Different methods for microalgae Isolation

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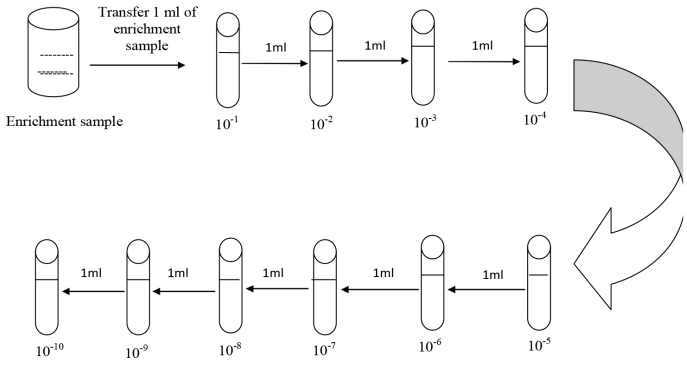
Once the decision of which microalgae needs to be cultured is taken, the first step is to isolate the pure culture. Even though the cultures are available from specialized culture collection centers, isolation of endemic strain is advisable because the strain can grow better in local environmental conditions. Collection of micro algal sample is the crucial step which can be done using plankton nets of mesh size 10, 30 and 120 μm. The collected micro algae need to be kept in sterile nutrient solution to keep them alive and allow them to grow. Some amount of the sample can be concentrated and preserved in 10% buffered formalin for the identification in the laboratory. While sample collection both biotic (light, water temperature, dissolved oxygen, dissolved carbon dioxide, nutrient concentration, pH, salinity) and abiotic factors (pathogen) at the sampling site need to be considered. Technique used for the isolation of micro algae includes technique of dilution, single cell isolation by micropipette and agar streaking.

i. Dilution Technique

This isolation technique is suitable for organisms which are abundant in samples and ineffective for rare organisms. This technique enables isolation of a single cell by repeated attempts. In dilution technique, inoculates large number of test tubes with culture media (dilution ranging from 10^{-1} - 10^{-10}). After inoculating, incubate the test tubes under controlled temperature and light. Examine the culture after 2-4 weeks by taking a small sample aseptically from each dilution tube. Uni-algal culture may grow in any of the higher dilution tubes. The success of the present technique is highly dependent on the



Serial dilution technique for isolation of micro algae

accuracy of a measured amount of cell culture during transfer from one medium to another. Axenic isolates are not possible with dilution technique because bacteria are more abundant than algae.

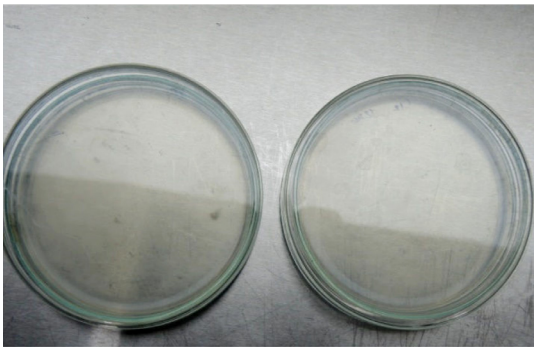
ii. Single cell isolation by micropipette

This is the most common isolation technique, which is performed with a pasture pipette or glass capillary under microscopic observation. These single cells are transferred to sterile droplets of water or suitable media. The technique requires expertise and precision. The cells can get damaged due to shear stress caused by micropipette or capillary tips. Caution is necessary for successful implementation of the present technique. Ultrapure droplets are required, especially for marine samples, to distinguish between microalgal cells and other particulate matters.

Isolation of single cell with the help of microscope

iii. Streaking cells along agar plates

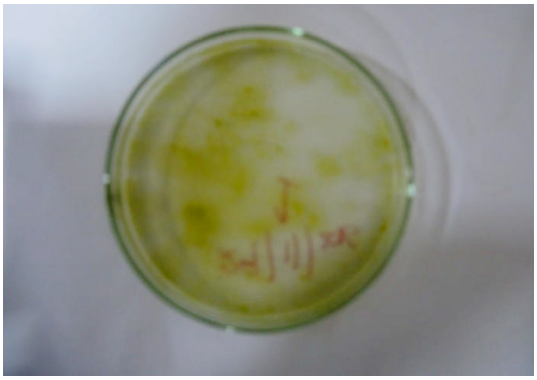
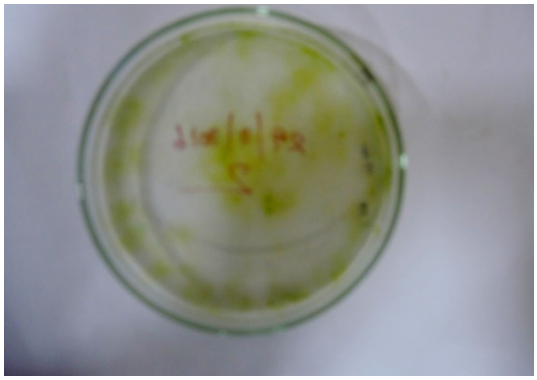
Isolation of cells on agar plate is an old and common method. For successful isolation onto agar the desired algae should be able to grow well on agar plates. For preparing the agar medium, 0.8 to 2 gm of agar is addedto a litre of natural filtered seawater in a conical flask. The flask containing agar is usually heated on a flame and boiled twice till the agar is dissolved in water. The culture media 'or' nutrients (solution A and B of Conway media or F2 media) are added to the agar



