INDIGENOUS MICROBES FOR SUSTAINABLE BIOPOLYMER SYNTHESIS: A COMPREHENSIVE OPTIMIZATION AND CHARACTERIZATION STUDY

Ph.D. Thesis

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I hereby certify that the work which is being presented in the thesis entitled **Indigenous Microbes for Sustainable Biopolymer Synthesis: A Comprehensive Optimization and Characterization Study** in the partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from June 2017 to May 2023 under the supervision of Dr. Kiran Bala, Professor, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

11.03.2024
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This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

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Prof. Kiran Bala

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DEDICATION

THIS THESIS IS DEDICATED TO MY FAMILY

SYNOPSIS

A. Introduction

Impetuous urbanization and population growth are driving increased demand for plastics to formulate impeccable industrial and biomedical commodities. The everlasting nature and excruciating waste management of petroleum-based plastics have catered to numerous challenges for the environment. However, just implementing various end-of-life management techniques for assimilation and recycling plastics is not a comprehensive remedy; instead, the extensive reliance on finite resources needs to be reduced for sustainable production and plastic product utilization. Several environmental and health-related issues are culminating every day due to extensive plastic production and usage, followed by infelicitous end-of-life management (Adane and Diriba 1993). Increasing invasion of plastic in our lives and environment and depleting fossil sources have led us to find an alternative of conventional plastic. One such alternatives can be polyhydroxyalkanoate commonly known as PHAs. PHAs are well studied and produced by various microorganisms including microalgae and purple non-sulphur bacteria. Despite of their versatility, high production cost and poor mechanical properties have been the reason of PHA lagging behind (Tan et al., 2021). Taking into consideration the prevailing plastic pollution and augmenting demand for plastic products, emphasizes on employing the biorefinery framework for cultivation of microalgae. Furthermore, while focusing on the biorefinery approach, it encompasses manoeuvring the sustainable conversion of cell biomass into a spectrum of bio-based

marketable products (bioplastics, biogas, bioethanol, pigments, protein, carbohydrates, and biofuels) to make microalgal biomass an economic-friendly and feasible feedstock for replacing the conventional plastic (Chandra et al., 2019). Photosynthetic microbial consortium (PMC) has been soaring in the recent years reason being the vast availability if nutrients and various by products. PMCs can utilize nitrate, phosphate, organic and inorganic carbon and carbon dioxide as well, which are often found in wastewater and atmosphere (Fradinho et al., 2014). Where the PMC provide a cost-effective production strategy, on the other hand uncontrolled growth of the microbes can often lead to lower volumetric productivity. One other major issue with commercialization of PHA is the high production cost, 50% of which constitute the substrate cost (Nguyenhuynh et al., 2021). Presence of organic carbon is a key component of PHA accumulation. Cheap alternatives for growth medium include industrial wastewater, oil-waste etc. Valorization of the waste would help in decreased overall cost of the production by making the polymer more competitive as their fossil counterparts (Hairudin et al., 2021). Scale-up and energy spent at production is the 2nd highest part of production cost. The synthesis of algae-based bioplastic comes with many challenges, and unravelling the complications needs to be the prime target for designing the upcoming research initiatives. A tailor-made consortia can be a solution towards fighting the challenges Selectively choosing the species compatible with

each-other can elevate the production of PHA at commercial level and easier to scale up to commercial level.

The following objectives were designed to achieve the desired output of the thesis:

Major Objective

Formulation of a tailor-made consortium of photosynthetic microorganisms for the sustainable production of biopolymer

Minor Objectives

i. **Biochemical profiling of indigenously isolated microalgal species**

ii. **(a) Nutritional source amelioration for enhanced PHA synthesis in microalgal hosts *via* two-stage cultivation**

    (b) Manoeuvring PHA accumulation in microalgae by employing various stress factors

iii. **Exploring indigenous purple non-sulfur bacteria for targeting PHA accumulation**

iv. **Algae-bacteria consortium for enhanced biopolymer production and its characterization**

v. **Scale-up study: Assessment of growth behavior of microalgae under semi-outdoor condition**

B. Summary of the results

The first objective towards our goal was to isolate microalgal species for nearby regions of Indore. Indore is situated in Madhya Pradesh, India, being in the central part of India, Indore receives high amount of solar radiation throughout the year. Global radiation Indore receives ranges from 13.50-

26.97 MJ/m^2/d throughout the year and it has a moderately extreme weather (2^o-45^oC) (Rajput et al., 2016). Water samples from different regions of Indore were isolated and identified using 18S rRNA and ITS sequencing. Further study of their growth profile and biochemical analysis (lipid, carbohydrate, protein, FAME) uncovered their potential. After studying the biochemical profiles, best species were selected for further optimization of PHA using 2-stage cultivation and mixotrophic growth.

The 2nd objective of the study was to screen host for PHA production. Three microalgal species namely *Coelastrella* sp., *Ettlia texensis* and *Pectinodesmus* sp. were screened for PHA production. Two-stage cultivation strategy was followed for the screening. With initial OD_{680} of 0.5, cells were cultivated for three days in various combinations, which are followings: For organic stress glucose (G), fructose (F), sucrose (S), mannose (Mn), maltose (Ma), acetate (A), (10 g/L) and glycerol (Gly), (5 g/L) were supplemented. For inorganic stress depletion and repletion of nitrate (0, 1.5, 5 g/L), phosphate (0, 0.04, 1 g/L) and NaCl in a fixed concentration of (5 g/L) was used. The codes are denoted as A) $P_{0.04}N_{1.5}$, B) $P_{0.04}N_0$, C) $P_{0.04}N_5$, D) $P_0N_{1.5}$, E) $P_1N_{1.5}$, F) $P_{0.04}N_{1.5}G_{10}$, G) $P_{0.04}N_{1.5}Ga_{10}$, H) $P_{0.04}N_{1.5}A_{10}$, I) $P_{0.04}N_{1.5}Ma_{10}$, J) $P_{0.04}N_{1.5}S_{10}$, K) $P_{0.04}N_{1.5}F_{10}$, L) $P_{0.04}N_{1.5}Mn_{10}$, M) $P_{0.04}N_{1.5}Gly_5$, N) $P_{0.04}N_{1.5}NaCl_5$ used where required. Their effect on PHA accumulation gave a surprising result along with that lipid, protein and carbohydrate profiles were also investigated. Galactose proved to be the best supplementation for PHB accumulation. All three

species were reported first time for PHA production and highest PHA accumulation was achieved 151.84 ± 12.08 µg/mg in *Coelastrella* sp. under **$P_{0.04}N_{1.5}Ga_{10}$** at 3rd day of cultivation.

In 2nd part of the objective, various stress was given to microalgal cells to increase PHA accumulation and best microalgal species were selected. Although optimum results were obtained at 3 days, extended cultivation was studied for up to 21 days to investigate the further accumulation of PHA with time with galactose as carbon source. Cultivated cells were harvested at different time period (7, 14, 21 Days). The highest PHA accumulation was found at 7th day (188.5±26.2 µg/mg) in *Coelastrella* sp., and highest biomass was as well. PHB accumulation declined on 14th and 21st day. Productivity of PHA at 7th day was still 1.9 folds less than at 3rd day. Hence further optimization studies were carried out at 3rd day. Furthermore, the effect of dark on PHA accumulation was also studied, where cells were grown in two different media with and without galactose and grown in complete dark condition. Highest PHA accumulation was observed in *E. texensis* with galactose supplementation, but it was 1.3 folds lower than when grown under (12:12) illumination.

Different cell concentration was also investigated (OD_{680} 1 & OD_{680} 2) for PHA accumulation. Along with increasing cell concentration, nutrient to cell concentration ratio increases which results in creating an energy deficit in cells to carry out normal activities like division. Biomass productivity was increased under higher initial inoculum, but PHA accumulation was

decreased. Highest PHA accumulation was in *E. texensis* at 1 cell density. Increasing initial cell density, positively affected biomass, and chlorophyll accumulation but PHA was not increased. Three galactose (5, 20, 30 g/L) concentration were used to observe the effect of carbon availability. Varying galactose concentration negatively impacted PHA accumulation in *Coelastrella* sp. and *Pectinodesmus* sp., whereas *E. texensis* was not majorly affected by the change. High concentration of galactose affected protein and lipid negatively in all the species. For further optimization combined effect of galactose with nitrate and phosphate variation was studied. Various combination of nitrate and phosphate along with galactose were supplemented to species to understand the accumulation pattern of PHA. Phosphate and nitrogen are major component of cells and responsible for major cell metabolism. Variation in their concentration can result in imbalance of cellular process resulting in diversion of energy towards PHA accumulation. Total depletion, moderate concentration (0.04g/L PO_4^{3-}; 1.5 g/L NO_3^-) and high concentration (1g/L PO_4^{3-}; 5g/L NO_3^-) was used in combination with Galactose (10 g/L). Highest PHA accumulation was found under $P_{0.04}N_5Ga_{10}$ in *E. texensis* (196.6±8.1 µg/mg). High nitrate with galactose was used in further optimization. Moving ahead, plant growth hormones (auxin, gibberellic acid, kinetin) were also supplemented to cells along with galactose as carbon source as microalgae have various similarity with plant cells. In the previous years, many studies have taken place where effect of plant growth hormone such as auxin, gibberellic acid and kinetin

have been investigated majorly to enhance lipid accumulation. Since the lipid and PHA pathways are intertwined, it can also trigger PHA accumulation in microalgae. Auxin and gibberellic acid were used in 0.05 g/L concentration whereas kinetin was used in 0.01 g/L. Growth hormones positively affected biomass and chlorophyll accumulation in all species. Highest biomass and chlorophyll were accumulated by *Pectinodesmus* sp. under $\mathbf{P_{0.04}N_5Ga_{10}Kin_{0.01}}$. Biomass composition including PHA was decreased under the influence of growth hormone. Highest PHA was accumulated under (Gibberellic acid) $\mathbf{P_{0.04}N_5Ga_{10}GAA_{0.05}}$ **(**142.1±3.0 µg/mg). Heavy metals (HM) are often present in wastewater and microalgae can remediate them from wastewater via either adsorption or absorption. If the cells absorb HM, it can trigger reactive oxygen species (ROS) which further can cause membrane lipid disruption and DNA damage (Cornelis et al., 2011), resulting in pool of ATP converted towards accumulation of various metabolic pathways. These responses depend on the interaction between cells and HM. At last, we studied effect of heavy metal on PHA accumulation. Heavy metals (Cu, Cr, Fe, Ag, Co) were used in 0.005 g/L concentration along with 5 g/L of nitrate and 10 g/L of galactose. All the heavy metals except Fe, negatively affected biomass and pigment production and cells could not grow. Highest PHA was accumulated under Fe supplementation in *E. texensis* (223.8±3.7 µg/mg). After the optimization, 162 folds increase in PHA accumulation was achieved as compared to control. *E. texensis* was selected the best species and was used

in further experiments. The best conditions were **$P_{0.04}N_5Ga_{10}Fe_{0.005}$, initial inoculum 0.5 at 680 nm cultivated under 12:12 photoperiod for 3 days.**

Polymer was extracted from the biomass of microalgae using solvent extraction (sodium hypochlorite) and physical and chemical properties of the polymer were studied. For doing so polymers were extracted from cells and various parameters like thermal, glass transition, melting temperature, molecular weight, polydispersity index etc. were studied. These properties helped in understanding the similarity in conventional plastic and produced polymer. Surface study was also performed for the same.

For 3rd objective, purple non-sulfur bacteria (PNSB) were isolated and optimized for PHA production. In the last several years purple bacteria have been studied for PHA accumulation. Various photosynthetic mixed cultures (PMC) majorly constitute of PNSB and microalgae. Growth profile of isolated strain PB_IIT_01 was studied and PHA accumulation at every 24 hrs was investigated for 4 days. For growth optical density was taken at 600 nm every 24 hrs was selected on the basis of highest PHA accumulation. Effect of VFAs (acetate, butyrate, propionate), and mixture of VFAs (2Ace:1But:1Prop) coupled with presence and absence of phosphate was studied for 4 days and harvested at every 24 hrs interval. The best PHA accumulation in PB_IIT_01 was under acetate supplementation without phosphate (95.3±3.1 µg/mg) at 3rd day of cultivation. Highest biomass was accumulated at 4th day under propionate supplementation with phosphate

(412.3±10.2 µg/mg). Acetate supplementation with OD_{600} 1, harvesting time 3rd day was selected for polymer extraction and characterization.

For 4th objective, A tailor-made consortium of selected microalgae and purple bacteria was created and grown under the best conditions determined during above mentioned objectives. *E. texensis* and PB_IIT_01 were cultivated together in $\mathbf{P_{0.04}N_5Ga_{10}Fe_{0.005}}$ medium, at initial concentration of OD_{680} 0.5 and OD_{600} 1 respectively. The cultures were cultivated at two photoperiods 12:12 and 24:00. The cultures were cultivated for 3 days and harvested every 24 hrs. The highest accumulation of PHA was 221.8±1.3 µg/mg at 3rd day under 12:12 photoperiod. Later, two media were designed to study the interaction further. Medium A was a BG-11 based medium, and Medium B was MSM based. These media were supplemented with 10 g/L of galactose, 4 g/L of acetate and 5 mg/L of Fe. The cells were cultivated in both medium individually and together with two photoperiods (12:12 & 24:00). Individually, *E. texensis* and PB_IIT_01 were able to grow and accumulate PHA better in medium A and medium B respectively. But the highest PHA was accumulated in consortium under Medium $A_{12:12}$ (357.3±0.6 µg/mg). This study has proven that the tailor-made consortium could perform better in the presence of multiple carbon source and accumulate higher PHA than individual microbes. The biomass was used to extract polymer and the characteristics were compared with the polymer extracted from *E. texensis* and PB_IIT_01 and found to be of higher quality.

As a last objective for establishing a biorefinery, the selected microalgae (*E. texensis*) were acclimatized for semi-outdoor scale up in the polyhouse at IIT Indore. Firstly, the microalgae were cultivated in open vessel in 5 L media for 3 generation (7 days) to let the cells acclimatize themselves for high temperature and light intensity. Preceding that, cells were transferred to a cheaper fertilized based media (FBM) containing urea and di ammonium phosphate (DAP) and artificial wastewater based medium (AWBM) in 5 L working volume open container in semi-outdoor condition as well as in 0.1 L working volume at laboratory scale and cultivated for 7 days and harvested at 3rd and 7th day. After harvesting, their biochemical profile was studied. The results were compared between FBM and AWBM at lab-scale and semi-outdoor conditions. The biomass production was higher in semi-outdoor condition depicting successful acclimatization of cells in semi-outdoor conditions. Protein accumulation was highest in FBM medium; however, carbohydrate and lipid accumulation were more in AWBM. Cells could not accumulate PHA under FBM due to the absence of organic carbon, whereas AWBM was proven better for high lipid, and carbohydrate accumulation. The culture of *E. texensis* species were further scale-up to 500 L working volume in 1000 L open High-Rate Algal Ponds (HRAPs). The cultures were mixed with propellers at 30 RPM for 10 min, twice a day. The cells were cultivated for 7 days and harvested at 3rd day and on 7th day. After harvesting, biochemical analysis and growth pattern analysis was performed. Highest biomass accumulation was 774.2±11.9

µg/mL under AWBM at 3 day. 493.4±34.4 µg/mg lipid was accumulated at 3rd day. Present findings confirm the successful growth of *E. texensis* in both FBM and WWBM under semi-outdoor conditions, leading to the accumulation of high biomass and precursors of value-added products such as lipid, carbohydrate, and protein. Above mentioned value-added precursors and use of inexpensive media can overcome the bottleneck for economically sustainable microalgae-based biorefineries.

C. Conclusions

Current study focuses on optimizing best conditions for production of biopolymer using a tailor-made consortium of microalgae and purple non-sulfur bacteria. Multi-parameter variation of organic carbon sources, harvesting period, photoperiod, initial cell density, concentration of carbon sources, combined effect of nitrate, phosphate, and galactose, plant growth hormone and heavy metals etc. At bench scale accumulated algal biomass of 600.9 µg/mL depicting the tolerance efficiency of studied species at different variables. Organic carbon supplementation with high nitrate and Fe supplementation ameliorated PHA content (223.8 µg/mg) and fetched high lipid content (78.7 µg/mg). PNSB accumulated 95.3±3.1 µg/mg PHA under acetate supplementation without phosphate. Whereas consortia of *E. texensis* and PB_IIT_01. produced 357.3±0.6 µg/mg PHA under BG-11 based medium with supplementation of glucose, acetate, and Fe. These results put forth a new strategy of making tailor-made consortium for higher PHA accumulation with mixed medium.

Under scale–up study at 1000 L raceway pond in AWBM, successfully produced biomass of 774.2 µg/mL along with high lipid content (493.4 µg/mg). This study puts forth novel microalgal species with a tailor-made consortium with PHA producing capacity without limiting biomass, and various precursors of value-added compounds which can be envisioned to establish a sustainable biorefinery.

Publication out of thesis

In Referred Journal

1. **Samadhiya, K**., Ghosh, A., Kashyap, M., Anand, V., & Bala, K. (2021). Bioprospecting of native algal strains with unique lipids, proteins, and carbohydrates signatures: A time dependent study. Environmental Progress & Sustainable Energy, e13735. https://doi.org/10.1002/ep.13735 **(Impact Factor: 2.8)**
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4. **Samadhiya, K.,** Ghosh, A., & Bala, K. (2023). Scaling up of native species for a sustainable microalgal biorefinery targeting different microalgal products. Algal Research, 75, 103246. https://doi.org/10.1016/j.algal.2023.103246 **(Impact Factor: 5.1)**
5. **Samadhiya, K**., Sonbhadra, S., Kaushik, A., & Bala, K. Manifesting microalgal biorefinery for production of Polyhydroxybutyrate and value-added products by exercising various stress factors. (Manuscript Under Preparation)
6. **Samadhiya, K.,** & Bala, K., Tailor-made consortium for high PHA accumulation using mixed carbon source (Manuscript Under Preparation)

Publication apart from thesis

1. Anand, V., Kashyap, M., **Samadhiya, K**., & Kiran, B*. (2018). Strategies to unlock lipid production improvement in algae.

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Abbreviations

CO_2 - Carbon dioxide

COD- Chemical oxygen demand

HRAP- High-rate algal ponds

Lcl-PHA – Long chain length polyhydroxyalkanoate

Mcl-PHA - Medium chain length polyhydroxyalkanoate

MMC - Mixed microbial culture

MW - Molecular weight

NADP- Nicotinamide adenine dinucleotide hydride

NADPH - Nicotinamide adenine dinucleotide phosphate hydride

PHA - Polyhydroxyalkanoate

PHB - Polyhydroxybutyrate

PHBH - Poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate)

PHBV - Polyhydroxy-butyrate-co-valerate

PMC - photosynthetic microbial culture

PNSB- Purple non-sulfur bacteria

PSB- Purple sulfur bacteria

Scl-PHA- short chain length Polyhydroxyalkanoate

SUP- Single use plastics

VFA - Volatile fatty acid

VSS- Volatile suspended solid

DO – dissolved oxygen

ITS - Internal Transcribed Spacer ITS

rRNA - ribosomal RNA (rRNA)

NCBI- National Center for Biological Information (NCBI)

FAME - Fatty acid methyl esters (FAMEs)

CHAPTER 1

A. Background and Motivation

The cataclysmic repercussions of disposable plastics on the environment have been a great deal of concern in today's scenario. Light weight, longer durability, and ease of access have elevated its importance in our life. It has gotten its claws in every aspect of our life, be it packaging, storage, toys, accessories, industrial equipment, medical devices, etc. The perennial nature of petroleum-based plastics instigates a conspicuous challenge for the ecological community in terms of their eradication and recovery. The recovery and recycling of plastic waste immensely depends on the method of disposal of plastic waste. The mixed disposal (presence of other waste such as electric waste or municipal waste) of plastic waste makes its recovery difficult and less energy-efficient (Devasahayam et al., 2019). Several environmental and health-related issues are culminating every day due to extensive plastic production and usage, followed by infelicitous end-of-life management (Adane and Muleta, 2011). Plastic in the oceans has cost us numerous aquatic lives. We all are aware of the "Great Pacific Garbage Patch", a study by Lebreton et al., (2017) has proved that the patch has been 16 times expanded since previous reports and many new garbage patches are accumulating in other oceans day by day. Landfills are affecting the natural flora of soil and creating a barrier between soil and ground which inhibits rain-water seepage into the ground thus reducing the groundwater level. Apart from the convenient and cheap plastic products, single-use plastics (SUPs) have been rapidly making a mark within our busy and evolving society. Not only plastics are polluting our environment but also when exposed to various environmental factors like ultraviolet rays, temperature, etc., they break down into microplastic (MP), and this phenomenon can occur not only to disposed plastic but also the ones still in use. The chemicals such as Phthalic acid esters that are used in the manufacturing of SUPs can transfer to the material contained within and travel to our food chain (Giacovelli et al., 2018; Chen et al., 2020).

The hike in plastic production has considerably surpassed any other human-made objects in the past 65 years (Geyer et al., 2017). The complex nature of petroleum-based plastic leads to the deterioration of the environment at the expense of economic benefits. A seismic shift in the environmental policies effectuated by the overwhelming and escalating plastic pollution has redirected the prevailing maneuvers towards the advancement of native low-carbon, circular economies, with strict landfill waste regulation, systematic recycling and recovery methodologies, and compulsory usage of bioplastics for the production of innumerable commodities (Kaeb et al., 2016; Elmarasi, 2017; Godfrey, 2019). In order to reduce energy requirements and carbon footprint, researchers all over the world envision the replacement of fossil-based plastic with carbon-neutral bio-based plastic for the betterment of the environment.

A.1 Comprehending Biopolymer

Biopolymers are biodegradable polymeric compounds composed of covalently bonded monomeric units. They are primarily derived from different biological sources such as plants, bacteria, microalgae, and photosynthetic bacteria (Xia et al., 2021). Major components of cells which includes protein, polysaccharides, lipids, and amino acids (Gonzalez-Gutierrez et al., 2010), have been discovered as the key constituents of bio-based biodegradable plastics. Their accumulation is triggered in the microbial cell when subjected to varying physicochemical perturbations (Samantaray and Mallick, 2015). Aliphatic polyesters, such as thermoplastic starch, polyhydroxyalkanoates (PHAs), polybutylene succinate (PBS), and polylactic acid (PLA), have been attributed as the building block of the bioplastic products currently in the market (Debuissy et al., 2018). Various bacterial strains including *Bacillus* sp., *Azatobacter* sp., *Alcaligens* sp., *Pseudomonas* sp., methylotrophs, *Cupriavidus necatar*, etc., as well as algal strains *Spirulina platensis*, *Nostoc muscorum*, *Synechococcus* sp., etc., have been explored extensively for the production

of bio-polymers (Lee, 1996; Steinbüchel and Füchtenbusch, 1998; Nishioka et al., 2001; Salehizadeh and Van Loosdrecht, 2004; Mallick et al., 2007; Toh et al., 2008; Babu et al., 2013; Hoarau et al., 2018).

The prevailing plastic pollution and augmenting demand for plastic products emphasizes employing the biorefinery framework for the cultivation of microorganisms. Furthermore, while focusing on the biorefinery approach, it encompasses maneuvering the sustainable conversion of cell biomass into a spectrum of biobased marketable products (bioplastics, biogas, bioethanol, pigments, protein, carbohydrates, and biofuels), to make microalgal biomass an economic-friendly and feasible feedstock for replacing the conventional plastic (Trivedi et al., 2015; Das et al., 2018; Chandra et al., 2019). Life cycle assessment (LCA) of several fossil-based and biobased plastics have postulated that the production and usage of the latter is advantageous over the former, primarily in terms of redemption of fossil reservoirs and attenuation of carbon emissions (Harding et al., 2007; Broeren et al., 2017). The bioplastics extracted from biomass feedstock and agricultural raw material tend to mimic various physical and mechanical properties of traditional plastics, and its substantial degradability by microbes has also been inferred by the researchers (Wu 2012; Kim et al., 2020; Rocha et al., 2020). In the current view, agricultural feedstocks such as wheat, sugar, potatoes, corn, rice, and soya are subjected to fermentation to produce different types of bioplastics. However, continuing this practice might adversely influence the provision of food all over the world. Thus, the need for cost-effective bioplastic raw material, including microbial biomass, emerges to be an effective solution for sustainable and feasible production of biodegradable bioplastic (Solaiman et al., 2006; Pakalapati et al., 2018). Polyhydroxyalkanoate (PHA) can be one such type of bioplastic that can be used to replace fossil based commercial plastic. Even though PHAs possess inimitable properties such as endurance for moisture and high heat, their market dispersion is hardly

visible. The price of PHA is currently around €5 /kg but is expected to drop once large-scale production facilities will be operational (van den Oever et al. 2017).

However, the concurrent and critical scrutinization of biopolymers had suggested significant obligations in their development and commercialization, including the higher cost of bio-based substrates, microbe cultivation methodologies, and downstream processing. Perhaps, rethinking and considering the synergized cost of PHA production and waste management, while focusing mainly on various inexpensive biological substrates required for improved microbial growth such as various effluents from industries, by-products of industries like whey or crude glycerol, or simply municipal waste (Tamang et al., 2019; Amaro et al., 2019; Wen et al., 2020) might give us a new perspective for economic and environmental evaluation of bioplastic regulatory framework comprising of its production, usage, and natural degradation. Carbon dioxide (CO_2), methane (CH_4), and syngas can also be utilized as an efficient substrate to produce biobased products, which further aids in the simultaneous sequestration of greenhouse gases as well as in wastewater treatment (Perez et al., 2020; Battashi et al., 2021; Sciarria et al., 2018).

A.2 Need of the hour- A Sustainable biorefinery

As defined by National Renewable Energy Laboratory, “Biorefinery is a facility that integrates conversion processes and equipment to produce fuels, power, and chemicals from biomass”. In elaboration to that, with increasing population and pollution bioeconomy (BE) is also an important aspect that is being focused nowadays. Bioeconomy or biobased economy is a powerful tool, enabling the generation of biobased products to decrease the use of fossil. While talking about biorefinery and bioeconomy, sustainability also comes to mind which completes the trio to rescue nature

from getting exploited. Hence having an integrated biorefinery can help in achieving our goal of a sustainable future.

B. Current status

B.1 Polyhydroxyalkanoate - A storage molecule emulating plastic characteristics

Polyhydroxyalkanoate (PHA) is a metabolite produced by several microorganisms as a storage product, which also acts as a shield against adverse environmental stress**.** Enhanced metabolic engineering methodologies have paved the way for exploring and exploiting various PHA biosynthesis pathways, which leads to the formation of different modified PHAs or bioplastics with specific material properties, beneficial for biomedical and industrial applications such as high-value medical equipment (including sutures, drug delivery, etc.) or low-value materialistic bioplastic (cosmetics, coatings, food packaging, etc.) (Keskin et al., 2017; Zhang et al., 2018).

Polyhydroxyalkanoate are essential building blocks for biodegradable plastic production. PHAs are classified into three categories based on their chain length, including short-chain-length PHAs (C3-C5), medium-chain-length PHAs (C6-C14), and long-chain-length PHAs (C14 and above) (Brandl et al., 1988; Steinbüchel and Wiese, 1992; Witholt and Kessler, 1999; McChalicher and Srienc, 2007). However, out of the different types of PHAs produced, PHB (poly-3-hydroxybutyrate), a short-chain-length PHA, has been the most widely characterized and explored bioplastic due to its early discovery in the year 1926 (Lemoigne, 1926). PHA copolymers are synthesized by bacteria when various substrates are introduced interactively and are anticipated to result in the formation of bioplastic composed of either of two types of monomers, i.e., 4-hydroxybutyrate (4HB) and 3-hydroxy valerate (3HV) (Figure 1) (Anderson and Dawes, 1990; Byrom, 1992).

The most common PHA is polyhydroxy butyrate (PHB). PHB has a high molecular weight and high crystallinity and shares similar physical properties with polypropylene. Co-polymer of PHB and PHV (polyhydroxy-valerate) have higher elasticity and varied applications. Commercialization of PHA started in the late 1980s when heterotrophic bacteria was solely used for production which resulted in higher cost and hence low popularity. In the current scenario high production cost is the bottleneck in the replacement of commercial fuel-based plastics by PHAs. The key factor of high production cost is raw material cost for heterotrophic organisms, aeration, aseptic conditions for genetically modified organisms (GMOs), and cost of downstream processing. To conquer these hurdles, extensive studies are going on, where converting waste agricultural or industrial products into PHAs is considered to control the substrate cost, different agricultural waste like molasses, and dairy industry waste like whey, etc. can be utilized as the cheap substrate to control the high production rates. On the path leading to attaining more sustainable production of PHAs, uncovering new photosynthetic organisms can be a milestone.

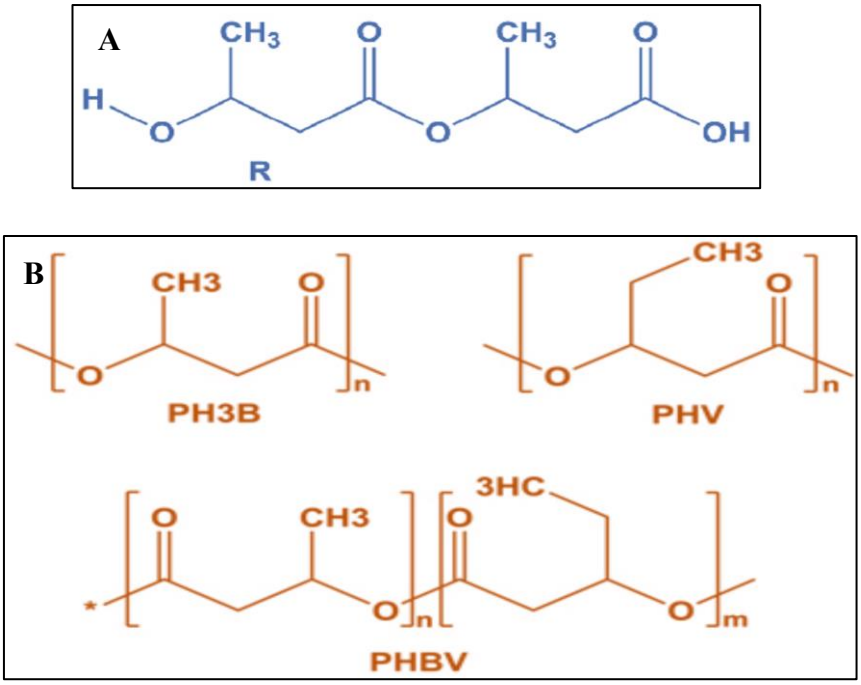


Figure 1: A) General structure of PHA, B) Structure of various types of PHA

B.1.1 PHA biosynthesis pathway

A brief illustration of the PHA accumulation mechanism is presented in Figure 2. Presence of an ample amount of nutrients to the microbes, the Krebs cycle leads to the production of a high quantity of CoA, which in turn obliterates enzyme PhaA, 3-keto thiolase to block the accumulation of PHA. This redirects the flux of acetyl-CoA towards the Krebs cycle to accomplish cell growth and energy production. Conversely, when nutrients including phosphorus and nitrogen are not sufficient and an excess of carbon is added to the microbes' growth environment, a low amount of CoA is produced, which cannot obliterate the PHA biosynthesis enzyme. Consequently, channelizing the acetyl CoA towards the PHA biosynthesis pathway stimulates PHA aggregation (Jung and Lee, 2000).

Furthermore, a comprehensive PHA biosynthesis pathway, as shown in Figure 2, comprehends the successive action of three prime enzymes (PhaA, PhaB, and PhaC) responsible for culminating three essential reactions. At first, enzyme PhaA, i.e., β-ketoacyl-CoA thiolase, eventuates the reaction between two acetyl-CoA molecules for acetoacetyl-CoA formation. Subsequently, an enzyme PhaB, which is NADPH-dependent acetoacetyl-CoA dehydrogenase, catalyzes the reduction of acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA monomer. Eventually, the enzyme poly-3-hydroxybutyrate polymerase, encoded as PhaC, instigates the polymerization of (R)-3-hydroxybutyryl-CoA monomer into poly-3-hydroxyalkanoate (PHA) (Madison and Huisman, 1999).

PHA synthesis is also reported to occur when fatty acids and sugar compounds are subjected to β-oxidation or de-novo fatty acid biosynthesis pathways (Figure 2) (Aldor and Keasling, 2003). The oxidation of carbon sources while neglecting the fatty acid β-oxidation pathway leads to the production of acetyl CoA and transacylase enzyme (PhaG), which further catalyzes the biosynthesis of PHA via the fatty acid de novo biosynthesis

pathway. Whereas, when oxidation of carbon sources via the fatty acid β-oxidation pathway takes place then enzyme, (R)-specific enoyl-CoA hydratase, PhaJ catalyzes the oxidization of enoyl-CoA to (R)-3-hydroxy acyl-CoA, which eventually acts as a target precursor for enzyme PhaC, PHA synthase, for PHA biosynthesis (Hoffmann et al., 2002; Muhammadi et al., 2015).

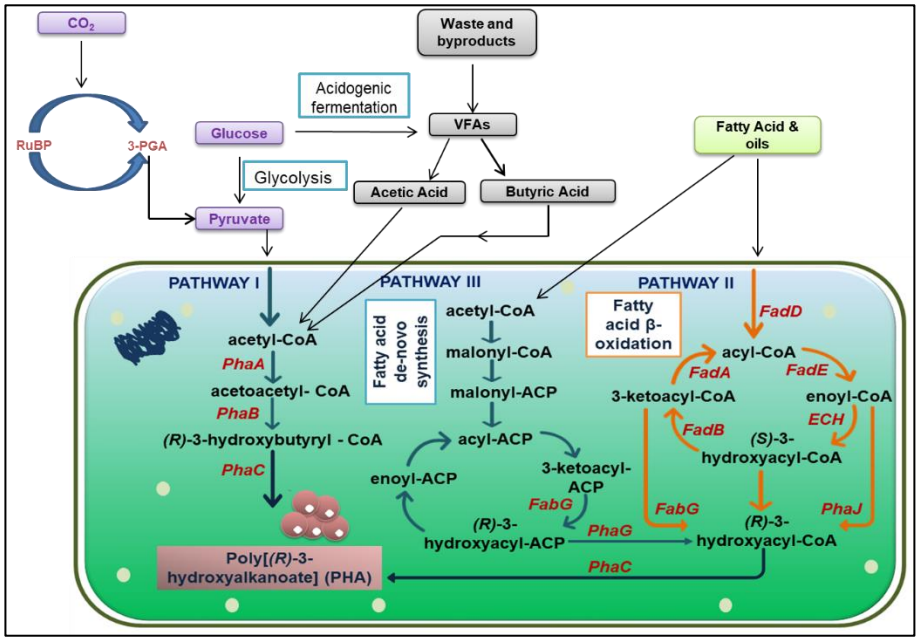


Figure 2: Pathways leading to PHA synthesis in photosynthetic microorganism (Samadhiya et al., 2022a)

There are several other intertwined pathways aside from the 3 major pathways discussed above. Various anabolic and catabolic reactions take place inside the cells, which contributes to the production of PHA using different precursors. PHB being the most studied scl-PHA does not limit the probability of production of numerous other PHAs. Raw material for conventional plastic is cheap to match the cost. Putative pathways have been a foundation of PHA synthesis strategies but to succeed in the battle between conventional plastic and biodegradable plastic, there is a need to look at some non-intuitive pathways and focus on those non-competitive substrates. Precursors like amino acids, VFAs, and even greenhouse gases can contribute to PHA production. Bacteria can accumulate up to 80 % of

their dry cell weight when fed with carbon sources (Budde et al., 2011). Several other bacteria can utilize different carbon sources and accumulate PHA, but valorization of these carbon sources is species-specific (García et al., 1999). A few other precursors could be volatile fatty acids such as acetate, butyrate, or propionate, amino-acids, gases like carbon dioxide or methane, etc. (Figure 2).

The significance of metabolic pathways in the production of any biotechnologically important metabolites is known worldwide; however, detailed know-how of every reaction, its reactants, and products along with the knowledge of enzymes involved needs to be gathered primarily before targeting the production of the metabolite of interest. PHA being a type of bioplastic precursor, the understanding of its biosynthesis pathway gives an insight into the nature of the catalyst involved, the role of varying physicochemical factors, and even the type of nutrient media required for diverting the carbon flux towards increased production of PHAs. Another scientific boom in the last decade has been the machine-learning approach. With the help of the already present database and pathways, various algorithms can be used and analyze the optimum culture conditions related to the selected host (Li et al., 2020). The genome-scale study can also help select the organism suitable for the specific application (Nobu et al., 2014; Nazem-Bokaee and Senger, 2015).

The diversion of carbon flux in response to diverse environmental stress conditions can be determined by studying the PHA production pathway and its understanding will further aid in optimizing the growth conditions of the microbe or will help in carrying out the genetic manipulation, while targeting up-regulation or down-regulation of genes involved in the formation of reactants, products, or an enzyme.

B.2 Photosynthetic factories in nature

Photosynthetic microorganisms are capable of converting sunlight into chemical energy. They often produce oxygen as a by-product (Nelson and Ben-Shem, 2004). The most known photosynthetic microorganisms are microalgae and cyanobacteria, where microalgae belong to the eukaryotic group whereas cyanobacteria belong to a prokaryotic group. Although some cyanobacteria have been reported as facultative anoxygenic photosynthetic microbe (Cohen et al., 1975). In anoxygenic photosynthesis, often organic acids (purple non-sulfur bacteria, PNSB) or hydrogen sulfide (H_2S) (Purple sulfur bacteria, PSB) are electron donors which do not result in the production of oxygen. As opposed to oxygenic phototrophs they do not contain chlorophyll or two photosystems, instead, they have carotenoid and bacteriochlorophyll and only contain one photosystem (Santoshi, 2016).

B.2.1 Algae as a source of value-added products

For years, photosynthetic organisms such as microalgae and cyanobacteria have been highly explored for their involvement in trapping solar energy and balancing atmospheric carbon (Ghosh and Kiran, 2017). The crucial life elements, including solar energy, CO_2, and water are processed through microalgae metabolism to form bioenergy products. Because of their rapid cell proliferation rate, microalgae's conversion efficiency is postulated to be 10 to 50 times higher than that of terrestrial plants. Thus, the microalgal land footprint required for bioenergy production is significantly less than plants. The most prevalent and topical agenda in the field of algal biotechnology is accomplishing the tremendous opportunities catered by algal biomass and its widespread biochemical portfolio. Due to their exceptional potential of assimilating photosynthetic carbon, microalgae are enunciated as a highly efficient form of cell biomass produced and have immense potential to engage in a clean energy future (Kiran et al., 2014). Economic constraints obviate many emerging commercial endeavors, and a

lot of such barriers can be conquered by incorporating a diligent conceptual configuration for augmenting the algal biomass values (Laurens et al., 2017). Comparatively, the commercialization of microalgal bioplastics is hindered by the high cost involved in traditional phototrophic cultivation techniques. Hence, presently, microalgae-derived bioplastic appears to be infeasible compared to conventional processes, but synergized cultivation of microalgae together with the treatment of wastewater paves the way for reduced commercialization cost. The production of microalgae-emanated bioplastics has been consummated through different research methodologies, including (1) amalgamation of microalgae biomass with bio-based or fossil-based plastics or their additives, (2) complete usage of microalgae biomass in the form of bioplastic, (3) improving the metabolite (a metabolite of interest) accumulation capacity of specific microalgal species along with its cost-effective extraction, (4) processing microalgae bioplastic while obeying the biorefinery model and lastly (5) administrating genetic engineering methodologies for the creation of proficient microalgal strain to synthesize an ideal bio-based plastic. Algae have the potential to grow in various conditions such as high salinity, nutrient depletion, organic and inorganic carbon supplementation, waste-water containing heavy-metals, fertilizers, various contaminants, etc. (Yang et al., 2015; Satpati et al., 2016; Anand et al., 2019; Ghosh et al., 2019). During their growth, they can accumulate various value-added products like, lipids, protein, carbohydrates, pigments, etc. (Anand et al., 2019). These products can be utilized in the production of various other by-products such as biodiesel, nutraceuticals, bioethanol, protein supplementation, and natural dyes. These products have a wide application at the present time. Biopolymers (PHA) has been recently reported in algal species, and extensive research has been going on in this direction for quite some time (Madadi et al., 2021). Cyanobacteria can accumulate PHA in photoautotrophic modes as reported previously under controlled conditions (4.5 %) or phosphate limitation (11

%) (Panda and Mallick, 2007). Many other reports have also established a relationship with mixotrophic and heterotrophic production of bioplastic by several cyanobacteria like *Nostoc muscorum*, *Synechocystis* sp., *Microcystis* sp., etc. (Table 1) (Bhati and Mallick, 2012; Gopi, 2014; Abdo and Ali, 2019). As a spin-off from algal biofuels, algae are recognized as proficient producers of various exclusive metabolites such as polyunsaturated fatty acid, carotenoids, phycobilin, polyhydroxybutyrate, etc., which are of commercial importance in pharmaceutical, nutritional, plastic, cosmetic, and other biotechnology-based industries (Laurens et al., 2017; Martosa et al., 2019; Arun et al., 2020).

Microalgae are now being approached by researchers very rapidly for PHA production as well (Roja et al., 2019; García et al., 2021). Bioplastic production from microalgae is considered to be an effective strategy for direct carbon entrapment (by mitigating CO_2 produced from flue gas), accompanied by the acquisition of imperative biochemical constituents, including lipids (Ho et al., 2014; Nakanishi et al., 2014; Anand et al., 2019), proteins (Schwenzfeier et al., 2011), carbohydrates (Ho et al., 2013b; Cea-Barcia et al., 2014), PHA (Roja et al., 2019), etc. that can act as potential bioplastic feedstock. Ultimately, establishing the fact that CO_2 sequestered by bioplastic-producing microalgae is directly getting entrapped in the form of polymer and not exiting into the environment (Abdul-Latif et al., 2020; Crocker et al., 2020).

In addition to that, supplementation of organic nutrients to activate the organism's heterotrophic metabolism, along with the addition of varying combinations of crucial nutrients, to accomplish the synthesis of aggravated PHA can effectuate a cost-effective cultivation model for microalgal bioplastic synthesis (Table 1) (Dietrich et al., 2017; Silva et al., 2017; Di Caprio et al., 2019b, 2019a; Karan et al., 2019; Abiusi et al., 2020). Conclusively, varying physical and chemical nutrient conditions of microalgae have been recognized to influence the quality and quantity of

metabolites being produced stupendously; thus, perturbing conditions can be exploited and scrutinized further for boosting the production of a metabolite of our interest, such as PHA (Gualtieri, 2001; De Morais and Costa, 2007; Hossain et al., 2008; Noreen et al., 2016). A perspective that starch-based bioplastics could be made from the starch-rich (49 % w/w) biomass of microalgae such as *Chlamydomonas reinhardtii* 11-32 A by exercising efficient plasticization through the twin-screw extrusion process was established (Mathiot et al., 2019).

Kato (2019) also reported that triacylglycerol-rich algal biomass of *Chlamydomonas reinhardtii* could be molded, in the crude form, directly into 7 mm bioplastic beads that can withstand compressive stress of up to 1.7 megapascals, thus excluding the cost of extraction and purification processes involved in the bioplastic production. Amidst all the metabolites produced, PHA is the highly explored and most expedient building block of microalgae bioplastic due to its easy biodegradation, catalyzed by enzymes (Shi et al., 2012; Noreen et al., 2016; Rahman and Miller, 2017; Karan et al., 2019; Beckstrom et al., 2020). The production of PHB from microalgae in the high-rate algal pond (HRAP) was analyzed along with their plasticization capacity, and it was concluded that out of two microalgal strains, *Microcystis aeruginosa* exhibited the highest PHB concentration. However, high-rate algal pond biomass dominated with *M. aeruginosa* showed the highest PHB concentration and was utilized to produce the bioplastic (Abdo and Ali, 2019).

Unraveling the novel native species might prove beneficial when speculating about the commercial production of PHAs. But using pure substrate and maintaining pure culture at a large scale for PHA production will still fall short from an economic point of view. Hence inducing non-intuitive pathways *via* physicochemical stress or adapting different approaches and applications of microalgae in PHA production is far-reaching (Chalima et al., 2017; Llamas et al., 2020).

Table 1: Algal species accumulating different forms of bioplastics

Microalgal Species	Type of Polymer	Accumulation (% CDW) [#]	References
Nostoc muscorum	PHB	31 – 69	(Sharma and Mallick, 2005; Samantaray and Mallick, 2014)
Phaeodactylum tricornutum	PHB	10.6	(Hempel et al., 2011)
Spirulina platensis	PHB	6.20	(Maheswari and Ahilandeswari, 2011)
Botryococcus braunii	PHB	20 – 60	(Kavitha et al., 2016a, 2016b)
Synechocystis sp. *PCC 6714*	PHB	37	(Kamravamanesh et al., 2017, 2018)
Chlorella fusca LEB11	PHB	17.4	(Cassuriaga et al., 2018)
Chlamydomonas reinhardtii	Starch	49	(Mathiot et al., 2019)
Microcystis aeruginosa	PHB	4.38	(Abdo and Ali, 2019)
Scenedesmus sp.	PHB	1 - 30	(García et al., 2021)

Abbreviations: *[#]%CDW, % Cell Dry Weight; PHB= Polyhydroxylbutyrate*

B.2.2 Purple bacteria- an aiding proteobacteria for bioresource recovery

Purple bacteria are anoxygenic bacteria, which means they do not produce oxygen during photosynthesis. They use either sulfur (purple Sulfur bacteria PSB) or hydrogen or ferrous ions (purple non Sulfur bacteria PNSB) as an electron acceptor during photosynthesis (Mothersole et al., 2018). They can be used for protein accumulation, heavy metal absorption as well as PHA

production (Talaiekhozani and Rezania, 2017; Alloul et al., 2019; Foong et al., 2019).

Both PSB and PNSB can accumulate PHA under autotrophic and heterotrophic conditions. In a study, wherein 12 strains containing 9 PNSB and 3 PSB have been exposed to organic and inorganic carbon as well as a mixture of both under nitrogen starvation and PHA accumulation was observed. Organic carbon yielded a higher accumulation of PHA as compared to inorganic carbon. *Rhodovulum visakhapatnamense* accumulated up to 30 % accumulation and *Rhodovulum sulphidopilum* when exposed to only acetate in seawater accumulated 9 % PHA (Higuchi-Takeuchi et al., 2016). PNSB prefers volatile fatty acids (VFAs) as a precursor for the production of PHA (Higuchi-Takeuchi and Numata, 2019). Acetate is considered the best precursor for PHB synthesis, having the least steps involved in the conversion. Other VFAs like propionate, butyrate, and valerate also have a high assimilation rate during the metabolism of PNSB. A proteomic study revealed that VFAs are responsible for the rapid and high accumulation of PHA in PNSB, however, bicarbonate is also important in the assimilation of valerate and other volatile fatty acids except for acetate in *Rhodospirillum rubrum* (Bayon-Vicente et al., 2020).

As it has been established before that purple bacteria favor VFAs over other carbon sources, studies have been conducted to find cheaper alternatives of VFAs from waste. Palm olive mill waste has high VFAs and chemical oxygen demand (COD) which was utilized by *Rhodopseudomonas* sp. S16-FVPT5 to accumulate 315 mg/L PHB and 2236 mL/L hydrogen (Carlozzi et al., 2019). They can also valorize high lignin-containing waste after thermal treatment and convert it into 21 % PHB of their dry cell weight (Allegue et al., 2021).

B.2.3 Mixed microbial culture (MMC)- a sustainable approach for commercial production

To accomplish the future agenda of worldwide usage of 100 % bio-plastic commodities, scientists are targeting bioplastic production from various microorganisms. MMCs have been widely explored for optimizing their culture conditions, growth, bioplastic accumulation potential, and downstream processing techniques. Despite the great potential of MMCs for bioplastic accumulation, their commercialization is economically not feasible. Thus, it is crucial to thoroughly explore the MMCs for their full potential in terms of PHA production as well as other applications to reduce the cost of scale-up and downstream processing. Tailor-made consortia of microbes can be one such approach in this direction. Therefore, more basic information on the mechanism involved behind the synthesis of precursors of bioplastic in microbes, different critical factors, and unique characteristics of individual microbes in PHA synthesis is required. The present status of bio-based plastic in the market needs to be contemplated to unfold the efficiency of microbes not yet explored for bioplastic production. Hence, the much-needed route exploration for the utilization of microbes, specifically bacteria, algae, and MMCs, in the form of productive cell factories for sustainable bioplastic production on a commercial scale along with the development of retail markets of the same is crucial. The utilization of pure microbial cultures for improved PHA production requires the supplementation of costly co-factors, vitamins, and organic substrates to strengthen the microbial metabolic processes, accompanied by the strict process control framework initiated by sterilization of equipment and growth media. All these parameters and requirements add-on to bioplastic production cost, thus obliterating the commercialization of bio-based plastic. In order to overcome these economical and technical hurdles, the administration of MMCs or photosynthetic microbial cultures (PMC) for the production of building blocks of bioplastic, such as

Polyhydroxybutyrate or polyhydroxy valerate, has been proposed. The mixed microbial culture utilization caters to the reduced biopolymer production cost owing to the consumption of inexpensive heterogeneous feedstocks complemented with the open cultivation conditions, neglecting the need for sterilization or stringent control regulations. Volatile fatty acids including, butyrate, propionate, acetate, and valerate obtained from the fermentation of sewage streams, are known to be the best precursors utilized by MMCs for PHA production (Albuquerque et al., 2011). Certainly, the financial supremacy and significance of mixed microbial cultures over pure cultures for PHA production have also been illustrated by the comparative financial analysis and life cycle assessment of MMC's PHA production (Gurieff and Lant, 2007). Nevertheless, apart from the economic outlook, wastewater, or sewage stream usage as an alternative to the nutrient substrate by MMC's along with the production of biodegradable plastic, have contributed to the diminished environmental footprint of bioplastic production.

However, to generate an elevated amount of PHA, the microbial genera present in the MMC needs to be specified and optimized. For the selection of microbes, the culture has been transiently fed by obeying the feast and famine regime, which comprises an intermittent feeding strategy, delineated as the enrichment of the culture with external carbon substrate (Feast) for a particular period; followed by the depletion of the carbon substrate (Famine) in the alternative time-period. These feast and famine cycles are repeatedly practiced, which favors cell growth, as well as accumulation of storage molecules, such as PHA during the feast cycle and later gets consumed during the famine cycle. Thus, microorganisms having high PHA storage capacity will ultimately survive and grow, becoming the microorganism of choice for inclusion in the MMCs (Dionisi et al., 2006; Serafim et al., 2006; Albuquerque et al., 2007, 2010b, 2010a; Oliveira et al., 2017).

Furthermore, the operating conditions and the composition of the substrate also play a significant role in the selection of diverse microbial species (Jiang et al., 2011c, 2011b, 2011a). Different researchers have worked on the varied substrate preferences of these organisms as presented in Table 2, which provides significant knowledge that might be used further to optimize PHA accumulation by tweaking the microbial community's assembly (Jiang et al., 2011a; Albuquerque et al., 2013; Marang et al., 2013; Pardelha et al., 2013; Carvalho et al., 2014). Subsequently, the system's operating parameters, including pH, temperature, feeding strategy, sludge retention time, a feast to famine ratio, and organic loading rate, have also been observed to significantly affect the PHA composition and content by influencing the culture conditions of mixed microbial culture (Dionisi et al., 2006; Johnson et al., 2009, 2010; Villano et al., 2010; Albuquerque et al., 2011; Jiang et al., 2011c; Chen et al., 2013; Wang et al., 2013; Carvalho et al., 2014).

Table 2: PHA accumulation by MMCs under the supplementation of varying substrates

Substrate	Bioplastic Monomer	PHA Accumulation	References
Crude Glycerol	PHB	49-60% cdw	(Dobroth et al., 2011)
Fermented Dairy Manure	P(3-HB-co-3-HV)	22.5-90.7% cdw	(Coats et al., 2016)
Fermented cheese whey with acetate pulse	P(3-HB-co-3-HV)	30% /VSS	(Fradinho et al., 2019)
Acidified hardwood spent sulfite liquor	P(3-HB-co-3-HV)	44.5% cdw	(Pereira et al., 2020)
Volatile Fatty acid	P(3-HB-co-3-HV)	44% cdw	(Guerra-Blanco et al., 2018)
Sucrose	P(3-HB-co-3-HV)	23.8 mg/ L/ Day	(Löwe et al., 2017)
Fermented Domestic Wastewater	P(3-HB-co-3-HV)	30.8% /VSS	(Almeida et al., 2021)

Abbreviations: #%CDW, % Cell Dry Weight; PHB= Polyhydroxylbutyrate, VSS= Volatile suspended solids; P(3HB-co-3HV), poly (3-hydroxybutyrate-co-3-hydroxy valerate)

As per the aforementioned literature, besides bacteria, algae have also been in the league of PHA production. Based on the facts, the algae-bacteria consortiums have been ascertained and reported to have the potential for wastewater treatment accompanied by the production of different biotechnologically essential bioenergy products (Ogbonna et al., 2000; Safonova et al., 2004; Van Iersel, 2009; Perez Garcia et al., 2010).

