Production of Bioplastic Materials by Microalgae and Cyanobacteria from Freshwater Sources

A Dissertation for

MIC-651 Discipline Specific Dissertation

16 credits

Submitted in partial fulfilment of Master's Degree

M.Sc. in Microbiology

by

ARZOO MULLA

Seat Number: 22P0420002

ABC ID: 173626279617

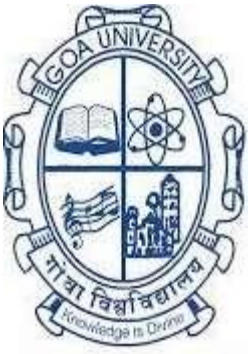
PRN: 201905943

Under the Supervision of

DR. LAKSHANGY CHARYA

School of Biological Sciences and Biotechnology

Microbiology Programme



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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Production of Bioplastic Materials by Microalgae and Cyanobacteria from Freshwater Sources" is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Lakshangy Charya and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Date: 08/04/2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Production of Bioplastic Materials by Microalgae and Cyanobacteria from Freshwater Sources"** is a bonafide work carried out by **Ms Arzoo Mulla** under my supervision in partial fulfilment of the requirements for the award of the degree of **Master of Science** in the Discipline Microbiology at the School of Biological Sciences and Biotechnology, Goa University.

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Microbiology Discipline

Date: 8/4/24

Dr. Bernard F. Rodrigues
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Place: Goa University

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Goa University, Goa-403206

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PREFACE

This piece of work has come to fruition after months of work, dedicated to taking a step for the environment. I've always believed that the Earth is full of bounties, and we are truly blessed to call this planet our home. It is in the hands of each one of us to take care of it. While reading up on the detrimental effects of pollution during my research, the horrifying reality of how our dependence on synthetic plastics have led to us literally choking the life out of our aquatic and terrestrial ecosystem, was really disturbing. The pollution caused due to improper disposal and inadequate waste management strategies has harmed the Earth immensely. The microorganisms existing around us, although unseen, have tremendous capabilities and roles. This was a very small and humble attempt of testing the capabilities of photoautotrophic microorganisms, the cyanobacteria and green microalgae, whose untapped potential could be explored and optimized to produce biodegradable plastics from their polyhydroxyalkanoate reserves. I hope this thesis report helps the readers to make environmentally conscious decisions and switch to a more sustainable lifestyle and implement proper measures of waste management. In the near future, hopefully sustainable biobased and biodegradable plastics will replace traditional plastics forever.

ACKNOWLEDGEMENTS

I take this opportunity to acknowledge everyone who helped me throughout this dissertation.

Foremost, I would like to express my utmost and eternal gratitude to Allah, my Creator, for the countless blessings that He showers upon me. I am thankful to my parents, for believing in me and for their endless support.

I would like to extend my sincere and deepest gratitude to my guide, Dr. Lakshangy Charya, Programme Director of Microbiology Discipline, SBSB, Goa University for giving me your valuable time and sharing your expertise and wisdom with me. It wouldn't have been possible without your guidance and supervision throughout each step of this dissertation study.

I express my sincere thanks and appreciation to all the teaching faculty of the Microbiology Discipline, Prof. Dr. Sandeep Garg, Dr. Milind Naik, Dr. Judith Noronha, Dr. Bhakti Salgaonkar, Dr. Trupti Asolkar and Dr. Lata Gawade for acknowledging my queries and helping me by sharing your expertise and providing your endless support. I am truly grateful to Dr. Priya D'Costa from the discipline of Marine Microbiology, SEAOS, for helping me acquire the necessary resources for my dissertation. I would like to thank Dr. Meghanath Prabhu, Programme Director of Biotechnology Discipline, SBSB, for letting me use the facilities of the Biotechnology department.

I sincerely thank all the non-teaching faculty of Microbiology Discipline for your constant support and generous help in fetching all the resources required throughout the course of this dissertation.

I acknowledge all the research scholars for your generous help, guidance, and advice throughout my study.

Special thanks and appreciation to my friends and dissertation colleagues who have been there for me since the start of my dissertation. Starting from accompanying me for sample collection to keeping me company during late hours, this really would be impossible without the hands-on and motivational support you all have provided me.

TABLES

FIGURES

ABBREVIATIONS USED

Standard Abbreviations

Entity	Abbreviation
Alpha	α
Beta	β
Dalton	Da
Degree celsius	°C
Gram	g
Kilo basepair	Kbp
Liter	L
Mega pascal	MPa
Micrometer	µm
Milligram	mg
Milliliter	ml
Millimeter	mm
Minutes	min
Molar	M
Nanometer	nm
Percentage	%

Other Abbreviations

Entity	Abbreviation
Allophycocyanin	A-PC
Blue-Green	BG
Carbon Dioxide	CO_2
C-Phycocyanin	C-PC
C-Phycoerythrin	C-PE
Dipotassium hydrogen phosphate	K_2HPO_4
Glass transition temperature	T_g
Long chain length	lcl
Medium chain length	mcl
Melting temperature	T_m
Nitrogen	N
Phosphorous	P
Polyhydroxyalkanoate	PHA
Polyhydroxybutyrate	PHB
Potassium dihydrogen phosphate	KH_2PO_4
Short chain length	scl
Sodium hydroxide	NaOH
Species	sp.
Water	H_2O

ABSTRACT

The use of conventional petrochemical plastics has been proven to be detrimental to the environment because of pollution as well as generation of microplastics. There have been multiple studies conducted to discover materials which are biodegradable and have properties which match those of plastics. Polyhydroxybutyrate is a good alternative proposed due to its properties of biodegradability and biocompatibility. It also has good mechanical properties, comparable to traditional polypropylene. Besides heterotrophic bacteria, cyanobacteria and microalgae have also been researched upon for autotrophic PHB production as a more sustainable alternative for mass production of PHB. 15 isolates from Goa were screened for PHB production under different nutritional conditions. The presence of PHB was observed by microscopy by staining techniques and visualization under UV. Deficiency of Nitrogen and Phosphorous, and presence of Glucose was found to stimulate the production of PHB in the isolates and are important factors in PHB production.

KEYWORDS: Microalgae, Cyanobacteria, PHAs, Bioplastic, Biodegradable, Sustainability

CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

Conventional plastics are one of the biggest pollutants contributing to land and ocean pollution. These plastics are derived from fossil fuels and hence are of non- renewable origin (Kuddus and Roohi, 2021). They are composed of hydrophobic long chain polymers of high molecular weight. These properties are a double-edged sword as they make the material durable and offer good tensile strength but also make plastics resistant to microbial degradation. Although plastics are versatile polymers and have favourable material properties like stability, durability and economic feasibility, the cons of using conventional plastics far outweigh the pros. The major problems of conventional plastics are depletion of oil sources, accumulation in the environment, their recalcitrance and the rise in greenhouse gas emissions and toxic gas emissions which comes with combustion of plastics (Mastropetros et al., 2022). The main types of plastics responsible are single-use, disposable plastics. Recycling is insufficient as it is impossible to recover the entirety of plastic refutes from the environment as well as it requires immense amount of energy to recycle the colossal quantities of waste.

Of the total plastic produced, only 9% has been recycled and 12% has been destroyed by incineration. The remaining 79% ends up as pollution in the environment with 10 million tonnes of new plastic entering our oceans each year. This continuous accumulation in strata of water bodies has been described as a new ecosystem - the platisphere (Agarwal et al., 2022). This “white pollution” is negatively affecting ecosystems, killing 1 million animals annually (Price et al., 2020).

By the year 2025, 11 billion tonnes of plastics have been predicted to accumulate in the environment (Dang et al., 2022). Because of its buoyant nature, the waste disperses across oceans and gets fragmented into smaller particles, forming macro- and microplastics. Microplastics have a very small size ranging from a few micrometers to 500 micrometers, and hence have large surface areas, which can adsorb

hazardous compounds like heavy metals and toxins and can penetrate the food chain and lead to bioaccumulation (Mastropetros et al., 2022). Microplastics pose a hazard to aquatic ecosystems as well as to public health.

Therefore, it is imperative to switch to environmentally friendly alternatives. The development of sustainable bio-based polymers is crucial in addressing the global environmental pollution caused by synthetic plastics production. Polyhydroxyalkanoates are bio-based, biosynthesized and biodegradable aliphatic polyesters made from hydroxyalkanoic monomers and are synthesized under physiological cell stress circumstances to serve as an alternative carbon and energy source for cells (Koller, 2020). They offer immense potential in terms of sustainability. PHB is a well-studied biopolymer under PHAs and is offered as a bioplastic alternative to conventional plastics.

While there is much research existing on bacterial PHAs, microalgae and cyanobacteria are also known to make PHAs. Autotrophic microorganisms derived polyhydroxyalkanoates have the potential for cost-effective production. Microalgae and cyanobacteria are a group of highly diverse and almost cosmopolitan organisms which can be used as potential cell factories for PHA thermoplastics production as they are primary producers which bind to and regulate carbon dioxide concentrations by performing photosynthesis and can thrive with minimal nutrient requirements and accumulate many useful metabolic compounds (Arias et al., 2020). They can also be cultivated in wastewaters (Singh et al., 2017). Cyanobacterial and microalgal polyhydroxyalkanoates offer several advantages over heterotrophic bacterial PHAs in terms of their production process and environmental impact.

The polymer having water-insoluble, non-toxic, biodegradable, and biocompatible properties makes it useful in biomedical and chemical industries(Yashavanth et al., 2021). With time, as the cost of production of PHB decreases, it can possibly replace traditional plastics completely as packaging material. Continued research and development in this field could significantly contribute to the reduction footprint and plastic burden and the transition towards a more sustainable and environmentally friendly future.

1.2. AIMS AND OBJECTIVES

Most of us are aware of the ill effects of petrochemical plastic use and disposal and how it has led to terrestrial and aquatic ecosystems, threatening the delicate balance of life. We remember the 3 Rs – Reduce, Reuse and Recycle for waste management, but it is also imperative to do our part for bringing about a revolutionary change which will help us to take a step forward in the road to sustainable development. Polyhydroxyalkanoates, a product of microbial metabolism, are an excellent bioplastic alternative. These are bio-based and biodegradable polymers, which can be composted or naturally degraded once they are disposed of. PHAs can be produced by both autotrophic and heterotrophic microorganisms. The property of autotrophic microorganisms such as microalgae and cyanobacteria to accumulate PHAs make them good candidates for the production of bioplastics as they can scavenge CO_2 and convert it to biomass and store PHAs intracellularly as a carbon and energy source. This not only helps to control the concentration of CO_2 in the atmosphere, but also leads to getting PHA yield without utilization of organic sources due to their low nutritional requirements. The bioplastics produced from these PHAs would be eventually utilized as a carbon source by other heterotrophic organisms for their growth.

This study aimed to isolate microalgae and cyanobacteria from freshwater sources. The isolates were then screened for the production of polyhydroxybutyrate, a well-studied biopolymer under PHAs. The yield of PHB can be enhanced under certain conditions such as deficiency of macronutrients like Nitrogen and Phosphorous as well as the presence of organic carbon substrate.

With this background, the following objectives were set in this study:

1. Isolation of axenic cultures of microalgae and cyanobacteria from freshwater sources
2. Screening of the isolates for the production of polyhydroxybutyrate
3. Checking the effect of certain nutritional conditions on the production of PHB

1.3. HYPOTHESIS

Utilization of cyanobacteria and microalgae for the production of bioplastic materials will be an economically viable and eco-friendly system as compared to heterotrophic microorganisms.

This is hypothesized due to the potential of cyanobacteria and microalgae to photosynthesize, utilizing the inorganic carbon source, carbon dioxide from the environment and converting it to polyhydroxybutyrate. These intracellular PHB stores can then be extracted and processed to produce bio-based and completely biodegradable plastics. Hence, offering a greener alternative to conventional bioplastic production using plant-based precursors or other microorganisms having organic nutritional requirements.

1.4. SCOPE

This study aims to isolate multiple photosynthetic microorganisms and check the effect of various nutritional conditions on the production of polyhydroxyalkanoates. Hence the isolates obtained from this research have potential which can be further explored, such as:

1. Optimization and standardization of the nutritional requirements in order to obtain bulk production of PHAs.
2. Characterization and testing of material properties of the polymer obtained.
3. Checking the biodegradation efficiency of the biopolymer.

CHAPTER 2: LITERATURE REVIEW

2.1. CYANOBACTERIA AND MICROALGAE

Algae are a group of photosynthetic organisms which are differentiated based on size into macroalgae and microalgae (Khan et al., 2018). Microalgae has been defined with some loose definitions in the past, like an organism possessing Chlorophyll a and an undifferentiated thallus (Richmond, 2004). This definition hence in a broad sense encompasses microscopic eukaryotic algae as well as members of *Cyanophyta,* which consists of prokaryotic autotrophic organisms. These organisms are ubiquitous in almost all aquatic ecosystems as well as on soil surfaces (Richmond, 2004). Around 200,000 to several million species of microalgae have been identified so far. They are small in size, mostly ranging from 5 -50 μm (Singh and Saxena, 2015). Microalgae may be devoid of cell wall or possess one. The microalgal cell wall is composed of cellulose microfibrillar layer which may be surrounded by an amorphous layer (Richmond, 2004). Green microalgae possess chloroplasts having Chlorophyll a and b pigments and have membrane bound organelles, they are members of *Chlorophyta* of algae and include genera such as *Chlorella, Scenedesmus, Coelastrum*, etc (Dang et al., 2022). Microalgae have a short doubling time and are one of the fastest growing autotrophs (Masojídek and Torzillo, 2014).

