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ORIGINAL ARTICLE

Microscope Assisted Uni-algal isolation through Dilution (MAU-D): a simple modified technique for tapping diverse cyanobacteria

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Abstract

Cyanobacteria are oxygenic photosynthetic microorganisms known for their agricultural and industrial importance. Unavailability of efficient and fast isolation and purification methods of cyanobacteria has impeded our understanding of cyanobacterial diversity. A number of techniques for isolation and purification of cyanobacteria are available, but most of them are cumbersome as well as time-consuming. In the present study, we modified and validated a uni-algal isolation technique named as Microscope Assisted Uni-algal isolation through Dilution (MAU-D) which used dilution of mixed algal population on slide and isolation of single type of cyanobacterial cells using light microscope. Using this technique, we obtained 81 cyanobacterial isolates belonging to various species from 19 different genera from soil and water samples collected from rice fields of Uttar Pradesh, India. This technique also resulted in isolation of six distinct genera, viz., *Cyanobacterium*, *Toxopsis*, *Desertifilum*, *Chroococcidiopsis, Halomicronema*, and *Alkalinema,* which were previously not reported from rice fields of India. Hence, the MAU-D technique presents a simple, comparatively fast method of isolation and purification of cyanobacteria which can help to isolate those cyanobacteria which are difficult to isolate through routine sub-culturing.

Keywords Cyanobacteria · Diversity · Isolation · Microscopy · Purification

Abbreviations

MAU-D Microscope Assisted Uni-algal isolation through Dilution

Introduction

Cyanobacteria or oxyphotobacteria have been one of the pioneer species of this planet and efforts have been made to understand its evolutionary, ecological, biochemical and taxonomic issues (Alvarenga et al. 2017; Wilmotte et al. 2018). Estimates show the diversity present among microalgae are in the range of 2,00,000 to several millions species in contrast to 2,50,000 species of higher plants (Norton et al. 1996). Among microalgae, cyanobacterial species may vary in number from 2780 to 4484 and may reach up to 8000 (Guiry 2012; Nabout et al. 2013; Guiry et al. 2014; Guiry and Guiry 2018). There are evidences that cyanobacterial diversity is under-represented due to lack of robust and efficient isolation techniques (Gerloff et al. 1950; Doan et al. 2011; Lee et al. 2014). Kozlov et al. (2016) proposed that on the basis of 16S rRNA gene sequences, only 170 genera of cyanobacteria have been described in contrast to 2998 bacterial genera (Parte 2018). With a rocky taxonomic history, cyanobacterial studies are already facing challenges and are undergoing scrutiny with respect to systematics of the phyla (Komárek et al. 2014). Cyanobacteria are widely distributed in diverse aquatic and terrestrial habitats ranging from soil, fresh and marine waters, tree barks, rice fields, the Antarctica, thermal springs, desert crusts, etc., and are known to exhibit unicellular to filamentous forms (Desikachary 1959; Garcia-Pichel et al. 2009). In particular, soil and water samples contain mixed algal population and separating single uni-algal form out of the mixed population is quite a tedious task. More than 70 years of algal research, especially in the area of isolation, purification,

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and maintenance of cyanobacteria, have passed, but till date, the research in the area has not been adequate. Cyanobacteria have associations with the bacterial and archaeal counterparts which makes it more difficult to isolate axenic cultures of cyanobacteria from any environmental samples (Thompson et al. 2012; Foster and Zehr 2019). Also, the traditional taxonomic studies based on morphological properties do not corroborate with their respective phylogenetic analyses (Hugenholtz et al. 2016). Despite a long history cyanobacteriology, there is still no comprehensive and easy method to purify mixed cultures of cyanobacteria. Different methodologies adopted by various algologists to isolate pure cyanobacteria from diverse environmental samples are listed in Table 1, but majority of these techniques are laborious, complicated, time-consuming, expensive, and requires great patience. Many of these methods require speciality chemicals and sophisticated instruments which may not be available everywhere (Elhai and Wolk 1988; Doan et al. 2011; Ali and Mirza 2017). Hence, much of the diversity works reported remain biased towards a few dominant genera of cyanobacteria due to lack of proper methods of cyanobacterial purification.

Owing to the large number of industrial bioactive products produced by cyanobacteria like lipids, pigments, vitamins, polysaccharides, antibiotics, restriction enzymes, etc., it becomes critical to develop an easy, time saving, and cost-effective method to tap into the realm of cyanobacterial world (Chakdar et al. 2012; Pagels et al. 2019; Patel et al. 2019). Importantly, discovery of new oxyphotobacteria may help ecologists in understanding the exact role of these microbes in the biogeochemical cycle of our planet which could address several climate change-related issues gripping the globe (Kulasooriya 2011; Banack et al. 2012 ; Walter et al. 2017).

