INSIGHT ON GREEN-BIOPOLYMER PRODUCTION VIA PHOTOSYNTHETIC MICROBES

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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Submitted in partial fulfilment of the

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **INSIGHT ON GREEN-BIOPOLYMER PRODUCTION VIA PHOTOSYNTHETIC MICROBES** in the partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE** 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 August 2020 to May 2022 under the supervision of Dr. Kiran Bala, Associate Professor, Department of Biosciences and Biomedical Engineering.

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.

05/05/22
Signature of the student
(Smrity Sonbhadra)

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

Signature of the Supervisor of M.Sc. thesis
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Smrity Sonbhadra

Dedicated to

My Beloved Family

ABSTRACT

Since the use of synthetic plastics has raised exponentially in the past few decades, there is an utmost importance of to find for an alternative. As a result, we have bioplastics, also termed as green polymers in the line, possessing similar characters to synthetic plastics as well as being environment friendly/biodegradable and non-toxic in nature as an additional boon. In this work, polyhydroxyalkanoates (PHAs), a type of bioplastics have been focussed, extracting from *E. texensis* (a microalgae) and a purple bacterial strain isolate. The two groups pof photosynthesizing microbes, i.e., microalga and the purple bacteria were first screened (based on their growth curves) from 7 available microalgae and 4 available purple bacterial strains in our lab. Once finalizing the species, these two candidate species were provided with different conditions like; presence of volatile fatty acids in the surrounding (source of external carbon), nitrate-phosphate variations, salinity, etc. to determine their impact on PHA accumulation in these groups of microbes. Based on the previous studies carried out in this lab, all of these factors were checked in the presence of external carbon source (galactose) in the surrounding of the microalgae, that was found to be best uptaken by them switching on to mixotrophic mode of nutrition and respond better. Also, harvesting time duration was optimized at which best results in terms of PHA accumulation was found both in microalga and purple bacteria. This study was done keeping in mind with the future prospect of scaling up the study with wastewater, determing the techno-economic analysis of the overall process and thereby finding the economic feasibility involved. As majority of these conditions are naturally available in wastewater, the study provides a novel and significant contribution in this field. As a result of which, better growth and PHA accumulation was found in absence of nitrates and phosphates than in control. VFAs had no significant effect on PHA accumulation, though they promoted photosynthetic pigment synthesis.

Salts negatively regulated bioplastics accumulation. And 72 hours was found to be the best harvesting time duration they maximum accumulated PHA. For purple bacteria, as they were grown for 15 days, two types of conditions were provided to them i.e., a stirring and a still condition. For the growth profile study of the 4 purple bacterial strains (PBYB, EB, EA and 4C), majority of them attained their decline phase after 10^{th} day and grew better in still condition (since are anoxygenic). Also, PBYB was found to be highest PHA accumulating bacteria among the 4. And on both 10^{th} and 15^{th} day of their growth, PHB was the major PHA (as compared to PHV) being accumulated by all the 4 strains in still condition. Also, in the presence of acetate (a VFA), PBYB accumulated highest PHA on 48^{th} hour. Future studies of growing these species at larger scale in raceway ponds with wastewater to determine the economic efficiency of the process is to be carried out further, that acclamitization of the species in that condition first, followed by their growth profile study and then determining PHA accumulation potential by these candidate species at larger scale.

LIST OF PUBLICATIONS

- Sangtani, R., **Sonbhadra, S**., Nogueira, R., & Kiran, B. (2022). Algal Biorefinery: A Paradigm to Sustainable Circular Bioeconomy. In *Zero Waste Biorefinery* (pp. 295-323). Springer, Singapore. (Book chapter)

- Samadhiya, K., **Sonbhadra, S**., Kiran, B., Optimization studies for sustainable biopolymer production from native species. (To be submitted)