Agar plates for streaking of micro algal culture

Streaking of micro algal culture on Agar plates

solution. The flask mouth is usually covered with Aluminium foil and then the flask is autoclaved for 15 minutes under 15 lbs pressure and 121°C temperature. The petri dishes are usually sterilized by keeping for 30 minutes at 150 °C in hot air oven. The solution C of culture media is added aseptically to the autoclaved agar flask once the temperature has been brought down to 40 °C. Then, agar plates are prepared aseptically by pouring the warm autoclaved agar into the sterile petri dishes near a Bunsen flame or in a laminar flow, followed by covering up the petri dishes and leaving them to cool for about 2 h.

Micro algae (*Nannochloropsis* sp.) colonies on agar plates isolated on agar plates

Isolation is accomplished by streaking the sample across the agar surface (0.8-2%). The streaking can be done with the help of 'loop' or spreader. After streaking, the agar plate is incubated until colonies of the cells appear. The isolated colonies are removed from the agar plate and can be further subcultured onto other agar plates. Axenic cultures without contamination can be produced with this method.

Growth dynamics of microalgae

The knowledge of microalgae growth dynamics is important for aquaculturist to know when to harvest the microalgae, to estimate growth rate and population doubling time. Generally, the microalgal culture follows a characteristic pattern of growth and follows five reasonably well defined phase of algal growth in batch culture.

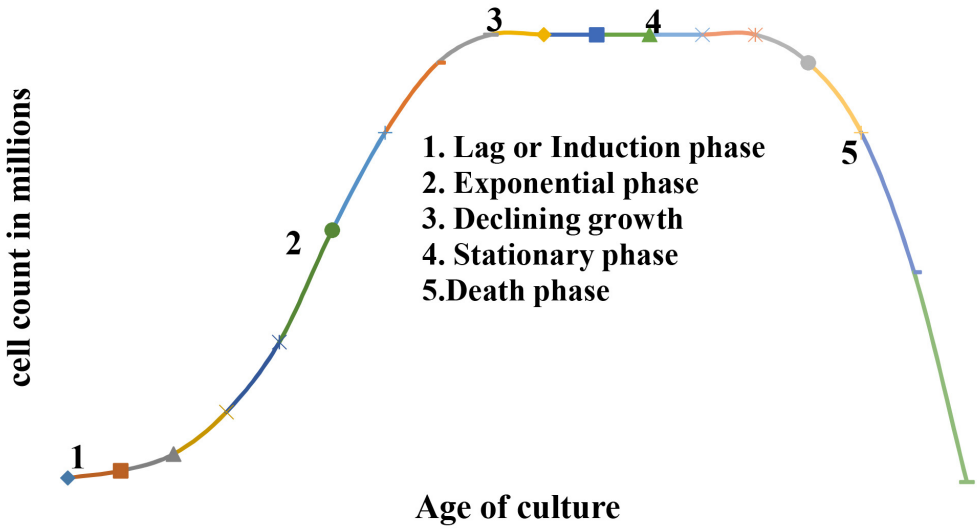
i. Lag or Induction phase

ii. Exponential phase

iii. Declining phase

iv. Stationary Phase

v. Death phase



Growth curve of micro algae (*Nannochloropsis* sp.)

i. Lag or induction phase

The Inoculation of culture into a new medium have to acclimatize with the surroundings or to the new physico- chemical conditions, so there will be no cell division for slight time thus the stage is known as lag or induction phase. The condition of the inoculum has a strong bearing on the duration of the lag phase. An inoculum taken from a healthy exponentially growing culture is unlikely to have any lag phase when transferred to fresh medium under similar growth physico-chemical conditions. In general the length of the lag phase will be proportional to the length of time the inoculum has been in phases 3 to 5. A lag phase may also occur if the inoculum is transferred from one set of growth conditions to another.

ii. Exponential phase

Once the inoculum is acclimatized to the new physico-chemical conditions, it starts multiplication and grows rapidly, thus this phase is known as exponential phase. The duration of exponential phase in cultures depends upon the volumeof the inoculum, the growth rate and the capacity of the medium and culturing conditions to support algal growth. The growth rate of a microalgal population is to measure the increase in biomass over time and it is calculated from the exponential phase. Growth rate is one important way of expressing the relative ecological success of a species or strain in adapting to its natural. Cell count and dry weight are common units of

biomass determination. Once the growth phase has been plotted (time on x-axis and biomass on logarthmic y-axis) careful determination of the exponential (straight line) phase of growth is needed. Two points, N_1 and N_2, at the extremes of this linear phase are taken and substituted into the equation (Levasseur *et al* 1993).

