CRYOPRESERVATION OF THE OUTDOOR ALGAL CULTURE

A Dissertation Report submitted

for the partial fulfilment of the Degree of Master of Science

By

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[M.Sc. Biotechnology]

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Master in Science

DEPARTMENT OF BIOTECHNOLOGY

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This is to certify that this training report entitled **"Cryopreservation of outdoor algal culture"** was successfully carried out by Miss Princy Patel towards the partial fulfilment of requirements for the degree of Master of Science in Biotechnology of Atmiya University, Rajkot. It is an authentic record of her own work, carried out by academic year of 2022-23. The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University.

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This is to certify that ***Ms. Princy Patel***, a student of M.Sc. Biotechnology from Atmiya Institute of Technology & Science has undergone training from 04 Jan 2023 to 31 Mar 2023. The Trainee has developed a project on "*Cryopreservation Of The Outdoor Algal Culture*".

The Trainee has shown keen interest in the training and was punctual and sincere during training.

We wish the trainee all the best for future endeavors.

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I hereby declare that the work incorporated in the present dissertation report entitled "**Cryopreservation of the outdoor algal culture"** is my own work and is original. This work (in part or in full) has not been submitted to any University for the award of any Degree or a Diploma.

Date: 31st March,2023 (Princy Patel)

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Cryopreservation of the outdoor algal culture

Objectives:

1. **Thawing of the cryo-preserved culture.**
2. **Culture acclimatization and study culture growth pattern.**
3. **Inoculum scale-up to support cultivation operation.**

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ABSTRACT

Cryopreservation of algal culture refers to the process of freezing and storing algal cells at very low temperatures (-196°C) in liquid nitrogen for long-term preservation. The purpose of cryopreservation of algal culture is to maintain the genetic stability of the culture and to ensure that the culture can be revived in the future for further study or use.

Cryopreservation provides a reliable and cost-effective method for long-term storage of algal cultures without the need for continuous sub-culturing, which can be time-consuming and labor-intensive. By preserving algal cultures in liquid nitrogen, using different cryoprotectant which prevent cell damage can ensure the availability of the culture for future research and development, even if the original culture is lost or contaminated.

Additionally, cryopreservation allows for the preservation of a diverse range of algal strains, including rare and endangered species, which might otherwise be lost due to environmental changes, habitat destruction, or other factors. Easy revival of the cryopreserved culture under controlled parameters affecting algal growth. Revival culture further support to scale-up of culture which is pure strain.

COMPANY INFORMATION

Reliance Industries Limited is an Indian multinational conglomerate company, Jamnagar. It has diverse business including energy, petrochemicals, natural gas, retail, telecommunications, mass media and textiles. Currently I am trainee at reliance Gagva site also known as RIL biofuel R & D Site. It covers area of 40 acres of land. This Pilot plant work to develop algae on sea water and covert into biofuel but currently it is working biorefinery from algae. Comprised of 150+ scientist and team of engineers , recently working on this. This technology takes carbon dioxide waste from refinery, then combines it with algae and sunlight to produce bio-crude oil that could one-day fuel carbon- neutral air travel.

INTRODUCTION

The ever-booming population of the globe, climate change, depletion of fossil fuels and ever increasing demand for food and energy are some of the paramount concerns of this century. The ever-surging dependence on conventional fuel sources has ignited an interest in securing alternative sustainable options when fossil fuels run dry. The prime external source of energy to earth is from the sun. A considerable proportion of this energy is saddled by developing oil yields to photo-synthetically convert sunlight-based energy into fuel. Experts across the globe have investigated yields, for example, sugar sticks for bioethanol, soybean, palm oil, and assault seeds for biodiesel to get what's to come requests through sustainable biomass. Apart from all these perks, these biofuels possess different downsides as well; one of them includes unavailability of sufficient inventory of the biomass required for fuel creation. Furthermore, this harvest competes with the assets expected for food security like rich land and freshwater. In the ongoing situation, just unambiguous parts or compounds of these oil crops/plants are used for the Biofuel age. Microalgae species are reported to have high efficiency for photosynthetic conversion of sunlight compared to the 1^{st} and 2^{nd} generation biofuel sources. Third generation biofuel is basically advanced algae-based biodiesel while fourth generation biofuels are created using petroleum-like hydro processing or advanced biochemistry.

Here, we are aware about the algae upstream processes like isolation and preservation of culture, culture cultivation in outdoor conditions and harvesting methods. So, here our sole purpose is to know all these processes for outdoor cultivation of algae and synthesize bio-products from it.

Why algae are preferred for biodiesel production?

- Comparatively higher oil content and quick biomass production.
- Autotrophic in nature
- Utilizes CO_2 as an energy source
- Uses environmental energy and hence the energy consumption is minimal
- Reduces greenhouse effect and global warming

OVERVIEW OF ALGAE

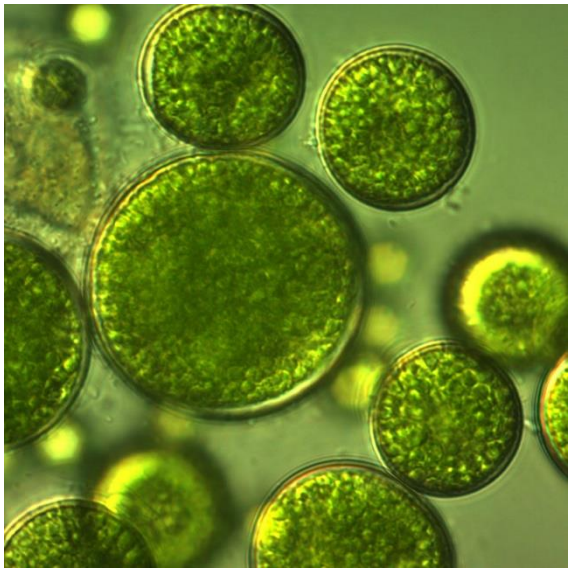


Figure 1 Microalgae cells

Microalgae comprises of a diverse group of microorganisms that can be found in water, soil, air, tree bark microhabitats, and in some cases even on animals. Microalgae are eukaryotic microorganisms containing pigments like chlorophyll, carotenoids, phycocyanin and astaxanthin that are bound as individual's cells or small colonies. Compared to other microbes and terrestrial plants, microalgae have several unique remarkable features. As the preliminary procedure involved in performing photosynthesis involves, absorbance of sunlight (photons) and assimilation of carbon dioxide from the atmosphere for biomass production.

Microalgae can be cultivated on unproductive land patches such as infertile arid, semi-arid lands and polluted soils that are not usable for conventional agriculture. These microorganisms can grow in saline water and even in nutrient-enriched wastewater. The same strain of microalgae can be replicated year around and can be harvested on daily basis.

Microalgae are photosynthetic species that make use of sunlight to convert nutrients present in the medium (i.e. water) into bioactive components in their cell structure. With regards to the suspension nature of the medium, microalgae growth can be administered and automated with better precision. Microalgae can be cultivated with three major sources, including water, sunlight, and CO_2. These resources are plentiful and affordable. Moreover, the resources required for cultivation of microalgae do not compete with conventional crops.

Nevertheless, the culture medium has to be nutrient-rich and must necessarily contain various salts essential for the growth of microalgae. However, these nutrients can be employed from household or industrial wastewater.

The microalgae biomass can be straightaway transformed into bio-fuel via four techniques.

1. Bio– chemical conversion
2. Thermochemical conversion
3. Trans esterification
4. Microbial fuel cell

The choice of selecting an appropriate process depends upon several parameters such as specification and the type of project, the availability of crude biomass feedstock, and the budget of the project.