Cyanobacteria are a group of Gram negative, highly morphologically and ecologically diverse group of oxygenic photosynthetic organisms and form the largest group of photosynthetic bacteria. Their size ranges from 0.5 to 100 μm in diameter. They can be found in both unicellular and filamentous forms. Cyanobacteria possess the pigment Chlorophyll a as well as certain proteins called phycobilisomes which are associated with the accessory pigments phycocyanin and phycoerythrin. The presence of these phycobilisomes is another key difference between microalgae and cyanobacteria, although a group of cyanobacteria called the *Prochlorophytes* possess both Chlorophyll a and b and lack phycobilisomes. Cyanobacteria can grow under photoautotrophy as well as photoheterotrophy. According to the Bergey’s

Manual, these organisms have been divided into five subsections based on their morphology, namely *Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales, Stegonematales* (Madigan et al., 2019; Willey et al., 2017). The characteristics of these subsections are summarized in table 2.1.(a). Cyanobacteria can produce a variety of metabolic products and are able to form a variety of structures associated with the function of storage, reproduction, and survival. Some of the storage reserve products of these microorganisms include glycogen, polyhydroxybutyrate, cyanophycin and polyphosphate inclusions (Price et al., 2020).

Table 2.1.(a) Cyanobacterial subsections and their characteristics (Willey et al., 2017)

Sub section	**Name**	**General Features**	**Reproduction and growth**	**Heterocysts**	**Representative genera**
I	*Chroococcales*	Unicellular rods or cocci, non-filamentous aggregates Non motile or swim without flagella	Binary fission, budding	Absent	*Chroococcus* *Gleocapsa* *Prochlorococcus* *Synechococcus*
II	*Pleurocapsales*	Unicellular rods or cocci, may be held together by aggregates. Some baeocytes are motile	Multiple fission to form baeocytes	Absent	*Pleurocapsa* *Dermocarpella* *Chroococcidiopsis*
III	*Oscillatoriales*	Filamentous, unbranched trichome with only vegetative cells Usually motile	Binary fission in a single plane, fragmentation	Absent	*Lyngbya* *Oscillatoria* *Prochlorothrix* *Spirulina* *Pseudoanabaena*
IV	*Nostocales*	Filamentous, unbranched trichome may contain specialized cells	Binary fission in a single plane, hormogonia	Present	*Anabaena* *Cylindrospermum* *Nostoc*

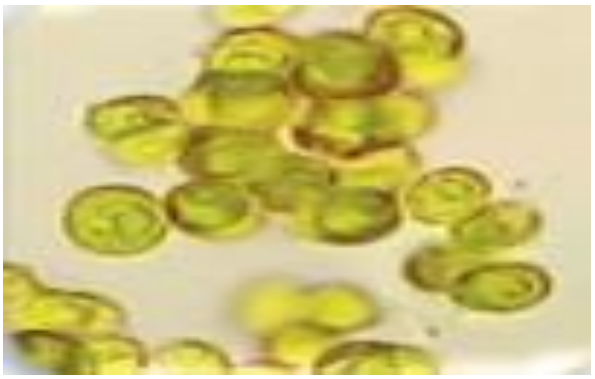
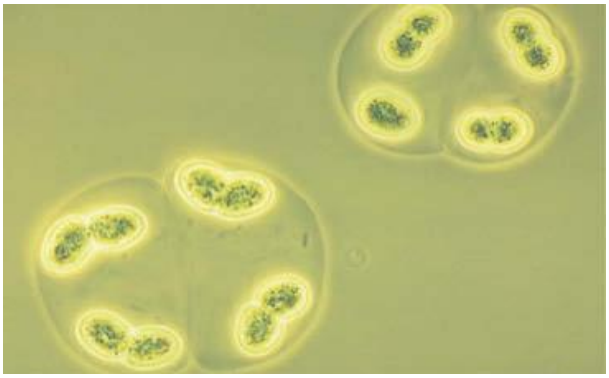
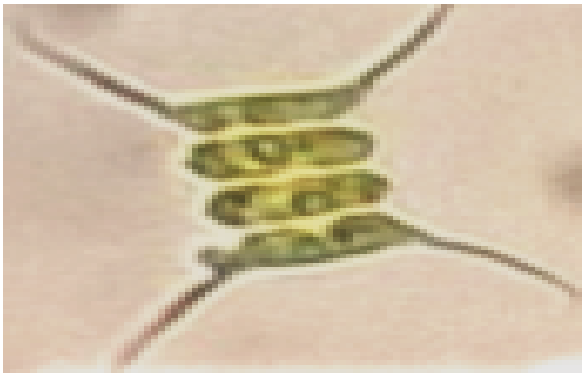
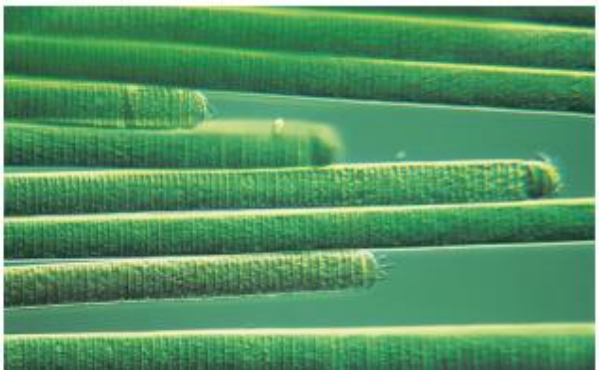
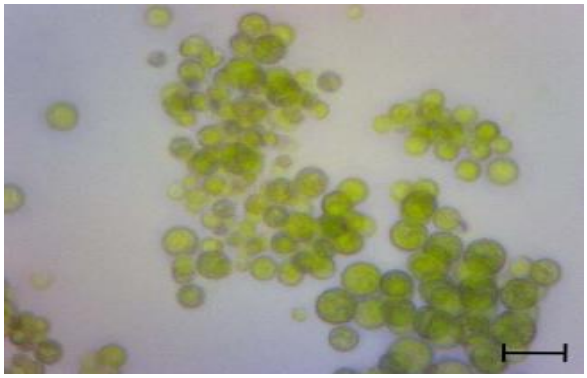
		Often motile, may produce akinetes	are formed by fragmentation		
V	*Stegonematales*	Filamentous trichomes either with branches or composed of more than one row of cells. May produce akinetes, have the greatest morphological complexity and differentiation in cyanobacteria	Binary fission in more than one plane, Formation of hormogonia	Present	*Fischerella* *Stigonema* *Geitlera*

Although the lines in literature are often blurred, there are many differences between these two photoautotrophic groups, which are highlighted in table 2.1.(b) with examples of some representative organisms of the two groups.

Table 2.1.(b) Key differences between *Cyanobacteria* and *Chlorophyta*

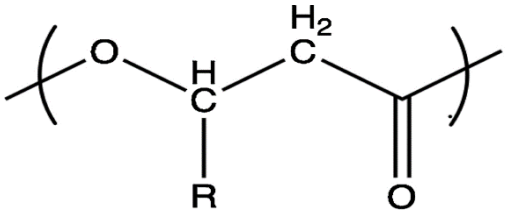
(Hachicha et al., 2022; Kavitha et al., 2016; Madigan et al., 2019; Selvaraj et al., 2021; Willey et al., 2017)

	Cyanophyta	*Chlorophyta*
Genome organization	Nucleoid	Nucleus
Membrane bound organelles	Absent	Present
Pigments	Chlorophyll a, β carotene, flavacene, echinenone, isozea-, zea-, myxo-, oscillaxanthin, APC, C-PC, C-PE	Chlorophyll a, b, α and β- carotene, lutein, zea-, viola-, loro- and neoxanthin
Flagellation	Unflagellated	2-4 flagella, isokont

Reproduction	Simple division, non-motile endospore, vegetative fragmentation, no sexual reproduction	Isogamy, anisogamy, oogamy, motile zoospore
Cell wall	Murein/ peptidoglycan, lack of cellulose	Contains cellulose, mannans, xylans, sitosterol; sometimes calcified
Examples	 *Chroococcus sp.*	 *Chlorella sp.*
	 *Gleocapsa sp.*	 *Scenedesmus sp.*
	 *Oscillatoria sp.*	 *Botryococcus sp.*

2.2. POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are a class of biodegradable aliphatic polyester molecules which are produced by a variety of autotrophic and heterotrophic microorganisms. They were first discovered in *Bacillus megaterium*, a Gram-positive heterotroph by French scientist Maurice Lemoigne. He observed intracellular granules of PHB in the bacterium (Mastropetros et al., 2022). Hydroxy acid monomers are joined by ester linkages i.e. the carboxyl group of one hydroxy acid unit forms an ester bond with the hydroxyl group of the adjacent monomeric unit to form these molecules (Singh et al., 2017). PHAs are synthesized by organisms under physiological stress conditions when there is excess of carbon. PHAs serve as an alternate source of energy and carbon to the cell. 155 PHA monomers with molecular weights ranging from 50×10^3 to 1×10^6 Da have been discovered so far. PHA monomers have been classified into 3 types based on the length of the carbon chain: short chain length (scl), medium chain length (mcl) and long chain length (lcl) PHAs (Mastropetros et al., 2022). Scl PHAs have a chain length of less than 5 carbon atoms, mcl PHAs have carbon atoms ranging from 5-14 carbons and lcl PHAs have more than 14 carbon atoms. The general structure of PHA is shown in figure 2.2. To mention, few of the common PHA monomers are 4-hydroxybutyrate, 3-hydroxybutyrate, 3-hydroxyvalerate, 3- hydroxypropionate, 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3- hydroxydodecanoate, and 3-hydroxytetradecanoate (Roja et al., 2019). Polyhydroxyalkanoates exhibit properties of high crystallinity (from 60-80%) and high melting temperature T_m ranging from 50 to 180°C (Mastropetros et al., 2022).



R = hydrogen	Poly(3-hydroxypropionate)
R = methyl	Poly(3-Hydroxybutyrate)
R = ethyl	Poly(3-hydroxyvalerate)
R = propyl	Poly(3-hydroxyhexanoate)
R = pentyl	Poly(3-hydroxyoctanoate)
R = nonyl	Poly(3-hydroxydodecanoate)

Figure 2.2. General molecular structure of PHAs [Source: (Price et al., 2020)]

2.3. POLYHYDROXYALKANOATE PRODUCTION IN MICROALGAE AND CYANOBACTERIA

More than 100 species of prokaryotic and eukaryotic photosynthetic microorganisms have been found to accumulate polyhydroxyalkanoates within their cells autotrophically (Mastropetros et al., 2022). Most commonly, poly-3-hydroxybutyrate molecules are stored within the cells. The PHB obtained from living cells is a 100 % biodegradable material and has properties like polypropylene. Heterotrophic bacteria are commonly employed in commercial PHB synthesis. Commercial production of PHB from bacteria may not be economically viable since it demands the use of expensive sugar substrates (30-40% of overall production costs), an uninterrupted supply of oxygen, and a high energy input. Bacterial-derived PHB is significantly more expensive than synthetic petroleum-based plastic and hence low-cost manufacturing technologies as an alternative is the need of the hour (Yashavanth et al., 2021). PHA producers are divided into two types based on when they synthesize PHA during their life cycle: either during normal growth phase or during starvation conditions with nutrient deficiency. Cyanobacteria are the sole prokaryotic species capable of producing PHB in both photoautotrophic and chemoheterotrophic conditions. (Agarwal et al., 2022)

Cyanobacteria offer cost-effective photoautotrophic synthesis of PHAs, making them a promising alternative to heterotrophic bacteria and higher plants. Several studies have found that limiting nutrients and adding precursors can boost the PHA output in cyanobacteria (Singh et al., 2017). PHB is the dominant internal storage component in cyanobacteria. Among 150 polyhydroxyalkanoates investigated, PHB was found to be the most common bioplastic in prokaryotic cyanobacteria. PHA production in cyanobacteria was initially detected in photoautotrophic conditions, but this can also be enhanced with organic carbon additions such glucose, acetate, citrate, xylose, and arabinose under photoheterotrophy (Roja et al., 2019). The Calvin Benson cycle is involved in the initial fixing of CO_2. The CO_2 is converted to glucose and then converted to pyruvate via glycolysis, ultimately giving acetyl CoA. The metabolic pathway involved in PHB biosynthesis consists of 3 sequential enzymatic reactions, which catalyze the

conversion of acetyl CoA to PHB. PhaA is a PHA specific β ketothiolase which forms acetoacetyl CoA by combining 2 molecules of acetyl CoA by a condensation reaction. PhaB being an acetoacetyl CoA reductase then reduces the acetoacetyl CoA to hydroxybutyryl CoA with the utilization of NADPH molecules. The final step is catalyzed by PHB synthase enzymes PhaEC, wherein the hydroxybutyryl CoA fatty acid monomer is linked to a growing chain of PHB molecule via an ester bond. The synthesis pathway is graphically explained in the figure 2.3. These PHB molecules tend to form amphipathic granules within the cell (Price et al., 2020; Roja et al., 2019; Singh et al., 2017).

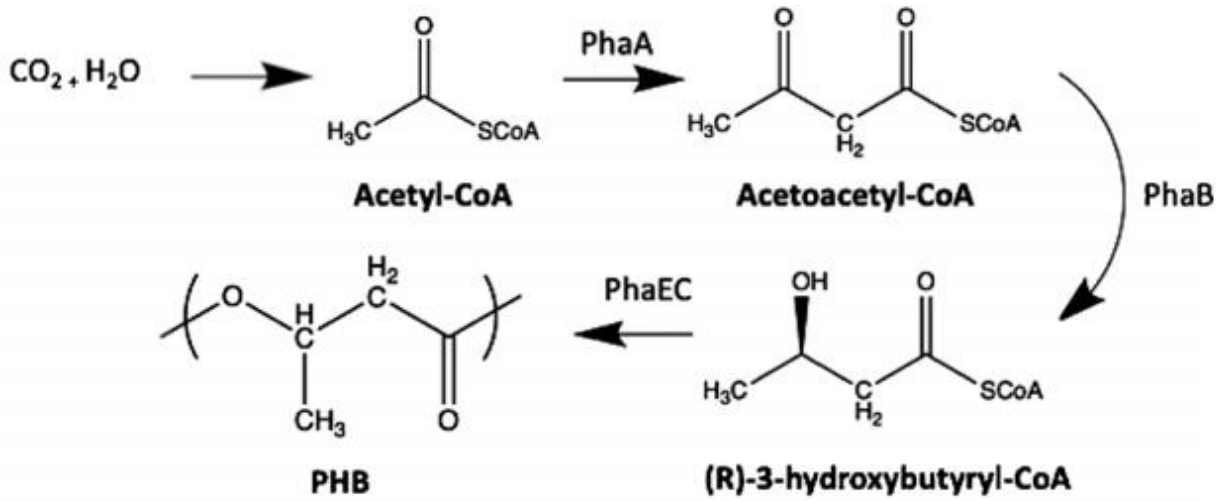


Figure 2.3. PHB synthesis metabolic pathway seen in *Synechocystis* sp. [Source: (Price et al., 2020)]

The PHB synthesis in both heterotrophic and autotrophic bacteria require the same genes *phaA, phaB* and *phaEC* and the process of synthesis is also similar. The difference lies in the position of the genes. In heterotrophs such as *Cupriavidus necator*, all the genes lie on the same operon whereas in Cyanobacteria such as *Synechocystis*, the genes are located approximately 500Kbp apart in two separate operons. The genes for PhaA and PhaB enzymes are present in the first loci and are putatively co-expressed and the genes for the heterodimeric enzyme PhaEC consisting of PhaE and PhaC are present in the second loci.

