediation of toxic compounds, in bio-control of pests, production of commercial and laboratory chemicals, restriction enzymes, pharmacological tools (Patterson, 1996) and potential therapeutic drugs for the management of diseases like cancer, asthma, diabetes (Skulberg, 2000) and in waste and effluent water treatment including the removal of harmful dyes from textile effluents (Fatma,1999; Prassana *et al*., 2000; Shah *et al*., 2001; Sadettin and Domez, 2006) etc.

To categorically place various cyanobacterial strains according to their potential applications, naturally, existing cyanobacteria need to be

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systematically isolated, purified and characterized. Isolation and purification procedures are time consuming and tedious in nature. Possible threats of contaminations in such purified cultures have led researchers to pay serious attention to methods of preservation of microorganisms, in order to enable successful and effective maintenance of these organisms in the laboratory conditions.

There are few established techniques for culture maintenance in the field of cyanobacterial research (Smith, 2004; Acreman, 1994; Syiem, 2005). A laboratory working with cyanobacteria employs methods such as batch cultures, agar slants, liquid nitrogen as well as storage at lower temperatures (-80ºC) using protectants such as glycerol, sucrose etc. for maintaining pure cultures for varying period of time. Most common being batch cultures and agar slants. Batch culturing requires regular transfer to fresh liquid media that enhances the chances of contamination. The most common procedure for maintaining cyanobacteria is by serial transfer of actively growing cultures to a fresh media on a regular schedule under suboptimal conditions (Lorenz *et al*., 2005). Some of the disadvantages associated with this method are the high risk of contamination and loss of characters, as well as, possible mislabeling or loss of cultures (Acreman, 1994). Also, there are reports of loss of phenotypic characters in several microalgal cultures during serial subculturing. Moreover, the genetic and phenotypic stability of the strains cannot be guaranteed over years of laboratory maintenance (Day *et al*., 2005). When maintained at optimal conditions strains do not need to express many of their characters as stresses of any form is not presented to them during routine maintenance in the laboratory (Smith, 2004). Other drawbacks of this method of serial subculturing is that it is labour intensive and requires consumables that are expensive and thus, limits the capacity of researchers to maintain a large number of strains.

Another widely used method for laboratory maintenance of cultures is by storing them in agar slants. This is done by inoculating pure cultures into a nutrient agar media which are solidified in sterile vials. However, this method of culture maintenance also suffers from the limitation of having a relatively short shelf-life and the jelly like consistency of agar changes over a period of time thus, subjecting the entrapped cultures to ample chances of contamination(s). In addition, such agar slants need to be stored in culture room or in refrigerators where they occupy valuable space.

To circumvent the above problems, we have introduced some simple changes in preparation of the nutrient agar. These modifications are reliable and reproducible. The end products of the process are dry agar flakes that can be conveniently stored at room temperature in sterile glass/plastic vials. Such immobilized cyanobacterial cells were viable for at least for a period of three years of storage and regenerated cultures remained pure with no apparent alterations in their attributes.

2. Materials and Methods

2.1 Organisms

Nostoc sp. and *Anabaena* sp. (Isolated from Shillong, Meghalaya), *(Mastigocladus* sp. (Jakrem hot spring, Meghalaya), *Fischerella* sp. (Stagnant pond water, Kolkata), *Cylindrospermum* sp. (Imphal rice field, Manipur) and *Plectonema* sp. (Pilani, Rajasthan) were chosen to study the effect of this preservation method on both heterocystous and non-heterocystous cyanobacteria. The isolated cyanobacteria were purified by pour plating method and axenic cultures obtained thereof were immobilized in nutrient agar.

2.2 Nutrient agar media

2.5% bacteriological agar with gelling temperature 40ºC was melted in 50 ml of BG-11_0 media for *Nostoc* sp., *Anabaena* sp., *Fischerella* sp. and *Cylindrospermum* sp.; BG-11_0 +5 mM sodium nitrate for *Plectonema* sp. (Rippka *et al.,* 1979) and D-medium for *Mastigocladus* sp. as described by Castenholz, 1981.

2.3 Immobilization

Exponentially growing (10 days old) cyanobacterial cells were concentrated by centrifugation at 2500 rpm for 5 minutes (100 ml of culture was concentrated to 10 ml; final concentration of chlorophyll *a* in this concentrate was kept at 50 µg/ml). Following this, the centrifuge tubes were vortexed mildly to mix the cells and then poured into the 40 ml of lukewarm nutrient agar solutions (final volume 50 ml; final chlorophyll *a* concentration was maintained at 10 µg/ml). This mixture was spread on flat bottom glass Petri dishes. These were left to solidify for 30 minutes in the laminar flow cabinet. Upon solidification, these were cut into (~0.5cm x 0.5cm) sized cubes. These cubes were air dried under aseptic condition in the laminar flow shelf for 24 h. (Fig. 1a).