The crucial stages of microalgae bio refinery are upstream and downstream processing. The upstream process mainly consists of microalgae cultivation. The raw materials involved in the upstream process are nutrients, water, light, and CO_2. The nutrients such as Phosphorous and Nitrogen determine the extent of growth of microalgae. An optimum amount of nutrient supply will ensure higher biomass production and a shorter maturation period. The source of illumination also affects the growth rate of microalgae.

Several studies were conducted that provides evidence for illumination via artificial lighting such as LED is more effective than direct sunlight for microalgae cultivation.

The downstream processing of microalgae biomass consists of harvesting, extraction, and purification of value-added products. The conventional extraction techniques include mechanical methods such as bead beating and blending, high-pressure homogenization, and ultrasound and chemical methods such as solvent extraction. Other processes such as freezing-thawing, autoclaving, and supercritical fluids have also been utilized. These processes are complex, involving a plethora of steps, and are costly. The economic trammel incurred due to these processes is enormous and extraction of various high-value products from microalga biomass should be economically feasible.

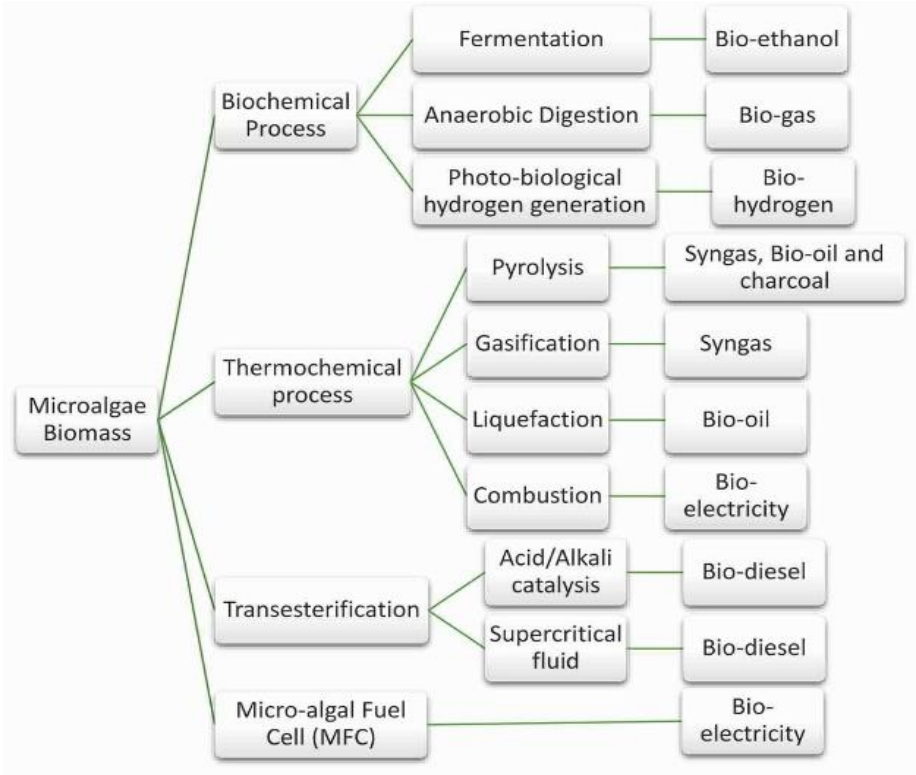


Figure 2 Techniques for microalgae biomass converted to biofuel

Microalgae biomass can be broadly divided into three fractions; oil, protein, and carbohydrate. Focus is on possible product streams to obtain numerous products from a single energy flow. The by-products or residual wastes obtained can be either recycled in the culture medium as nutrients or used to produce power in the form of combined heat and power (CHP) plant in the bio-refinery.

UPSTREAM PROCESS

Field collection

Various types of habitats such as salt water, fresh water bodies are surveyed. Water tests with noticeable microalgae populace are gathered from lakes. Inspection of enormous collections of new water happens at various destinations along the waterfront. Assortments are made for the top and lower part of the water at every area in order to determine and decide the predominant microalgae species in every space.

Isolation

Isolation of cells of microalga is the technique whereby a cell is picked from the sample using a micropipette or glass capillary under microscopic observation and in a sterile condition. These single cells are then transferred to sterile droplets of water or suitable media. This technique requires expertise and precision. Several techniques are used for isolation such as serial dilution followed by the streak plate method, pour plate method, spread plate method, etc.

Serial Dilution: Serial dilution is a process through which the concentration of organisms is systematically reduced through successive re-suspension in a fixed volume of liquid diluent.

Streak Plate method: Streak plate technique is used to grow organism on a growth media surface so that individual organism colonies are isolated and sampled.

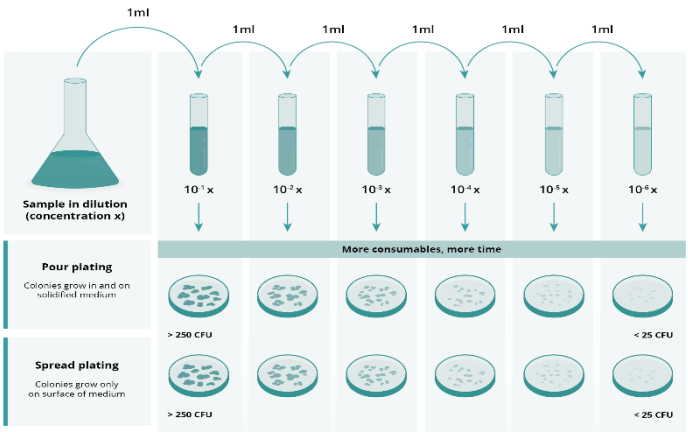


Figure 3 Serial dilution, streak plate and pour plate method

Pour Plate method: Pour plate method usually encompasses the method of choice for counting the number of colonies forming organisms present in a liquid specimen since the sample is mixed with the molten agar medium.

Spread plate method: The spread plate method is a technique to plate a liquid sample containing microorganisms so that they are easy to count and isolate. A successful spread plate method will have a countable number of isolated colonies evenly distributed on the plate.[2]

Culture preservation techniques

The primary aim of culture preservation is to maintain the organism alive, uncontaminated, and without variation or mutation, that is, to preserve the culture in a condition that is as close as possible to the original isolate.

Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure culture free from contamination. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination.

Since repeated subculturing is time consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition, there is a risk of genetic changes as well as contamination. Therefore, it is now being replaced by some modern methods that do not need frequents subculturing. These methods include refrigeration, paraffin method, cryopreservation, and lyophilization (freeze drying), etc.

Preservation technique	Application
Formalin	Preserve colour as well as morphological organism. Useful for long - term storage. Formalin is a good preservative for green algae, cyanobacteria, dinoflagellates, etc.
Glutaraldehyde	Formalin is good preservation for green algae, cyanobacteria, dinoflagellates etc.
Iodine	Widely used for short-term preservation. Help to keep cell shape intact form.
Formalin Acetic acid- Alcohol (FAA)	It is a mixture of formalin, glacial acetic acid, and alcohol. Used to preserve flagella organisms.
Gelatin	Preserve the cell structure and colour of the microalgae. If kept at frozen (-20 degree

	Celsius) conditions, the algal sample can be stored for a long period.
Lyophilization (freeze - drying)	The alga is frozen and then dehydrated at low temperature in a high vacuum. The water is removed by sublimation.
Cryopreservation	Liquid nitrogen at -196 degree Celsius is used to preserve certain algal species.

Table 1 Preservation techniques and application

Here, we used cryopreservation method for long term storage of pure culture. Cryopreservation method is reliable and cost-effective method for long term storage. Using different cryoprotectant effectively store culture at higher concentration (5-10%) slurry with one pure strain with high revival chance of cells.