Under this background, the present work was aimed at developing a method that would not only save time but also much effort and cost which goes into the isolation studies of cyanobacteria. While working on the improvement of the existing isolation methods, we have come up with an easy, time saving, cost-effective and reliable modified technique to isolate uni-algal cyanobacterial cultures. Using this technique, we obtained diverse genera of cyanobacteria including few not reported earlier from similar ecological niches from India.

Materials and methods

Sampling sites

Soil and water samples were collected from two different locations of India (Table 2). Water and soil samples were stored in sterile plastic bottles at 4 °C and further used for isolation and purification of cyanobacteria. Physicochemical parameters like pH and EC were recorded following standard procedure for each sample type (Jackson 1959).

Sample enrichment

BG11 media were used for sample enrichment and isolation of cyanobacteria from different soil and water sample. The pH of the medium was set 8.0 to 8.5 before autoclaving. 1 g of soil from each sample was suspended in sterile BG11 medium (100 ml) with and without nitrogen source and mixed to homogenise. 10 ml of water sample was dissolved as such in 90 ml of sterile BG11 medium. The suspension containing soil and water was allowed to grow in algal growth chamber (Atlanta Drugs & Chemicals, Kolkata, India) under 50–55 µmol photons m^{-2} s^{-1} light intensity and 16:8 light and dark cycle at 27 ± 1 °C for 10–15 days (Stanier et al. 1971).

Standardization of the isolation technique and morphological characterization

It was conceptualized that serial dilution of mixed culture followed by microscopic observation can result in isolation of higher number of uni-algal isolates. Routinely followed methods of serial dilution and incubation theoretically lower the chance of getting diverse uni-algal cultures due to over population of dominant species resulting in competition for nutrients. However, allowing the cyanobacteria present in sample to proliferate in number for a first phase of growth followed by dilution and microscope assisted pick up of cells will allow isolation of diverse uni-algal cyanobacteria.

To achieve this, the process was tested as follows- 20 µl of mixed culture (obtained after 10–15 days of growth) was pipetted out with micropipette and placed on slide (which was wiped with 70% ethanol). The slide was observed at 100X magnification under light microscope (Olympus, Japan). A number of sterile water droplets (15 µl) was placed on the slide. 5 µl of culture suspension was picked up from culture droplet and mixed to one of the 15 µl of sterile water droplet and the process was followed for series of dilutions and was observed under 100X magnification. The droplet containing uni-algal form was pipetted out from slide and collected into sterile glass tube containing 2 ml of BG 11 medium. A few repetitions of the slide dilution of the mixed algal growth, observation under microscope, and pipetting out uni-algal droplets were carried out to isolate maximum possible diversity. The tubes containing single alga were incubated under same conditions mentioned above. After 10–15 days, the uni-algal forms were observed under microscope (400 and 1000X), and morphological observations were recorded and provisionally characterized according to the keys described by Desikachary (1959). The frequency

Table 1 A summary of available techniques for isolation of cyanobacteria

Techniques	Description	Remarks	Described by
Alginate bead method	Mixed algal cells were trapped in the alginate beads. The alginate beads were suspended in 200 µl BG11 medium in 96 well plate and subsequently the beads were teased partially and incubated under controlled conditions. After incubation, cultures were observed under microscope	7 out of 20 slides showed isolated cultures	(Ali and Mirza 2017)
Streaking	Samples were collected from water bodies and diluted. Different dilutions were plated and allowed to grow. After growth, cultures were re-streaked on sterile plate containing various growth medium and incubated under controlled conditions. The procedure was repeated until the single colony of cyanobacteria was obtained	Different cyanobacteria as well as green microalgae were isolated. The technique was laborious and time-consuming as it required several months for isolation and identification	(Lee et al. 2014)
Flow cytometric cell sorting	The cell sorting technique basically utilized cell size and chlorophyll content	96 lipid rich microalgae were isolated from 5 different samples	(Doan et al. 2011)
Nutrient saturated glass fibre filter	This method was employed for axenization of algal culture. 90 mm glass fibre filters were sterilized by fitting in glass petri plates and saturated with BG 13 media. Plates were inoculated and incubated under light. The grown cyanobacteria were treated with imipenem to remove bacterial contaminants	Achieved axenic cultures nearly 80% of the time with single treatment. This method could isolate the cyanobacteria that is not able to grow on solid agar medium	(Ferris and Hirsch 1991)
Ultrasonic cavitation	Culture suspension in a 25 ml tube was subjected to cavitation in ultrasonic cleaner. The fragments of the cultures were observed under microscope. Washed the fragments by centrifugation (1000 X g, 5 min). Subsequently, a series of tenfold dilution made to resuspend the cyanobacteria and $1/10^{th}$ ml from this was placed on the solid agar and incubated under light. After few days, blue green microcolonies appearing on the plate were viewed under dissecting microscope. Finally the tiny agar cube embedded with blue green colony transferred in fresh liquid medium	A sophisticated ultrasonication unit was required. The method also required precise knowledge regarding resonance frequency to generate cavitation effect. Chances of cell damage due to ultrasonication could not be ruled out	(Wolk 1988)
Repeated sub-culturing	Heavy algal suspension was collected from lake and small portion of it was inoculated into large number of flasks or glass tubes containing Chu's 10 medium. The growth however occurred was mixed algal culture but desired organism from culture was selected for additional sub-culturing. Uni-algal cultures were obtained by repeated sub-culturing	20 cyanobacteria species were isolated and identified by this method	(Gerloff et al. 1950)