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LIST OF ABBREVIATIONS

PE- Polythene

PP- Polypropylene

PVC- Polyvinylchloride

PLA- Polylactides

PHA- Polyhydroxyalkanoates

PHB- Polyhydroxybutyrate

PHV- Polyhydroxyvalerate

PGLA- Polylactic-co-glycolic acid

CDW- Dry cell weight

LPC- Lipid, protein, carbohydrate

SPV- Sulpho-phospho schechvanillin

FID- Flame ionization detector

MSM- Minimal salt media

MCTs- Microcentrifuge tubes

VFAs- Volatile fatty acids

DO- Dissolved oxygen

BG-11- Blue green-11

OD- Optical density

TEA- Techno-economic analysis

Chapter 1

Introduction

Synthetic petroleum-based plastics have been in use since the early 1940s. By then, the slope for their demands has never declined. They have immensely become an integrated part of the exponentially growing human population so that we start our day by holding on to a plastic brush to switching off the plastic buttons for lights before going to bed. Plastics possess highly desirable properties as they are light, flexible, robust, durable, etc.[1,2] We come across their use in a much more significant way than we can think of. The primary raw materials for most of these synthetic plastics are crude oil and natural gas. Some common examples of these synthetic plastics are PE (polythene), PVC (polyvinylchloride), PP (polypropylene), etc. However, the flaws associated with synthetic plastics cannot be ignored, including the production of toxic products followed by their incineration to being resistant to degradation, thus leading to the piling of the copious quantity of these non-degradable substances in and around our planet.

"Bioplastics," also known as the green-polymers, have emerged as an attractive alternative to synthetic plastics. Bioplastics are polymers produced naturally by certain microorganisms under various environmental conditions (carbon-rich, nutrient deficient, etc.) [3]. In addition to the properties exhibited by synthetic plastics, they show some surplus advantages of being biocompatible, non-toxic, bio-degradable, eco-friendly, etc., in nature. Some common examples of bioplastics include PLA (polylactides), carbohydrate polymers, PHAs (polyhydroxyalkanoates), etc.

1.1 What are PHAs (Polyhydroxyalkanoates)?

1.1.1 Structure of PHA:

Polyhydroxyalkanoates are lipid molecules synthesized as insoluble intracellular storage materials in many microorganisms under stress

conditions. Maurice Lemoigne, a French scientist, first identified a PHA molecule (in the form of PHB, polyhydroxybutyrate) in 1926 in a bacterium *Bacillus megaterium* [4]. The ester bond is formed by linking the (-COOH) carboxylic group of a monomer with the (-OH) hydroxyl group of a neighboring monomeric unit [5].

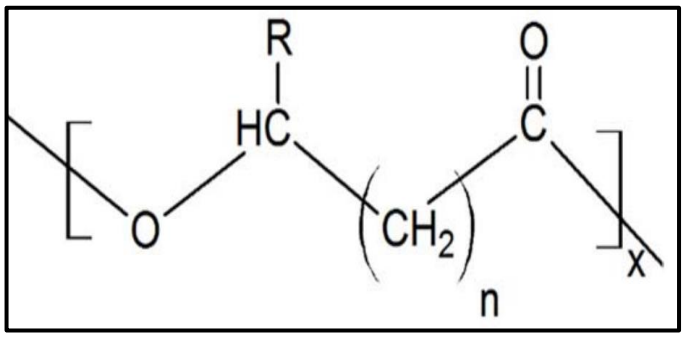


Fig. 1.1: Chemical structural formula for PHA. (n could vary from 1 to 4, x ranges from 100 – 300 000, R-alkyl side chain) [6]

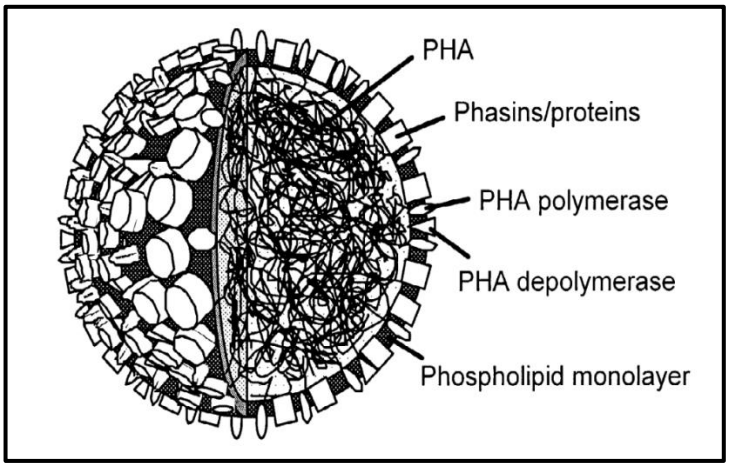


Fig. 1.2: Granular structure of PHA molecule [4]

1.1.2 Classification of PHA

PHAs can be classified in the following ways:

I. Based on the chain length and the number of carbon atoms in the monomeric unit, PHAs are categorised as scl (short-chain length) having 3-5 carbon atoms, e.g., P(3HB), P(4HB), P(3HB-co-3HV); mcl (medium-chain length) having 6-14 carbon atoms, e.g., P(3HHx), P(3HO), P(3HHx-co-3HO) and lcl (long-chain length)

having greater than 14 carbon atoms, these are found rare in nature and also are of not so much interest [7,8].

