

**EXTRACTION OF BIOSURFACTANT FROM MARINE BACTERIA AND STUDIES
ON FLOCCULATION OF MARINE MICROALGAE**

Submitted in Partial Fulfillment of the requirement for the award of a Bachelor
of science degree in Biotechnology

By

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**SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

**Accredited with Grade “A” by NAAC | 12B STATUS BY UGC | APPROVED BY
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of who **HEMAPRIYA.K (Reg.NO - 40770018)** carried out the project entitled **"EXTRACTION OF BIOSURFACTANT FROM MARINE BACTERIA AND STUDIES ON FLOCCULATION OF MARINE MICROALGAE"** under my supervision from September 2022 to April 2023.

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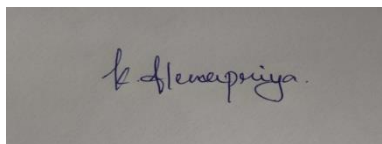
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DECLARATION

I, **HEMAPRIYA.K (Reg.NO - 40770018)** hereby declare that the Project Report entitled “**EXTRACTION OF BIOSURFACTANT FROM MARINE BACTERIA AND STUDIES ON FLOCCULATION OF MARINE MICROALGAE**” done by me under the guidance of **Dr. S M.INDUMATHI** (internal guide) and **Dr. D. INBAKANDAN** (external guide) at **CENTRE FOR OCEAN RESEARCH, COL. Dr. JEPPIAAR RESEARCH PARK, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** is submitted in Partial fulfillment of the requirements for the award of Bachelor of Science and Technology degree in **Biotechnology**

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ABSTRACT

In an effort to search for an efficient and environmentally friendly harvesting method of marine microalgae by chemical and organic methods of flocculation. Microalgae have been extensively studied for the production of various valuable products. The application of microalgae for the production of renewable energy has also received increasing attention in recent years. However, the high cost of microalgae biomass harvesting is one of the bottlenecks for the commercialization of microalgae-based industrial processes. Considering harvesting efficiency, operation economics, and technological feasibility, flocculation is a superior method to harvest microalgae from mass culture. This article reviews the latest progress of various microalgae cell harvesting methods via flocculation, emphasising the current progress and prospect in environmentally friendly bio-based flocculation. Harvesting microalgae through bio-based flocculation is a promising component of the low-cost microalgae biomass production technology.

Keywords: Microalgae, Flocculation, Surfactant, GC-MS, TLC

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CHAPTER – 1

INTRODUCTION

1. ALGAE

Algae are found in the fossil record dating back to approximately 3 billion years in the Precambrian. They exhibit a wide range of reproductive strategies, from simple, asexual cell division to complex forms of sexual reproduction. Algae are important as primary producers of organic matter at the base of the food chain. They also provide oxygen for other aquatic life. In tropical regions, coralline algae can be as important as corals in the formation of reefs. Seaweeds are larger algae that live in the marine (salt or brackish water) environment. Kelps are large brown seaweeds in the genera *Pelagophycus*, *Laminaria*, *Macrocystis*, etc. Algae may contribute to mass mortality of other organisms, in cases of algal blooms but their ecological roles as oxygen producers and as the food base for almost all aquatic life, algae are economically important as a source of crude oil and as sources of food and a number of pharmaceutical and industrial products for humans. The taxonomy of algae is contentious and subject to rapid change as new molecular information is discovered. The study of algae is called phycology, and a person who studies algae is a phycologist. The size range of the algae spans seven orders of magnitude. Many algae consist of only one cell, while the largest have millions of cells. In large, macroscopic algae, groups of cells are specialized for specific functions, such as anchorage, transport, photosynthesis, and reproduction; such specialization indicates a measure of complexity and evolutionary advancement. Algae form organic food molecules from carbon dioxide and water through the process of photosynthesis, in which they capture energy from sunlight. Similar to land plants, algae are at the base of the food chain, and, given that plants are virtually absent from the oceans. In addition to making organic molecules, algae produce oxygen as a by-product of photosynthesis. Algae produce an estimated 30 to 50 percent of the net global oxygen available to humans and other terrestrial animals for respiration.

1. TYPES OF ALGAE

Algae can reproduce sexually, asexually, or by a combination of both processes through the alternation of generations. The types which reproduce asexually divide

naturally (in the case of single-celled organisms) or release spores that may be motile or non-motile. Algae that reproduce sexually are generally induced to produce gametes when certain environmental stimuli - including temperature, salinity, and nutrients - become unfavorable. These algae species will produce a fertilized egg or zygote to create a new organism or a dormant zygosporangium that activates with favorable environmental stimuli.