Specific growth rate (μe)

The specific growth rate of microalgae was calculated using the equation:

μ = ln (N2 / N1) /(t2-t1)

where μ= specific growth rate (d^{-1}), N1 and N2 are the number of cells. mL^{-1} at the time t1 and t2, respectively.

Duplication time

The time for the number of cells (cells. mL^{-1}) of microalgae to double in number was calculated

using the equation

$t_g = \ln 2/ \mu$

where tg (days) is the time for cell duplication and μe is the specific growth rate, and ln2 is the natural logarithm of 2 (approx. 0.693).

Division rate, $k = \mu / \ln 2$

The volumetric productivity, Px (cell L^{-1} h^{-1}) = $C_x \mu$

where Cx (cell mL^{-1}) refers to maximum cell density achieved from the batch cultivation.

iii. Declining phase

When the death of the cells will exceed the multiplication of the cells in a culture, then the phase is known as declining phase. This phase generally occurs in culture when either a specific requirement for cell division is limiting or something else is inhibiting reproduction. The biomass is often very high and exhaustion of a nutrient salt, limiting carbon dioxide or light limitation becomes the primary cause of declining growth. At low cell densities too much CO_2 may lower the pH and depress growth. CO_2 limitation at high cell densities control any further biomass increase to be linear rather than exponential (with respect to time) and proportional to the input of CO_2. Light limitation at high biomass occurs when the cells absorb most of the incoming irradiation and individual cells shade each other. This is known as self-shading.

Microalgae are generally well adapted to surviving conditions of low incident light and may survive for extended periods under these conditions; however the growth will be linear rather than exponential.

iv. Stationary phase

After the arrested growth, the culture will be stationary without any further cell division. The net growth of the culture becomes zero during this phase and within an hour's culture cells may undergo dramatic biochemical changes. The nature of the changes depends upon the growth limiting factor. Nitrogen limitation may result in the reduction in protein content and relative or absolute changes in lipid and carbohydrate content. Light limitation might affect the pigment content of most species and changes in fatty acid composition. Light intensities that were optimal for growth in the first 3 phases can now become stressful and lead to a condition known as photo inhibition. It is important to note that while the measured light intensity within the culture will decrease with increasing biomass, if the incident illumination is maintained relatively high then a large proportion of cells may become stressed, leading to photo inhibition and the culture can be pushed into the death phase. This is especially more if the culture is also nutrient stressed. It is preferable for many species to further reduce the incident light intensity when cultures enter stationary phase to avoid photo inhibition. The lowering of temperature combined with lower irradiance can reduce stress in many species. The longer the cells are held in this condition the longer the lag phase will be when cells are returned to good growth conditions for sub culture.

v. Death phase

This is last phase of growth dynamics, when the cells may lose its viability and start to die. The death phase of culture is generally very rapid, thus the term 'culture crash" is often used for this phase. The cultures of some species will loose their pigmentation and appear washed out or cloudy, whereas cells of other species may lyse (no recognizable cells) but the culture colour will be maintained. The latter is an important consideration and one reason why colour should not be relied upon to gauge culture health. Bacteria which may have been kept in check during exponential and early stationary phase may "explode" as cell membrane integrity becomes progressively compromised or leaky and a rich carbon source for bacterial growth is released. Free pigment and bacterial growth are further reasons why measures of turbidity or fluorescence should not be used beyond early stationary phase as surrogate biomass indicators, or especially as indicators of culture health.

Occassionally cell growth of some species can reoccur after a culture has apparently died. In this instance most vegetative cells will have died, and possibly most of the bacteria, releasing nutrients back into the media. Then either the very few remaining vegetative cells or more likely germination of cysts or temporary cysts will be able to fund this secondary growth.

Maintanance of algae culture

Algal multiplication is normally dependant of various steps like culture water sterilization, nutrient enrichment, inoculation of new culture with the pure algal strain, microscopic observations for growth of the algal cells and finally mass culture of algae in larger containers. **Stock culture** provides reservoir of algae cells from which large cultures can be initiated. Stock cultures are kept in small volume containers. S**ubculture** involves inoculating some cells from old stock culture to fresh culture medium, so that the cells can continue to grow, devide and healthy. If subculturing is not done, cells in the stock culture will die eventually. Precaution maust be taken while transfering the stock culture, so that contaminants from air should not enter into the stock culture.