II. Depending upon the type of monomeric units as PHAs are of 2 kinds; homopolymer (having similar monomeric units), e.g., poly(3-hydroxybutyrate), poly(3-hydroxyoctanoate), and copolymer (having different monomeric units), example, poly(3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-co-3HV)), etc. [9]

1.2 PHA Synthesis Pathways

The type of PHA being generated depends mainly on the carbon source available in the environment of the host organism. There are 3 commonly known pathways for PHA synthesis (Fig. 3). In the first pathway, 2-acetyl-CoA, produced from either of the sources (sugars, amino acids, or fatty acids), is transformed into hydroxybutyric-coA involving the enzymes acetoacetyl-CoA reductase and β-ketothiolase. Hdroxybutyryl-coA is further polymerized by PHA synthase to give PHB. This particular pathway is mainly responsible for the scl type of PHA. The II pathway converts fatty acids into enoyl-coA, which is further converted to R-3-hydroxyacyl-coA by the enzyme R-3-hydroxyacyl-CoA hydratase. Subsequently, PHA synthase catalyzes PHA production from R-3-hydroxyacyl-coA. This pathway deals with mcl-type PHA production. The III pathway involves in situ fatty acid synthesis wherein 3-hydroxyacyl-ACP is converted to 3-hydroxyacyl-coA that further produces PHA, catalyzed by the enzyme 3-hydroxyacyl-acyl carrier protein-CoA transferase [10].

Furthermore, many genes, as well as enzymes, take part in PHA biosynthesis [11]. These three are reported as the essential enzymes, viz., PhaA (β-ketothiolase), PhaB (acetoacetyl-CoA reductase), and PhaC (PHA synthase) [12]. Moreover, 3 classes of PHA synthase enzymes have been stated: Type I, Type II, and Type III. Where type I synthase enzyme produces PHA polymers with molecular weights ranging from 500,000 to several million kDa, Type II synthase is responsible for making PHAs with molecular weights from 50000–500000 Da, and

Type III is known to produce PHA molecules with molecular weights ranging in between to those synthesized by Type I and II synthases [11].

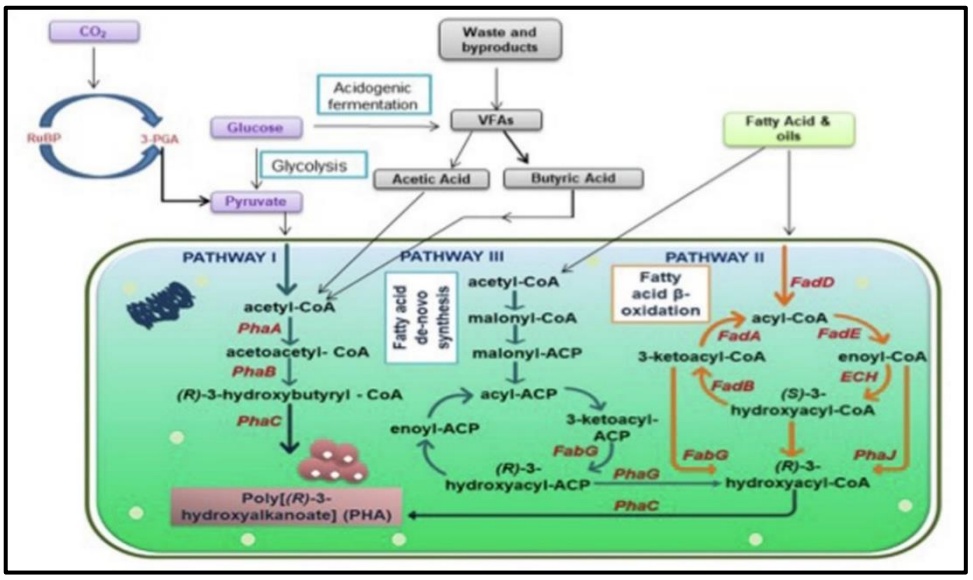


Fig. 1.3: Schematic diagram of PHA biosynthetic pathways found in microbes [13]