Algae can be categorized into seven major types, each with distinct sizes, functions, and colors. The different divisions include:

- Euglenophyta (Euglenoids)
- Chrysophyta (Golden-brown algae and Diatoms)
- Pyrrophyta (Fire algae)
- Chlorophyta (Green algae)
- Rhodophyta (Red algae)
- Paeophyta (Brown algae)
- Xanthophyta (Yellow-green algae)

2. MARINE ALGAE

Marine algae are ancient organisms: in a recent discovery, a group of scientists in India found a pair of 1.6-billion-year-old fossils that appear to contain red algae, which may be the oldest plant-like life discovered on Earth. About 12,000 species of marine algae have been described to date. Marine algae are plant-like organisms that generally live attached to rock or other hard substrata in coastal areas: red and brown algae are almost exclusively marine, while green algae are also common in freshwater (rivers and lakes), and even in terrestrial (rocks, walls, houses, and tree bark in damp places) situations. But marine algae are not plants. Marine algae are used in many countries as a source of food, in pharmacology, cosmetics, agriculture, and wastewater treatment, but also for industrial applications, such as the production of biogas.

3. FRESHWATER AND MARINE ALGAE

Freshwater algae are the algae that live in freshwater. Generally, algae are simple, aquatic, plant-like organisms. Therefore, they do not have true roots, stems, and leaves. Most algae are microalgae that are microscopic. Other algae grow like

filaments or mats. Algae contain chlorophyll in order to undergo photosynthesis. More importantly, algae produce oxygen through photosynthesis. They absorb nutrients and heavy metals from water, purifying water. They are also valuable indicators of environmental quality as algae are sensitive to pH, temperature, and nutrient levels.

Furthermore, algae are abundant in slower streams rather than in fast rivers. There are three types of freshwater algae: green algae, red algae, and diatoms. In general, green algae look like green hair flowing inside the water such as in *Spirogyra*. *Audouinella* is an example of red algae that especially grows in shaded places. Usually, diatoms form phytoplankton along with cyanobacteria forming slimy layers on rocks.

Marine algae are the types of algae that grow in the sea. The most complex forms of marine algae are seaweed. Seaweed is essentially macroalgae that exclusively grows in marine habitats. In addition, there are four types of marine algae: green algae, red algae, brown algae, and phytoplankton. Of these, brown algae, also known as dusky plants, are the most common type of seaweed with a brown or yellow-green colour. The appearance of seaweed resembles non-arboreal, terrestrial plants.

Moreover, seaweeds have root-like structures called holdfasts, anchoring algae to the surface. Their plant body is a thallus and the leaves are known as blades. Further, the strips are the stem-like structures in seaweeds. Generally, large brown algae are the kelp. Kelp is the most commonly consumed seaweed, containing many important vitamins and minerals such as vitamin K, vitamin A, vitamin C, folate, vitamin E, vitamin B12, vitamin B6, niacin, thiamine, riboflavin, pantothenic acid, magnesium, iron, iodine, calcium, sodium, phosphorus, and zinc.

1.2 FLOCCULATION

Flocculation refers to the aggregation of unstable and small particles through surface charge neutralization, electrostatic patching, and/or bridging after the addition of flocculants. Flocs formation allows for separation (or recovery) by simple gravity-induced settling or any other conventional separation method. The flocculation process is simple and efficient and has been extensively investigated as a promising strategy for harvesting various algal species

As the flocculant plays a major role in the flocculation harvesting process, the discovery of a highly efficient and cost-effective flocculant has forever remained a challenge in most studies. Nowadays, the use of conventional inorganic metal salts such as aluminium sulphate and ferric sulphate has been reduced due to high dosage and biomass contamination. Various natural and synthetic organic flocculants have been designed and developed to improve flocculation efficiency. However, the former has high production costs and a short shelf-life while the latter has adverse effects on harvested biomass and non-biodegradability, due to their petroleum origins. Metal cations released from the electrode under direct electric current conditions are able to electrostatically attract almost all types of algal cells, resulting in efficient flocculation. Significant efforts are being directed to prevent electrode/biomass fouling and to reduce systemic/electric costs for large-scale algae harvesting. Nanoparticles in either single or hybrid forms decorated with various cationic chemicals have been employed for rapid algae separation and/or multi-functionalities such as cell disruption and lipid extraction. This approach although highly efficient, is expensive and is mostly limited to laboratory-scale studies. Spontaneous aggregation of algal cells under specific conditions and the use of a self-flocculating microorganism can be considered sustainable and environment-friendly. However, species-specific reactivity, availability of low-cost bio-flocculant microorganisms, and process scale-up should be properly considered for practical applications. Ideally, in addition to excellent harvesting efficiency and promising scalability, the industrial flocculant should satisfy the demands for recyclability, low toxicity, low-cost material, and a massive production process.

Many review articles are available on the use and mechanism of specific flocculants/techniques, but a comprehensive comparison of various flocculation-based methods including classical inorganic chemicals and newly engineered nanoparticles is limited. We herein classify the flocculation techniques that have been studied and applied in the algal refinement process, into five categories which are auto-flocculation, bio-flocculation, chemical flocculation, particle-based flocculation, and electrochemical flocculation. The main objective of this review is to introduce the readers to these flocculation techniques and the key issues in their commercialization. The review thus provides an updated overview of the recent technical developments and progress in this field. Further research directions have also been proposed to

direct the development of flocculation harvesting processes suitable for large-scale algae applications.