According to the literature, the highest energy-intensive prerequisite of mixed microbial cultures recognized is the supply of proper aeration for fulfilling the requirement of oxygen (an electron acceptor) because the PHA content accumulated in an aerobic environment (75%) is substantially high as compared to the PHA content gathered in anaerobic/aerobic environment (37%). However, the requirement of aeration ultimately increases the operation cost involved in the PHA production by mixed microbial cultures (Dionisi et al., 2004; Dai et al., 2007; Rosso et al., 2008; Bengtsson, 2009; Albuquerque et al., 2010b).

While looking into the PHA production efficiency of pure bacterial species and mixed microbial consortia, an example of *Ralstonia eutropha* can be considered. *R. eutropha* independently under the supplementation of VFAs derived from food waste is known to accumulate approximately 52% PHA copolymer (Bhatia et al., 2019). Although *R. eutropha* cannot assimilate sucrose in the form of carbon sources, however when synthetic mixed microbial consortia composed of *Bacillus subtilis* and *R. eutropha* are grown in the presence of sugarcane sugar then it leads to the accumulation of about 66 % PHA copolymer. Therefore, it suggests the importance of MMCs over the pure bacterial culture for PHA production (Bhatia et al., 2018). The biosynthesis of PHB by mixed microbial culture supplemented with crude glycerol (obtained as a byproduct from the production of biodiesel) was evaluated by a group of scientists and illustrated that due to the deficiency of macronutrients in the presence of crude glycerol, accumulation of PHB is elated. They projected that on scaling up, about

20.9 tons of PHB could be produced every year by MMCs while processing 10 million gallons of biodiesel every year (Dobroth et al., 2011).

Therefore, to vanquish this obligation, Fradinho et al. (2013a) culminated the enrichment of photosynthetic mixed culture comprising bacteria and algae consortia by observing the accumulation of PHA by bacteria in the feast cycle followed by its consumption in the famine cycle while using the oxygen generated by the photosynthetic algae, thus excluding the need of aeration provided externally. The PHA accumulated by algae-bacteria consortia was reported to be 20 % PHA per volatile suspended solid (VSS) when the culture was supplemented with acetate in the form of a carbon source.

It has been proven by previous studies that oxidation of reducing molecules like NADP and NADPH is required for the conversion of carbon source into PHA. In the absence of oxygen, these reducing molecules will never be oxidized, and carbon source conversion to PHA will not be initiated. Hence, justifying the quintessence of photosynthetically mixed culture (composed of algae and bacteria consortia) for ameliorated PHA production (Fradinho et al., 2013a, 2014, 2016).

Weiss et al. (2017) designed a synthetic consortium comprising a photoautotroph and a chemoautotroph. The synthetic consortia were light-driven, and the heterotrophic *Halomonas boliviensis* bacterium was capable of metabolizing sucrose secreted by algae, *Synechococcus elongatus* CscB. It was reported that the bacteria in consortia were able to accumulate about 31% dry cell weight of PHB, with a productivity of 28.3 mg PHB $L^{-1}D^{-1}$ (Kourmentza et al., 2017; Weiss et al., 2017). In a nutshell, an upcoming paradigm of embracing the importance of MMCs for the production of bioplastic, especially PHA, should be practiced, to ensure sustainable and economically feasible bioplastic production on a commercial scale.

On summarizing the positive impact of photoautotrophic cultivation of algae, i.e., no specific requirement of agricultural land, high CO_2 sequestration ability, photosynthetic efficiency, and treatment of waste material prove to be economically proficient over the heterotrophic growth of bacteria. However, even maintaining axenic cultures of algae on a large scale has been known to be economically infeasible due to the maintenance cost of the optimal physicochemical parameters for the production of PHA. Therefore, to overcome all the challenges discussed previously, MMCs composed of either different chemoautotrophic bacteria or of algae and phototrophic bacteria need to be practiced and explored further for making the PHA production process economically feasible. Not only do different microbes participating in the mixed consortia need to be investigated for their PHA yield but varying physical and chemical parameters affecting the accumulation capability of the consortia also need to be optimized for obtaining supreme yield keeping in view the large-scale production of bioplastic.

The evaluation needs to be done to explore the biopolymer production capability of pure strains as well as MMCs. The biorefinery approach for the production of bacterial bioplastic along with other essential bioproducts derived from bacteria needs to be scrutinized and exercised to make the process techno-economically feasible. Additionally, systems analysis incorporating life cycle assessment (LCA) and techno-economic analysis (TEA) must be investigated for determining the sustainability of bacterial biopolymer production.

Photosynthetic microbial consortium (PMC) has been soaring in recent year's reason being the vast availability if nutrients and various by-products. PMCs can utilize nitrate, phosphate, organic and inorganic carbon, and carbon dioxide as well, which are often found in wastewater and the atmosphere (Fradinho et al., 2014). Where the PMC provides a cost-effective production strategy, on the other hand, uncontrolled growth of the

microbes can often lead to lower volumetric productivity. One other major issue with the commercialization of PHA is the high production cost, 50% of which constitutes the substrate cost (Nguyenhuynh et al., 2021).

B.3 Factors influencing bioplastic precursor accumulation

Establishing a sustainable and commercial process for bioplastic production has two main components: quantity and quality of accumulated precursor. High quantity will ensure the sustainability of the process but on the other hand, if the properties which determine the equivalence of bioplastic with conventional plastic, will be surpassed, it will fail to make its place in the market. Properties like crystallinity, glass transition temperature, thermal stability, melting temperature, etc. determine the quality of the polymer (Ten et al., 2015). Homopolymers often suffer from the disadvantage of being brittle hence, the production of copolymers is sought to overcome the hurdle (Bhati and Mallick, 2015). In this section, factors that affect the accumulation of bioplastic precursors have been discussed.

B.3.1 Strain selection

Primarily, strain selection plays a very important role in determining the percentage and type of bioplastic precursor being produced. The chemical and physical properties of the bioplastic have been analyzed to be highly dependent on the type of PHA accumulated by different strains. *Alcaligens latus* has been reported to produce scl PHA whereas mcl PHA is commonly produced by *Pseudomonas putida* (Wang and Lee, 1997; Sun et al., 2007). While scaling up, native strains are proven to be a better choice to withstand outer conditions.

B.3.2 Organic nutrients

Another important factor that affects the PHA accumulation potential of microorganisms is the composition of nutrient media. The deficiency of essential nutrients or supplementation with waste (rich in carbon sources,

amino acids, or fatty acids) has been delineated to influence PHA production. Even the mechanical properties of PHA material get modified based on the nutrients present in the media and it eventually characterizes the use of bio-based material in different fields of biotechnology. PHB yield of *Bacillus megaterium* subjected to various physicochemical parameters was investigated by Mohanrasu et al. (2020) who concluded that optimization of parameters such as pH, nitrogen, and carbon sources need to be optimized for each bacterial species for the productive accumulation of good quality PHA.

Various nutrients and physicochemical factors play a role in the biosynthesis pathway of PHA accumulation (Figure 2). Three key enzymes of PHA accumulation are phaA (keto thiolase), phaB (Acetoacetyl reductase), and phaC (PHA synthase). Several copies of the PHA synthase gene have been reported in over 30 genera of bacteria. All these genes produce different types of PHA synthase, which determines the conversion of substrate into PHA. It also determines the molecular weight of the PHA being produced (Rehm and Steinbüchel, 1999). The molecular weight of PHA determines the crystallinity, and thermal stability of bioplastics (Laycock et al., 2014). PHA synthase has 3 classes that produce PHA with different molecular weights. Class I of PHA synthase produces high molecular weight PHA ranging from 500 kDa to a few million, class II produces 50 kDa to 500 kDa, whereas class III PHA synthase product's molecular weight ranges in between the two classes (Rehm and Steinbüchel, 1999). High molecular weight also increases young's modulus which determines the elasticity of the bioplastic (Domínguez-Díaz et al., 2015). Few studies have reported the influence of different carbon sources on molecular weight. *Paraburkholderia xenovorans* LB400, was grown in 3 different carbon sources namely, glucose, xylose, and mannitol. The study concluded that PHB produced with glucose and xylose had similar molecular weight, which is near to commercially available PHB, whereas PHB produced in

mannitol supplementation had double molecular weight as compared to control and other carbon sources. The study also concluded that carbon sources not only affect the quantity of PHA production but also the molecular weight of bioplastic (Sanhueza et al., 2020).

Furthermore, the PHB accumulation capacity of carbon-sequestering microalgae, *Botryococcus braunii* has been evaluated and validated statistically by optimizing different parameters, including temperature, pH, and substrate concentration of sewage wastewater. To some extent, these process parameters were found responsible for diverting the carbon flux towards the enhanced production of PHB. The usage of sewage wastewater as an inexpensive nutrient substrate for algae-based bioplastic production indicated that this strategy could be adopted at an industrial scale for economically feasible and beneficial large-scale PHB production. Microalgae, *B. braunii* was claimed and recommended as a potential candidate for PHB production (around 20 % of dry algal weight), which can be used in the pharmaceutical industry. NADH plays a major role in the assimilation of pentose and hexose sugars. Activation of glucose transporters releases NADH, which in turn activates the accumulation of PHA via inducing the conversion of acetyl co-A (Kavitha et al., 2016a, 2016b).

Likewise, *Chlorella fusca* LEB 111 microalgae have also been examined experimentally for improved PHB production by optimizing the requirement of pentose sugar substrate, photoperiod, and light intensity factors, which contribute significantly to the accumulation of PHB. It was demonstrated that 28 μmol photons/m^2-s of light intensity, 6 hrs exposure to light, and xylose supplementation induced the microalgae to synthesize 17.4 % (w/w) PHB. Therefore, deviation in the light periodicity integrated with varying luminous intensities and involvement of pentoses in the metabolic pathway has been proven to stimulate and boost the PHB accumulation capacity of *Chlorella*. The uptake of organic carbon is highly

dependent on photoperiod and light intensity. The light phase triggers the enzymes responsible for autotrophic growth hence blocking the utilization of carbon sources but during the dark cycle of mixotrophy, it starts assimilating organic carbon hence converting the flux towards PHA synthesis (Cassuriaga et al., 2018).

B.3.3 Inorganic nutrients

Inorganic nutrients such as nitrates, phosphate, salts, etc. play an important role in PHA accumulation (Koller, 2020). They are often found in wastewater in bulk amounts and can be used as a cheap nutrient source for growth. While working with pure culture limitation of nitrogen and phosphate can trigger a chain reaction resulting in the disruption of cellular activities (Alipanah et al., 2018; Ghosh et al., 2019). When working with the mixed culture maintaining the ratio of carbon, nitrogen, and phosphate (C/N/P) is a critical factor in regulating PHA accumulation. In a study done on MMCs, different ratios of C/N were tested and revealed that a lower C/N ratio enabled high PHA accumulation whereas low PHA was accumulated under a high C/N ratio (Rojas and Fajardo, 2021). Thus, successfully curating nutrient requirements for cultures can profoundly impact PHA accumulation.

B.3.4 Heavy metals

Heavy Metals (HM) are metals that have high density and atomic weight. Commonly known heavy metals are copper (Cu), iron (Fe), silver (Ag), chromium (Cr), lead (Pb), cobalt (Co), etc. These heavy metals are most commonly present in industrial wastewater, or battery industries (Ajiboye et al., 2021). Many reports have shed light on the role of HM in increasing lipid accumulation in microalgae (Kizilkaya et al., 2012; Nanda et al., 2021). HM can also increase the accumulation of polysaccharides in some species (De Philippis et al., 2001). Although there are very few reports available linking PHA and HM, previous reports have postulated that during

the interaction with HM, ROS are generated which create an energy imbalance and trigger the accumulation pathways for various molecules (Koller, 2017; García et al., 2021). Purple bacteria have also been reported to have an interaction with a wide range of heavy metals (Grattieri et al., 2022).

B.3.5 Plant hormones

Plant hormones have shown the enhancement of overall cellular metabolism, biomass production, and alleviation of oxidative stress by regulating ROS mechanisms in several microalgal species due to their close relationship with plants (Allaf, 2013). The plant growth promoters like GA_3 showed to elicit lipid accumulation by 1.9 folds than in control, and with ethephon hormone, protein accumulation got enhanced by 3.5 folds to control in the microalga *Chlorella pyrenoidosa* (Du et al., 2017); in *Chlamydomonas reinhardtii*, the combined effect of plant growth promoters (auxins, gibberellins, and cytokinins) promoted the overall cell growth, their division and thereby positively regulating the biodiesel production (Woong Kim et al. 2013); in another study, the synergistic effect of plant growth promoters- auxins (indole-3-acetic acid (IAA), indole-3-butyric acid and indole-3-propionic acid (IPA)) and cytokinin (benzylaminopurine and (BAP) and thidiazuron (TDZ)) in microalga *Desmodesmus* sp. JS07 resulted in enhanced cell growth, biomass, and lipid accumulation (Singh et al., 2020)

Despite having any reported connection with the PHA pathway in organisms plant hormones can be used to enhance PHAs due to the intertwined pathway of lipid and PHA accumulation. Irrespective of the significance of either of the microbes, i.e., bacteria or algae in the production of bioplastic, the accumulation of metabolite in the cell along with the growth of microorganisms has been delineated to depend highly on various physicochemical parameters and environmental conditions present

at the time of cultivation of microbes. Physical and chemical parameters such as type of fermentation, type of growth conditions, time of harvesting, aeration, presence or absence of oxygen, pH, temperature, light intensity as well as the composition of nutrient media have been studied immensely by researchers for optimizing maximum biomass growth and PHA accumulation of microbe of interest. However, the type of microbe and its strain influences the quality and quantity of the bioplastic produced. Individual microbial cultures will limit the quality and quantity of produced bioplastic, therefore different algal and bacterial strains need to be studied and the influence of various influential parameters needs to be optimized.

B.4 Scale-up and downstream processing of microbial biopolymer in pure cultures

Although the microbial community has been determined as a proficient candidature for bioplastic production; however, just optimizing the growth and accumulation of bioplastic precursors at the lab-scale is not sufficient for the commercialization of bioplastic material. Scaling up of lab-scale parameters on the pilot scale has frequently let down, the reason being the added factors related to the type of pilot-scale plant. Pilot-scale plants include two categories: closed bioreactors and open pond systems.

Closed bioreactors are often utilized for upscaling axenic cultures. It is easy to maintain purity and controlled conditions in closed reactors, but additional costs to operate the reactor has a negative impact on the cost of the final product (Alam et al., 2020). Another factor adding to the cost is the substrate. Using pure substrate at the pilot scale is not feasible from an economic point of view. To overcome these factors, many studies have been performed and introduced various cheap substrates for PHA production (Bhagat et al., 2020; Riedel and Brigham, 2020). Operational conditions like agitation and aeration impacts growth as well as PHA accumulation inside the reactor. *Halomonas campisalis* MCMB-1027 was grown in a 14

L reactor and accumulated 41 % PHA under optimum agitation and aeration. Optimized dissolved oxygen level (DO) further increased accumulation up to 56 %. This optimization helped develop a correlation for upscaling up to 120 L fermenters (Kshirsagar et al., 2013). Optimizing all the operational conditions can be time-consuming, and model-based approaches can resolve limitations (Papapostolou et al., 2019). Closed photobioreactors are often used for photosynthetic microorganisms. Flue gases can be used as a substrate to trigger PHA accumulation (Troschl et al., 2017). A semi-continuous tubular photobioreactor was used to grow *Synechocytis* sp. CCALA192, CO_2 was used as substrate and 12.5 % PHB was accumulated in the semi-continuous mode (Troschl et al., 2018). Byproducts like glycerol, grape pomace, molasses, etc. can also serve the purpose (Nighat Naheed, 2012; Follonier et al., 2015; Volova et al., 2019).

High-rate algal ponds often used to grow microalgae, utilizes waste water as substrate (Chakravarty et al., 2010; Simon et al., 2016; Abdo and Ali, 2019). It has a low operational cost as compared to closed reactors but maintaining axenic cultures is a tough task, especially when wastewater has high COD and VFA content. Scaling up mixed culture is far better due to low maintenance and wide range availability of substrate choices and in the tailor-made consortium, microorganisms based on their attributes and compatibility can be selected for soaring PHA production.

Even pilot-scale production of PHB accumulated within the cyanobacteria has been known to be difficult due to the contamination-related challenges that eventually hinder the purity of the type of bioplastic produced (Troschl et al., 2018). It has been postulated that a distinct strategy needs to be devised for the large-scale cultivation of bioplastics. Thus, while optimizing upstream processing steps at the lab scale, the pilot scale approach should also be investigated extensively, followed by the investigation of an appropriate extraction strategy. A sustainable bioplastic purification process that is cost-effective, has a lower impact on the environment and higher

conversion efficiency as compared to the traditional methodology. As the cost of different steps of bioplastic downstream processing elevates the overall bioplastic manufacturing cost, therefore detailed scrutinization of different types of extraction and purification techniques taking part in the process should be considered for facilitating the commercialization of bio-based plastics.

Downstream processing has also been known to contribute substantially to determining the economic status of bioplastic production owing to the high accumulation of PHA inside the cell and lower yield of product extracted; therefore, different extraction and purification strategies also need to be investigated to make the process economically feasible and environmentally sustainable (Lorini et al., 2021).

While extracting and purifying bioplastic precursors from the microbe, several factors need to be considered for discerning the strategy to be adopted for economical downstream processing. Parameters such as the type of PHA-producing microbe, yield and type of bioplastic, and the purity of the product to be extracted help in deciding the extraction procedure. Eventually, the extraction and purification methodology employed, not only influences the type of bioplastic material produced but also delineates the environmental and economic considerations of the same (Koller et al., 2013).

A comparative analysis of various strategies available for PHA extraction has been done by Kurian and Das (2021). In the following study, people mentioned chemical and biological extraction processes and concluded biological process is better than the chemical approach. Another study with a similar aim suggested choosing the extraction process based on end product (Kunasundari and Sudesh, 2011).

Additionally, life cycle assessment and techno-economic analysis of various PHA recovery processes have been performed and illustrated that the usage

of solvent leads to a hike in the production cost of bioplastic and should be persuaded only while employing solvents obtained from biorefinery setup or should be performed when the absolute form of bioplastic precursor is required. Likewise, the economic and environmental impact of every methodology involved in the downstream processing of bioplastic precursors needs to be exercised for attaining sustainable bioplastic production design (del Oso et al., 2021).

B.5 Existing commercial status quo of bioplastics

The global upsurge and advancement in the bioplastic market are attributed to its sustainable production protocol (compared to conventional plastic) and its widespread applications in different industrial fields such as food services and packaging. Moreover, increasing awareness regarding the benefits of biocompatible and biodegradable plastic, formulation of government norms to stimulate and acknowledge eco-friendly techniques, and ever-augmenting fossil-fuel prices have invigorated the commercial diversion towards the production of bioplastic elements.

The worldwide spread of the bioplastic business is still subordinate compared to the petroleum-based plastic market. Different bioplastic-producing plants are situated in different parts of the world, including Italy, Brazil, the USA, China, and others. Indeed, various industries are in the business of bioplastic production, especially PHA thermoplastic material. Table 3 summarizes few companies producing biopolymer for various application using PHA as raw material.

Table 3: PHA based commercial products and their applications

Product	Monomer	Application	Manufacture	Reference
Mirel™ P1003, P1004	PHB	Injection Moulding, Paper Coating	Metabolix Inc.	Ulprospector (2023, May 21)
Nodax®	PHA	Straw, Cups, Lids, Diaper Lining, Toys, trash bags etc.,	Danimer Scientific	Danimerscientific (2023, May 21)
Minerv-PHA™	PHA	Automobile, Electronics, Packaging, Beverages, fibers, pharma	Bio-On	Bio-On (2023, May 21)
ENMAT Y1000, Y1000P, Y3000, Y3000P	PHB, PHBV	Injection molding, films, fibers, water treatment	TianAn Biopolymer	TianAn (2023, May 21)
TephaFlex®	P4HB	Sutures, medical mesh, surgical films	Tepha Inc.	fi-tech (2023, May 21)

It has been evident that microalgae have proved to be a superlative candidate for the production of bioplastic but need to be explored further in order to achieve commercial production of bioplastic emanating from microalgae independently or in consortium with bacteria. A substitute for a plastic bottle was also prepared with the composition of red algae powder and water (Verlinden et al., 2007; Singh et al., 2017; Alcântara et al., 2020; Chia et al., 2020; Price et al., 2020; Shafqat et al., 2020). Therefore, commercialization of bioplastic emanating from algae can be achieved by investigating the bioplastic production potential of various algal species along with the metabolic engineering and evaluation of multiple aspects influencing the accumulation of bioplastics. However, acquiring a more profound understanding of the know-how related to downstream processing as well as the participation of algae-derived plastics need to be the prime

concern of the researchers, as they contribute considerably to the production cost of commercialized plastic.

C. Relevance of the research

The challenges faced in the process of production of biopolymers include the need for expensive carbon sources, operational costs to maintain the process control parameters, low yield of a metabolite of interest, and difficulty in achieving considerable productivity as well as downstream processing steps. Whereas the usage of photo-synthetic organisms is still in its early course, they have immense leeway for achieving a greater place in bioplastic industries. Over the past few decades, microalgae and cyanobacteria have also been under surveillance for evaluating their potential for bioplastic precursor accumulation or for direct usage in bioplastic production. Establishing a biorefinery for sustainable PHA production has two major bottlenecks: 1) Production cost, and 2) The quality of the polymer.

To overcome these hurdles, the first step was to isolate novel native strains to make scaleup easier and cost-effective (no need to maintain favorable conditions). These strains will be easy to acclimatize in an open pond system which is more cost-effective than closed photobioreactors. Open ponds have low production as well as operational cost and are more feasible for life cycle assessment (LCA) or techno-economic analysis (TEA) (Costa et al., 2019).

Keeping in mind the various pros and cons of PHA production *via* various microbes individually, a consortium approach seems to be a light in the dusk. In recent years, different mixed microbial cultures are under observation to uncover their bioplastic production potential, either by tweaking certain culture conditions or by metabolic engineering approach. The practice of utilizing photosynthetic MMCs composed of bacteria and

algae consortia or different photosynthetic bacteria, accompanied by the consumption of wastewater or byproducts of various production processes like molasses, grape pomace, olive pomace, crude glycerol, etc., as a substitute for pure organic carbon source, will prove to be an efficacious perspective for the production of economically feasible, environmentally sustainable and biodegradable bioplastic. PHA produced by MMCs can also have a greater composition for better composition and mechanical properties.

There has been an increase in mixed microbial cultivation for PHA accumulation. While working with mixed microbial cultures (MMCs), different types of substrates can be used for production, decreasing the cost of production but often have the irregular presence of microbes which can result in the production of the varied product and cannot be scaled up as a commercial product. Another step to cut the cost was to design a tailor-made consortium to maintain the properties of polymer while not having to worry about the high cost to cultivate pure strains.

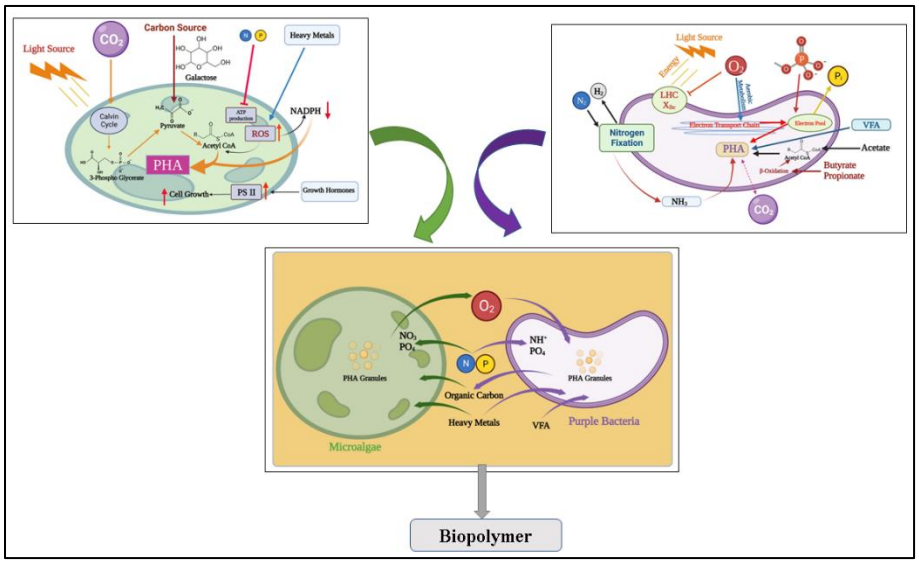


Figure 3: An illustration of tailormade Consortium for PHA production

The major steps taken to make the process more cost-effective and sustainable, an integrated biorefinery concept was adopted and along with

PHA, other value-added products like, protein, carbohydrate, lipid, and pigment production were also studied.

Figure 3 illustrates the proposed tailormade consortium for PHA production utilizing two photosynthetic microorganisms i.e., microalgae and PNSB. These microbes, despite being photosynthetic have different metabolic activities and photosynthetic mechanisms. In addition to that in various mixed cultures they have been reportedly growing in a symbiotic relation together which can be utilized in a tailormade consortium for better PHA accumulation. Keeping the nutrient requirements in mind for both organisms, optimization could take place to enable their full potential for PHA accumulation.

D. Research objective

This research has been designed, to unearth new photosynthetic microbes from Indore (Madhya Pradesh, India), that are capable of accumulating PHA. Elucidating their capacity to maximum and finally introducing a tailor-made consortium for the accumulation of PHA for a sustainable biorefinery approach. This approach might help in maintaining the quality of the polymer without increasing the high cost of cultivation for pure cultures.

The following objectives were designed to achieve the desired output of the thesis:

Major objective

Formulation of a tailor-made consortium of photosynthetic microorganisms for the sustainable production of biopolymer

Minor objectives

i. **Biochemical profiling of indigenously isolated microalgal species**

ii. **(a) Nutritional source amelioration for enhanced PHA synthesis in microalgal hosts *via* two-stage cultivation**

(b) Manoeuvring PHA accumulation in microalgae by employing various stress factors

iii. **Exploring indigenous purple non-sulfur bacteria for targeting PHA accumulation**

iv. **Algae-bacteria consortium for enhanced biopolymer production and its characterization**

v. **Scale-up study: Assessment of growth behavior of microalgae under semi-outdoor condition**

The above-mentioned objectives were achieved and explained in upcoming chapters.

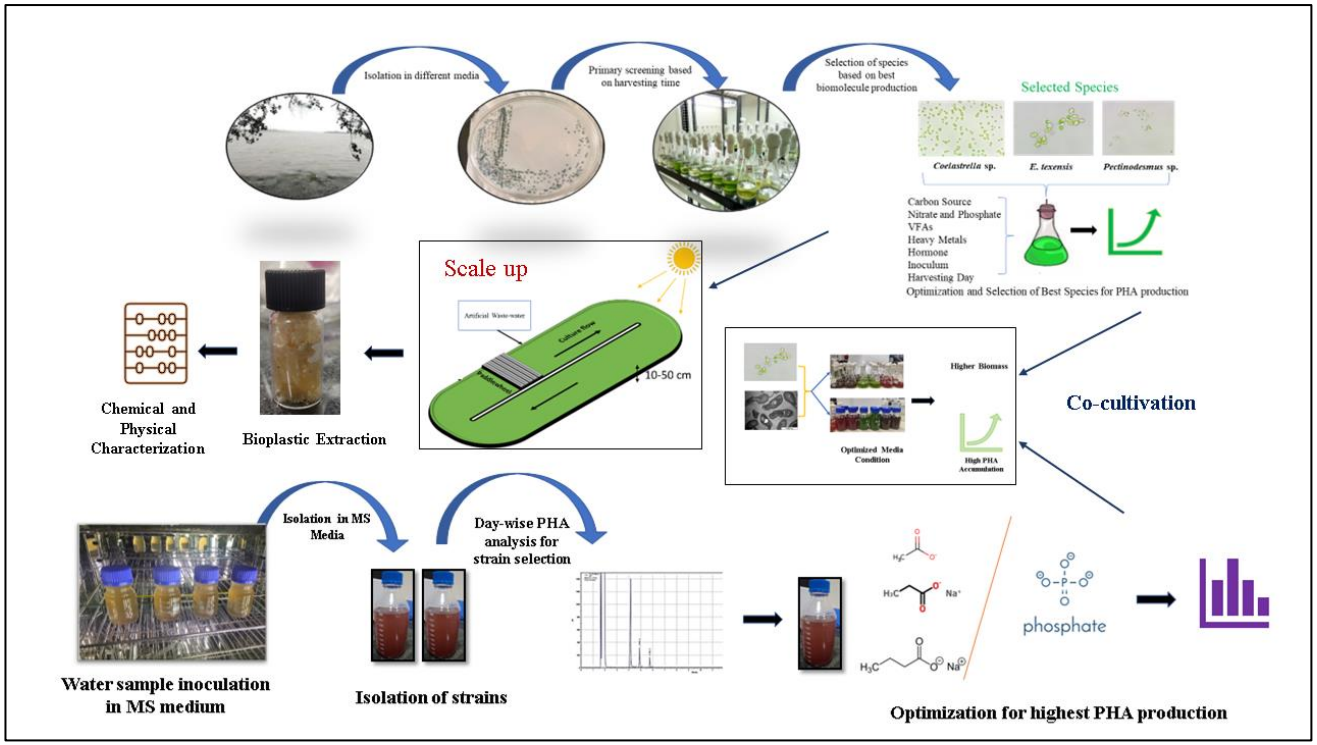
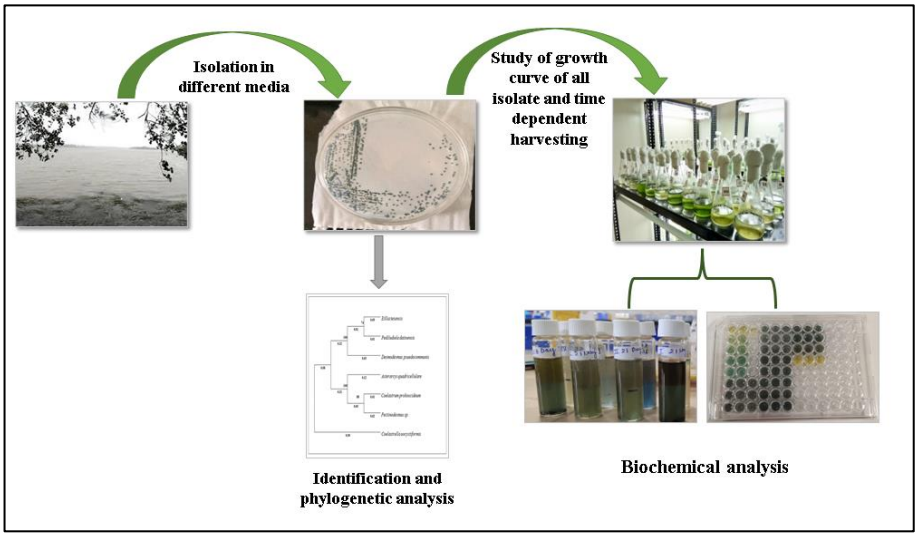


Figure 4: Schematic overview of the experimental approach for biopolymer production

CHAPTER 2

Biochemical profiling of indigenously isolated microalgal species



A. Introduction

Microalgae have been present on this earth's environment for over a billion year as photosynthetic autotrophs. They are photoautotrophic microorganisms believed to be originated from the protists. They have abundance of chlorophyll and synthesize their food by photosynthesis using inorganic nutrients like nitrate, phosphates, sunlight and atmospheric CO_2 (Moestrup, 2006). These can be both terrestrial as well as aquatic and can grow in freshwater i.e. *Chlorella* sp., *Nannochloropsis* sp., *Monoraphidium* etc. (Thao et al., 2017); marine water i.e. *Nannochloropsis oculata, Chlorella vulgaris, Nannochloropsis salina, Rhodomonas salina* etc. (Mohseni et al., 2020; Thomas et al., 2020; Zamani-Ahmadmahmoodi et al., 2020) and wastewater as well i.e. *Chlorella sorokiniana*, *Micractiniumpusillum, Chlorella variabilis* etc. (Park et al., 2015). Surrounding environment (pH, temperature, presence of different salts) of microalgae exert significant effect on its metabolic pathways that lead to the synthesis of the different metabolites. Researchers have explored these variations in surrounding environment of microalgae during their growth period to identify and produce high value metabolites like ω-3 poly-unsaturated fatty acids (Hong et al., 2012), vitamins like riboflavin, niacin, folate and vitamin B_{12} (Edelmann et al., 2019). The microalgal biomass can be processed to extract certain commercially viable products like biodiesel, poly-unsaturated fatty acids (PUFAs), proteins, bio-ethanol and pigments like asta-xanthin, beta-xanthins etc. (Feng et al., 2011; Metz et al., 2001; Suganya et al., 2016)

Various microalgal biomass components (proteins, lipids and carbohydrates) can be utilized to manufacture certain important commercially valuable products like methane, bioethanol, bio-hydrogen, biopolymer and biodiesel. However, this process is not yet economically sustainable due to lower yields of proteins, lipids and carbohydrates from the algal biomass. Therefore, understanding and exploring the biochemical profile of each microalgal species is a first

step towards the production. Within past few years, multiple studies have tried to identify various strategies and methods to explore and enhance either lipid accumulation (Carvalho and Malcata, 2005; Atreyee Ghosh et al., 2019; Pancha et al., 2015), or accumulation of various pigments (Mulders et al., 2014), or certain value-added secondary metabolites (Cheng et al., 2016; De Philippis et al., 2001; Goiris et al., 2012; Hashimoto, 1954; Plaza et al., 2010; Robert and Iyer, 2018) in microalgal cells. However, only a handful of reports are available which compare entire biomass profile of various microalgal species at different growth phases (Zhu et al., 1997). It is also imperative to understand the fact that the accumulation of the various biomass components in a microalgal cell is much greater than mere energy storage as these components lead to the synthesis of other important biomolecules in the cell. Therefore studying the entire biomass composition in a microalgae cell would help us to use these species much more efficiently in context to various metabolites production (Karemore and Sen, 2016). Further, the biochemical constituents (proteins, lipids and carbohydrates) in a microalgal cell can be enhanced by manipulating the growth conditions. Amongst various factors (temperature, light intensity, nutrients, salinity), cultivation time is a critical factor deciding the proportion of biomass constituents in a microalgae species (Anand et al., 2019; Ghosh et al., 2021;Mansour et al., 2003; Zhu et al., 1997). Therefore, the storage and production of these chemical constituents need to be studied at various cultivation time and growth phases to decide upon biomass harvesting time and reduce costs in biofuel production.

It is also equally important to isolate native species and screen them based on target chemical constituent. This would help us to identify species and further use them for scale-up approach (Chew et al., 2017). Till date very few indigenous species of microalgae have been isolated and being tried for lipid, protein or carbohydrate production at commercial scale. With this aim, the present study focussed on exploring the native species of Indore region. Indore has wide window of temperature variation i.e., 12.4 °C to 39.2 °C (climatedata.org), which

gives a better opportunity to understand the variation in the strains caused by the change in temperature and of course resulting changes in the biochemical profile of the strains. It is important to identify the suitable species for bio-refinery applications based on their growth pattern and biochemical constituents. Apart from biodiesel, high carbohydrate producing species can be further channelized for bio-ethanol production, whereas high protein accumulation can help us find an alternative source of proteins. Various harvesting time has enabled us to unlock the true potential of synthesis of above-mentioned products by these isolates to attain maximum productivity to minimize the cost of favoured products. All the species used in the study have not been previously reported to produce biopolymer (Polyhydroxyalkanoate; PHA). Extensive study of their growth and biochemical profile could enabled screening of probable producers of biopolymer especially lipid profiling since lipid and PHA accumulation pathways are intertwined hence high lipid producing species can accumulate PHA under stress or supplementation conditions (Koller, 2017). Furthermore, other by-products of the microalgae could help reduce the overall cost of the production hence enabling a sustainable biorefinery.

B. Material and methods

B.1 Isolation and molecular characterization

Microalgal species used in this study have been isolated from nearby areas of Indore, Madhya Pradesh (Central India). Water samples from various regions of Lal bagh, Indore (site A 12.9507° N, 77.5848° E), Shitala mata waterfall (site B 22.4042° N, 75.6099° E), Narmada River, Maheshwar (site C 22.1759° N, 75.5872° E), Kshipra River, Ujjain (site D 23.1907° N, 75.7642° E) were collected aseptically. A sewage treatment plant was selected (site A) to isolate and explore robust species helpful in wastewater treatments. The rest of the three sites were selected based on the flow rate of water. To reduce the cultivation costs species which do not require shaking during their growth can become excellent candidates. Two separate growth media were used to facilitate the

isolation of different species belonging to different classes of algae i.e. BG-11 (Stanier et al., 1971) and WC medium (Guillard & Lorenzen, 1972). BG-11 broth comprised of 1.5 g sodium nitrate, 0.04 g di-potassium hydrogen phosphate, 0.075 g of magnesium sulphate heptahydrate, 0.036 g of calcium chloride, 0.006 g citric acid, 0.006 g ferric ammonium citrate, 0.001 g EDTA (di sodium salt), 0.02 g sodium carbonate, 2.286 mg boric acid, 1.81 mg manganese chloride, 0.222 mg zinc sulphate, 0.039 mg sodium molybdate dihydrate, 0.079 mg copper sulphate pentahydrate, 0.0494 mg cobalt (II) nitrate hexahydrate per liter. pH was maintained 7.4 using diluted acid or base. WC broth comprised of 85.01 mg sodium nitrate, 8.71 mg di-potassium hydrogen phosphate, 36.97 mg of magnesium sulphate heptahydrate, 36.76 mg of calcium chloride dihydrate, 12.60 mg sodium bi-carbonate and trace element consisting of 4.36 mg EDTA (di sodium salt), 1 mg boric acid, 0.18 mg manganese chloride tetrahydrate, 0.022 mg zinc sulphate heptahydrate, 0.006 mg sodium molybdate dihydrate, 0.01 mg copper sulphate pentahydrate, 0.01 mg cobalt chloride hexahydrate, 3.15 mg ferric chloride hexahydrate. Vitamins 0.1 mg thiamine-HCl, 0.5 µg biotin and 0.5 µg cobalamin were added after autoclaving. pH of the growth media was maintained at 7.4 using diluted acid or base. For isolation of new algal species 150 mL Erlenmeyer flasks containing 60 mL of broth and 10% water samples were inoculated in aseptic conditions. Flasks were kept under the illumination of 3000 lux in 12:12 hour photoperiod at 27±5 °C temperature (Kaushik, 1987).

Once visible growth was attained in the flasks, the samples were purified using sector plate technique on agar plate of each medium containing 1.5% agar to obtain individual colonies. The cultures were morphologically identified using Olympus microscope under 40x or 100x magnification. Characterization of strains at genetic level was done by National Collection of Industrial Microorganisms (NCIM), Pune using Internal Transcribed Spacer (ITS) and 18S ribosomal RNA (rRNA) primers. Sequence obtained were BLAST against National Centre for Biological Information (NCBI) database and then aligned

using clustalW and phylogenic tree was constructed using Maximum Likelihood method through MEGAx version 10.1.7 software with 500 bootstrap resampling (Kumar et al., 2018; Tamura and Nei, 1993).

B.2 Growth and maintenance of cultures

Once pure cultures were obtained and identified, they were maintained in BG-11 broth at pH 7.4 under illumination of 3000 lux with 12:12 hrs photoperiod at 28 ±5 °C temperature. The seed cultures were grown for 10 days, and the experimental flasks were inoculated from this culture. The initial cell density was kept 0.1 at 680 nm and were cultivated till 28th day. Growth was measured every 48 hrs and biomass was harvested at four different time intervals: 7th, 14th, 21st and 28th days. After harvesting, algal biomass was analyzed for chlorophyll, lipids, proteins, carbohydrates and fatty acid methyl esters (FAMEs).

B.3 Growth kinetics

Growth was measured every 48 hrs by taking absorbance at 680 nm using DR-6000 HACH spectrophotometer which was further used to calculate biomass production through a linear graph between OD (Optical density) vs dry biomass. Furthermore, biomass productivity, specific growth rate and division time was calculated following equations given by Pancha et al., (2015) and Guillard (1973).

$$P = \frac{(X_2 - X_1)}{(t_2 - t_1)} \quad (1)$$

P is biomass productivity in µg/mL/day, whereas X_2 and X_1 represents biomass concentration at day t_2 and t_1 respectively.

$$\mu = \frac{\ln \frac{N_t}{N_0}}{T_t - T_0} \quad (2)$$

Here μ is specific growth rate (Per day), N_t and N_0 represents cell densities at time T_t and T_0.

$$Division\ Time = \frac{0.69}{\mu} \quad (3)$$

Division time is represented in days.

B.4 Biomass compositional analysis

To study and understand the carbon channeling in different species throughout their growth cycle, detailed biochemical study was done.

Protein estimation was done using a modified micro-biuret method (Chen and Vaidyanathan, 2013). A known amount of lyophilized biomass was taken and 1 mL lysis buffer comprising of 1N sodium hydroxide in 25% methanol was added. Tubes were vortexed for 30 seconds and incubated at 80 °C for 15 minutes in water-bath. After centrifugation, supernatant containing crude protein was mixed with biuret reagent (0.21% Copper sulphate in 30% NaOH) in 2:1 ratio and incubated in dark for 15 minutes. Absorbance was taken at 310 nm in microplate reader. Protein concentration in sample was calculated using calibration curve made with bovine serum albumin (BSA) purchased from Himedia.

Carbohydrate estimation was performed using anthrone test Roe, (1979). A known amount of lyophilized biomass was taken, and 1 mL distilled water was added to make a suspension. 4 mL anthrone reagent (100 mg anthrone and 1 gm thiourea in 75% sulfuric acid) was added in diluted algae sample and tubes were again vortexed. After incubation, the tubes were cooled on ice and absorbance was taken at 620 nm. Concentration was calculated using standard curve prepared with D-Glucose (Himedia).

Chlorophyll estimation was done using protocol given by Arnon, (1949). 5 mL sample was centrifuged, and the pellet was washed with distilled water. 5 mL of 95% ethanol was then added and kept in boiling water. Absorbance was taken by Hach spectrophotometer at 645 nm and 663 nm with 95% ethanol as a blank.

For lipid estimation, SPV method was used Mishra et al., (2014). Known amount of lyophilized biomass was taken in a tube and 100 µL distilled water was added and vortexed. 2 mL sulfuric acid was added to the tubes and kept at 100 °C for 10 minutes. After cooling, 5 mL vanillin reagent

(0.6 gm vanillin, 10 mL absolute ethanol, 90 mL distilled water and 400 mL ortho-phosphoric acid) was added in each tube and incubated at 37 °C for 15 minutes. Absorbance was taken at 530 nm. Concentration was calculated using calibration curve made with different concentrations of glyceryl trioleate.

B.5 FAME profiling

For FAME profiling, dried biomass was derivatized into FAMEs for gas chromatography (GC) analysis using direct transesterification method given by (Lewis et al., 2000; Ríos et al., 2013), which is a less time consuming method for FAMEs conversion directly from microalgal biomass. In this method, extraction and transesterification of lipid takes place in one step followed by separation of FAMEs. GC–FID (Flame Ionization Detector) analysis was performed using Agilent GC 7890B with ZB-FFAP column. The calculation for each component was done with comparison to internal standard (IS) peak area i.e. Methyl nona-decanoate (C19:0) (Breuer et al., 2013). Components were identified by comparing retention time with a standard pattern given by 37 Component FAME Mix (Sigma-Aldrich).

B.6 Confocal microscopy using Nile red dye

Confocal microscopy was done for 21-days old culture. 1 mL algal suspension was washed 3-4 times with distilled water and volume was made up 1 mL with distilled water in 1 mL cell suspension 330 µL dimethyl sulfoxide (25% v/v DMSO) and 15 µL Nile red solution (0.1 mg/mL in acetone) was added. Micrographs were captured using confocal laser scanning microscope using 530 nm and 604 nm excitation and emission light filters respectively (Zhang et al., 2016) (Multi Photon Laser Scanning Microscope FV1200MPE, IX83 Model, Olympus). Fluorescence intensity of lipid droplets was calculated using ImageJ software (version 2.1, U.S. National Institutes of Health, Bethesda, Maryland, USA), to corroborate the neutral lipid data.

B.7 Scanning electron microscopy

Scanning electron microscopy was performed for 21-days old cultures to visualize their morphology and cell structure. Lyophilized samples were spread on a carbon tape and pasted to the stub, and to increase the conductivity, the cells were coated with copper for 2 minutes. The micrograph was recorded by ZOEL Microscope, at 5kV and 5000x magnification with working distance of 4.5 mm Bhowmick et al., (1980).

B.8 Statistical analysis

All experiments were performed in 3 replicates and average values along with standard deviations are reported in this manuscript. One-way ANOVA analysis was done using Microsoft excel 2016 (Lamotte, 1976).

C. Results and discussion

C.1 Isolation and molecular characterization of algae species

Molecular identification of isolated species was done using 18S rRNA and ITS primers. The sequences obtained ranged from 680 to 921 base pairs (bps) (Table 4). For identification of genus and species name, nBlast was performed. The sequences were submitted to NCBI and Accession numbers were obtained. All seven isolated and purified strains (*Coelastrum proboscideum* (MZ317357*), Desmodesmus pseudocommunis* (MZ317347)*, Asteararcys quadricellulare* (MZ323982)*, Ettlia texensis* (MZ323986)*, Pediludiela daitoensis* (MZ326029)*, Pectinodesmus* sp. (MZ317353)*, Coelastrella* sp. (MZ323977) belong to freshwater green microalgae, Chlorophyceae related to seven different genera, ranging from 90-100% sequence similarity (Table 4).

Table 4: Taxonomic identification of isolated species with partial ITS and 18S rRNA gene sequence analysis

Isolation Site	Coordinates	Sequence Length	Similarity (%)	Closest Relative
Site A (Lal Bagh Indore)	12.9507° N 77.5848° E	692	99	*Coelastrum proboscideum*
Site A (Lal Bagh Indore)	12.9507° N 77.5848° E	761	99.6	*Coelastrella* sp.
Site B (Shitala mata waterfall)	22.4042° N 75.6099° E	941	97	*Desmodesmus pseudocommunis*
Site B (Shitala mata waterfall)	22.4042° N 75.6099° E	875	100	*Asterarcys quadricellulare*
Site B (Shitala mata waterfall)	22.4042° N 75.6099° E	680	89.8	*Pectinodesmus* sp.
Site C (Narmada River, Maheshwar)	22.1759° N 75.5872° E	940	99	*Ettlia texensis*
Site D (Kshipra River Ujjain)	23.1907° N 75.7642° E	922	99	*Pediludiela daitoensis*

The evolutionary relationship was assessed using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). This analysis included seven nucleotide fragments. Codon positions contained 1st+2nd+3rd+Noncoding positions. 500 bootstrap re-sampling was performed.

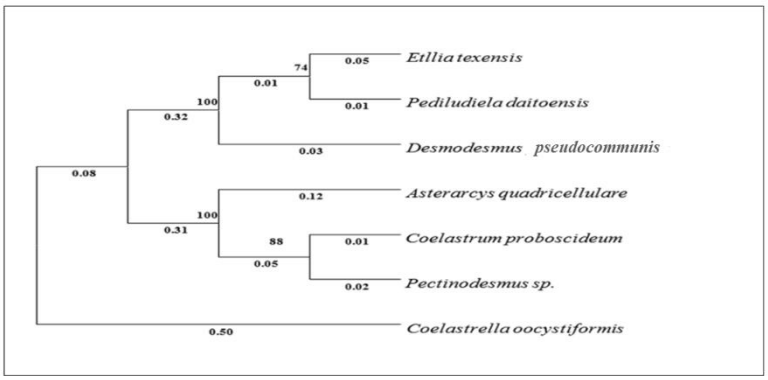


Figure 5: Phylogenetic tree showing the relationships amongst microalgal species sequences based on the 18S rRNA gene (Maximum Likelihood method and Tamura-Nei model) constructed using MEGA X

The tree has shown internal relationship amongst isolates. *E. texensis* and *P. daitoensis* are the closest and have the same ancestor as *D. pseudocommunis*. Similarly, *C. proboscideum, Pectinodesmus* sp. and *A. quadricellulare* also shared a common ancestor but they are more evolved from each other. First inter-node depicted one common ancestor for all the isolates, which later on multifurcated and 2401 positions were found in final dataset (Figure 5) (Kumar et al., 2018).

C.2 Growth rate and pigment production All the isolates have shown stationary growth on the 28th day (Figure 6). Isolates were harvested at a constant interval of 7 days till 28th day to study biochemical and growth profiles. The transition state of exponential-stationary phase was identified at 21st day, which also have shown all promising results. Biomass productivity (µg/mL/day), specific growth rate (per day) and division time (day) on 21st day is tabulated in Table 5, which is indicating highest biomass productivity of *A. quadricellulare* (51.3±2.0 µg/mL/day) i.e., 1.4 folds higher ($P<0.01$) than the lowest biomass producer *Pectinodesmus* sp. (36.9±0.2 µg/mL/day) (Table 5).

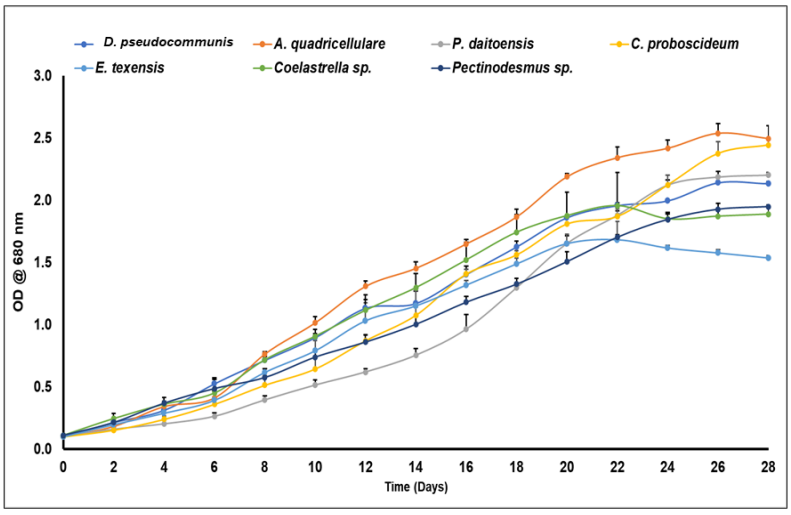


Figure 6: Graphical representation of O.D. values with respect to time for various microalgal species

In a study done by Yildirim et al. (2014), *E. texensis* yielded 436 mg/L biomass under phototrophic conditions, which is 1.3 folds less than current study as observed on 14th day (582.9 mg/L) (Yıldırım et al., 2014).

Table 5: Growth kinetics analysis of isolated microalgal species on 21st day

Species	Biomass Productivity (µg/mL/Day)	Specific Growth Rate (Per day)	Division Time (Days)
D. pseudocommunis	42.5±0.5***	0.1±0.0**	4.9±0.1**
A. quadricellulare	51.3±2.0**	0.2±0.0**	4.6±0.1
P. daitoensis	40.7±0.8**	0.1±0.0*	5.0±0.1**
C. proboscideum	40.5±1.4*	0.1±0.0**	5.0±0.0**
E. texensis	46.6±4.8*	0.2±0.0*	4.7±0.2ns
Pectinodesmus sp.	36.9±0.2	0.1±0.0	5.1±0.0**
Coelastrella sp.	47.7±1.8**	0.2±0.0**	4.7±0.1ns

Significant differences (one-way ANOVA) are marked as *(P<0.1), ** (P<0.01), *** (P<0.0001) and ns (not significant) with respect to lowest Biomass productivity, specific growth rate and division time

Chlorophyll accumulation was increased exponentially till the 21st day and on 28th day when cells were in late stationary phase (Figure 6), chlorophyll content was decreased for the studied species (Figure 7).

On 21st day, highest chlorophyll accumulation was observed for *A. quadricellulare* (33.1±0.6 µg/mL) which is 1.6 folds higher (P<0.0001) than *D. pseudocommunis* (21.8±0.8 µg/mL) (Figure 7).

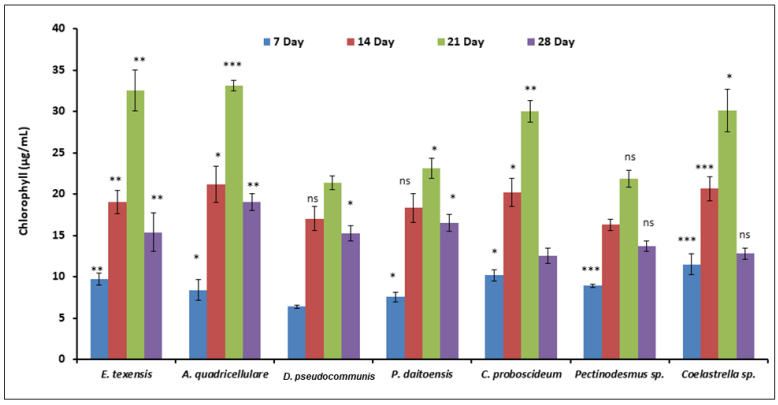


Figure 7: Chlorophyll content (µg/mL) of algal species with varying time. Significant difference (one-way ANOVA) is mark as ns (non-significant), *P <0.05, **P<0.01 and ***P<0.0001 as compared to lowest chlorophyll producing species

Chlorophyll is an indicator of photosynthetic activity and increasing chlorophyll indicates exponentially growing cells, high biomass productivity and fastest growth indicates high photosynthetic activity in *A. quadricellulare*, hence highest chlorophyll content at the peak of its growth.