1.3 Characteristics and Applications of PHAs

PHA, being a bioplastic, is in huge demand and has a broad range of applications because of its characteristic properties [8]. They can be summarized as follows;

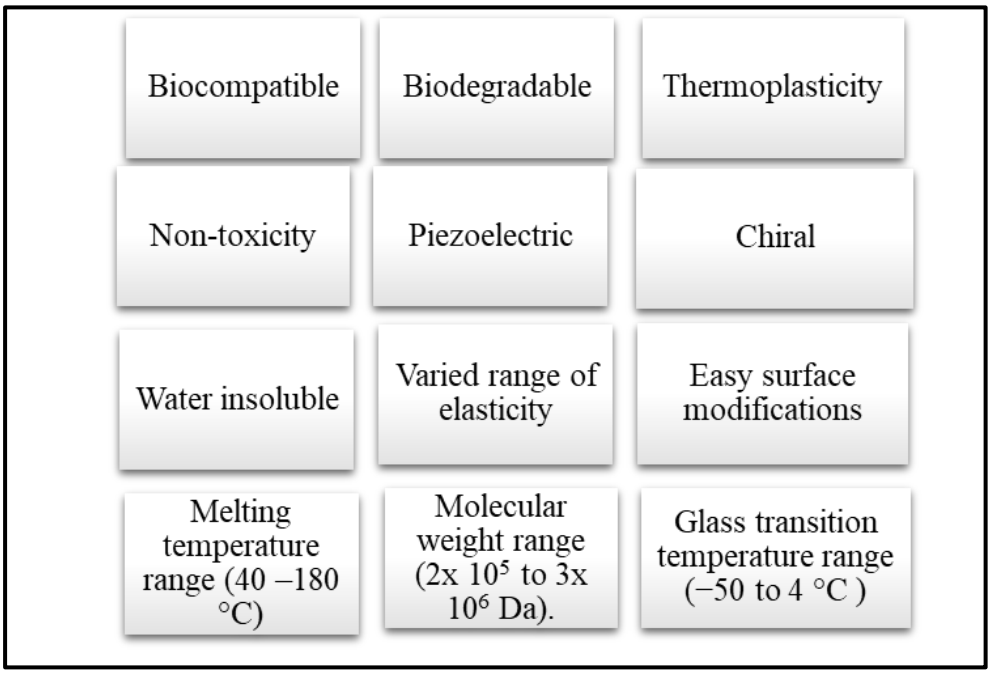


Fig. 1.4: Characteristic properties of PHA

PHAs are being used in different sectors for many other applications. For instance, being biocompatible and non-toxic allows them to be used as a bio-implant in the body without owing to the risk of causing inflammations and other infections. To this day, PGLA (polylactic-co-glycolic acid) is the only FDA-approved polymer that can be administered in the human body. PHB can replace such synthetic polymers in health industries. Also, nowadays, they are popularly being used in surgical needles, sutures, stents, orthopedic pins, drug-delivery carriers, famine hygiene products, and various other such innovative products in the medical sector[[14]]. They are used as packaging materials, paper coatings, smart materials in material industries, biofuel additives, etc. [[15]]

Since PHAs exhibit similar physicochemical, thermal & mechanical behaviors as synthetic plastics, hence can be considered an attractive and sustainable replacement for petrochemical polymers, as represented in Table 1[[16]].

Table 1.1: Comparison of properties of scl-PHAs, mcl-PHAs and their copolymers with polypropylene (conventional synthetic plastics) [[17]]

	Homopol-ymer (scl-PHAs)	**Homop-olymer (mcl-PHAs)**	**Copolymer P(3HB-co-3HV)**	**Polypropylene**
(T_m) Melting Temperature (°C)	179	80	137-170	176
(Tg) Glass transition Temperature (°C)	4	-40	10- (-6)	-10
(Y) Young's modulus of elasticity (in GPa)	3.5	NA	0.7-2.9	1.7
Elongation to break (%)	40	300	30-38	38
Tensile strength (in Mpa)	5	20	Up to 690	400

Chapter 2

Literature review

A wide range of microorganisms (including prokaryotic microbes like bacteria, purple sulfur bacteria, photosynthesizing cyanobacteria, eukaryotic microalgal species, etc.) is used to study bioplastics production.