Apart from PHB which is accumulated photoautotrophically, under conditions of mixotrophy or heterotrophy, polyhydroxyvalerate can be synthesized by growing the culture in the presence of valerate, and co-polymers of PHA such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) can be obtained by adding acetate and valerate as substrates. Among microalgae, the genera *Chlorella* and *Botryococcus* have been reported to naturally accumulate PHB in their cells (Price et al., 2020). *Chlamydomonas reinhardtii* is another microalgal strain which has the intrinsic *phaA* gene but lacks the other genes, but a recent finding of the same species isolated from an Indian lake has depicted PHB production of 206 mg L^- with the supplementation of D-xylose (Sirohi et al., 2021). PHA production can be stimulated or enhanced by tweaking the culture conditions. Limitation of elements Nitrogen and Phosphorous enhanced PHA accumulation in cyanobacteria (Afreen et al., 2021; Bhati et al., 2010). The effect of the deficiency of these elements has been researched upon for photoautotrophic, mixotrophic as well as heterotrophic production of PHA. For mixotrophic and heterotrophic production, an organic carbon source such as glucose, acetate, propionate or valerate is supplemented in the growth medium. (Carpine, 2018; Carpine et al., 2019; Samantaray and Mallick, 2015; Troschl et al., 2017).

2.4. TYPES OF WASTE USED AS SUBSTRATES FOR PHA PRODUCTION

Cyanobacterial and microalgae derived bioplastic production is currently not economically feasible due to the high costs associated with culture equipment and water requirement and because most strains have PHB yield content lower than heterotrophic bacteria. Despite the lower PHB yield, it is still far more feasible to have photoautotrophic production of PHB due to free availability of sunlight and carbon dioxide as energy and carbon sources (Mariotto et al., 2023). Utilization of waste materials as substrates for the cultivation is a way to reduce the operational costs for sustainable large-scale production (Price et al., 2021). Cyanobacteria and microalgae are excellent nutrient scavengers, having a nutrient removal efficiency and hence a variety of waste can be used as a substrate for their growth. Agricultural residues

can be used as a substrate for culturing cyanobacteria and microalgae on a large scale, which will reduce the input costs as well as the nutrients from the agro-waste can be utilized completely before their disposal. Wastewater is also a preferred nutrient source due to high nutrient concentration as well as the hazardous heavy metals present can be utilized and bioremediated by the microalgal cultures (Mastropetros et al., 2022). Aquaculture wastewater, secondary waste effluent from sewage treatment, poultry waste, tannery, industrial effluents, anaerobically digested food industry effluents as well as CO_2 emissions from industries can be valorized (Arias et al., 2020; Gradíssimo et al., 2020; Senatore et al., 2023; Sood et al., 2015). Domestic primary wastewater has also been tested as a growth medium for cyanobacterial cultivation (Price et al., 2021)

Some common genera which have been utilized for wastewater valorization include *Synechocystis salina*, *Synechocystis* sp. 6803*, Aulosira fertilissima, Synechococcus* sp., *Phormidium* sp., *Nostoc muscorum* Agardh, *Botryococcus braunii* (Arias et al., 2020; Gradíssimo et al., 2020; Kavitha et al., 2016; Price et al., 2021; Senatore et al., 2023)

2.5. PHB PRODUCTION AS A SOLUTION FOR PLASTIC POLLUTION

PHAs in general have gained prominence over other bioplastic materials due to their chemical structure diversity and distinctive material properties such as glass transition temperature (T_g) and melting temperature (T_m), Young's modulus and tensile strength, which come close to certain types of traditional plastic polymers such as polypropylene. Poly-3-hydroxybutyrate has reduced permeability to gaseous components and water vapour from the atmosphere and is also highly resistant to hydrolytic degradation and water. An overview of the comparison of properties between plastics and bioplastics is given in table 2.5.

The process of PHA degradation begins once they come in contact with soil, marine sediment or compost. PHB biodegradation by aerobic process gives CO_2 and H_2O and the anaerobic degradation products are CO_2 and CH_4 (Balaji et al., 2013). From an environmental point of view, once disposed, high density PHA plastics do not float, rather they sink and submerge and are decomposed by surface biogeochemical processes. This is a stark difference because the PHB can act as a source of carbon for aquatic organisms, rather than leading to pollution which results due to mass disposal of conventional plastics, leading to generation of fragmented microplastics and garbage patches in the oceans (Agarwal et al., 2022; Dang et al., 2022; Madadi et al., 2021; Markl et al., 2019; Mastropetros et al., 2022; Singh et al., 2017).

Table 2.5. Overview on the properties of conventional and biodegradable plastics.

(Markl et al., 2019; Singh et al., 2017)

Property	Conventional Plastics (PP)	Biodegradable Plastics (PHB)
Microbial degradability	Non-degradable	Degradable
Renewability	Non-renewable	Renewable
Sustainability	Non-sustainable	Sustainable
Greenhouse gas emission	High	Low
Crystalline melting point T_m (°C)	176	175
Crystallinity (%)	70	80
Molecular weight (Daltons)	2×10^5	5×10^5
Glass transition temperature T_g (°C)	-10	4
Density (g/cm^3)	0.905	1.250
Tensile strength (MPa)	38	40
UV resistance	Poor	Good
Solvent resistance	Good	Poor

2.6. APPLICATIONS OF PHA IN VARIOUS FIELDS AND INDUSTRIES

Microalgal and cyanobacterial PHA biopolymers have diverse applications across various industries. They can be used to manufacture biodegradable water-resistant surfaces. They have considerable potential for applications in the fields of pharmaceuticals for retarded drug release as well as PHA monomers have potential therapeutic effects and can be utilized as drugs. 3-hydroxybutyrate and its derivatives have been found to have an inhibitory influence on cell apoptosis. In agriculture, they are used as mulch films and for regulated discharge of plant growth regulators and fertilizers, as well as for herbicides and pesticides. Methyl esters of 3-hydroxybutyrate and mcl 3-alkanoate can be used as biofuels. PHAs are biocompatible and are widely used in the medical field for developing absorbable sutures, bone plates, bone marrow scaffolds, tendon repair devices, surgical pins, spinal fusion cages, ocular cell implants, vascular grafts, pericardial patches, closure of atrial septal defect. They have various tissue engineering applications. PHAs can be utilized for manufacture of disposals such as razors, serving trays, diapers and sanitary products, cosmetic packaging products, utensils, upholstery and compostable packaging, bags (Agarwal et al., 2022; Dang et al., 2022; Mastropetros et al., 2022; Roja et al., 2019; Samantaray and Mallick, 2015; Singh et al., 2017; Singh et al., 2019).

CHAPTER 3: METHODOLOGY

3.1. SAMPLING

The freshwater samples were collected from areas of Moira, Aldona, Chicalim, Udear and Siolim. The sampling was done over the months of August to December 2023. All the samples were collected in clean dry plastic bottles. The bottles were first rinsed with the water at the sampling site before collecting the water sample.

3.2. ISOLATION, PURIFICATION, AND MAINTENANCE OF PURIFIED ISOLATES

3.2.1. Preparation of media

The medium used for the isolation of microalgae and cyanobacteria was Blue Green (BG-11) medium (pH 7.5) using stock solutions (Appendix I) prepared separately (Gopi, 2014; Noronha, 2021; Rippka et al., 1981) The medium was filter sterilized using 0.22 μm nitrocellulose filter and stored at 4°C. The liquid broth was used for growing the cyanobacteria/ microalgae and BG-11 agar plates were prepared for isolating cultures from the broth.

3.2.2. Isolation of cyanobacteria/microalgal cultures

Water sample (5 ml) was inoculated in 100 ml of sterile BG-11 medium and incubated in an area illuminated with sunlight at room temperature. Each sample was inoculated in triplicates. After sufficient growth, 2 ml of broth from the master flasks were subcultured in 50 ml fresh BG-11 broth. When the medium turned greenish due to cyanobacterial/ microalgal growth, a loopful of the broth was streaked onto sterile BG-11 agar plates and maintained at room temperature in a well-lit area till green colonies

appeared on the plates. The flasks and plates were then kept on a stand illuminated by cool white fluorescent lights to provide uniform illumination for a light: dark period of 14:10 hours. The flasks were swirled gently by hand each day (Balaji et al., 2012; Gopi, 2014).

3.2.3. Purification of the isolates

The BG-11 plates were supplemented with 100 µg/ml Cycloheximide (Appendix I) to reduce fungal contamination (Gopi, 2014). Green colonies having different morphological characteristics were chosen and carefully picked up from the master plates and streaked onto fresh BG-11 plates. Repeated streaking was performed multiple times until pure isolates were obtained. For filamentous cultures, the filaments were serially diluted in sterile tissue culture titre plates till the highest dilution contained no heterogenous growth. A single filament was inoculated in 30 ml of BG-11 medium in cuboidal shaped sterile containers to allow more surface area for growth and incubated till sufficient growth was achieved.

3.2.4. Maintenance of isolates

Isolates were maintained by periodic transfer onto fresh plates. The isolates were also inoculated in sterile glass vials having 8 ml of sterile BG-11 medium as well as in test tubes. Glycerol stocks of the isolates were also prepared using 70% glycerol and stored at -20°C.

3.3. MORPHOLOGICAL CHARACTERIZATION OF THE ISOLATES

The isolates from the BG-11 plates and cuboidal containers were selected for morphological characterization.

3.3.1. Colony characteristics

The selected isolates were streaked onto sterile BG-11 agar plates and incubated at room temperature in an illuminated area for 30-45 days. The colony characteristics of the grown colonies were then recorded in terms of size, shape, colour, margin, opacity, consistency, and elevation (Mousa et al., 2018).

3.3.2. Light Microscopy

The wet mount of the purified isolates (both colonies and filaments) was prepared on clean, grease-free glass slides and covered with a cover slip. The slide was then observed under the microscope under 10X, 40X and under oil immersion lens (100X)

3.3.3. Scanning Electron Microscopy (SEM)

The isolates were processed for SEM under sterile conditions by making a smear on the SEM slides and air dried. The samples were then fixed with 2.5% Glutaraldehyde overnight (Hernández Mariné et al., 2004). The following day, the slides were washed carefully with sterile distilled water and then subjected to dehydration series with acetone (20%, 40%, 60%, 80%, 90% and 100%). The samples were then sputter coated and observed under 3000-6000X magnification depending on cell size.

3.4. PIGMENT EXTRACTION OF THE ISOLATES

The isolates were grown by inoculating 5 ml in 100 ml until the flasks turned fully green and enough cell density was obtained. 40 ml of this culture broth was then centrifuged, and the cell pellet obtained was used for extraction of pigments, namely Chlorophyll, Carotenoids and Phycobilins. (Jaiswal et al., 2018). Solvents were prepared to extract each type of pigment and 2-3 ml of solvent was added: 95% methanol for chlorophyll, 85% acetone for carotenoids and 0.05 M phosphate buffer (pH 7.5) for phycobilins (Appendix II). For chlorophyll extraction, the tubes were subjected to 60°C for 30 mins after addition of

methanol. For carotenoids and phycobilins extraction, the tubes were subjected to repeated freezing and thawing until the pigments oozed out and pellet was colourless.

Absorption of extracted pigments was noted spectrophotometrically at 650 and 665 nm for chlorophyll, 450 nm for carotenoids and 562, 615, 652 nm for phycobilin pigments.

3.5. TESTING THE PRODUCTION OF PHB UNDER DIFFERENT NUTRITIONAL CONDITIONS

The production of PHB was checked under 4 different conditions: basic BG-11, N-deficient BG-11, P-deficient BG-11, and BG-11 supplemented with Glucose as organic carbon source. Stocks for N-deficiency and P-deficiency BG-11 were prepared (Balaji et al., 2012; Panda et al., 2006). For BG-11 supplemented with glucose, 1% glucose solution was separately autoclaved and added to the molten BG-11 agar before pouring (Appendix I). Agar plates of each medium were prepared, and the culture was spot inoculated in triplicates and allowed to grow under a well-illuminated area for more than 30 days to allow PHB accumulation.

3.6. SCREENING OF THE ISOLATES FOR POLY-HYDROXY BUTYRATE (PHB) PRODUCTION

The screening of the isolates for PHB production was done by Microscopy (Sudan Black B staining) and by Nile Blue A staining for broth culture and plate assay method.

3.6.1. Sudan Black B Screening

A smear of the culture was prepared on clean, grease free glass slides and heat fixed. The smear was then treated with Sudan Black B solution (Appendix II) for 10 minutes. The slides were immersed in xylene till complete decolourization and then counterstained with Safranine solution (Appendix II). The slides

were air dried and observed under oil immersion (100X) for black intracellular granules (Ansari et al., 2016).

