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Determining the Reliability of Commoner Purification Methods and Mono-culturing Techniques for Microalgae

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Competing interests

The authors have declared that no competing interests exist.

Abstract

Background: The reliability of the purification process and monoculture technique for microalgae was investigated in this study. The study involves the isolation of microalgae from the environment of different localities to get the axenic culture of microalgae and test the reliability of other commonly used techniques. The vast usage of microalgae in industry and for the welfare of mankind demands the mono and pure culture of microalgae. Contaminants in the growth medium can alter the products which causes significant losses.

Materials and Methods: Morphological study of any strain highly demands pure culture, so different techniques are being used, but only some techniques are reliable.

Results: From the investigation, not a single technique is present which is entirely reliable, as contaminants are freely present in the universe so there are great chances of contamination.

Conclusion: Comparatively single cell picking and dilution to extinction are effective methods for investigation purposes. These techniques are more reliable when combined with other techniques, for example, the addition of antibiotics or the use of ultraviolet radiation and washing methods compositely used with the above-mentioned techniques gave fruitful and reliable results.

Key words: Dilution to Extinction, Microalgae Single Cell Purification, Monoculture

Introduction

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can thrive in very harsh conditions and grow quickly due to their simple multicellular or unicellular structures (Y. Li, Horsman, Wang, et al., 2008; Y. Li, Horsman, Wu, et al., 2008). Microalgae exist in both aquatic and terrestrial habitats. It has been estimated that almost 30,000 species of microalgae have been described and studied till now and more than 50,000 species are expected to be present (Li, Horsman, Wu, et al., 2008). By the process of photosynthesis, algae convert sunlight into chemical energy and reproduce (Larkum et al., 2020; Z. Wang et al., 2022; Xie et al., 2022; Xiong et al., 2023).

Microalgae are microscopic with chlorophyll and other photosynthetic pigments. The group of organisms which fulfill this definition are polyphyletic and have great diversity. Eukaryotic microalgae were developed from the endosymbiosis process. According to primary endosymbiosis, eukaryotes engulfed the cyanobacterium and became able to run photosynthesis in the structure which was named as plastid. According to the secondary endosymbiosis process, eukaryotes engulfed other photoautotrophic eukaryotes (Keeling, 2004; Larkum et al., 2020; Sato, 2020). Microscopically, algae variably consist of unicellular to colonial to fibrous form of cells. Microalgae are classified as prokaryotes that consist of cyanobacteria and blue-green algae or into eukaryotes, which include a range of diatom, green, and red-green growth among others. Growth is influenced by climatic changes (Bhat et al., 2022; Demirbas, 2010).

The photosynthetic pigments and morphological characteristics of the cells have distinguished microalgal species. Sometimes molecular techniques are very useful for their identification, particularly for the pico-sized fraction that has very rare morphological features that are used for identification (MacKeigan et al., 2022; Not et al., 2007; Xu et al., 2022). The phycologists have also developed molecular techniques for use as an indirect method of detection. These indirect methods are used to detect nucleic acids, carbohydrates, toxins, and proteins from microalgae. DNA-based approaches have been developed extensively, and this has expanded the understanding of molecular systematics, genetic diversity, and evolution for all organisms' not just microalgae (Bott et al., 2010; Kudela et al., 2010; Medlin & Kooistra, 2010). The goal of purification methods is to obtain a viable culture of a single species, free of all other species "contaminants" whether eukaryotes, prokaryotes, or viruses. The idea of pure cultures undoubtedly started at the time of Koch and Pasteur about bacteria. Extended next to eukaryotes, it first produced the term unialgal for single-species cultures of algae, and if cultures had no detectable contaminants, they were called pure or axenic. The term gnotobiotic is usually used for individuals of larger species reared free of microorganisms or parasites (Pokorny et al., 2022; Saxena et al., 2021). Some algae are nutritionally partially or wholly organo-heterotrophic, bacteri- vorous, planktivorous, or even carnivorous, it may be necessary to supply food as live or killed cultures of bacteria, other algae, or protists. Such fed cultures are sometimes called bixenic. Other cultures are beyond simple definition. For example, how should a culture of *Pinnularia* with endosymbiotic bacteria be regarded (B-Béres et al., 2023; Gabed et al., 2022; Schmid, 2003a, 2003b). Though the axenic monoculture of microalgae is most complicated in an open culture system, preliminary research on axenic conditions is the first step to getting axenic growth of microalgae under simple controlled conditions.