Outdoor Cultivation

Algae culture is a form of aquaculture involving the farming of species of algae. The species of algae that are intentionally cultivated fall under the category of microalgae (also referred to as phytoplankton, microphytes, or planktonic algae). The two most commonly used methods of microalgae cultivation are open cultivation systems, such as open ponds, tanks, and raceway ponds, and controlled closed cultivation systems using different types of bioreactors.

- **Open pond cultivation**

Open pond cultivation has been one of the oldest and simplest ways to cultivate microalgae on commercial scale. Open ponds are widely used in the industry due to their relatively cheaper construction, maintenance, and operation cost. Other advantages of using an open pond system include simplistic operation and maintenance, low energy demand, and a case to scale up. There are a few types of open pond, which includes natural water such as lakes, ponds, and artificial water bodies such as circular and raceway ponds. In some cases, a container such as a tank can also be used to culture microalgae. The open pond system has the upper hand of being the most cost-efficient cultivation system. However, despite the large cultivation area, cultivation of microalgae from natural water has a relatively lower cell concentration, and thus a highly efficient harvesting method is required. Another issue that surfaces in an open pond cultivation system is the possibility of contamination which causes the products to be toxic and unusable.

1. Circular pond:

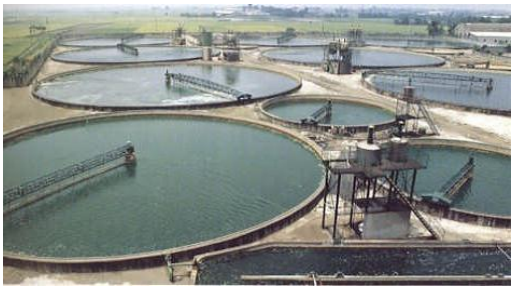


Figure 4 Circular pond

Circular ponds are the first artificial pond to be used in large-scale cultivation of microalgae. This cultivation system derived its name from its circular-shape based culture tank, which typically has a depth of 30-70 cm and a width of 45 m along with a rotating agitator located at the centre of the pond. The rotating agitator is being used to ensure efficient mixing and prevent sedimentation of algae biomass. However, the design of this cultivation system is restricted by its size since a bigger pond might introduce stronger water resistance, and therefore causes

mechanical strain on the parts of the agitator. However, the demerits involved in such systems accounts for high energy usage in the agitation process and high construction cost.

2. Raceway pond:

Figure 5 Raceway Pond

Raceway pond is one of the most frequently used open pond types for the cultivation of microalgae. It consists of a series of closed- loop channels around 30cm deep and paddlewheel which enables recirculation of microalgae biomass to ensure equal distribution of nutrients and prevent sedimentation of microalgae biomass. Raceway ponds have been perceived as one of the best open pond cultivation designs available accounting to its better energy efficiency, as a single paddlewheel is sufficient enough to properly agitate a 5-hectare raceway pond.

- **Photo bioreactor (PBR)**

Photo bioreactor is a bioreactor system used to culture photoautotrophs such as microalgae in an enclosed system which does not allow a direct interchange of material between the culture and environment. A Photo bioreactor is able to overcome several constraints faced commonly by open pond culture design. First, the size bioreactor is more compact compared to an open pond, therefore providing more efficient land usage. Second, the system provides a closed and highly controlled growth condition for the culture, thus able to produce a contamination free, single-strain microalgae culture. In addition to this, the highly superintended culture condition can also translate into elevated nutrient and metabolic efficiency which leads to higher biomass production per unit of substrate. However, the bottleneck of practical usage of photo bioreactor is bounded to its scalability due to abundant design flaws, rendering it uneconomical to be used in large-scale production. Moreover, highly restrained growth condition of Photobioractor always comes with soaring capital and operating costs.

1. Flat panel PBR:

Figure 6 Flat panel PBR

A plate reactor simply consists of inclined or vertically arranged translucent rectangular boxes which are often partitioned into two parts to affect an agitation of the reactor fluid; generally these boxes are arranged into a system by linking those connections. They are also used for making the process of filling/emptying, introduction of gas and conveying nutritive substances. The introduction of the flue gas mostly occurs at the bottom of the box to ensure that the carbon dioxide has enough time to interact with algae in the reactor fluid. Typically, these plates are illuminated from either sides and have a high light penetration. Drawbacks of the flat plate design are the limited pressure tolerance and unreasonably high space requirements.

2. Tubular PBR:

Figure 7 Tubular PBR

A tubular reactor incorporates vertical or horizontal arranged tubes, connected to a pipe system. The algae suspended fluid can circulate in this tubing. The tubes are normally made from transparent plastics or borosilicate glass and the uninterrupted circulation is kept up by a pump at the end of the system. The introduction of gas takes place at the end/beginning of the tube system. This method of introduction of gas results in carbon dioxide deficiency and high concentration of oxygen at one end of the unit ultimately making the process inefficient. The growth of microalgae on the walls of the tubes can inhibit the penetration of the light as well.

3. Bubble column PBR:

Figure 8 Bubble column PBR

A bubble column photo-reactor consists of vertical arranged cylindrical columns comprising of transparent material. The introduction of gas takes place at the bottom of the column and introduces a turbulent stream to enable an optimum gas exchange. The bubbling also acts as a natural agitator. Light is typically sourced from outside the column, however present-day designs introduce lights within the column to raise light distribution and penetration.

Advantages and disadvantages of different types of cultivation techniques

Production system	Advantage	Limitations
Raceway Pond	Relatively cheap Easy to clean Utilised non-agriculture land	Poor biomass productivity. Large area of land required. Limited to few strain. Culture easily contaminated.
Tubular Pond	Large illumination surface area. Suitable for outdoor culture Relatively cheap	Fouling, Requires large land space.
Flat Plate PBR	Readily tempered Suitable for outdoor culture	Some degree of wall growth
Column PBR	Compact high biomass transfer Low energy consumption Good mixing and easy to sterilize	Small illumination area. Expensive compare to open pond. Sophisticated construction.

Table 2 Advantages and disadvantages of cultivation techniques

DOWNSTREAM PROCESS

Harvesting is the step of prime importance in microalgae development. Several studies have suggested that it makes up 20-30% of the total production cost due to high energy demand and capital cost. In general, all harvesting techniques aim to eliminate as much culture media from the microalgae biomass to facilitate the next downstream processing such as the extraction of bioactive compounds. Plethora of harvesting methods have been used to collect biomass, viz. includes filtration, centrifugation, flocculation, and flotation. For some circumstances, a combination of two or more techniques is employed to further upsurge the harvesting efficiency.

- **Filtration**

Figure 9 Filter assembly

The filtration process employs a semipermeable membrane that can retain microalgae on the membrane and at the same time allows the liquid media to pass through, leaving the algal biomass behind to be collected. This method can harvest a high concentration of cells from the medium of differing pore size and the filter membrane enables the system to suit the needs of different microalgae and is able to handle the more delicate species that are prone to damage due to shearing. However, this method is very susceptible to fouling and clogging and therefore needs frequent changes of fresh filter or membrane that might accord to significant processing cost.

- **Centrifugation**

Figure 10 Centrifuge

Centrifugation operation segregate microalgae cells from the culture media based on density of each component and particle size utilizing centrifugal force as its principle force of separation. This technique has impressive cell harvesting efficiency, but the process is time-consuming and energy-intensive at the same time. Moreover, high gravitational force used in centrifugation might cause cellular damage rendering it unfavourable for certain applications since the sensitive nutrients might be lost. Several different types of centrifugal systems have been operational in the industry; these include dock stack centrifuges, perforated basket centrifuges, imperforated basket centrifuges, decanters, and hydro cyclones.