Growth, heterocyst frequency, nitrogenase activity, photosynthetic oxygen evolution and respiratory oxygen consumption study in regenerating samples.

Periodically few of the agar flakes were introduced to liquid growth media. The cyanobacterial filaments grew out into the media within seven days (Fig. 1b). The regenerated cultures were then assessed on the tenth day for growth measured as increase in chlorophyll *a* concentration (Mac Kinney, 1941). Heterocyst frequencies were counted under Olympus BX 51 microscope. Nitrogenase activities were measured using acetylene reduction protocol (Stewart *et*

al., 1967) and photosynthetic and respiratory activities were studied as described by Robinson *et al*., 1982.

3. Results and Discussion

This study reports retention of various characters in cyanobacterial cells that were immobilized in nutrient agar and were regenerated after three years of storage. Fig. 1c is a scanning electron micrograph (JEOL JSM 6360, SAIF, NEHU) of freshly embedded cyanobacteria filament in agar. Entrapment in agar did not have any drastic stress on the cyanobacterial morphology. The cells in the filament retained their shape and size. The dried agar cubes were stored for three years before they were introduced to fresh liquid media. Thus, cyanobacteria embedded within the dried agar matrix did not have any access to any form of fluid and/or nutrient forcing them to remain dormant throughout the storage period. However, when put into liquid nutrient media, the immobilized cells regenerated quickly into viable healthy filaments (Fig. 1b, d) that emerged out of the agar matrix and rapidly populated the medium. Fig. 1e-h shows viable filaments of regenerated cyanobacteria in culture media.

For this study, representatives of different groups of cyanobacteria (*Nostoc, Anabaena* and *Cylindrospermum* sp. for filamentous heterocystous type; *Mastigocladus* and *Fischerella* sp. for filamentous branched heterocystous type and *Plectonema* sp. for filamentous non-heterocystous type) have been used. Inclusion of *Mastigocladus* isolate from hot spring was intentional to ascertain any adverse effect of immobilization and preservation of this thermophilic cyanobacterium at temperature other than their optimal growth temperature in nature (~60ºC). A comparative analysis of various characters in regenerated cells to their free-living counterparts is presented in Table 1.