C.3 Biochemical profiling of native algal strains

For a better understanding of carbon partitioning in isolates lipids, carbohydrates, and protein were analyzed in terms of µg/mg dry cell weight, which is graphically represented in Figure 8. *P. daitoensis* has shown highest protein accumulation (205.8±2.6 µg/mg dry cell weight or 20.6%) which is 2.1 folds higher ($p<0.0001$ significantly) than the lowest one i.e., *E. texensis* (98.6±3.3 µg/mg dry cell weight or 9.9%) on 21st day. In a study done in 2013 by Salim et al. *E. texensis* has accumulated 29.0±1.4 µg/mg protein which is 3folds less than the present study (Salim et al., 2013). During the growth cycle, all isolates have shown increase in protein accumulation up to 21st day which is the transitioning point of exponential phase to stationary phase or late exponential phase (Figure 6), cells are in highly active state currently hence this has become the peak point of high accumulation whereas in stationary phase cells start to enter senescence phase hence protein is decreased on later day. A similar study by Zhu et al., has also presented the findings that highest protein accumulation occur during exponential phase (Zhu et al., 1997). The present study has first time reported biochemical profiles of *P. daitoensis*. Protein accumulation was found to be ranging from 98.6-205.8 µg/mg dry cell weight on 21st day in these species (Figure 8). Protein synthesis can be very well linked with the cell growth and active division (Myers, 1980). The enzymes as well as the metabolites important for active growth during the exponential phase need high protein precursors. Storage energy compounds like lipids and carbohydrates also require enzymes, which are synthesized using protein precursors.

Coelastrella sp. was found to produce highest carbohydrates on 21st day (303.0±1.1 µg/mg dry cell weight or 30.03%), which is 3.6 folds higher than lowest carbohydrate accumulator, *D. pseudocommunis* (Figure 8). Carbohydrate accumulation was found to be increasing in all the species till 21st day (late log phase), whereas on 28th day (Figure 8) it decreased except for *D. pseudocommunis*. Similar trend was observed in the research done by Zhu et al., (1997) where they compared biochemical profiling of *Isochrysis galbana* at 2 different growth phases and observed that carbohydrate production was higher in stationary phase as compared to exponential phase. In a study performed on *Chlorella* species by Warburg, it was proven that during photo-autotrophic growth, limitation of nutrient or light can trigger carbohydrate accumulation (Warburg, 1928). In current study, cells were grown in a batch culture for 28 days, in this period cells have multiplied by up taking the nutrients from medium, with increase in cultivation days cell density has also increased which can be a switch to trigger carbohydrate synthesis. Carbohydrate is a storage component in microalgae, which can be used as raw material for various products, such as bioethanol, bio-butanol, etc. The overall carbohydrate production in these species ranged from 85.0-303.0 µg/mg (8.5-30.3%) cell dry weight on 21st day (Figure 8).

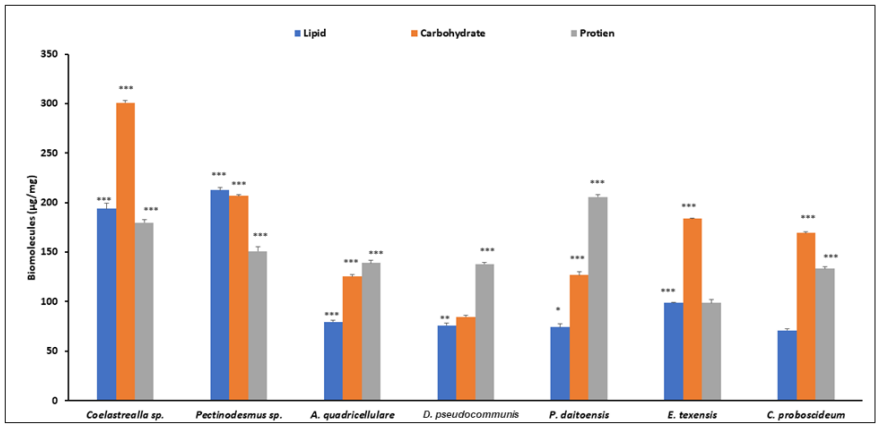


Figure 8: Biochemical Profiles (lipids, carbohydrates and proteins) of algal species in terms of µg/mg dry cell weight on 21st day of growth. Significant difference (one-way ANOVA) is marked as *P <0.05, **P<0.01 and ***P<0.0001 as compared to lowest produced

A comparative summary of similar studies (photoautotrophic growth) and reported biochemical composition has been presented in Table 6, there are fewer studies which focus on the entire biochemical profiling of these species. These results can help in screening of high carbohydrate producers, which can be optimized further for feedstock or for bioethanol production in future. Lipid production potential of microalgae is an important target in context to biodiesel applications. In the present study, highest total lipids were produced by *Pectinodesmus* sp. (212.7±2.5 µg/mg dry cell weight or 21.3%) on 21st day, whereas the least amount was produced by *C. proboscideum* (70.8±1.5 µg/mg dry cell weight or 7.1%), which is 3 folds less than *Pectinodesmus* sp. ($P<0.0001$) (Figure 8). The lipid content obtained by *Pectinodesmus* sp. is 29% higher, than a recent study done on *Pectinodesmus* sp. F13 under photoautotrophic condition in different medium and 7% higher than *Pectinodesmus pectinatus* under photoautotrophic cultivation (Gong and Huang, 2020; Rocha et al., 2017) (Table 6).

Table 6: Lipid, protein and carbohydrate data of reported species with respect to present study

Species	Lipid (µg/mg)	Carbohydrate (µg/mg)	Protein (µg/mg)	References
Pectinodesmus sp.	212.7±2.5	207.8±1.6	151.0±4.3	Present study
	180	180	70	(Rocha et al., 2017)
	150	NA	NA	(Gong & Huang, 2020)
Coelastrella sp.	193.9±5.2	303.0±1.1	179.7±3.1	Present study
	350	NA	NA	(Tonon et al., 2002)
	220	NA	NA	(Sakthi et al., 2020)
	NA	NA	180 mg/mL	(Li et al., 2018)
	250	NA	NA	(Luo et al., 2016)
A. quadricellulare	79.6±1.4	125.4±1.6	139.0±2.9	Present study
	258.0±0.0 (mg/L)	NA	NA	(Sangapillai & Marimuthu, 2019)
	300	NA	NA	(Chaudhary & Khattar, 2019)
	100	320	180	(Karthikeyan & Thirumarimurugan , 2017)
E. texensis	98.6±0.8	184.1±1.3	98.6±3.3	Present study
	180	NA	NA	(Seo et al., 2017)
Desmodesmus sp.	75.8±2.8	85.0±1.8	138.0±1.3	Present study
	200	NA	NA	(Singh et al., 2020)
	176	NA	NA	(Rugnini et al., 2018)
P. daitoensis	74.3±3.4	126.6±2.6	205.8±2.6	Present study
C. proboscideum	70.8±1.5	165.2±4.5	133.3±2.0	Present study

Total lipid accumulation was found in the range of 70.8-212.7 µg/mg (7.1%-21.3%) in current study (Figure 8). *A. quadricellulare* despite having high biomass accumulation and high growth rate, have shown lower lipid accumulation whereas *Pectinodesmus* sp. and *Coelastrella* sp. have shown high lipid accumulation. These species can be further utilized in either biodiesel production or as ω-3 or ω-6 fatty acid supplement, depending on their fatty acid profile which is discussed in section C.4, or can be used to induce biopolymer production, which shares the metabolic pathway with fatty acid synthesis (Figure 2; chapter 1).

It is very important to study the complete growth cycle to understand the lipid synthesis kinetics and selecting the right harvesting period for maximum productivity as well as the right fatty acid composition of

lipids produced by microalgae to converge its fate into bio-refinery approach.

C.4 Fatty acid methyl esters (FAME) profiling using gas chromatography-flame ionization detector (GC-FID)

Maximum total lipid accumulation was observed on the 21st day for all the studied strains. Presence of unsaturation and carbon chain length, inversely and directly proportional to fuel properties like cetane number (CN), heat of combustion (HG), cloud point (CP) and pour point (PP) etc. (Knothe, 2005). Highest TFA content was found in *Pectinodesmus* sp. (115.7 µg/mg or 11.6%) which is approximately 83% (Figure 9, Table 7) higher than *P. pectinatus* (Rocha et al., 2017) on 21st day. Further analysis of FAME profiling of *Pectinodesmus* sp. has shown that it has high SFA and MUFA content which facilitate high value biodiesel properties (Figure 9).

FAME profiling at each growth phase also supported in estimating the ratio of saturated and unsaturated fatty acids which indicated that at 7th day *Pectinodesmus* sp. has high PUFA % which is desirable for omega fatty acids production whereas on 14th, 21st and 28th day FAME profiles indicating towards biodiesel preference (Figure 9).

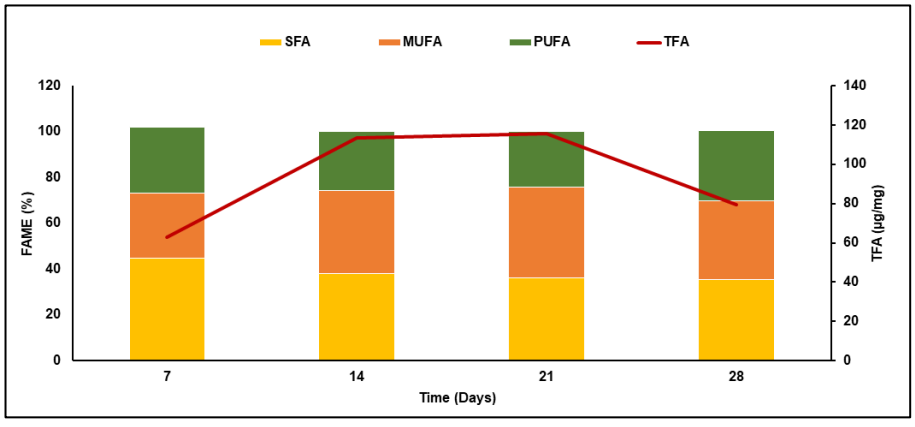


Figure 9: Comparative analysis of percent Saturated fatty acids (SFA), Mono-unsaturated fatty acid (MUFA), Poly unsaturated fatty acids (PUFA) and Total fatty acid (TFA) accumulation in high lipid accumulating species, *Pectinodesmus* sp. with varying time

High amount of saturated fatty acid and lower amount of unsaturated fatty acids is an indicator of good quality biodiesel. Degree of

unsaturation not only affects a number of properties which decide the quality of biodiesel but also increases the emission of hydrocarbons and nitrous oxides (Benjumea et al., 2011). Carbon partitioning differs at each growth phase due to variable environmental as well as nutritional factors. Therefore, assessing the FAME profile at each phase becomes imperative. Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (18:1), linoleic acid (LA) (C18:2) and α-linolenic acid (C18:3) were found to be the most prevalent fatty acids for all the species which are also important in determining fuel properties. *D. pseudocommunis* have shown good amount of linoleic acid 30.83%, 28.28% and 26.61% on 14th, 21st and 28th day. Linoleic acid is a precursor of ω-6 fatty acid pathway which produces arachidonic acid (C20:4) which is a high value fatty acid.

Table 7: Total fatty acid (TFA) content in microalgal species (µg/mg) at different interval of times

Species	7th Day	14th Day	21st Day	28th Day
A. quadricellulare	38.5	47.5	50.0	47.9
D. pseudocommunis	25.3	41.7	74.8	34.3
P. daitoensis	33.3	42.1	46.5	41.8
E. texensis	31.7	33.4	51.9	16.1
C. proboscideum	38.3	46.5	52.3	51.5
Coelastrella sp.	62.4	75.8	109.5	42.6
Pectinodesmus sp.	63.0	113.3	115.7	79.5

Whereas *P. daitoensis* and *Pectinodesmus* sp. will be more suitable for biodiesel production (Table 7). With time, FAME % of individual fatty acid is found to be changing, which can be due to change in nutrient availability with varying time period. Saturated fatty acid is most likely to be synthesized during stress or in presence of reactive oxygen species (ROS) (Anand et al., 2019). Whereas unsaturated fatty acids are more likely to be synthesized when cells are not under stress and have healthy environment for growth (Udayan et al., 2018). There are many factors

which affect FAME profiling including genetic makeup and metabolic pathways. A similar kind of study was done by Tonon et al., where they studied the FAME profiling of four microalgae at two different time intervals: exponential and stationary phase. In three out of four microalgae i.e. *Nannochloropsis oculata, Phaeodactylum tricornutum* and *Thaslassiosira pseudonana*, PUFA production was higher in exponential phase and saturated fatty acids in stationary phase, whereas in fourth microalgae, *Pavlova lutheri* the trend was opposite (Tonon et al., 2002). This may be due to the expression of varying genes at different levels of growth in one species, which is unique for each species. It is important to explore FAME profile with varying time period and nutritional set up for different species as they have their own signatures.

C.5 Microscopic imaging

Lipids are stored inside the cells as lipid droplets, which were observed by staining with Nile red using confocal microscopy. On the 21st day, lipid droplets can be visualized inside cells as shown in (Figure 10.1), supporting lipid accumulation as revealed through biochemical analysis with colorimetric assay. When the cells reach the 21st day in same medium, nutrients start depleting which suppresses the growth and can trigger lipid storage in cells. Biomass productivity of *A. quadricellulare* is found to be 1.4 folds higher than *Pectinodesmus* sp., which further supports previous studies as biomass production is inversely proportional to lipid synthesis in microalgae. Fluorescence intensity of lipid droplets were found to be highest in *Pectinodesmus* sp. indicating highest neutral lipid or TAG accumulation inside the cells (Figure 10.2).

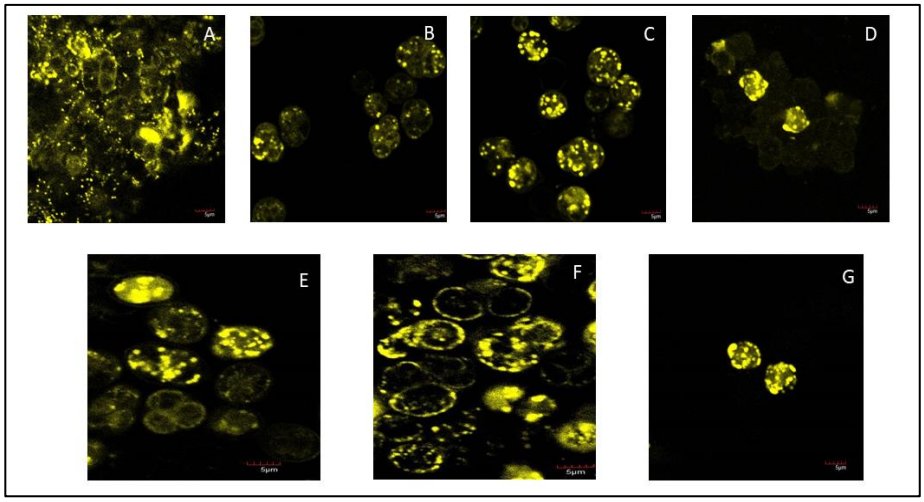


Figure 10.1: Confocal images showing lipid droplets on 21st day (highest lipid accumulation day) (A) *Pectinodesmus* sp. (B) *D. pseudocommunis* (C) *P. Daitoensis* (D) *A. quadricellulare* (E) *C. proboscideum* (F) *E. texensis* (G) *Coelastrella* sp.

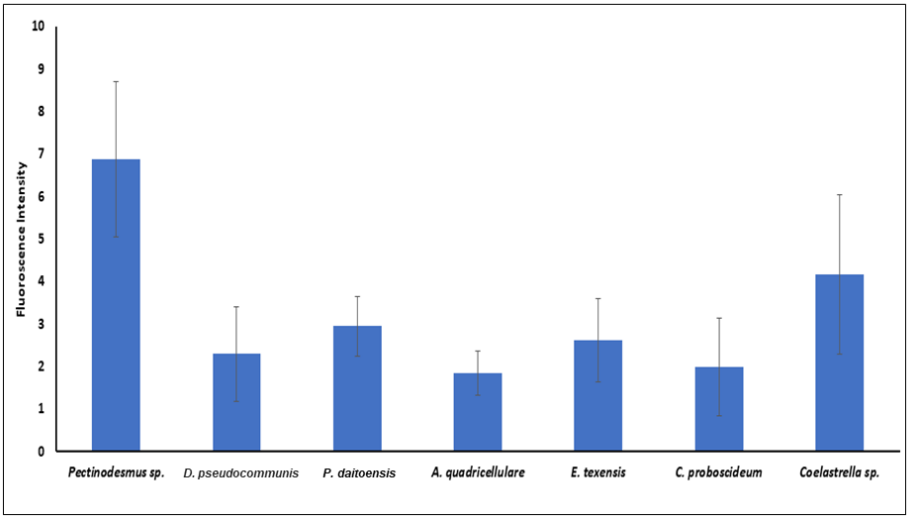


Figure 10.2: Bar Graph showing fluorescence intensity of various lipid droplets in species on 21st day

Scanning electron microscopy was done for visualization of morphological characteristics of each species. The morphology of *E. texensis* was observed as elongated ellipsoidal whereas *D. pseudocommunis, A. quadricellulare, P. daitoensis and C. proboscideum* is found to be spherical to round. *Pectinodesmus* sp. have shown long needle like ends and separated cells than the other species which are clustered together (Figure 11).

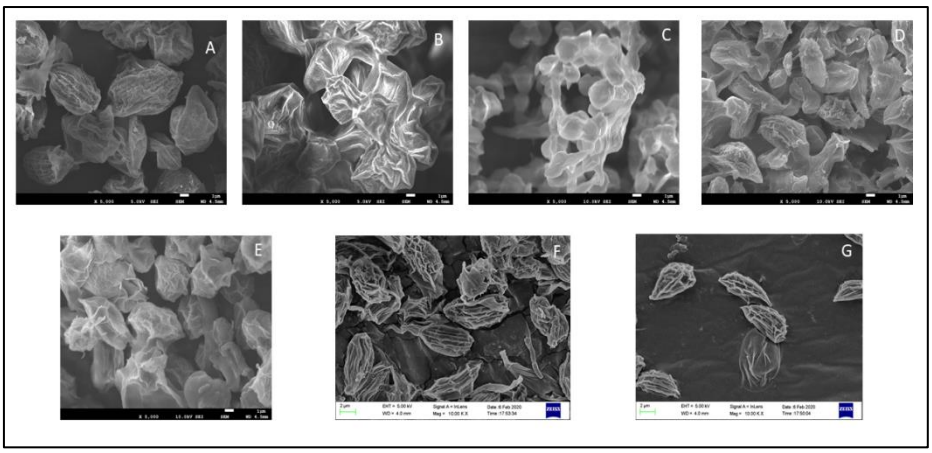


Figure 11: Scanning Electron microscopy (SEM) Images of (A) *C. proboscideum* (B) *E. texensis* (C) *D. pseudocommunis* (D) *P. daitoensis* (E) *A. quadricellulare* (F) *Coelastrella* sp. (G) *Pectinodesmus* sp.

D. Conclusions

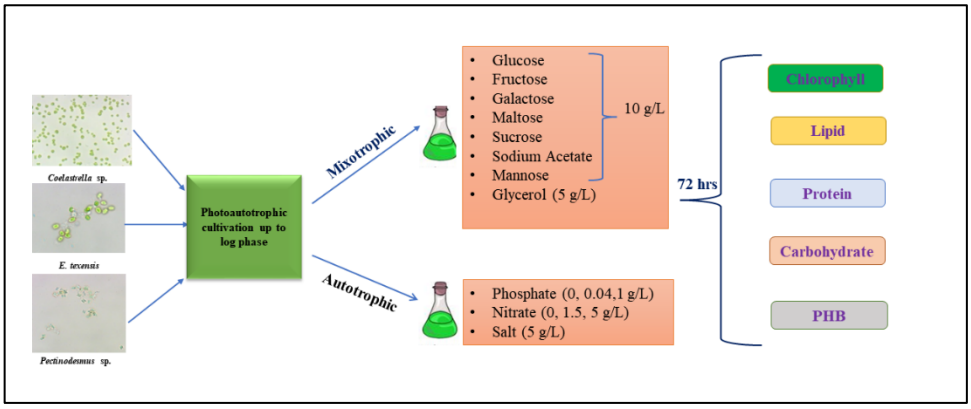
Aim of this study was to screen indigenous species as high lipids, carbohydrates and proteins producers and study their growth pattern and respective compositional analysis. Seven native microalgal species were isolated, identified and compared for total proteins, carbohydrates, lipids and pigment synthesis, biomass accumulation at different time intervals. Isolating microalgal strains from local habitats provide us with species that can adapt easily to specified native conditions and therefore are the most suitable for pilot-scale cultivation. The native species are easier to cultivate in the open environment which reduces the upstreaming cost. Four isolation sites were chosen by keeping their water quality in mind. Site A was a sewage treatment plant which helped us isolating robust species with ability to treat wastewater and produce high value products as a by-product. The rest of the 3 sites were freshwater reservoir, where 2 are the main rivers of Madhya Pradesh and one is stagnant water source. The study was performed at laboratory scale and various spectrometric and microscopic techniques have been used to yield the results and collect the supportive data. *A. quadricellulare* has shown highest biomass productivity (51.3±2.0 µg/mL/day) along with chlorophyll content of 33.1±0.6 µg/mL on 21st day ($P<0.01$).

Pectinodesmus sp. has shown highest total lipid and TFA accumulation, proving it as a good biodiesel candidate. *D. pseudocommunis* with good PUFA content can be an idea candidate for omega fatty acid production. Highest carbohydrates and proteins have been accumulated by *Coelastrella* sp. and *P. daitoensis*, respectively. Results of this study have shown a promising way to select algae for production of biomolecules like lipids, proteins and carbohydrates to target derivative products like, bioethanol, biodiesel, bioplastics etc. Some species like *P. daitoensis* and *D. pseudocommunis* have not yet been studied for effect of growth in photoautotrophic cultivation to the best of our knowledge. With increasing age of the culture, certain products are accumulated in the organism which differ from one-another. Studying the growth profiles of these species with respect to time helped in finding out potential candidates of biodiesel and bioethanol for bioenergy like high lipid and carbohydrate producer and alternative protein source which is ever demanding in the current scenario of increasing population. Identification of native potent producers of biomolecules is steppingstones to target up scaling and commercialization.

Based on the lipid profiling three highest lipid producers to be precise *Pectinodesmus* sp., *Coelastrella* sp., and *E. texensis* were selected for further screening of PHA accumulation under the supplementation of organic and inorganic nutrients such as phosphate, nitrate and salinity. Organic carbon plays a major role in triggering PHA accumulation pathways. A two-stage approach was used for screening the most potent factor initiating PHA accumulation in microalgal species. This approach enabled high productivity of PHA and various by-product in microalgae. Next chapter shows a two-stage cultivation approach for screening of various nutritional requirement for PHA accumulation in microalgal species. Comparison between mixotrophic and autotrophic mode of cultivation was assessed to understand the triggering of metabolic pathways to accumulated PHA. A detailed analysis will unravel various connections between growth and carbon partitioning in isolates to build a sustainable bio-refinery using native strains.

CHAPTER 3 (A)

Nutritional source amelioration for enhanced PHA synthesis in microalgal hosts *via* two-stage cultivation



A. Introduction

Plastic flew into our lives in the early 19th century and never left owing to its numerous desirable characteristics such as durability, lightness, high resistance to degradation, robustness and widespread domestic and industrial applications. Increasing population and global demand have fueled plastic industries which in turn have given us unimaginable plastic pollution dwelling in our ocean and landfills, which is considered a "poorly reversible pollutant" (MacLeod et al., 2021). Although better management and disposal techniques are making an impact in combating plastic pollution, superseding conventional plastic with naturally degrading polymers would also boost the odds of winning the battle against the "poorly reversible pollutant".

Polyhydroxybutyrate (PHB) is a short chain polymer often found in bacteria as a storage molecule (Marjadi & Dharaiya, 2018). It is not only a good alternative for our everyday fossil-based plastic but fields like biomedical industries and cosmetic industries can also be benefitted from this alternate form (Parvizifard and Karbasi, 2020; Pavelková et al., 2020; Shishatskaya et al., 2006). It was first reported by Lemoigne in 1926 (Lemoigne, 1926). Although PHA production was first reported in bacteria, microalgae and cyanobacteria have also been identified as potent producers of PHA. Cyanobacteria and microalgae both have been reported for PHA production. Still, under natural conditions (phototrophic cultivation) highest PHA accumulation has been reported to be <8% (Gopi, 2014). In contrast, when grown in heterotrophic or mixotrophic mode and with nutrient depletion, they can accumulate up to 69% of their dry cell weight (Bhati and Mallick, 2012, 2015). *Nostoc muscorum*, a nitrogen fixating cyanobacteria, have been reported for the production of 8.4% w/w production of PHB in controlled photoautotrophic conditions. In contrast, when grown heterotrophically, for 21 days in the presence of 0.4% Acetate, it accumulates the highest of 28% w/w PHB (Bhati and Mallick, 2012). *Synechocystis* sp., when grown under acetate supplementation, resulted in a 10% accumulation

of PHA (Hein et al., 1998). Microalgae have not been studied extensively for the production of PHB, but few recent studies have reported up to 30% accumulation of PHB under organic carbon supplementation and nutrient stress (García et al., 2021). Carbon di oxide (CO_2), as an inorganic carbon, can also induce PHB production in microalgae. In a study by Das et al., the author presented a 27% accumulation of PHB by *Chlorella pyrenoidosa* with CO_2 as an organic carbon source (Das et al., 2018). Apart from organic carbon sources, glycerol is also a great source for PHB accumulation. Glycerol not only contains carbon backbone, in its crude form, it also contains volatile fatty acids (VFAs) and other nutrients that can trigger PHB biosynthesis in organism. Being a by-product of the biodiesel industry, it can establish mass-energy balance forming a sustainable alliance between biodiesel and PHB industry (Kumar et al., 2021). Studies aimed towards comparing microalgal species for PHA producing capacity are scarce. Additionally, only a few have addressed the compositional profile of PHAs in context to their properties which is imperative for two purposes: firstly, to evaluate if the PHAs obtained from microalgae are in comparison to the existing polymers, and secondly, to provide an insight into the probable mechanisms that can be manipulated to enhance the synthesis of biopolymers in microalgae. Thus, the present study provides a species as well as nutrient-based comparison on the synthesis of PHAs of different microalgae, with an aim to verify their potential use towards replacing fossil fuel-based plastics. In this work different combinations of nitrate, phosphate and carbon (both organic and inorganic sources) were tested on three native microalgal species (Bhati and Mallick, 2015; Samadhiya et al., 2021).

In this study we employ two-stage cultivation for selected species to achieve maximum accumulation of PHB without harming biomass accumulation. Many studies before have suggested that due to high initial cell density, two-stage cultivation prevents contamination in heterotrophic growth (Aziz et al., 2020; Nagappan et al., 2019; Yen and Chang, 2013).

The present study puts forth a species-based comparison with three native microalgal strains and 13 different nutrient combinations with nitrate, phosphate, and carbon to reckon the superlative combination of strain and nutrients for PHA production as well as precursors of energy products.

B. Material and methods

B.1 Experimental conditions

Three microalgal strains, *Coelastrella* sp., *Ettlia texensis* and *Pectinodesmus* sp. were isolated from native region of Indore, and identification was done by NCIM, Pune (Samadhiya et al., 2021) and were cultivated in BG-11 medium (Stanier et al., 1971) at 27±5 ºC under 3000 lux illumination, 12:12 hrs photoperiod (Kaushik, 1987) and re-cultivated every 10^{th} day. For inducing PHA accumulation in these three strains, various nutrient stress, salinity and organic carbon supplementation were provided individually.

Glucose, fructose, sucrose, galactose, maltose, mannose, and sodium acetate were supplemented at 10 g/L concentrations denoted as G_{10}, F_{10}, $S_{10,}$ $Ga_{10,}$ M_{10}, Mn_{10} and $A_{10,}$ respectively. Glycerol, nitrogen, and sodium chloride (NaCl) were supplemented at 5 g/L concentrations denoted as Gly_5, N_5 and $NaCl_{5,}$ respectively. Nitrate and phosphate depleted medium were denoted as N_0 and $P_{0,}$ and supplementation of phosphate at 1 g/L concentration was denoted by P_1 .The codes are denoted as A) $P_{0.04}N_{1.5}$, B) $P_{0.04}N_0$, C) $P_{0.04}N_{5,}$ D) $P_0N_{1.5}$, E) $P_1N_{1.5}$, F) $P_{0.04}N_{1.5}G_{10}$, G) $P_{0.04}N_{1.5}Ga_{10}$, H) $P_{0.04}N_{1.5}A_{10}$, I) $P_{0.04}N_{1.5}Ma_{10}$, J) $P_{0.04}N_{1.5}S_{10}$, K) $P_{0.04}N_{1.5}F_{10}$, L) $P_{0.04}N_{1.5}Mn_{10}$, M) $P_{0.04}N_{1.5}Gly_5$, N) $P_{0.04}N_{1.5}NaCl_5$ for various combination in this study and used where required.

BG-11 without any organic carbon source or nutrient repletion/depletion was used as a control medium. All the culture flasks were inoculated with 0.5 initial cell density at 680 nm (OD_{680nm}) from 10-day old seed culture. The cultures were cultivated for 72 hrs. Growth was periodically

monitored by taking absorbance at 680 nm. Harvesting was done by centrifugation at 7500 rpm for 5 minutes, and dried biomass was obtained by lyophilization. Lyophilized biomass was used for biochemical composition and PHA analysis.

All the experiments were performed in triplicates and repeated at least twice.

B.2 Growth kinetics and pigment analysis

Cell growth was measured by monitoring absorbance at 680 nm right after inoculation and after 72 hrs using DR-6000 HACH spectrophotometer. Biomass production (μg/mL) was calculated using a linear graph between absorbance and dry biomass. Since the growth rate of cells is directly proportional to biomass produced, specific growth rate and division time were determined. Growth kinetics can be studied by various models available in the literature. In this study, we have used the models proposed by Guillard, (1973) and Pancha et al., (2015).

Chlorophyll analysis was performed by the method given by Arnon, (1949).

B.3 Biomass composition analysis

Biomass obtained at the end of the experiment was analyzed for three major cell components i.e., lipid, protein, and carbohydrate. These macromolecules were quantified using the spectrophotometric method. The Anthrone method was used to determine total carbohydrate concentration (Roe, 1955). For the determination of total protein, micro-biuret method was used (Chen and Vaidyanathan, 2013). For analysis of total lipid, Sulpho Phospho Vanillin (SPV) method was used (Mishra et al., 2014).

B.4 PHA estimation using Gas Chromatography

Polyhydroxy butyrate (PHB) and poly hydroxy valerate (PHV) are two classes under scl PHA biopolymer (Marjadi and Dharaiya, 2018). For estimating its concentration in cells, gas chromatographic (GC) analysis

was performed using method given by Oehmen et al. (2005). Known amount of lyophilized biomass was taken into a sample preparation vial and 2 mL of acidic methanol was added (3% H_2SO_4 in methanol). 50 mg/100 mL benzoic acid was added as internal standard. In the same tube 2 mL chloroform was also added. Samples were incubated at 100 ºC for 3.5 hrs. After cooling down the sample, 1 mL distilled water was added and mixed vigorously. After layer separation, the lower layer was used for GC analysis.

Agilent GC 7890B equipped with ZB-FFAP column was used for PHB analysis. The temperature of the injector and detector were kept at 180 ºC, and 260 ºC respectively, and the initial oven temperature was kept at 120 ºC. Helium was used as carrier gas. Peaks were identified using standards.

B.5 Statistical analysis

The PCA (Principal component analysis) figures were plotted using RStudio with R software. R programming was employed to compute the data and plot the graphs in PCA. The maximum variance was determined in the entire data set and according to the Kaiser Concept (Eigen vectors having more than 1) the principal components were selected along with the major contributing factors at different treatment conditions. Statistical significance between the data sets in the experimental responses was computed using one-way analysis of variance (ANOVA).

C. Results and discussion

C.1 Phototrophic vs mixotrophic growth under different nutrient supplementation

In the present study, microalgae were subjected to different concentrations of NaCl, phosphate and nitrate supplementation/depletion, and organic carbon individually to study the accumulation of PHB in these species. Experimental setup included a two-step cultivation methodology. Cells were grown in phototrophic

mode till log phase was achieved and then transferred into the nutrient sets. Maximum growth was observed in terms of chlorophyll accumulation (Figure 12) which was found to be maximum (26.1±1.0 µg/mL) in *Pectinodesmus* sp. under mixotrophic growth with galactose supplementation (10 g/L). This species yielded biomass of 2196.6±60.6 µg/mL which is 7 folds higher than the control (unmodified BG-11 media). Galactose is an epimer of glucose that is often found in milk in the form of lactose, a disaccharide which can take part in glycolysis by converting into glucose-1-phosphate (Cohn and Segal, 1973). This leads to the production of ATP, energy currency of cell, which help the cells pace the metabolism resulting in a higher growth rate (0.7±0.0 per day) and higher biomass productivity 627.4±21.1 µg/mL/day. *Coelastrella* sp. also accumulated the highest biomass (591.3±17.5 µg/mL) under galactose supplementation, whereas *E. texensis* chose glucose as its primary carbon source producing 290.9±5.7 µg/mL biomass.

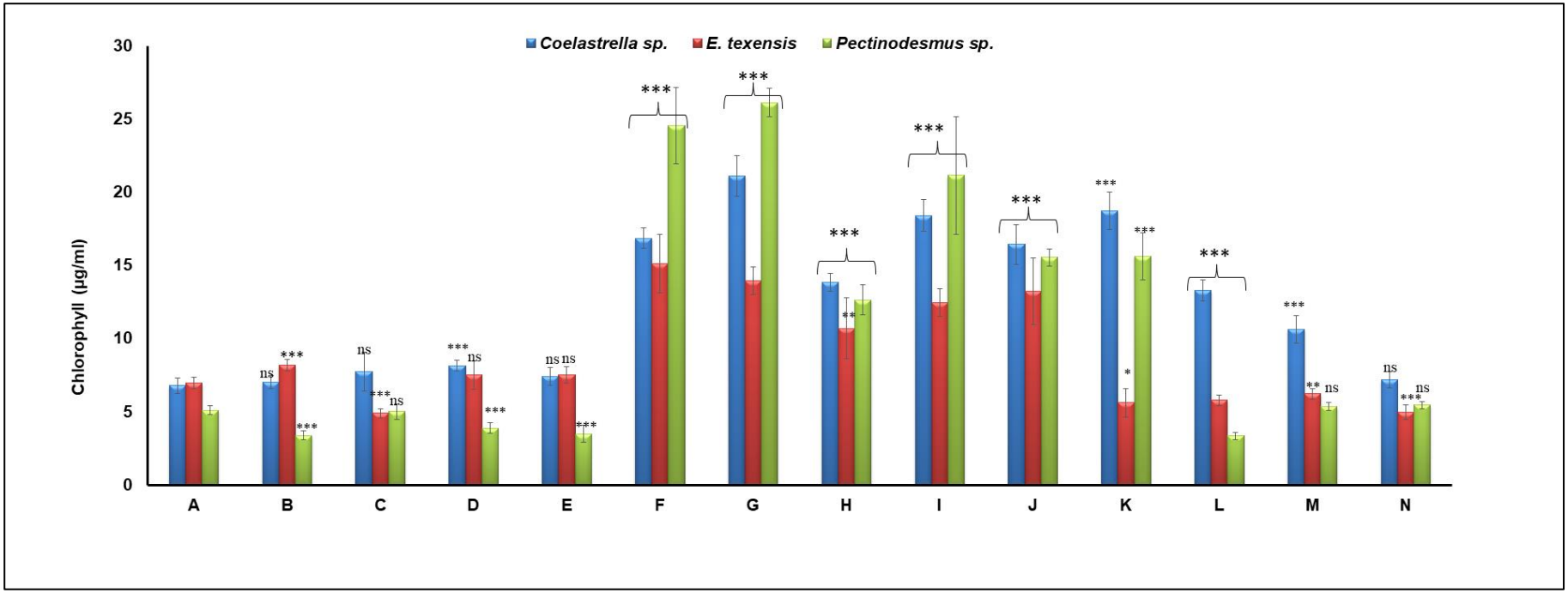


Figure 12: Chlorophyll accumulation (µg/mL) depicting cell growth at 72 hrs after treatment. Significance of data is computed, and p value is shown as ns= not significant, *= P≤0.05, **=P≤0.01, ***= P≤0.001

The codes are denoted as A) $P_{0.04}N_{1.5}$, B) $P_{0.04}N_0$, C) $P_{0.04}N_5$, D) $P_0N_{1.5}$, E) $P_1N_{1.5}$, F) $P_{0.04}N_{1.5}G_{10}$, G) $P_{0.04}N_{1.5}Ga_{10}$, H) $P_{0.04}N_{1.5}A_{10}$, I) $P_{0.04}N_{1.5}Ma_{10}$, J) $P_{0.04}N_{1.5}S_{10}$, K) $P_{0.04}N_{1.5}F_{10}$, L) $P_{0.04}N_{1.5}Mn_{10}$, M) $P_{0.04}N_{1.5}Gly_5$, N) $P_{0.04}N_{1.5}NaCl_5$

Nitrate and phosphate limitation in combination B and D, respectively, caused biomass loss in *Pectinodesmus* sp., which resulted in a significant decrease in chlorophyll pigment as compared with control (Figure 12).

C.2 Effect of salinity, nutrient starvation and mixotrophic growth on biomass composition

C.2.1 Carbohydrate accumulation

Microalgae can store various carbohydrates during their course of growth. Lower molecular weight carbohydrates are preferred over higher once like monosaccharides and linear chain carbohydrates. The genetic makeup of each algae can also influence the accumulation (Raven and Beardall, 2003). The highest carbohydrate accumulation in the study was obtained by *Coelastrella* sp. (257.1±29.7 µg/mg DCW) under fructose supplementation, which is significantly 2.5-folds higher than control (p value ≤0.001) and 5.5 folds higher than acetate (p value ≤0.001) supplementation (Table 8). Nitrate and phosphate starvation also increased carbohydrate synthesis. *E. texensis* and *Pectinodesmus* also accumulated 1.5 folds and 1.7 folds higher carbohydrates, then control under fructose and glucose supplementation respectively. *Coelastrella* has also shown increased carbohydrate accumulation under NaCl supplementation (Table 8). Carbohydrate accumulation under mixotrophic growth conditions severely decreased, which can be explained by presence of external organic source available for metabolic activities. The two pathways responsible for carbon assimilation in microalgae Embden Meyerhof Pathway (EMP) and the Pentose Phosphate Pathway (PPP) generate a high amount of energy enough for exserting positive impact on cell growth, thus limiting carbon accumulation as a storage compound (Perez-Garcia et al., 2011). Respiration and metabolism of carbohydrates is an integral part of microalgae but change in nutrient composition can cause carbon shift and channelization of carbon flux towards another anabolic pathway. Nutrient shifts like phosphate and nitrate repletion or depletion can

either trigger accumulation of carbohydrates in the form of monosaccharides or polysaccharide. There are several reports connecting lipid and protein enhancement using nitrate and phosphate manipulation (Dean et al., 2008; Ghosh et al., 2019). Carbon flux often depends on the type of nitrogen source present such as starch accumulation can be escalated if nitrate is present as a nitrogen source. Comprehension about the role of phosphorus is scarce. Additionally, intracellular phosphate concentration is imperative than medium phosphate concentration. Salts majorly inhibit RubisCO activity which in turn can escalate carbohydrate accumulation in some algal species (González-Fernández and Ballesteros, 2012).

Table 8: Carbohydrate and protein (µg/mg DCW) accumulation in three microalgal strains after 72 hrs treatment

	Carbohydrate			Protein		
	***Coelastrella* sp.**	***E. texensis***	***Pectinodesmus* sp.**	***Coelastrella* sp.**	***E. texensis***	***Pectinodesmus* sp.**
$P_{0.04}$ $N_{1.5}$	102.4±6.7	140.2±18.1	89.3±7.9	544.1±37.2	446.5±35.4	395.2±31.6
$P_{0.04}$ N_0	88.4±11.4*	151.2±20.5^{ns}	97.5±14.5^{ns}	279.8±26.0***	541.5±50.5***	385.5±25.9^{ns}
$P_{0.04}$ N_5	98.0±5.5^{ns}	152.2±14.8^{ns}	108.9±7.6***	460.0±18.5***	439.7±16.7^{ns}	455.8±24.4**
P_0 $N_{1.5}$	103.4±5.9^{ns}	147.4±17.3^{ns}	91.2±16.9^{ns}	239.1±29.0***	540.5±69.8**	397.7±17.4^{ns}
P_1 $N_{1.5}$	78.4±8.0***	151.4±10.9^{ns}	89.6±5.1^{ns}	729.1±46.3***	457.6±35.9^{ns}	441.8±20.0**
$P_{0.04}$ $N_{1.5}$ G_{10}	147.0±8.3***	215.4±19.9^{ns}	154.7±32.2***	433.8±37.9***	250.0±19.4***	358.1±47.2*
$P_{0.04}$ $N_{1.5}$ Ga_{10}	72.0±7.9***	110.4±13.4***	79.9±5.1**	443.0±44.5***	506.7±36.2*	331.8±33.8***
$P_{0.04}$ $N_{1.5}$ A_{10}	46.7±4.0***	106.3±8.4**	67.4±6.0***	330.2±18.9***	461.2±38.2^{ns}	284.4±39.1***
$P_{0.04}$ $N_{1.5}$ Ma_{10}	67.2±9.3***	87.7±8.4***	57.7±8.1***	572.3±51.6***	502.3±53.0*	391.9±23.5^{ns}
$P_{0.04}$ $N_{1.5}$ S_{10}	74.2±6.1***	85.2±11.3***	61.6±9.2***	685.1±55.1***	617.9±68.8***	443.5±50.9^{ns}
$P_{0.04}$ $N_{1.5}$ F_{10}	257.1±29.7***	216.0±16.8***	100.9±16.0*	455.8±35.3***	447.2±33.1^{ns}	477.9±50.7**
$P_{0.04}$ $N_{1.5}$ Mn_{10}	103.9±8.8^{ns}	121.8±11.2***	58.0±6.8***	338.0±33.2***	460.8±42.5^{ns}	422.0±58.1^{ns}
$P_{0.04}$ $N_{1.5}$ Gly_5	95.1±9.8^{ns}	84.3±11.0**	77.9±8.0*	367.3±37.5***	266.9±34.0***	393.2±54.1^{ns}
$P_{0.04}$ $N_{1.5}$ $NaCl_5$	139.9±14.0***	142.5±8.3***	71.7±10.6**	456.5±44.0***	470.0±42.0^{ns}	455.6±31.1**

Significance of data is computed, and p value is shown as ns= not significant, *= P≤0.05, **=P≤0.01, ***= P≤0.001

C.2.2 Protein accumulation

Protein is a major building block of cells and often contributes to 30-60% of the cell components. The nutrient shift can severely affect accumulation of both structural and functional protein inside cells (Procházková et al., 2014). In the present study highest protein accumulation was shown by *Coelastrella* sp. under high phosphate at 1 g/L supplementation (729.1±46.3 µg/mg DCW), which is 1.3-folds (p value ≤0.001) higher than control, followed by sucrose (685.1±55.1 µg/mg DCW) (p value ≤0.001) and maltose (572.3±51.6 µg/mg DCW) (p value ≤0.001) respectively. *E. texensis* accumulated 617.9±68.8 µg/mg DCW protein under sucrose, whereas *Pectinodesmus* sp. had the highest protein accumulation under high nitrate supplementation (Table 8). Nitrate has always played a significant role in protein accumulation due to its abundance in protein structure. Even so often, high nitrate is corroborated with high protein accumulation, and nitrate depletion triggers the lipid accumulation pathway. But phosphates also have a focal influence on protein accumulation. Phosphate depletion limit cell growth, whereas increased phosphate can upsurge cell growth, thus increasing functional protein concentration inside cells (Hemaiswarya et al., 2011). Rosche et al., proposed and proved that in higher plants, sucrose can activate protein accumulation and serve as raw material in protein production (Rosche et al., 2005).

C.2.3 Lipid accumulation

The carbon flux of oleaginous microalgae often channelizes towards lipid accumulation when even a slight change in media happens (Anand et al., 2019; Ghosh et al., 2019, 2021). Nitrate, phosphate, and salinity are vastly studied components for lipid enhancement in microalgae, whereas mixotrophy has also proven to be fruitful in its course. In the present study, NaCl has induced the highest lipid in *Coelastrella* sp. (228.9±22.2 µg/mg DCW) (p value ≤0.001) due to salinity stress. A 1.8-folds increase in lipid as compared to control followed by glucose (p value ≤0.001) supplementation and nitrate (p value ≤0.001) depletion in

Coelastrella (Table 9). On the contrary, *E. texensis* has mixotrophically accumulated 1.4-folds lipid accumulation under maltose (219.8±26.7 µg/mg DCW) (p value ≤0.001) followed by mannose (p value ≤0.001) and acetate (p value ≤0.01) (Table 9).

Lipid synthesis pathway is linked with carbohydrate metabolism. Glycolysis and TCA produce various raw product for lipid biosynthesis like malonyl co-A and acetyl co-A, which later fuel the lipid biosynthesis pathway (Ghosh et al., 2021). Nitrate and phosphate limitation as well as salinity stress, causes accumulation of reactive oxygen species (ROS), which causes disruption of cell growth and energy assembly, and cells divert their carbon flux towards lipid accumulation (Anand et al., 2019).

PCA analysis established the fact that protein concentration in biomass had a close correlation with enhanced biomass accumulation in *Coelastrella* sp., indicating that improved protein concentration could also be linked to high biomass (Figure 13 B). In *E. texensis,* biomass and lipid concentration were closely related (Figure 14 B), whereas in *Pectinodesmus* sp. a close correlation between biomass and carbohydrate concentration was observed (Figure 15 B). The biplots in Figure 13, 14 and 15 show dots and vectors which represent sample scores and variable loadings for principal component. The farther the vectors are from the principal component origin, the greater is the influence they exert on a principal component. Furthermore, these plots also explain the correlation between the variables: a small angle denotes a positive correlation, a large angle displays a negative correlation, and a 90° angle is suggestive of no correlation between two variables.

Furthermore, from the biplots it was evident that NaCl and the absence of nitrate and phosphate impacted lipid concentration to a greater extent in *Coelastrella* sp. In *E. texensis* presence of glucose and fructose with nitrate and phosphate supplementation heavily impacted the increase in carbohydrate concentration in the biomass. In *Pectinodesmus* sp.

supplementation of acetate as organic carbon highly influenced the increase in lipid accumulation.

Table 9: Polyhydroxybutyrate (PHB) and Lipid (µg/mg DCW) accumulation in three microalgal species after 72 hrs of treatment

	PHB			Lipid		
	***Coelastrella* sp.**	***E. texensis***	***Pectinodesmus* sp.**	***Coelastrella* sp.**	***E. texensis***	***Pectinodesmus* sp.**
$P_{0.04}$ $N_{1.5}$	1.3±0.1	1.4±0.1	1.1±0.1	126.1±14.7	159.3±22.2	149.7±16.5
$P_{0.04}$ N_0	0.9±0.1**	0.8±0.1**	2.1±0.2***	206.5±14.9***	133.7±24.5*	104.0±22.2***
$P_{0.04}$ N_5	0.6±0.1***	1.3±0.1^{ns}	1.8±0.0***	159.0±10.3***	120.2±30.6**	112.4±12.9***
P_0 $N_{1.5}$	1.1±0.2^{ns}	1.1±0.3^{ns}	1.8±0.1***	182.1±15.4***	160.8±30.6^{ns}	110.4±22.3**
P_1 $N_{1.5}$	1.0±0.3**	1.2±0.1^{ns}	1.5±0.2*	196.5±18.3***	165.0±23.1^{ns}	95.9±13.0***
$P_{0.04}$ $N_{1.5}$ G_{10}	29.7±1.8***	3.7±0.2***	2.7±0.2***	211.4±19.1***	174.7±14.5^{ns}	129.6±23.0^{ns}
$P_{0.04}$ $N_{1.5}$ Ga_{10}	151.8±12.1***	135.5±11.7***	85.6±3.8***	135.6±26.3^{ns}	138.3±23.4^{ns}	108.0±19.1***
$P_{0.04}$ $N_{1.5}$ A_{10}	79.3±6.1***	18.2±2.2***	1.9±0.7^{ns}	154.5±15.0***	196.2±17.2**	105.6±14.7***
$P_{0.04}$ $N_{1.5}$ Ma_{10}	146.2±8.2***	11.9±1.9***	4.7±2.6*	121.1±17.8^{ns}	219.8±26.7***	99.3±13.0***
$P_{0.04}$ $N_{1.5}$ S_{10}	150.8±8.5***	134.6±8.0***	103.8±26.2***	133.3±23.6^{ns}	181.7±17.3^{ns}	105.5±13.9***
$P_{0.04}$ $N_{1.5}$ F_{10}	78.6±4.1***	116.8±6.1***	70.8±9.0***	157.7±34.3*	142.1±23.8^{ns}	99.3±14.5***
$P_{0.04}$ $N_{1.5}$ Mn_{10}	23.4±1.8***	84.5±14.0***	47.5±3.8***	153.2±20.1**	196.0±18.8**	144.3±22.3^{ns}
$P_{0.04}$ $N_{1.5}$ Gly_5	69.2±5.1***	41.8±2.6***	2.2±0.1***	183.9±29.7***	120.8±12.4***	110.5±17.1***
$P_{0.04}$ $N_{1.5}$ $NaCl_5$	1.0±0.3^{ns}	1.1±0.3^{ns}	0.7±0.1**	228.9±22.2***	148.3±43.0^{ns}	104.1±14.9***

Significance of data is computed, and p value is shown as ns= not significant, *= P≤0.05, **=P≤0.01, ***= P≤0.001

In a previously reported study, PCA was performed to evaluate the relationships and correlations between the chemical and biochemical compositions of five different microalgal and cyanobacterial species where lipid, protein and specific growth rate did not demonstrate good correlation with PC1 (PC1 expressed 47.9% of the overall variance) and protein and specific growth rate were close to PC2 but with negative coordinates (PC2 expressed 27.9% of the variance) (Perendeci et al., 2019). Yet in another study done on *C. vulgaris*, PCA has shown a strong

correlation between organic carbon supplementation in the form of glucose, growth rate, biomass production and lipid accumulation (Arora and Philippidis 2021).

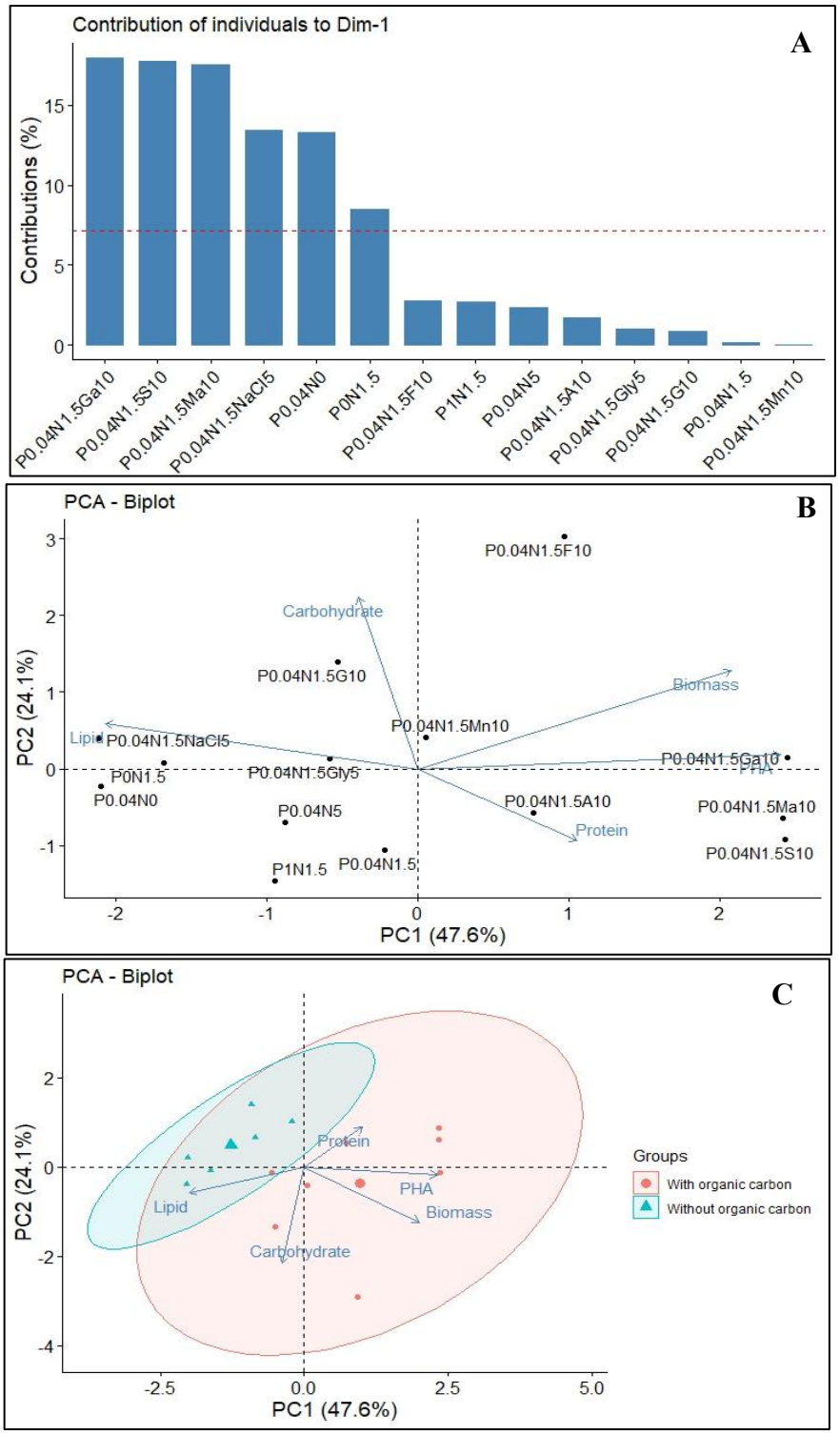


Figure 13: Bars in figure (13 A) are the contribution of individuals (nutrient combinations) to principal component 1. PCA biplots (B, C) show loadings of biomass components (variables or vectors) of *Coelastrella* sp. across different combinations of nutrients. PCA biplots (PC1 and 2) of (B) and (C) shows the magnitude of variance and contribution to variables where ordination axes (based on Eigen values) explaining the variance across the combinations of nutrients applied

C.3 Effect of nutrient, salinity, and organic carbon on Polyhydroxybutyrate accumulation

Polyhydroxybutyrate or PHB is polymer, representing the same qualities as conventional plastics (Maheshwari and Ahilandeswari, 2009). In the present work, we have investigated phototrophic and mixotrophic accumulation of PHB in 3 microalgal species. The highest PHB was accumulated in *Coelastrella* sp. which was 151.8 ±12.1 µg/mg DCW under galactose supplementation, followed by sucrose and maltose (Table 9), *E. texensis* and *Pectinodesmus* sp. also got the highest PHB under galactose and sucrose supplementation, respectively. We have achieved up to a 109-folds increase in PHB accumulation as compared to control (p value≤0.001). Galactose assimilation in microalgae could be enabled by Leloir pathway which enable the phosphorylation of galactose molecule making it available for metabolic activities in microorganisms (Chroumpi et al., 2022; Cohn and Segal, 1973). In comparison with phototrophic cultivation medium, mixotrophy triumphs in PHB accumulation. The highest accumulation in phototrophic mode is with nitrate stress in *Pectinodesmus* sp. (2.1±0.2 µg/mg DCW) (p value≤0.001).