Several techniques for obtaining axenic cultures of microalgae are being practiced in parallel with rapid advancement in algal research. Various techniques have been developed for the axenic growth of microalgae as sub-culturing (Fernandez-Valenzuela et al., 2021; Mantzorou & Ververidis, 2019; Vu et al., 2018), serial dilution, micro pipetting, ultra-sonication, phototaxis, chemicals, ultraviolet radiation (Foo et al., 2023; Gasulla et al., 2010; Lee et al., 2021; Mantzorou & Ververidis, 2019). The present study was planned to determine the reliability of the purification processes and monoculture techniques that are being practiced worldwide.

Materials and Methods

Sample collection and isolation of microalgae

Water samples were collected from natural water bodies including ponds, rivers, and damp soil from district Kasur and were brought to the Applied and Environmental Microbiology Laboratory, Department of Wildlife and Ecology, UVAS, Ravi Campus, Pattoki, for the isolation and pure culture of microalgal strains employing different purification processes and monoculturing techniques. Sample was taken 100 mm below the water surface according to (Andersen & Kawachi, 2005).

Planktonic Biomass

The planktonic biomass was assessed by subtracting TDS from TS by the following equation.

$$\text{Plankton Biom} = \text{Total solids} - \text{Total dissolved solids}$$

Mono-culturing techniques

The methods performed for monoculture involved use of agar plates, differential centrifugation and dilution to extinction

Single cell picking

Single cell picking was performed with the help of a microscope and micropipette.

Procedure

A droplet was placed on a microscope slide and examined under the light microscope at 10×, 40× and 100× magnifying powers. The micropipette tip was filled with a small amount of sterilized water which acted as a cushion for picked cells. A single cell was picked using a micropipette while examining through microscope to ensure the required cell is picked and transferred to a 15 mL tube containing sterilized culture medium. The cell was also transferred to solid media to get growth in 7 days. The procedure was repeated several times to get a monoculture of microalgae.

Culture media

Stock solutions of BG 11 and BBM and working culture media were prepared according to the recipes in Table 1 and 2.

Table 1: Composition of BG-11 Medium

Stock Solutions for BG-11	
Stock 1 (g/L)	
Chemical	Required for g/L
Na ₂ Mg EDTA	0.1g
Ferric Ammonium citrate	0.6g
Citric ACID. 1H ₂ O	0.6g
CaCl ₂ .2H ₂ O	3.6g
Stock 2 (g/L)	
MgSO ₄ . 2H ₂ O	7.5g
Stock 3 (g/L)	
K ₂ HPO ₄ . 3H ₂ O OR K ₂ HPO ₄	4 or 3.05g
Stock 5 (g/L)	
H ₃ BO ₃	2.86g
MnCl ₂ . 4H ₂ O	1.81g
ZnSO ₄ . 7H ₂ O	0.222g
CuSO ₄ . 5H ₂ O	0.07g
CoCl ₂ .6H ₂ O	0.050g
NaMoO ₄ . 2H ₂ O OR MoO ₄ (85%)	0.391 or 0.018g
Basic BG-11 medium	
Stock 1	10L
Stock 2	10L
Stock 3	10L
NaNO ₃	1.5g
Na ₂ CCO ₃	0.02 g
Stock 5	10 mL

Preparation of agar plate

Agar plates were prepared by mixing the 1.8 g agar for 100 ml medium. The solution was boiled on a hot plate to dissolve the agar in the medium. The mixture was autoclaved at 150°C for sterilization. After which the agar medium was cooled and poured to clean and sterilize plates in laminar flow. The agar plates were stored in the refrigerator for 24 hours before these were used.