Table 1 (continued)

Techniques	Description	Remarks	Described by
Single cell isolation by micropipette	This method targeted on picking up single cell using a Pasteur pipette or glass capillary and further they are sequentially washed in sterile droplets either on a Petri plate or a glass slide. The droplets are observed under microscope and those containing "single cell" are transferred to fresh medium for growth	Pasteur's pipette or ultrathin glass pipette or glass capillary with tubing system is used in this method to pick single cells which demands high skill and precision for picking up cells. For using this method, also considerable time needs to be invested for production of micropipettes to be used for isolation. In this method, droplets containing "single cell" is targeted which is prone to cell injury and revival/growth upon transfer to fresh medium is low and unpredictable	(Andersen and Kawachi 2005)
Microscopic Assisted Uni-algal isolation through Dilution (MAU-D)	Collected samples were grown in BG11 medium with and without nitrogen source and allowed to grow under appropriate conditions. Mixed algal culture then diluted on slide in a series of different sterile water droplets one after another and observed under 100 X magnifications. The droplets showing uni-algal presence was pipetted out and mixed with 2 ml of BG11 medium in sterile glass tube. The cultures were then incubated under appropriate conditions for further studies	The technique requires no special efforts or instrumentation; it just requires knowledge of handling simple microscope. In this method droplet/s containing "single type" of cyanobacterium (not "single" cyanobacterium/cell) is targeted. As the number of cells in the droplets is high, the chances of revival and growth of cells upon transfer to fresh medium is also high. Simple micropipette is used in this method to pick up the dilution droplet containing single type of cells (obviously many in numbers!) which is easy to handle The technique produced more than 81 uni-algal cultures from different samples belonging to distinct cyanobacterial genera in less than a month	Present study

Table 2 Different sampling sites, geographical location, and physicochemical properties

Sr. No	Sampling sites	Source of samples	Geographical location of sampling sites	Physicochemical Properties of the Sampling Sites	
				pH	EC ($\mu Scm^{-1)}$
1	Kushmaur, Mau, U.P	Soil from Paddy Field	25°53.942'N and 83°29.257'E	9.5	190
2	Kushmaur, Mau, U.P	Soil from Pigeon pea Field	25°54.017'N and 83°29.300'E	9.6	135
3	Kushmaur, Mau, U.P	Soil from Garden	25°53.815'N and 83°29.303'E	10.5	99.7
4	Alipur, Mau, U.P	Soil from Paddy Field	25°90.619'N and 83°47.860'E	8.7	248
5	Mau, U.P	Water from Pond	25°95.183'N and 83°55.231'E	8.6	1500
6	New Jirang, Ri Bhoi, Meghalaya	Soil from Paddy Field	25°55.077'N and 91°34.605'E	7.7	56

(%) of obtaining uni-algal cultures from each sample was calculated as (No. of tubes found to have uni-algal culture as observed under microscope after complete incubation/No. of tubes inoculated with tentative uni-algal cultures) × 100. The isolates were deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-NBAIM, Mau, India and accession numbers were obtained.

Comparison of MAU-D method with other methods for isolation of cyanobacteria

Comparison of MAU-D method was done with other frequently used isolation techniques such as: Repeated subculturing method as described by Gerloff et al. (1950), Single cell isolation method as described by Andersen and Kawachi (2005), and Streaking method as discussed by Lee et al. (2014) using the soil sample collected from paddy field of Alipur, Mau, U.P., India. Growth medium and conditions were same as described in the section *Sample Enrichment*. Purified cyanobacterial isolates obtained from different isolation techniques were identified as described in the subsequent sections.