- **Flocculation**

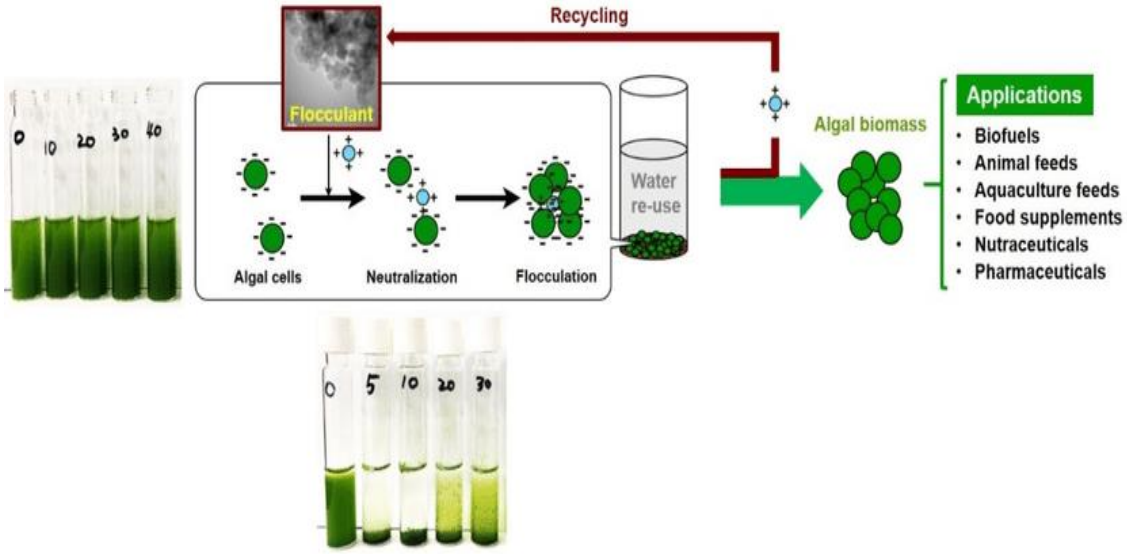


Figure 11 Floculation

Flocculation is a process where free-floating unicellular microalgae cells aggregate together to form a larger particle known as floc by the inclusion of flocculating agent to eradicate the surface charge of cells. Flocculating agents can be grouped primarily into two types, namely chemical flocculants, and bio-flocculants. Cheap and readily available chemical flocculants such as iron and aluminium salts have been greatly used in the industry. However, the chemicals are not eco-friendly accounting to their high toxicity, and they must be removed by additional treatment processes which further adds to the production cost. Bio-flocculants on the other hand are much safer and eco-friendly as compared to their chemical counterparts. They are also economical to be used, and typically there is no pre-treatment required before further downstream processing of microalgae and recycling of culture media. Most of the bio-flocculants utilized are biopolymers such as acrylic acid and chitosan that exist naturally or are produced artificially.

- **Flotation**

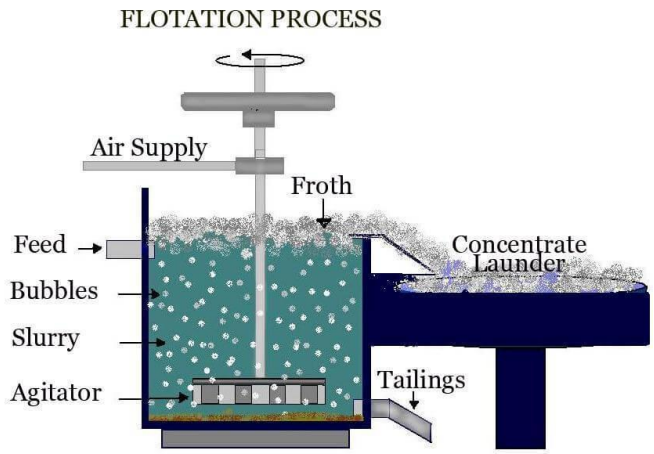


Figure 12 Flotation process

Flotation utilizes minute bubbles which cling on microalgae cells to enhance the floating of cells on the surface of the culture media for easy harvesting. The advantages bundled with the flotation system include: relatively high harvesting efficiency, ease of operation, and high processing throughput at low cost.

There are three main types of flotation agitator systems that generate air bubbles required by using different mechanisms. The dissolved air flotation system generates air bubbles by the means of saturating the culture with compressed air and then discharging the culture at atmospheric conditions. This method has been proactively used in wastewater treatment but is hindered by exclusively high costs due to power consumption and usage of chemicals. Dispersed air flotation on the other hand uses a sparger to generate air bubbles which in turn have lower energy demand when compared to dissolved air flotation. The third method is electro-flotation, which deploys electrolysis operation to generate micro-bubbles from its electrode to trap free floating microalgae. Apart from harvesting, this method also permits simultaneous cell disruption

operation when an alternating current is being used. However, the respective system is extremely energy-consuming and periodic replacement of electrodes is required due to fouling which might thereafter surge the production cost.

- **Sedimentation**

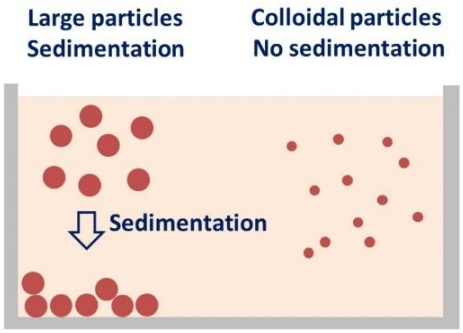


Figure 13 Sedimentation process

Sedimentation refers to the settling of particles, such as algae cells, in a liquid. In algal cultivation, sedimentation can be a useful process for separating algae from the growth medium. Sedimentation can occur naturally due to gravity or can be facilitated by centrifugation or filtration.

Algae can be separated from the growth medium by allowing the culture to settle and then removing the liquid above the settled algae. This method is commonly used for large-scale cultivation of algae for biofuels or other industrial applications.

There are several factors that can affect the sedimentation rate of algae, including the size and shape of the algae cells, the density of the growth medium, and the strength of the gravitational field. Some algae species may be more prone to sedimentation than others, and the sedimentation rate can also be influenced by the conditions in which the algae are grown, such as light intensity, nutrient availability, and temperature.

To maximize the efficiency of sedimentation, it is important to maintain optimal growth conditions for the algae and to carefully control the rate of sedimentation to avoid damage to the cells. In addition, other methods such as centrifugation or filtration may be used in conjunction with sedimentation to achieve a higher degree of separation and purification of the algae.

Advantages and Disadvantages of different Harvesting methods

Process	Advantages	Disadvantages
Filtration	Less expensive, wide variety of filters and membranes available.	Require periodic backwashing, time-consuming, highly dependent on algal spices; best suited to large algal cells. Clogging or fouling is an issue.
Flotation	Cost-efficient and quicker than sedimentation	Use of chemical depends on suspended particles, is less reliable, microalgae species specific, has high consumption, and high capital cost
Centrifugation	Quick, highly efficient, good recovery	Expensive due to high energy consumption and high capital costs
Sedimentation	Low cost, the potential for use as a first stage to reduce energy input and cost of subsequent stages	Slow separation, final concentration may be low, may not be suitable for *Nannochloropsis* cells
Microfiltration/Ultrafiltration	Capable to handle *Nannochloropsis* cells, very efficient and reach up to 98% dewatering can be used as pre-treatment prior to centrifugation	High operating costs and membrane fouling

Table 3 Advantage and disadvantage of harvesting method

CROP PROTECTION

Most of the algae that have been successfully cultivated at scale to date rely on culture conditions that are inhospitable to most pests (e.g., Dunaliella or Spirulina). Alternatively, for other species some form of batch culture is used to minimize contamination and product loss. Both of these approaches reduce the requirement for crop protection processes. In order to cultivate other desirable strains that require high saline conditions and more neutral pH ranges, more options for crop protection will be required.[9]

Some of the oldest and best studied methods for crop protection include the use of relatively simple chemical compounds to treat infected systems. The overall goal is to identify compounds that can be added to production systems at concentrations that kill, inhibit, or mitigate contamination by deleterious species. Ideally, chemical treatments will not be detrimental to algal production, adversely impact downstream use of the biomass, or add excessive additional cost. This balance can be difficult to achieve and limit the utility of these approaches. Although many chemical treatments can both be used in prophylaxis and interdiction, extensive and continued use of chemical agents can lead to desensitization and resistance in the targeted pest species. Here, we discuss the variety of chemical treatments and their effectiveness on prophylaxis and interdiction of algal cultures.