1. TYPES OF FLOCCULATION

1. Auto-Flocculation

In auto-flocculation, suspended algal cells spontaneously aggregate, forming large flocs, which induce their simple gravitational sedimentation. This phenomenon has been observed in various algal species, particularly under non-ideal culture conditions such as changes in pH and cultural aging. Both alkaline and acidic conditions have been reported to reduce the intensities of the negative surface charge of algal cells, thereby promoting their self-aggregation. Under alkaline conditions above pH 9, the changes in the surface charge of algal cells are mainly attributable to the significant secretion of protective extracellular polymers. Under acidic conditions, fluctuating dissociations of carboxyl and amine groups in the algal cell wall can cause changes in surface charge

2. Bio-Flocculation

Bio-flocculation is performed by adding a self-flocculating microorganism or its extracellular biopolymer to the culture broth to harvest non-flocculating, target algae. Bio-flocculants include bacteria, fungi, yeasts, or self-flocculating algae as well as their exudate-rich culture supernatants. Since no chemical is required in this process similar to the case of auto-flocculation, the bio-flocculation method can also be considered a sustainable and environmentally friendly technique for algal biomass harvesting. A bio-flocculant- microorganism can be prepared by co-culturing with target algae or culturing separately in a different bioreactor, before performing the intended use. Although the mechanism of bio-flocculation has not been clearly elucidated, it is believed that it is mainly a function of the reactivity of the extracellular biopolymer and/or the direct adsorption of the self-flocculating microorganisms on the target algae

3. Chemical Flocculation

Chemical flocculation of algae occurs due to charge neutralization and electrostatic bridging between the suspended algal cells and the applied flocculant, resulting in floc formation and subsequent sedimentation. Multivalent inorganic chemicals, biopolymers, or inorganic-organic hybrid polymers have been extensively used as

algae-harvesting flocculants. Aluminum sulfate and ferric chloride are the most popular inorganic flocculants for wastewater clarification and algal biomass recovery. Chitosan, cationic starches, modified tannins, and polyacrylamides are examples of organic polymers that are widely used. The harvesting efficiency of both organic and inorganic flocculants depends largely on their physicochemical properties such as solubility and electronegativity, as well as the operating conditions, such as dosage and algal solution characteristics (e.g., cell density, pH, and ionic strength). It should be noted that the sizes of the flocs formed through charge neutralization with conventional inorganic chemicals are generally small, requiring high dosages for algae flocculation. On the other hand, the bridging and sweeping reactions between polymeric flocculants and algal cells can lead to the formation of larger-sized flocs, thereby promoting efficient biomass recovery at a relatively low dosage.

4. Particle-Based Flocculation

Particle-based can potentially circumvent some drawbacks of conventional chemical flocculation such as bio-toxicity and difficulties related to chemical recovery. For these purposes, particle-based flocculants should be designed to be more efficient, recoverable, and/or have multi-functionalities other than algae recovery, such as cell disruption and lipid extraction. Therefore, numerous research efforts have devoted effort towards the development of new and optimal nano/micro-particle-based flocculants. This section summarizes the recent progress in algae harvesting using nano/micro-particle-based flocculants, namely amino clay (AC)- based particles, magnetic particles, and more advanced multi-functional or recyclable particles.

5. Electrochemical Flocculation

Electrochemical algae harvesting is generally carried out by passing a direct electrical current through electrodes into a culture broth wherein algal cells act as negatively charged colloids. There are two types of electrodes, "sacrificial electrodes", whose metal ions are released into the aquatic environment, and "non-sacrificial electrodes" with non-reactive anodes and cathodes. The electrical current in an aqueous solution can cause a water-electrolysis reaction through either the sacrificial or non-sacrificial electrodes, which would release hydrogen and oxygen gases from the cathode and anode electrodes, respectively. In this review, the electrochemical flocculation (ECF)

process is discussed for the following three aspects: the sacrificial electrode, the non-sacrificial electrode, and electro-flotation.

3. Biosurfactant

Biosurfactants are active compounds produced at the microbial cell surface or excreted, reducing surface and interfacial tension. Microbial surfactants offer several advantages over synthetic ones, such as low toxicity and high biodegradability, and remain active at extreme pH and salinity. Biosurfactants are produced by bacteria, yeasts, and filamentous fungi. The composition and yield of biosurfactants depend on bioreactor characteristics, pH of the medium, nutrient composition, agitation, oxygen availability, and temperature. Solid-state fermentation conditions allow the use of alternative substrates such as industrial or agricultural by-products to reduce costs. Thus, there is industrial interest in developing large-scale production processes for these compounds. The largest market for biosurfactants is the oil industry; however, the applications are distributed among the various industrial sectors, such as the agriculture, cosmetics, and food industries. Based on this, the current chapter reports the processes involved in biosurfactant production.