Molecular characterization and sequencing

Total genomic DNA was isolated using Nucleo-pore® gDNA Fungal/Bacterial kit (Genetix Biotech Asia Pvt. Ltd, Delhi, India) following the manufacturer's protocol with slight modifications. The primer pairs used were Cya 359F-(5'GGGGAATYTTCCGCAATGGG3') and Cya 781R(a)-(5'GACTACTGGGGTATCTAATCCCATT3') targeting 16S rRNA gene (Nübel et al. 1997). Amplification of 16S rRNA gene was achieved as described by Nubel et al. (1997). Bacteria-specific universal primers 27F and 1492R were also used to cross-check ten random samples of the purified cultures for the presence of signal from contaminating bacteria using PCR amplification (Edwards et al. 1989; Rai et al. 2015). Sequencing of the amplified PCR product was done by outsourcing the samples to Biokart India Pvt Ltd. The sequence electropherograms (.abi files) were analyzed through FinchTV (https://www2.le.ac.uk/offices/itservices/ithelp/my-computer/programs/finchtv) for the presence of contaminating sequence and quality of sequencing results. Curated sequences were used for BLAST search using EzBiocloud server (http://www.eztaxon.org) for determination of identity (Yoon et al. 2017). The partial 16S rRNA gene sequences were submitted to NCBI GenBank.

Results

Cyanobacterial isolation and characterization

As the technique used microscopy and dilution of algal population on slides, it was named as Microscope Assisted Uni-algal isolation through Dilution (MAU-D) method. Figure 1 represents a schematic diagram of the MAU-D technique for isolation of uni-algal form from mixed culture. The isolation technique resulted in 81 uni-algal cyanobacterial cultures from various samples containing mixed cyanobacterial population (Fig. 2a–c). The frequency of obtaining uni-algal cultures for each sample was as follows: (1) Soil from paddy field, Kushmaur, U.P.: 80%; (2) Soil from pigeon pea field, Kushmaur, U.P.: 85.7%; (3) Soil from garden, Kushmaur, U.P.: 80%; (4) Soil from paddy field, Alipur, U.P.: 92.85%; (5) Water from pond, Mau, U.P.: 83.3%; (6) Soil from paddy field, Ri Bhoi, Meghalaya: 92.30%. Table S1 presents the different morphotypes belonging to various species of 19 different genera, identified on the basis of 16S rRNA gene homology and morphological features. Distinct ecological niches have been detailed in this table along with respective diversity tapped. Table S2 presents details of various cultural and morphological attributes of the isolates. Using the MAU-D technique, both unicellular and filamentous types of cyanobacteria were isolated (Fig. 3a–f). Unicellular forms included: *Cyanobacterium*, *Chroococcidiopsis*, *Geminocystis*, *Chlorogloeopsis*, *Limnococcus, Aphanothece,* and *Gloeothece,* while the filamentous forms included: *Desertifilum*,