In the last decade, various studies have consented to mixotrophy and heterotrophy for PHB accumulation in cyanobacteria as well as microalgae (Chaogang et al., 2010; García et al., 2021; Kamravamanesh et al., 2018; Panda and Mallick, 2007; Sharma and Mallick, 2005; Troschl et al., 2020). The studies have also suggested that nutrients present in the medium might also have a positive or negative impact depending on the concentration and duration of the exposure. For instance, in a study done by Samantaray and Mallick, lower concentration of NaCl were able to increase PHB accumulation, but high NaCl concentration had a negative impact due to increased flux around photosystem I and improved photophosphorylation led to a decrease in PHB accumulation (Samantaray and Mallick, 2015).

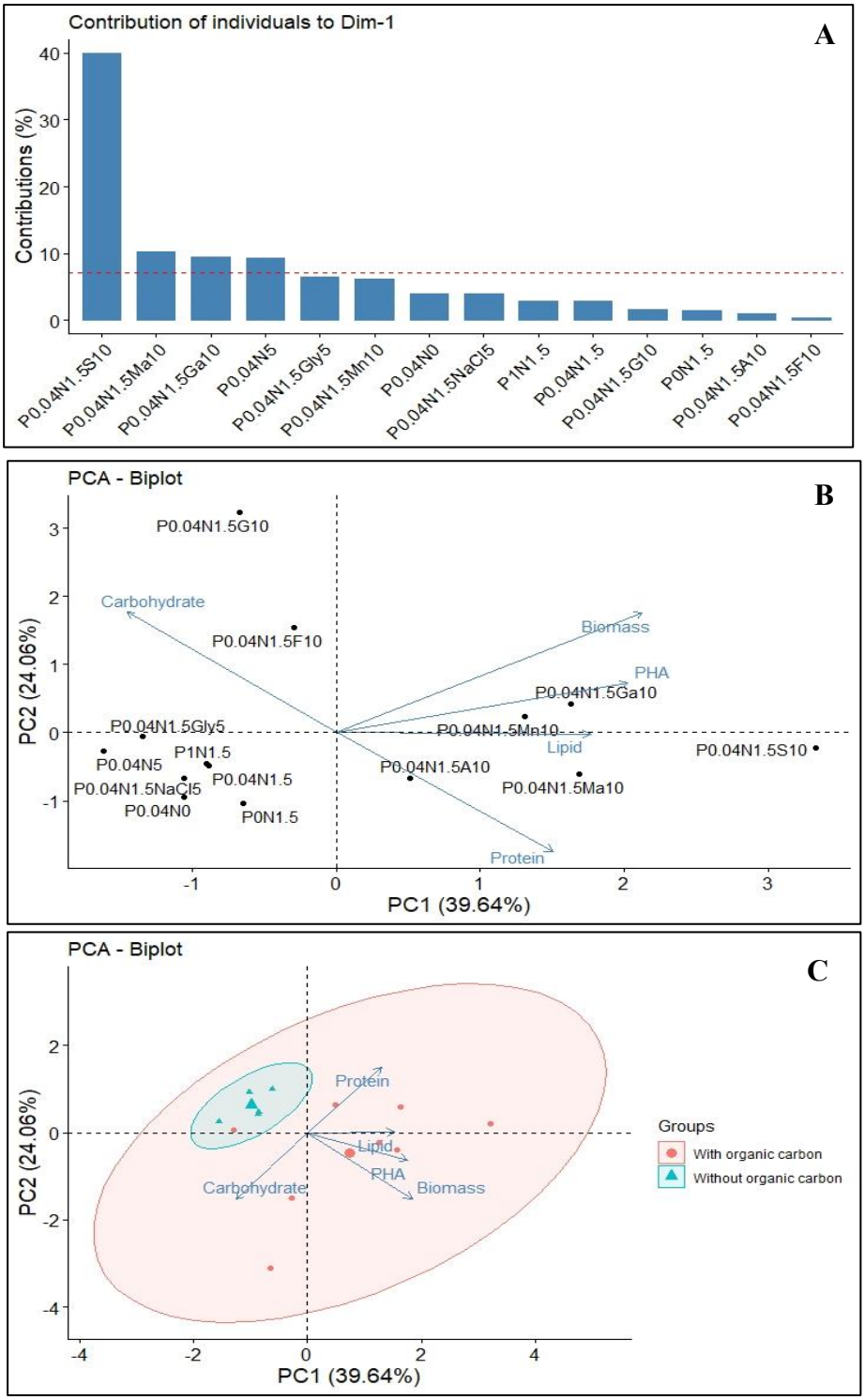


Figure 14: Bars in figure (14 A) are the contribution of individuals (nutrient combinations) to principal component 1. PCA biplots (B, C) show loadings of biomass components (variables or vectors) of *E. texensis*. across different combinations of nutrients. PCA biplots (PC1 and 2) of (B) and (C) shows the magnitude of variance and contribution to variables where ordination axes (based on Eigen values) explaining the variance across the combinations of nutrients applied

Salt stress is often linked with triggering lipid accumulation, and owing to the shared metabolic precursors, lipid and PHB accumulation pathways are often contradicting to each other (Khozin-Goldberg and Cohen, 2006). Nitrogen and phosphate limiting conditions have also been reported to incline towards the lipid biosynthesis pathway *via* production of Glutamine oxoglutarate aminotransferase (GOGAT) (Kilham et al., 2003).

PHB accumulation just like lipid accumulation is elicited by intensification of NADPH/NADH. Organic carbon like galactose, sucrose and maltose can enter glycolysis and TCA cycle producing much higher amount of NADPH and precursors like acetyl co-A, which is then reduced into acetoacetyl co-A in ß-oxidation pathway followed by a series of steps including three major enzymes, ß-ketothiolase, Acetoacetyl reductase and PHA synthase, resulting in high PHB accumulation. The mentioned carbon sources are present in natural products like molasses, dairy wastewater and grains present as a cheap source for PHB production. Sucrose and galactose both can be chosen as raw product for large scale production of PHB, galactose being the epimer of glucose and being a monosaccharide is more easily available to cells for uptake and channelizing (Cohn and Segal, 1973). In the present study, galactose yielded highest biomass productivity (627.4±21.1 µg/mL/day) as well as highest PHB productivity (90.7±0.2 µg/mL/day) in *Pectinodesmus* sp. and *Coelastrella* sp. respectively.

PCA analysis results presented in Figure 13 A, 14 A and 15 A explain the participation of treatment (different nutrient combinations) in explaining the variance (Table 10). Out of 13 combinations used, the maximum impact was contributed by $P_{0.04}N_{1.5}Ga_{10}$ in *Coelastrella* sp. and *Pectinodesmus* sp. influencing biomass components and PHA production (Figure 13 A and 15 A).

Table 10: Principal component analysis depicting the amount of variation each principal component (PC) captures from the data for all the three species

Dimension	Eigen value			Variance (%)			Cumulative Variance (%)		
	Coelastrella sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pettiness's* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.
Dimension 1	2.4	2.0	1.5	47.6	39.6	30.3	47.6	39.6	30.3
Dimension 2	1.2	1.2	1.4	24.1	24.1	28.6	71.7	63.7	58.9
Dimension 3	0.9	1.0	1.0	17.5	20.2	19.9	89.3	84.0	78.8
Dimension 4	0.4	0.7	0.9	8.5	13.3	17.6	97.8	97.3	96.4
Dimension 5	0.1	0.1	0.2	2.2	2.7	3.6	100	100	100

In *E. texensis*, $P_{0.04}N_{1.5}S_{10}$ contributed the maximum to PC1. PCA analysis confirmed that the biomass and PHA concentration under nutrient variation were closely related for all the three species and were influenced by presence of organic carbon concentration, nitrate (1.5 g/L of $NaNO_3$) and phosphate (0.04 g/L of K_2HPO_4). A previous study that similarly investigated the PCA ordination of organic carbon sources, reported an increase in biomass upon sucrose supplementation and displayed homogeneity of data compared to other carbon sources (Oliveira et al., 2021). Additionally, it was observed that in *Coelastrella* sp. and *Pectinodesmus* sp. PHA production was negatively correlated to lipid synthesis and closely related to protein synthesis. On the other hand, in *E. texensis* PHA production was closely related to lipid synthesis. Therefore, in *E. texensis* both lipid production and PHA production can proceed together under the presence of organic carbon, nitrate and phosphate. Various studies have similarly reported the effect of these nutrient concentrations on lipids, carbohydrate and protein content of the microalgal biomass. But these vary considerably depending on the microalgal species. Certain studies reported on *Chlorella* sp. (Kozłowska-Szerenos et al., 2004) and *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006) showed close association of lipid accumulation and phosphorous concentration in the

culture medium. A low phosphorous culture medium increased the lipid accumulation. The carbohydrate content *Dunaliella parva* showed significant dependency on nutrient supplementation and increased under high-phosphorous conditions (Said, 2009). Since the protein content of the biomass majorly comprises of intracellular pool of nitrogen, it is heavily impacted by the supplementation of nitrogen in the culture medium. In contrast, studies conducted on algal species like *Prorocentrum donghaiense* showed no difference to the protein content under various nutrient variations (Zhao et al., 2009), reinforcing the idea of differential impact to nutrient variation based on algae species. Cluster plots depicted by Figures 13 C, 14 C and 15 C clearly showed that all the biomass components and biomass production were highly impacted by organic carbon with maximum influence on biomass and PHA production.

Therefore, these results are suggestive of the fact that organic carbon sources present in natural products can make the biopolymer production process using microalgae both economical and sustainable and can subsequently replace conventional plastic.

This study, therefore, provides us with three new species which have not been previously explored for PHA production and additionally proves the suitability of organic carbon found in naturally available sources such as molasses, dairy wastewater, and poultry litter for high PHA productivity. Elaborated biomass profiling for chlorophyll, lipids, carbohydrate and proteins also paves a new passageway for microalgae-based biorefinery for a more sustainable approach.

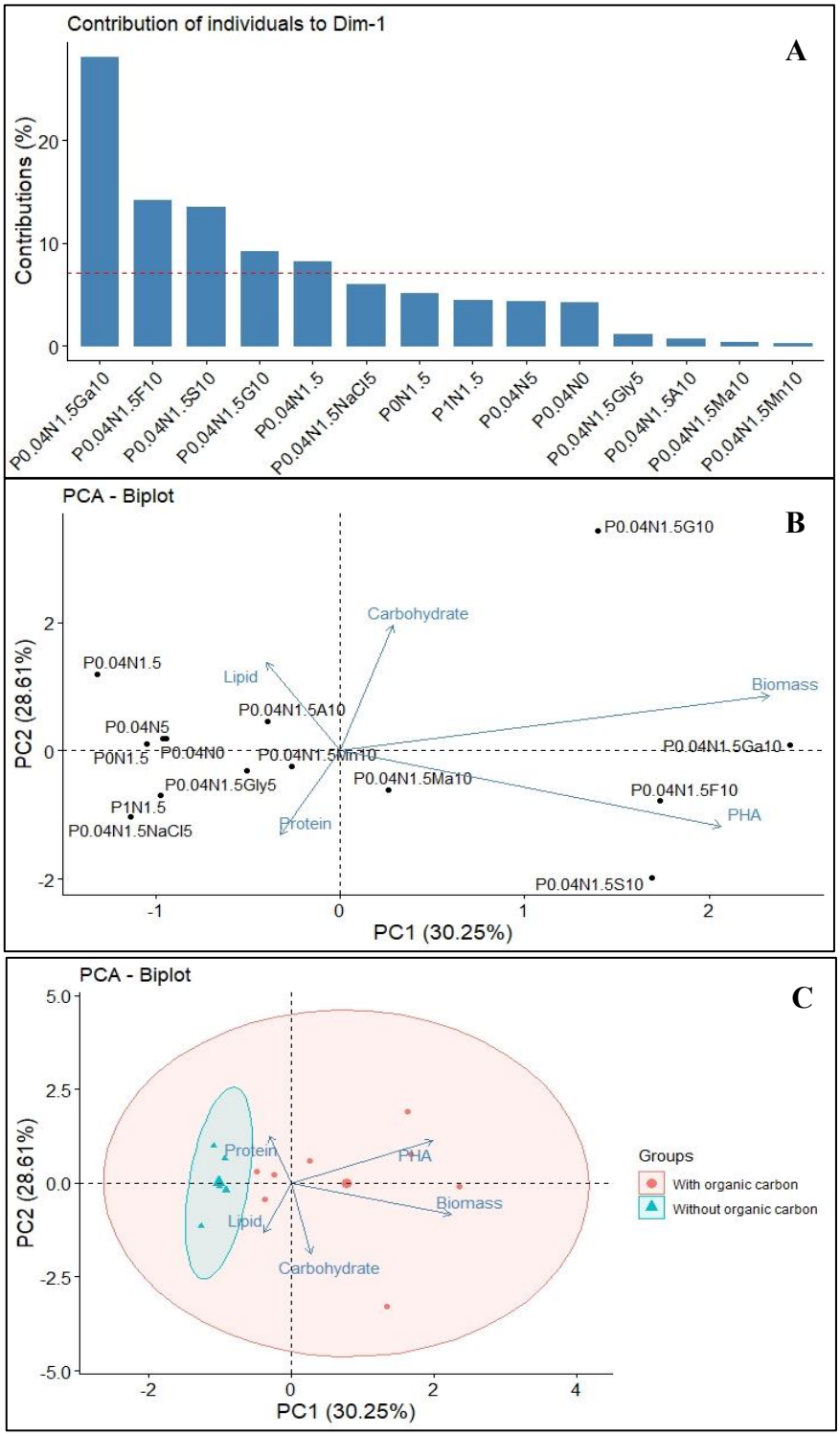


Figure 15: Bars in figure (15 A) are the contribution of individuals (nutrient combinations) to principal component 1. PCA biplots (B, C) show loadings of biomass components (variables or vectors) of *Pectinodesmus* sp. across different combinations of nutrients. PCA biplots (PC1 and 2) of (B) and (C) shows the magnitude of variance and contribution to variables where ordination axes (based on Eigen values) explaining the variance across the combinations of nutrients applied

D. Conclusions

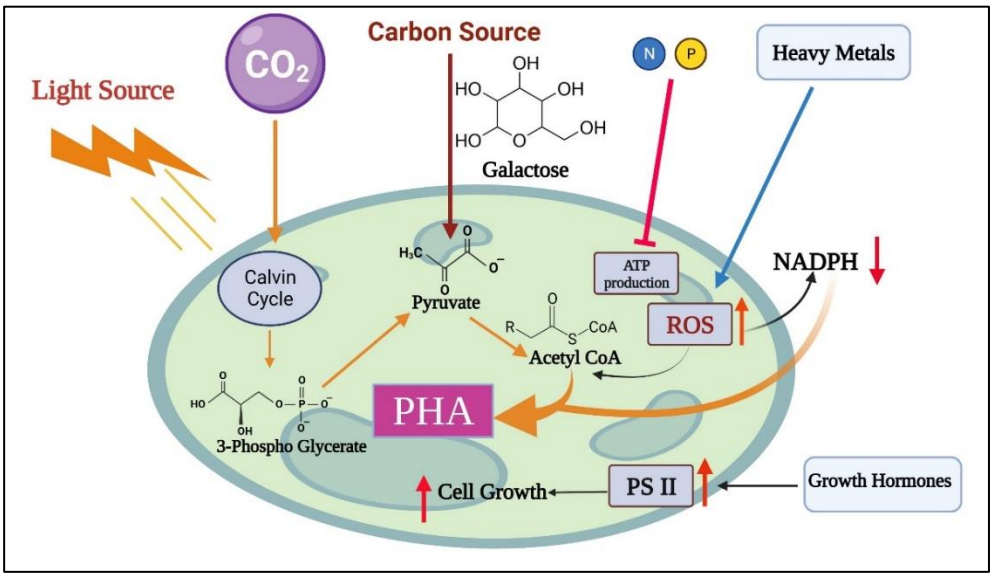
In the present study, the individual effects of nitrate ($NaNO_3$), phosphate (K_2HPO_4), salt (NaCl), and organic carbon sources, i.e., glucose, fructose, galactose, sucrose, maltose, mannose, acetate, and glycerol, were studied in three native microalgae (*Coelastrella* sp., *E. texensis* and *Pectinodesmus* sp.) for accumulation of PHB, biomass, chlorophyll, and biomass composition (lipid, protein, carbohydrate) *via* two-stage cultivation. Results are statistically analyzed using PCA and one-way Anova, which suggested the correlation between various combinations used for the study as well as different cell components. For example, negative correlation in lipid and PHB establishes the fact that these two pathways are not only correlated but are negatively influence each other (Figure 13 C and 15 C).

This study aided in analyzing the individual and combined effects of different components for PHB production in each of the three microalgal species without compromising biomass production and laid a foundation for microalgae-based production of PHB without any genetic manipulation. Galactose can be used as PHB inducer in the three isolates, without interfering with biomass to fetch. With this study we established the importance of presence of C-source in PHB accumulation in microalgae.

Further optimization for PHB accumulation in selected species could be helpful in gaining high PHB accumulation as well as understanding the role of various other factors important in PHB accumulation in microalgae. Next chapter includes the optimization process for PHA accumulation capacity of microalgal species under varied concentration of C-source, duration, initial cell density, heavy metals, hormones or conjoint effect of nitrate and phosphate with carbon source. These factors are chosen by keeping the wastewater composition in mind for future usage of wastewater as a cheap medium for microalgae.

CHAPTER 3 (B)

Manoeuvring PHA accumulation in microalgae by employing various stress factors



A. Introduction

Since the early 1940s, the slope for using petroleum-derived plastics has been rising exponentially. Plastics have become a key component in everyone's life, from waking up in the morning to going to sleep at night plastic has submerged everywhere (Anjum et al., 2016). Though the properties exhibited by these synthetic plastics become highly irreplaceable, one cannot neglect the potential harms and dangers they possess, including their non-degradable nature and unsustainable property of petroleum, from which these synthetic polymers are derived. As a matter of concern, the accumulation of these plastics in different environmental components sets the sovran threat, affecting the lives of many species present across the planet. The day is not far from when we humans, the creator of this issue, will have to suffer from the consequences caused by plastic to the extreme (Barnes et al., 2009). Therefore, it becomes quite important to focus more efficiently on an alternative for these synthetic plastics with no further delay. Towards this, PHAs (Polyhydroxyalkanoates), a type of bioplastics, have recently gained copious attention as an alternative for synthetic plastic. PHAs are the ester molecules that are known to accumulate intracellularly in several microbial species as a storage molecule in response to stress. These ester bonds are formed by linking one monomer's carboxylic group to its neighboring monomer's hydroxyl group. Some common examples of PHA molecules include PHB (polyhydroxybutyrates), PHV (polyhydroxyvalerates), P(3HB-co-3HV), i.e., poly(3-hydroxybutyrate-co-3-hydroxyvalerate), etc. (Tan et al., 2021). These molecules are of great interest to us as their properties align with conventional plastics by being tough, resilient, flexible, and robust, etc. They also possess a few added benefits, including biodegradability, biocompatibility, and environmental-friendly nature (Kai and Loh, 2014). Due to the possession of a diverse range of properties, these bioplastics stand as a promising candidate to be used widely in different fields, including the packaging industries, biofuels additives, medical industries such as

sutures, stents, implants, drug-delivery carriers, etc. (Keskin et al., 2017; Paulraj et al., 2018; Samrot et al., 2021). PHAs have been reported to accumulate in photosynthetic microbes naturally under certain conditions, especially the microalgal PHAs are rising as a trend in the era of bioplastics. Since microalgae possess extra advantage over other heterotrophic bacteria, this makes the microalgal cells a potent microbial to be studied further. For example, in addition to accumulating PHA molecules, they also produce proteins, lipids, and carbohydrates as their basic cellular biochemical composition in considerable amounts with a minimum requirement of nutrients (Samadhiya et al., 2022a). They have the potential to grow in wastewater, valorizing the waste to produce many other value-added products like pigments, vitamins, nutraceuticals, biofuels, etc., leading to the concept of biorefinery (Di Caprio et al., 2019). PHA producing pathways in microalgae can be maneuvered using various stress and supplementation factors to trigger the PHA accumulation process (Samadhiya et al., 2022b). In the present work, three native microalgae have been optimized and screened for PHA production using various stress including growth hormone, heavy metals and conjoint nutrient effect.

B. Material and methods

B.1 Strains and maintenance of cultures

Three native strains of microalgal species, viz., *Coelastrella* sp., *Ettlia texensis* and *Pectinodesmus* sp. were selected for the study. The cells were cultivated up to log phase and transferred into different medium to study the effect of various stress and supplementation on PHA as well as lipid, protein and carbohydrate accumulation.

B.2 Experimental setup

In this study, a two-stage cultivation method was applied to study the effect of various parameters on microalgal cells. Strains were cultivated up to log-phase and then transferred into the different medium at 0.5 initial OD_{680nm}. Thus, maintaining the initial OD_{680} of 0.5, with 10 g/L

galactose as carbon source with BG-11 medium under 27 ± 5 °C temperature and a light intensity of 3000 lux illumination, and photoperiod of 12:12 hrs for cultivation of microalgae were selected as the best PHA production conditions from previous study (Samadhiya et al., 2022a). The same conditions were followed throughout the study unless stated otherwise. The experiment was carried out in 250 mL conical flask with 100 mL cultures. All the experiments were performed in biological triplicates and repeated at least twice. Various effects studied for PHA accumulation are as follows:

A time course study was designed to study the effect of cultivation time on PHA accumulation. The cells were inoculated (OD_{680nm} 0.5) in BG-11 medium with 10 g/L galactose and cultivated for 21 days. These were harvested on every 7^{th} day and the biomass was investigated for PHA accumulation. BG-11 medium without galactose was used as a control. The following codes were used: $P_{0.04}N_{1.5}Ga_{10}$ and $P_{0.04}N_{1.5}$ for with and without galactose respectively, where N represents $NaNO_3$ (Sodium Nitrate) in 1.5 g/L concentration and P represents K_2HPO_4 (Di potassium hydrogen phosphate) in 0.04 g/L concentration. Harvesting time is represented as T (T_7, T_{14}, T_{21}).

Microalgal cells were grown in complete darkness for 72 hrs to understand the effect of absence of light with and without galactose as a carbon source. Codes were used as follows: $P_{0.04}N_{1.5}Ga_{10}$Dark, and $P_{0.04}N_{1.5}$Dark depicting presence and absence of galactose respectively in dark.

Initial cell density 1 and 2 at 680 nm was used. BG-11 medium without carbon source was used as a control. Cells were cultivated and harvested at 72 hrs. Codes used were as follows: $P_{0.04}N_{1.5}Ga_{10}CD_1$ and $P_{0.04}N_{1.5}Ga_{10}CD_2$ depicting initial cell density 1 and 2 in the presence of galactose. Similarly, in the absence of galactose $P_{0.04}N_{1.5}CD_1$ and $P_{0.04}N_{1.5}CD_2$ representing cell density.

Three different galactose concentrations were used in this study, 5 g/L, 20 g/L and 30 g/L (10g/L already used, chapter 3a). The effect of varied concentration on cell survivability and PHA accumulation was studied. Codes used were as follows: $P_{0.04}N_{1.5}Ga_5$, $P_{0.04}N_{1.5}Ga_{20}$ and $P_{0.04}N_{1.5}Ga_{30}$ for concentration of galactose 5 g/L, 20 g/L and 30 g/L respectively.

For this study five different combinations of nitrate, phosphate and galactose were used in three different concentrations of nitrate and phosphate (0, 1.5, 5 g/L nitrate & 0, 0.04, 1 g/L for phosphate). Codes were used as follows: $P_0N_0Ga_{10}$, $P_{0.04}N_0Ga_{10}$, $P_0N_{1.5}Ga_{10}$, $P_1N_{1.5}Ga_{10}$, and $P_{0.04}N_5Ga_{10}$.

IAA and GAA were used in 0.05 g/L concentration whereas Kin was used in 0.01 g/L concentration. Codes were used as follows: $P_{0.04}N_5Ga_{10}IAA_{0.05}$, $P_{0.04}N_5Ga_{10}GAA_{0.05}$, and $P_{0.04}N_5Ga_{10}Kin_{0.01}$.

Five heavy metals; copper (Cu), chromium (Cr), silver (Ag), cobalt (Co), and iron (Fe) were used in 0.005 g/L concentration to see the impact on PHA accumulation in microalgae. The following codes were used for the study: $P_{0.04}N_5Ga_{10}Cu_{0.005}$, $P_{0.04}N_5Ga_{10}Cr_{0.005}$, $P_{0.04}N_5Ga_{10}Co_{0.005}$, $P_{0.04}N_5Ga_{10}Ag_{0.005}$, and $P_{0.04}N_5Ga_{10}Fe_{0.005}$.

B.3 Growth kinetics and pigment analysis

Cell density measurement was performed by measuring the OD at 680 nm wavelength, using the DR-6000 HACH spectrophotometer. Growth kinetics analysis was done using models given by Guillard (1973) and Pancha et al., (2015).

Chlorophyll analysis was done using a method given by Arnon (1949).

B.4 Biochemical compositional analysis

After harvesting, cells were washed twice with distilled water and dried by using lyophilizer. Biochemical profiling was done from the obtained dry algal biomass sample for three key cell macromolecular composition i.e., lipid, protein, and carbohydrate via spectrophotometric analysis method. For determining total carbohydrate concentration, anthrone

method was used, using glucose as a standard (Roe, 1955). Micro-biuret method was used to determine the total protein concentration of cells by using bovine serum albumin (BSA) as a standard (Chen and Vaidyanathan, 2013). Sulpho-phospho vanillin (SPV) method was used for determining total lipid concentration, using glyceryl trioleate as a standard (Mishra et al., 2014).

B.5 PHA estimation using gas chromatography

To estimate the concentration of PHB (Polyhydroxy butyrate) and PHV (polyhydroxy valerate), the two commonly known classes of short chain length PHA molecules in microalgal cells, gas chromatographic (GC) analysis was used following the method published by Oehmen and co-workers (Oehmen et al., 2005).

B.6 Statistical analysis

Principal Component analysis or PCA is a tool to analyze multivariate data. Among the experiments performed in this chapter, NP variation and heavy metal supplementation have given positive results in terms of PHA accumulation. Hence only the variables and parameters used in these two experiments were used for PCA. PCA was performed using R studio version 4.3.0.

B.7 Extraction and characterization of biopolymer

After the optimization, the best optimized conditions were selected to grow the selected microalgal species and cultivated biomass for polymer extraction. The biomass was harvested and lyophilized. Lyophilized biomass was used for biopolymer extraction using solvent extraction (Tamang et al., 2019). After extraction, the polymer was subjected to mass-spectrometry using gas chromatography equipped with EI- mass spectrometer (Perkin-Elmer Clarus GC680-SQ8T MS). Thermal properties of the polymer were analyzed using thermogravimetric analysis (TGA). Molecular weight and polydispersity index were investigated using Gel Permeable chromatography (GPC) equipped with RI detector. Sample was analyzed at sophisticated instrumentation

center for applied research and testing. The polymer was dissolved in tetrahydrofuran (THF) and used for analysis. For surface analysis scanning electron microscopy (SEM) was performed. Films were made using a 1 % solution of polymer in chloroform (W/V) and casted onto a glass plate and further dried in oven at 50°C for 3 hrs.

C. Results and discussion

C.1 Time course study on microalgal species

Cells were grown under galactose supplementation (mixotrophic) and without galactose supplementation (autotrophic) for 21 days. Longer cultivation duration allows the cells to metabolize the nutrients better and accumulate more storage compounds including PHA, lipid, and carbohydrates.

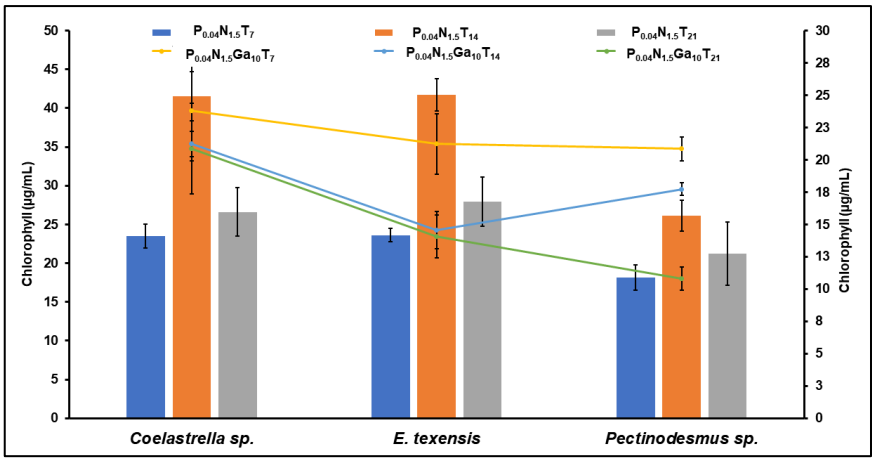


Figure 16: Chlorophyll accumulation (µg/mL) representing photosynthesis activity for microalgal species cultivated for 21 days by harvesting at every 7th day

Comparison of the cultures shows that the microalgal growth in mixotrophic conditions was higher than autotrophic growth at every time point (7th, 14th and 21st day) Figure 16. The highest chlorophyll accumulation during the cultivation was under autotrophic conditions in *E. texensis* on 14th day (41.7±2.1 µg/mL) whereas under mixotrophic condition, highest chlorophyll was under *Coelastrella* sp. on 7th day (23.8±1.5 µg/mL). Under autotrophic conditions cells have been seen to produce increase in chlorophyll accumulation till 14th day and a decline 21st day in all species, whereas in mixotrophic cultivation there was a

continuous decline in chlorophyll accumulation was observed in all the selected species (Figure 16). A similar trend of chlorophyll accumulation has been seen in other reports where mixotrophy and autotrophy has been compared. Chlorophyll production was halted in many mixotrophic cultures because the cells readily uptake available organic carbon in the medium rather than performing photosynthesis hence chlorophyll is not increased despite of higher growth rate (Cheirsilp and Torpee 2012; Adesanya et al., 2014; Mohammad Mirzaie et al., 2016).

Table 11: Cell kinetics of microalgal cells cultivated for 21 days by harvesting at every 7th day

Species	Days	Biomass Productivity (µg/mL/day)		Specific Growth Rate (Per day)		Division Time (Days)	
		$P_{0.04}$ $N_{1.5}$	$P_{0.04}N_{1.5}$ Ga_{10}	$P_{0.04}$ $N_{1.5}$	$P_{0.04}N_{1.5}Ga_{10}$	$P_{0.04}$ $N_{1.5}$	$P_{0.04}N_{1.5}$ Ga_{10}
***Coelastrella* sp.**	**7**	44.7±1.7	220.1±4.6	0.2±0.0	0.3±0.0	4.4±0.1	2.0±0.0
	14	55.7±2.0	131.5±3.2	0.1±0.0	0.2±0.0	5.4±0.1	7.1±0.1
	21	49.0±2.0	107.5±4.4	0.1±0.0	0.1±0.0	7.1±0.1	5.3±0.1
E. texensis	**7**	54.1±3.6	179.2±10.4	0.2±0.0	0.3±0.0	3.8±0.1	2.1±0.1
	14	50.0±0.3	146.8±12.4	0.1±0.0	0.2±0.0	5.5±0.0	3.6±0.1
	21	55.0±1.5	88.3±3.1	0.1±0.0	0.1±0.0	6.6±0.1	5.5±0.1
***Pectinodesmus* sp.**	**7**	51.4±3.1	199.5±11.8	0.2±0.0	0.4±0.0	3.3±0.1	1.8±0.0
	14	38.7±2.0	141.0±2.4	0.1±0.0	0.2±0.0	5.4±0.1	3.3±0.0
	21	38.8±1.4	95.4±1.8	0.1±0.0	0.1±0.0	6.8±0.1	4.9±0.0

Although the autotrophic mode had more chlorophyll accumulation, higher growth rate was observed in mixotrophic mode of cultivation (Table 11). Biomass productivity refers to the rate at which microalgae accumulate biomass over a certain period, highest biomass productivity under mixotrophic cultures was observed in *Coelastrella* sp. at 7th day (220.1±4.6 µg/mL/day), whereas on 14th day under autotrophic cultivation (55.7±2.0 µg/mL/day) Table 11. which was 4 folds less than mixotrophic mode. Additional organic carbon sources allow the microalgae to access more stored nutrients and energy sources during

dark phase growth leading to an increase in growth rate. When microalgae are grown under autotrophic conditions, they can only use carbon dioxide (CO_2) and other inorganic nutrients in the culture medium, which can limit their growth rate, in contrast, when microalgae are grown under mixotrophic conditions, they can also metabolize organic carbon sources through glycolysis and the Krebs cycle, leading to increased respiration rate and enhances growth rate of microalgae, which can lead to faster growth rates. Additionally, microalgae can also use the organic carbon source from media, reducing the need for carbon dioxide fixation through Calvin cycle (Zhan et al., 2017). Biomolecules were also studied and revealed that while protein accumulation was higher in autotrophic mode, lipid carbohydrate and PHA accumulation was higher in mixotrophic mode with some exception (Table 12). Highest carbohydrate accumulation was found under the *Pectinodesmus* sp. at 14th day under mixotrophic mode (285.0±10.9 µg/mg) which was 1.3 folds higher than highest accumulation under autotrophic mode at 14th day in *E. texensis*. In a study carried out on *Chlorella* sp. it was observed that diphosphate-fructose-6 -phosphate-1-phosphotransferase (PFP) was upregulated in the presence of carbon source which increases the gluconeogenesis thus increasing carbohydrate accumulation during mixotrophic cultivation as opposed to down regulation of glycolytic enzymes (Cecchin et al., 2018). Highest lipid accumulation under mixotrophic mode was in *Pectinodesmus* at 14th day (223.8±8.2 µg/mg), which was 1.6 folds higher than the control. The highest lipid in autotrophic mode was also accumulated in *Pectinodesmus* sp. On the 7th day. Highest protein accumulation was observed under autotrophic mode in all three species which also corresponds with the chlorophyll accumulation data. Maximum protein accumulation was found in *Coelastrella* sp. With the 837.9±25.3 µg/mg protein accumulation at 14th day in autotrophic condition which was 1.6 folds higher than the highest mixotrophic accumulation at 7th day by the same species. Previous studies have revealed that presence of organic carbon is beneficial as well key component in some cases for the accumulation of

PHA in microalgae (Touloupakis et al., 2021; Samadhiya et al., 2023). Availability of carbon source increases the production of NADPH, which helps in driving the carbon flux towards PHA accumulating pathways.

Table 12: Biomass composition of microalgal cells cultivated for 21 days by harvesting at every 7th day

Biomolecules	Days	*Coelastrella* sp.		*E. texensis*		*Pectinodesmus* sp.	
		$P_{0.04}$ $N_{1.5}$	$P_{0.04}N_{1.5}Ga_{10}$	$P_{0.04}$ $N_{1.5}$	$P_{0.04}N_{1.5}$ Ga_{10}	$P_{0.04}$ $N_{1.5}$	$P_{0.04}N_{1.5}Ga_{10}$
Carbohydrate (µg/mg)	**7**	133.2±2.8	148.3±5.7	194.1±5.0	238.7±13.4	137.5±5.8	209.5±9.8
	14	212.7±25.3	207.6±16.4	223.0±14.1	246.0±10.0	109.6±4.7	285.0±10.9
	21	202.2±8.9	178.7±13.7	201.3±11.3	232.5±9.4	110.0±6.8	183.8±11.4
Protein (µg/mg)	**7**	681.1±28.4	515.4±59.8	517.1±27.0	443.5±25.2	514.2±30.3	361.0±18.9
	14	837.9±25.3	427.4±15.9	758.3±22.6	239.2±14.1	577.6±23.7	291.9±14.5
	21	415.1±27.3	194.9±11.6	423.6±40.2	245.6±17.7	497.8±17.4	218.9±11.0
Lipid (µg/mg)	**7**	142.0±9.3	162.8±6.4	162.3±7.0	203.2±8.9	254.0±7.4	175.9±14.6
	14	243.9±20.2	204.5±13.7	132.9±11.3	163.8±11.2	139.44±10.0	223.77±8.2
	21	131.7±7.7	99.0±8.4	130.9±11.4	117.2±9.8	109.1±8.6	168.9±12.7
PHA (µg/mg)	**7**	2.5±0.0	188.5±26.2	1.2±0.1	137.5±2.1	1.2±0.0	128.3±6.8
	14	2.4±0.1	168.2±10.7	1.5±0.1	137.4±3.5	1.2±0.0	125.2±4.9
	21	2.4±0.0	133.9±4.8	1.6±0.1	73.2±0.6	1.1±0.1	121.3±7.6

A similar pattern was also observed for the time course study. Highest PHA accumulation was found under mixotrophic mode at 7th day (188.5±26.2 µg/mg) in *Coelastrella* sp. that was 75 folds higher than the autotrophic mode cultivation at the same day (2.5±0.0 µg/mg). Reported time course study have revealed increase in PHA accumulation with increasing cultivation time (Ansari and Fatma, 2016). On the contrary, this study has shown a decreasing pattern of PHA accumulation (Table 12). The reason could be the two-stage cultivation, where cells are in log-phase when inoculated in medium and readily available to consume C-source and store it as PHA and other storage molecule, nevertheless when there as decrease in the carbon pool inside the medium, cells can

start utilizing the stored molecule for energy generation thus decline in the PHA concentration was seen (Koller, 2020). Although kinetics was not studied, lower PHA productivity was observed due to lower PHA accumulation rate in microalgal cells. In our previous study we concluded that galactose supplementation for 72 hrs (3 days) resulted in 151.8 ± 12.1 µg/mg DCW of PHA, which concludes to productivity of 50.6 µg/mg/day PHA, whereas current study has a productivity of 26.9 µg/mg/day which was 1.9 folds lower that previously reported (Samadhiya et al., 2022a). Therefore, 72 hrs was selected as the optimum harvesting time.

C.2 Effect of cultivation under dark

When microalgal species were grown in dark with an external carbon source supplementation, i.e., a heterotrophic condition, promoted the biomass productivity than without carbon sources (chemoautotrophy) significantly in all three of them, being highest in *Coelastrella* sp. (68.4±16.2 µg/mL/day) (Table 14). Lowest biomass productivity was observed in *Pectinodesmus* sp. (10.7±0.5 µg/mL/day) in the absence of galactose.

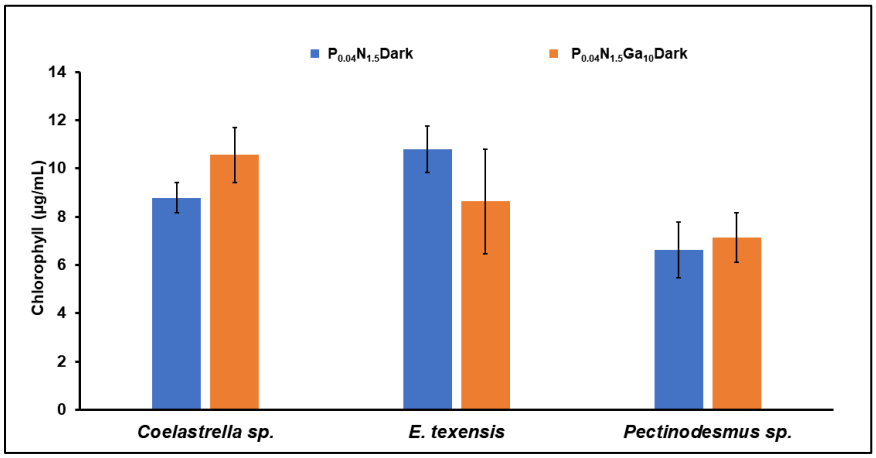


Figure 17: Chlorophyll accumulation in microalgal species grown in dark conditions with and without carbon source for 72 hrs

This can be explained by the fact that the heterotrophic mode leads to the inactivation of Calvin cycle and an active operation of pentose phosphate pathway (PPP) that allows higher generation and saving of energy producing molecules (ATP, NADPH), thus making the cells to

utilize and channelize the energy and carbon for cell growth, higher biomass yield, and enhanced accumulation of storage molecules (Yang et al., 2000; Perez-Garcia et al., 2011). On comparing the photopigment synthesis, *Coelastrella* sp. and *Pectinodesmus* species showed an expected trend bearing a higher level of chlorophyll in chemoautotrophic mode than in heterotrophic mode as shown in Figure 17. Highest chlorophyll was accumulated by *E. texensis* (10.8±1.0 µg/mL). This can be explained by the fact that the microalgal cells in the presence of an external carbon source in the dark (heterotrophic nutrition) do lack the need of photosynthesis process and the need to prepare any food, thereby, eliminating the necessity to synthesize chlorophyll pigment (Ellis et al., 1975).

A similar trend was also observed for specific growth rate and division time in all the three microalgal species (Table 13). With a heterotrophic mode, significant rise in specific growth rate was found, showing highest specific growth rate in *Pectinodesmus* species (0.3±0.0 per day) and lowest in the absence of galactose, i.e., the chemoautotrophic mode the in *Coelastrella* sp. (0.1±0.0 per day). This is possible as growing microalgae in the absence of light provided with carbon source in the media, eliminates the need of light absorption, penetration, food preparation, and thereby greatly enhancing the probability of increased cell density and biomass production (Chen, 1996). Accordingly, the division time for the three species was also determined, which was in accordance with the specific growth rate and biomass productivity data, being lowest for *Pectinodesmus* sp. (2.3±0.2 days) in heterotrophic mode and highest in *Coelastrella* sp. (10.2±0.8 days) in chemoautotrophic mode.

Table 13: Cell kinetics of microalgal species cultivated for 72 hrs grown under dark condition

Species	Biomass Productivity (µg/mL/day)		Specific Growth Rate (Per day)		Division Time (Day)	
	$P_{0.04}$ $N_{1.5}$Dark	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark	$P_{0.04}$ $N_{1.5}$Dark	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark	$P_{0.04}$ $N_{1.5}$Dark	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark
***Coelastrella* sp.**	11.6±0.9	68.4±16.2	0.1±0.0	0.3±0.1	10.2±0.8	2.5±0.4
E. texensis	30.8±5.0	54.4±8.7	0.2±0.0	0.3±0.0	4.2±0.6	2.8±0.3
***Pectinodesmus* sp.**	10.7±0.5	55.7±8.2	0.1±0.0	0.3±0.0	8.0±0.4	2.3±0.2

Table 14 represents the biochemical profile for the microalgal species, the effect of heterotrophic mode on carbohydrate accumulation led a positive response, being highest in *E. texensis* (153.2±12.2 µg/mg), following a lowest accumulation in chemoautotrophic mode in *Pectinodesmus* sp. (109.8±38.7 µg/mg). This was possible as in the heterotrophic mode absence of photosynthesis happening in the microalgal cells. The carbohydrate metabolism is governed by the PPP rather than the glycolytic pathway which is thought to be more energy-driven, and hence, yielding higher carbohydrates in cells (Cheng et al., 2022). With respect to lipids, two of the species (*E. texensis* and *Coelastrella* sp.) responded positively to heterotrophic mode, yielding higher lipid concentration in the presence of glucose. But *Pectinodesmus* species showed a decrease in lipid accumulation potential in heterotrophic mode as compared to chemoautotrophic mode. Highest lipid was found to be accumulated by *E. texensis* (123.33±39.05 µg/mg) with galactose in the media in dark. Protein accumulation was not favored with external mode supplementation. For instance, lowest protein was found to be accumulated in *E. texensis* (390.0±53.0 µg/mg) in the presence of galactose, whereas maximum protein synthesis was found in *Pectinodesmus* sp. (506.8±26.2 µg/mg) in the absence of galactose. For *Coelastrella* sp., there was not any significant difference observed in protein accumulation with and without galactose in the media, being 420.0±64.3 µg/mg and 436.3±75.1 µg/mg respectively. There could be a few possible reasons attributing to this fact, firstly, in

heterotrophic mode of nutrition, the microalgal cells might not be synthesizing the essential enzymes required for forming amino acids (proteins) and secondly, the major storage molecules accumulated in the microalgal cells, that have been reported are lipids and carbohydrates and not proteins. These factors suggest an overall shift in metabolic flux from protein to lipid synthesis (Patel et al., 2022). Lower synthesis of pyruvate was also seen (since, PPP is more common pathway in heterotrophic mode), which serves as a precursor for several non-essential amino acids, and overall decrease in proteins could be observed (Good and Zaplachinski, 1994).

Table 14: Biomass composition of microalgal cells cultivated for 72 hrs under dark

Biomolecules	Combination	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.
Carbohydrate (µg/mg)	**$P_{0.04}$ $N_{1.5}$Dark**	121.6±9.0	134.7±7.2	109.8±38.7
	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark	122.9±11.9	153.2±12.2	120.7±7.9
Protein (µg/mg)	**$P_{0.04}$ $N_{1.5}$Dark**	436.3±75.1	496.4±20.9	506.8±26.2
	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark	420.0±64.3	390.0±53.0	434.9±46.5
Lipid (µg/mg)	**$P_{0.04}$ $N_{1.5}$Dark**	106.9±39.1	109.7±17.0	99.8±16.9
	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark	117.8±29.0	123.3±9.1	80.0±10.7
PHA (µg/mg)	**$P_{0.04}$ $N_{1.5}$Dark**	1.5±0.0	8.7±0.3	2.1±0.1
	$P_{0.04}$ $N_{1.5}$ Ga_{10}Dark	37.1±4.1	107.5±4.8	26.8±0.4

Heterotrophic mode had a positive impact on PHA accumulation as well in all the three microalgal species. Highest PHA was found to be synthesized in *E. texensis* (107.5±4.8 µg/mg) in the presence of galactose in dark which was significantly higher than that being synthesized in the absence of galactose (8.7±0.3 µg/mg) i.e., nearly 12 folds increase. In the absence of light microalgal cells are unable to sequester CO_2, thus cells cannot produce RubisCO which can be then carry out the cascade of enzymatic reaction for PHA accumulation therefore, cells were unable to accumulate PHA in the absence of

external carbon source (Cecchin et al., 2018; Samadhiya et al., 2023). PHA accumulation was observed lowest in *Coelastrella* sp. (1.5±0.0 µg/mg) in chemoautotrophic mode. As these lipids and PHA can be categorized as storage molecules, they can be well expected to get accumulated in abundance when provided with the heterotrophic mode (Yang et al., 2000; Perez-Garcia et al., 2011). Overall productivity of PHA under dark cultivation was lower than that of in the previous study (Samadhiya et al., 2022a), hence mixotrophic mode of cultivation was preferred for further optimization of the process.

C.3 Effect of initial cell density

Both autotrophic and mixotrophic conditions, microalgae use chlorophyll to capture light energy and convert it into chemical energy through photosynthesis. When comparing OD_1 (lower optical density) to OD_2 (higher optical density), the concentration of chlorophyll increased in both autotrophic and mixotrophic conditions (Figure 18), as the microalgae are growing and synthesizing more chlorophyll to support their increased metabolic activity (Cecchin et al., 2018). Highest chlorophyll accumulation in mixotrophic and autotrophic conditions was in *E. texensis* (51.9±5.4 µg/mL) and *Coelastrella* sp. (54.4±4.4 µg/mL) respectively. Additionally, as the microalgae reached higher densities, they become more efficient at harvesting available light, which could plausibly lead to increase in the chlorophyll concentration.

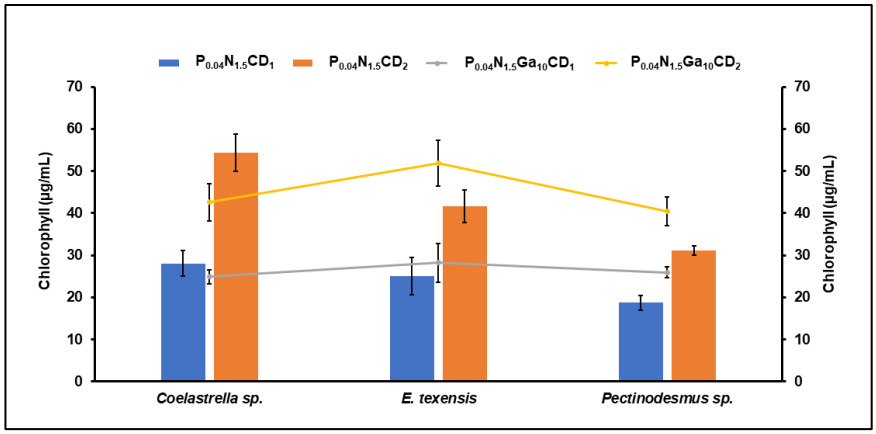


Figure 18: Chlorophyll accumulation in microalgal species grown for 72 hrs at two different cell densities

While comparing the OD_2 to OD_1, biomass productivity increased in both mixotrophic and autotrophic conditions, but mixotrophic conditions yielded higher biomass productivity and growth (Table 15). This was mainly due to the dense culture wherein microalgae will have the access to more resources, such as nutrients and light, that can result in increase in the photosynthetic activity which can support increased growth and biosynthesis (Ghosh et al., 2019). There was no clear trend observed in division time as OD increases, as it can be influenced by a variety of factors, such as the specific microalgal species, growth conditions, and nutrient availability (Li et al., 2021).

For *Coelastrella* sp., it has been observed that OD_2 mixotrophic conditions result in the highest carbohydrate accumulation of 179.9±11.4 µg/mg. This was likely because *Coelastrella* can utilize both organic carbon and inorganic carbon sources for growth, and in higher initial inoculum with mixotrophic conditions down regulated glycolytic enzymes resulting in high carbohydrate accumulation in cells (Cecchin et al., 2018). In *E. texensis*, carbohydrate accumulation was highest in the OD_2 autotrophic conditions (167.1±7.3 µg/mg), indicating that the organism primarily relies on inorganic carbon sources for growth.

Table 15: Cell kinetics of microalgal cells cultivated for 72 hrs with different cell densities

Combinations	Biomass Productivity (µg/mL/day)			Specific Growth Rate (Per day)			Division Time (Day)		
	Coelastrella sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.
$P_{0.04}$ $N_{1.5}CD_1$	55.7±8.8	58.2±16.4	23.1±8.1	0.2±0.0	0.2±0.0	0.1±0.0	4.3±0.5	4.0±0.9	7.2±2.2
$P_{0.04}$ $N_{1.5}CD_2$	61.4±13.2	107.5±12.0	53.5±16.9	0.1±0.0	0.2±0.0	0.2±0.0	5.6±1.0	3.3±0.3	4.4±1.1
$P_{0.04}N_{1.5}Ga_{10}CD_1$	207.1±25.9	279.8±28.5	135.8±31.1	0.4±0.0	0.5±0.0	0.5±0.0	1.7±0.1	1.4±0.1	1.4±0.1
$P_{0.04}N_{1.5}Ga_{10}CD_2$	260.4±28.8	374.2±30.8	376.3±41.9	0.4±0.0	0.5±0.0	0.5±0.0	1.9±0.1	1.5±0.1	1.4±0.1

This may be due to its metabolic pathway, where it was able to utilize CO_2 through the Calvin cycle. *Pectinodesmus* sp. does not show a specific trend for carbohydrate accumulation, which may suggest that its metabolic pathway for carbohydrate synthesis was more flexible and adaptable to different growth conditions.