2.1 PHA Production from a Bacterial Source

2.1.1. Eubacteria:

Many bacterial populations have been reported to be an affluent source of PHA accumulation. Some common examples include *Novosphingobium nitrogenifigens* Y88, giving a yield of 81% of their total (dry cell weight) CDW as PHB providing glucose in supplementation [18]. *Pseudomonas putida* KT2440 producing PHAs 48.8% of CDW under oleic acid supplementation [17]; *Pseudomonas extremaustralis* 14-3b, using sodium octanoate as a C- source synthesized 35.80% PHA of their CDW [19].

Though these bacteria can produce a considerable amount of PHAs, they have certain limitations. The overall process of PHA production is cost-intensive and demands high energy input for harvesting, polymer extraction, excess of good quality carbon source supplementation, etc. To overcome these challenges, researchers are now trying to explore photosynthetic microbial species for the same.

2.1.2. Photosynthetic Bacteria:

Photosynthesizing prokaryotic bacteria include purple bacteria and cyanobacteria. These bacterial populations were studied for many decades and yet left to be explored further. Purple bacteria are prokaryotic anoxygenic (do not require oxygen) bacteria with certain photosynthetic pigments, allowing them to perform photosynthesis. PSB (purple sulfur bacteria utilizes hydrogen sulfides as electron donors) while PNSB (purple-non sulfur bacteria) typically utilizes hydrogen (H)

as electron donors. Some of these purple sulfur and purple non-sulfur bacteria (PNSB) ascertained for this purpose encompass; *Rhodobacter sphaeroides* RV, producing different concentrations of PHAs on providing various carbon sources, such as 40% with acetate, 12% with lactate [20], *Rhodospirillum rubrum* producing 46.8% of PHB with β-Hydroxybutyrate as a carbon source [21], PHB accumulation by various Rhodovulum species (PNSB) has been examined by Takeuchi et al., [22] ranging PHB yield from 2-30% using seawater to reduce cultivation costs. Cyanobacteria, also called BGA (blue-green algae), possess photopigments similar to plants (like chlorophyll a) that make them capable of performing photosynthesis. Some common cyanobacterial species being explored for PHA production purposes include; *Nostoc muscorum* yielding 22% of PHA in a nutrient-deficient environment (nitrates and phosphates) and xylose and glucose as carbon sources[23], *Synechocystis sp.* giving 38% of PHA provided styrene as carbon source and nitrogen-deficient environment [24], *Spirulina* sp. with 13.4% of PHA [25].

2.1.3. PHA from Microalgae

Microalgae are the eukaryotic microscopic photosynthesizing species that are now being extensively studied as a platform for bioplastic accumulation. Though currently, biopolymers production from microalgae is not cost-effective, certain assets associated with microalgae make it an attractive candidate organism to be explored and studied further. Some of the associated benefits with microalgae could be stated as; their ability to perform photosynthesis help in (Carbon dioxide) CO_2 sequestration, thereby regulating CO2 levels in the atmosphere; they are known to accumulate lipids in high amounts, show a high growth rate, and can even grow in wastewaters using nutrients (nitrates, phosphates) from it fulfilling the dual purpose of wastewater treatment as well as their growth, wherein this algal biomass could be further utilized to process multiple product streams(Fig. 5), including the high and low value-added products (e.g., biofuels, biogas, bioplastics, nutraceuticals, vitamins, pigments, etc.) promoting the

concept of circular economy, making the overall system much more economically sustainable [26,27]. Hence this biorefinery model for microalgal biomass could make it a much more feasible resource [28,29]. Some common studies involving microalgae as a source of PHA production include; *Chlorella fusca* giving 17.4% PHA [30], *Scenedesmus* sp. yielding about 29.92% of PHA [31], etc.

2.1.4. PHA Production using Microbes Grown in Waste-water

Different waste waters and industrial effluents are a rich source of metals, salts, and organic sources that can be used as a medium to grow and cultivate microbes [32, 33]. Certain studies have shown that bacterial and microalgal species can thrive in waste-water constituents and accumulate high biomass, which can be further processed to yield value-based products for a more sustainable biorefinery approach in combination to waste water remediation [34]. For instance, *Arthrospira* species are grown in waste water with a dual purpose of biodiesel, biogas, and other value-added products production and integrating it to waste-water treatment, hence significantly reducing the overall cost of the process [35]. Microalgae Consortium (MC), mainly consisting of *Scenedesmus* and *Desmodesmus* species (~80%), was used to grow in wastewater that helped in removing nitrates and phosphates from it and in turn producing high protein content (~48%). Biomass produced in the process was further blended with glycerol to produce bioplastic [36]. Due to high volumes of wastewater and cost constraints, often mixed cultures are preferred over pure cultures. In a study, mixed microbial cultures were used to study their PHA accumulating potential by growing them in fruit waste water. They got pretty positive results with about 80.5% of PHA as a maximum yield [37]. A mixed photosynthetic culture (MPC) comprising of microalgae and bacterial consortium was used, growing the culture in oxygen-deficient (famine and feast regime) which led to an accumulation of nearly 20% PHA content of their biomass [38]. In another study, *Nannochloropsis*, a microalga was grown using corn starch followed by mixing with petroleum-based bioplastic that was