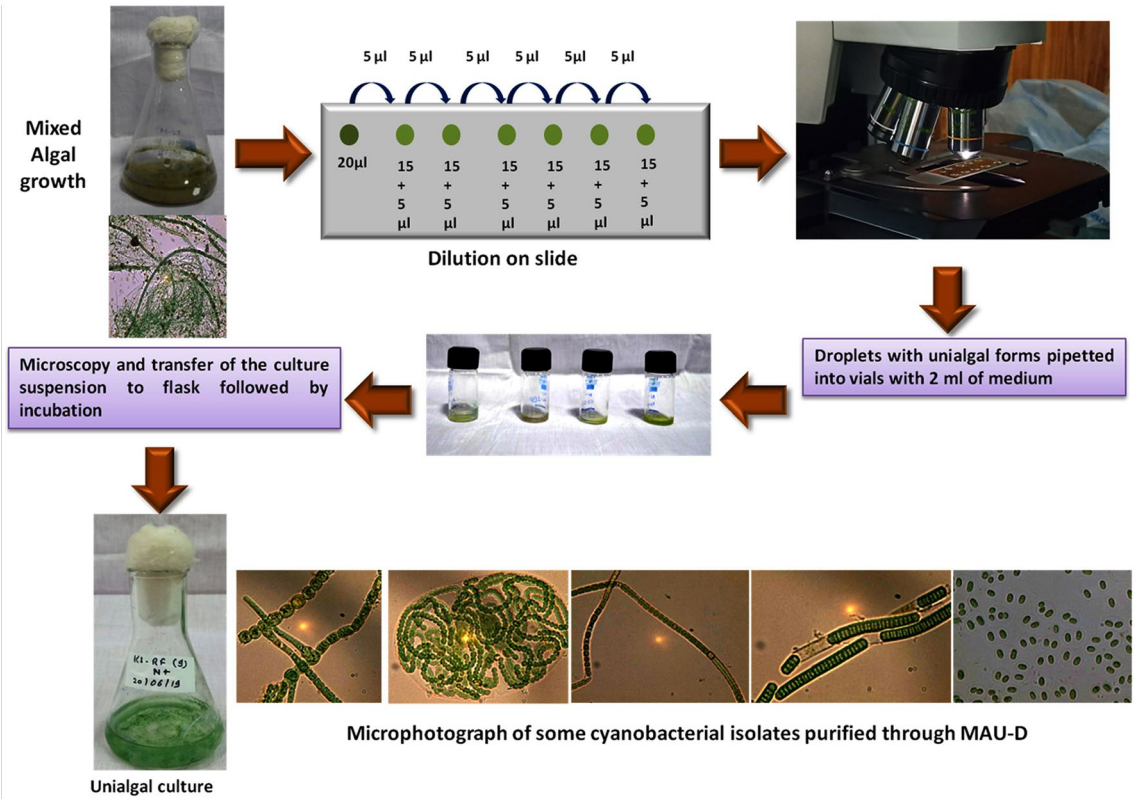

Fig. 1 Illustration of Microscope Assisted Uni-algal isolation through Dilution (MAU-D) technique for isolation and purification of cyanobacteria

Lyngbya, *Phormidium*, *Hapalosiphon*, *Aulosira*, *Toxopsis*, *Trichormus*, *Halomicronema, Nostoc*, *Alkalinema*, *Leptolyngbya,* and *Scytonema*. The present study also reported a few distinct genera (*Cyanobacterium*, *Toxopsis*, *Desertifilum*, *Chroococcidiopsis*, *Geminocystis*, *Halomicronema*, *Chlorogloeopsis*, *Alkalinema, Limnococcus*) those were not frequent or not reported earlier from their respective niche in India. All the cultures were deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC), Maunath Bhanjan, India.

Comparison of MAU-D method with other methods for isolation of cyanobacteria

From the soil sample collected from paddy field of Alipur, Mau, U.P., highest (13) number of cyanobacterial isolates was retrieved following MAU-D method, followed by Streaking method (07), Single cell isolation method (05), and Repeated sub-culturing method (04). The cyanobacteria belong to *Nostoc, Limnococcus,* and *Lyngbya* which could be obtained through MAU-D method; however, those could not be isolated by other methods. On the other hand, isolate belonging to *Fortiea contorta* obtained through streaking and single cell isolation method could not be obtained by MAU-D method (Table 3; Table S3). The frequency of obtaining uni-algal cultures using other three methods ranged from 36 to 70% in comparison to ~ 93% for MAU-D method.

Test of axenicity for pure isolates

Ten purified random cultures which were cross examined by bacteria-specific 16S rRNA gene primers (27F and 1492R) and the analyses showed the absence of any bacterial 16S rRNA gene sequence signal (Fig. S1). Electropherograms confirmed the presence of only cyanobacteria as bacterial contamination would have been reflected by mixed peaks upon sequencing with bacteria-specific universal primers. The identities also matched with the results obtained with cyanobacteria-specific primers used earlier (Table 4).

Discussion

Earlier, groundbreaking works were done in this field during the 1950s and 60s by Gerloff et al. (1950); Allen (1952); Kratz and Myers (1955); Hughes et al. (1958); Van Baalen (1962); Jones et al. (1973); Allen and Gorham

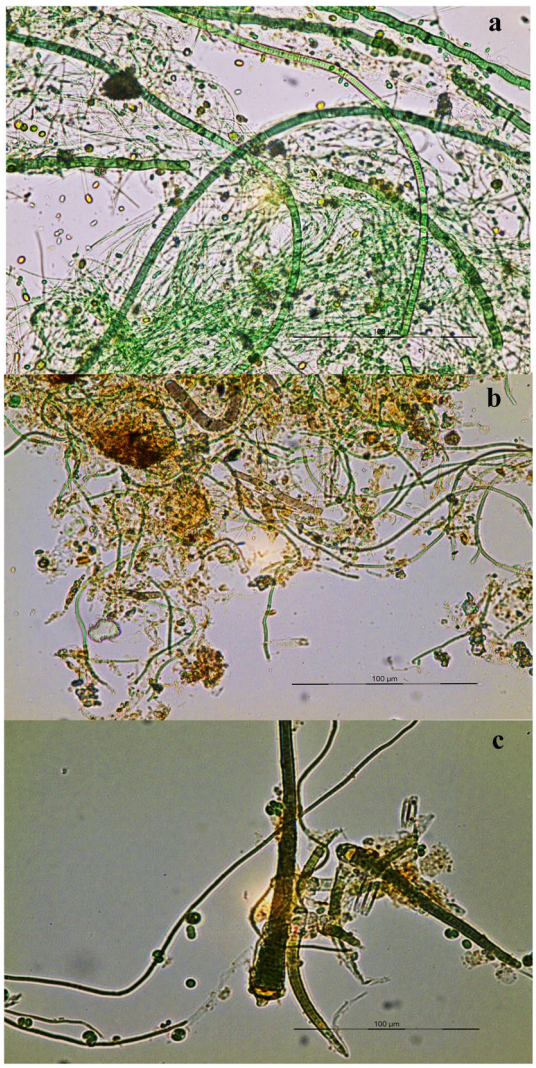

Fig. 2 Mixed cyanobacterial population present in **a** Paddy field (Kushmaur) soil; **b** Garden soil; **c** Pigeon pea field soil