For protein accumulation, it was highest in higher initial cell density, under autotrophic conditions in *Coelastrella* (653.6±45.5 µg/mg). In *E. texensis* protein accumulation was highest in OD_1 under mixotrophic conditions (675.6±45.6 µg/mg), and in *Pectinodesmus*, protein accumulation was highest in OD_2 under mixotrophic conditions (675.7±57.4 µg/mg) (Table 16). Mixotrophic conditions increased the protein accumulation in cells by upregulating enzymes involved in amino-acid metabolism (Cecchin et al., 2018). However, *E. texensis* and *Pectinodesmus* showed the highest protein accumulation in different initial cell density clearly indicating different metabolic activities in different species under same cultivation conditions (Salim et al., 2013; Gong and Huang, 2020).

Table 16: Biomass composition of microalgal species cultivated for 72 hrs grown under different cell densities

Species	(µg/mg)	$P_{0.04}$ $N_{1.5}CD_1$	$P_{0.04}$ $N_{1.5}CD_2$	$P_{0.04}N_{1.5}Ga_{10}CD_1$	$P_{0.04}N_{1.5}Ga_{10}CD_2$
***Coelastrella* sp.**	**Carbohydrate**	143.5±5.5	111.5±9.1	129.5±4.5	179.9±11.4
	Protein	622.2±27.1	653.6±45.5	576.9±40.3	522.5±35.6
	Lipid	77.2±5.6	92.8±10.8	79.0±11.0	76.7±8.4
	PHA	4.2±0.4	2.5±0.1	127.4±3.8	84.9±8.1
E. texensis	**Carbohydrate**	149.0±6.6	167.1±7.3	148.1±5.8	133.2±3.6
	Protein	596.9±44.9	552.1±50.0	675.6±45.6	496.3±43.0
	Lipid	109.8±15.2	117.1±12.6	82.5±17.0	78.5±9.4
	PHA	1.9±0.2	4.2±0.6	132.6±4.2	99.8±4.2
***Pectinodesmus* sp.**	**Carbohydrate**	139.2±6.1	141.7±7.0	148.1±7.3	142.8±4.5
	Protein	544.0±49.3	419.7±37.6	556.0±32.0	675.7±57.4
	Lipid	75.7±4.7	110.3±17.1	99.6±13.5	93.8±12.9
	PHA	0.9±0.1	0.7±0.0	73.1±3.6	49.5±4.8

Lipid accumulation was found to be highest in the OD_2 autotrophic conditions in *Coelastrella* (92.8±10.8 µg/mg) and *E. texensis* (117.1±12.6 µg/mg), higher cell densities could interfere with the light penetration and nutrient availability, creating a scarcity of nutrients. Thus, lipid accumulation pathways can be triggered more in higher cell

densities as compared to lower cell densities (Salim et al., 2013; Minhas et al., 2016; Li et al., 2021).

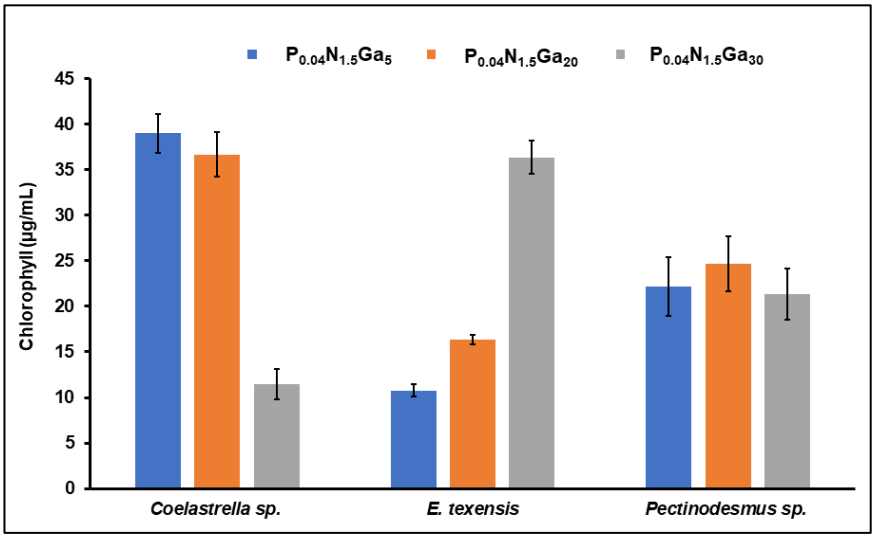


Figure 19: Chlorophyll accumulation in microalgal species grown for 72 hrs in three varying concentrations of galactose

PHA accumulation was observed highest in OD_1 mixotrophic conditions in all the three organisms, which may be due to the more availability of organic carbon sources for PHA synthesis in lower cell density (Li et al., 2021). The higher accumulation of PHA in mixotrophic conditions as compared to autotrophic conditions could be attributed to the organism's ability to utilize both organic and inorganic carbon sources for growth, which may have led to higher carbon assimilation and PHA accumulation (Koller, 2020). The highest PHA accumulated in *E. texensis* (132.6±4.2 µg/mg) which amounts to 45.3 µg/mg/day as opposed to 50.6 µg/mg/day, that was higher than the previous selected cultivation conditions with 0.5 OD (Samadhiya et al., 2022a). Further optimization was continued with 0.5 OD_{680nm} for initial inoculum.

C.4 Effect of varied concentration of galactose

Varying galactose concentrations (5, 20, and 30 g/L) and evaluating on different parameters, the three-candidate species responded in the following manner. From Figure 19, it was evident that chlorophyll initially kept on increasing with an increase in galactose concentration from 5g/L to 20g/L. But at very high galactose concentration chlorophyll

content decreased significantly. It is possible that with external sugar supplementation, the microalgal cells switch to mixotrophic mode of nutrition, intaking food directly from external source and eliminating the need to sequester CO_2 followed by carbon fixation using Calvin cycle, thus leading to a reduction in photosynthesis rate (Ellis et al., 1975; Perez-Garcia et al., 2011; González-Fernández and Ballesteros, 2012). Highest chlorophyll in *Coelastrella* and *Pectinodesmus* species was obtained with lower galactose concentration (5 g/L) and for *E. texensis* it was found to be highest at a moderately high galactose concentration of 20 g/L.

Table 17: Cell kinetics of microalgal cells grown for 72 hrs under different galactose concentration

Combinations	Biomass Productivity ((µg/mL/day)			Specific Growth Rate (Per day)			Division Time (Day)		
	Coelastrella sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella sp.*	*E. texensis*	*Pectinodesmus* sp.
$P_{0.04}N_{1.5}Ga_5$	213.9±9.3	182.2±16.1	122.3±2.2	0.6±0.0	0.5±0.0	0.5±0.0	1.3±0.0	1.3±0.1	1.4±0.0
$P_{0.04}N_{1.5}Ga_{20}$	240.1±24.6	227.7±20.2	150.3±17.9	0.6±0.0	0.6±0.0	0.5±0.0	1.2±0.1	1.2±0.1	1.3±0.1
$P_{0.04}N_{1.5}Ga_{30}$	189.3±19.5	221.5±23.5	139.1±1.8	0.5±0.0	0.6±0.0	0.5±0.0	1.3±0.1	1.2±0.1	1.3±0.0

As per Table 17, the highest biomass productivity was found in *Coelastrella* sp. 240.1±24.6 µg/mL/day with a galactose supplementation of 20 g/L and lowest was observed in *Pectinodesmus* species at low galactose concentration of 5 g/L (122.3±2.2 µg/mL/day). Moreover, among the three, *Coelastrella* sp. corresponded to synthesize highest chlorophyll and yielded highest biomass productivity.

For all the three-candidate species, maximum specific growth rate was found with galactose present in the media of 20 g/L being highest (0.6±0.0 per day) for both *E. texensis* and *Coelastrella* sp. and 0.5±0.0 per day for *Pectinodesmus* sp. This can be understood by the fact that the microalgal cells in the provision of optimum food supply (carbon source) grew well, showing an elevated growth rate and bearing a lower division time, which further declines on providing carbon sources in abundance. As the extra carbon sources could be utilized in forming

storage molecules, that would be utilized as energy deriving compounds in later stages of growth (Cheng et al., 2022). Therefore, it can be understood that the excess carbon in the media results in a shift of the overall metabolic flux by utilizing the sugars in secretion and accumulation of storage compounds (carbohydrates and lipids) rather than utilizing it for their growth and biomass enhancement. For instance, excess carbon (in the form of galactose here) is incorporated into microalgal cells and metabolized into triacylglycerols (TGAs), a storage oil (González-Fernández & Ballesteros, 2012; Anand et al., 2018).

Table 18: Biomass composition of microalgal species cultivated for 72 hrs under varied concentration of galactose

Species	(µg/mg)	$P_{0.04}N_{1.5}Ga_5$	$P_{0.04}N_{1.5}Ga_{20}$	$P_{0.04}N_{1.5}Ga_{30}$
***Coelastrella* sp.**	Carbohydrate	82.2±5.8	127.2±8.0	122.1±21.4
	Protein	442.0±36.8	447.0±53.6	339.9±62.0
	Lipid	110.4±17.2	102.2±6.4	93.9±7.2
	PHA	51.9±1.6	64.5±1.0	42.0±2.6
E. texensis	Carbohydrate	115.3±16.8	135.8±5.9	163.4±11.3
	Protein	586.5±11.1	422.4±9.2	396.8±41.8
	Lipid	109.4±43.6	145.9±11.8	73.8±9.8
	PHA	114.1±5.6	120.3±2.6	130.1±3.0
***Pectinodesmus* sp.**	Carbohydrate	85.0±10.1	102.7±8.9	111.8±6.3
	Protein	440.3±19.1	341.0±18.8	272.8±22.6
	Lipid	72.2±8.5	104.9±11.8	110.7±7.6
	PHA	35.3±4.8	60.5±6.8	55.9±9.8

Table 18 can be referred for carbohydrate accumulation, with increasing galactose concentration, from 5 g/L to 30 g/L in the media, the carbohydrate accumulation potential also kept on increasing and was found to be maximum at 30 g/L galactose concentration for *E. texensis* i.e., 163.4±11.3 µg/mg. Furthermore, galactose as a carbon source can be expected to get converted into glucose 6-phosphate via the Leloir pathway which can then be metabolized to yield pyruvate through the glycolysis pathway, where these pyruvate acts as a potential precursor of acetyl-CoA, subsequently entering the TCA cycle producing energy

other carbohydrate derived molecules (Frey, 1996; Chroumpi et al., 2022).

E. texensis also accumulated highest protein (586.5±11.1 µg/mg), highest lipid (145.9±11.8 µg/mg) as well as highest PHA (150.3±2.6 µg/mg) at moderately high (20 g/L) galactose concentration. Here, these molecules (lipids, PHAs and carbohydrates) can be expected to be synthesized as storage molecules formed during sufficiently high levels of food in the surrounding environment (Yang et al., 2000; Cheng et al., 2022). The lowest carbohydrate was accumulated in *Coelastrella* sp. (82.2±5.8 µg/mg) and for lipid and PHA, minimum yield was found in *Pectinodesmus* species, i.e., 72.2±8.5 µg/mg and 35.3±4.8 µg/mg respectively, all obtained at minimum galactose concentration (5 g/L). *Coelastrella* sp. showed a highest carbohydrate accumulation at a moderately high concentration of galactose (20 g/L galactose). On the other hand, minimum protein (272.8±22.6 µg/mg) was obtained in *Pectinodesmus* sp. at high galactose concentration of 30 g/L. Furthermore, protein accumulation was found to be gradually decreasing with increase in galactose concentration as compared to the control. So, for all the three species, increase in galactose concentration did not favor an elevation in protein levels. This can be explained as the mixotrophy condition (provision of external carbon source in media) results in a shift of microalgal metabolism towards carbohydrates and lipid synthesis, rather than accumulating proteins or amino acids formation (Patel et al., 2022). Moreover, the carbon sources were up taken by the microalgal cells following the mixotrophic mode, which are used for producing energy derived component (like, ATP and NADPH) *via* pentose phosphate pathway (PPP), or results in the incorporation of those carbon molecules into TGA molecules as storage compounds (Perez-Garcia et al., 2011; Adesanya et al., 2014). For *E. texensis* and *Pectinodesmus* species, lipids concentration was found to be considerably high on increasing the galactose concentration from 5 g/L to 20 g/L. However, *Coelastrella* sp. showed a decrease in lipid accumulation with gradual increase in galactose concentration. With

respect to PHA accumulation by these microalgal species, the gradual increase in galactose concentration did not significantly lead to increase in PHA accumulation. 30 g/L of galactose in the media triumphed PHA accumulation (130.1±3.0 µg/mg), which was considerably higher than the lower concentration of galactose (5, 20 g/L). However, the overall productivity was still lower than that of previous study (Samadhiya et al., 2022a). 10 g/L concentration of galactose was chosen for further optimization.

C.5 Conjoint effect of nitrate and phosphate with galactose supplementation

In a previous study it was established that high PHA accumulation in the selected microalgae was not possible without the presence of external C-source. Varied C/N ratio can be a critical factor in triggering PHA accumulation in microbes (Steinbiichel et al., 1992; Jiang et al., 2018; Koller, 2020). Phosphate is a key component for PHA metabolism in microbes as well (Bhati and Mallick, 2015). In this section we will discuss the conjoint effect of nitrate and phosphate with galactose concentration. Nitrogen is a key component of protein moiety and a critical factor in protein synthesis, as well as chlorophyll production in algal cells (Ellis et al., 1975).

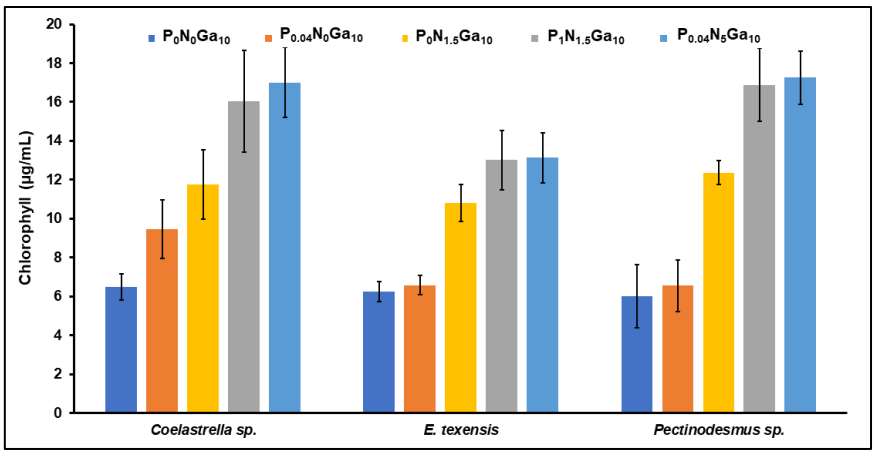


Figure 20: Chlorophyll accumulation in microalgal cells grown for 72 hrs under conjoint effect of nitrate phosphate variation and galactose supplementation

Highest chlorophyll was accumulated in *Pectinodesmus* under high concentration of nitrate and moderate concentration of phosphate (17.3 ±1.7 µg/mL). The combinations depleted of nitrogen were not able to accumulate high amount of chlorophyll (Figure 20). Phosphate being the key component of DNA and cell highest growth and biomass productivity was under high phosphate concentration (Alipanah et al. 2018; Sanz-Luque et al. 2020). Highest biomass productivity was under 1g/L concentration of phosphate in *Coelastrella* sp. (336.2±13.3 µg/mL/day). Where high nitrate and phosphate gave higher growth rate and biomass productivity, absence of phosphate resulted in lower values (Table 19).

Table 19: Cell kinetics of microalgal species cultivated for 72 hrs under conjoint effect of nitrate, phosphate variation with galactose

Combinations	Biomass Productivity (µg/mL/day)			Specific Growth Rate (Per day)			Division Time (Day)		
	Coelastrella sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.
$P_0N_0Ga_{10}$	34.8±2.3	27.8±2.9	31.7±4.7	0.2±0.1	0.2±0.0	0.2±0.0	4.2±1.2	4.6±0.4	3.3±0.4
$P_{0.04}N_0Ga_{10}$	25.1±3.9	54.7±4.2	48.7±5.8	0.1±0.0	0.3±0.0	0.3±0.0	5.3±0.7	2.7±0.2	2.5±0.2
$P_0N_{1.5}Ga_{10}$	113.0±11.5	89.8±11.1	50.0±3.8	0.4±0.0	0.4±0.0	0.3±0.0	1.8±0.1	2.0±0.2	2.4±0.1
$P_1N_{1.5}Ga_{10}$	336.2±13.3	229.3±9.2	184.5±10.2	0.7±0.0	0.6±0.0	0.6±0.0	1.0±0.0	1.2±0.0	1.2±0.0
$P_{0.04}N_5Ga_{10}$	231.0±12.3	217.3±10.5	160.6±9.9	0.6±0.0	0.6±0.0	0.6±0.0	1.2±0.0	1.2±0.0	1.2±0.0

Nitrogen is a key factor for protein synthesis, wherein nitrogen repleted conditions achieved higher concentration of protein accumulation in all three species whereas depletion of nitrates resulted in significant decrease in protein synthesis in microalgae (Zarrinmehr et al., 2020). Highest protein was accumulated in *E. texensis* (668.9± 25.2 µg/mg) under 5 g/L of nitrate supplementation (Table 20). Nitrogen also plays a key role in lipid biosynthesis. Several researchers have reported that absence of nitrogen in growth medium triggers lipid accumulation in microalgal cells (Ghosh et al., 2019; Fernandes and Cordeiro, 2022). In the present study, highest lipid was accumulated under the absence of both nitrate and phosphate (199.1± 15.9 µg/mg) in *E. texensis*.

Highest carbohydrate was accumulated under 5 g/L concentration of nitrate resulting in 167.3± 16.8 µg/mg carbohydrate (Blanco and Blanco, 2017; de Carvalho Silvello et al., 2022). Interestingly high nitrate and phosphate concentration triggered PHA accumulation in all three microalgal species, resulted in highest PHA accumulation in *E. texensis* sp. under 5 g/L concentration of nitrate (196.6± 8.1 µg/mg) (Table 20).

Table 20: Biomass composition of microalgal species cultivated for 72 hrs under conjoint effect of nitrate, phosphate variation with galactose

Species	(µg/mg)	$P_0N_0Ga_{10}$	$P_{0.04}N_0Ga_{10}$	$P_0N_{1.5}Ga_{10}$	$P_1N_{1.5}Ga_{10}$	$P_{0.04}N_5Ga_{10}$
***Coelastrella* sp.**	**Carbohydrate**	114.9±11.6	122.8±15.1	104.7±5.6	51.6±5.5	51.3±6.4
	Protein	311.3±120.9	315.6±140.8	261.1±23.3	441.8±36.3	645.6±52.9
	Lipid	186.5±17.0	141.5±14.7	116.7±14.0	101.1±10.4	93.2±8.4
	PHA	12.4±1.7	6.0±0.8	58.2±5.2	159.6±9.3	179.5±4.9
E. texensis	**Carbohydrate**	150.0±4.5	124.1±6.4	127.6±9.6	137.3±3.7	156.5±8.4
	Protein	336.4±21.5	365.1±15.0	418.5±38.7	476.5±29.5	668.9±25.2
	Lipid	199.1±15.9	142.9±12.8	113.9±11.8	109.4±10.8	143.5±15.2
	PHA	11.5±1.2	11.1±0.7	27.4±1.5	177.9±4.1	196.6±8.1
***Pectinodesmus* sp.**	**Carbohydrate**	102.0±11.1	119.1±13.7	115.3±9.1	131.4±10.5	167.3±16.8
	Protein	264.3±17.4	357.1±24.0	355.7±25.9	424.4±40.3	544.3±30.5
	Lipid	174.3±22.0	120.3±9.8	107.2±6.0	59.8±4.8	68.0±5.8
	PHA	3.7±0.6	10.0±0.8	35.7±3.7	56.7±4.9	73.7±4.4

Abundance of nitrogen and phosphorus in culture medium allows microbes to grow at higher pace and high nitrogen also increases the protein and enzyme production. In recent study, high PHA have been achieved in all species under high nitrogen and phosphate supplementation, which contradicts many reported studies for PHA accumulation (Sun et al., 2007; Bhati and Mallick, 2015; Koller, 2020). The presence of different genes and genetic makeup can affect these metabolic processes. High nitrogen can help microalgae produce necessary enzymes; enzymes used in PHA biosynthesis are not nitrogen dependent hence presence of nitrogen does not negatively affect these. The presence of phosphate is also crucial for the conversion of galactose

into glucose -1-6 phosphate for it to be readily available by cells to get assimilated into PHA producing pathways (Cohn and Segal, 1973; Frey, 1996). Nitrogen and phosphorus are abundantly present in various wastewater which can be utilized as a cheap medium and eutrophication of water bodies can be prevented (Johnson et al., 2010; Koller, 2018; Troschl et al., 2020). High nitrogen supplementation has given highest PHA productivity (65.3 µg/mg/day). Hence for further optimization 5 g/L nitrate was used in the medium.

C.6 Effect of plant growth hormone with galactose supplementation

Plant growth hormones are responsible for growth and cellular activity in plant cells, and due to the similarities in microalgae and plants, their effects are extensively studied in microalgae as well. Auxin, gibberellic acid and kinetin were selected for this study and supported microalgal growth with highest productivity in *E. texensis* under gibberellin supplementation with galactose (308.0±20.5 µg/mL/day) (Table 21).

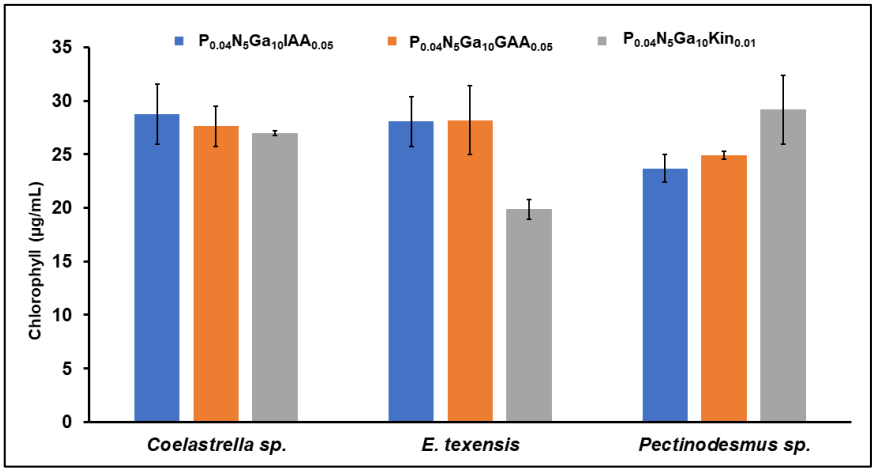


Figure 21: Chlorophyll accumulation in microalgal cells cultivated for 72 hrs under the influence of growth hormones

However highest chlorophyll accumulation was observed under kinetin supplementation in *Pectinodesmus* sp. (29.2±3.2 µg/mL) (Figure 21) various other reports have supported the trend. Kinetin acts as an antioxidant reducing stress in the cells and increasing growth and photosynthetic activity (Kokkiligadda et al., 2017; Udayan et al., 2018).

Table 21: Cell kinetics of microalgal species cultivated for 72 hrs under conjoint effect of growth hormone with galactose

Parameters	Species	$P_{0.04}N_5Ga_{10}IAA_{0.05}$	$P_{0.04}N_5Ga_{10}GAA_{0.05}$	$P_{0.04}N_5Ga_{10}Kin_{0.01}$
Biomass Productivity (µg/mL/day)	***Coelastrella* sp.**	197.0±8.5	199.9±33.9	197.64±2.0
	E. texensis	193.9±47.4	308.0±20.5	263.9±38.9
	***Pectinodesmus* sp.**	185.5±4.8	180.0±16.9	197.9±15.8
Specific Growth Rate (Per day)	***Coelastrella* sp.**	0.5±0.0	0.5±0.1	0.5±0.0
	E. texensis	0.5±0.1	0.7±0.1	0.6±0.0
	***Pectinodesmus* sp.**	0.6±0.0	0.6±0.1	0.6±0.1
Division Time (Days)	***Coelastrella* sp.**	1.3±0.0	1.3±0.1	1.3±0.0
	E. texensis	1.3±0.2	1.1±0.1	1.1±0.1
	***Pectinodesmus* sp.**	1.2±0.0	1.18±0.15	1.1±0.1

Plant growth hormones have been reported to increase lipid accumulation in microalgae (Piotrowska-Niczyporuk and Bajguz, 2014; Salama et al., 2014; Zhang et al., 2021). Due to the intertwined pathways of lipid and PHAs, their effect was also investigated (Rehm et al., 2001). But unfortunately, lipid and PHA accumulation both decreased under supplementation. One reason could be the high nitrogen supplementation coupled with hormone triggering the higher growth and chlorophyll accumulation rather than lipid and PHA biosynthesis. Highest PHA was accumulated under gibberellic acid accumulation which was 1.4 folds less than only high nitrogen and galactose supplementation (Table 19,21). Highest lipid and carbohydrate were synthesized in *E. texensis* under auxin (102.4±9.9 µg/mg) and kinetin (155.8±4.7 µg/mg) respectively. Highest protein production was under kinetin supplementation in *Pectinodesmus* sp. (503.8±42.8 µg/mg) (Table 22). Since phytohormones did not increase the PHA accumulation any further, they were not supplemented in the next experiment.

Table 22: Biomass composition of microalgal species cultivated for 72 hrs under conjoint effect of growth hormone with galactose

Species	(µg/mg)	$P_{0.04}N_5Ga_{10}IAA_{0.05}$	$P_{0.04}N_5Ga_{10}GAA_{0.05}$	$P_{0.04}N_5Ga_{10}Kin_{0.01}$
***Coelastrella* sp.**	**Carbohydrate**	90.8±5.1	88.5±5.1	104.9±6.9
	Protein	491.7±35.7	394.3±18.1	330.0±14.8
	Lipid	93.7±7.5	46.1±5.0	86.4±5.7
	PHA	23.3±1.6	136.3±7.4	18.2±1.1
E. texensis	**Carbohydrate**	141.4±5.6	131.9±7.5	155.8±4.7
	Protein	482.2±60.4	438.9±15.6	486.3±41.3
	Lipid	102.4±9.9	72.0±7.6	63.5±7.4
	PHA	13.4±0.9	142.1±3.0	140.4±2.1
***Pectinodesmus* sp.**	**Carbohydrate**	95.9±4.4	95.8±8.8	98.6±7.2
	Protein	443.3±22.9	428.3±24.7	503.8±42.8
	Lipid	80.6±7.1	82.4±7.7	79.0±10.4
	PHA	11.8±0.6	77.3±1.0	28.8±1.5

C.7 Effect of heavy metals

Heavy metals are abundantly present in the industrial wastewater, often leading to cytotoxicity in the biological treatment (Kizilkaya et al., 2012; Pradhan et al., 2019; Kinuthia et al., 2020; Sharma et al., 2021; Srivastava et al., 2022). Although heavy metals are toxic to microalgae, low concentrations of such metals can promote their growth and increase the concentration of storage products such as proteins and fatty acids. Additionally, some studies have reported the potential of heavy metals to stimulate PHA accumulation. In this study, all heavy metals except iron (Fe) negatively affected growth and chlorophyll production of microalgae.

Highest biomass productivity (220.9±21.2 µg/mL/day) and chlorophyll production (21.6±2.0 µg/mL) was under Fe supplementation in *Coelastrella* sp. (Figure 22, Table 23). Microalgal cells have higher tolerance towards Fe as compared to other microalgae and have similar trend has been reported in other studies (Cameron et al. 2018). $FeSO_4$ has been utilized which has Fe (II) ions that are more soluble but less

stable hence readily converts into Fe (III). High iron assimilation has been seen in microalgae.

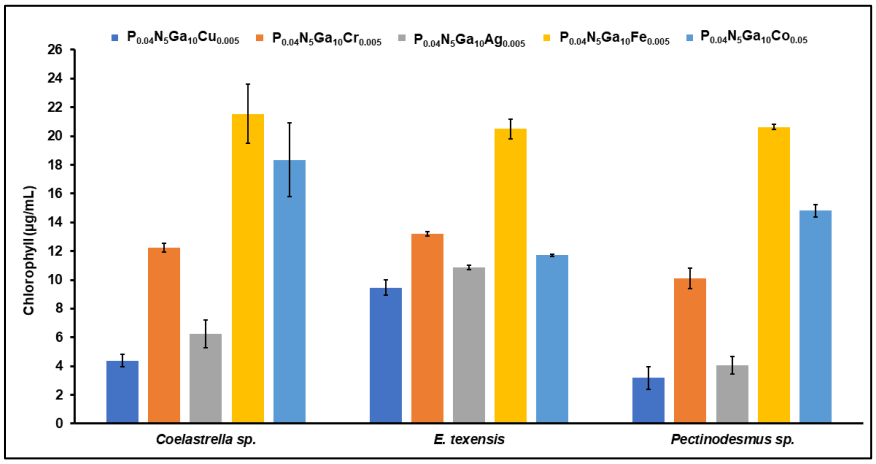


Figure 22: Chlorophyll accumulation in microalgal cells grown for 72 hrs under heavy-metal and galactose supplementation condition

Fe can enter microalgal cells and induce an ROS (reactive oxygen species) response that can trigger lipid biosynthesis. Iron nanoparticle synthesis has been also widely investigated (Sakthi et al. 2020; Rana and Prajapati 2021).

Table 23: Cell kinetics of microalgal species cultivated for 72 hrs under conjoint effect of heavy metals with galactose

Parameters	Species	$P_{0.04}N_5Ga_{10}Cu_{0.005}$	$P_{0.04}N_5Ga_{10}Cr_{0.005}$	$P_{0.04}N_5Ga_{10}Ag_{0.005}$	$P_{0.04}N_5Ga_{10}Fe_{0.005}$	$P_{0.04}N_5Ga_{10}Co_{0.005}$
Biomass Productivity (µg/mL/day)	*Coelastrella* sp.	12.9±0.2	35.1±0.9	16.7±0.2	220.9±21.2	36.8±0.9
	E. texensis	15.7±0.8	48.2±0.8	15.5±1.5	200.5±12.0	32.2±0.6
	Pectinodesmus sp.	9.6±1.0	23.8±3.5	13.4±1.5	211.2±14.3	37.2±0.6
Specific Growth Rate (Per day)	*Coelastrella* sp.	0.1±0.0	0.2±0.0	0.1±0.0	0.6±0.0	0.2±0.0
	E. texensis	0.1±0.0	0.2±0.0	0.1±0.0	0.6±0.0	0.2±0.0
	Pectinodesmus sp.	0.1±0.0	0.2±0.0	0.1±0.0	0.6±0.1	0.2±0.0
Division Time (Days)	*Coelastrella sp.*	11.4±0.6	4.0±0.5	8.3±0.4	1.2±0.1	3.9±0.5
	E. texensis	8.5±0.2	3.2±0.2	9.9±0.6	1.3±0.0	4.1±0.1
	Pectinodesmus sp.	13.1±0.1	4.7±0.2	6.6±0.6	1.1±0.1	3.0±0.6

Highest lipid and carbohydrate were produced in *Pectinodesmus* under silver (Ag; 212.5±5.5 µg/mg) and chromium (Cr; 230.4±10.8 µg/mg) respectively. Microalgae have been studied extensively to produce silver nanoparticles, these molecules can also generate ROS effect inducing

lipid production in microalgae (Kashyap et al., 2019, 2021; Romero et al., 2020). Highest protein was synthesized in *E. texensis* producing 576.9±10.8 µg/mg under copper supplementation. Copper has been known to produce ROS and induce lipid accumulation while risk of protein assembly being destroyed. In some species Cu is known to induce the production of proline synthesis, which can act as a protectant for protein production assembly thus generating higher protein concentration (Zhang et al., 2008; Cameron et al., 2018; Fathi et al., 2020). Fe supplementation has been highly sought after to produce PHA (Foong et al., 2019; García et al., 2021).

Table 24: Biomass composition of microalgal species cultivated for 72 hrs under conjoint effect of heavy metals with galactose

Species	(µg/mg)	$P_{0.04}N_5Ga_{10}Cu_{0.005}$	$P_{0.04}N_5Ga_{10}Cr_{0.005}$	$P_{0.04}N_5Ga_{10}Ag_{0.005}$	$P_{0.04}N_5Ga_{10}Fe_{0.005}$	$P_{0.04}N_5Ga_{10}Co_{0.005}$
***Coelastrella* sp.**	**Carbohydrate**	106.7±7.2	206.1±25.1	140.3±16.1	77.4±124.1	124.1±6.9
	Protein	551.0±27.3	371.7±24.5	489.6±20.6	467.2±31.1	524.31±52.3
	Lipid	104.0±8.4	49.6±3.8	178.0±4.0	41.1±3.5	85.9±6.4
	PHA	7.3±0.5	1.63±0.12	1.0±0.1	192.0±8.6	3.9±0.02
E. texensis	**Carbohydrate**	130.0±6.6	209.3±18.8	132.7±7.5	132.2±12.3	147.4±12.6
	Protein	576.9±48.4	331.7±35.0	492.1±23.5	406.8±18.5	532.4±54.0
	Lipid	64.8±3.8	105.9±6.5	165.1±7.5	78.7±7.0	86.4±9.8
	PHA	1.0±0.1	7.0±0.4	1.1±0.1	223.8±3.7	1.1±0.1
***Pectinodesmus* sp.**	**Carbohydrate**	143.0±7.5	230.4±10.8	152.3±8.7	102.0±4.2	122.3±8.4
	Protein	539.3±57.6	419.3±26.4	426.1±34.5	442.2±37.3	250.8±26.0
	Lipid	68.9±7.7	120.3±12.4	212.5±5.5	91.4±7.4	138.0±10.7
	PHA	4.1±0.9	1.0±0.2	1.4±0.1	93.1±4.6	1.7±0.1

Although no direct relation between PHA and iron have been established, Fe being a trace element can increase the bioactivity of microbes making a positive ATP pool to produce PHA when present with other nutrient such as C-source (Chidambarampadmavathy et al., 2015; Rana and Prajapati, 2021). Highest PHA was also produced by *E. texensis* under Fe supplementation (223.8±3.7 µg/mg) which was 1.7 folds higher than previously reported (Table 24) (Samadhiya et al., 2022a). Similar results have been reported in *Scenedesmus* sp. and

purple photosynthetic bacterium as well (Foong et al., 2019; García et al., 2021).

C.8 Nutrient variable analysis for accumulation of biomass, PHA and energy precursors using PCA

PCA was performed for cumulative effect of NP variation and galactose as well as heavy metal supplementation on microalgal cells. Since these two effects have shown high variance in PHA accumulation in microalgae, they were chosen for the analysis. The multivariate analysis depicts the up to 84% variance was explained using dimension 1 and 2. The Eigen value higher than 1 was selected to perform the PCA (Table 25).

Table 25: Eigen value, variance, and co-variance analysis of microalgal species

Dimension	Eigen value			Variance (%)			Cumulative Variance (%)		
	Coelastrella sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.	*Coelastrella* sp.	*E. texensis*	*Pectinodesmus* sp.
Dimension 1	3.0	2.2	2.8	60.1	44.6	55.2	60.1	44.6	55.2
Dimension 2	1.0	1.1	1.4	20.3	22.0	28.7	80.4	66.6	83.9
Dimension 3	0.8	1.0	0.4	16.1	19.4	8.7	96.5	85.9	92.6
Dimension 4	0.1	0.7	0.3	2.4	13.4	6.6	98.9	99.3	99.2
Dimension 5	0.1	0.0	0.0	1.1	0.7	0.8	100	100	100

The bar diagram explains the contribution of variance in the PCs by the variables. The red dotted line depicts the threshold for average contribution, bar below this line indicates lower or no contribution. For *Coelastrella* sp. only three variables have contributed to the variance, whereas for *E. texensis* and *Pectinodesmus* five factors contributed towards variance (Figure 23A, 24A, 25A). PCA-Biplots (score and vector plot) is represented in Figure 23B, 24B, 25B. The dots represent variables whereas the vector represents the output categories. Variables having less than 90° have close correlation whereas large angled variables have negative correlations. The distance from origin also represents the quality of the vectors, longer the distance higher the quality.

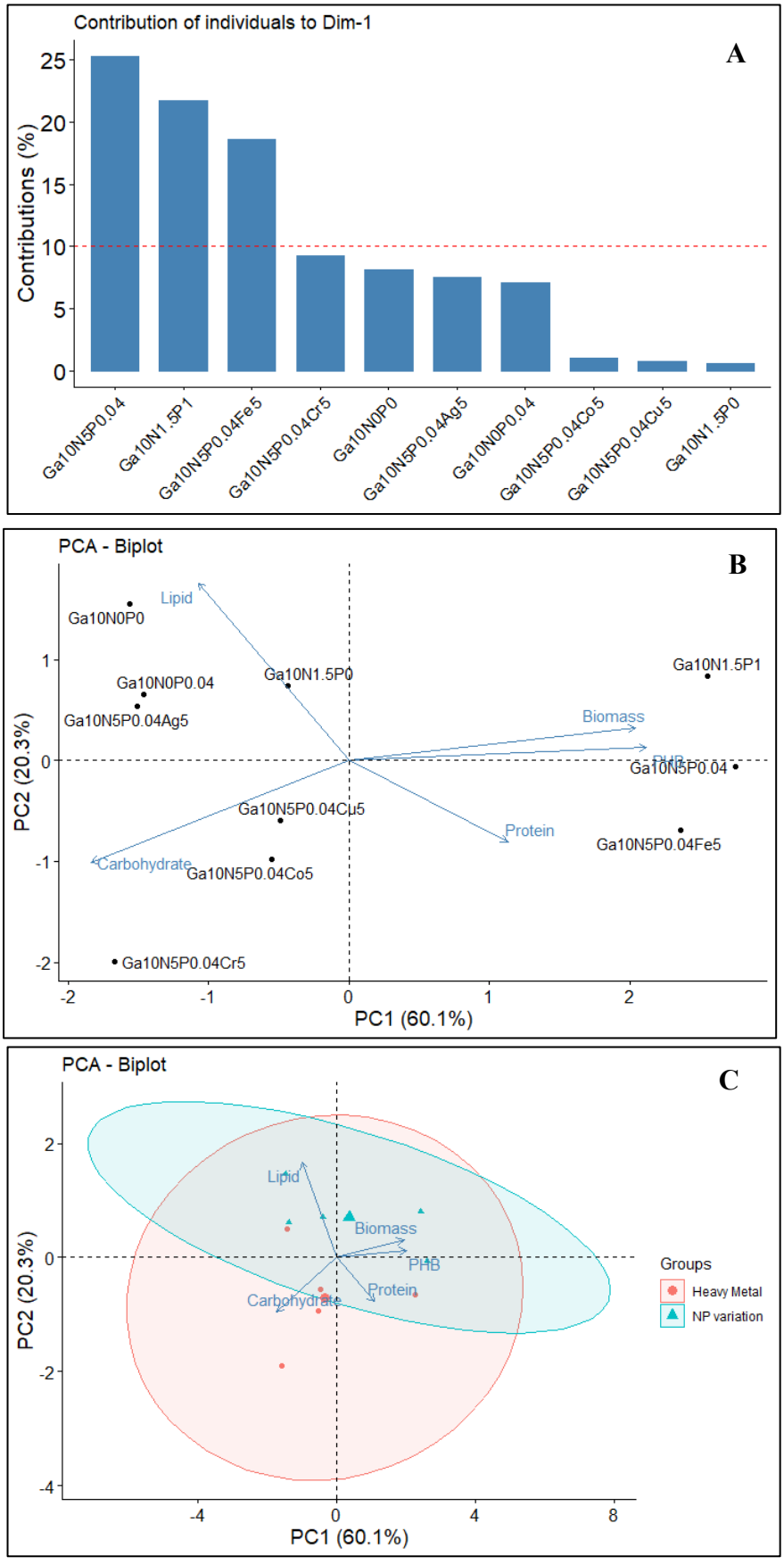


Figure 23: Bars in figure (A) are the contribution of individuals (nutrient combinations) to principal component 1. PCA biplots (B, C) show loadings of biomass components (variables or vectors) of *Coelastrella* sp. across different combinations of nutrients

For all three species biomass and PHB is groped closely which shows a close positive co-relation between these factors. Whereas lipid and protein are negatively co-related or not correlated with each other. The dots representing the treatments and their placement in the quadrants show the factor they influence the most. For instance, PHB and biomass is majorly influenced by high concentration of nitrate and phosphate along with iron supplementation. Lipid was influenced by absence or nitrate, carbohydrate with Cr. The position of these dots also represents the intent of their influence on the variables (Mutale-joan et al. 2020).

PCA-Bioplot represented in Figure 23C, 24C, 25C is a combination of vector and clustered plot. The vectors position in the positive quadrants shows a positive influence whereas negative influence is shown by the placement in the lower left quadrant. The variables are clustered together based on the treatment exerted on the cells which is represented by dot (heavy metal) and triangle (NP variation). The magnitude of circle or ellipse shows the variance exerted on the data through these variables. In *Coelastrella* sp. and *Pectinodesmus* sp. Carbohydrate is not influenced by NP variation effect where as in *E. texensis* all the variables are influenced and show statical significance (Jolliffe, 2019).

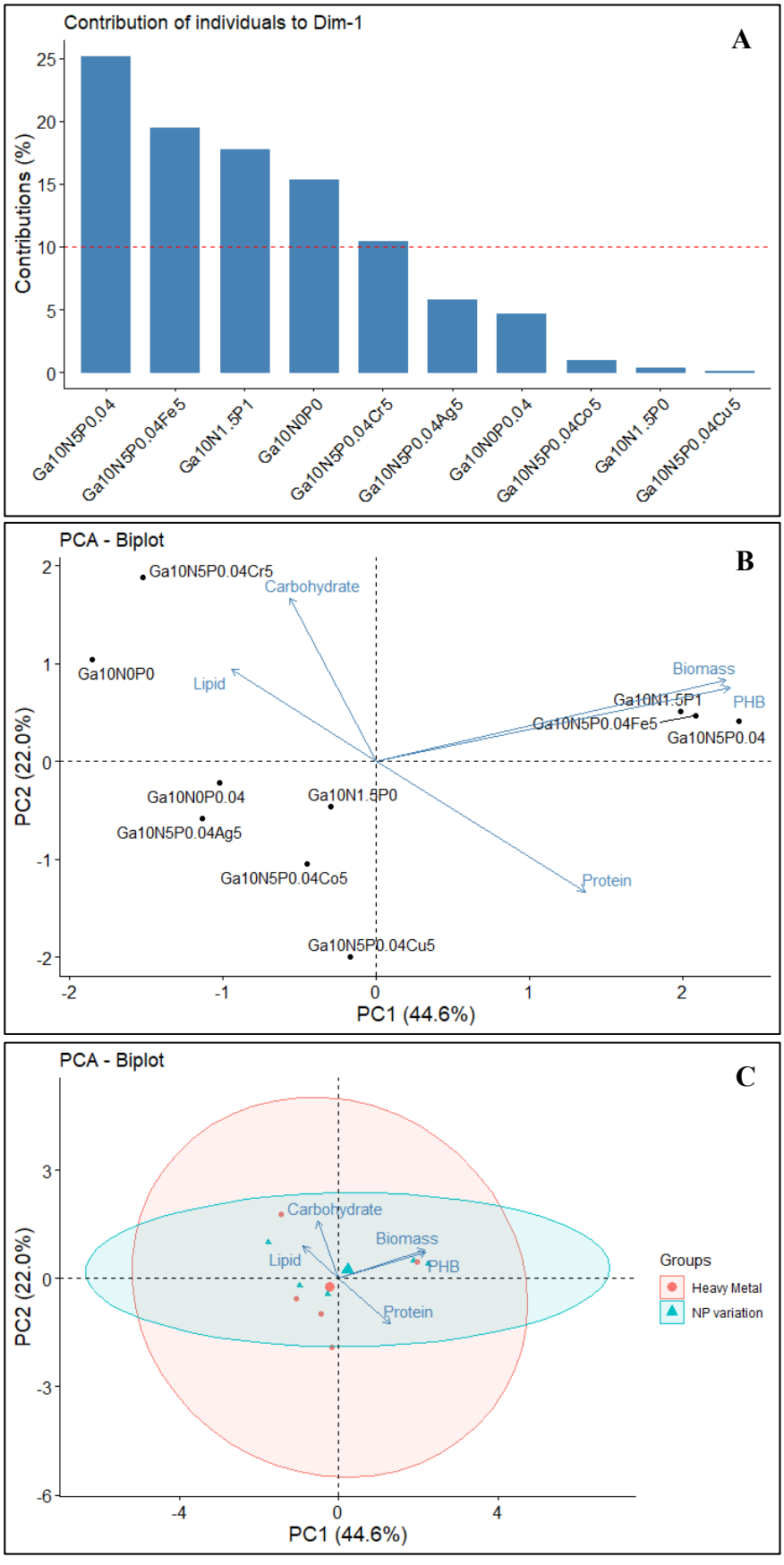


Figure 24: Bars in figure (A) are the contribution of individuals (nutrient combinations) to principal component 1. PCA biplots (B, C) show loadings of biomass components (variables or vectors) of *E. texensis* across different combinations of nutrients

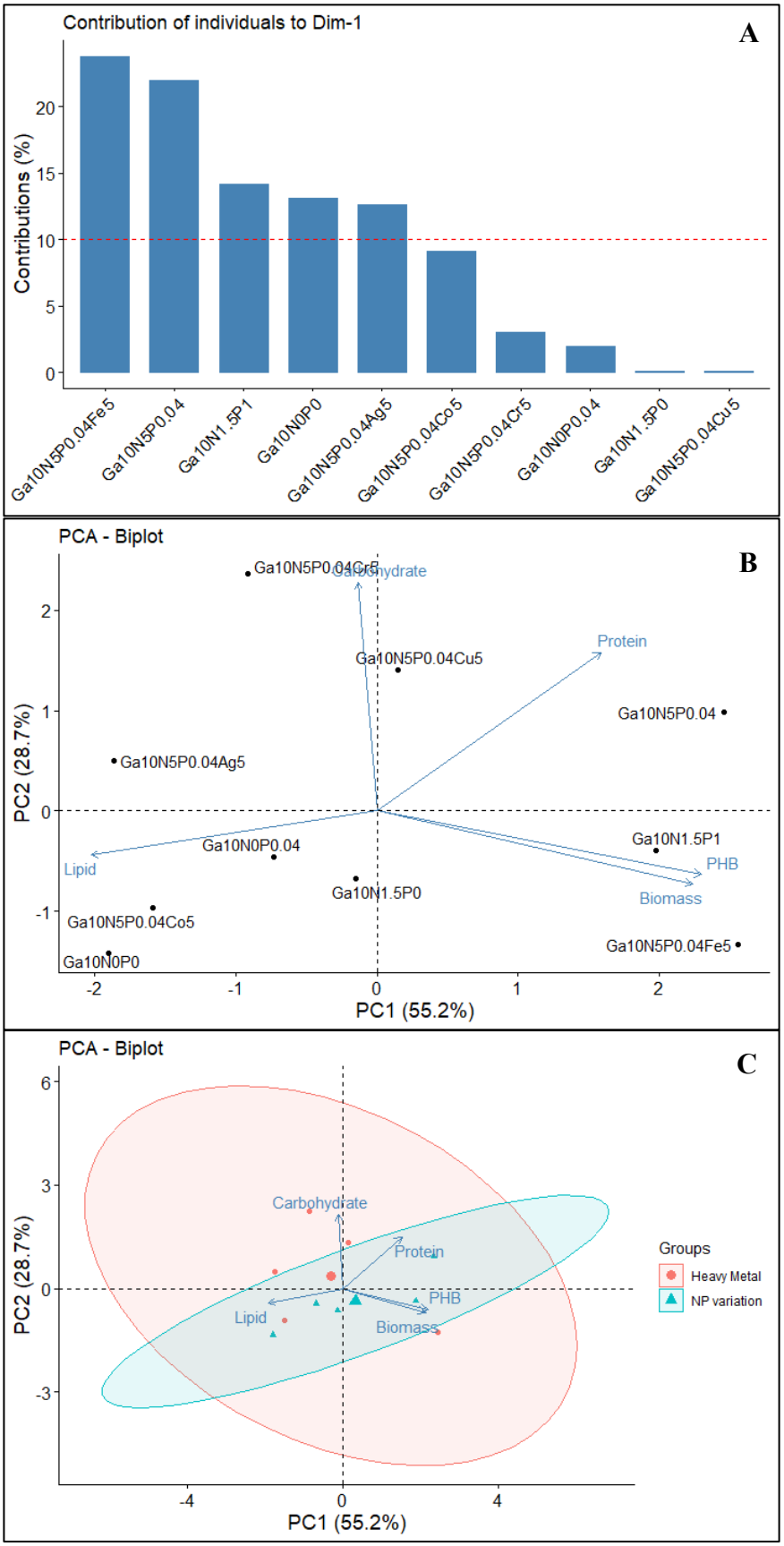


Figure 25: Bars in figure (A) are the contribution of individuals (nutrient combinations) to principal component 1. PCA biplots (B, C) show loadings of biomass components (variables or vectors) of *Pectinodesmus* sp. across different combinations of nutrients

C.9 Characterization of biopolymer:

Figure 26 shows the SEM image of polymer film. A homogenous structure can be revealed from the micrograph with a few protrusions on the surface, which shows the complexity of the matrix (Vahabi et al., 2019).

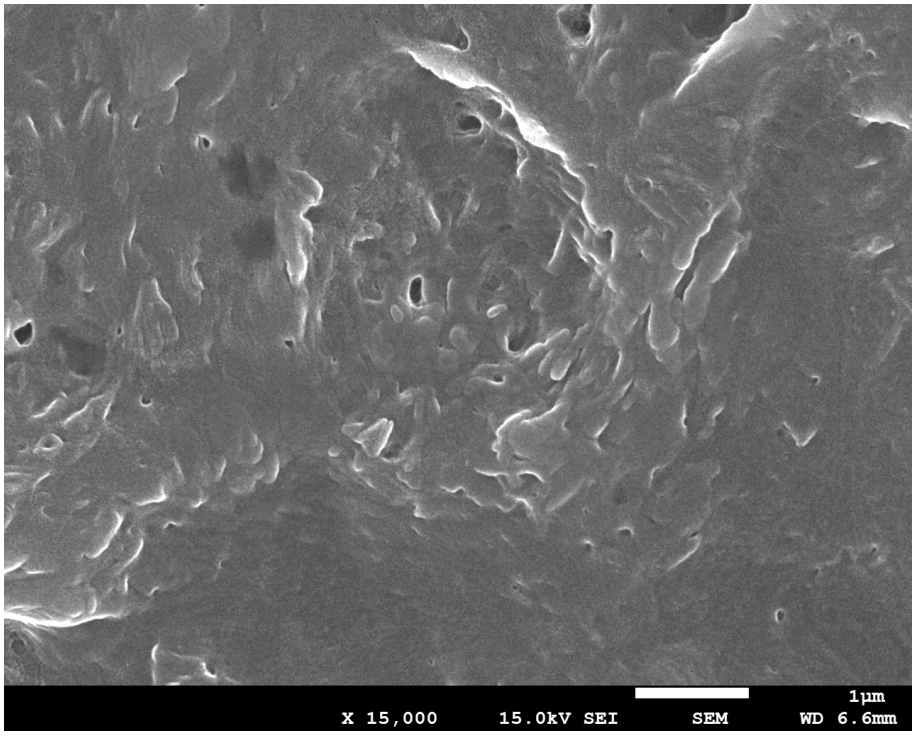


Figure 26: Scanning Electron microscopic analysis for surface study of polymer sheet (15,000x magnification)

Thermogravimetric analysis (TGA) of the polymer is represented as a graph in Figure 27. Figure 27A represents three step degradation of the polymer. The first degradation of polymer started at 141°C, which accounts for the main degradation process, whereas maximum degradation temperature was 215.9°C (Table 26, Figure 27B). Second step degradation started at 239.4°C, followed by 358.8°C (Figure 27A) (Aldas et al., 2020).