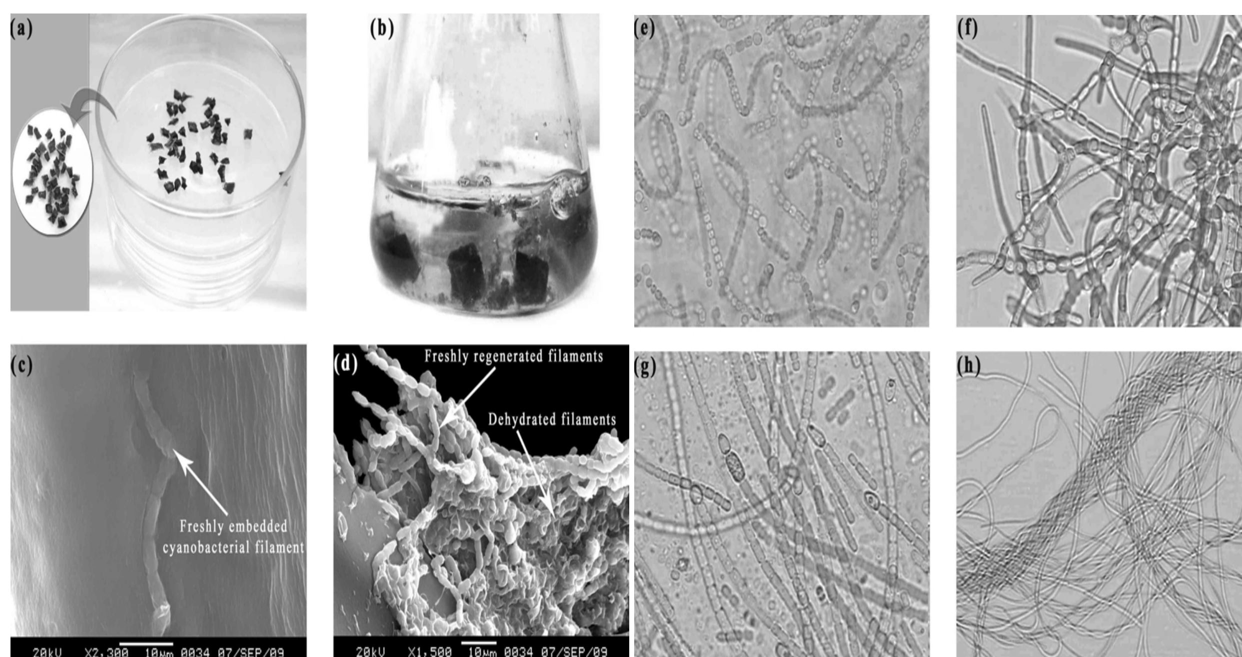

Fig. 1(a). Air dried agar cubes; (b): Regeneration of cyanobacterial filaments from agar cubes after three years of preservation; (c): Freshly embedded cyanobacterial filaments in agar; (d): Scanning electron micrograph of cyanobacterial filaments regenerating from agar cubes when introduced to fresh media; (e-h) Healthy growth of *Nostoc* sp., *Mastigocladus* sp., *Cylindrospermum* sp., *Plectonema* sp. Respectively in culture media after regeneration from immobilization.

Table 1. Comparative study of various parameters of cyanobacteria in control culture (maintained in batch cultures as free-living organisms) and in filaments regenerated after three years from dehydrated agar. (All data were generated as the mean of three independent sets of experiments)

Organisms	Growth (μg chl *a* ml^{-1})		Heterocyst frequency (%)		Nitrogenase activity (nmol C_2H_4 produced μg^{-1} chl *a* h^{-1})		Photosynthetic activity (nmol O_2 produced μg^{-1} chl *a* h^{-1})		Respiratory activity (nmol O_2 consumed μg^{-1} chl *a* h^{-1})	
	C	R	C	R	C	R	C	R	C	R
***Nostoc* sp.**	3.4	3.1	8.4	7.5	11.4	11.2	439	420	413	378
***Anabaena* sp.**	4.2	4.2	8.7	7.7	12.2	11.2	440	415	413	367
***Mastigocladus* sp.**	2.8	2.5	9.2	7.3	5.3	4.5	280	218	390	324
***Fischerella* sp.**	2.5	2.4	5.3	4.7	3.1	2.9	311	235	465	421
***Cylindrospermum* sp.**	3.1	2.9	6.1	5.8	3.5	3.6	364	347	389	354
***Plectonema* sp.**	4.3	4.0	-	-	12.0	10.9	553	520	334	311

C- Control free-living samples; R-regenerated samples

Characters that were studied were found to be retained in cells regenerated from agar flakes. All parameters were comparable to their free-living counterparts (Table 1). The growth was instantly closer to their control. This may be due to the fact that as soon as the stress in the form of dehydration was lifted, the immobilized cells underwent vigorous cell divisions in the presence of optimum nutrient availability. The heterocyst frequency, however, was less by 5 to 21% in various cyanobacterial strains studied. The reduction in heterocyst number was reflected as reduced nitrogenase activity in all isolates except in *Nostoc* sp. This was expected as nitrogenase enzyme is located in heterocysts. Comparison of photosynthetic oxygen evolution and respiratory oxygen consumption showed that these values were also compromised in the cells regenerated after three years of storage. However, complete recovery in all these attributes was seen in the very second transfer of cells to specific growth media after regeneration from immobilization. No loss of viability and characters were noticed in *Mastigocladus* cells that were not maintained at their ideal growth temperature while under preservation. Cells that were regenerated from the immobilized cells in the subsequent transfers in liquid media were similar in every respect to their free-living controls. Thus, keeping cyanobacterial cells stored in dried agar seems to be an excellent method of preservation as such cells retain complete viability and native characters. Cells can be preserved using this protocol up to a period of three years. At this point, it must be mentioned that reduction seen in any characters at the end of three years was even lesser when cells were regenerated after a shorter duration of storage. The modification that was brought about by us in preparation of nutrient agar have the advantages of ease of handling and ensuring substantially long-term storage of cyanobacteria in dry form. Storage in this method also significantly reduces the probability of cross-contamination that one encounters while maintaining liquid batch cultures of many cyanobacteria with similar nutrient requirements in close proximity. Strains that require specific conditions when maintained in liquid growth medium (such as *Mastigocladus* sp. Requiring temperature to be maintained around 45°C) could also be kept viable in agar cubes at room temperature thus making this method of preservation reliable, convenient and user friendly.

4. Conclusion

In conclusion, we would like to mention that in our laboratory we are preserving a sizable number of purified strains as agar flakes. Whenever necessary, a purified strain is regenerated from the flakes. We find that the process is very simple and convenient for storing cyanobacterial strains. This has also allowed us to cut down the use of a large number of glassware and maximize space utilization in the culture room. Above all, organisms preserved were found to retain their native characters. Retention of native characters in *Mastigocladus* included in the study also suggests that no special treatment is needed while maintaining strains with otherwise specific requirements for their growth and maintenance.

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