1. Types of biosurfactant

Biosurfactants are classified into five major groups:

1. Glycolipids, like rhamnolipids produced by *Pseudomonas aeruginosa* and sophorolipids from *Candida*
2. Lipopeptides produced by *Bacillus subtilis*
3. Lipopolysaccharides, from *Acinetobacter calcoaceticus*
4. Phospholipids, obtained from *Corynebacterium Lepus*
5. Fatty acids and neutral lipids

CHAPTER 2

LITERATURE SURVEY

2.1 INTRODUCTION

Microalga is an economical and potential raw material of biomass energy [3], because it does not require a large area of land for cultivation, exhibits a short growth period, possesses a high growth rate, and contains more high-lipid materials than food crops [4-6]. In general, an algal biomass production system includes growing microalgae in an environment that favours the accumulation of target metabolites and recovery of the microalgae biomass for downstream processing [7]. However, due to the small size (5~50 μm), negative surface charge (about -7.5~-40 mV), and low biomass concentration (0.5~5 g/L) of microalgae cells, harvesting microalgae biomass from growth medium is a challenge [8], which accounts for more than 30% of the total production cost from algae to biodiesel [9, 10]. Therefore, it is necessary to develop effective and economic technologies for the harvesting process.

There are currently several harvesting methods, including mechanical, electrical, biological, and chemical-based [11]. In mechanical-based methods, microalgae cells are harvested by mechanical external forces, such as centrifugation [12], filtration [13], sedimentation [14], dissolved air flotation [15, 16], and usage of attached algae biofilms and ultrafiltration membranes [17, 18]. Electrical-based methods are based on electrophoresis of the algae cells [19, 20]. Because of the negative charge of microalgae cells, they can be concentrated by being moved in an electric field [16, 21]. Biological based methods are flocculation caused by extracellular polymeric substances such as polysaccharides and proteins, originating from microalgae and microorganisms [22]. Chemical-based methods mainly refer to chemical flocculation induced by inorganic and organic flocculants. Electrolytes and synthetic polymers are typically utilized [23].

2.1.1 INORGANIC FLOCCULATION

Iron and aluminium salts form positively charged hydroxides in aqueous media that destabilize negatively charged algal cells, inducing algae flocculation and subsequent precipitation. Aluminium sulphate ($\text{Al}_2(\text{SO}_4)_3$), commercially known as "alum", is widely

used in algae harvesting processes and wastewater treatments. It was reported that 1.2 g/L alum enables a harvesting efficiency of 85.6% for marine alga *Tetraselmis sp.* of 3 g/L cell density. Despite the high doses required for algae harvesting, the relatively low cost of alum makes it a potential choice for bulk biomass recovery in biofuel production. However, it should be noted that this method is generally not recommended for algal bio refinery processes that utilize whole cells for multiple bio-products including biofuel and biochemical, since excess aluminum is a well-known carcinogen and also known to cause Alzheimer's disease.

Ferric sulfate ($\text{Fe}_2(\text{SO}_4)_3$) and ferric chloride (FeCl_3) have been used for algae harvesting as well. Harvesting efficiencies of 93.8% and 87.3% were reported for marine alga *N. oculata* using 0.4 g/L $\text{Fe}_2(\text{SO}_4)_3$ and 0.6 g/L FeCl_3 , respectively, after treatment for 180 min. However, $\text{Fe}_2(\text{SO}_4)_3$ is not preferred for large-scale applications due to its strong and corrosive reactivity. This challenge could be resolved if $\text{Fe}_2(\text{SO}_4)_3$ is efficiently removed and recycled after being used for algae harvesting. Recently, Kim et al. reported that $\text{Fe}_2(\text{SO}_4)_3$ could be reused three times for harvesting *Chlorella*, Topd very high flocculation efficiencies of ~98% biomass could be achieved each time; further, the biomass could be used directly in the post-lipid extraction process. $\text{Fe}_2(\text{SO}_4)_3$ from algae flocs was effectively released under low-pH conditions by adding sulfuric acid.

Positively charged calcium salts and magnesium hydroxide are able to neutralize negatively charged algal cells in addition to their pH-increase effect, thus inducing algae flocculation . For instance, 60 mg/L CaO was reported to harvest a *C. vulgaris* biomass of 1.5 g/L cell density with an efficiency of 85% . In addition, 80 mg/L of CaCO_3 -rich eggshell (dissolved in 1% HCl solution) has been reported as an effective flocculant (99% efficiency) for harvesting *C. vulgaris* of 2.3 g/L after treatment for 20 min at pH 6. $\text{Mg}(\text{OH})_2$ at 145 mg/L has been reported to induce 90% precipitation in *Chlorella sp.* at a pH of 10.7. Interestingly, $\text{Mg}(\text{OH})_2$ can be effectively be recovered (up to 95%) from harvested biomass if the pH is properly controlled (reduced to 8) .

Various inorganic flocculants have been frequently used in algae biofuel applications, but the release of such chemicals into the environment is a critical environmental and health concern.

2.1.2 BIOSURFACTANT Algal–

Bacterial Bio-Flocculation

Bacteria generally co-exist during conventional algae cultivation under reactor-scale and/or outdoor conditions. Their interactions are species-specific and can be either mutually benefiting or inhibiting according to the nutritional and environmental conditions. Interestingly, some algae-associated bacteria such as *Flavobacterium*, *Terrimonas*, and *Sphingobacterium* have been proven to promote the formation of large flocs which can settle thereby enhancing algae flocculation. Axenic culture of *C. vulgaris* with bacteria exhibited high harvesting efficiency of ~94%, while an axenic (bacteria-free) *Chlorella* culture showed only 2% flocculation efficiency. Bacterium *Solibacillus silvestris* isolated from activated sludge has been reported to secrete a less-toxic bio-flocculant (composed of 75.1% proteoglycan and 24.9% protein), inducing electrostatic bridging and patching between algal cells. However, this bio-flocculant (dosage, 0.2 g/L) showed only a relatively low harvesting efficiency of 76.3% for marine alga *N. occulata*. Fast growth rates of bacteria can be beneficial relative to other microbial flocculants such as yeast and fungus. However, species-specific reactivity and environmental safety issues should be carefully considered and resolved for large-scale applications.