used to accumulate bioplastics in an (algae/bioplastic) ratio of 14/80 % [39]. Similarly, various other microalgal and bacterial species and their consortium could be utilized to remediate different waste-water streams and channel their potential to accumulate bioplastics and other value-added products.

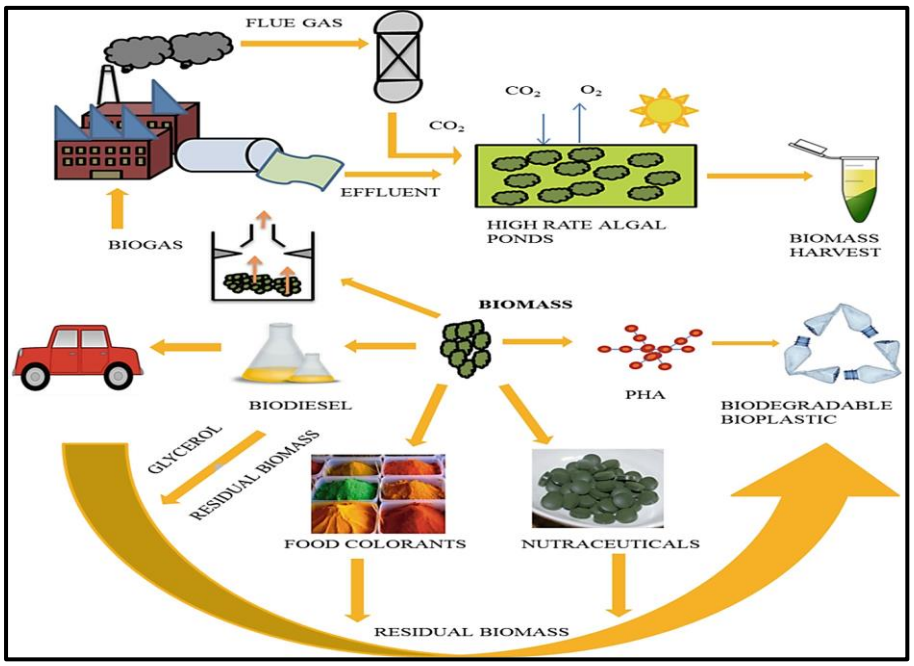


Fig. 2.1: A pictorial representation for the circular economy via the sustainable bioeconomic process for value-added products generation from microalgae [23]

2.1.5. High Rate Algal Ponds (HRAPs)

High rate algal ponds also known as the raceway ponds that are artificially designed to perform the scale-up studies. They are shallow and provided with a cylindrical shape, having curves at the ends that gives proper turbulence to the pond algalculture and further aided with low-power paddle wheel which is used for maintaining proper aeration and mixing of components (sunlight, air, nutrients) present in the pond [40]. They form an innovative and affordable approach to growing algae, and sometimes bacteria get sufficient sunlight exposure (since they are shallow). These can also be used to grow the algal/bacterial culture providing wastewater as the nutrient media, well circulating in the pond.

They are widely being utilized at present to carry out scale-up experiments that also help to evaluate the TEA (techno-economic analysis) of the overall process [41]. For instance, *Chlamydomonas reinhardti* have been grown and cultivated in raceway ponds for scale-up studies for maximizing biofuels and bioproducts at a larger scale from lab to fields [42], *Spirulina platens* was cultivated for high biomass production in raceway ponds utilizing underground water, etc. [43].