Sr. No.	Chemical Additives	Tested Against	Effective amount	Advantages	Disadvantages
1	Copper Sulphate	*Brachionus calyciflorus* (Rotifers)	0.1-1.5 ppm in 100 mg/l dry weight	High heavy metal concentrations found in many waste water treatment facilities	inhibits algal growth, limits photosynthetic efficiency, inhibits colony formation of S.obliquus, heavy metal pollutan
		Colpoda sp.,(ciliates)	10 mg/L		
2	Peraacetic acid	Grazers	25 ppm	Degradable, several oxidizers, known	High cost for peracetic acid,EPA warns against

				biocides, ozone micro bubbles can spur algal CO2 uptake	use >1.3 ppm for peracetic acid ,50ppm or more than that kill algae
3	Chlorine dioxide	virus and animal plankton	3 mg/l	Degradable , several oxidizer , known biocides	Clo2 is algicides
4	Hydrogen peroxide	Ciliates	150-200 mg/l	Rapid decomposition , light sensitive	Light sensitive
5	Quinine sulphate	*Colpado sp.*	20 mg/l	effective against variety of grazers without harming algae , long lasting in water system	Expensive, toxic to human health , environmentally harmful do to developing resistance
		Vorticella sp.	20 mg/l		
		Grazers	10 mg/l		
		Brachionus calyciflorus (Rotifers)	17 µm		
6	Rotenone	*Brachionus calyciflorus (Rotifers)*	0.074 µm	degrades to nontoxic chemicals, algae are largely insensitive, effective against marine and fresh water	Rapidly degrades in sunlight, extremely toxic to insects and aquatic life , harmful to human in concentrated doses
		Brachinus rotundiformis	0.13 µm		
		Brachionus manjavacas	0.26 µm		
		Oxyrrhis sp.	1.3 µm		
		Euplotes	5 µm		
7	Lugol's iodine	Euplotes, Oxyrrhis	0.3-1 ml per 100 ml sample	Potent grazer inhibition	Too expensive, too toxic for algae
8	Methylene blue	Euplotes, Oxyrrhis	100 mg/l stock sol.	Potent grazer inhibition	Too expensive, too toxic for algae

			Add 50 ml in 1 gm		
9	Toluidine blue	Inhibit Blue green algae	10 µm p-toluidine per disk	Blue green algae inhubiter,slight inhibit diatoms	expensive, too toxic for algae
10	Ivermectin	Euplotes, Oxyrrhis	5.7 mg/l	Potent grazer inhibition	Toxic for target environmental effects

Table 4 Crop protecting chemicals its effective amount with advantage and disadvantages

BULK CRYOPRESERVATION OF GREEN ALGAE

Cryopreservation or cryo-conservation is a process where biological material - cells, tissues, or organs - are frozen to preserve the material for an extended period of time. At low temperatures (typically −80 °C (−112 °F) or −196 °C (−321 °F) using liquid nitrogen) any cell metabolism which might cause damage to the biological material in question is effectively stopped. Cryopreservation is an effective way to transport biological samples over long distances, store samples for prolonged periods of time, and create a bank of samples for users. Molecules, referred to as cryoprotective agents (CPAs), are added to reduce the osmotic shock and physical stresses cells undergo in the freezing process. Some cryoprotective agents used in research are inspired by plants and animals in nature that have unique cold tolerance to survive harsh winters, including trees, wood frogs, and tardigrades.

- **STEPS INVOLVED IN CRYOPRESERVATION**

1. The culture should be cryopreserved when it reaches exponential growth phase.
2. Cryopreservation procedure done in a dark condition.
3. Check the concentration (around 5-10 %v/v) and the purity of culture.
4. In dark, put the concentrated culture in a container and add CPA(Cryoprotectant) on a volume basis. Mix the culture properly with CPA.
5. Incubate the algal culture slurry and cryoprotectant mixture for several time(1 to 2 hrs.).
6. Incubate the Slurry in a bag.
7. Label the bags with the details of the cryopreservation.
8. As the cryoprotectant added culture is in large amounts (approximately 1kg or more then that) gradually not to increase the temperature.
9. First keep at 4 °C . Followed by 0°C . -20°C then at a temperature of-80 degrees and then finally at -196 °C.