3.6.2. Isolate Screening for PHB Production from Broth Cultures

1 ml of the culture broth from an old culture (60 days) was added in microfuge tubes and pelleted by centrifuging at 10000 rpm for 10 minutes. The supernatant was discarded and the 0.5 ml of ethanolic Nile Blue A stain (0.05% w/v) (Appendix II) was added to the pellet (Balaji et al., 2013; Prabhu, 2010). The tubes were vortexed to dislodge the pellet and the tubes were incubated in the dark for 20 minutes. The tubes were centrifuged again at 10000 rpm for 10 minutes and the Nile Blue A supernatant was carefully decanted. The tubes were then placed on a UV transilluminator and exposed to UV to observe for bright orange fluorescence which indicates the presence of PHB.

3.6.3. Plate Assay Method

The isolates were spot inoculated (5 µl) on BG-11 agar plates in triplicates. Different variants of BG-11 agar were used (BG-11, N-deficient BG-11, P-deficient BG-11, BG-11 supplemented with glucose) (Balaji et al., 2012; Panda et al., 2006). The plates were incubated at room temperature in an illuminated area for a light: dark period of 14:10 hours. After about 30 days the plates were flooded with 0.05% (w/v) Nile Blue A in Ethanol and incubated in the dark for 20 minutes. The stain was decanted and exposed to UV to observe for bright orange fluorescence which is indicative of PHB production (Prabhu, 2010).

4. ANALYSIS AND CONCLUSIONS

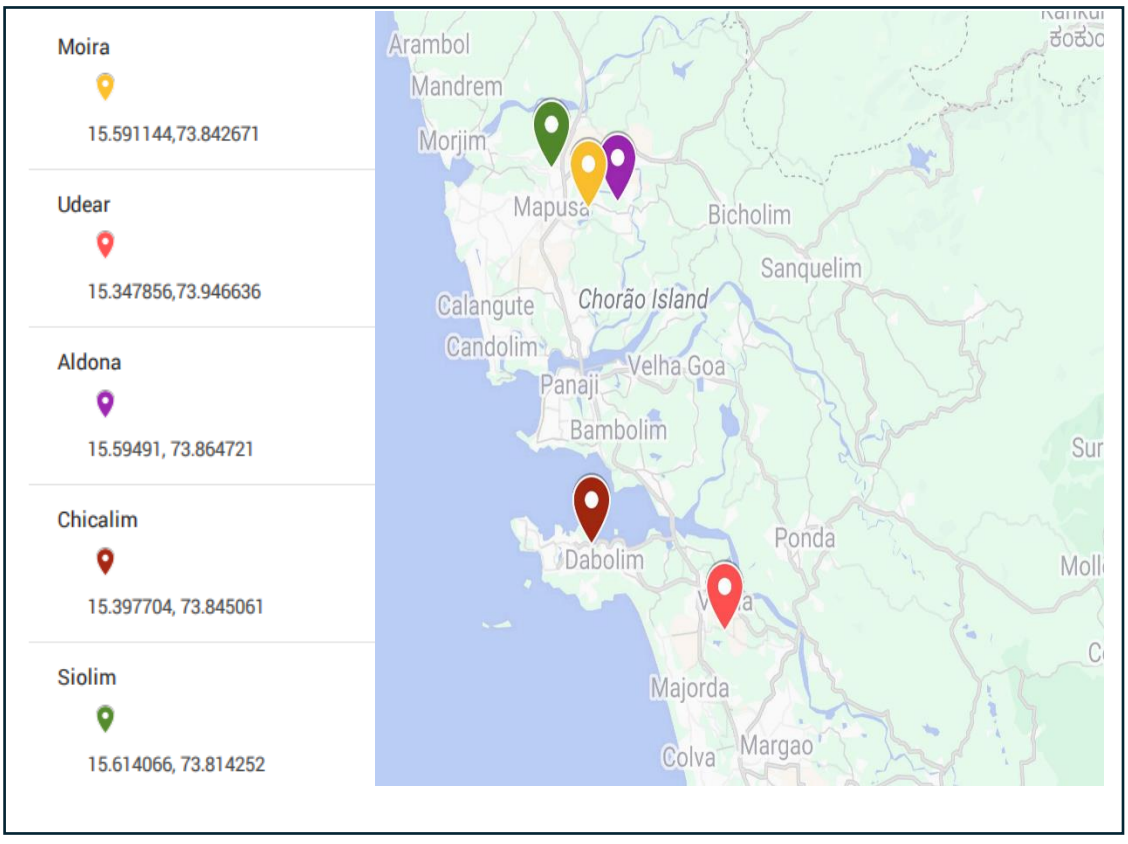
4.1. SAMPLING

The water samples were collected from the areas of Moira, Aldona, Chicalim, Udear and Siolim. Only freshwater samples were chosen for this study, which included wells, ponds, springs, and paddy field water sample. Sampling was done over the course of months from August to December 2023. The details of the sampling sites are given in the table 4.1. The map of the sampling sites can be seen in figure 4.1. (a) with the images at the site of sampling.

Table 4.1. Sampling sites used for isolation of microalgae/cyanobacteria.

Area	Latitude	Longitude	Type
Moira	15.591144	73.842671	Well
Aldona	15.59491	73.864721	Pond
Chicalim	15.397704	73.845061	Paddy field
Udear	15.347856	73.946636	Spring
Siolim	15.614066	73.814252	Well

(a)



(b)

Figure 4.1.(a) Map of the sampling sites in Goa and (b) Images from the location of sampling in (i) Moira (ii) Aldona (iii) Chicalim (iv) Udear (v) Siolim

4.2. ISOLATION OF CYANOBACTERIAL AND MICROALGAL CULTURES

The samples collected were inoculated in the BG-11 broth medium and after sufficient growth, purification was subsequently carried out as seen in figure 4.2. by streaking and serial dilution. In total, 15 cultures were successfully isolated, out of which 10 were purified by repeated streaking on BG-11 agar and the remaining 5 were purified by serial dilution methods. The isolates were coded as AIM1-AIM15 and the details of the isolate and its source are tabulated in the table 4.2. The isolates were maintained by preparing glycerol stocks and by inoculation in glass vials.

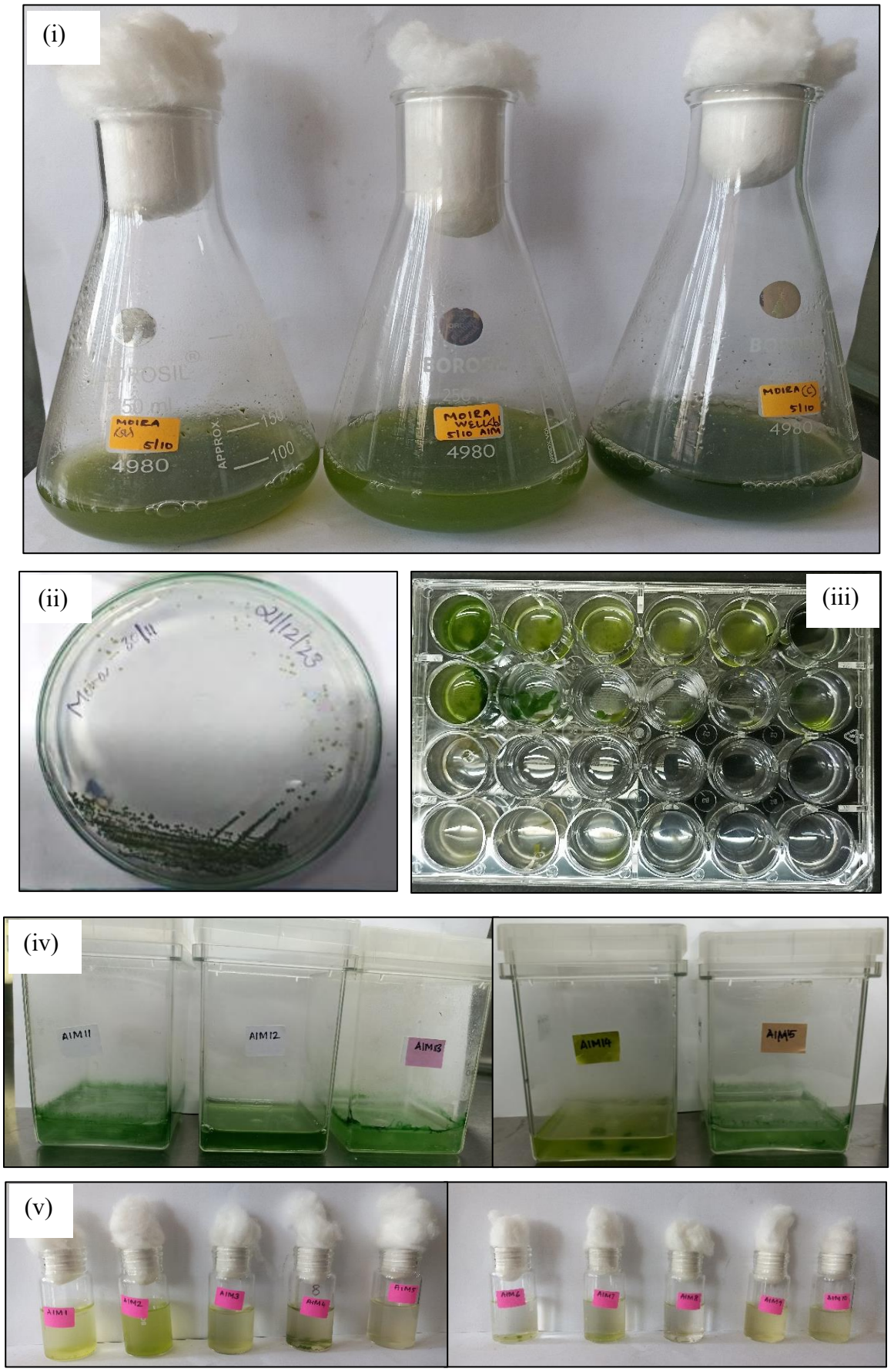


Figure 4.2.(i) BG-11 medium after 30 days of incubation (ii) Master plate (iii) Well titer plates used for purifying filamentous cultures (iv) Cuboidal containers used for growth of filamentous isolates (v) Glass vials used for maintenance of isolates

Table 4.2. The isolates obtained and their source

Isolate	Source
AIM1	Moira
AIM2	Moira
AIM3	Moira
AIM4	Aldona
AIM5	Aldona
AIM6	Udear
AIM7	Siolim
AIM8	Aldona
AIM9	Moira
AIM10	Udear
AIM11	Chicalim
AIM12	Chicalim
AIM13	Udear
AIM14	Chicalim
AIM15	Chicalim

All the collected samples contained photoautotrophic microorganisms. Out of the five samples used for isolation, most isolates were obtained from Moira well sample – AIM1, AIM2, AIM3 and AIM9. The Chicalim paddy field sample showed the presence of mostly filamentous growth which were then further purified to get the isolates AIM11, AIM12, AIM14 and AIM15. AIM4, AIM5 and AIM8 were isolated from Aldona pond sample. Isolates AIM6, AIM10 and AIM13 were sourced from Udear spring sample. AIM7 was isolated from Siolim well sample.

4.3. MORPHOLOGICAL CHARACTERIZATION OF THE ISOLATES

4.3.1. Colony characteristics

The colony characteristics of the isolates AIM1- AIM10 purified on BG-11 agar are noted in table 4.3.1

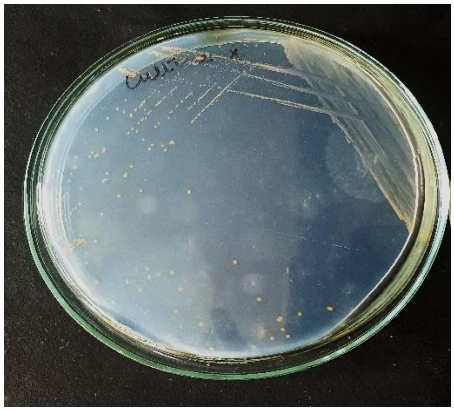
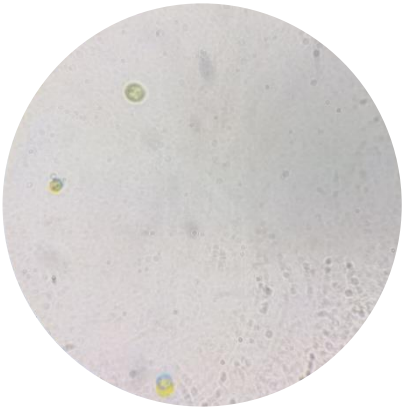
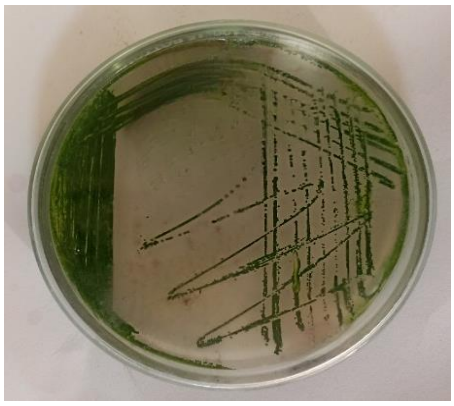
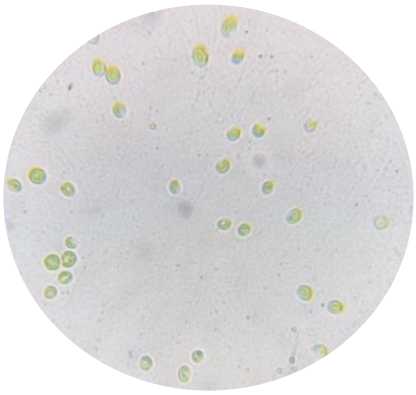
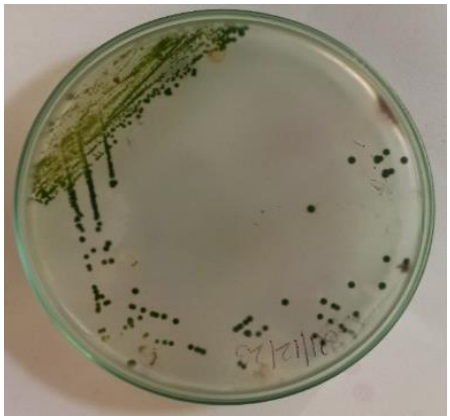
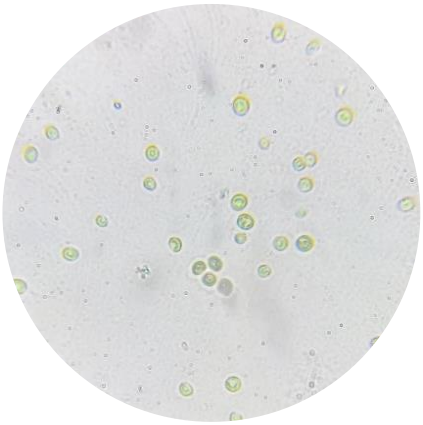
Table 4.3.1. Colony characteristics of the isolates AIM1 – AIM10