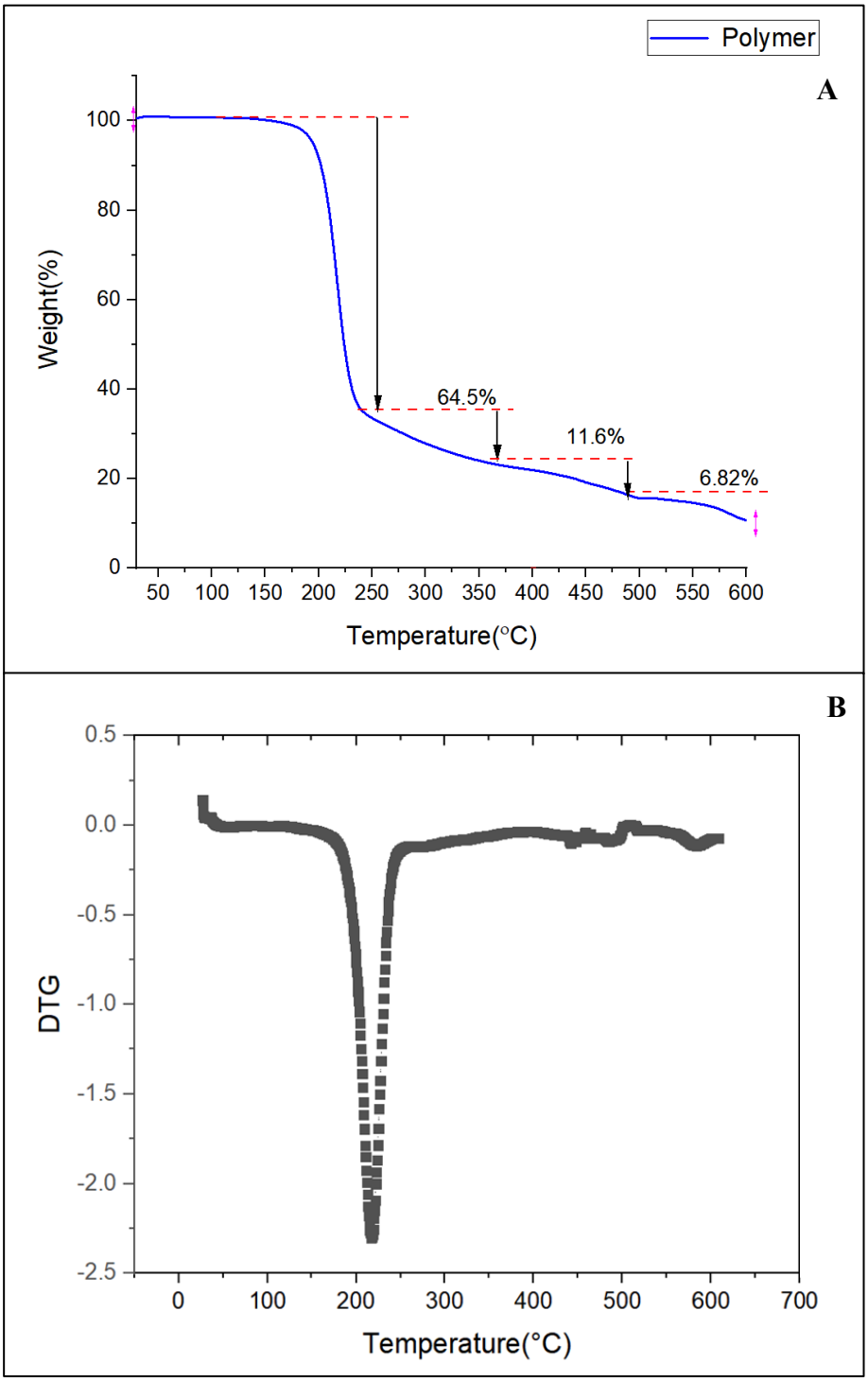


Figure 27: A) Thermogravimetric analysis (TGA) B) and derivative curve (DTG) of polymer showing thermal degradation

Molecular weight analysis is a key property which determines the physical and mechanical characterization of a polymer. High molecular weight polymers are often sought after due to their effect on crystallinity and elasticity (Rehm and Steinbüchel, 1999; Samadhiya et al., 2022b). The molecular weight of the polymer was 1332 kDa with average molecules of 766 kDa. Higher molecular weight in polymers is favored since there can be a loss in molecular weight during the extraction process using sodium hypochlorite (Hahn et al., 1994). The molecular weight of the polymer produced in this study is higher than the commercially and other reported polymers, which gave this polymer more desirable for commercial applications (Tanadchangsaeng and Yu, 2012; Hernández-Núñez et al., 2019). Polydispersity index (PDI) represents the uniformity of molecules in a polymer. PDI value 1 indicates a monodisperse polymer whereas increase in its value represents increases the variance of Mw distribution in a polymer (Tsuge, 2016).

Table 26: Physical properties of extracted polymer

Properties	Values
Molecular Weight (Mw) (kDa)	1332
Poly Dispersity Index (PDI)	1.7
Z Average (Mz)	2706
Number Average (Mn)	766
Maximum Degradation Temperature (T_m °C)	215.9

Current study presents a polymer with a polydispersity index of 1.7 which is lower than reported PDIs. Lower PDIs are preferred for polymers as they impact the properties such as flexibility and toughness,

but it also depends on the polymer type and other factors (Rane and Choi 2005). According to a study PHB polymers when molded can show a lower molecular weight and higher PDI, caused by the thermal effect but having a high molecular weight and lower PDI would positively impact the characteristics of produced biopolymer (Lynd et al., 2008; Gilbert et al., 2009; Malinconico et al., 2014). The polydispersity index is a key factor in determining the mechanical properties of polymer, which is often ignored, but can be utilized to mold the polymer into a better version for commercial applications (Gentekos et al., 2019).

D. Conclusions

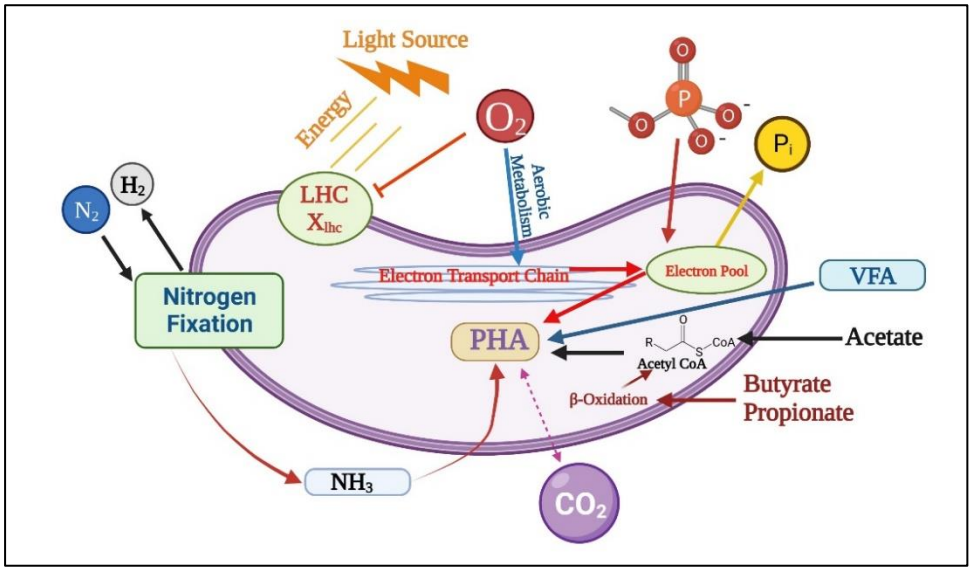
This chapter extensively investigates the various factors for enhanced PHA production in three native microalgae: *Coelastrella* sp., *E. texensis*, and *Pectinodesmus* sp. The stress and supplementation used in this chapter are often present in wastewater in abundance or in trace amount. The effect of these parameters on microalgae helped us in screening the best microalgae sp. suitable for the PHA production in wastewater for future studies. This study also provides a deep understanding of these factors on other bio macromolecules such as lipid, carbohydrate, protein and pigments as well as growth kinetics, which are important for establishing a microalgal biorefinery. Additionally, a 1.7 folds increase in PHA production was found in comparison to the previous study and 142 folds increase from control conditions, indicates that the factors used in the study have positively impacted PHA accumulation. Heavy metal stress also helped in visualizing the tolerance of microalgae towards HM and the positive impact on lipid, and carbohydrate accumulation in accordance with PHA. PCA analysis further supported the data and correlation among factors were explained using PC1 and PC2. The characterization study of synthesized polymer revealed the important characteristics, ensuring the applicability of produced polymer. *E. texensis* had proven to be the best microalgal species for PHA accumulation under various stress and producing an ultra-high molecular weight polymer with low polydispersity. Further pilot scale

studies would be designed for scale-up using wastewater for a sustainable biorefinery. *E. texensis* would be used as sole microalgae for PHA accumulation in formulation of a tailor-made consortium with a photosynthetic bacterium. The medium conditions would be used for creating a suitable medium for tailor-made consortium.

The next chapter explains the isolation and screening details of purple non-sulfur bacteria from Indore. The bacteria isolated was explored for growth curve analysis, time dependent PHA accumulation. Further it explores the PHA accumulation capacity of the isolated PNSB three VFAs along with phosphate variations in a time dependent study that would help in optimizing the parameters for high PHA synthesis in the other microbe in a tailor-made consortium. The varied nutrient supplementation would also help in further formulating the medium for consortium.

CHAPTER 4

Exploring indigenous purple non-sulfur bacteria for targeting PHA accumulation



A. Introduction

With increasing population, there is a hike in consumer demand now more than ever. In a recent article by Times of India, India's plastic waste generation is projected to have a 10 times higher growth by the year 2030 (2023, January 12). These frightening figures can be a projection from 3 to 5 years ahead from now but there is a giant problem coming towards and we must start preparing, before it gets out of hand. To battle the upcoming challenges with plastic pollution we can opt for the alternative option to plastic such as Bioplastics. Bioplastics are polymers often originated from microorganisms and biodegradable in nature (Suriyamongkol et al., 2007). Polyhydroxyalkanoate consists of a carbon backbone and based on the number of carbons they can be divided in to three categories: Short chain length (3- 5 carbon), medium chain length (6-14 carbon) and long chain length (15 and more carbon). These can also be divided on the basis of the composition: Homopolymer (containing only one type of chain) such as polyhydroxy Butyrate (4C), Polyhydroxy valerate (5C) and heteropolymer or copolymer (containing different types of chain) such as Poly-β-hydroxybutyrate - co-β-hydroxy valerate (PHBV) (Koller, 2020). In the last two decades, PHA has emerged as the leading bioplastic majorly focusing on Polyhydroxy Butyrate (PHB) (Koller et al., 2010; Albuquerque et al., 2013; Fradinho et al., 2014; Bhati and Mallick, 2015; Koller, 2018). The applications of these biopolymers can be assessed by various physical, chemical and mechanical properties, for instance, glass transition temperature and degree of crystallinity is responsible for the elasticity of the polymer. Short chain length polymers have high crystallinity as their counterparts. In the case of mechanical properties, three major properties to focus on are tensile strength, young's modulus and elongation at break, which represents rigidity, hardness and flexibilities. These three properties can be altered when the position of the side chains is changed (Ilyas et al., 2020; Kumar et al., 2020). PHBs

have been FDA approved since 2007 for surgical and dental uses (Zhang et al., 2018).

Biopolymer is a giant rock under the ocean, where only the tip is exposed. There is a lot to explore and with the untiring efforts of researchers soon the whole world will be able to reap the fruits of the tree "Microbial Biopolymer". Purple non-sulfur bacteria are one such microbe that can fight this battle with rising plastic mountains. Purple non-sulfur bacteria are photosynthetic bacteria, belonging to the protobacteria group. They have been reported for various applications such as biopolymer production, hydrogen production, fuel cells, and bioremediation of nutrients and various heavy metals such as chromium, selenium, lead etc. (Adessi et al., 2016; Talaiekhozani and Rezania, 2017; Touloupakis et al., 2021). *Rhodospirillum rubrum* can produce copolymer of PHA under VFA supplementation and has been reported for the remediation of heavy metals selenium and cadmium (Bayon et al., 2020; Brandl et al., 1989; Monroy and Buitron, 2020). *Rhodopseudomonas*, and *Rhodobacter* sp. both have been reported PHA production under various VFAs including acetate, succinate, malate, pyruvate, and heptanoate (Carlozzi & Sacci, 2001; Monroy & Buitron, 2020; Kranz et al., 1997). They have also been reported to remediate wastewater including Swine wastewater, municipal wastewater, winery and dairy wastewater to produce high value compounds (Kim et al., 2004, Bengtesson et al., 2017, Corona and Buitron 2021; Kars and Alparslan, 2013).

Purple non-sulfur bacteria have been extensively studied for PHA production as pure culture as well as mixed culture (Fradinho et al., 2014; Alloul et al., 2019). Photosynthetic mixed cultures are often dominated by PNSB (Ogbonna et al., 2000). Their anaerobic or facultative anaerobic nature, wide array of application and adaptability makes them a perfect candidate for the formation of a tailor-made consortium.

B. Material and methods

B.1 Isolation of PHA producing bacteria and maintenance of the cultures

Various photosynthetic mixed cultures (PMC) majorly constitute of PNSB and microalgae. Purple bacteria are often found in shallow water ponds, so water samples were collected from a Shallow Pond outside IIT Indore.

Minimal salt media (MSM) was used to isolate the PHA producing bacteria (Fradinho et al., 2014). One liter of MSM consist of 0.4 g Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 0.8 g Sodium chloride (NaCl), 0.55 g Ammonium chloride (NH_4Cl), 0.1 g Calcium chloride dihydrate ($CaCl_2.2H_2O$), 2.45 g Sodium acetate (CH_3COONA), 0.085 g Di potassium hydrogen phosphate (K_2HPO_4), 0.065 Potassium di hydrogen phosphate (KH_2PO_4), 10 mL Iron citrate solution (1 g/L), 2 mL trace element solution. pH was adjusted to 6.5 using mild acid or base. In 100 mL reagent bottle (RB) 100 mL media was taken and 10% sample was inoculated and kept in Being plant growth chamber at 30^oC under 3000 lux in continuous illumination. RB was manually inverted 2-3 times, twice a day for mixing. Cells were isolated using sector plate method (Katz, 2008).

B.2 Experimental set-up

Effect of shaking was studied on the growth profile as well as PHA accumulation of the isolated strain. Cultures were grown in MSM medium. Initial optical density was kept at 0.2, under continuous illumination at 3000 Lux. Optical density was measured at 600 nm every 24 hrs. PHA accumulation was studied at different time interval (every 24 hrs) till 4th day after inoculation under still conditions. The code used for the growth media is indicated as $TP_{0.15}Amm_{0.55}A_{2.45}$, where TP indicates the concentration of K_2HPO_4 and KH_2PO_4, Amm indicates ammonium chloride and acetate is denoted by A. The concentrations are indicated in g/L.

Two stage cultivation was used for the experiments. Cells were inoculated in modified MSM, which consist of 0.022 g Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 0.044 g Sodium chloride (NaCl), 0.006 g Calcium chloride dihydrate ($CaCl_2.2H_2O$), 0.005 g Di potassium hydrogen phosphate (K_2HPO_4), 0.004 Potassium di hydrogen phosphate (KH_2PO_4), 0.5 mL Iron citrate solution (1g/L), 0.11 mL trace element solution. pH was adjusted to 6.5 using mild acid or base. Initial cell density was kept at 1 at OD_{600nm} and three volatile fatty acids (VFAs) acetate C_2, propionate C_3, butyrate C_4, and mixture of VFAs (2Ace:1But:1Prop) were used at a 4000 mg/L. Two different experiments with phosphate repleted and depleted conditions were performed with above mentioned VFAs. Codes used for this chapters are presented in the Table 27.

Cells were cultivated for 4 days under still condition 100 mL culture was taken out every 24 hrs till 4th day. Dissolved oxygen (DO) is the amount of oxygen present in surrounding aqueous environment. The amount of DO impacts the synthesis of various products in microbes. The cultures were used for pH and DO estimation. DO was measured in supernatant of purple bacteria cultures using HACH DO meter. For pH estimation pH meter was submerged in cell suspension and the pH was recorded.

Optical density ($OD_{600\ nm}$) of cultures was then measured to investigate the growth of PNSB. Biomass production (µg/mL) was calculated using OD vs Dry weight linear curve prepared for each individual species. After measuring cell density, cultures were centrifuged at 7500 rpm, and pellet was again washed with distilled water and lyophilized. Lyophilized cells were used for PHA accumulation test using GC-FID.

Table 27: Medium codes used in the chapter

Codes	Description
$TP_{0.15}Amm_{0.55}A_{2.45}$	MSM growth medium for PNSB, Acetate (2.5 g/L) 0.15 g/L Phosphate and 0.55 g/L Ammonium
$TP_{0.15}Amm_{0.55}A_{2.45}T_0$	MSM growth medium for PNSB 0th day, Acetate (2.5 g/L) 0.15 g/L Phosphate and 0.55 g/L Ammonium
$TP_{0.15}Amm_{0.55}A_{2.45}T_1$	MSM growth medium for PNSB 1st day, Acetate (2.5 g/L) 0.15 g/L Phosphate and 0.55 g/L Ammonium
$TP_{0.15}Amm_{0.55}A_{2.45}T_2$	MSM growth medium for PNSB 2nd day, Acetate (2.5 g/L) 0.15 g/L Phosphate and 0.55 g/L Ammonium
$TP_{0.15}Amm_{0.55}A_{2.45}T_3$	MSM growth medium for PNSB 3rd day, Acetate (2.5 g/L) 0.15 g/L Phosphate and 0.55 g/L Ammonium
$TP_{0.15}Amm_{0.55}A_{2.45}T_4$	MSM growth medium for PNSB 4th day, Acetate (2.5 g/L) 0.15 g/L Phosphate and 0.55 g/L Ammonium
$TP_{0.09}Amm_0A_4$	MSM Accumulation medium for PNSB with Acetate (4 g/L) 0.09 g/L Phosphate and 0 g/L Ammonium
$TP_{0.09}Amm_0Pr_4$	MSM Accumulation medium for PNSB With Propionate (4 g/L) 0.09 g/L Phosphate and 0 g/L Ammonium
$TP_{0.09}Amm_0B_4$	MSM Accumulation medium for PNSB with butyrate (4 g/L) 0.09 g/L Phosphate and 0 g/L Ammonium
$TP_{0.09}Amm_0 A_2Pr_1B_1$	MSM Accumulation medium for PNSB Mixed Acetate (2 g/L), Propionate (1 g/L), Butyrate (1 g/L) 0.09 g/L Phosphate and 0 g/L Ammonium
$TP_0Amm_0A_4$	MSM Accumulation medium for PNSB with Acetate (4 g/L) 0 g/L Phosphate and 0 g/L Ammonium
$TP_0Amm_0Pr_4$	MSM Accumulation medium for PNSB With Propionate (4 g/L) 0 g/L Phosphate and 0 g/L Ammonium
$TP_0Amm_0B_4$	MSM Accumulation medium for PNSB with butyrate (4 g/L) 0 g/L Phosphate and 0 g/L Ammonium
$TP_0Amm_0 A_2P_1B_1$	MSM Accumulation medium for PNSB Mixed Acetate (2 g/L), Propionate (1 g/L), Butyrate (1 g/L) 0 g/L Phosphate and 0 g/L Ammonium

B.3 Polyhydroxyalkanoate analysis

Polyhydroxyalkanoates are accumulated as PHA granules inside cells. They are a type of biopolymer which can be analysed using GC-FID by

converting into methyl esters. For converting PHAs into FAME (fatty acid methyl esters), method given by Oehmen et al. (2005) was used.

B.4 Multivariate statistical analysis

Principal component analysis (PCA) was performed as statistical analysis. The data obtained in this chapter was divided into 4 different groups namely, acetate, propionate, butyrate and a mixture of butyrate. PCA was performed using RStudio version 4.3.0.

B.5 Solvent extraction for polymer and characterization

PHA is accumulated in cells in a form of granules. These granules have a polyester core which is surrounded by lipids and protein which acts as a protective shield against cellular components. For their extraction from cells 40% Sodium hypochlorite was added in cells and were incubated for 3 hrs at room temperature and centrifuged at 7500 rpm for 10 minutes. Pellet was washed twice with 70% isopropanol and 70% ethanol and dried overnight in oven (Tamang et al., 2019). Extracted polymer was used for physical and chemical characterization, to determine its properties.

B.5.1 Polymer film surface study

Films of polymer were made using 1% polymer in chloroform and casted onto glass plates. Films were further dried in oven at 50^{o}C for 3 hrs (Savenkova et al., 2000). Field Emission Scanning Electron Microscope (FE-SEM) was used to study the surface morphology of polymer film. Before SEM analysis films were pasted on the stub with carbon tape to increase conductivity and coated with Copper (Cu) for 2 minutes. Micrographs were recorded using (FE-SEM, Supra55 Zeiss) at 5kV and 10000x magnification (Bhowmick et al., 1980).

B.5.2 Thermal properties

Thermal gravimetric analysis was used to determine the weight percentage degradation and maximum degradation temperature. Origin

version 9.1 was used derivatization and plot preparation (Savenkova et al., 2000).

B.5.3 Molecular weight determination (MW)

Molecular weight of extracted polymer was determined using gel permeation chromatography (GPC) equipped with RI detector. Samples were analysed at Sardar Patel Centre for Science and Technology (SICART), Anand, Gujrat India. Polymer was dissolved in tetra hydro furan (THF) and used for analysis.

C. Results and discussion

C.1 Isolation of Purple non-sulphur bacteria

Samples were inoculated in the MS medium. After 7 days of cultivation the medium turned green showing algal growth, whereas after 20 days the medium turned reddish purple (Figure 28 A, B).

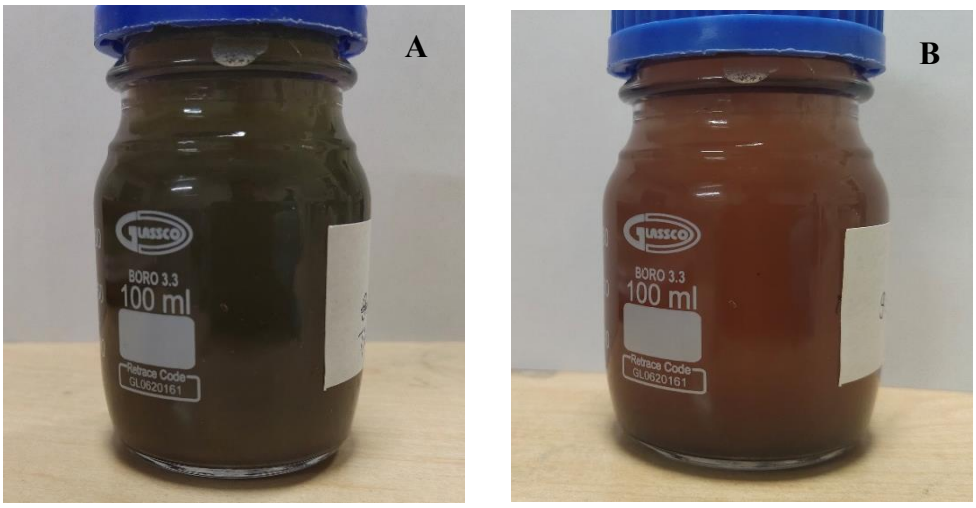


Figure 28: Isolation of bacteria in Minimal Salt medium (MSM) from the sample collected from outside pond of IIT A) 7 Days of Cultivation, B) 20 days of cultivation

After reddish purple color was visible, culture was inoculated in plates containing 1.5% agar, sector plate streaking was used. After separate colonies were visible on plate, individual colonies were transferred to another plate using a nichrome wire loop and used as an inoculum for

mother cultures. The isolated colonies were orangish red in color and smaller in size (Figure 29) (Higuchi-Takeuchi et al., 2016a).

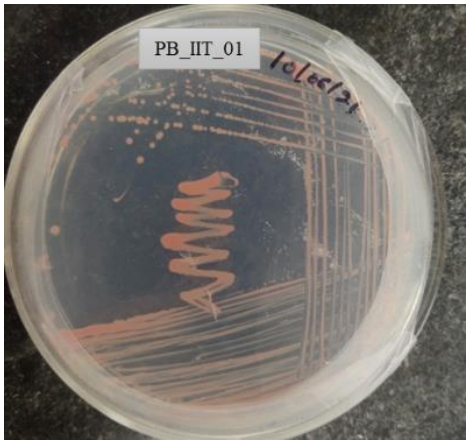


Figure 29: Isolated Colonies of Purple Non-Sulphur Bacteria

Based on these preliminary tests, the isolate was identified as purple-non sulfur bacteria and would be referred to as PB_IIT_01 (Bergey, 2001).

Purple non-sulphur bacterium (PNSB) can be facultative anaerobic, gram-negative and belongs to proteobacterium family. It is anoxygenic bacteria, which do not use oxygen (O_2) as an electron donor during photosynthesis, instead hydrogen (H_2), or organic acids can be used. Hence they do not produce O_2 during the photosynthesis (Vethanayagam and Krishnamurthy, 1995). It has been extensively studied for PHA and hydrogen production (Bianchi et al., 2010; Adessi et al., 2016; Alloul et al., 2019).

C.2 Effect of stirring on growth

The isolated strain was cultivated for 15 days, and the growth pattern was studied in two different cultivation conditions: stirring and still. Stirring is often used for bacterial cultures to ensure mixing of nutrient and aeriation of cultures (Duetz and Witholt, 2001; De Almeida et al., 2010). PB_IIT_01 being a facultative anaerobe could survive in both in

the presence and absence of oxygen. In Figure 30, a growth pattern of PB_IIT_01 can be observed, showing the growth during cultivation period of 15 days.

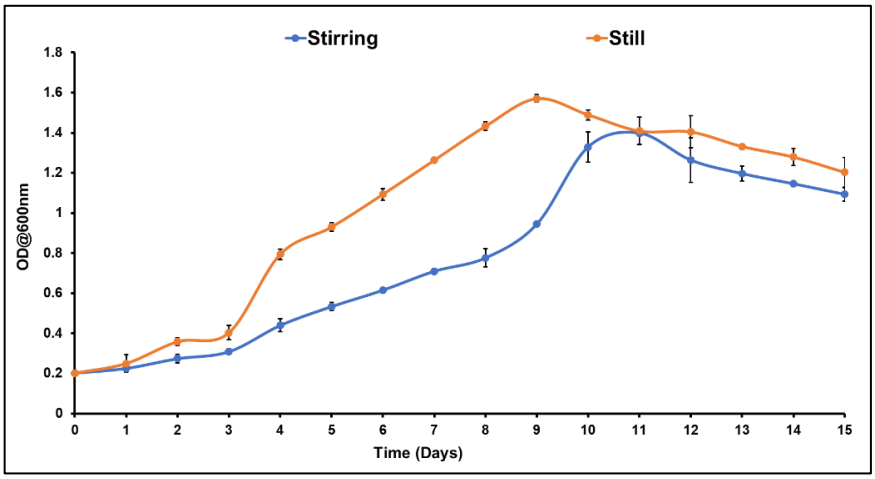


Figure 30: Optical density against cultivation time depicting growth curve of PB_IIT_01

A lag phase can be observed during first three days of growth, log phase of the species was of 7 days, during which still cultivation mode resulted in higher growth rate. Whereas in stirring conditions the growth rate was less. Stationary phase was reached on 9th day under still conditions whereas in stirring conditions it was on 10th day. A decline phase was observed straightaway, under both cultivation mode. Due to higher growth rate in still conditions were selected as the best cultivation condition for PB_IIT_01.

C.3 Time course study of PHA accumulation in PB_IIT_01

After the growth curve study, the cells were subject to PHA accumulation in the growth medium with acetate as carbon source. Cells were able to accumulate PHA from the very first day of their growth. Highest accumulation of PHA was on 2nd day where cells accumulated 8.2 µg/mg per dry biomass. After second day, there was a decline in the PHA accumulation where cells could not accumulate more PHA and

subsequently the concentration decreased (Figure 31). Acetate is one of the precursors of PHA accumulation pathways, and abundance of acetate in the growth media, could lead to the storage of PHA under cells.

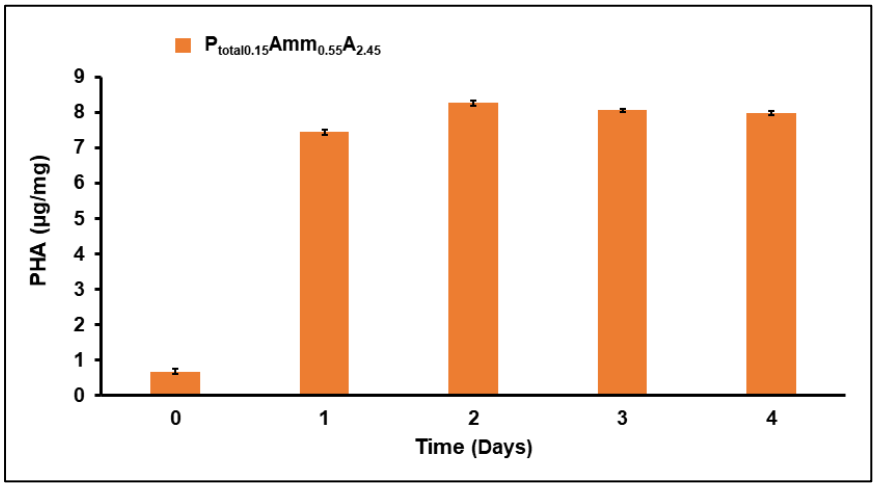


Figure 31: PHA accumulation against cultivation time under growth media supplementation

However, C/N ratio is also one of the major influences when it comes to triggering the storage compounds. In addition, abundance of nutrient in the growth medium supported the cell's metabolic activities, resulting in higher growth rate as compared to high PHA accumulation.

C.4 Effect of volatile fatty acids coupled with varied phosphate conditions on biomass accumulation

Volatile fatty acids (VFAs) such as acetate, butyrate, and propionate are often found in acidified industrial wastewater, and makes a major part of COD of wastewater. Acetate is the smallest organic acid, which is also a component of the growth medium, so cells are accustomed to the presence of acetate and able to take up and utilize it. On the other hand, limitation of nutrients in the accumulation medium, seized the growth of cells in the accumulation medium. Highest biomass accumulation was achieved under propionate supplementation (412.3±23.0 µg/mL) on 4^{th} day, followed by on 3^{rd} day (396.4±13.6 µg/mL) under phosphate replete conditions which is 1.5 times higher than initial concentration (Figure 32 A). Phosphate being the key component of DNA (deoxyribonuclease), plays an important role in cell growth and division.

Higher biomass accumulation has been observed in cultures, grown in medium containing phosphate as compared to medium depleted of phosphate. Propionate can be converted into propionyl Co-A which can then be incorporated into TCA cycle producing energy for higher biomass accumulation. Butyrate can have lower consumption rate in the cells resulting in the lowest biomass accumulation (Fradinho et al., 2014). Phosphate depletion could seize the biomass production resulting in lower biomass accumulation, but presence of organic acids helps the cells survive and grow at a lower rate. Highest biomass production in phosphate replete conditions were with co-substrate supplementation at 4th day (357.7±21.5 µg/mL) followed by propionate at 4th day (336.8±15.4 µg/mL) (Figure 32 B).

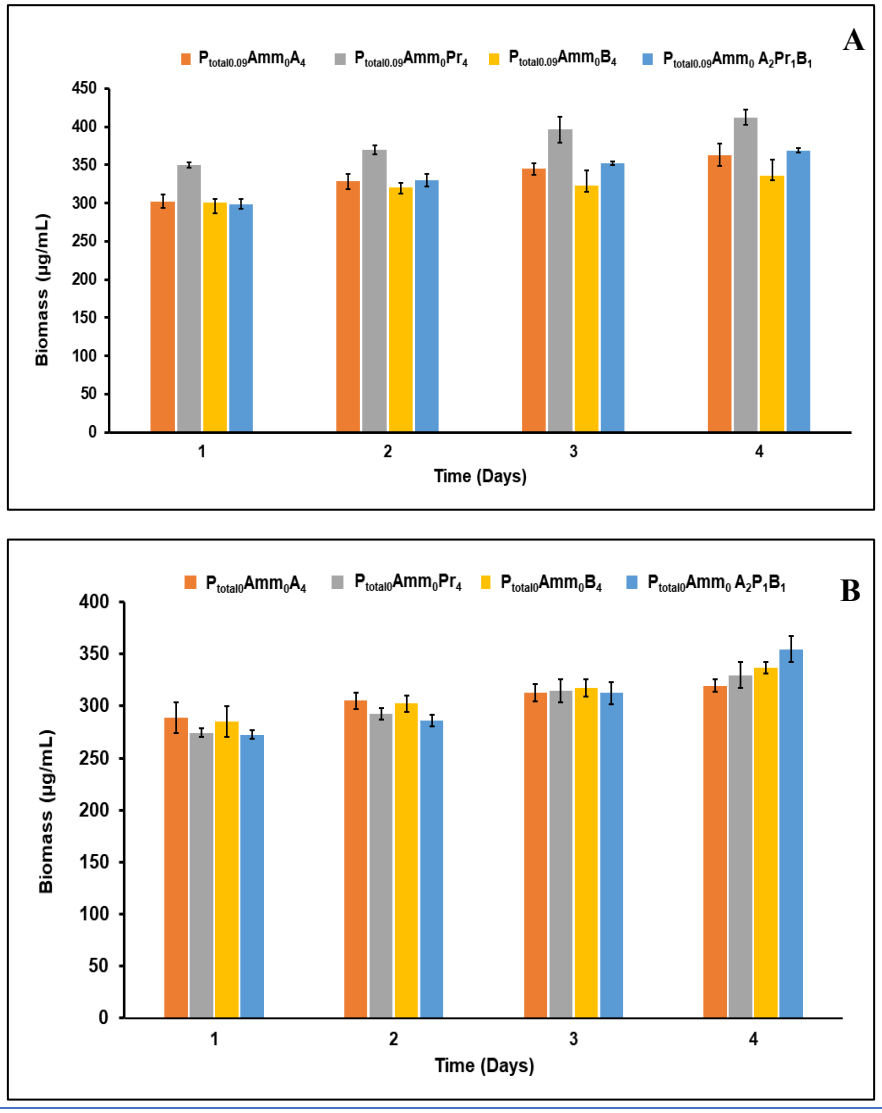


Figure 32: Biomass accumulation in PB_IIT_01 under VFA supplementation A) Phosphate repletion, B) Phosphate depletion

In the absence of phosphate, conversion of propionate or butyrate is much more energy consuming as opposed to co-substrate where presence of acetate enables the better consumption of other VFAs resulting in higher biomass accumulation. However during the conversion of acids, ATP breakdown generated a small amount of inorganic phosphate enabling a slower growth rate even in the complete absence of phosphate (Alloul et al., 2019).

C.5 Effect of volatile fatty acids coupled with varied phosphate conditions on PHA accumulation

The experiment was designed to increase the PHA accumulation in PB_IIT_01 by creating the energy deficit by reducing the nutrient quantity. Many reports have concluded that nutrient limitation mainly nitrogen and phosphates trigger PHA accumulation (Lenz and Marchessault, 2005). While cultivated in the growth medium, cells could grow better but couldn't accumulate high amount of PHA and soon a decline in PHA accumulation was seen (Figures 31, 33). Nutrient limitation seizes the cell growth resulting in redox imbalance, creating a pool of acetyl co-A and NADPH which could positively influence storage molecule synthesis, mainly PHAs (Johnson et al., 2010). PHA also serves as a storage carbon reserve in bacteria which can be utilized in the events of nutrient limitation leading to energy shortage (Fradinho et al., 2016; Silva et al., 2017). An increasing trend was observed in PHA accumulation under all VFA supplements till 3rd day, after that a decrease was observed at 4th day. Highest PHA accumulation was observed under acetate supplementation under the absence (95.3±3.1 µg/mg) and presence (94.7±3.1 µg/mg) of phosphate (Figures 34 A, B). It was 11 folds higher than PHA accumulation in growth medium. The difference between PHA accumulation under presence and absence of phosphate was not huge under acetate supplementation considering the rapid uptake of acetate inside the cells and feasibility of acetate assimilation into pathways leading to higher accumulation of PHA. PHA accumulation was lowest under propionate, considering the high energy

uptake for its assimilation into the synthesis pathway. A study investigating the PHA accumulation in acidified waste-water suggest the affinity of microbes towards acetate for PHA production indicating lower PHA yield under supplementation of propionate or butyrate (Tamang et al., 2021). Conversion of acetate into acetyl Co-A takes place under acetyl-Co synthase (ASC). External supply of acetate would increase the rate tricarboxylic acid cycle (TCA), thus increasing the rate of PHA synthesis. A study on acetate induced PHA accumulation revealed the increased expression levels of isocitrate dehydrogenase which converts isocitrate into 2-oxoglutarate a step in TCA cycle which prove that acetate supplement could increase rate of TCA cycle. It also shows the highest PHA accumulation under acetate supplementation as oppose to other carbon source (Higuchi-Takeuchi and Numata, 2019).

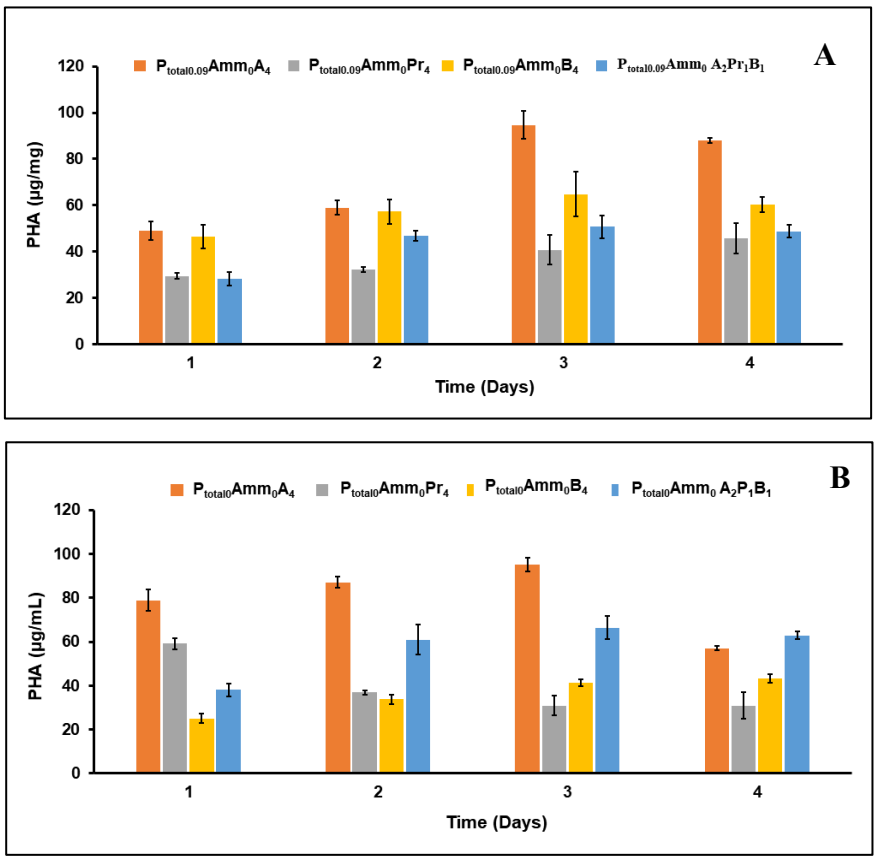


Figure 33: PHA accumulation in PB_IIT_01 under VFA supplementation A) Phosphate repletion, B) Phosphate depletion

Another pathway found in *R. sphaeroides,* puts forth the possibility of direct assimilation of acetate resulting in higher PHA production. Ethyl-malonyl Co-A pathway (EMC), which suggests that conversion of acetate into intermediates of TCA cycle could takes place resulting in higher rate of TCA cycle as well as acetyl Co-A. Among supplemented VFAs acetate has also been reported to be the best VFA supplementation for PHA accumulation in PB_IIT_01. (Bianchi et al. 2010; Luo et al., 2018; Montiel-Corona and Buitrón, 2022). PHA accumulation reported in this study is higher than previously reported study under acetate supplementation under similar cultivation conditions (Carlozzi and Sacchi, 2001; Touloupakis et al., 2021; Montiel-Corona and Buitrón, 2022).

C.6 Effect of volatile fatty acids coupled with varied phosphate conditions on pH and dissolved oxygen (DO)

Apart from C/N ratio, dissolved oxygen concentration also plays a major role and is an indicator of PHA accumulation in microbial systems. pH of the environment surrounding microbial cells have a major effect since enzyme activity is highly fluctuate due to change. pH and DO were investigated every day during the experiment and represented in Figure 34. An increase in pH was observed during the cultivation (up to 9.7). Increased pH can interfere with the uptake rate of C-sources by increasing the energy required for their transformation (Filipe et al., 2001; Montiel-Jarillo et al., 2017). DO often used in mixed culture cultivation to track the activity of the microorganisms. High DO indicate the absence of carbon source whereas decrease in DO is associated with the high metabolic activities in microorganisms (Tamang et al., 2019; Tamang and Nogueira, 2021).

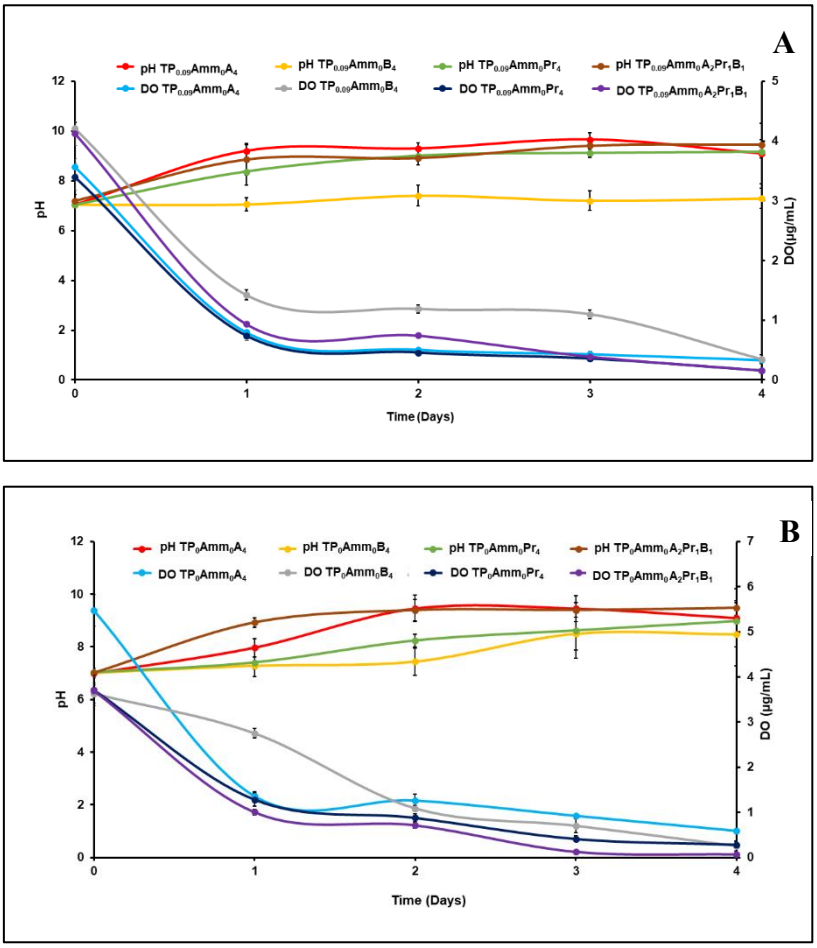


Figure 34: Dissolved oxygen (DO) and pH in PB_IIT_01 cultures under VFA supplementation A) Phosphate repletion, B) Phosphate depletion

During batch conditions, concentration of substrate cannot be controlled or replete thus having a direct indicator of the presence of carbon can clarify the carbon metabolism to avoid the degradation of accumulated PHA under energy crisis. In the present study DO drop drastically after inoculation, which shows the rapid uptake of VFAs. Thus, resulting in the PHA accumulation. In a recent study relation between DO and PHA accumulation was studied concluding that even in low DO concentration PHA accumulation can take place whereas nitrification process is halted. Indicating the importance to also take an account of DO while the PHA production process (Wang et al., 2019).

C.7 Principal Component analysis of the results obtained

PCA revealed that 87% of variance in the data could be explained using PC1 and PC2. Eigen value more than 1 has more predictive power hence dimension 1 was chosen as the key dimension to understand the contribution of various factors in the variance (Table 28) (Abdi and Williams, 2010).

Table 28: Eigen value and variance for the 4 dimensions of PCA

Dimensions	Eigenvalue	Variance (%)	Cumulative variance (%)
Dimension 1	2.8	71.1	71.1
Dimension 2	0.6	15.8	87.0
Dimension 3	0.3	7.9	94.9
Dimension 4	0.2	5.1	100

Figure 36 depicts the contribution of individual medium composition at various days. The red dotted line determines the average contribution of factors on variance. Bars higher than red dotted line showed the composition having higher degree of importance for the component whereas bars lower than the line hold lower significance (Lid and Planning, 1993).

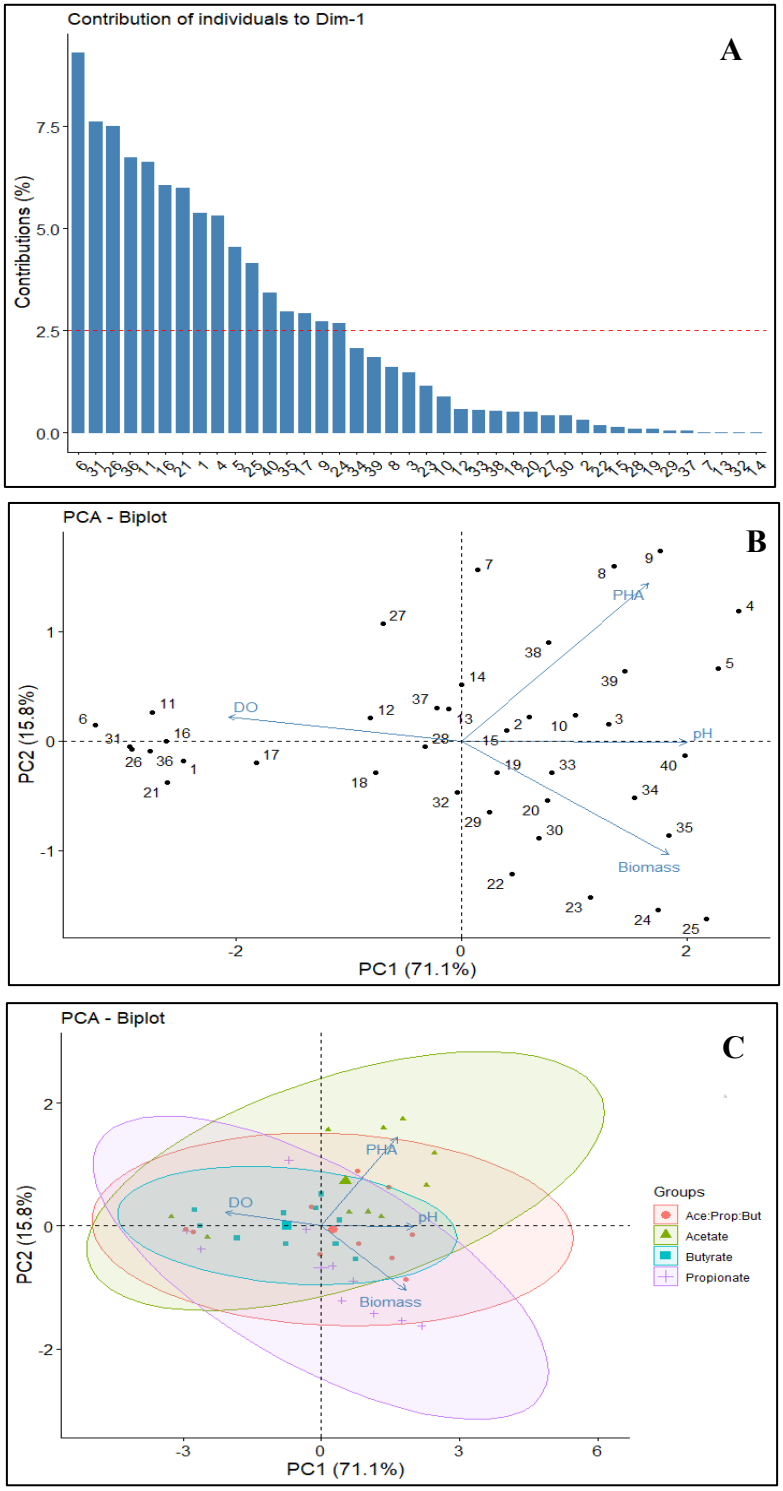


Figure 35: Bars in figure (A) are the contribution of individuals (combinations) to principal component 1. PCA biplots (B, C) show loadings of PHA, biomass, pH and DO (variables or vectors) of isolate PB_IIT_01 across different combinations of nutrients

Codes shown in figure 36 A &B are as follows: 1- $TP_{0.09}Amm_0A_4t_0$, 2- $TP_{0.09}Amm_0A_4t_1$, 3- $TP_{0.09}Amm_0A_4t_2$, 4- $TP_{0.09}Amm_0A_4t_3$, 5- $TP_{0.09}Amm_0A_4t_4$, 6- $TP_0Amm_0A_4t_0$, 7. $TP_0Amm_0A_4t_1$, 8. $TP_0Amm_0A_4t_2$, 9. $TP_0Amm_0A_4t_3$, 10. $TP_0Amm_0A_4t_4$, 11. $TP_{0.09}Amm_0B_4t_0$, 12. $TP_{0.09}Amm_0B_4t_1$, 13 $TP_{0.09}Amm_0B_4t_2$, 14. $TP_{0.09}Amm_0B_4t_3$, 15. $TP_{0.09}Amm_0B_4t_4$, 16. $TP_0Amm_0B_4t_0$, 17. $TP_0Amm_0B_4t_1$, 18. $TP_0Amm_0B_4t_2$, 19. $TP_0Amm_0B_4t_3$, 20. $TP_0Amm_0B_4t_4$, 21. $TP_{0.09}Amm_0P_4t_0$, 22. $TP_{0.09}Amm_0P_4t_1$, 23. $TP_{0.09}Amm_0P_4t_2$, 24. $TP_{0.09}Amm_0P_4t_3$, 25. $TP_{0.09}Amm_0P_4t_4$, 26. $TP_0Amm_0P_4t_0$, 27. $TP_0Amm_0P_4t_1$, 28. $TP_0Amm_0P_4t_2$, 29. $TP_0Amm_0P_4t_3$, 30.

$TP_0Amm_0P_4t_4$, 31. $TP_{0.09}Amm_0A_2Pr_1B_1t_0$, 32. $TP_{0.09}Amm_0A_2Pr_1B_1t_1$, 33. $TP_{0.09}Amm_0A_2Pr_1B_1t_2$, 34. $TP_{0.09}Amm_0A_2Pr_1B_1t_3$, 35. $TP_{0.09}Amm_0A_2Pr_1B_1t_4$, 36. $TP_0Amm_0A_2Pr_1B_1t_0$, 37. $TP_0Amm_0A_2Pr_1B_1t_1$, 38. $TP_0Amm_0A_2Pr_1B_1t_2$, 39. $TP_0Amm_0A_2Pr_1B_1t_3$, 40. $TP_0Amm_0A_2Pr_1B_1t_4$. (These codes are explained in section B.2). t indicates time in days.

Biplot depicted the significance of each factor in accordance with its variables. It also showed the correlation among factors. For instance, PHA and biomass both are positively correlated with pH whereas DO is negatively correlated with rest of three. The length of vector from origin indicates the strength of the vectors.

The combination present nearby PHA, pH and biomass vector indicate the variation in the vectors. Location of the scores or dot vectors also indicated their strength in the correlation (Figure 35B). The clustered biplot indicates the similarity of vectors under the influence of various VFAs. The data generated in this chapter was divided into 4 groups based on the VFA supplemented in the culture medium. The phosphate was not considered a factor in division due to its lower effect on PHA accumulation. In Figure 35 C, it is evident that presence of acetate inevitably increased PHA accumulation in PB_IIT_01, whereas biomass was more influenced by propionate supplementation. The spread of the vectors in the clustered biplot indicate the variance due to each VFA (Chen et al., 2022).

C.8 Polymer extraction and characterization studies

The extracted polymer was characterized using GC-MS and revealed that PHB concentration was higher in the polymer. Surface analysis revealed a bumpy structure followed by the irregular shapes on the surface showing an irregular distribution of the monomers (Figure 36). This could be due to the lower percentage of PHV in the co-polymer. studies have shown a more regular and smoother surface with higher amount of PHV in the polymer (Bhati and Mallick, 2012).

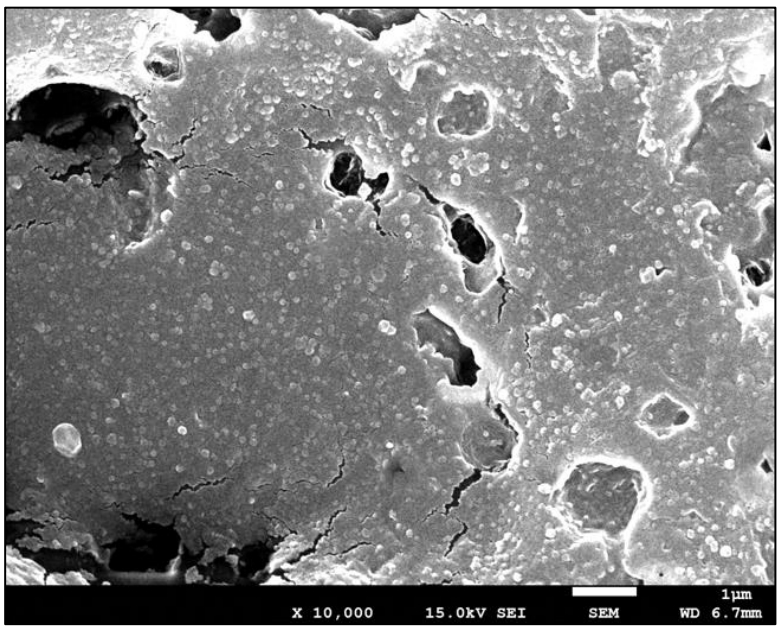


Figure 36: Surface analysis of polymer using Scanning Electron Microscopy (10,000x)

The thermal analysis of the polymer shows the thermal degradation of the polymer (Figure 37A). It shows three step degradation. Maximum degradation temperature of the polymer was calculated using derivatized thermal degradation map 210.4 °C (Figure 37B).