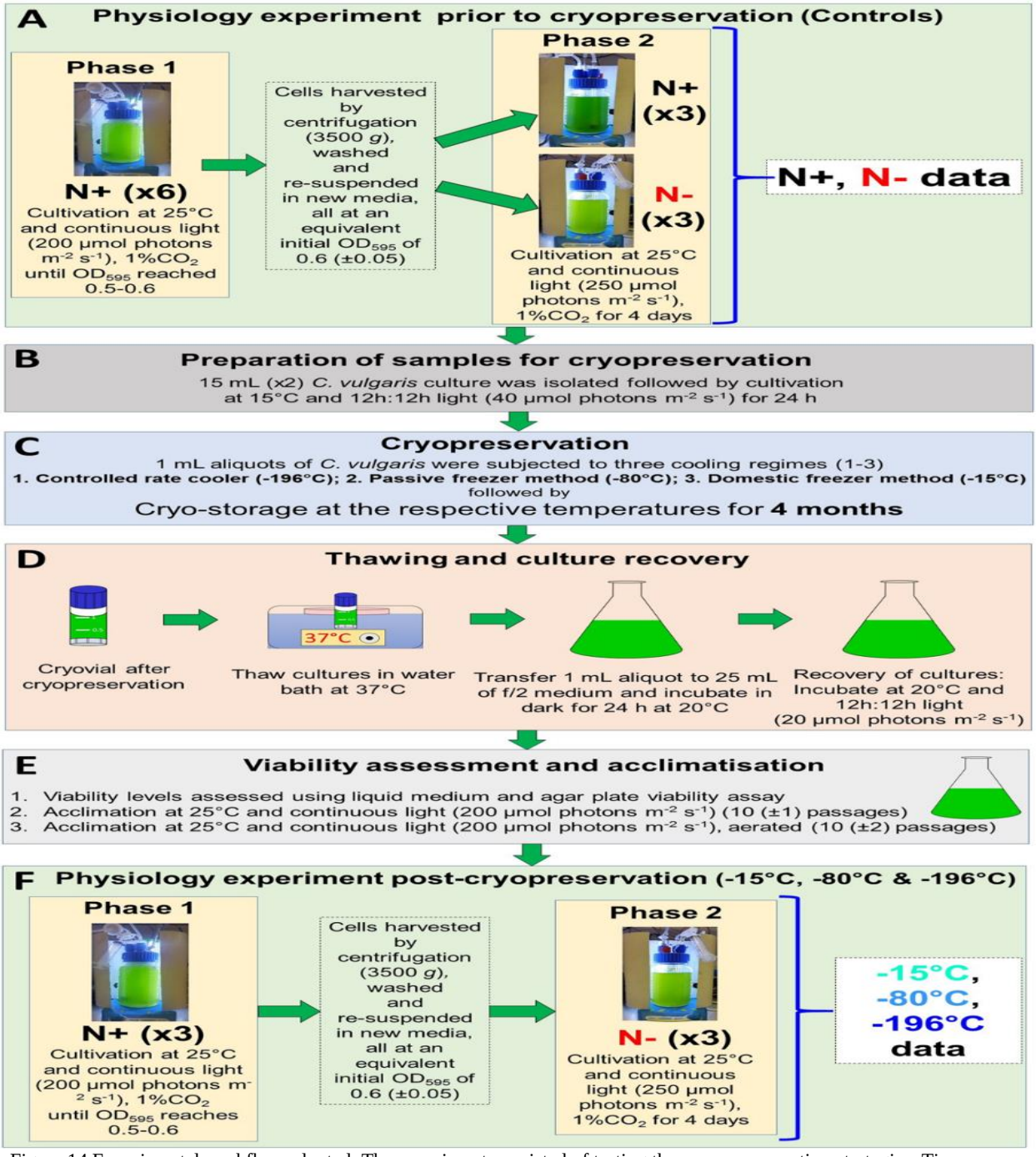


Figure 14 Experimental workflow adopted. The experiment consisted of testing three cryopreservation strategies. Tis was preceded by a physiological experiment to assess functionality prior to cryopreservation and consisted of growth in two phases (A), and subsequent preparation of samples for cryopreservation (B). the cryopreservation step (C) was then followed by thawing and culture recovery (D) and viability assessment and culture acclimatisation (E), before repeating the physiological experiment on the cultures post-cryopreservation in two phases (F) to test for functionality

- **STEP INVOLVED IN REVIVAL OF CRYOPRESERVED CULTURE:**

1. The revival is carried out in the dark incubation in the laboratory at the time of revival.
2. The culture bags are removed from the freezer and kept for thawing at room temperature.
3. Prepare commercial media for inoculation in small pond.
4. All procedure occur in dark condition and bags open directly in pond for culture revival. Till culture adapted to the natural environment condition. Culture should not be directly contact with sunlight. Culture first adapted to sunlight to give them light low intensity to sunlight intensity.
5. The culture is exposed to direct sunlight after the culture is adapted to the environment.
6. Revival of the culture is observed after inoculation. By increasing dividing cell, cell shape and cell colour we know culture was healthy or not.
7. After thawing of the culture revival is observed by taking OD of the culture and doing microscopy or other parameters.

- **CULTURE ACCLIMATIZATION**

Culture acclimatization is the process of gradually adapting a culture of living organisms to new environmental conditions, such as changes in temperature, humidity, light, or nutrients. The goal of acclimatization is to help the organisms adjust to their new environment and maintain their health and viability.

Algal culture acclimatization is the process of gradually adapting a culture of algae to a new environment, such as changes in light, temperature, pH, nutrients, and salinity. The acclimatization process is important for maintaining the health and viability of the algae and ensuring their successful growth and reproduction.

Acclimatization of algae can be achieved through several methods, including:

- Gradual changes in environmental conditions: When introducing a new algal strain to a culture or transferring it to a new environment, it is important to make gradual changes in environmental conditions over time to avoid sudden shock. For example, a change in light intensity or temperature should be made slowly over several days.
- Monitoring and adjusting nutrient levels: Algae require specific nutrient levels for optimal growth and reproduction. Acclimatization involves monitoring and adjusting the nutrient levels to match the new environment. This includes adjusting the ratio of nitrogen to phosphorus and other trace elements.
- Salinity adaptation: Algae that grow in marine environments may need to be adapted to different salinity levels when transferred to freshwater environments, or vice versa. This can be achieved by gradually changing the salinity levels in the culture over time.
- pH adjustment: Algae can be sensitive to changes in pH, so adjusting the pH of the culture to match the new environment is critical for acclimatization.
- Light adaptation: Algae require specific light conditions for optimal growth. When transferring algae to a new environment with different light conditions, acclimatization can involve gradually increasing or decreasing the light intensity or changing the light spectrum.

  Overall, acclimatization is a critical step in maintaining the health and viability of algal cultures and ensuring their successful growth and reproduction in new environments.

➢ CULTURE PARAMETERS EFFECT THE GROWTH OF ALGAE

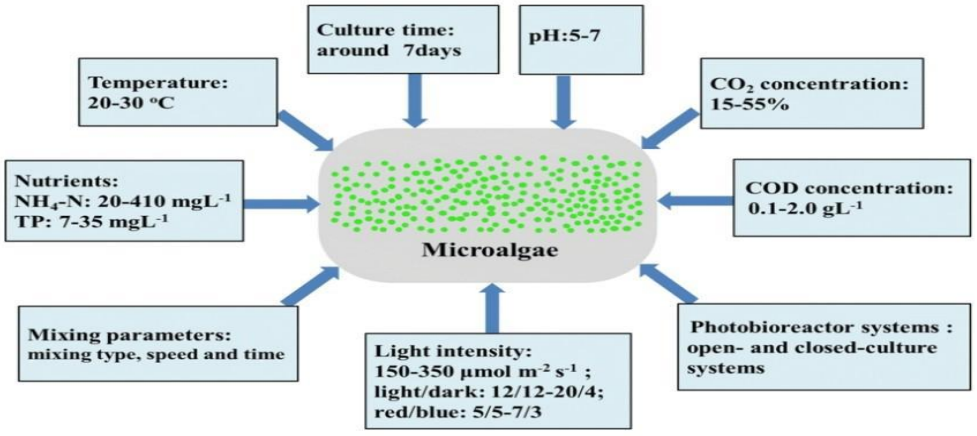


Figure 15 Parameters affecting algae growth

- Light Intensity and Quality

Availability of the light is the primary factor in the growth and productivity of photosynthetic microorganisms. As light is the ruling energy input for photosynthetic microorganisms, it must be maximized for better output. However, excess light especially coupled with sub-optimal temperature or high oxygen levels can adversely affect the photosynthetic apparatus.

Therefore, by the adequate design of its geometry and orientation, the light supply to the cultivation system must be optimized. The growth of microalgae is determined by the photosynthetic rate, which is a direct function of the irradiance to which the cells are exposed to inside the culture. As light intensity enhances, photosynthesis in microalgae also rises until it reaches a maximum rate at the saturation point.

- Temperature

Temperature is considered as one of the most significant environmental factors that influence algal growth rate, cell size, biochemical composition, and nutrient requirements. Microalgae culture absorbs heat by radiation from the light source, resulting in increase of temperature in the culture. The optimal temperature for microalgae growth ranges from 20°C to 35°C, although some mesophilic species can endure up to 40°C. Below the optimal temperature, the yield of the strain gets reduced, while on the other hand overheating of the cultures have been identified to be critical since it can damage the cells. Therefore, seasonal variations, which lead to temperature variations during the day/night cycle, have significant impact on microalgae cultivation.

- Nutrients

Conditions of nutrient limitation affect a considerable variation in the biochemical composition of microalgae. An ideal culture medium for microalgae must contain inorganic elements such as Phosphorus (P), Nitrogen (N), and Iron (Fe), among others, which may vary according to the cultivated species. Thus, the most important nutrients or macronutrients for autotrophic growth are carbon (C), nitrogen (N), and phosphorus (P).

- Carbon Dioxide (CO2)

Carbon dioxide (CO2) is another vital factor that is involved in the growth of microalgae. To produce 1 kg of biomass, microalgae requires 1.8 to 2.0 kg of CO2. Considering this ratio, the

amount of CO2 present in the air is 0.03% which is not adequate to provide the necessary gas pressure in the culture to promote high productivity.

Thus, for elevating photosynthetic efficiency in their growth, it is necessary to supply carbon, either in the form of salts, such as bicarbonate or by injection of CO2-rich air in the culture.