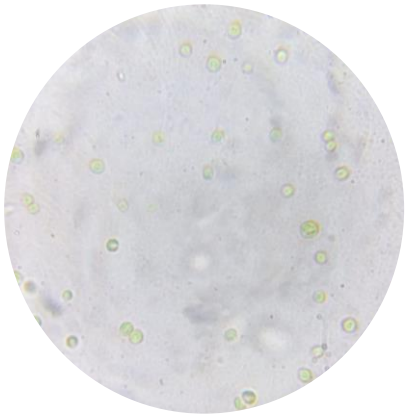
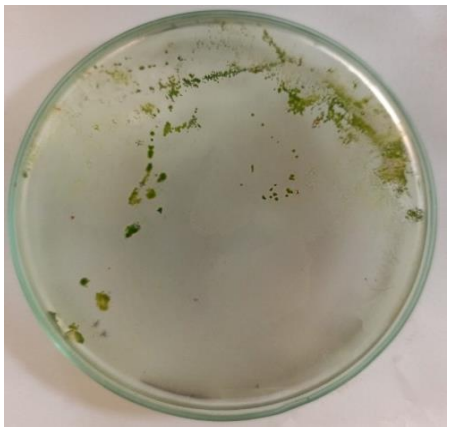
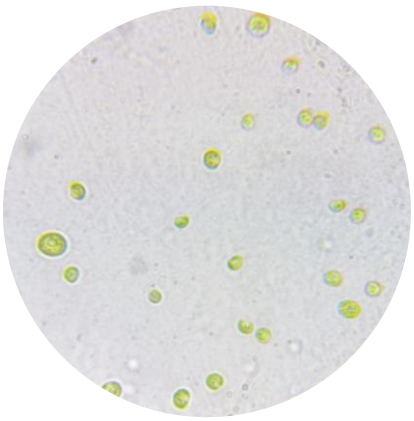
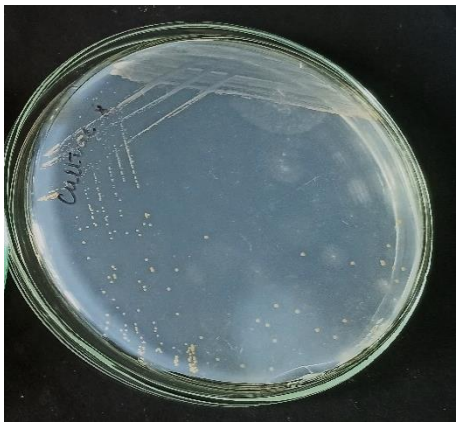
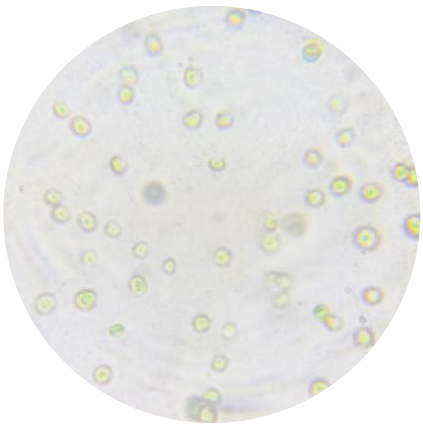
Isolate	**AIM1**	**AIM2**	**AIM3**	**AIM4**	**AIM5**	**AIM6**	**AIM7**	**AIM8**	**AIM9**	**AIM10**
Medium	BG-11	BG-11	BG-11	BG-11	BG-11	BG-11	BG-11	BG-11	BG-11	BG-11
Time	45 days	45 days	45 days	45 days	45 days	45 days	45 days	45 days	45 days	45 days
Temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature
Size	1 mm	2 mm	1.5 mm	3 mm	1 mm	1.5 mm	1 mm	3 mm	2 mm	1 mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Dark green	Dark green	Grass green	Dark green	Olive green	Grass green	Dark green	Forest green	Yellowish green	Dark green
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Flat	Convex	Convex	Raised	Convex	Raised	Convex	Umbonate	Convex	Raised
Consistency	Viscid	Butyrous	Viscid	Butyrous	Butyrous	Viscid	Butyrous	Friable	Viscid	Butyrous

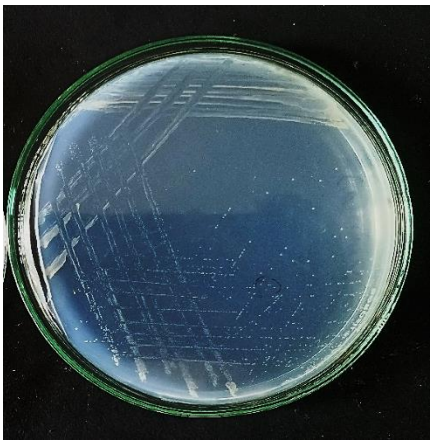
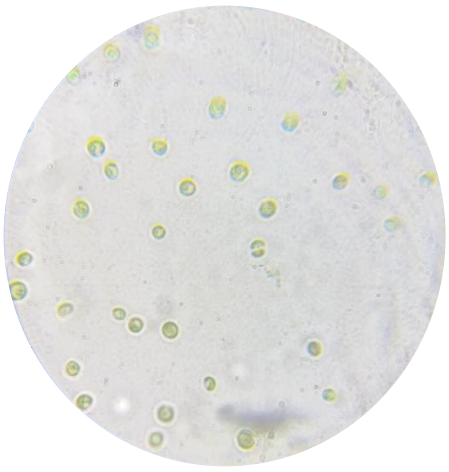
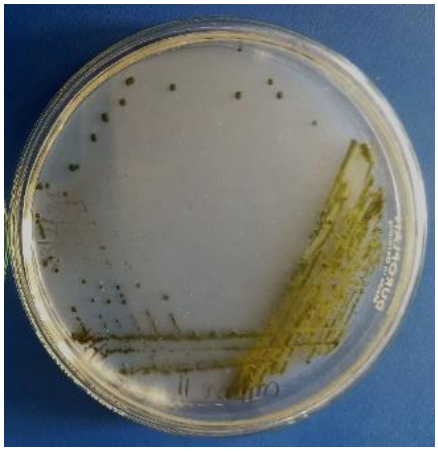
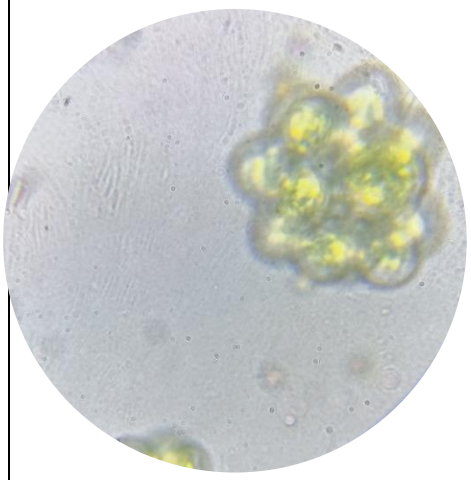
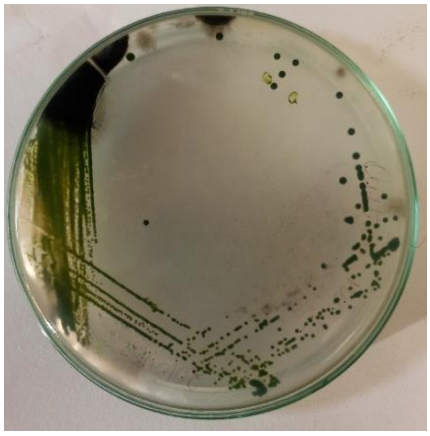
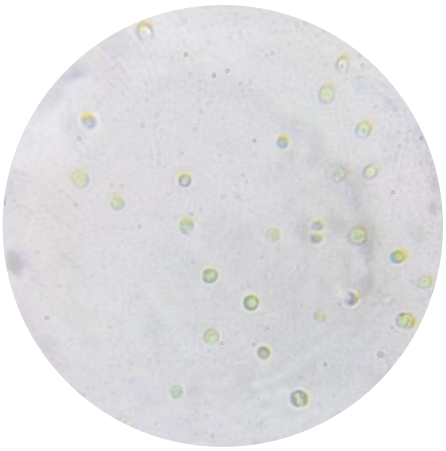
4.3.2. Light Microscopy

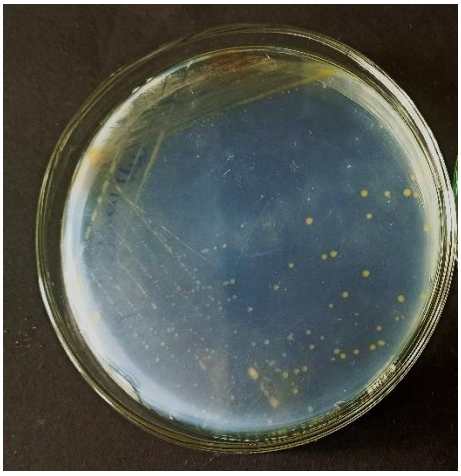
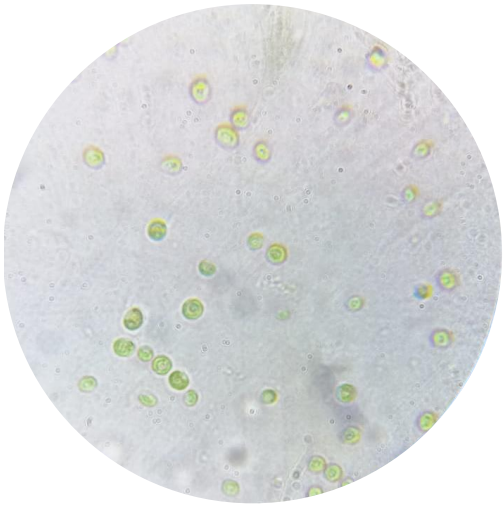
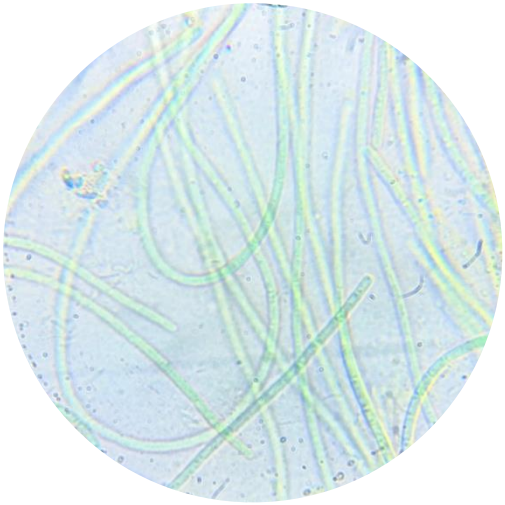
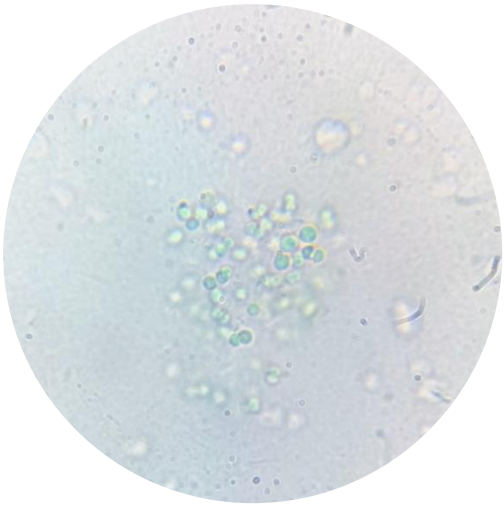
The morphology of the pure isolates AIM1-AIM15 was noted by observing the wet mount under the microscope under 10 X, 40 X and oil immersion lens (100 X). The images under oil immersion lens for each isolate are tabulated in table 4.3.2.