CHAPTER 3

AIM AND OBJECTIVES

1. AIM

- To study the flocculation of different microalgae for various applications

2. OBJECTIVES

- To culture marine microalgae
- To harvest algal biomass by various flocculation methods.
- To optimize the flocculent and its concentration
- To quantify the extracted algal biomass
- To mass produce the algal biomass.

CHAPTER 4

MATERIALS AND METHODS

1. ALGAE CULTURE MAINTENANCE

Nannochloropsis occulata from stock culture and was cultivated in 1 L culturing flask used in sterile f/2 guillard's medium and Conway medium. The filtered sterilized seawater was enriched with the required quantity of f/2 guillard's medium and Conway medium. The pH was maintained and autoclaved at 121°C for 1hr. The filtered sterilized vitamins were added after cooling and the culture maintained under 5,000 lux illuminated at 12:12 h light and dark condition for 7 days at room temperature.

1. Growth kinetics

The effects of pH and illumination time on microalgae growth were studied before conducting the growth kinetic. Many growth kinetic models are available; among these, the logistic model uses simple calculation for studying microbial growth, because it is independent of substrate consumption and is highly suitable for autotrophic culture of microalgae. The rate of growth of cells is directly proportional to the cell mass concentration at the given time. When the cell mass reaches the stationary phase, the growth rate ceases. A gradual decrease is observed in the late exponential phase or when the cells are near the stationary phase. The advantage of this model is that it facilitates the exponential phase and endogenous metabolic phase.

2. COMPOSITION AND PREPARATION OF GUILLARD'S F/2 MEDIUM

Stock Solution:

Table 4.1 Composition of Guillard's F/2 medium stock solution

CHEMICALS	CONCENTRATION (g/L)
NaNO ₃	75 g/L
NaH ₂ PO ₄ .H ₂ O	5 g/L
Na ₂ SiO ₃ .H ₂ O	30 g/L

Trace Metal Solution:

Table 4.2 Composition of Guillard's F/2 medium trace metal solution

CHEMICALS	CONCENTRATION (g/L)
FeCl ₃ .6H ₂ O	3.15 g
Na ₂ EDTA.2H ₂ O	4.36 g
CuSO ₄ .5H ₂ O	9.8 g
Na ₂ MoO ₄ .2H ₂ O	6 g
ZnSO ₄ .7H ₂ O	22 g
CoCl ₂ .6H ₂ O	10 g
MnCl ₂ .4H ₂ O	108 g

4.1.3 COMPOSITION AND PREPARATION OF CONWAY MEDIUM

Trace Metal Solution:

Table 4.3 Composition of Conway medium trace metal solution

CHEMICALS	CONCENTRATION (g/L)
Potassium nitrate	100 g
Monosodium phosphate	20 g
EDTA disodium	45 g

Boric acid	33.4 g
Ferric chloride	1.3 g
Manganese chloride	0.36 g

Solution B:

Table 4.4 Composition of Conway medium stock solution

CHEMICALS	CONCENTRATION (g/L)
Zinc chloride	4.2 g
Cobalt chloride	4 g
Copper sulfate	4 g
Ammonium molybdate	1.8 g

4.2 BACTERIA CULTURE MAINTENANCE

Marine isolated bacteria were cultured in laboratory environment. These bacteria were grown in LB broth and Zobell Marine Broth. The broth is autoclaved for 1 hour at 121°C. After cooling, the bacteria strains are added, and the culture flask is kept at room temperature and incubated for 2-3 days to attain the required growth.