(1981). These methods were able to yield axenic cultures and really accelerated the algological research, but majority of them required very long time (months!!) just to isolate a few cultures of cyanobacteria. The use of antibiotics like imipenem, neomycin, cycloheximide, etc. also worked up to some extent, but only a selected group of bacterial contaminants could be eliminated (Ferris and Hirsch 1991; Choi et al. 2008). Moreover, the time and cost remained an issue with such isolation methods. Streaking methods were easy to perform, but it took weeks and sometimes months to get uni-algal axenic cultures and, at the same time, agar posed as hurdle due to inability of many cyanobacteria to grow on agar (Ferris and Hirsch 1991; Lee et al. 2014). Many other isolation techniques required the use of special equipments or materials like nylon filtration tubes, vacuum-driven filtration apparatus, flow cytometer, ultra-sonicators, etc. (Elhai and Wolk 1988; Vázquez-Martínez et al. 2004; Doan et al. 2011; Shiraishi 2015). All these issues together impeded the pace of advancement in algological studies. From our own professional experiences, we have observed that in India, very few people (in comparison to other microbial groups!!) are working on cyanobacteria and the major issue is the lengthy work to purify cultures and maintaining their axenicity.

Enrichment of soil and water sample results in isolation of distinct cyanobacterial isolates. Rippka (1988) reported that if crude material contained a high percentage of larger filamentous forms, it would be better to separate the later. Kostikov et al. (2001) found that inoculation and cultivation of small portion of the soil sample (initial sample) in nutrient media resulted in obtaining mixed cultures of cyanobacteria which were further purified. It has been suggested that aqueous samples those are too dilute to isolate colonies directly on agar plates should be enriched in a liquid medium before isolation (Waterbury 2006). Basically, all these method suggested that an initial enrichment is quite necessary to increase the population size of cyanobacteria before isolating and purifying them. So keeping these in mind, in the present study, we have developed Microscope Assisted uni-algal isolation through Dilution (MAU-D) technique (involving an initial enrichment followed by isolation of uni-algal cells under microscope) which is a simple, time saving, and inexpensive modification of single cell isolation method to isolate uni-algal cyanobacterial cultures. The range for the frequency of obtaining uni-algal cultures using MAU-D was 80–92% which was quite high. Andersen and Kawachi (2005) reported a single cell isolation method using glass capillary or Pasteur pipette where the cells were sequentially washed in sterile droplets and observed under microscope to find droplets containing single cells. When three other methods, viz., repeated sub-culturing, single cell isolation, and streaking method, were compared to MAU-D, it was found to be more advantageous in terms of number of different cyanobacteria retrieved from the same sample and frequency of obtaining uni-algal cultures. Many of the cyanobacteria like *Aulosira, Hapalosiphon, Alkalinema, Halomicronema,* and *Aphanothece* could be isolated using all the techniques, but a few cyanobacteria like *Limnococcus, Lyngbya* sp. could be obtained from the same sample using the MAU-D method only. This may be due to the initial enrichment which might have helped to populate such cyanobacteria which were otherwise low in number in the original sample. Although MAU-D method, is quite similar