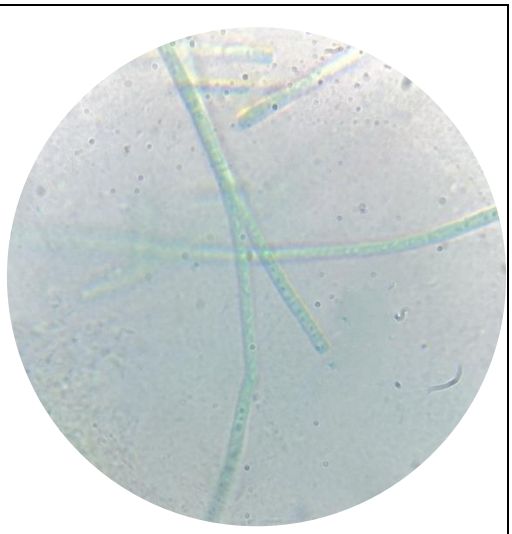
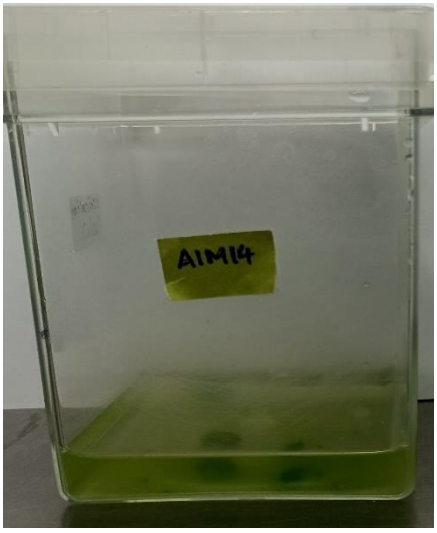
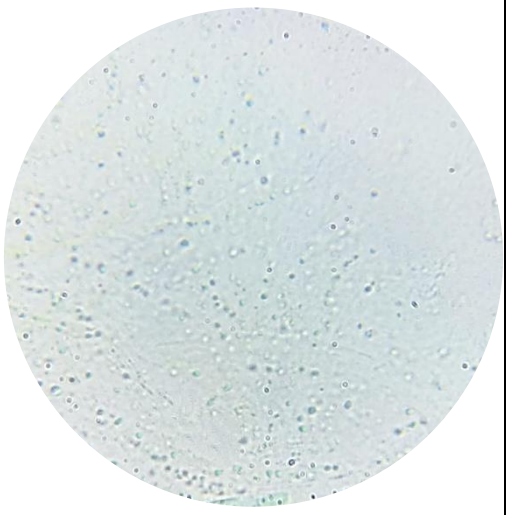
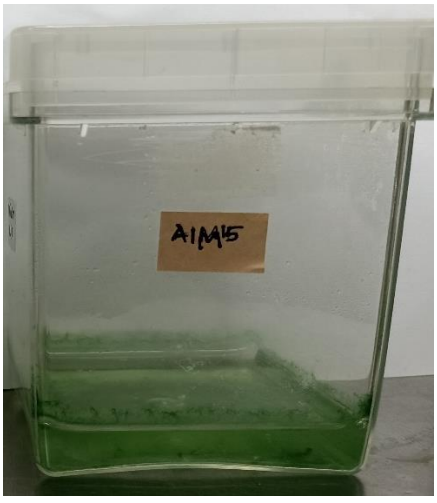
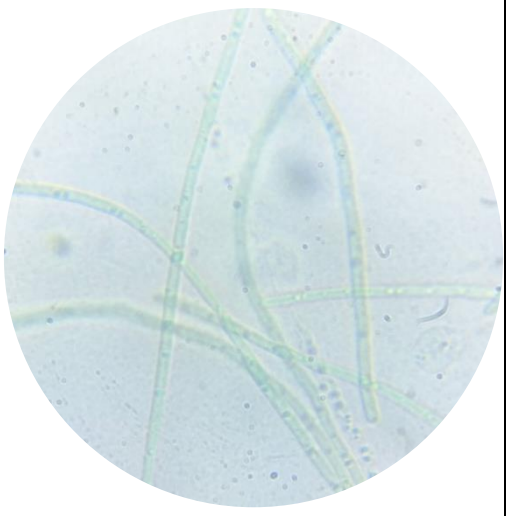
Table 4.3.2. Morphological characteristics of the isolates AIM1-AIM15

Culture	Morphology	BG-11 plate/ container	Microscopy (1000X)
AIM1	Unicellular		
AIM2	Unicellular		
AIM3	Unicellular, some diploid		

AIM4	Unicellular, some diploid		
AIM5	Unicellular		
AIM6	Unicellular		

AIM7	Unicellular		
AIM8	Colonial		
AIM9	Unicellular		

AIM10	Colonial (cells in chains)		
AIM11	Filamentous		
AIM12	Unicellular, some diploid		

AIM13	Filamentous		
AIM14	Unicellular		
AIM15	Filamentous		

After performing light microscopy at 1000X magnification, the cultures were characterized based on cell morphology. The isolates AIM1, AIM2, AIM5, AIM6, AIM7, AIM9 and AIM14 showed unicellular cell morphology. AIM3, AIM4 and AIM12 showed both unicellular and diploid morphology. The isolates AIM8 and AIM10 showed colonial forms. AIM11, AIM13 and AIM15 showed filamentous morphology. The isolates AIM11, AIM12, AIM13, AIM14 and AIM15 appeared bluish green under the microscope.

4.3.3. Scanning Electron Microscopy (SEM)

The isolates were processed for SEM and were observed under a magnification range of 3000-6000X depending on cell size. The images obtained are categorized in the figure 4.3.3.

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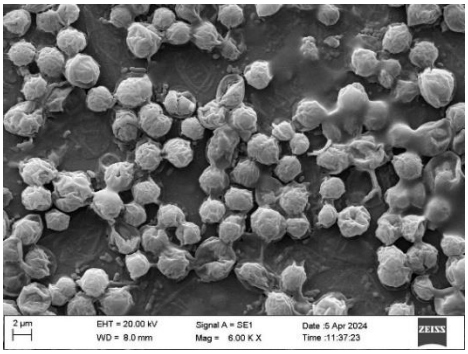
<td> 
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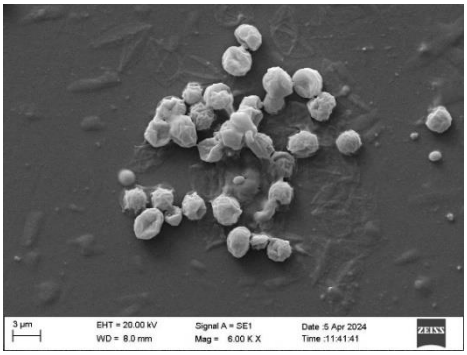
<td> 
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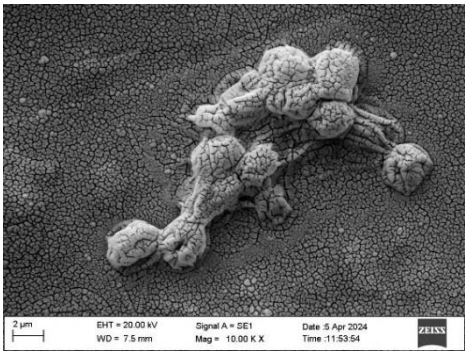
<td> 
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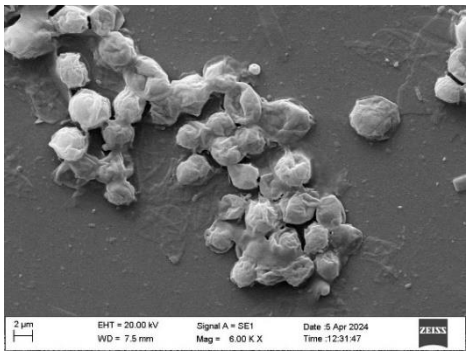
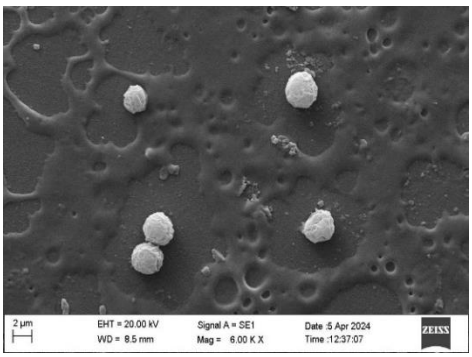
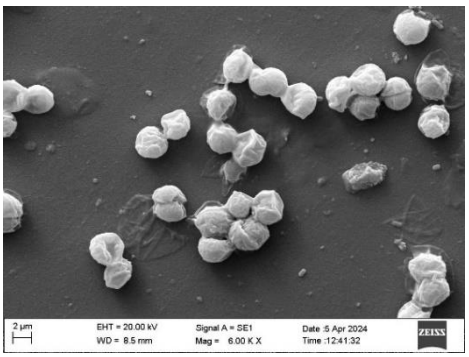
<td> 
AIM 4</td>
<td> 
AIM 5</td>
<td> 
AIM 6</td>
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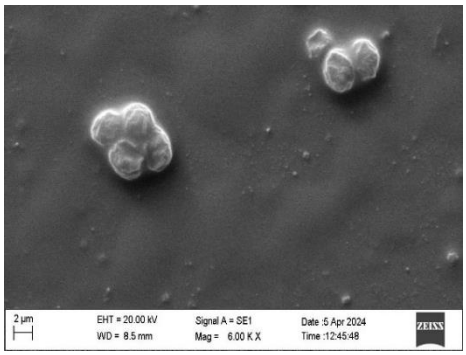
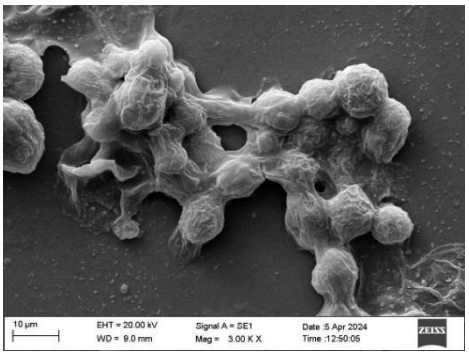
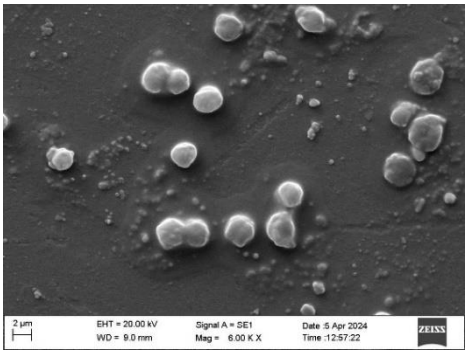


AIM 7



AIM 8



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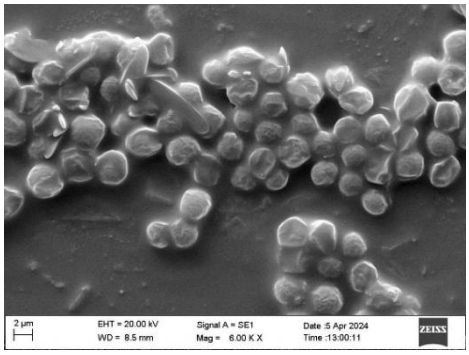

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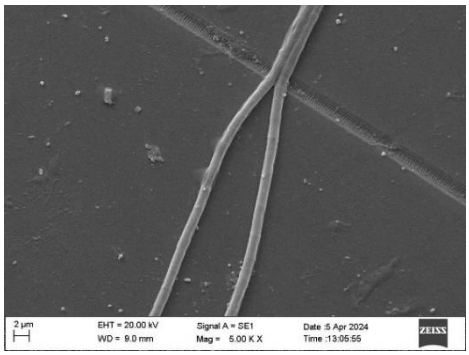
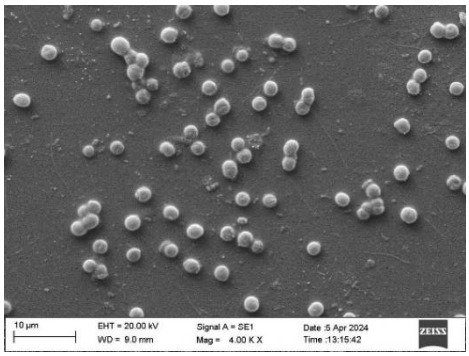
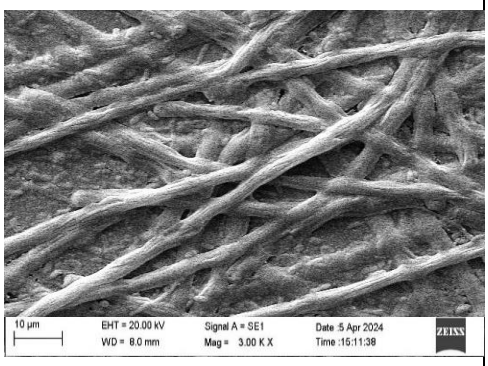


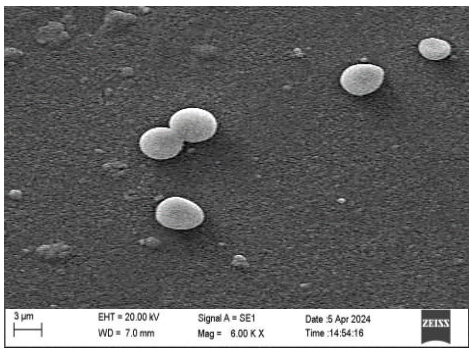
AIM 11



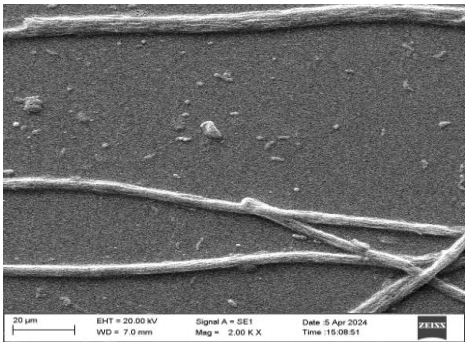
AIM 12



AIM13



AIM14



AIM15

Figure 4.3.3. SEM images of the isolates AIM1-AIM15

As shown in figure 4.3.3., the morphology of the isolates varied from solitary cells and filaments to clusters of cells or filaments. AIM1, AIM2, AIM3, AIM4, AIM6, AIM8, AIM10 and AIM13 were found

in clusters. AIM5, AIM7, AIM9 and AIM12 were solitary cells, whereas AIM11 and AIM15 were solitary filaments. Some isolates which also displayed diploid morphology are AIM6, AIM7, AIM9, AIM12 and AIM14. The approximate diameter size of the isolates under SEM was: AIM1 (4 µm), AIM2 (3 µm), AIM3 (4 µm), AIM4 (4 µm), AIM5 (4 µm), AIM6 (4 µm), AIM7 (10 µm), AIM8 (10 µm), AIM9 (3 µm), AIM10 (4 µm), AIM11 (2 µm), AIM12 (2.5 µm), AIM13 (4 µm), AIM14 (6 µm) and AIM15 (7 µm).

4.4. PIGMENT EXTRACTION OF THE ISOLATES

All the 15 isolates were processed for the extraction of the pigments Chlorophyll, Carotenoids and Phycobilins to roughly identify the cultures as cyanobacteria or microalgae. The data obtained has been tabulated in table 4.4.