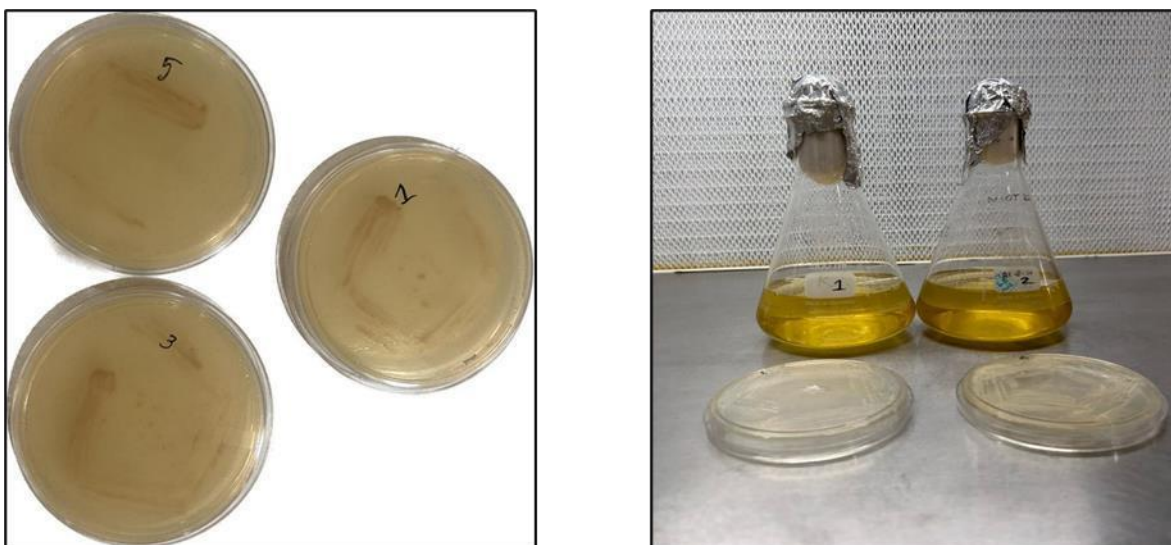


Fig 4.1 Marine bacteria culture inoculation

4.3 Surfactant extraction

Surfactants used in the microalgae sector have received considerable interest. One of the key impediments to cost-effective mass production and industrial-scale manufacture of microalgae has been identified as the harvesting process, which accounts for 20-30% of total biomass production costs [38]. Several techniques have been developed for microalgae harvesting, including but not limited to sedimentation, filtration, centrifugation, flocculation, flotation, and electrophoresis [39]. Sedimentation is less costly but is time-consuming and the concentration of harvested. Surfactant-assisted extraction of microalgal cell contents

Due to most microalga-based products, such as lipids, fatty acids, pigments, and some hydrophilic compounds being intracellular, the extraction of intracellular products is another major challenge for the microalgae industry. The extraction methods of intracellular substances usually include extraction from dry microalgae and extraction from wet microalgae. The costs of dewatering and drying of microalgae culture are very high, which account for 20-30% of the total energy costs in microalgae.

Cells were separated by centrifuge at 10,000rpm for 15 min. Supernatant filtered using a 0.45µm membrane. The PH of the filtrate was adjusted to 2 by adding 3M HCl. The resultant solution was maintained in response to allowing precipitate of surfactin by 12hrs. The precipitate was collected by centrifugation at 10,000 for 15 min at 45°C to obtain the crude surfactin. Purification - the crude surfactin was dissolved in deionized

H₂O & it was extracted 3 times by using an equal volume of dichloromethane. Bacteria are cultured in Zobell Marine Broth for 2-3 days. Cells were separated by centrifuging at 7,000 rpm for 12mins at 25°C. The supernatant is transferred into the flask and heated for 1hr. Then the pH was adjusted to 2 by adding 1N HCl to precipitate. Stored at 4°C in the refrigerator overnight. An equal volume of ethyl acetate is added. Separation of lipid by crude extraction method. Purification- the pure rhamnolipid was extracted from the crude by rotator evaporator process.

4. Characterisation of surfactant

1. TLC

TLC was found to be a preferable technique to paper chromatography due to the ease in quantitatively recovering the pigments from its adsorbents. TLC requires less sample, is less tedious, and produces chromatograms with sharper resolutions. Inorganic adsorbents such as chloroform and methanol were found to be the most efficient stationary phases for thin layer chromatography utilisation of silica gel as their stationary phase and managed to separate all of the plant pigments except for some minor components.

6.6 g of silica powder was dissolved in 15 ml of distilled water and stirred continuously with a magnetic stirrer in a beaker. The silica gel is carefully poured into a sterilised glass slide, dried in a hot air oven for one hour. A few drops of the sample culture are placed at one end of the TLC plate. Mobile phase is then placed in a beaker with solvent, chloroform, and methanol (2:1 ratio), and the TLC plate is placed into the beaker with solvent. For band visualisation a straining dip is used. 95% of ethanol is dissolved in 1ml of acetic acid and 0.3 g of ninhydrin. R_f value was determined

4.4.2 UV–visible analysis

Absorbance spectra of all plants, including algae, are maximal in the blue (and to a lesser extent, the red) portion of the visible spectrum. The qualitative UV-Vis spectra showed a sharp peak and the nature of the secondary metabolite corresponds to a specific wavelength at which the band is obtained. The UV-visible spectrum of *Chlorella vulgaris* shows two sharp peaks of phenolic compound. A similar peak for phenolic compounds was not observed in *Nannochloropsis oculata*. Various forms of chlorophyll have an absorption maximum at different wavelengths. All organisms able

to conduct photosynthesis have chlorophyll a, which absorbs red light at approximately 662 nm and violet light at approximately 430 nm. Chlorophyll b absorbs strongly the flame light at 642 nm and blue light at 453 nm algae with more complex chlorophyll systems absorb light in wider ranges, and in that manner, the process of photosynthesis was much more effective.