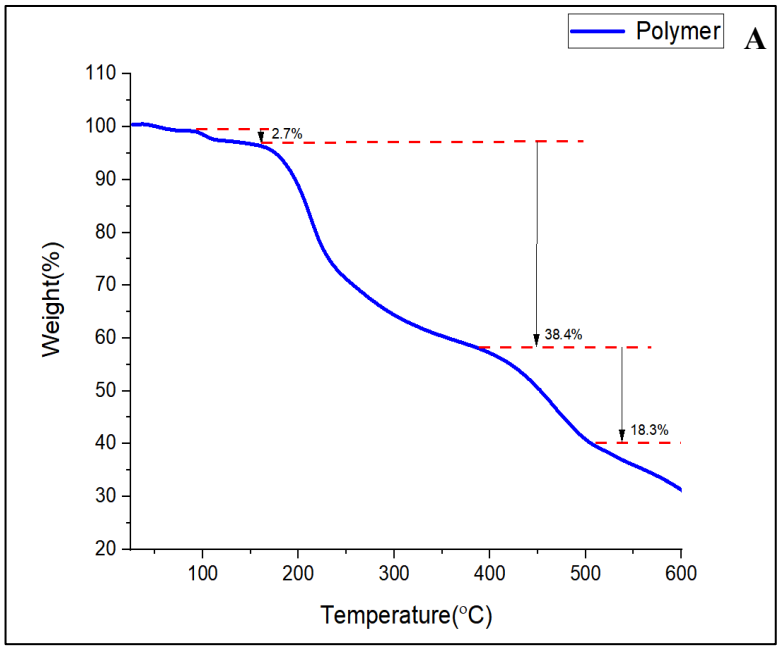


Figure 37 (A): Thermogravimetric analysis (TGA)

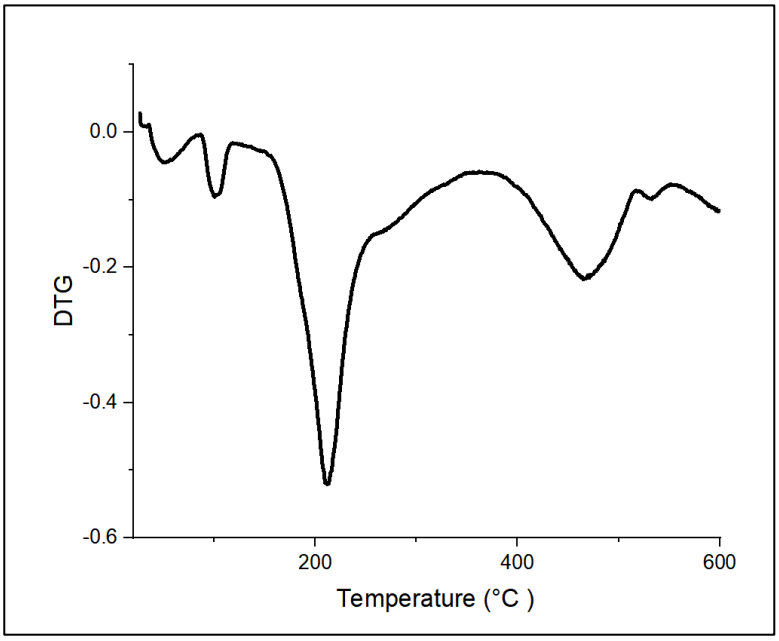


Figure 37 (B): and derivative curve (DTG) of polymer showing thermal degradation

Gel permeation chromatography revealed the molecular weight of polymer that was 1182 kDa. The produced polymer was a high molecular weight polymer which is reported to be produced in marine purple bacteria. The molecular weight of polymers holds the utmost importance due to their impact on mechanical properties linked to their applications. An important enzyme PhaZ has been reported to decompose PHA inside cells thus resulting in low molecular weight polymer. On the other hand, nitrogen limited conditions have been reported to decrease the production of the gene responsible enabling the production of higher molecular weight polymer (Higuchi-Takeuchi et al., 2016b). PhaC is also responsible for the enabling of higher chain formation during PHA synthesis (Sim et al., 1997). The Polydispersity Index (PDI) shows the uniformity of the molecules present in the polymer. PDI of 1.5 indicated the uniform distribution of molecules (Table 29). High molecular weight and lower PDI than literature prove the merit of this study indicating production of a better polymer which could be scale up for commercialization (Tsuge, 2016).

Table 29: Physical properties of extracted polymer

Properties	Values
Molecular Weight (MW) (kDa)	1185
Poly Dispersity Index (PDI)	1.5
Z Average (Mz)	1924
Number Average (Mn)	815
Maximum degrading Temperature (°C Tm)	210.37

D. Conclusions

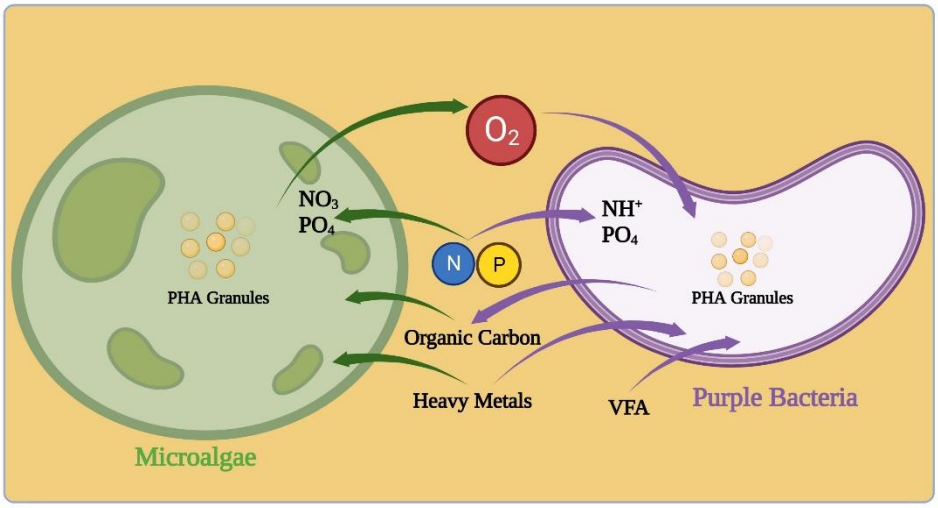
In this chapter purple non-sulphur bacterium was isolated from native regions of Indore to synthesize a tailor-made consortium of photosynthetic microorganisms. Different cultivation conditions revealed that isolate PB_IIT_01 preferred static mode of growth due to its facultative aerobic nature. PHA accumulation studies in growth medium (higher nutrient availability) have shown low PHA accumulation. Which led to the cultivation of cells under nutrient limited condition varied with presence and absence of phosphate. The highest accumulation of PHA was under acetate supplementation with phosphate depletion followed by presence of phosphate with acetate which was 11 folds higher than synthesized under growth medium supplementation. Acetate has been the most preferred C-source for PHA accumulation due to the higher assimilation capacity and the intermediatory effect with TCA cycle. However, phosphate did not seem to have higher impact on PHA accumulation in PB_IIT_01. This chapter also confirms the superiority of acetate as a carbon source than propionate and butyrate. DO and pH was also investigated to understand their relationship with PHA accumulation and prevent degradation of PHA. Nitrogen deficiency has played a key role in PHA accumulation

as well as the molecular weight determination of polymer. A high-molecular weight polymer was produced, PhaZ enzyme could be responsible which is suppressed under nitrogen deficiency. Investigation done in this chapter pave the way for the formulation of tailor- made consortium of photosynthetic microorganism for higher PHA accumulation.

Next chapter shows the formulation of a tailor-made consortium containing photosynthetic microorganism; highest PHA accumulating microalgae *E. texensis* which was optimized in chapter 3 (B), with isolate PB_IIT_01 form this chapter. The culture conditions optimized in previous chapter and this chapter will lay the foundation for the nutritional requirement of consortium.

CHAPTER 5

Algae-bacteria consortium for enhanced biopolymer production and its characterization



A. Introduction

Polyhydroxyalkanoate or more commonly known as PHAs have been present and explored by researchers for decades (De Almeida et al., 2010; De Koning, 1995; Lemoigne, 1926; Muhammadi et al., 2015; Samadhiya et al., 2023; Steinbüchel and Lütke-eversloh, 2003). PHA based products are now also reaching into commercial market and available in form of various products (Koller and Mukherjee, 2022). Even after decades of research high production and downstream processing still keeps the cost of PHA based polymer significantly higher than the commercial plastic (Kurian and Das, 2021). A major contribution to the cost of a final product is pure cultures as a host. More than 90% of the companies use pure heterotrophic bacteria as a host for microbial PHA production (Koller and Mukherjee, 2022). Although photosynthetic microbes have been investigated for PHA production and have obtained high PHA content as well yet their scale up into commercial is far-fetched (Ansari and Fatma, 2016; Bhati and Mallick, 2016; Samadhiya et al., 2022b; Touloupakis et al., 2021). The main bottleneck of commercialization of these species is the cost associated with pure culture cultivation (Wang et al., 2022). Mixed cultivation of two or more organisms can solve the problem associated with the cost of pure culture cultivation. Mixed cultures have been studied for various application including wastewater remediation, heavy metal removal and production of biopolymer utilizing wastewater (Muñoz et al., 2006; Tamang et al., 2019a; Tamang and Nogueira, 2021). Consortium of bacteria-bacteria, algae-bacteria and algae-algae have been investigated in the previous years and have successfully yielded desired product or waste remediation (Dhaouefi et al., 2022; Jiang et al., 2021; Nayak et al., 2022). Although mixed cultures seem to be the a savior for commercial production, incompatible consortium can lead to competition between microbes for nutrients that can result in lower production of desired products (Khatami et al., 2022).

In recent years photosynthetic mixed cultures (PMCs) have also gain popularity evidently as a biopolymer producers (Fradinho et al., 2016;

Reis et al., 2003). These PMCs are often present in nature and enriched using feast-famine growth regime to filter PHA accumulating microbes to make the consortium more productive (Fradinho et al., 2016; Nguyenhuynh et al., 2021). Algae and purple bacteria are two abundant organism present in this consortium (Fradinho et al., 2013). Microalgae and purple non-sulfur bacteria (PNSB) are photosynthetic organisms but the mechanism of photosynthesis differ from in both, where microalgae have oxygenic photosynthesis, PNSB do not produce oxygen during photosynthesis, they utilize organic acid or hydrogen as an electron donor (Mothersole et al., 2018). Microalgae and purple bacteria can form a mutualistic symbiotic relationship with each other by sharing and exchanging nutrients as well as complementing each other's metabolic activities. For instance, microalgae and purple bacteria both contain different chlorophyll i.e., chlorophyll a, b, and bacteriochlorophyll, respectively. These pigments can harvest different and absorb different spectrum of light which enables the consortium to have high light availability for photosynthesis (Simkin et al., 2022). Purple bacteria also possess nitrogenases which enables nitrogen fixation. These nutrients can be exchanged with in consortium. Oxygen can play an important role as well, wherein microalgae can provide oxygen to PNSB for their metabolic activity and organic acids can be utilized by microalgae (Fradinho et al., 2013). PMCs can be a good alternative to pure cultures but untargeted growth of microbes results in low yield of PHA and maintaining a feast and famine condition could not be feasible for upscaling (Fradinho et al., 2021; Samadhiya et al., 2022b). Thus, a tailor-made consortium could be an answer to reduce the untargeted growth as well as cost associated with pure culture. In this chapter a tailor-made consortium was developed, which will consist of high PHA producing microalgae and PNSB strains, a high initial inoculum (two-stage cultivation) of both microbes was used to prevent growth of other microbes. Biopolymer produced with this consortium was extracted and characterized to understand the effect of consortium on biopolymer

properties. First time in this study, the two microorganisms were cultivated together.

B. Material and methods

B.1 Maintenance of cultures

Microalgae *E. texensis*, and PNSB isolated PB_IIT_01 were used for consortium. Microalgae mother cultures were maintained in BG-11 medium (Kaushik, 1987). PNSB isolate was maintained in MSM (Fradinho et al., 2014).

B.2 Experimental setup

B.2.1 Time course study of consortium under high nutrient supplementation

The best PHA accumulation conditions were used for consortium. Microalgae and purple bacteria were first inoculated in the BG-11 medium with iron, galactose, and high nitrate supplementation ($P_{0.04}N_5Ga_{10}Fe_5$). The initial cell density of microalgae was kept at 0.5 OD_{680nm} and for purple bacteria 1 OD_{600nm} was selected. This was determined based on the division time of purple bacteria and microalgae to avoid the competition of nutrient between two microbes. The cells were grown under two different photoperiods i.e., 12:12 and 24:00. The following codes were used to denote the combinations $P_{0.04}N_5Ga_{10}Fe_5LD_{12:12}$, $P_{0.04}N_5Ga_{10}Fe_5LD_{24:00}$, here LD indicates light duration. Other codes have been explained in the previous chapter (chapter 3B). These cultures were cultivated for 3 days, followed by cells were harvested at the interval of every 24 hrs. PHA accumulation, biomass and chlorophyll estimation was performed to determine the best harvesting day.

B.2.2 Formulation of culture medium for the tailor-made consortium

Two different media were formulated to assess the growth and PHA accumulation capacity of the consortium. The best conditions obtained in previous chapters were utilized here to formulate the medium. The first medium A was based on BG-11 composition which also contained Fe in 0.005 g/L, nitrate in 5 g/L, acetate at 4 g/L and galactose at 10 g/L, this medium is denoted as medium A $LD_{12:12}$ for 12:12 photoperiod, and Medium A $LD_{24:00}$ for continuous light period. Medium B contained composition of MS medium for accumulation containing Fe in 0.005 g/L, nitrate in 5 g/L, acetate at 4 g/L and galactose at 10 g/L as well. The photoperiod codes were used same as above. The cells were also cultivated for the 3 days. A comparison study between pure and co-culture was carried out. Microalgae and PNSB were also cultivated in medium A and medium B.

B.3 Growth and chlorophyll estimation

Optical density at 600 nm and 680 nm was recorded to understand the growth pattern of purple bacteria and microalgae, respectively. For consortia both 600 nm and 680 nm wavelength were used. Chlorophyll a & b were also estimated to analyze the amount of microalgal cells. Chlorophyll estimation was done by hot extraction method given by Arnon (1949). Biomass was determined gravimetrically. After harvesting, cells were centrifuged at 7500 rpm and washed with autoclaved distilled water 2-3 times. Pellet was then lyophilized, and dry cells were weighed to determine total biomass.

B.4 Polyhydroxyalkanoate (PHA) analysis

PHA was determined using GC-FID. The sample were prepared using the method given by Oehmen et al., (2005). Benzoic acid was used as an internal standard and the concentration of monomers was determined using PHB-PHV copolymer as an external standard.

B.5 Multivariate analysis of results using PCA

Principal component analysis was employed for multivariate analysis. It reduces the variance of data and produces the correlation between variable and data of the experiment. PCA was performed using RStudio V4.3.0.

B.6 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was used to visualize the cross sections of microalgae, purple bacteria, and their interaction in consortium. Cross sections of cells unlocked the door to understanding the effect of various stress on their internal structures. TEM also helped in visualizing the interactive impact of purple bacteria and microalgae on each other. TEM samples were prepared at sophisticated analysis instrumentation facility (SAIF) at AIIMS Delhi. The live cells were first fixed in 2.5% glutaraldehyde and 2% paraformaldehyde overnight at 4 ^{o}C. cells were than centrifuged and fixatives were replaced with 0.1M phosphate buffer and samples were sent to AIIMS Delhi. The samples were fixed using various chemical agents and the cells were entrapped in the resin and thin cross-sections of the sample were cut and stained using titanium. The samples were analysed at ICGEB, Delhi.

B.7 Solvent extraction for polymer and characterization

PHA is accumulated in the cells in a form of granules. These granules have a polyester core which is surrounded by lipids and protein which acts as a protective shield against cellular components. For their extraction from cells 40% sodium hypochlorite was added and were further incubated for 3 hrs at room temperature and centrifuged at 7500 rpm for 10 minutes. Pellet was washed twice with 70% isopropanol and 70% ethanol and dried overnight in oven (Tamang et al., 2019b). Extracted polymer was used for physical and chemical characterization, to determine its properties.

B.7.1 Polymer film surface study

Films of polymer were made using 1% polymer in chloroform and casted onto glass plates. Films were further dried in oven at 50 ºC for 3 hrs (Savenkova et al., 2000). Field Emission Scanning Electron Microscope (FE-SEM) was used to study the surface morphology of polymer film. Before SEM analysis films were pasted on the stub with carbon tape to increase conductivity and coated with copper (Cu) for 2 minutes. Micrographs were recorded using (FE-SEM, Supra55 Zeiss) at 5kV and 10000x magnification (Bhowmick et al., 1980).

B.7.2 Thermal properties

Thermal gravimetric analysis (TGA) was used to determine the weight loss percentage and maximum degradation temperature. Origin version 9.1 was used derivatization and plot preparation (Savenkova et al., 2000).

B.7.3 Molecular weight determination (MW)

Molecular weight of extracted polymer was determined using gel permeation chromatography (GPC) equipped with RI detector. Samples were analysed at Sardar Patel Centre for Science and Technology (SICART), Anand, Gujrat India. Polymer was dissolved in tetra hydro furan (THF) and used for analysis.

C. Results and discussion

C.1 Effect of cultivation duration on the consortium

The consortium contained *E. texensis* and PB_IIT_01 in 1:2 ratio. The time course study enabled to understand the mutualistic relationship between microalgae and PNSB. The cells were inoculated and cultivated under two photoperiods 12:12 and 24:00. Figure 38 indicated the day-wise chlorophyll and biomass accumulated under the medium composition $P_{0.04}N_5Ga_{10}Fe_5$ by consortium. There was an increasing trend in biomass and chlorophyll accumulation. Highest biomass (1780.1±16.8 µg/mL) and chlorophyll (25.7±0.3 µg/mL) accumulated

on the 3rd day of cultivation. 12:12 light and dark period achieved higher biomass as compared to continuous illumination (24:00).

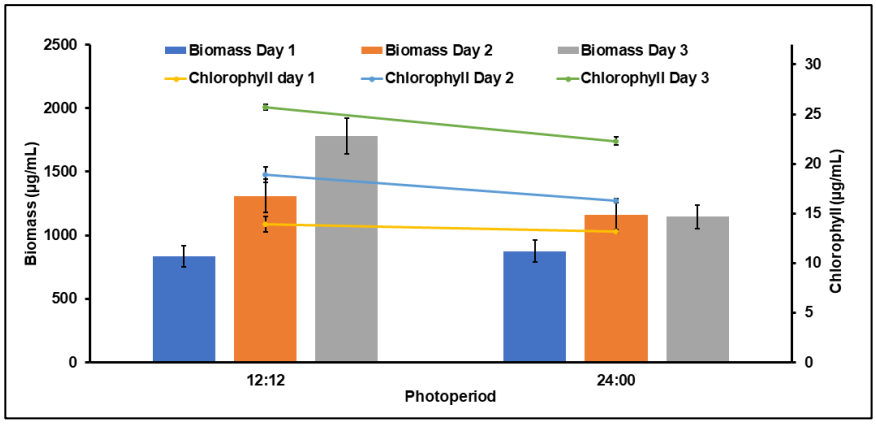


Figure 38: Biomass and chlorophyll accumulation in tailor-made consortium under $P_{0.04}N_5Ga_{10}Fe_{0.05}$ medium supplementation

There was a 1.6 folds increase in biomass and chlorophyll at 72 hrs under 12:12 photoperiod as compared to continuous illumination. Elongated photo exposure could damage the photosynthetic apparatus thus reducing the amount of pigment. Reduced activity leads to decreased biomass production. Continuous illumination could also result in photooxidative effect, reactive oxygen species (ROS) could be released and harm the enzymes or protein associated with photosynthetic apparatus. Photosystem I and II works simultaneously, light harvesting complex (LHC) captures quanta which is then transferred to thylakoid membrane for further processing the pigment and protein involved in the process uses energy in the form of ATP and NADPH, decrease in the energy could result in loss of quanta which in turn can damage the photosystem (Nelson and Ben-Shem, 2004). Owing to a broader wavelength capacity of anoxygenic photosynthesis, PNSB could help reduce the stress exerted with prolonged exposure of light on consortium (Santoshi, 2016).

C.2 Effect of cultivation duration on PHA accumulation

Biopolymers are often accumulated by microorganisms as a storage molecule. These can be breakdown and used as energy source for the growth in the adverse conditions. The time course study was determined to understand the accumulation pattern of PHA in the consortium. Since the consortium consisted of microalgae and PNSB, their compatibility could affect the overall PHA. However, it was found that high PHA was accumulated at 3rd day and then the PHA started to decline (data not shown). Highest PHA (221.8±1.3 µg/mg) was accumulated under 12:12 light & dark cycle on 3rd day which was 2.2 folds higher than continuous illumination (Figure 39).

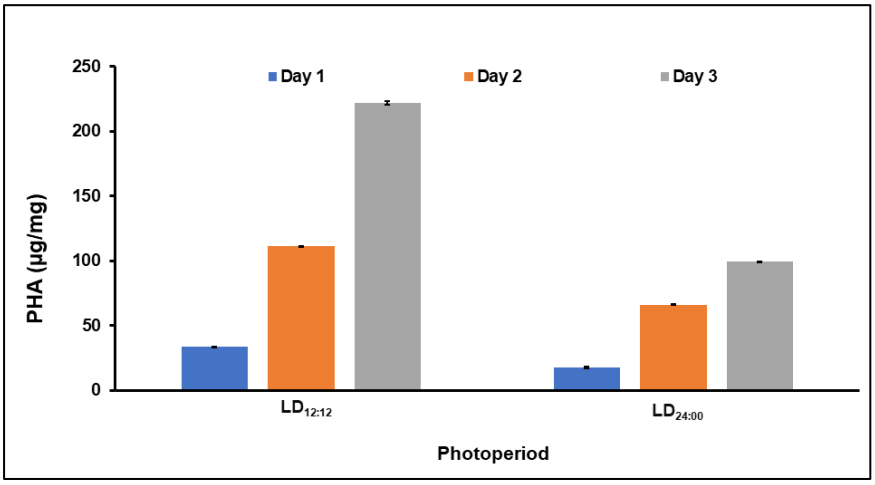


Figure 39: PHA accumulation in tailor-made consortium under $P_{0.04}N_5Ga_{10}Fe_{0.05}$

Continuous light could affect the photosystem of consortium exerting high stress which could result in lower PHA accumulation. However, a photoperiod containing a dark and light phase give the cells a speck of time to undergo the metabolic pathways and utilizing the energy produced during light cycle. Dark phase enables consumption of external carbon in spite photosynthesis. Energy produced by utilizing carbon source could be used for acetate uptake by PNSB, that would trigger PHA accumulation pathway (Samadhiya et al., 2022a). In a study, researchers observed a 2 folds increase in specific PHA productivity, while the consortium were grown under light and dark period as compared to continuous illumination (Fradinho et al., 2013).

Moreover, the consortium was cultivated without C-source as well and was able to accumulate PHA (18.8±1.1 µg/mg under 12:12 illumination and 11.5±0.1 µg/mg under 24:00 illumination).

C.3 Optimization of medium for consortium to induce high PHA accumulation

In the previous section the consortium was grown in a medium which was suitable for microalgae for PHA accumulation (chapter 3B). The previous section confirmed that the consortium was able to mutually grow together and could also produce PHA. But the produced PHA was not higher than the PHA produced by microalgae. Chlorophyll content suggested that the microalgae dominated the growth in the provided medium rather than PNSB. Another reason could be the absence of volatile fatty acids (VFAs). Previous reports have suggested that VFAs play a major role in triggering high PHA accumulation in PMCs. To overcome that two different combinations of medium were formulated based on the previous experiments (chapters 3B, 4). A comparative investigation between pure cultures and consortium was also conducted.

C.3.1 Effect of new formulated medium on growth and pigment production in pure cultures *vs* consortium

The two formulated media were based on BG-11 and MSM. Due to lower nutrient concentrations MSM based medium did not positively influence the biomass accumulation under pure or consortium cultures (Figure 40). The consortium accumulated higher biomass under medium A as compared to medium B due to high nutrient conditions. On the other hand, photoperiod did not affect the biomass accumulation noticeably. In pure cultures PNSB and microalgae both have shown similar growth pattern, but microalgae were able to achieve higher biomass under 12:12 photoperiod as compared to 24:00 (Figure 40).

Microalgae (1203±72.4 µg/mL) have shown 2.8 folds increase in biomass as compared to medium B (425.0±66.7 µg/mL).

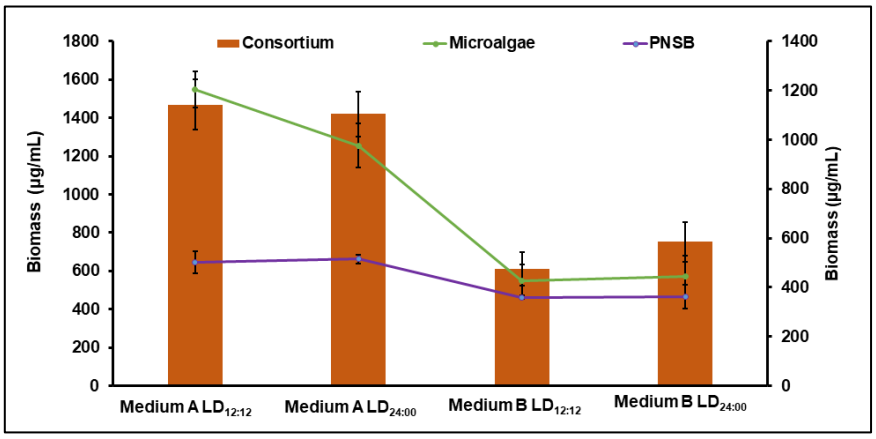


Figure 40: Biomass accumulation in Tailor-made consortium under medium A and B

Consortium have shown 2.4 folds increase in biomass under medium A as compared to medium B. PNSB and consortium (1.03- and 1.02-folds increase respectively) have not exhibited elevated effect between photoperiods whereas microalgae exhibited (1.2 folds decrease under continuous illumination).

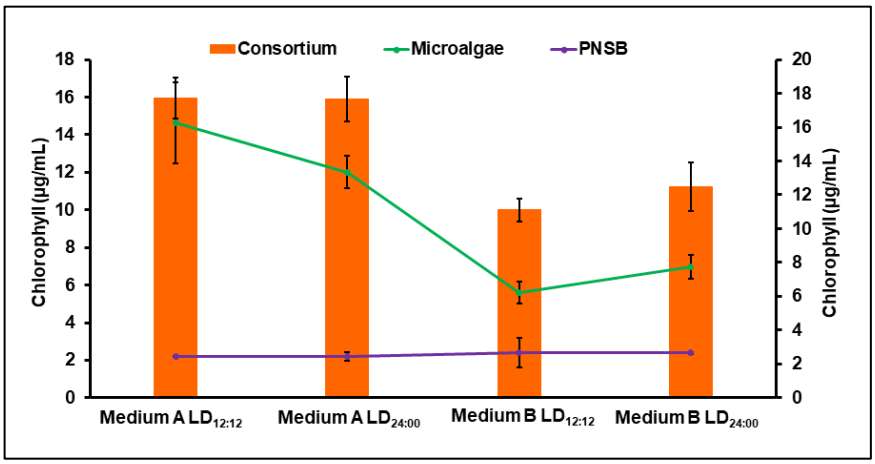


Figure 41: Chlorophyll accumulation in tailor-made consortium under medium A and B

The chlorophyll content has shown a similar trend as biomass accumulation. Highest chlorophyll was accumulated (16.3±2.4 µg/mL) by microalgae under 12:12 photoperiod. Whereas consortium accumulated less than microalgae (Figure 41). This observation sheds light on the mutualism of consortium where PNSB could absorb the high amount of light present in continuous illumination, this could prevent

photodegradation and thus maintaining the biomass accumulation during consortium growth. Prolonged exposure could result and formation of ROS that leads to damage of photosynthetic apparatus resulting in lower biomass. However, a consortium of algae and PNSB could absorb light of different wavelength thus not competing or getting high photo radiation could aid other cell to perform better during continuous illumination.

C.3.2 Effect of new formulated medium on PHA production in pure cultures *vs* consortium

Microalgae and purple bacteria have been a part of photosynthetic mixed cultures which are used for PHA production using wastewater as a medium. In this chapter we were able to successfully cultivate them together in two different media with two photoperiods. These consortia have preferred BG-11 based medium due to the presence of high nutrition content despite MSM based medium for growth and pigment production. When investigated for PHA accumulation consortium could accumulate 1.6 folds and 5 folds higher PHA than pure cultures when supplemented with medium A under 12:12 photoperiod. Although there is a slight decrease in PHA accumulation under continuous illumination (Figure 42). Under medium B condition microalgae and consortium could not accumulate high PHA but purple bacteria accumulated 123.9±11.4 µg/mg PHA under 12:12 illumination. Medium A increased 1.6-folds PHA accumulation in consortium as compared to previous medium addition of acetate could help PNSB with more PHA accumulation which could increase the total PHA accumulation in the consortium. Although medium B could increase the PHA accumulation

in PNSB, but low biomass production could not fulfil the high cost associated with production.

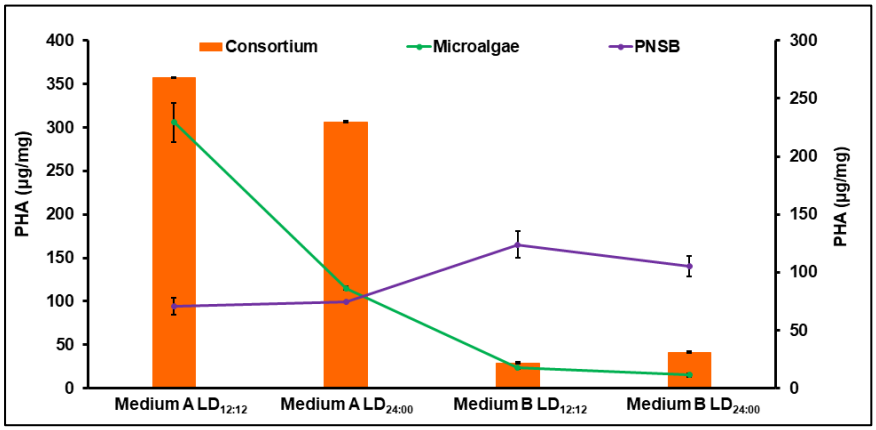


Figure 42: PHA accumulation in tailor-made consortium under medium A and B

Addition of acetate in the BG-11 based growth medium, increase PHA content by consortium by 1.6 folds. *E. texensis* have shown low PHA content when cultivated under acetate supplementation (Samadhiya et al., 2022a), nevertheless PB_IIT_01 have preferred acetate as a C-source over other VFAs (chapter 4). Acetate uptake leads to higher concentration of acetyl-Co-A the precursor of PHA accumulation (Figure 2, chapter 1). PMCs cultivated in wastewater under continuous illumination were able to accumulated 20% PHA which is 1.5 times lower that accumulated by tailor-made consortium of *E. texensis* and PB_IIT_01 (Fradinho et al., 2013). The dark phase of 12:12 photoperiod induces activity of nitrogenases by ceasing the TCA activity leading to high PHA accumulation (Monroy and Buitrón, 2020; Montiel Corona et al., 2017).

C.3.3 Microscopic analysis of consortium and pure culture using TEM analysis

Transmission electron microscopy (TEM) has been used as a diagnostic tool in pathology as well as for understanding the ultrastructure of cells. It provides a cross-sectional analysis, with a 2-dimentional image (Graham and Orenstein, 2007). TEM analysis revealed the ultrastructure of microbes (Figure 43). We could also observe the interaction of PNSB

and microalgae in Figure 43 C & D. The morphology of PNSB was similar to rod shaped elongated cells. TEM analysis is often used to visualize the PHA granules inside PNSB cells. Reports have suggested that granules are present in cytoplasm and can be seen as a round body which can be shown in Figure 43 A (Çetin et al., 2006; Higuchi-Takeuchi et al., 2016; Simon et al., 2016). The microalgae were grown in medium containing galactose and Fe (iron). Iron is a heavy metal; these can attach itself to the outer membrane or could also increase the permeability of cell wall. Although microalgae have been reported to have a higher tolerance for Fe it could still affect the outer structure of cell (Cameron et al., 2018). The Figure 44 B, showed the irregular shape of cell wall which could indicate the effect of Fe (Eldalatony et al., 2016).

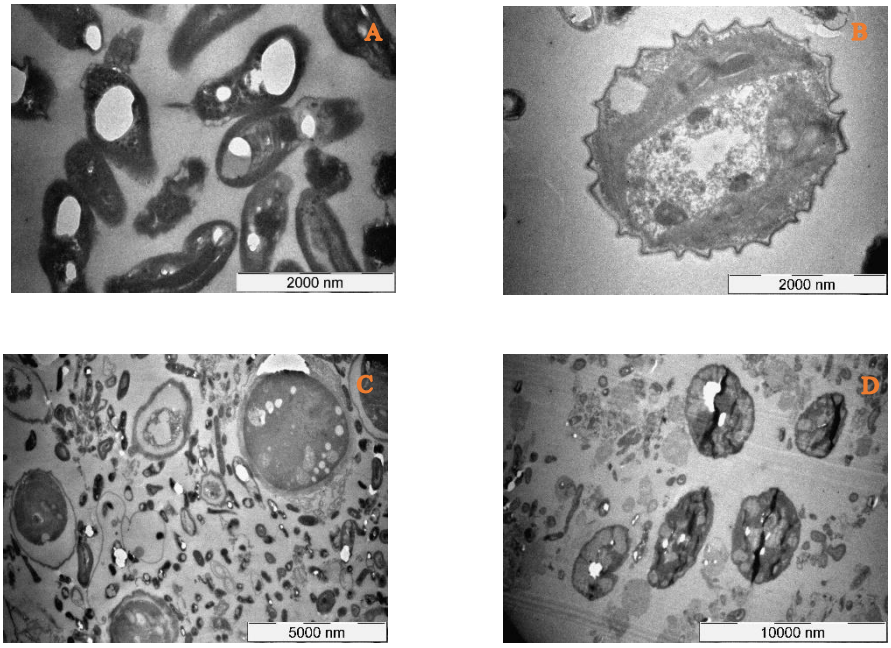


Figure 43: Transmission Electron Microscopy (TEM) analysis of A) PB_IIT_01 cultivated in medium B $LD_{12:12}$, B) *E. texensis* cultivated in medium A $LD_{12:12}$, C) Consortium in medium A $LD_{12:12}$, D) Consortium in medium B $LD_{12:12}$. Cells were cultivated for 3 days

TEM images of consortium revealed the size difference between microalgae and PNSB. Figure 44 C also entails the healthier cell wall of microalgae and higher population of PNSB rather than Figure 43 D. The consortium of PNSB and microalgae could symbiotically help each

other by exchanging metabolic product or PNSB could have absorbed Fe heavy metal which could result in healthier microalgal cells (Grattieri et al., 2022; Sakarika et al., 2020). Figure 43 D shows cells with irregular cell shapes and lower population of PNSB. This could happen due to the composition of medium B, which has lower nutrient composition as compared to medium A. This data also corroborates with the biomass and PHA accumulation data (Figures 40, 42).

C.4 Multivariate analysis of results *via* principal component analysis

PCA was performed with chlorophyll, PHA and biomass production among microalgae, purple bacteria and consortium. Table 30 displays Eigen values, variance and cumulative variance of the data. The Eigen value of dimension 1 indicates that 81.4% variance could be explained with this dimension. In combination with that eigen value more than 1 displays greater significance of the data hence the dimension 1 was chosen for further analysis of PCA (Abdi and Williams, 2010).

Table 30: Eigen value, variance and cumulative variance of PCA analysis

Dimension	Eigen Value	Variance (%)	Cumulative Variance (%)
Dimension 1	2.4	81.4	81.4
Dimension 2	0.4	14.3	95.8
Dimension 3	0.1	4.2	100

The bar plot in Figure 44 A, displays the various combination and their contribution in dimension 1. The red dotted line present in the bar graph indicates average contribution.

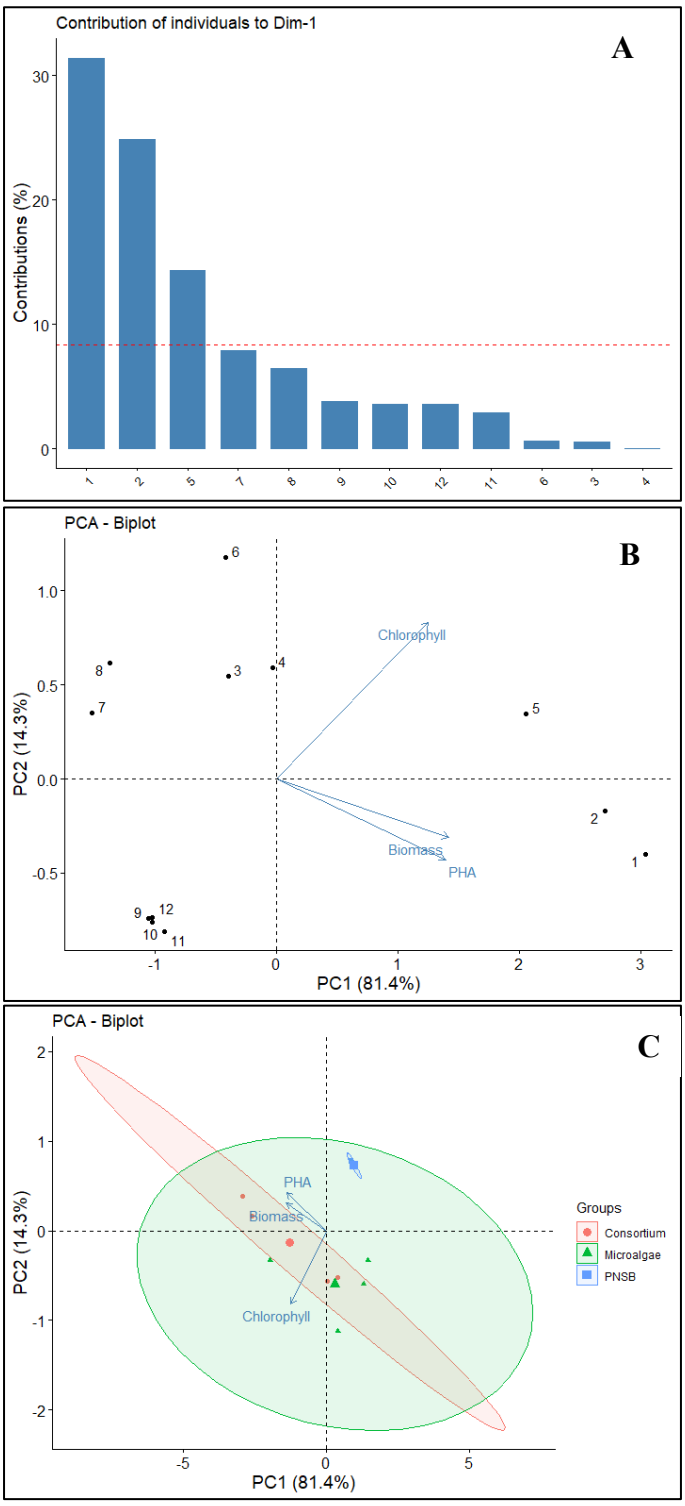


Figure 44: Bars in figure (A) are the contribution of individuals (combinations) to principal component 1. PCA biplots (B, C) show loadings of PHA, biomass, pH and DO (variables or vectors) across different combinations of nutrients

Codes shown in Figures 44 A & B are as follows: 1- Consortium Medium A $LD_{12:12}$, 2- Consortium Medium A $LD_{24:00}$, 3- Consortium Medium B $LD_{12:12}$, 4- Consortium Medium B $LD_{24:00}$, 5- Microalgae Medium A $LD_{12:12}$, 6- Microalgae Medium A $LD_{24:00}$, 7- Microalgae Medium B $LD_{12:12}$, 8- Microalgae Medium B $LD_{24:00}$, 9- PNSB Medium A $LD_{12:12}$, 10- PNSB Medium A $LD_{24:00}$, 11- PNSB Medium B $LD_{12:12}$, 12- PNSB Medium B $LD_{24:00}$

The 3 combinations contributing towards dimension one are consortium in medium A & B and microalgae in medium A at 12:12 photoperiod that contributed to more than 80% of the varince. These combination also produced highest amount of PHA, chlorophyll and biomass (Figures 40-42).

The biplot consisted of vectors and score plot, vectors indicating outputs and dot scores indicating variables. Four quadrants of biplots indicate various significance of the variables positioned. Lower left quadrant displays the most significant contribution, where as lower right qudrant contains the least significant variables. Position from origin also indicates the significant of the data, most faraway indicating greater significance (Figure 44 B). PHA and biomass were closely related to eachother showing a lower angle between them. This biplot corraborate the results obtained in the compositional analysis (Mutale-joan et al., 2020).

Cluster biplot has shown interesting result indicating higher variation of output in microalgae and consortium data whereas PNSB has not shown any significance to variance. The amplitude of ellipse and circle have shown more significance for dimension one. On the contrary, absence of variance in PNSB output majorly in chlorophyll production put PNSB out of the correlation with consortium and PNSB (Lid and Planning, 1993).

C.5 Characterization of polymer synthesized using a tailor-made consortium

The extracted polymer was characterized using GC-MS and revealed that PHB and PHV both monomers were present in the polymer. The composition of polymer is often dependent on the substrate and host cells. The medium consisted of two different carbon source and a symbiotic cultivation of microalgae and PNSB produced a copolymer, that are reported to be more sophisticated and desired for commercial applications (Bhati and Mallick, 2015).

Surface analysis revealed a porous structure with some scaffolds. Polymer surfaces can show various different structure based on the dispersion of monomers and phases and interphases caused by the evaporation of solvent during drying process (Figure 45). Similar structure was observed by Panaitescu and coworkers where the polymer was extracted from *Pseudomonas* (Altaee et al., 2016; Panaitescu et al., 2018).

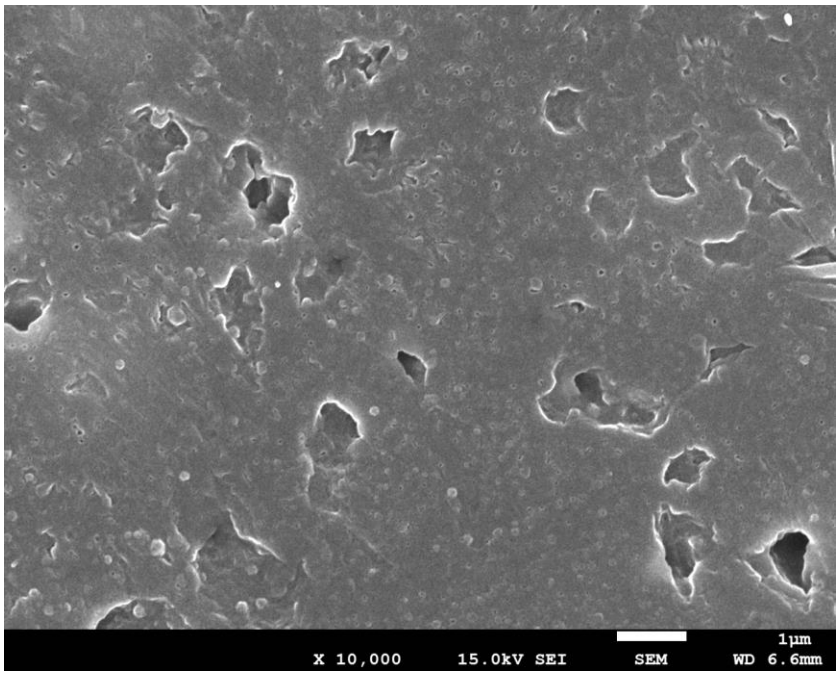


Figure 45: Surface analysis of polymer using Scanning Electron Microscopy (10,000x)

The thermal degradation curve of the polymer was made using the TGA data (Figure 46 A). It showed a four-step degradation; first two steps showed a total of 3.3% degradation which may arrive due to moisture content followed by a third and fourth step indicating polymer degradation. Maximum degradation temperature of the polymer was calculated using derivatized thermal degradation map which was 207.3 °C (Figure 46 B).

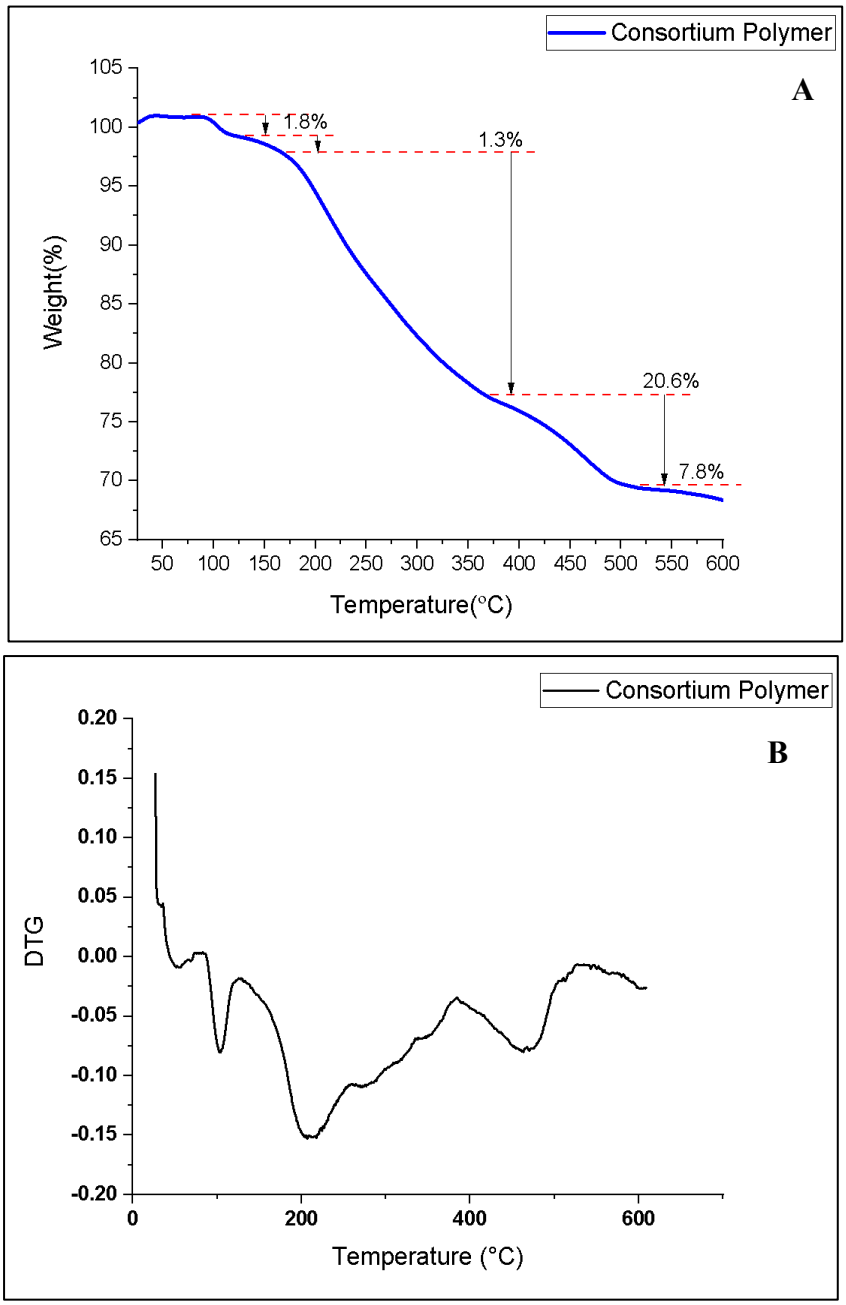


Figure 46: A) Thermogravimetric analysis (TGA) B) and derivative curve (DTG) of polymer showing thermal degradation

Molecular weight of polymer was analyzed using gel permeation chromatography, MW of polymer was found to be $5.6x10^3$ kDa (Table 31). The Mw keeps the polymer in ultra-high molecular weight category (Higuchi-Takeuchi et al., 2016). MW is a key quality of polymer while considering it for commercialization due to its link to mechanical properties (Domínguez-Díaz et al., 2015; Mcchalicher and Srienc,

2007). PhaZ enzyme has been reported to decompose PHA inside cells thus resulting in low molecular weight polymer on the contrary PhaC enable high chain length molecules (Higuchi-Takeuchi et al., 2016; Sim et al., 1997). Mw of polymer produced by consortium has attained higher molecular weight compared to polymer produced by microalgae and PNSB (chapters 3B & 4).

Table 31: Physical properties of extracted polymer a tailor-made consortium

Properties	Values
Molecular Weight (MW) (kDa)	5962
Poly Dispersity Index (PDI)	1.7
Z Average (Mz)	37733
Number Average (Mn)	846
Maximum degrading Temperature (°C Tm)	207.3

The Polydispersity Index (PDI) shows the uniformity of the molecules present in the polymer. PDI of 1.7 indicated the uniform distribution of molecules (Table 31). High molecular weight and lower PDI than literature prove the merit of this study indicating production of a better polymer which could be scale up for commercialization (Tsuge, 2016). Being a high Mw polymer, the distribution of monomer was higher which could also be visualized on the surface of polymer (Figure 46).

D. Conclusions

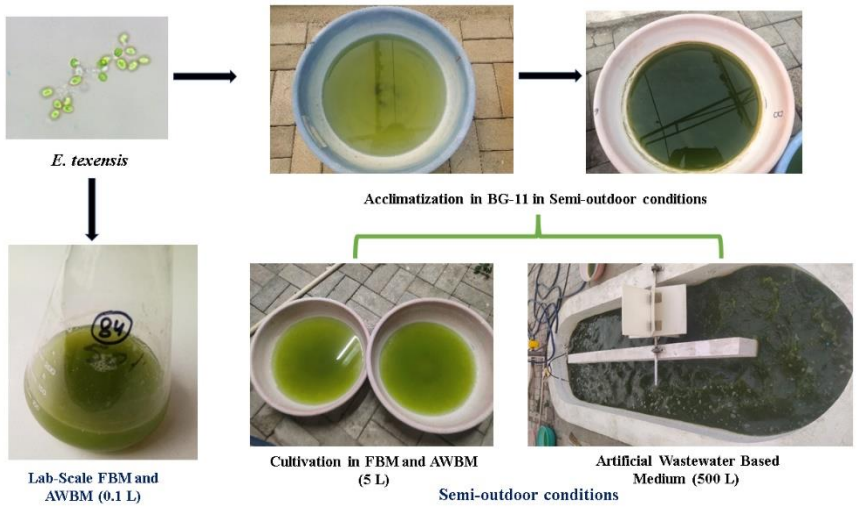
This chapter illustrates a tailor-made consortium consisting of microalgae *E. texensis* and PNSB isolate PB_IIT_01. The consortium was successfully grown under the medium formulated with the optimized conditions from chapter 3B and chapter 4. A comparison study between pure culture and mixed culture was carried out as well indicating the higher PHA accumulation under co-cultivation. Two

different photoperiods were analyzed in combination with two different media, proving a light dark ratio of 12:12 favoring PHA and biomass accumulation. A 1.6-fold increase in PHA accumulation was achieved, thus proving the successful application of consortium. The consortium also accumulated 1.5 folds higher PHA accumulation than previously reported PMC systems. TEM analysis illustrates symbiotic relation between microalgae and purple bacteria when cultivated together. PCA analysis confirmed that consortium positively affected PHA, biomass and chlorophyll content whereas purple bacteria have shown a lesser effect on the accumulation. Biopolymer produced from consortium was an ultra-high molecular weight polymer and has a higher Mw than the pure cultures. To best of authors' knowledge this tailor-made consortium with a formulated medium is novel and has not been used yet for the production of PHA. This chapter also contains the TEM images of *E. texensis* which have not been published yet. This novel approach of tailor-made consortium could be utilized for scale-up process without the need of feast-famine regime of PMCs that is time consuming and do not guarantee the high quality or quantity of polymer.

Next chapter demonstrates scale-up cultivation of *E. texensis* microalgae under semi-outdoor conditions. A comparison between fertilizer based medium and wastewater-based medium is done to understand the effect on production of biomolecules. These media could pave the way for a wastewater-based biorefinery for the production of energy precursors.