Table 4.4. Pigments present in isolates AIM1- AIM15

Isolate	Pigments		
	Chlorophyll	Carotenoids	Phycobilins
AIM 1	+	+	-
AIM 2	+	+	-
AIM 3	+	+	-
AIM 4	+	+	-
AIM 5	+	+	-
AIM 6	+	+	-
AIM 7	+	+	-
AIM 8	+	+	-
AIM 9	+	+	-
AIM 10	+	+	-
AIM 11	+	+	+
AIM 12	+	+	+
AIM 13	+	+	+

AIM 14	+	+	+
AIM 15	+	+	+

Based on the data obtained by pigment extraction, it can be inferred that AIM 1-10 are tentatively microalgae due to the absence of phycobilins and AIM 11- 15 are tentatively cyanobacteria as they show presence of phycobilin pigments. The data obtained from light microscopy and Sudan Black B staining also supports this statement. The pigment extraction set for each pigment is shown in figure 4.4.

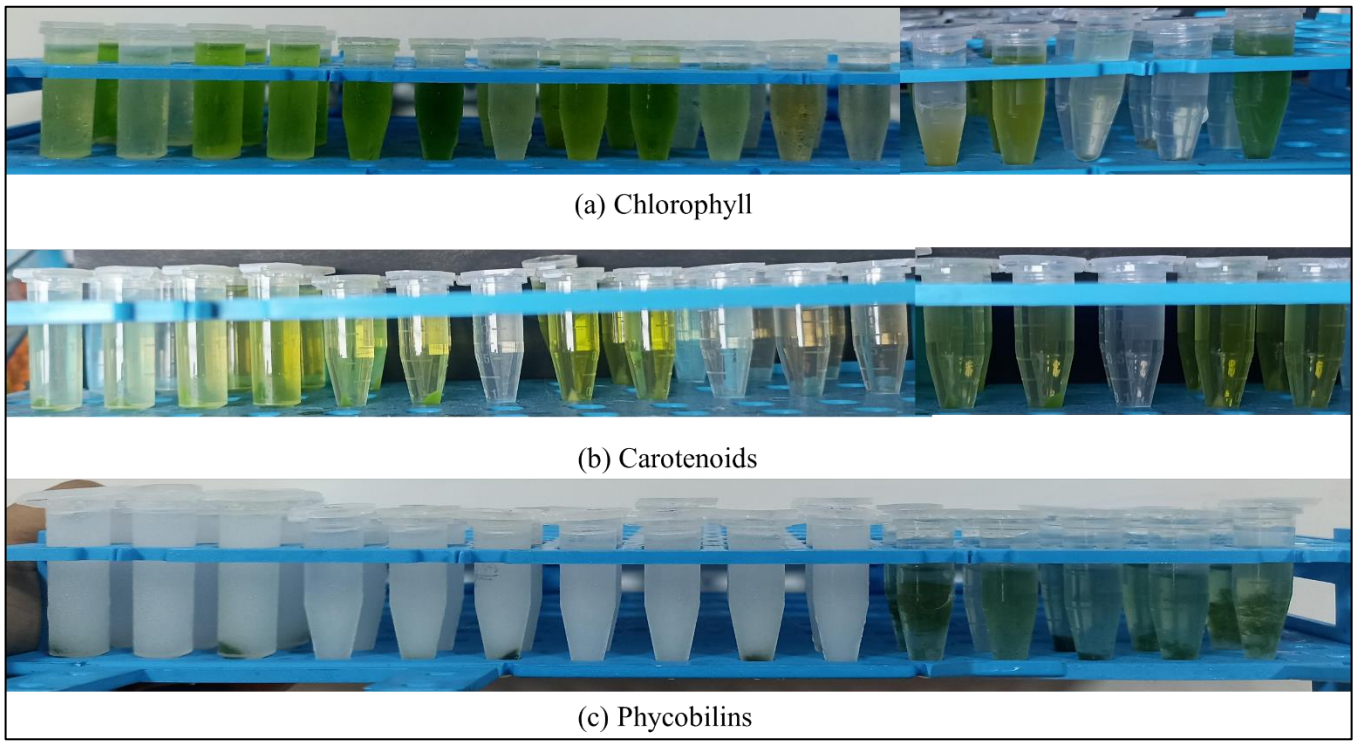


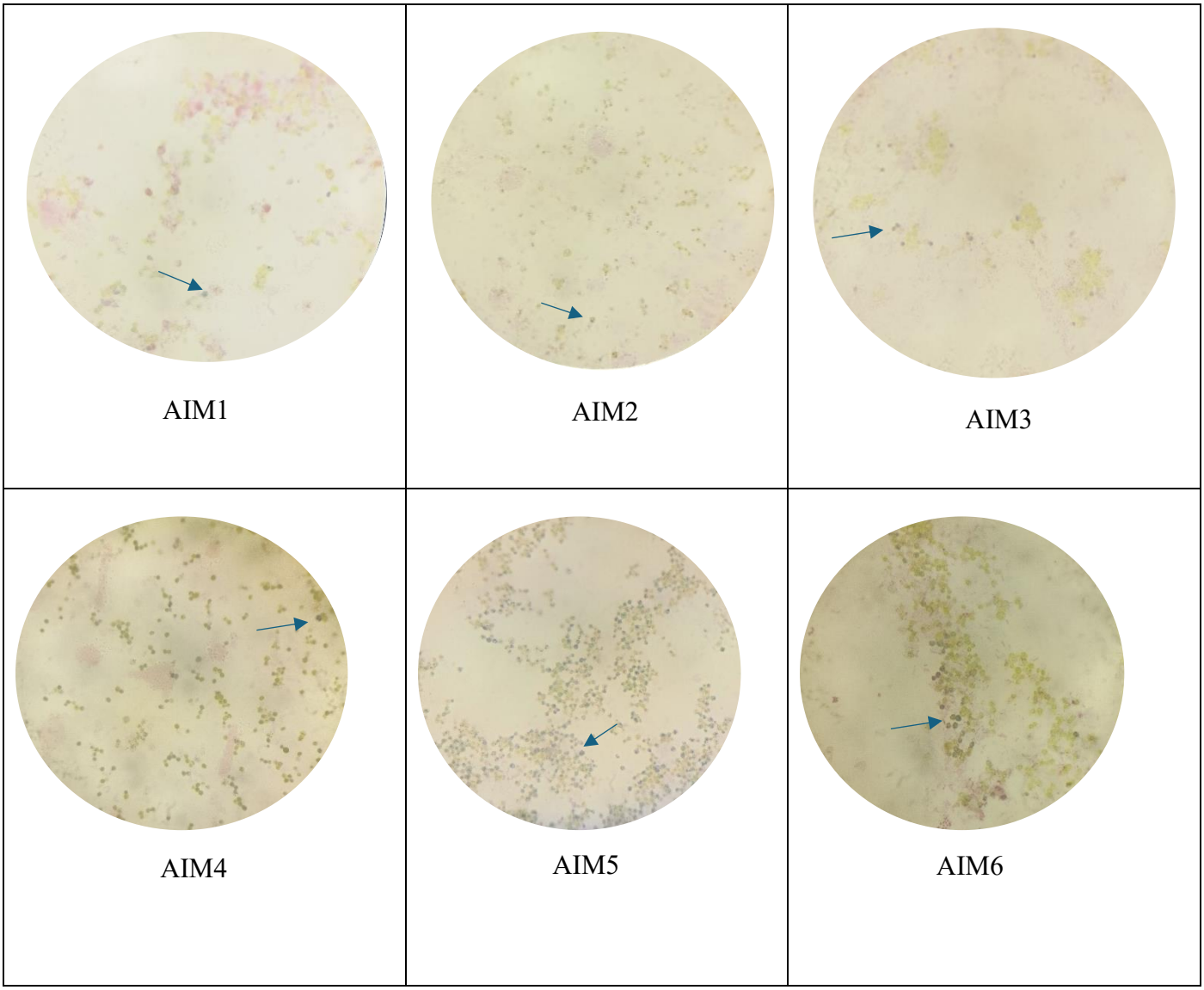
Figure 4.4. Pigment extraction set (a) Chlorophyll (b) Carotenoids (c) Phycobilins of the isolates AIM1-AIM15 (from left to right)

4.5. SCREENING OF THE ISOLATES FOR POLY-HYDROXY BUTYRATE (PHB) PRODUCTION

The screening of the isolates AIM 1-15 was done by microscopy (Sudan Black B staining) and by using Nile Blue A for broth culture and plate assay method.

4.5.1. Sudan Black B staining

Upon observing the slides after performing the staining procedure, black intracellular granules were observed in the isolates which are indicated by the blue arrows in the figure 4.5.1.



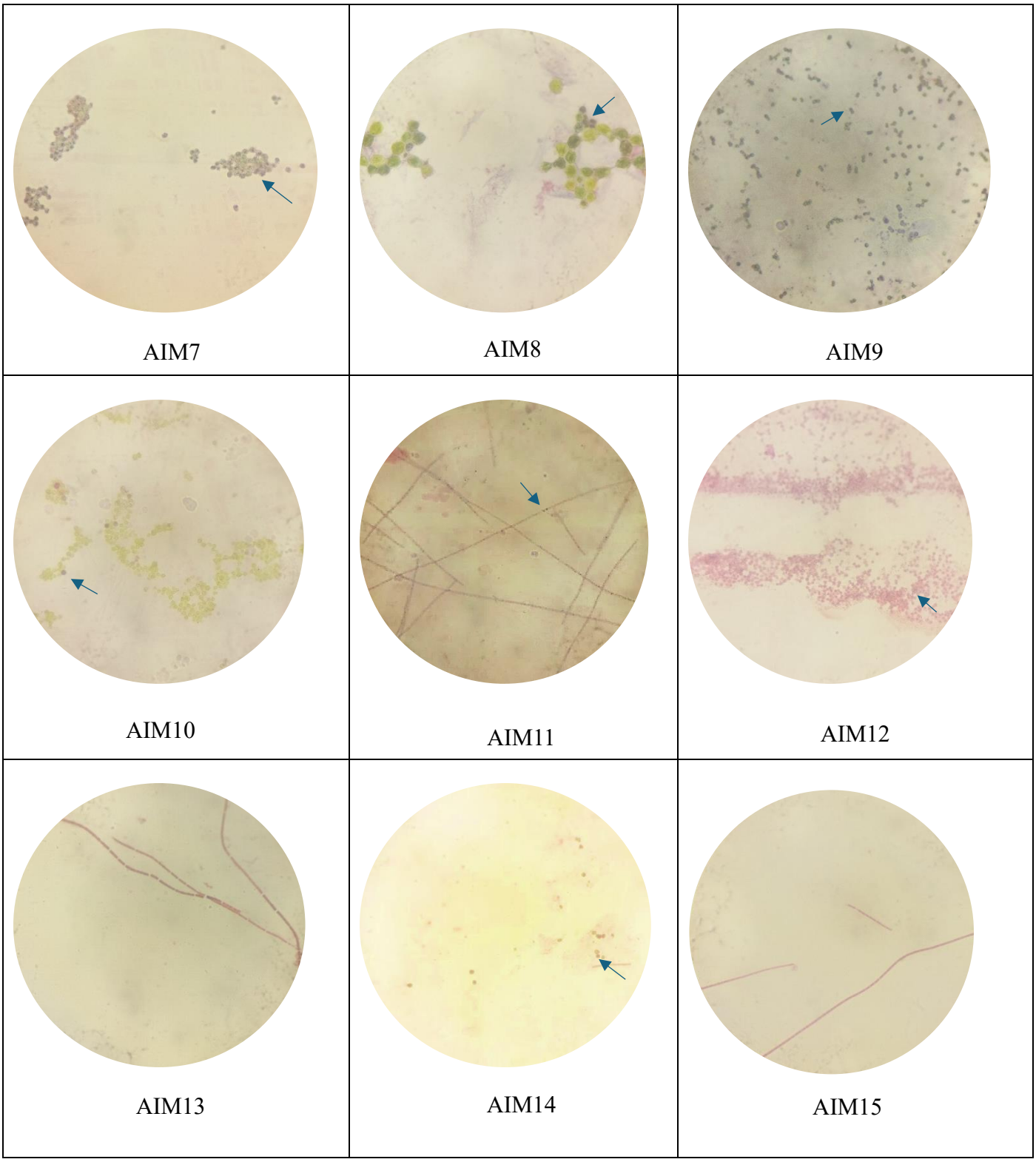


Figure 4.5.1. Images of the isolates AIM1-AIM15 under 100X lens after Sudan Black B staining

The isolates AIM1-AIM10 were not counterstained with safranine which further supports that the isolates could be microalgae and were not stained due to possible differences in the cell wall composition.

Intracellular granules were not observed in AIM13 and AIM15 which indicates that they might not be PHB producers. AIM4, AIM5, AIM7, AIM9 and AIM11 showed more quantities of black granules within their cells, and the isolates AIM 2 and AIM 10 showed least granules.

4.5.2. Isolate Screening for PHB Production from Broth Cultures

Culture broth was treated with Nile Blue A stain and the cell pellet was observed under UV transilluminator and presence of PHB was visualized by bright orange fluorescence as seen in figure 4.5.2.