Streak plate procedure

The agar plates were used for streaking under sterile conditions. The lid of the growth tube was opened, and the sterilized inoculation loop was touched with the sample. A Streak was made using the inoculum on the agar in the quadrant. The process was repeated for remaining three quadrants to get another streak on the same plate at 90° of the previous quadrant. After streaking, the inverted plate was incubated in growth chamber at 26°C under light for 7 days, followed by examination of growth pattern. Single cells were picked and transferred to new plate to obtained single cell culture.

Table 2: Composition of BB medium

Stocks	g/400 mL
NaNO ₃	10.0g
MgSO ₄ . 7H ₂ O	3.0g
NaCl	1.0g
K ₂ HPO ₄	3.0g
KH ₂ PO ₄	7.0g
CaCl ₂ . 2H ₂ O	1.0g
Stocks	g/1000mL
Trace Elements	
ZnSO ₄ . 7H ₂ O	8.82g
MnCl ₂ .4H ₂ O	1.44g
MoO ₃	0.71g
CuSO ₄ . 5H ₂ O	1.57g
Co(NO ₃) ₂ . 6H ₂ O	0.49g
H ³ BO ³	1.42g
EDTA	50.0g
KOH	31.0g
FeSO ₄ . 7H ₂ O	4.98g
H ₂ SO ₄ (conc.)	1.0 mL
Combine the following stock solutions per liter of medium.	
Stock 1-6	10 mL
Stock 7-10	10 mL

Spreading plate procedure

In spread plate colonies, micropipette tips were autoclaved, and 0.03 mL inoculum was transferred to the agar plate under sterilized conditions. A sterilized spreader made by bending the pasture tube in an “L” shape, was used to spread the inoculum on the agar plate. Finally, the inverted plates were incubated in the growth chamber at 26°C under light for 7 days, followed by growth pattern examination. The required colony was picked for further growth.

Differential centrifugation

Filtered natural water with algal samples were centrifuged at 3000 rpm. The supernatant was discarded, and the pellets were resuspended using the distilled water stirred for 3 minutes, and again re-centrifuged. The process was repeated three to four times. The pellets were used as inoculum for liquid and solid media to establish algal growth in a growth chamber for a week.

Dilution-to-extinction method

In this method, 9 mL distilled water in 12 labelled capped tubes were autoclaved for sterilization. The first tube was supplemented with 1 mL algae water sample under sterilized condition. After 5 minutes tube was gently shaken gently, and 1 mL sample was transferred to the second tube under sterilized condition. A serial dilution of the algal sample was prepared following the serial dilution method with the probability to get

single cell in any tube. The dilutions were used for inoculation of agar plates.

Purification processes

Axenic culture means the unialgal culture free of the other organism. The axenic algal culture can be developed using antibiotics, UV treatment and use of different antimicrobial agents. Broad-spectrum antibiotics like amoxiclav (Droop, 1967; Guillard, 2005) can inhibit bacterial contamination while establishing algal culture. An addition of 2mg antibiotic/10ml of growth media serves the purpose of axenic algal culture.

Most algae are resistant to UV light compared to bacterial cells. A 5ml microalgae sample was exposed to UV light for 5 minutes and later used to inoculate the solid and liquid culture medium under the sterilized condition followed by growth observation after 7 days.

Besides antibiotics, other antimicrobial agents like 70% Ethanol, can eliminate the bacterial infection from the sample under study.