3. ATR-FTIR

The FTIR spectra of the microalgal biomass represented the alcohol group (OH), a carboxyl group (COOH), amino (NH₂), and other groups associated with organic compounds. The peak in the high-intensity region indicated the presence of lipids in biomass, suggesting that the lipid peak in this region is due to the symmetrical and asymmetrical stretching vibration of CH₂. Different peaks of alkane groups, aldehyde groups, ester groups, and carboxylic groups were recorded. The presence of saturated and unsaturated hydrocarbons was marked by the peaks of alkane and alkene groups. An abundance of carbohydrates, proteins, and lipids was predicted by the corresponding peaks in functional groups such as aldehyde, carboxyl, and ester groups.

4. GC-MS

Gas chromatography-mass spectrometry (GC-MS) is an analytical method that combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample. TLC are rapid and versatile technique used for the analytical characterization of compounds. The compounds are identified from peaks obtained in the spectra and by retention time.

4. FLOCCULATION

1. Chemical Flocculation

500ml samples (*Nannochloropsis occulata* and *Chlorella vulgaris*) were taken from each culture in two different beakers respectively. 0.5g of FeCl₃ and AlSO₄ were added. The samples were kept undisturbed for 5-6 hrs for the aggregation of the microalgae. Then the remaining phase of the non-algal solution is removed and the algal sludge is collected in falcon tubes. The falcon tubes are centrifuged at 8000 rpm, 10 min, and 4°C twice to remove the seawater content. This process of centrifugation is carried out

to avoid the crystallization of algae while drying in a hot air oven. Then algal biomass is dried and weighed.

4.4.2 Surfactant based flocculation

500ml samples (*Nannochloropsis occulata*) were taken from each culture in two different beakers respectively. The samples were kept undisturbed for 5-6 hrs for the aggregation of the microalgae. Then the remaining phase of the non-algal solution is removed and the algal sludge is collected in falcon tubes. The falcon tubes are centrifuged at 8000 rpm, 10 min, and 4°C twice to remove the seawater content. This process of centrifugation is carried out to avoid the crystallization of algae while drying in a hot air oven. Then algal biomass is dried and weighed.

CHAPTER 5

RESULTS

5.1 MICROALGAE CULTIVATION

The culture growth of the examined algal species differed and was dependent on the photoperiod applied. Continuous illumination stimulated the growth of *Nannochloropsis occulata* and *Chlorella vulgaris* more efficiently. Photoperiod is also important in terms of the economic aspect of algal biomass is produced with a supply of light from artificial sources. There are several reports on the influence of the photoperiod on the productivity and growth rate of *C. vulgaris* and *Nannochloropsis occulta*.



Fig.5.1 Marine microalgae culture

5.2 BACTERIAL CULTIVATION

The strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* are inoculated into a conical flask containing LB broth and Zobell Marine Broth. After incubating the conical flask for 24-72 hours growth will be large. Growth on a minimal medium can take longer depending on the nutrient sources provided.

5.3 SURFACTANT EXTRACTION

The biosurfactant was produced by marine bacteria in LB broth under controlled conditions. Cells are centrifuged and collected. pH was maintained by adding an equal amount of HCl (in order to precipitate). The crude biosurfactant was obtained using a rotary evaporator under a vacuum.



Fig 5.2 Surfactant extraction

4. CHARACTERISATION OF SURFACTANT

1. THIN LAYER CHROMATOGRAPHY

Thin layer chromatography revealed the presence of 5-6 pigment fractions extracted from the isolated green algal strains. Chlorophyll a and β -carotene are the dominant pigments present in the green algal strains. The identified pigments were xanthophyll, chlorophyll b, chlorophyll a, Anthocyanin, and β -carotene their R_f values were 0.41, 0.48, 0.54, 0.60, 0.73 respectively.



Fig 5.3 Thin layer chromatography for the extracted surfactant

5.4.2 UV-vis analysis

UV-vis analysis for the extracted surfactant indicated multiple bands in the range of 200 - 700 nm. This indirectly emphasizes the presence of various compounds in the crude surfactant.

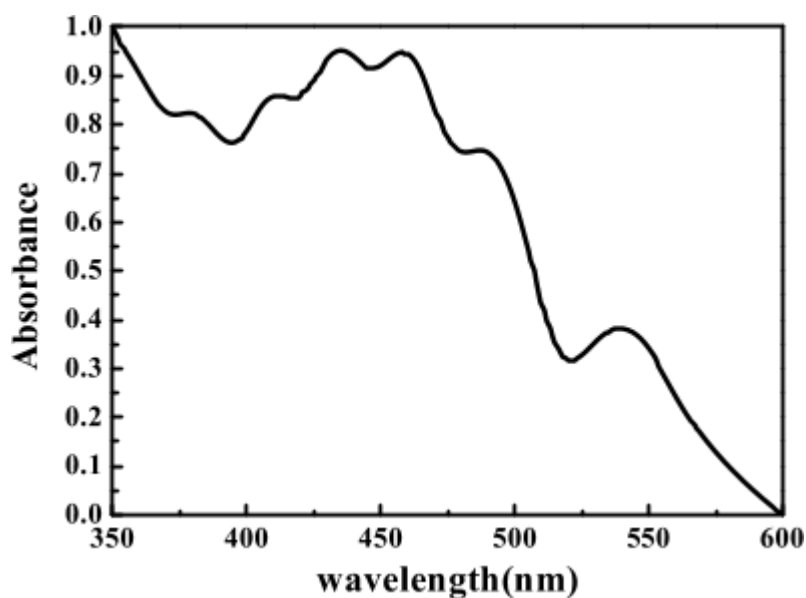


Fig 5.4 UV-vis data for the extracted crude surfactant

5.4.3 ATR-FTIR

The extracted crude surfactant was subjected to ATR-FTIR analysis to determine the presence of various functional groups in the compound. The analysis interpreted the presence of amine, hydroxyl, ester and carboxyl groups in form of peaks at various ranges.