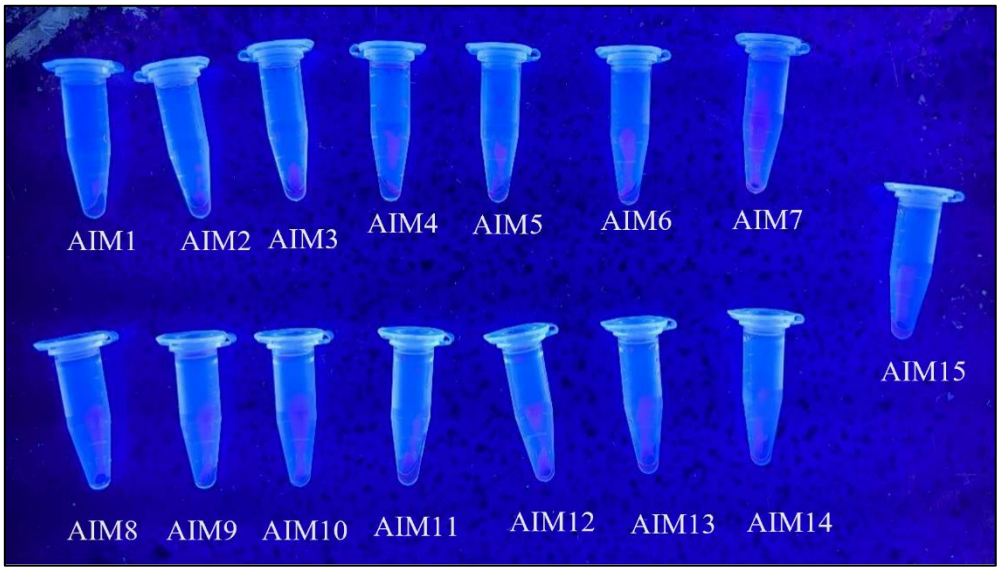
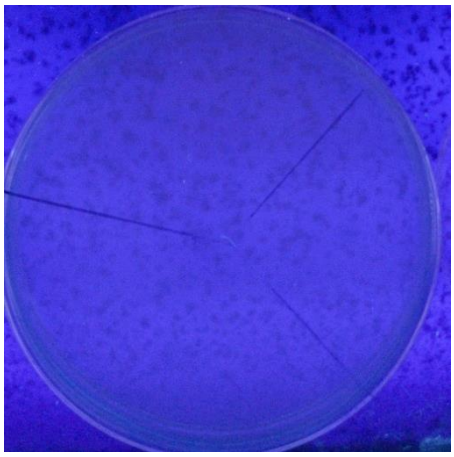
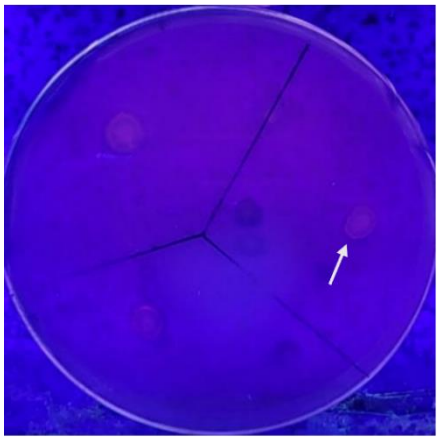
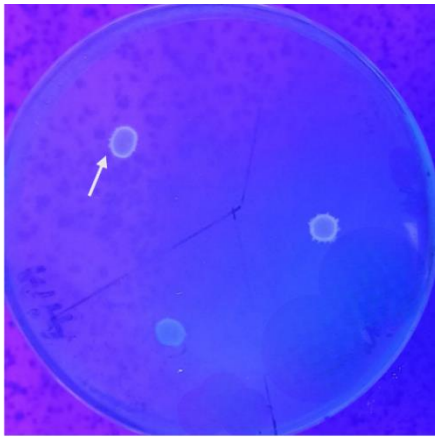


Figure 4.5.2. Isolates from broth screened for PHB accumulation by Nile Blue A staining

The isolates which were intensely fluorescent are AIM4, AIM5, AIM6, AIM7, AIM8, AIM9, AIM10, AIM11, AIM12, AIM13 and AIM15. AIM2, AIM3, AIM14 showed mild orange fluorescence and AIM1 showed negligible fluorescence. Since the cultures used were 60 days old, it can be said that PHB accumulation in the isolates occurs slowly over late logarithmic or stationary phase of the growth.

4.5.3. Testing the Production of PHB under different Nutritional Conditions

The spot-inoculated isolates AIM1- AIM15 on basic BG-11, N-deficient BG-11, P-deficient BG-11, and BG-11 + Glucose solid media were checked for PHB accumulation by plate assay method using Nile Blue A staining. The orange fluorescence is indicated by arrows (figure 4.5.3.)

<table>
<tr>
<td>BG-11</td>
<td> 
Control plate</td>
</tr>
<tr>
<td>P-deficient BG-11</td>
<td> 
AIM 8
 
AIM 12</td>
</tr>
</table>

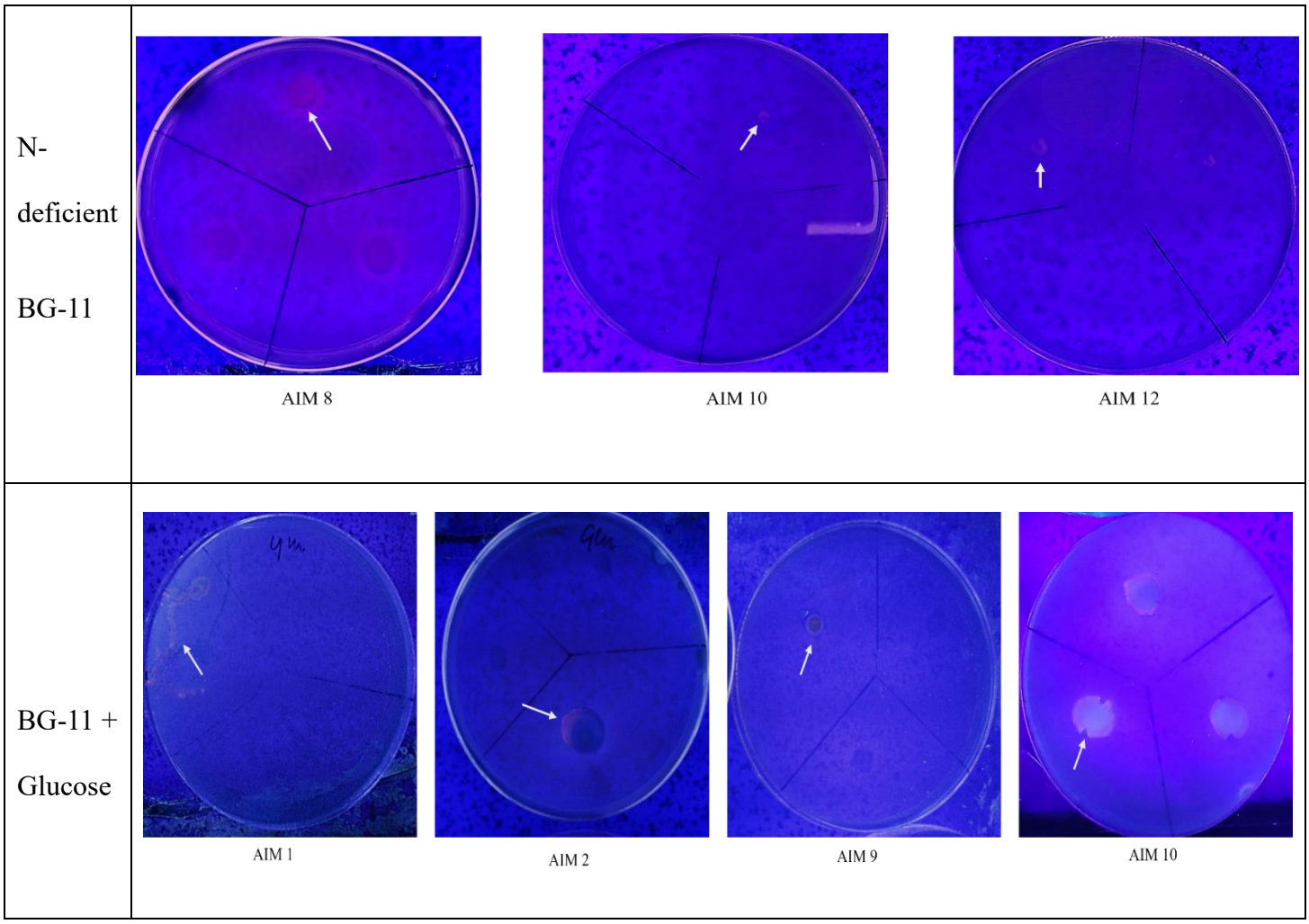


Figure 4.5.3. Nile Blue A assay plates under UV transilluminator

Table 4.5.3. Presence of PHB in the Isolates

Isolates	Growth Medium			
	Basic BG-11	N-deficient BG-11	P- deficient BG-11	BG-11 with 1% Glucose
AIM 1	-	-	-	+
AIM 2	-	-	-	+
AIM 3	-	-	-	-
AIM 4	-	-	-	-
AIM 5	-	-	-	-
AIM 6	-	-	-	-
AIM 7	-	-	-	-

AIM 8	-	+	+	-
AIM 9	-	-	-	+
AIM 10	-	+	-	+
AIM 11	-	-	-	-
AIM 12	-	+	+	-
AIM 13	-	-	-	-
AIM 14	-	-	-	-
AIM 15	-	-	-	-

Based on the results of plate assay in the table 4.5.3., we can observe that accumulation of PHB was stimulated by the nutrient deprivation conditions. In the same amount of time (30 days) the isolates on BG-11 agar did not accumulate PHB granules but under Phosphorous deficiency, isolates AIM8 and AIM12 showed bright orange fluorescence upon UV exposure. Deficiency of Nitrogen also stimulated PHB production in 3 isolates AIM8, AIM10 and AIM12. Growing the cultures under mixotrophic conditions also had a positive effect on production of PHB, as BG-11 supplemented with Glucose was found to stimulate PHB production in 4 isolates: AIM1, AIM2, AIM9 and AIM10. The combined effect of these conditions on the isolates can be further researched upon.

The unicellular isolates AIM8 and AIM10 which are possible microalgal species and the tentatively cyanobacterial isolate AIM12 could produce PHB under most of these conditions and hence have good potential to be used as the PHB producer organisms for future studies.

4.6. CONCLUSION

To conclude, in the present study, 15 autotrophic isolates were obtained from various sites in North and South Goa districts. These isolates were morphologically characterized by performing colony characteristics, light microscopy and by scanning electron microscopy. These isolates were also tentatively characterized as microalgae or cyanobacteria based on the presence or absence of Phycobilin accessory pigments. Isolates AIM1-AIM10 appear to be microalgae and isolates AIM11-AIM15 appear to be cyanobacteria.

These isolates were screened for production of PHB, a common biopolymer under polyhydroxyalkanoates under various nutritional conditions. The granules were visualized by staining using Sudan Black B and Nile Blue A. Screening of the culture broth showed that all isolates were positive for PHB accumulation. For the plate assay, the parameters of Nitrogen and Phosphorous deficiency were found to enhance the production of PHB in some of the isolates. Glucose was also found to stimulate PHB accumulation in some of the cultures. These isolates have shown the potential for PHB production under the parameters used, which must be further standardized with respect to incubation time, growth conditions and nutritional requirements for efficient production of PHB on a large scale.

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APPENDIX I

Composition of media

Stock solutions required for preparation of BG-11 medium.

Stocks	Concentrations
Stock 1	5X (g/200 ml)
Na_2EDTA	0.1
Ferric ammonium citrate	0.6
Citric acid	0.6
$CaCl_2.2H_2O$	3.6
Stock 2	5X (g/200 ml)
$MgSO_4.7H_2O$	7.5
Stock 3	5X (g/200 ml)
$K_2HPO_4.3H_2O$	4.0
Stock 5 (Micronutrients)	2X (g/500 ml)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$CuSO_4.5H_2O$	0.079
$CoCl_2.6H_2O$	0.050
$NaMoO4.2H_2O$	0.391

The stocks 1, 2 and 3 of 5X concentration were sterilized by autoclaving for 10 minutes. Stock 5 of 2X concentration was filter sterilized. The stocks were stored at 4°C.

Stock solutions required for preparation of modified BG-11 medium.

1. N-deficiency BG-11 medium

N- deficiency medium	
Stock 1	5X (g/200 ml)
Na_2EDTA	0.1
Ferric citrate	0.6
Citric acid	0.6
Same BG-11 Stock 2, 3 and 5 are used	

2. P-deficiency BG-11 medium

P- deficiency medium	
Stock 3	5X (g/200 ml)
KCl	4.0
Same BG-11 Stock 1, 2 and 5 are used	

The stocks of 5X concentration were sterilized by autoclaving for 10 minutes and stored at 4°C.

For basic BG-11 medium of 1 litre volume, the following stock combination was used.

Stock Solution	Per litre of medium
Stock 1 (5X)	2 ml
Stock 2 (5X)	2 ml
Stock 3 (5X)	2 ml
Stock 5 (2X)	0.5 ml
$NaNO_3$	1.5 g
Na_2CO_3	0.02 g

For BG-11 agar, 1.6% agar was used as high agar concentrations are inhibitory for cyanobacterial and microalgal growth.

For N-deficient BG-11, NaNO3 was omitted.

For Glucose supplemented BG-11 medium, same stock volumes were used as mentioned for basic BG-11 medium. A separate solution of 1 % glucose was prepared and sterilized separately and added to the BG-11 molten agar prior to pouring the medium into sterile petriplates.

The stocks prepared for modified BG-11 were used in the same volume proportions. The pH of the medium was adjusted to 7.5 using 1 M NaOH.

Preparation of the medium and filter sterilization was done under sterile conditions.

Cycloheximide stock

Cycloheximide	0.1 g
Distilled water	10 ml

The cycloheximide powder was weighed and added to sterile distilled water under sterile conditions and then the stock solution was filter sterilized using a 0.22 µm syringe filter. The sterile stock was then dispensed (1 ml) into sterile Eppendorf tubes and stored at 4°C.

APPENDIX 2

Composition of reagents

0.3 % Sudan Black B solution

Composition	g/100 ml
Sudan Black B	0.3 g
70% Ethanol	100 ml

0.5 % Safranine solution

Composition	g/100 ml
Safranine	0.5 g
Distilled water	100 ml

95% Methanol

Composition	Volume per 100 ml
Methanol	95 ml
Distilled water	5 ml

85% Acetone

Composition	Volume per 100 ml
Acetone	85 ml
Distilled water	15 ml

0.05 M Phosphate buffer (pH 7.5)

Composition	g/100 ml
0.1 M K_2HPO_4 solution	
K_2HPO_4	1.742 g
Distilled water	100 ml
0.1 M KH_2PO_4 solution	
KH_2PO_4	1.361 g
Distilled water	100 ml

To get 0.05 M Phosphate buffer, mix 50 ml of 0.1 M K_2HPO_4 solution and 0.1 M KH_2PO_4 solution. Adjust the pH to 7.5 with 1 M NaOH using a pH meter.

0.05% (w/v) Ethanolic Nile Blue A solution

Composition	g/100 ml
Nile Blue A	0.05 g
Absolute Ethanol	100 ml