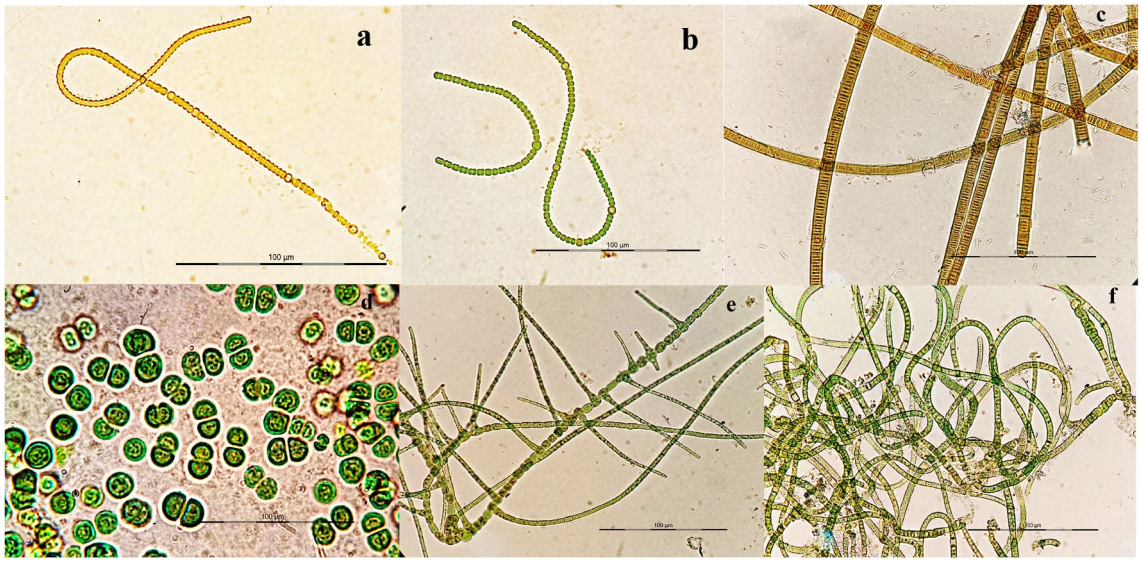

Fig. 3 Some of the cyanobacterial isolates obtained using MAU-D technique **a** *Aulosira laxa* K58; **b** *Nostoc* sp. K60; **c** *Lyngbya hieronymusii* K81; **d** *Limnococcus* sp. K71; **e** *Hapalosiphon* sp. K50; and **f** *Scytonema* sp. K55

to the method described by Andersen and Kawachi (2005), MAU-D method is distinctly different in few aspects like (1) serial dilution is used; (2) auto pipettes are used which are easy to handle, and (3) chances of cell injury is low (as the droplet generally contains many cells of single type) leading to high probability of revival in fresh medium, while in case of single cell isolation using capillary, the chances of cell injury due to capillary forces are high. Series of dilution with microscopic observations offer greater chances to obtain unialgal cultures. Less number of sub-culturing associated with MAU-D increases the probability of getting the cyanobacteria which are otherwise difficult to isolate through routinely followed repeated subcultures. Recently, Šulčius et al. (2017) reported a single cell pick up method which involved a setup having a micromanipulator attached with an inverted microscope. Though effective, this method involved complex instrumentation making the method costly along with the intricacies in handling an invert microscope–micromanipulator system. However, the MAU-D method did not require an inverted microscope; instead, a simple light microscope can be used. Another single cell sorting technique was available which used the fluorescence of chlorophyll present in cyanobacterial populations which could be monitored by flow cytometry (Reckermann 2000; Crosbie et al. 2003). But such techniques require knowledge of flow cytometry, trained man power. MAU-D technique follows simple rule of dilution and observation under microscope. It is worthy to mention that it is never possible to tap the entire cyanobacterial diversity following a single method of isolation, medium, or growth conditions. It is evident from the present study that using different isolation methods with same medium and growth conditions, it is possible to isolate different types of cyanobacteria.

Most significant observation we made after using this technique for isolation was the presence of genera like *Cyanobacterium*, *Toxopsis*, *Desertifilum*, *Chroococcidiopsis, Halomicronema*, and *Alkalinema* which have not been reported from rice fields of India yet (Anand and Kumar Hopper 1987; Nayak et al. 2004; Nayak and Prasanna 2007; Prasanna et al. 2009; Selvi and Sivakumar 2012; Singh et al. 2014; Adhikari and Baruah 2015; Srivastava et al. 2015; Vijayan and Ray 2015; Borah et al. 2016; Dash et al. 2017; Debnath et al. 2017). A recent study of cyanobacterial diversity from rice fields of Maharashtra reported 137 species from 35 genera with *Anabaena* and *Oscillatoria* as predominant genera (Ghadage and Karande 2019). However, none of the six newly reported genera in the present study were documented by them. Srivastava et al. (2009) characterized the molecular diversity of cyanobacteria from the rice fields of eastern Uttar Pradesh (Azamgarh, Mirzapur, Jaunpur, Chandauli, and Varanasi) which is well known to be affected with salinity problem. In this study, they mainly reported *Anabaena, Nostoc, Aulosira, Tolypothrix, Hapalosiphon, Oscillatoria, Phormidium, Lyngbya, Rivularia,*

Table 3 Comparison of different isolation techniques

	Repeated sub-culturing	Single cell isolation by micropipette	Streaking	MAU-D
Reference	(Gerloff et al. 1950)	(Andersen and Kawachi 2005)	(Lee et al. 2014)	This study
Duration required to obtain uni-algal cultures	Three growth cycles of 12–15 days each	One growth cycle of 15–20 days	Three growth cycles of 12–15 days each	One growth cycle of 15 days
No. of uni-algal cyanobacteria retrieved	04	05	07	13
Frequency (%) of obtaining uni-algal cultures	70.58	66.70	36.36	92.85
Identity of the cyanobacteria retrieved	*Aulosira laxa*, *Hapalosiphon* sp., *Alkalinema pantanalense*, *Halomicronema* sp.	*Aulosira laxa*, *Fortiea contorta*, *Aphanothece* sp., *Alkalinema pantanalense*, *Halomicronema* sp.,	*Aulosira laxa*, *Hapalosiphon* sp., *Fortiea contorta*, *Alkalinema pantanalense*, *Halomicronema* sp., *Leptolyngbya* sp., *Aphanothece* sp.	*Aulosira laxa*, *Hapalosiphon* sp., *Nostoc* sp., *Alkalinema pantanalense*, *Halomicronema* sp., *Leptolyngbya* sp., *Aphanothece* sp., *Limnococcus* sp., *Lyngbya wollei*, *Lyngbya hieronymusii*