The microalgae growth was treated with 70% Ethanol for 10 minutes. For this purpose, the culture was taken in eppendorf tubes, centrifuged to 10000 rpm for 3 minutes. The supernatant was discarded, and the eppendorf was filled with 50 and 60 % ethanol to resuspend the microalgal pellet, for 3 minutes followed by re-centrifuged. The sample was treated 3 times before it was used to inoculate the liquid and solid culture medium followed by growth observation after 7 days.

Results

Mono-culturing techniques

Single cell picking

A single cell was picked by using the micropipette under the microscope and transferred to a rinsing droplet. After rinsing many times single cell was transferred to agar media. Many trials were repeated for different cells. Some trails were unfruitful due to damage to the cell during rinsing and transferring. After 7 days the growth of colonies was prominent, each colony originated from a single CFU. Single cell picking is reliable for mono-culturing, but minor bacterial contamination was noticed during handling (Figures 1 and 2).

Agar plates

In streak Plate, the plate was streaked with an inoculating loop under aseptic condition. The initial streak contained the intense colonies but at the end of the streak, colonies were apart and separated. These colonies were further picked (Figure 3).

In spreading method, 0.3ml inoculum having different biomass concentration of microalgae was spread on agar plates. The number of colonies were directly proportional to the biomass concentration of microalgae and inversely proportion to the dilution factor of the sample. Clear colonies were obtained during this approach.

Differential centrifugation

In gravity separation and differential centrifugation, only larger organisms were separated. Growth of the microalgae did not show uniformity in colonies and bacterial colonies were noticed in petri plates. This method was not reliable for monoculture and axenicity in first attempt, however, repeating the process by discarding the supernatant and addition of sterilized water showed fruitful results.

Dilution-to-extinction method

Different dilutions of microalgae growth were made, and each dilution of 0.3 ml was spread on the agar plates. Dilution to extinction technique growth resulted in separate colonies (Figure 4). This technique was effective for monoculture but not reliable for axenic culture as the abundant growth of bacteria were observed as compared to microalgae. Conjugation of this technique with washing with distilled water and use of antibiotic was more effective for axenic and monoculture.

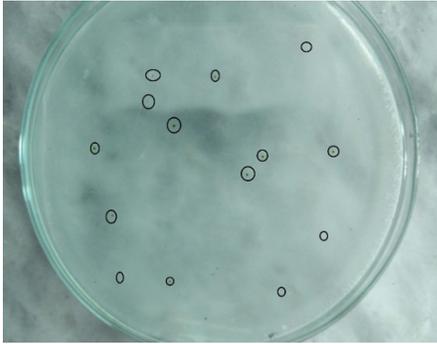


Figure 1: Single cell picked and transferred to agar medium

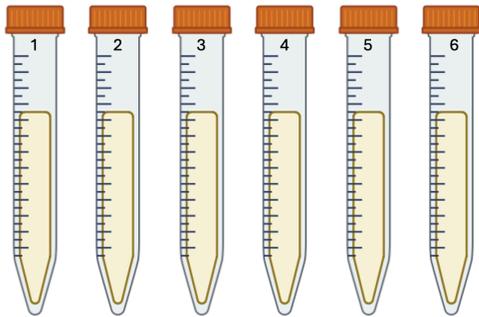


Figure 2: Single cell picked and transferred to BG11 broth



Figure 3: microalgae streaking on agar medium

Purification processes

Microalgae treatment with amoxiclav antibiotic for 24 hours showed bacteria free growth with no effect on microalgal growth. Exposure of the microalgal growth to UV light for 5 minutes showed an effective result for axenicity and did not show a negative effect on the microalgae and chlorophyll (Figure 5). The use of 50% ethanol as an antimicrobial agent produced

axenic culture but it greatly affected the microalgal cell. It destroyed many microalgal cells and affected their viability.