- Hydrogen Potential (pH) and Salinity

The pH has eminent significance in microalgae cultures, because, besides affecting the microalgae themselves, it determines the solubility of minerals and CO2 in the medium. Several factors such as composition and buffering capacity also play a dominating role in development of microalgae.

- Mixing

The mixing plays a key role in the balance of gases and pH of the system. Enough turbulence of microalgae cultures minimizes the existence of gradients that can restrict the performance of the cells. Thus, mixing diminishes the gradient of nutrients in the culture broth, avoids cell sedimentation in the system, and forces the cells to move between dark to light zones, eventually enhancing the rate of photosynthesis. It helps to facilitate heat transfer and avoid thermal stratification by ensuring all cells of the population have unvarying average exposure to light and nutrients.[1]

- **INOCULUM SCALE UP AND STUDY CULTURE GROWTH PATTERN**

Inoculum scale-up and studying culture growth patterns are critical steps in large-scale algal cultivation. Here are some key considerations:

Inoculum preparation and scale up:

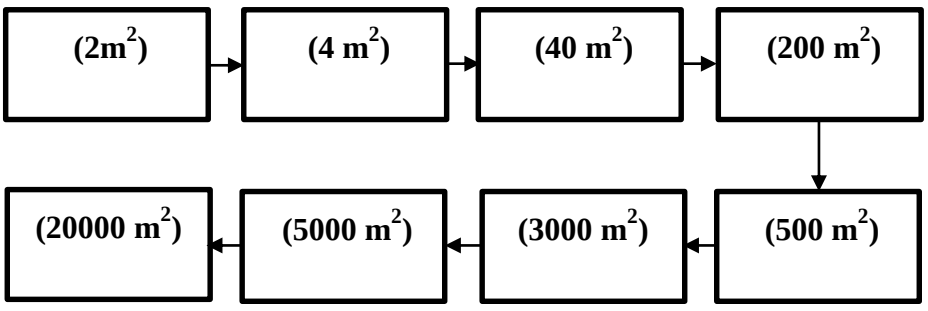


Figure 16 Pond arrangement

The inoculum should be prepared from a cryopreserved culture that has been for revival of culture. The inoculum should be grown in a small volume of medium to reach a high density before it is added to the larger culture vessel.

inoculum development done in inoculum ponds. First of all culture inoculate in 2m2 and leave this for 6-7 days to adopt the environmental conditions and increase culture cell density. Then transfer into4 m2.and follow the process as mention up to 500m2. It's takes 25-30 days to reach at this scale now culture is ready to grow on production pond transfer the culture into 3000 m2 pond and follow the process mention above. After some day cell density increase cell transfer into 5000 ms2 ponds and finally into 20000 m2ponds. This three ponds are knows as production ponds.

- **MONITORING AND MAINTENANCE OF CULTURE**

Culture health check with different method and equipment and maintain by performing spectrophotometry, microscopy , CHNS, nutrient analysis with different method , Total solid , total suspended solid , total dissolve solid and ash free dry weight health of culture checked.

- Spectrophotometry: Spectrophotometry is a technique used to measure the amount of light absorbed or transmitted by a sample as a function of the wavelength of the light. It is widely used in scientific fields to determine the concentration or purity of a substance in a culture. The basic principle of spectrophotometry involves shining a beam of light through a sample solution and measuring how much of the light is absorbed by the sample. The amount of light absorbed is proportional to the concentration of the substance in the solution, and this relationship is described by the Beer-Lambert law. To perform spectrophotometry, a spectrophotometer is used, which is a device that produces a narrow beam of light of a specific wavelength and measures the intensity of the light before and after it passes through the sample. The difference in intensity is used to calculate the amount of light absorbed by the sample, which is then used to determine the concentration of the substance in the solution. From this growth rate is measured.
- Microscopy: Using phase contrast microscope on different magnifying lenses like 4x, 10x, 40x clumps of algae, ciliates, flagellates, diatoms, tetraselmis, health of algae cells and contamination and ratio of cell and contamination observed.

- FlowCAM: FlowCAM is an imaging flow cytometer that is used to analyse particles and microorganisms in fluids. It is a powerful tool for studying aquatic environments, including culture of algae. The FlowCAM uses a combination of flow cytometry and microscopy to capture high-resolution images of particles and microorganisms as they flow through a fluidic system. The system includes a high-speed camera that captures images of each particle as it passes through a laser beam, which is used to detect the size and shape of the particle. The images captured by the FlowCAM are then analyzed using image analysis software, which can provide information about the particle's size, shape, and other physical characteristics. This allows to identify and quantify different types of microorganisms and cell health and size in the culture. The FlowCAM is particularly useful for studying microorganisms that are difficult to culture in the lab, as it allows to visualize and analyse them in their natural environment.
- Nutrient analysis : Nutrient analysis of phosphorus, ammonia, urea and other trace metals like iron are occur to know the amount of nutrient present in the culture and used by the algae.. Accornding to the calculation nutrients added in the pond.
- TS, TSS and TDS: By performing total solid, total suspended solid and total dissolve solid contain present in the culture are identify.
- Ash free dry weight: It is use for accurate measurement of biomass, better understanding of algal composition and calculate the specific growth rate and biomass productivity which is measure in gm/liter and on the basis of biomass aerial productivity identify.
- CHNS: Amount of carbon, hydrogen, nitrogen and sulphur present in culture were identify.

Performing all above techniques culture health, organic and inorganic content, contamination in the culture, algae cell health identify and according to results further process of harvesting done.

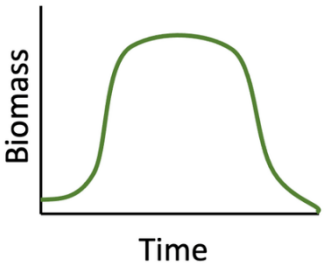
➢ POND OPERATION METHODS

There are three general methods to grow algae that are across a spectrum.

1. Batch culture
2. Semi continuous batch culture
3. Continuous culture.

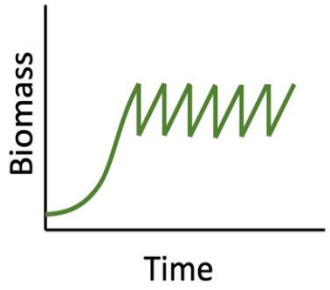
BATCH CULTURE

In the batch culture you have a finite volume of media in which the cells grow. You do not add any new media. Over time the biomass looks like this plot. The culture experiences lag-phase, exponential growth, stationary phase, and then decline.



Graph1Time vs Biomass

SEMI-CONTINUOUS BATCH CULURE

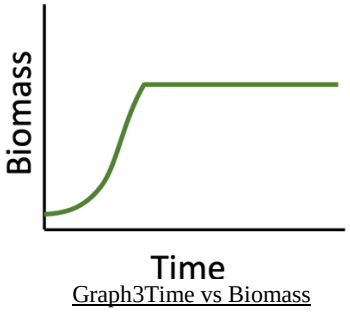


Graph2Time vs Biomass

In the semi-continuous batch culture. The culture is harvested every day to week and new media is added to the container. For the greatest amount of biomass produced, the harvesting is done at the highest biomass possible before the culture enters the stationary phase of growth. The culture experiences the lag phase and exponential phases. If done correctly, it never entered stationary phase or decline. Over time this type of culture method could experience pathogens or weed algae growing in the media, so monitoring of the culture is important.

CONTINOUS CULTURE

Continuous culture is a most efficient way to create biomass. This culture method is also called a chemostat, or a turbidostat (when the density is held constant). In this method, a continuous supply of media is added to the culture. With the culture volume held stationary, every new mL of media yields a new ml of product. You could view this a semi-continuous batch culture, where the harvest phase is every second. [10]



Graph3Time vs Biomass

But here we are follow batch method for revival and scale up of cryopreserved culture. Algae ponds are a popular method for cultivating algae for various purposes such as biofuel, animal feed, nutritional supplements and culture revival. To maximize algae production, many operations use batch mode, which involves growing algae in a series of batches or cycles.