Fischerella, Gloeothece, and *Aphanothece* with *Nostoc* and *Aulosira* predominant in saline soils. In another study, Tiwari et al. (2001) showed that rice fields of Allahabad, Varanasi, Pratapgarh, Bareilly, and Basti were inhabited by members of *Pseudanabaena, Limnothrix, Phormidium, Microcoleus, Oscillatoria, Lyngbya*, and *Plectonema*. Literature survey suggested that majority of the above -cited studies used routine sub-culturing techniques with or without minor modifications. In this study, we report six additional genera from the paddy fields of salinity affected areas of Mau- a district of eastern Uttar Pradesh, India. It is worthy to mention that majority (*Cyanobacterium, Desertifilum, Halomicronema*, and *Alkalinema*) of the six new genera reported in this study were tolerant to high salinity and have originally been reported from saline/hypersaline habitats (Abed et al. 2002; Moro et al. 2007; Dadheech et al. 2012; Vaz et al. 2015). This finding highlights the fact that the MAU-D method could isolate diverse cyanobacteria which were otherwise difficult to obtain through routine sub-culturing techniques leading to isolation of only few dominant cyanobacterial groups. Another interesting aspect of the MAU-D method was revealed by the molecular analyses of cyanobacterial isolates sequenced with bacteria-specific primers. The absence of bacteria-specific signals in the cyanobacterial DNA indicated the axenicity of the ten random samples. Molecular analyses can become ambiguous and complicated for the identification of uni-algal cyanobacteria due to DNA contamination and its unusual degree of genomic streamlining with its associated microorganisms, particularly bacteria (Erwin and Thacker 2008; Ran et al. 2010; Tripp et al. 2010).

Although we did not carry out any optimization, the mixed algal population was diluted by fivefold at every dilution step instead of tenfold dilution which could also be done if the algal growth was very high. Dilution at micro-volumes actually resulted in higher probability to get single cyanobacterial cultures in droplets. Despite framing the methodology conceptually, we could successfully establish and validate a simple method with much precision than many of the earlier reported methods. The present method had three distinct advantages: (1) simplicity for execution; (2) comparatively fast (may be completed in two cycles (15 days each) of growth); (3) ability to yield uni-algal cultures with a better representation of existing diversity which is difficult to with routine sub-culturing techniques. (4) Revival rate of cultures in fresh medium is comparatively higher as the method can also result in axenic cultures, but we believe that it may not be the case always. Although, we have tested this method on soil and water samples, it should be equally applicable for other samples too.

Table 4 Comparison of identification of ten random samples using cyanobacteria-specific and bacteria-specific primers

Sl. no	Isolate no	Identity based on cyanobacteria-specific primers [Cya359F & Cya781R(a)]	Identity based on bacteria-specific primers [27F & 1492R]
1	K50	*Hapalosiphon* sp.	*Hapalosiphon* sp.
2	K51	*Nostoc* sp.	*Nostoc* sp.
3	K53	*Aulosira laxa*	*Aulosira* sp.
4	K54	*Cyanobacterium aponinum*	*Cyanobacterium aponinum*
5	K55	*Scytonema* sp.	*Scytonema* sp.
6	K56	*Nostoc carneum*	*Nostoc* sp.
7	K57	*Aulosira laxa*	*Aulosira* sp.
8	K58	*Aulosira laxa*	*Aulosira* sp.
9	K59	*Aulosira laxa*	*Aulosira* sp.
10	K95	*Lyngbya hieronymusii*	*Lyngbya* sp.

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Authors contribution HC: contributed in conceptualization of the work. SV has optimized the methodology and validated the study. SV and SYB have investigated the study and prepared the original draft. HC, SV, SYB, PC, NS, and SD have contributed in writing—reviewing the draft. HC and AKS have supervised the study and edited the final draft and gave final approval of this version to be published. All authors read and approved the final manuscript.

Availability of data and materials Gene sequences are available in NCBI, Cultures are available in National Agriculturally Important Microbial Culture Collection (NAIMCC) at ICAR-NBAIM, Mau, Uttar Pradesh, India, and other information are available with the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interests.

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