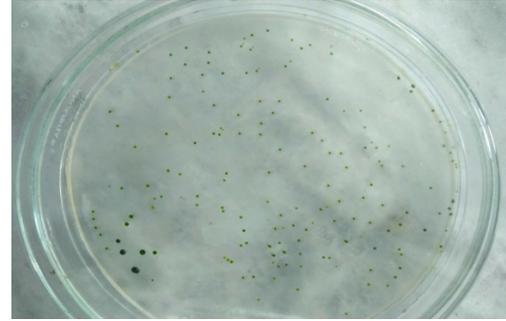


Figure 4: Dilution to extinction growth



Figure 5: Microalgae exposure to UV

Discussion

The purpose of the study was to test the reliability of commonly used monoculture techniques and purification processes. Protocols for different techniques were followed according to (Andersen & Kawachi, 2005) for microalgae growth according to standard methods prescribed earlier (ASTM, 2000; Barros et al., 2019; Hossain & Mahlia, 2019).

Environmental factors greatly affect biological activities. A critical range of environmental factors are valuable for sustainable life. Most of the environmental parameters were suitable for proper growth and acted as the model for the laboratory growth of microalgae. Temperature is an important and basic component that allows the microalgal biomass to flourish at the proper rate. The main environmental components derive the microalgae growth light, water temperature, nutrient concentration, salinity, pH (Mutanda et al., 2011), and CO₂ concentration as it is a basic requirement for microalgae which depends on the aeration during laboratory trials. The temperature was (Wang et al., 2007) 26°C suitable for growth which was comparable to the study of ranging from 22 to 30°C.

Single cell picking through a smooth tip of the micropipette is favorable method as irregular tips damage the cell structure, and the cell loses its viability. It also depends on the sterility of the environment. Sometimes contaminations can occur through the micropipette tips disturbing the single cell transfer (Parvin et al., 2007; Weiskirchen et al., 2023). Sterilized water sucking before picking the cell was effective as it works like a cushion for receptive cells and prevents them from damage. Single cell

picking is the reliable technique for axenic and monoculture for the microalgae however, it can be more reliable by composite usage of this technique along with other techniques like the cell washing (Huang et al., 2021; Parvin et al., 2007).

The agar plates method is the beneficial for monoculture of microalgae however, this method does not provide axenic culture, and bacterial contaminations have been reported (Andersen & Kawachi, 2005). In the streak plate method, the far point to the start of the streak sets colonies apart from each other. But there were bacterial colonies. BBM and BG11 medium has shown best growth of *C. vulgaris* (Ilavarasi et al., 2011; Parvin et al., 2007). The dilution of algal growth also gives separate and distinct colonies of microalgae, which were further grown to get the axenic culture of microalgae (Vu et al., 2018).

Differential centrifugation used to separate the large organism from the sample. Centrifugation was effective in combination with washing, antibiotic, and agar plates. The centrifugation washing and exposure to antibiotics gave the axenic colonies of microalgae (Parvin et al., 2007).

Dilution to extinction is the most used technique in microalgae study. Mare's use of dilution was not so effective as the number of bacteria in the environment was huge. Serial dilution was the more effective to give clear and bacteria free culture (Piredda et al., 2017; Sena et al., 2011).

Use of antibiotics and UV radiations were found reliable methods as these did not affect the growth of microalgae and eliminated the bacterial strains. Use of 70 percent ethanol on the other hand affected both bacterial and algal cells (Leng et al., 2020; C. Li et al., 2022).

Conclusion and Recommendations

In the presence study the suitability and reliability of different microalgal culturing techniques was determined. The findings of this study suggests that single cell picking is the most suitable method to establish monoculture of microalgae.

Authors' contributions

SS and MAr contributed to the conceptual framework, methodology, and manuscript preparation. MM was involved in data collection, experimental work, and manuscript drafting. ZS, NS, and MArS contributed to data analysis and interpretation. AFT and AT assisted with the statistical analysis and manuscript review. MAm provided additional expertise in methodology and manuscript revision. All authors read and approved of the final manuscript.

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