CHAPTER 6

Scale-up study: Assessment of growth behavior of microalgae under semi-outdoor condition



A. Introduction

India is a country with the most arable land and more than 40% of its population depends on agriculture for their livelihood. The agricultural sector uses the most freshwater, and chemicals that are used to boost crop productivity, such as pesticides, agrochemicals, sediments, organic matter, drug residues, and fertilisers, are extremely detrimental to both surface water and subsurface water. Agricultural discharge is thought to be the most toxic of the various discharges that enter water bodies. Due to the growing population, contaminated water is used in both commercial and agricultural settings. The growing population and consumer demands have led farmers to use more fertilizers without understanding the need or utilization capacity of the soil. India has shown an annual increase of 6% in the past few decades and this trend is projected to double by the year 2050 (FAO, 2018). The most used chemical fertilizers in India are urea and diammonium phosphate (DAP). There is a huge gap between available nitrogen/phosphorus and the use of nitrogen/phosphorus by the crops which led to most of the amount staying on the upper surface of the soil that travels down with rainfall or irrigation to groundwater or waterbodies which causes a high concentration of nitrates and phosphates in waterbodies that led to eutrophication (Bera et al., 2021; Móring et al., 2021; Randive et al., 2021). Apart from agriculture, a large part of the population depends on cattle farming for their livelihood, majorly for milk production. India is the largest milk producer globally, but its dairy sector generates 9 times as much wastewater (chemical oxygen demand; COD) per metric cube of milk production as it does milk. COD is made up of milk traces high in lactose and galactose sugars (*Economic_survey_2021_2022*).

Algal biomass is an effective strategy for avoiding the use of costly, environmentally harmful chemicals in wastewater treatment. However, its practical application is limited due to the cost of raw materials and harvesting and processing limitations. Microalgae are microorganisms that are capable of converting CO_2, GHGs, and nutrients into

carbohydrates, protein, and lipids while performing photosynthesis and generating oxygen. They have been widely used in the creation of value-added products such as biodiesel, biopolymer, nutraceuticals, food supplements, fertilisers, and animal feed, as well as for the remediation of wastewater. However, there are still difficulties in commercialising microalgal goods due to high raw material costs and subpar scale-up performance. Several wastewater types, including municipal and industrial wastewater, have been reported to be treated by microalgae. Microalgae could not only remediate the wastewater it was also able to produce various by-products such as lipids, protein, biodiesel, etc. using the nutrients from the waste (Do et al., 2019; Heredia Falconí et al., 2021; Nivetha et al., 2019; Khan et al., 2022; Kiran et al., 2014; Passero et al., 2015; Passero et al., 2014). Despite the benefits of microalgae in utilizing various substrates to produce value-added products, the scaling-up process remains a challenge for researchers worldwide (Kumar and Singh, 2019). Most of the optimization studies happen at the lab-scale, where the species performs well and produces a large amounts of value-added products, but when these lab-scale studies are transferred to the pilot scale studies, it often leads to decreased productivity (Zhang et al., 2016). The key reason for decreased productivity during the scaling up of production is the adaptation of species to the outdoor environment or maintaining certain cultivation conditions with a high cost of raw materials (Nagarajan et al., 2021).

Employing varying composition of different wastewater streams as a source of growth media for algal biomass has proven to be a difficult task. The concept of using algae as a biorefinery to remove beneficial nutrients from various wastewater streams with complicated chemical compositions is the main aim of this paper, as is the possible use of the produced algal biomass for bioenergy and bioactive chemicals. The present chapter aims to focus on the successful scale-up of *E. texensis* species isolated from nearby regions of Indore in semi-outdoor conditions (Samadhiya et al., 2021). Furthermore, this study also compares the biochemical signatures of the species cultivated in

fertilizer based medium (FBM) and wastewater based medium (WWBM) at two different volumes.

B. Material and methods

B.1 Species maintenance and cultivation conditions

B.1.1 Maintenance of species at lab-scale conditions

Microalgae *Ettlia texensis* was isolated from central India (Indore, Madhya Pradesh) and was identified using ITS and 18S rRNA at NCIM, Pune (Samadhiya et al., 2021). BG-11 medium was used for cultivation and pH was maintained at 7.4 ± 0.2 (Kaushik, 1987). Initial inoculum for the mother culture was kept at 0.1 OD_{680}. It was grown for 10 days under 27 ± 5 °C, and 3000 ± 500 lux and cultivated till log phase was achieved (10 days). The purity of the cultures was checked with light microscope at 40x at regular intervals.

B.1.2 Maintenance in semi-outdoor conditions

E. texensis strain was cultivated for three generations under semi-outdoor conditions to make sure that cells were acclimatized to the environmental conditions of the semi-outdoor. The temperature and light variations are shown in Figure 47.

B.2 Experimental setup and medium composition

B.2.1 Medium composition

Fertilizer-based media (FBM) was prepared using fertilizer-grade urea and DAP. Nitrate and Phosphate were used in the same concentration as present in BG-11 medium (Kaushik, 1987). Tap water was used for preparing media and the pH was kept at 7.4.

For artificial wastewater-based media (WWBM), galactose (1000 mg/L) was used as a carbon source and the composition of artificial waste-water was followed as per Mittal et. al., (2022).

B.2.2 Experimental setup

In the experiment under laboratory conditions, the cells were inoculated in fertilizer-based media and artificial wastewater-based media (Section B.2.1). The initial optical density was kept 0.5 at 680 nm and the volume

was 0.1 L. The combination was coded by $FertV_{0.1}$ and $ArtV_{0.1}$ respectively. The cells were cultivated for 7 days, and the harvesting was done on 3rd day and 7th day.

For the cultivation in semi-outdoor conditions, the acclimatized cells were first grown in 5 L open containers. For fertilizer-based media, the codes were depicted as $FertV_5$ and for artificial wastewater-based media $ArtV_5$ was used. The initial OD was also kept at 0.5 at 680 nm. The cells were cultivated for 7 days, and the harvesting was done on the 3rd and 7th day.

Upon the comparison of lab-scale conditions and semi-outdoor conditions, artificial wastewater provided better results as compared to FBM. Hence, for the 1000 L facility (500 L working volume) artificial wastewater was used as the medium for cultivation ($ArtV_{500}$). To avoid the settlement of media components and cells, impellers were employed at 30 rpm for 10 min twice a day. To compensate for the surface evaporation, and to maintain the cells to medium volume ratio, 5 L tap water was added every day. Initial inoculum, cultivation period, and harvesting period were kept same as above.

B.3 Growth monitoring and kinetics study of cells

Growth monitoring was assessed by measuring the absorbance at 680 nm using a HACH spectrophotometer (DR6000). Total biomass production was calculated using OD vs dry weight calibration curve. Biomass productivity and specific growth rate were studied to understand the cell kinetics during the experiments. Models given by Guillard (1979) and Pancha et al., (2015) were used.

B.4 Biomass composition analysis

After harvesting, the cells were rinsed thoroughly with distilled water (DW) and freeze-dried using lyophilizer. Dried biomass was utilized for biochemical analysis.

Freeze-dried biomass was taken in a known quantity to analyze carbohydrates using the Anthrone method (Roe, 1955). The

concentration of carbohydrates was calculated by plotting the calibration curve using glucose as a standard. For protein analysis, freeze-dried biomass was taken to perform the micro-biuret test (Chen and Vaidyanathan, 2013). Bovine serum albumin (BSA) was used to prepare the standard calibration curve for the quantification of protein in dried biomass. For lipid analysis dried biomass was analyzed using the SPV method given by Knight and co-workers (Mishra et al., 2014). Glyceryl trioleate (Triolein) was used for quantifying lipids in dried biomass using a calibration curve.

B.5 Principal component analysis (PCA)

Data obtained in this chapter was statistically analyzed by using PCA. PCA analyzes different variables used in the study and correlates them with each other to obtain the relation between variables as well as the results acquired. It also analyzes the contribution of various components within the outputs of the study. RStudio 4.3.0 was used to perform the PCA between biomass, protein, carbohydrate, and lipid production.

C. Results and discussion

C.1 Growth monitoring and cell kinetics

This study was designed for compliance of two intents, to monitor the growth of *E. texensis* in FBM and WWBM, and to investigate their behavior at the lab-scale as well as in semi-outdoor conditions. Under laboratory conditions, *E. texensis* cells were able to grow successfully in FBM, showing a biomass production of 205.6 ± 22.7 µg/mL on the 7^{th} day of cultivation. However, the specific growth rate (SGR) decreased up to 50% from 3^{rd} to 7^{th} day. *E. texensis* have shown lower growth and biomass accumulation in $FertV_{0.1}$ as compared to BG-11 medium (235.8±7.5 µg/mL). Nitrogen and phosphorus, two important nutrients were present in FBM, but other nutrients such as magnesium (Mg), calcium (Ca), as well as essential micronutrients were absent. As a result, the growth of the cells was slow, but they were able to grow in

the medium. On the other hand, biomass production in artificial wastewater ($ArtV_{0.1}$) was higher compared to the BG-11 medium.

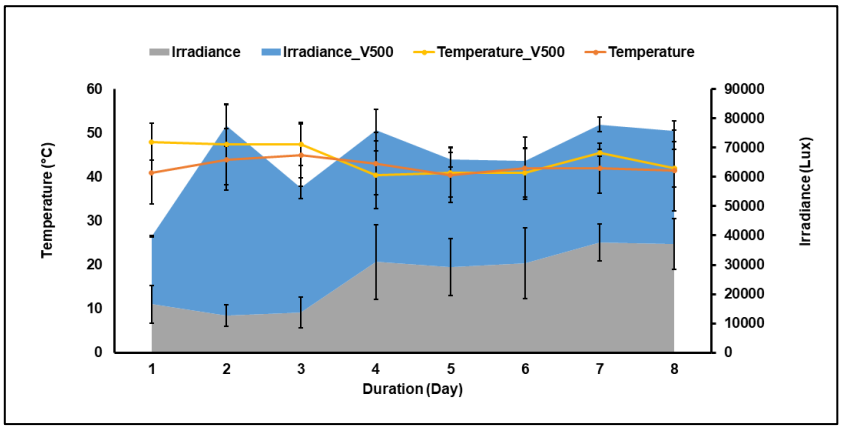


Figure 47: Irradiance (Lux), and temperature (°C) for semi-outdoor condition for cultivation period

#Irradiance_V500 and Temperature_V500 corresponds to the temperature for 500 L culture volume

On the 3rd day of cultivation, the cells had biomass productivity (BP) of 60.5 ± 12.6 µg/mL/day, which was 2 times higher than BG-11 medium (30.0 ± 2.3 µg/mL/day), and 3.7 times higher than FBM (16.5 ± 4.5 µg/mL/day). There was a steep decrease in SGR on the 7th day of cultivation under FBM ($FertV_{0.1}$) as well as artificial WWBM ($ArtV_{0.1}$) (Table 32). While comparing the growth behavior of the cells under laboratory conditions, WWBM exhibited high BP and SGR as compared with FBM (Table 32). A study conducted with *Desmodesmus abundans* with synthetic wastewater medium, reported the biomass productivity of 47.0 mg/L/day which is 1.3 times less concerning the current study under laboratory conditions (Table 32) (Prasad et al., 2018).

After the successful cultivation of cells under FBM and WWBM, cells were transferred to grow in semi-outdoor conditions. To ensure the cells were acclimatized to the conditions (high temperature and light intensity; Figure 47), the cells were grown in BG-11 medium for 3 generations. The biomass was transferred to FBM and WWBM, at a volume of 5 L, denoted as $FertV_5$, and $ArtV_5$ respectively. The growth pattern of cells in FBM was opposite in semi-outdoor conditions as compared to laboratory conditions (Table 32). BP and SGR increased

1.9 and 1.5 times respectively, at the 7th day of cultivation (Table 32). Whereas in $ArtV_5$, the highest biomass accumulation was observed on the 7th day of cultivation (459.4 ± 24.0 µg/mL), BP and SGR decreased up to 1.6 times on the 7th day. While comparing the growth of cells in FBM and WWBM, cells were grown better in WWBM, due to the higher COD present. The cells were successfully able to grow in the semi-outdoor condition, despite high temperature and light intensity (Figure 47). Cells have shown good SGR and BP, even higher in the case of WWBM, this denotes that the cells were easily adaptable to the outdoor environment, making them a better candidate for scale-up process.

Table 32: Cell kinetics of *E. texensis* cultivated under controlled (0.1 L), and semi-outdoor (5L and 500 L) conditions for fertilizer and wastewater-based media

Duration	Combination	Biomass (µg/mL)	Biomass Productivity (µg/mL/day)	Specific Growth Rate (Per day)
	$FertV_{0.1}$	194.3±13.5	16.5±4.5	0.10±0.02
	$Art_{0.1}$	326.3±37.8	60.5±12.6	0.27±0.04
3rd Day	**$FertV_5$**	163.1±6.9	6.1±2.3	0.04±0.01
	Art_5	314.5±25.5	56.6±8.5	0.26±0.03
	Art_{500}	774.2±11.9	56.4±15.2	0.26±0.05
	$FertV_{0.1}$	205.6±22.7	8.7±3.3	0.05±0.02
	$Art_{0.1}$	377.2±71.5	33.2±10.2	0.13±0.03
7th Day	**$FertV_5$**	226.2±13.2	11.6±1.9	0.06±0.01
	Art_5	459.4±24.0	36.0±3.4	0.16±0.01
	Art_{500}	729.3±48.8	22.5±2.6	0.10±0.01

#$FertV_{0.1}$ = Fertilizer based media under controlled condition in 0.1 L volume; $FertV_5$= Fertilizer based media under semi-outdoor condition in 5 L volume; $ArtV_{0.1}$= Wastewater based media under controlled condition in 0.1 L volume; $ArtV_5$= Wastewater based media under semi-outdoor condition in 5 L volume; $ArtV_{500}$= Wastewater based media under semi-outdoor condition in 500 L volume

To further measure the scalability of the cells, *E. texensis* was cultivated in 1000 L capacity ponds with a working volume of 500 L with the best

medium suitable for biomass production i.e., WWBM. For this process, the high-rate algal ponds (HRAP) system present at the institute's semi-outdoor plants were used with high temperature and light intensity (Figure 47). Continuing with the trend, the highest biomass was accumulated on 3rd day of cultivation under $ArtV_{500}$ combination (774.2±11.9 µg/mL) whereas there was 2.5 times decrease in the BP and 2.6 times decrease in SGR. As compared to lab-scale conditions 3.4 times higher BP and 2.6 times SGR was achieved (Table 32). *E. texensis* have shown the highest growth in semi-outdoor condition, which depicts the higher threshold for temperature and light. But the interesting thing to note in the results was that on the 7th day of cultivation the SGR dropped drastically, indicating that the long exposure to high temperature and intensity limits the photosynthetic activity of *E. texensis* (Jeong et al., 2012). Previous research has also demonstrated that nutrient limitation can hamper cells' ability to contest high light intensity (Ghosh et al., 2021). Additionally, long exposure to high temperatures can result in ATP scarcity and denaturation of the protein involved in photosynthetic activity (Raven and Geider, 1988). Despite these limitations, *E. texensis* was able to sustain high temperatures as well as high light intensities. These results ensure a successful scale-up of the species *E. texensis* under high temperature and light intensity with artificial wastewater making it a suitable candidate for establishing a biorefinery.

C.2 Biomass composition

Biomass composition is the key component when considering scale-up of the process for any biorefinery approach. High biomass productivity alone is not enough unless the biomass can be transformed into various value-added products to balance the economic implementation for the biorefinery or any scale-up process (Kim et al., 2020). Microalgal biomass consists of three important molecules namely, carbohydrate, protein, and lipid (Alam et al., 2020). The abundance of these molecules in microalgae is a boon for the sustainable production of various

products like biodiesel, biohydrogen, biopolymer, nutraceutical, food supplements, and animal feedstock, etc.(Anand et al., 2019; Costa et al., 2018; de Carvalho Silvello et al., 2022; Soares et al., 2019).

C.2.1 Carbohydrate synthesis under different media and cultivation conditions

Carbohydrate is one of the most studied biomolecules produced by microalgae with many applications in fields such as energy, feed, fuel, nutraceutical, and drug discovery (Atreyee Ghosh et al., 2019; Guihéneuf & Stengel, 2013; Kaushik et al., 2023; Nagappan et al., 2019; Samadhiya et al., 2021). In the current study, during lab-scale cultivation, the highest carbohydrate was accumulated under WWBM on 3rd day of cultivation, whereas there was no significant change in carbohydrate accumulation in FBM (Table 32).

Table 33: Biochemical analysis of biomass obtained by cultivation of *E. texensis* under controlled (0.1 L), and semi-outdoor (5L, 500 L) conditions for fertilizer and wastewater-based media

Duration	Combination	Carbohydrate	Protein	Lipid
3rd Day	**Fert$V_{0.1}$**	108.8±5.9	602.4±35.2	291.7±15.7
	Art$_{0.1}$	172.8±15.0	361.7±30.5	319.0±22.6
	FertV_5	136.8±4.7	384.6±55.4	321.9±14.0
	Art$_5$	205.5±10.1	336.7±17.5	448.8±9.7
	Art$_{500}$	186.0±18.2	419.6±18.4	493.4±34.4
7th Day	**Fert$V_{0.1}$**	107.1±5.6	581.1±54.6	351.1±21.0
	Art$_{0.1}$	128.1±16.4	449.6±26.8	420.9±15.2
	FertV_5	160.9±8.9	366.4±30.3	308.6±25.2
	Art$_5$	158.2±13.1	435.4±33.7	269.8±27.3
	Art$_{500}$	200.4±33.2	385.6±25.8	311.1±18.0

#Fert$V_{0.1}$ = Fertilizer based media under controlled condition in 0.1 L volume; FertV_5= Fertilizer based media under semi-outdoor condition in 5 L volume; Art$V_{0.1}$= Wastewater based media under controlled condition in 0.1 L volume; ArtV_5= Wastewater based media under semi-outdoor condition in 5 L volume; ArtV_{500}= Wastewater based media under semi-outdoor condition in 500 L volume

1.6 folds higher accumulation of carbohydrates was achieved under WWBM, with a decreasing pattern observed with time under lab-scale conditions. Under semi-outdoor condition, $ArtV_5$ accumulated the highest carbohydrate accumulation (205.5 ± 10.1 µg/mg biomass), which was 1.3 times higher than the highest carbohydrate accumulated under FBM (Table 33). Under 500 L working volume, the carbohydrate content did not vary for the 5 L volume. The nutrient composition between FBM and WWBM varied, the former has high nitrogen as compared to the latter, which in turn negatively affect the microalgal accumulation of carbohydrate. Zhu et al., conducted a study on *Chlorella zofingiensis* and established that nutrient limitation exclusively nitrogen, has a positive effect on carbohydrate accumulation. Nitrogen works as a gatekeeper for the flux of carbon and its depletion can result in accumulation of high lipid or carbohydrate (Markou et al., 2012; Zhu et al., 2014). The high temperature in semi-outdoor conditions also plays an important role. When studying the effect of temperature on photosynthesis, it was revealed that high temperature decreases the F_m/F_v ratio which represents the efficiency of photosystem II. It also simultaneously affects the activity of Rubisco, causing photoinhibition to elicit the accumulation of storage molecules such as carbohydrates and lipids inside the cells. *E. texensis* has already shown the ability to withstand high light intensity and temperature, which makes it fitting for the upscaling of the process (Raven and Geider, 1988; Salvucci and Crafts-Brandner, 2004).

C.2.2 Protein synthesis under different media and cultivation conditions

Proteins are primary elements of the cell and hold an important role, but their production is also influenced rapidly by encountering any stress or limitation. Microalgal protein also makes a good vegan protein as food and feed (Amorim et al., 2021). Cells grown in FBM exhibited higher protein content as compared to WWBM (Table 33), likely due to the high presence of nitrogen in FBM, which can activate protein

accumulation by increasing the production of essential amino acids. Highest protein synthesis occurred on 3rd day under FBM supplementation (602.4 ± 35.2 µg/mg biomass) which was found to be 1.7 folds more than the lowest protein accumulation under WWBM supplementation at the same time point (361.7 ± 30.5 µg/mg biomass) under lab-scale conditions. The same amount of protein was accumulated by *Scenedesmus* sp. grown in outdoor conditions (Olsen et al., 2021). It was interesting to observe that the highest protein accumulation was observed in the $ArtV_5$ combination on 7th day of cultivation (435.4 ± 33.7 µg/mg biomass), followed by $ArtV_{500}$ on 3rd day of cultivation (419.6 ± 18.4 µg/mg biomass). Microalgal species such as *Scenedesmus* and *Spirulina* are grown in outdoor conditions accumulated 15-41 % proteins throughout the year under wastewater or FBM, which is equivalent to protein content under WWBM (Cardoso et al., 2020; Haider et al., 2023). Protein accumulation was found to increase with increasing the cultivation time, however, in $ArtV_{0.1}$ and $ArtV_5$ an inverse trend was observed (Table 33). A decrease in protein accumulation can be related to the decreasing nitrate level over time. Additionally, high temperatures and light intensities can cause photoinhibition that results in decreased protein accumulation (Amorim et al., 2021; Salvucci and Crafts-Brandner, 2004).

C.2.3 Lipid synthesis under different media and cultivation conditions

Microalgae have been extensively studied for their lipid content, which has had promising applications in the biofuel and nutraceutical industries in previous decades. In the present study, the highest lipid accumulation under lab-scale conditions was observed at 7th day (420.9 ± 15.2 µg/mg biomass) under WWBM supplementation, whereas the lowest lipid accumulation was 1.4 times lower under FBM on 3rd day (291.7 ± 15.7 µg/mg biomass). The present study reports twice the lipid accumulation as compared to the study carried out by Prasad et al., under laboratory conditions (Prasad et al., 2018). *E. texensis* cells were very

prompt in accumulation of lipid when supplemented with WWBM in semi-outdoor conditions, with the highest accumulation of 493.4 ± 34.4 µg/mg biomass of lipid under $ArtV_{500}$ followed by 448.8±9.7 µg/mg biomass in $ArtV_5$. Under semi-outdoor conditions, lipid accumulation decreased with cultivation time, while under lab-scale conditions, lipid accumulation increased with time (Table 33). Nutrient depletion, higher carbon flux, high light intensity, and temperature are positively correlated with lipid accumulation in microalgae, whereas long-term exposure of stress can damage the photosynthetic assembly thus decreasing the lipid accumulation (Benjumea et al., 2011; Ghosh et al., 2021; Ghosh, et al., 2021a; Salvucci & Crafts-Brandner, 2004). The current study has shown 4.5 times higher lipid productivity than, reported by Sharma et al. for *Chlorella minutissima* grown in outdoor conditions using FBM with CO_2 bubbling (Sharma et al., 2021).

6.3.4 Statistical analysis using principal component analysis (PCA)

Principal component analysis (PCA) is a statistical technique used to analyze large data sets with multiple variables by assessing their variance and co-variance in relation to the variable (Abdi & Williams, 2010). The technique chooses principal components based on their ability to express the largest possible variance in the data set. For example, principal component 1 (PC1) represents the largest possible variance. Subsequently, PC2 explains the 2nd largest variance in the data unrelated to PC1.

Table 34: Eigen value and variance among different dimensions, for principal component analysis for the output

Dimension	Eigen Value	Variance (%)	Cumulative Variance (%)
Dimension 1	2.3	58.0	58.0
Dimension 2	0.9	21.9	79.8
Dimension 3	0.6	16.0	95.9
Dimension 4	0.2	4.1	100

The data is converted into a series vector and the variance in the data is explained by the Eigenvector (direction of the variance), and Eigenvalue (magnitude of the variance). Eigenvalue is a scaler non-zero vector, which is used to analyze the correlation. In this study, the Eigenvalue of the data set represents 69.6 % of the cumulative variance i.e., 69.6% of the variance can be explained by dimensions 1 and 2 (Table 34).

The scree plot represents the contribution of principal components (PCs) in the variance of data and the contribution from most to least (Figure 48 A). It represents the individual contribution of variables to PCs, the marking of the red dotted line represents the minimum contribution in the variance from the individuals, whereas bars going higher these lines represents important group responsible for variance. The PCA biplot represented in Figure 48 B depicts the correlation between the individual combinations as well as the correlation between various outputs. The distance of the variable from the origin of the PC represents the prominence of the variable for PCs. However, the angle between the vectors predicts the correlation with each other. Figure 48 B represents the Cosine square plot or Corr plot or factor map, which represents the quality of variables on dimensions. Here dark brown color shows a high Cos2 value whereas light-yellow color depicts a low Cos2 value. A gradient of the color is represented by the scale on the right Y-axis of the map. Positively correlated variables are grouped together, and negatively correlated groups are put apart on the map. As proof, biomass is positively correlated with dimension 3 and is well represented because of its position far away from the origin. Whereas lipid is strongly correlated to dimension 2 but the position of biomass and lipid is opposite to each other on the biplot.

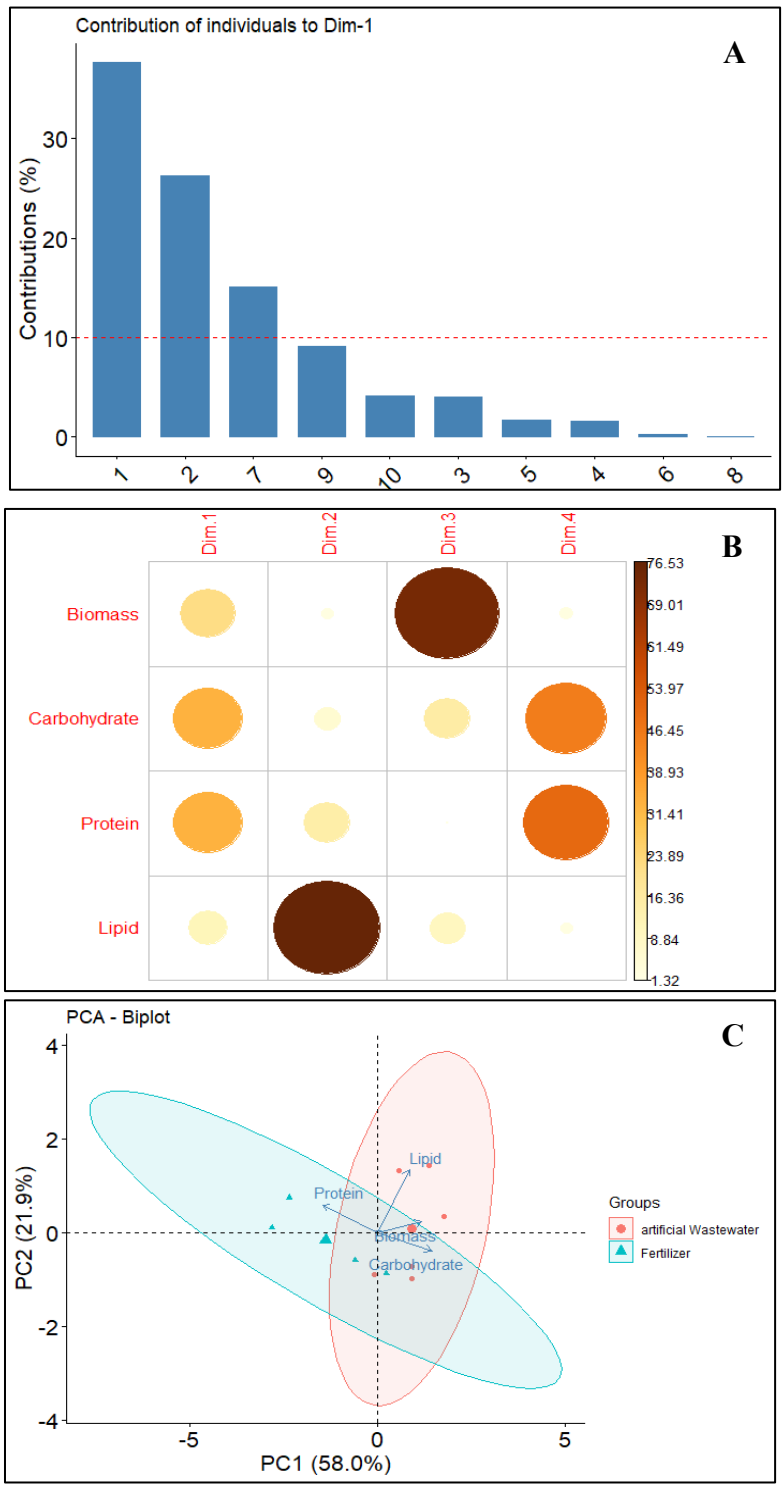


Figure 48: A) Bar plot representing contribution of each individual with respect to dimension 1; B) Cosine square plot or Corr plot represents impact of variable on different dimensions of the PCA; C) Cluster Biplot represents the covariance and correlation

#$FertV_{0.1}$ = Fertilizer based media under controlled condition in 0.1 L volume; $FertV_5$= Fertilizer based media under semi-outdoor condition in 5 L volume; $ArtV_{0.1}$= Wastewater based media under controlled condition in 0.1 L volume; $ArtV_5$= Wastewater based media under semi-outdoor condition in 5 L volume; $ArtV_{500}$= Wastewater based media under semi-outdoor condition in 500 L volume

1. $FertV_{0.1}$ at 3rd day; 2. $FertV_{0.1}$ at 7th day; 3. $ArtV_{0.1}$ at 3rd day; 4. $FertV_{0.1}$ at 7th day; 5. $FertV_5$ at 3rd day; 6. $FertV_5$ at 7th day; 7. $ArtV_5$ at 3rd day; 8. $ArtV_5$ at 7th day; 9. $ArtV_{500}$ at 3rd day; 10. $ArtV_{500}$ at 7th day

Lipid and biomass both showed a positive correlation with biomass and carbohydrate but a negative correlation with each other, whereas lipid exhibited a no correlation with protein and carbohydrate, and these two were negatively correlated with each other. The Biplot also predicted the influence of various treatments on these variables. The output of the chapter was clustered into two different groups, fertilizer based, and wastewater based. The clustered biplot graph has shown that lipid was strongly co-related with WWBM whereas other biomolecules were higher under FBM. To illustrate the point, the protein was majorly influenced by FBM, and WWBM mainly affected the lipid accumulation (Section B.2.2; Table 33).

D. Conclusions

The findings from this chapter can be summarized into three major aspects. Firstly, successful growth of *E. texensis* cells was established under the supplementation of both FBM and WWBM at lab-scale and in semi-outdoor conditions. The highest protein production was found in FBM while lipid production was more robust in WWBM, as shown in Table 33. Secondly, the cells of *E. texensis* exhibited exceptional growth kinetics and resilience in semi-outdoor conditions, resulting in improved biomass production (Table 32). Further, scale-up of the culture to a 500 L volume under WWBM enhanced cell kinetics and biomass composition, and the cells were able to tolerate high temperature and light intensity and could still flourish (Figure 47). Lastly, the results of PCA uncovered various correlations between variables and compositions that were consistent with the observed trends. Present findings confirmed the successful growth of *E. texensis* in both FBM and WWBM under semi-outdoor conditions, leading to the accumulation of high biomass and precursors of value-added products such as lipid, carbohydrate, and protein. Above mentioned value-added precursors and use of inexpensive media can overcome the bottleneck for economically sustainable microalgae-based biorefineries. Further

studies could focus on increasing the production of biopolymers by using wastewater having high COD, such as dairy or starch industry wastewater. Here we have shown the first report on analysis and cultivation of *E. texensis* at 500 L volume in semi-outdoor conditions with high temperature and light intensity.

CHAPTER 7

Conclusions and future prospective

A. Conclusive remarks

Increasing population and urbanization deflects on the plastic utilization as well as production. Exponential growth of online shopping and food delivery systems in urban area have seen a rapid growth in use of single use plastic and their disposal. Conventional plastics not only take a long time to degrade but also fossil fuel is consumed in its production. To combat the ever-increasing plastic problem an alternative polymer is a need of the hour. Polyhydroxyalkanoates or PHAs can be such alternative biopolymer that posses' similar properties as conventional plastic. After decades of research there are few commercial products available, yet the cost is much higher than conventional plastic. Some companies had to stop the production owing to higher production cost. The organisms used for commercial production are heterotrophic or methanotrophs pure culture. Sterilization and maintenance of pure cultures bears a major part of production cost. Mixed cultures could be and answer to the bottlenecks in replacing commercial plastic, however mixed cultures pose other niche of untargeted growth of microbe resulting in lower product yield or fluctuating properties of polymer. The key properties which maintain the mechanical properties are molecular weight and composition of monomers. These two physical properties affect the mechanical properties thus affecting the overall quality of the polymer being produced. A tailor-made consortium of photosynthetic microbes could solve help overcome the limitation of current state of biopolymers.

In the present work a tailor-made consortium was formulated for the production of PHA using microalgae and purple bacteria. These two microbes are predominantly present in the photosynthetic mixed cultures and produce PHA while valorising waste. A scale-up study of microalgae for the production energy precursors also took place to understand the growth pattern in semi-outdoor conditions. To formulate the tailor-made consortium, indigenous microalgae and purple non sulfur bacteria were isolated. These native microbes had the ability to

grow under the weather condition of Indore M.P. which will aid during the scaleup study. Various factors influencing the growth, PHA and energy precursor molecules were studied to optimize the best conditions for PHA accumulation in microalgae. For PNSB growth conditions, nutrient requirements, and supplementation of various VFAs were investigated and best conditions were finalized. After screening and selecting best species, conditions and nutrient requirement, a tailor-made consortium containing two photosynthetic microbes i.e., *E. texensis* and isolate PB_IIT_01 was cultivated in different media and photoperiod to achieve high PHA content. During this work 35.7% PHA accumulation in a tailor-made photosynthetic consortium under mixed carbon source and Fe supplementation. Multiparameter studies were statistically analysed using PCA, that helped in understanding the correlation between various nutrient and stress factors. Following sections elucidate the summary of each objective proposed in present work.

A.1 Biochemical profiling of indigenously isolated microalgal species

Seven indigenous microalgae were isolated, identified and phylogenetically analyzed from Indore M.P. Isolated species belonged to Chlorophyceae family belonging to green microalgae. The growth pattern, kinetics and biochemical profile was investigated for 28th day or till stationary phase under BG-11 supplementation. Seven species showed different growth patterns and accumulation of storage compounds. *Asterarcys quadricellulare* had shown the highest biomass productivity 51.0±2.0 µg/mL/day with highest chlorophyll (33.1±0.6 µg/mL) on 21st day. Highest carbohydrates and proteins have been accumulated by *Coelastrella* sp. and *P. daitoensis* respectively. Isolates have produced different ratios of PUFAs and MUFAs that act as a raw material for biofuels or nutraceuticals. Morphological analysis revealed the structure of isolates. Isolates such as *Pediludiela daitoensis* and *Desmodesmus pseudocommunis* had been explored for the first time for

biochemical screening. Three best lipid producing species explicitly *Pectinodesmus*, *Coelastrella* and *E. texensis* were selected and screened for PHA accumulation in next chapter.

A.2 Nutritional source amelioration for enhanced PHA synthesis in microalgal hosts *via* two-stage cultivation

For further screening of isolates, PHA accumulation was studied by employing a two-stage cultivation and a phototrophic *vs* mixotrophic approach. Cells were cultivated in various C-sources (Glucose, galactose, fructose, sucrose, mannose, maltose, acetate, glycerol) and varying concentration of inorganic nutrients (N, P, NaCl). Along with PHA, precursors of value-added products such as lipid protein and carbohydrate were also investigated. Mixotrophic growth was proven to be better for the production of biomass, PHA, and carbohydrate whereas lipid and protein accumulation was highest under autotrophic accumulation. Highest PHA content was achieved under galactose supplementation $P_{0.04}N_{1.5}Ga_{10}$ in *Coelastrella* sp. (151.8 ±12.1 µg/mg). *Pectinodesmus* sp. produced 2196.6±60.6 µg/mL biomass under galactose supplement. Highest carbohydrate (257.1±29.7 µg/mg) was accumulated under fructose supplementation $P_{0.04}N_{1.5}F_{10}$ in *Coelastrella* sp. Highest lipid (228.9±22.2 µg/mg) and protein (729.1±46.3 µg/mg) were accumulated under NaCl and high nitrate supplementation in *Coelastrella* sp. respectively. It was interesting to note that two out of three species preferred galactose as C-source for high PHA accumulation whereas *Pectinodesmus* preferred sucrose for highest PHA accumulation. A 116 folds increase in PHA was achieved using two stage cultivation as compared to control medium.

To summarize this chapter mixotrophic growth was proven to accumulate high PHA rather than autotrophic growth. Galactose was selected as the preferred carbon source for further optimization in the next chapter. Utilization of galactose by microalgae for PHA accumulation indicated that these species can also be cultivated in dairy wastewater having high COD resulting from presence of lactose.

A.3 Maneuvering PHA accumulation in microalgae by employing various stress factor

After selecting the carbon sources for high PHA accumulation, various other parameters such as cultivation duration, presence of light, concentration of galactose, initial inoculum concentration, cumulative nitrate and phosphate effect with galactose, phytohormone and heavy metals were investigated in order to optimize the best nutrient combination and conditions for PHA accumulation as well as selecting the best microalgal species. After exercising various stress factors, two factors were able to increase PHA accumulation. These factors are cumulative effect of N, P and galactose and heavy metal supplementation. High concentration of nitrate coupled with galactose ($P_{0.04}N_5Ga_{10}$) elevated PHA synthesis in *E. texensis* by 1.5 folds (196.6±8.1 µg/mg). Heavy metals except Fe negatively affected PHA, whereas Fe ($P_{0.04}N_5Ga_{10}Fe_{0.005}$) elevated PHA accumulation by 1.2 folds then highest nitrate and galactose supplementation (223.8±3.7 µg/mg). Highest lipid production was achieved under Ag ($P_{0.04}N_5Ga_{10}Ag_{0.005}$) supplementation in Pectinodesmus sp. (212.5±5.5 µg/mg). 200.5±12.0 µg/mL/day biomass was produced under the same combination in *E. texensis*, which proves that PHA could be accumulated under the nutrient combination without negatively affecting biomass productivity. High productivity is the key to a cost-effective production process. An overall 160 folds increase in PHA accumulation was achieved in *E. texensis* after optimization of various nutrient and stress factors. Thus *E. texensis* and the nutrient combination ($P_{0.04}N_5Ga_{10}Fe_{0.005}$) were selected for the formulation of tailor-made consortium. Biomass produced under this nutrient supplementation was extracted and characterized. The molecular weight of polymer was $1.3x10^3$ kDa which is categorized as high molecular weight polymer and higher that reported polymers.

A.4 Exploring indigenous purple non-sulfur bacteria for targeting PHA accumulation

As the third objective of this work, a photosynthetic purple non-sulfur bacterium was isolated from near IIT Indore using minimal salt medium (PB_ IIT_01). Based on colony morphology, microscopic analysis and Gram's nature of the cell it was identified as purple non-sulfur bacteria (PNSB). Growth nature and PHA accumulation were investigated and a comparative analysis of stirring and still cultivation conditions were studied. Still conditions were chosen owing to higher growth of isolate. PHA synthesis under growth medium was investigated at different time interval for 4 days and at 3rd day highest PHA (8.2 µg/mg) was produced and after 3rd day the PHA content decrease. To overcome lower PHA accumulation in PB_IIT_01, a modified MS medium was used as accumulation medium which was then supplemented with three different volatile fatty acids (VFAs) i.e., acetate, butyrate and propionate. A mixture of these three VFAs in 2:1:1 ratio was also supplemented. In combination with these VFAs absence and presence of phosphate was investigated as well. Highest PHA accumulation (95.3±3.1 µg/mg) was achieved under acetate supplementation under the absence of phosphate ($TP_0Amm_0A_4$) on 3rd day. Highest Biomass (412.3±23.0 µg/mL) was achieved under propionate supplementation with phosphate ($TP_{0.09}Amm_0Pr_4$) on 4th day. An 11.6 folds increase was achieved during the PHA optimization. PCA analysis revealed that DO and pH impose a negative and positive correlation respectively with PHA accumulation. Biopolymer produced was extracted and characterized. The extracted polymer has a high molecular weight and a higher maximum degrading temperature as compared to microalgal polymer.

The nutrient combination finalized in this chapter was used for formulation of a tailor-made consortium in the next chapter. Interaction between microalgae and purple bacteria were studies for enhanced PHA accumulation.

A.5 Algae-bacteria consortium for enhanced biopolymer production and its characterization

Optimization and screening from previous chapters were utilized in formation of a tailor-made consortium. This consortium has *E. texensis* and PB_IIT_01 isolate in 1:2 ratio. These taxonomically different species were selected for PHA accumulation to limit the inter species out competing and include larger metabolic pathways contained in these two. Two different media were formulated based on best combination achieved by microalgae and PNSB optimization. The consortium accumulated 357.3±0.3 μg/mg PHA under supplementation of medium A in 12:12 photoperiod. An overall increase of 32 folds was achieved as compared to control. A comparative analysis also revealed 1.6 and 5 folds more PHA accumulation than compared to microalgae and PNSB under same supplementation. Symbiotic growth relation of microalgae and PNSB was visualized using TEM analysis where the microalgal cells were more health with PNSB present in medium as compared to pure cultures. Consortium preferred BG-11 based medium with light dark photoperiod, for higher PHA accumulation. Biopolymer produced from consortium is an ultra-high molecular weight polymer and has a higher Mw than the pure cultures. This novel approach of tailor-made consortium could be utilized for scale-up process without the need of feast-famine regime of PMCs that is time consuming and do not guarantee the high quality or quantity of polymer.

A photosynthetic tailor-made consortium could help to overcome the bottlenecks of scaleup of mixed culture and decrease the cost associated with pure culture and maintenance. To understand the impact of scaleup in semi-outdoor conditions, *E. texensis* was investigated. A comparative analysis with fertilizer and wastewater based medium for production of energy precursors were studied in the last chapter.

A.5 Scale-up study: Assessment of growth behavior of microalgae under semi-outdoor condition

The findings from this chapter can be summarized into three major aspects. Firstly, successful growth of *E. texensis* cells was established under the supplementation of both FBM and WWBM at lab-scale and in semi-outdoor conditions. The highest protein production was found in FBM while lipid production was more robust in WWBM. Secondly, the cells of *E. texensis* exhibited exceptional growth kinetics and resilience in semi-outdoor conditions, resulting in improved biomass production. Further, scale-up of the culture to a 500 L volume under WWBM enhanced cell kinetics and biomass composition, and the cells were able to tolerate high temperature and light intensity and could still flourish. Lastly, the results of PCA uncovered various correlations between variables and compositions that were consistent with the observed trends. Present findings confirmed the successful growth of *E. texensis* in both FBM and WWBM under semi-outdoor conditions, leading to the accumulation of high biomass and precursors of value-added products such as lipid, carbohydrate, and protein. 1.7 folds increase in lipid concentration (493.4±34.4 μg/mg) was achieved under 500 L pond cultivation in WWBM as compared to lab-scale cultivation under same medium. Above mentioned value-added precursors and use of inexpensive media can overcome the bottleneck for economically sustainable microalgae-based biorefineries. Further studies could focus on increasing the production of biopolymers by using wastewater having high COD, such as dairy or starch industry wastewater. Here we have shown the first analysis and cultivation of *E. texensis* at 500 L volume in semi-outdoor conditions with high temperature and light intensity.

B. Future perspectives

The present work focused on formulating a tailor-made consortium of native photosynthetic microbes for biopolymer production. A multi-objective screening and optimization for maximizing the biomass

productivity, energy precursors profile and biopolymer production. The correlation between various stress and nutrients and their interactive effects were investigated. Based on the findings of this research work, certain future directions for further research in this field are suggested as follows:

- A time dependent biochemical profile could unravel various storage compounds and precursors of energy and value-added products such as biodiesel, biopolymer, or nutraceuticals.
- The findings of this work will help in finding out various other unexplored microalgal species for mixotrophic growth to attain higher biopolymer synthesis and various trigger of nutrient in microalgae, which have not yet been explored to its full potential.
- A photosynthetic tailor-made consortium would not only reduce the untargeted growth of microbes in a PMCs and reduce the cost of fermentation, but it will also remove the need to enrich PMCs using feast and famine condition which is a time consuming and not a permanent process.
- Characterization of polymer would also enlighten the importance of substrate, host and their impact on the physical properties of polymer which in turn affect the mechanical properties of the biopolymer. Parameters such as PDI are often neglected yet very important to mold the polymer for a desired application.
- A scale-up study of *E. texensis* under the semi-outdoor condition under fertilizer and wastewater-based media would help understanding the impact on outdoor condition on microalgae and feasibility of achieving higher biochemical profiles from a biorefinery perspective.

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Kanchan Samadhiya

Biotechnologist

Born on 17.07.1993 in Gwalior India

Single

About me:

- Motivated and hardworking Algal Biotechnologist
- Experience in Microbial Biotechnology
- Experience in Gas chromatography
- Critical thinker and goal-oriented
- Strong learning ability
- Good inter-personal skills
- Highly experienced in verbal and written communication skills and scientific writing
- Sincere and optimistic with the team's vision
- Proficient in English and MS-office

WORK EXPERIENCE

10.2023-Present	**Leibniz University Hannover, Germany** Post-doctoral Fellow *(Kanal Detective)* Monitoring of Multi resistant bacteria in sewer systems • qPCR

- Bacterial isolation
- Wastewater monitoring
- ddPCR

07.2023-10.2023 **Leibniz University Hannover, Germany**
SING Visiting Fellow
(Biohydrogen: A carbon-neutral energy vector from Purple Non-Sulfur Bacteria)

- Anaerobic Cultivation of PNSB
- Gas analysis using GC-TCD
- Biochemical analysis

06.2017 – 06.2023 **IIT Indore India**
Research Scholar

- Bioprocess engineering and downstream processing
- Isolation, identification and maintenance of microalgae, cyanobacteria and purple non-sulphur bacteria
- Microscopic Techniques such as: Light Microscope, Confocal Microscope, Scanning Electron Microscope and Transmission Electron microscope
- Biochemical analysis and method optimization
- Gas chromatography and GC-Mass spectroscopy method development
- Polyhydroxyalkanoates (PHA), Lipid, Carbohydrate and Protein production in various microbes
- Co-cultivation of microalgae and purple non-sulphur bacteria
- Scale up of microalgal culture in semi-outdoor condition
- Polymer extraction and characterization
- Principal Component Analysis (PCA) using R
- Practical classes for Master's student as a Teaching Assistant
- Supervision of Master's Theses

08.2022 – 12.2022	**Leibniz University Hannover, Germany** Visiting Researcher • Microscopic Analysis of purple non-sulphur bacteria • Gas chromatography and GC-Mass spectroscopy method development • Biohydrogen production by purple non-sulphur bacteria • Biochemical tests for nutrition uptake
04.2019 - 06.2019	**Leibniz University Hannover, Germany** Visiting Researcher • Analysis of Polyhydroxyalkanoate using Microscopy and Gas Chromatography Analysis • Preliminary screening of potential PHA producers
08.2016 - 05.2017	**Choithram College of Professional Studies Indore** Assistance Professor • Undergrad and Postgrad classes for Biotechnology, Zoology and Environmental Studies • Undergrad and Postgrad practical classes for Biotechnology and Zoology
09.2015 - 03.2016	**CSIR- National Chemical Laboratory Pune** Research Trainee Thesis Title: "Biochemical Characterization of Long Chain Poly Unsaturated Fatty Acids (LCPUFAs)" • Maintenance of Diatom and Fungi cultures • Gene isolation from Fungi: DNA, RNA isolation, PCR for gene amplification, Colony PCR, Transformation etc. • Optimization of PCR conditions • Gas Chromatographic analysis of Fatty Acid Methyl Esters (FAMEs)

EDUCATIONAL QUALIFICATION

06.2017 – till date	**Indian Institute of Technology Indore India** Doctor of Philosophy (Ph.D.) in Biotechnology Thesis title "Indigenous Microbes for Sustainable Biopolymer Synthesis: A Comprehensive Optimization and Characterization Study" Course Work: CGPA 8.12/10
07.2014 - 05.2016	**Maharaja Ranjit Singh College of Professional Studies Indore, India** Master of Science in Biotechnology Percentage: 75%
07.2012 - 06.2014	**Government Kamla Raja Girls Post Graduate (Autonomous) College Gwalior, India** Bachelor of Science in Biotechnology Percentage: 61%

AWARDS

2023	**Small Immediate Need Grant** Indo-German Science and Technology Center
2022	**DAAD "A new passage to India" Mobility Fellowship** Leibniz University Hannover, Germany
2019	**ip@LUH Visiting Fellowship** Leibniz University Hannover, Germany

SKILLS

Software	MS office (Proficient)
Languages	English (Proficient) Hindi (Native)

PUBLICATIONS (First Author)

2023	Scaling up of native species for a sustainable microalgal biorefinery targeting different microalgal products. Algal Research, 75, 103246. https://doi.org/10.1016/j.algal.2023.103246

	Effect of acute vs chronic stress on Polyhydroxybutyrate production by indigenous cyanobacterium. *International Journal of Biological Macromolecules*, 227, Pages 416-423. doi.org/10.1016/j.ijbiomac.2022.12.177
2022	Newly isolated native microalgal strains producing Polyhydroxybutyrate and energy storage precursors simultaneously: Targeting microalgal biorefinery. *Algal Research*, 62, 102625. doi.org/10.1016/j.algal.2021.102625
2021	Bioprospecting of native algal strains with unique lipids, proteins, and carbohydrates signatures: A time dependent study. *Environmental Progress & Sustainable Energy*, e13735. doi.org/10.1002/ep.13735
	Insightful advancement, and opportunities for microbial bioplastic production. *Frontiers in Microbiology*, 3755. doi.org/10.3389/fmicb.2021.674864
PUBLICATIONS (Co-Author)	
2022	Multi-objective tailored optimization deciphering carbon partitioning and metabolomic tuning in response to elevated CO_2 levels, organic carbon and sparging period. *Environmental Research*, 204, 112137. doi.org/10.1016/j.envres.2021.112137
	A strategy for lipid production in Scenedesmus sp. by multiple stress induction. Biomass *Conversion and Biorefinery*. 3037-3047. doi.org/10.1007/s13399-021-01392-2
2021	Synthesis, characterization, and application of intracellular Ag/AgCl nanohybrids biosynthesized in Scenedesmus sp. as neutral lipid inducer and antibacterial agent. *Environmental Research*. doi.org/10.1016/j.envres.2021.111499

	Maximizing intrinsic value of microalgae using multi-parameter study: conjoint effect of organic carbon, nitrate, and phosphate supplementation. *Clean Technologies and Environmental Policy*, 1-13. doi.org/10.1007/s10098-021-02192-y
2020	The use of response surface methodology for improving fatty acid methyl ester profile of Scenedesmus vacuolates. *Environmental Science and Pollution Research*, 27(22), 27457-27469. doi.org/10.1007/s11356-019-07115-5
	Screening of microalgae for biosynthesis and optimization of Ag/AgCl Nano hybrids having antibacterial effect. *RSC advances*, 9(44), 25583-25591. https://doi.org/10.1039/C9RA04451E
2019	Salinity driven stress to enhance lipid production in Scenedesmus vacuolates: A biodiesel trigger? *Biomass and Bioenergy*, 127, 105252. https://doi.org/10.1016/j.biombioe.2019.05.021
2018	Strategies to unlock lipid production improvement in algae. International *Journal of Environmental Science and Technology*, 16(3), 1829-1838. doi.org/10.1007/s13762-018-2098-8
Workshop and Conferences	
February, 2023	**5-day hands on training workshop "Characterization and quantification of emerging contaminants"** 05.02.2023-10.02.2023 IIT Indore • Organized the workshop • Training of GC-MS for characterization of contaminant to participants
February, 2022	**Research Industrial Conclave 2022** Scientific Quiz and Poster Presentation

- First position under Scientific Quiz, Health Informatics

June, 2021

10th International Conference on Algal Biomass, Biofuels and Bioproducts (Algal BBB) online

Poster Title: "Unearthing promising indigenous producer of biopolymer from native sites of central India

November, 2018

Workshop on "New Frontiers in Algal Omics" organized by ICGEB Delhi

19th-30th November 2018

EXTRACURRICULAR ACTIVITIES

05.2018-06.2018

Vigyan Jyoti Program at Indian Institute of Technology Indore (IIT Indore)

Knowledge, Personality and Skill development activities for aspiring school-girls'

- Mentoring and introducing a female student to the world of Microbiology
- Scientific activities to peak student's interest in science

09.2015-06.2016

Microbiologist Society India (https://microbiosociety.com/)

President, Indore Chapter

- Organization of various college level activities as an introduction to microbiology

07.2012 - 07.2014

National Cadet Corps, India (https://indiancc.nic.in/)

Senior Under Officer

- Discipline Training
- Leadership Training
- International and national camp participation
- Republic Day Camp 2012

	• Prime Minister Rally 2012 • Supervision of Cadets from Group level • Participation in Cultural exchange programs
2009 - 2014	**Surya Foundation India (**www.suryafoundation.org) Group Leader • Training of yoga, self-defense and naturopathy • Leadership Training • National personality development camp participation and organization
2008-2014	**Vivekanand Kendra Kanyakumari India (**https://www.vrmvk.org/home-1) Volunteer • Training of yoga, self-defense and naturopathy • Cultural and National Building Exercise • National personality development camp participation and organization

ADDITIONAL INFORMATION

Reference 1	Prof. Kiran Bala PhD Thesis Supervisor Associate Professor, Department of Biosciences and Biomedical Engineering Indian Institute of Technology Indore Simrol, Khandwa Road Indore M.P. India (453552) Email: kiranb@iiti.ac.in Phone: +91 731 6603266
Reference 2	Prof. Dr. Ing. Regina Nogueira Post-doctoral Supervisor Institute for Sanitary Engineering and Waste management Leibniz University Hannover Welfengarten 1, 30167 Hannover Email: nogueira@isah.uni-hannover.de Phone: +49 511 7623371

Indore, 15.12.2023