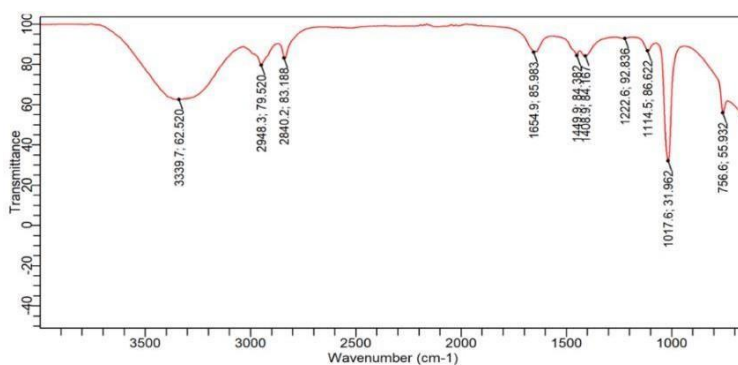


Fig 5.5 ATR-FTIR data for the extracted crude surfactant

5.4.4 GC-MS

Gas chromatography techniques was employed to determine the presence of various compounds in the extracted crude surfactant. The compounds vary in the composition which can be interpreted by observing various major and minor peaks.

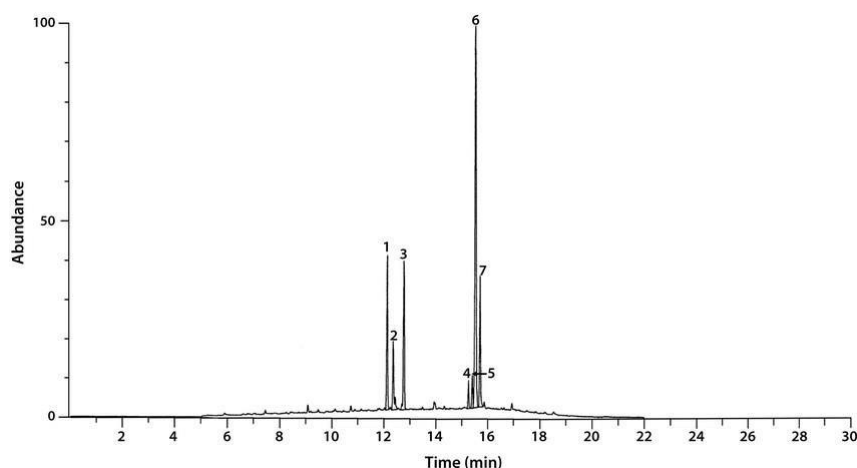


Fig 5.6 GC-MS peaks for the extracted crude surfactant

5. Flocculation

1. Chemical based flocculation

Two flocculants were used to extract the algal biomass. While using chemical flocculant, the flocculation efficiency was less and it took more time to settle down the particle. The flocculation efficiency and biomass extracted was listed down below:

Table 5.1 Flocculation efficiency of the chemical flocculants

S.No	Flocculant used	Organism	Flocculation efficiency	Biomass extracted
1.	FeCl ₃	<i>N. oculata</i>	89%	125 mg/100 ml
2.	AlSO ₄	<i>N. oculata</i>	91%	114 mg/100 ml

5.5.2 Surfactant based flocculation

Surfactant extracted from the marine bacterial source was subjected to the flocculation of marine microalgae. When added to the microalgae culture, cells started to aggregate readily, and dispersion happened comparatively faster than other chemical

flocculants used in this study. The flocculation efficiency and the extracted biomass was listed below.

Table 5.2 Flocculation efficiency of the surfactant

S.No	Flocculant used	Organism	Flocculation efficiency	Biomass extracted
1.	Surfactant	<i>N. oculata</i>	96%	250 mg/100 ml



Fig 5.7 Extracted biomass using surfactant-based flocculant

CHAPTER 6

CONCLUSION

Marine microalgae and *Nannochloropsis oculata* and *Chlorella vulgaris* were successfully cultivated in F2 medium and Conway medium supplemented with seawater. Therefore, high biomass productivity can supply high demand for biomass production or other value-added products which on the other hand will reduce the production cost. Due to that reason, *Nannochloropsis oculata* and *Chlorella vulgaris* is selected for mass production of microalgae biomass. The functional groups present in algae biomass have been confirmed by FTIR spectral analysis. The FTIR analysis of dry algae clearly indicated the presence of lipid groups, alcoholic groups, and carboxyl groups. FTIR studies could be used to estimate the approximate lipid proportion of a given microalga; therefore, a combination of FTIR offers an excellent tool for the characterization of microalgae.

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