In batch mode, algae are grown in a pond for a set period, typically ranging from a few days to a few weeks, depending on the specific strain and growth conditions. After this period, the pond is drained, and the algae are harvested. The pond is then cleaned and prepared for a new batch.

Batch mode has several advantages for algae pond operation. First, it allows for greater control over the growth conditions, as each batch can be tailored to the specific needs of the algae strain. Second, it minimizes the risk of contamination, as each batch is grown in a clean pond. Finally, it can be easier to manage than continuous mode, where algae are grown continuously in the same pond, as it allows for easier monitoring and adjustments to the growth condition.

RESULT AND DISCUSSION

- The optical density vs ash-free dry weight in large scale algal cultivation can provide insights into the growth and productivity of algae. Optical density is a measure of the amount of light absorbed by a culture of algae, which is proportional to the concentration of cells in the culture. Ash-free dry weight, on the other hand, is a measure of the biomass of the algae culture that remains after burning off any organic material. In general, as the culture of algae grows and the concentration of cells increases, the optical density will also increase. At the same time, the ash-free dry weight will also increase as the algae accumulate more biomass. The relationship between optical density and ash-free dry weight can provide useful information about the health and productivity of the algal culture. For example, if the optical density is increasing rapidly but the ash-free dry weight is not keeping pace, it could indicate that the algae are not growing efficiently and may be experiencing stress or nutrient limitations. Alternatively, if the ash-free dry weight is increasing rapidly but the optical density is not increasing at the same rate, it could indicate that the algae are becoming more denser or clumped together, which could affect their

ability to absorb nutrients and light. In general, a healthy and productive algal culture will show a strong correlation between optical density and ash-free dry weight, indicating that the algae are growing efficiently and accumulating biomass at a consistent rate.

- PAR refers to the range of wavelengths of light (typically 400-700 nm) that are absorbed by photosynthetic pigments in plants and algae. The PAR value is a measure of the intensity of light within this range that is available for photosynthesis.

The OD of the algal culture also increases, up to a certain point. Beyond that point, the OD may remain constant or start to decrease due to photoinhibition or other factors that can limit algal growth.

The optimal PAR value for algal cultivation varies depending on the specific type of algae and the growth conditions. However, in general, it is important to maintain the PAR value within a suitable range to maximize algal growth and minimize photoinhibition. This can be achieved by adjusting the light intensity and/or the culture density to maintain a balance between the available PAR and the ability of the algal cells to utilize it for photosynthesis.

Overall, the OD vs PAR graph provides valuable information for optimizing algal cultivation conditions and can help researchers and practitioners to achieve higher algal productivity and biomass yields.

CONCLUSION

In conclusion, cryopreservation is an effective method for long-term storage of algal cultures, and revival of cryopreserved algal cultures is a crucial step in maintaining and propagating these cultures.

Revival of cryopreserved algal cultures typically involves thawing the cryopreserved bags containing the frozen culture, transferring the culture to a suitable growth medium, and providing appropriate environmental conditions for growth. Revival of cryopreserved culture is successfully done by giving them suitable environment.

Overall, cryopreservation and revival of algal cultures is an important tool for preserving and maintaining valuable algal strains for future research and industrial applications.

REFERENCE

1. Khan, M. I., Shin, J. H., & Kim, J. D. (2018). The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. In Microbial Cell Factories (Vol. 17, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12934-018-0879-x
2. Fernandez-Valenzuela, S., Chávez-Ruvalcaba, F., Beltran-Rocha, J. C., San Claudio, P. M., & Reyna-Martínez, R. (2021). Isolation and Culturing Axenic Microalgae: Mini–Review. The Open Microbiology Journal, 15(1), 111–119. https://doi.org/10.2174/1874285802115010111
3. Mutanda, T., Naidoo, D., Bwapwa, J. K., & Anandraj, A. (2020). Biotechnological Applications of Microalgal Oleaginous Compounds: Current Trends on Microalgal Bioprocessing of Products. In Frontiers in Energy Research (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fenrg.2020.598803
4. Tan, J. sen, Lee, S. Y., Chew, K. W., Lam, M. K., Lim, J. W., Ho, S. H., & Show, P. L. (2020). A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids. *Bioengineered*, *11*(1), 116–129. https://doi.org/10.1080/21655979.2020.1711626
5. Koyande, A. K., Show, P. L., Guo, R., Tang, B., Ogino, C., & Chang, J. S. (2019). Bio-processing of algal bio-refinery: a review on current advances and future perspectives. In *Bioengineered* (Vol. 10, Issue 1, pp. 574–592). Taylor and Francis Inc. https://doi.org/10.1080/21655979.2019.1679697
6. Uduman, N., et al., Dewatering of microalgal cultures: a major bottleneck to algaebased fuels. Journal of renewable and sustainable energy, 2010. 2(1): p. 012701.
7. Barros, A.I., et al., Harvesting techniques applied to microalgae: a review. Renewable and Sustainable Energy Reviews, 2015. 41: p. 1489-1500.
8. Singh, G. and S. Patidar, Microalgae harvesting techniques: a review. Journal of environmental management, 2018. 217: p. 499-508.
9. Pugazhendhi, A., et al., A review on chemical mechanism of microalgae flocculation via polymers. Biotechnology Reports, 2019: p. e00302.

10. Singh, G. and S. Patidar, Microalgae harvesting techniques: a review. Journal of environmental management, 2018. 217: p. 499-508.
11. Springer Nature Switzerland. (2019). Agronomic Practices for Photoautotrophic Production of Algae Biomass. www.springer.com. 2019, from https://doi.org/10.1007/978-3-030-25233-5-4
12. Kapoore, R. V., Huete-Ortega, M., Day, J. G., Okurowska, K., Slocombe, S. P., Stanley, M. S., & Vaidyanathan, S. (2019). Effects of cryopreservation on viability and functional stability of an industrially relevant alga. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-38588-6
13. https://algaeresearchsupply.com/blogs/news/batch-semi-continuous-and-continuous-cultures

FIGURE REFERENCE

Figure no.	**Reference**
1.	https://theconversation.com/microalgae-is-natures-green-gold-our-pioneering-project-to-feed-the-world-more-sustainably-170158
3.	https://www.neutecgroup.com/resource-library/microbiology-lab-automation/application-notes/223-serial-dilution-a-nightmare-for-the-microbiologists
4.	https://www.researchgate.net/figure/Circular-open-pond-system-Making-Biodiesel-Books-2018-Raceway-ponds-are-the-most_fig5_330102589
5.	https://www.intechopen.com/chapters/62060
6.	https://en.wikipedia.org/wiki/Photobioreactor
7.	https://en.wikipedia.org/wiki/Algae_bioreactor
8.	https://bioprocessintl.com/downstream-processing/separation-purification/continuous-solids-discharging-centrifugation-a-solution-to-the-challenges-of-clarifying-high-cell-density-mammalian-cell-cultures/
9.	
10.	https://www.mdpi.com/2076-3417/9/15/3069

11.	https://www.911metallurgist.com/blog/froth-flotation-process
12.	https://kenkidryer.com/2020/05/10/flocculation-treatment/
13.	https://www.nature.com/articles/s41598-019-38588-6
14.	https://www.researchgate.net/figure/Culture-parameters-affecting-the-growth-of-microalgae_fig3_341715446
15.	https://www.researchgate.net/figure/Culture-parameters-affecting-the-growth-of-microalgae_fig3_341715446