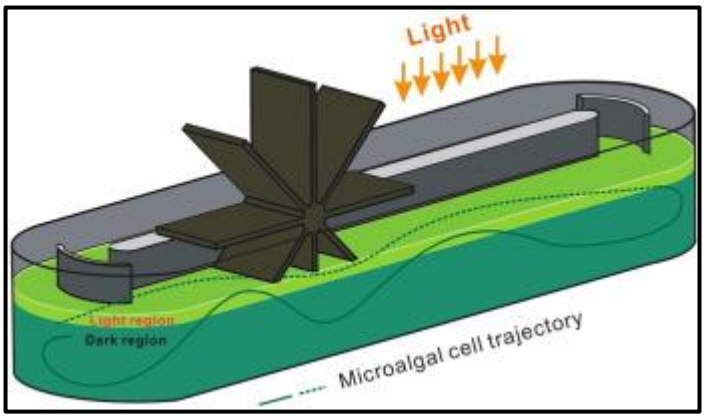


Fig. 2.2: Raceway ponds showing basic design and construction [44]

2.1.6. Selected Microalgal Species for the Study : *Ettlia texensis*

Ettlia texensis is a green microalgal species generally found in freshwater lakes. They have been known to possess a high growth rate and have been reported as a rich source of various lipids and saturated and unsaturated fatty acids accumulation [45].

This species was isolated from Narmada river Maheshwar in our lab for experimental purposes. The taxonomic classification of *E. texensis* is as follows:

Division: Chlorophyta

Class: Chlorophyceae

Order: Chlamydomonadales

Family: incertae sedis

Genus: *Ettlia*

Species: *texensis*

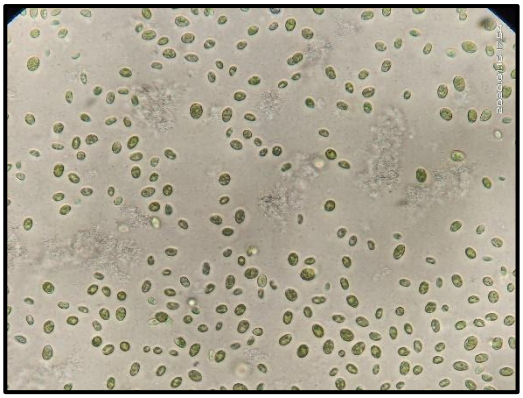


Fig. 2.3: Microscopic image of *E. texensis*

2.1.7. Different Modes of Nutrition in Microalgae:

Though microalgal cells are capable of performing photosynthesis and preparing their own food, some are also known to switch on to utilize external carbon sources as their food.

On this basis, there are three types of nutrition microalgae [46] are known to exhibit, which are as follows;

I. **Autotrophic mode:** This is the most common and well-known mode of nutrition for chlorophyll-bearing algae or plants. Here, the microalgal cells entirely depend on photosynthesis they perform for their food and primary metabolism. It is performed in the presence of light.

II. **Mixotrophic mode:** Here, the microalgal cells, in the presence of light, perform photosynthesis and prepare their own food, but in while dark (i.e., in the absence of light) and in the supplementation of an external C- source, they become capable of utilizing that external C-source and uptake the already available food (carbon sugars) from their environment, thereby switching on to the mixotrophic mode of nutrition. For instance, *Chlamydomonas reinhardtii* switched on to a mixotrophic mode of nutrition uptaking acetic acid from the medium in the absence of light while grown in a photobioreactor [47], *Desmodesmus sp.* showed enhanced growth and nutrient removal capacity in the mixotrophic mode of their nutrition grown in piggery wastewater [48].

III. **Heterotrophic mode:** In this mode, certain microalgal cells, when grown in the complete absence of light and external C-source supplementation, uptake the readily available food from their environment and are not known to perform photosynthesis in that case. This enables them to correspond to high metabolic diversity, which can be exploited well to attain circular economic approach [49]. Some common examples include *Scenedesmus obliquus* (a green alga) well adapted to heterotrophic mode utilizing external C-sources [50], *Chlorella minutissima* (grown in industrial wastewater) was capable of uptaking acetate, glycerol, and glucose as external C-source [46], etc.

Chapter 3

Hypothesis and significance of study

3.1. Hypothesis

In order to get the best results for a study, a potential candidate species is of utmost importance. So, as the initial part of the study, screening of potent microalgal and purple bacterial species needs to be done first and foremost. For triggering PHA accumulation in microorganisms, introducing organic carbon sources in their growth environment can be fruitful, provided they have an indigenous pathway for PHA production. The two-stage cultivation method applied in this study provides an opportunity to shock the cells by acute stress, which makes them switch their metabolic pathway towards PHA production. Cultivation duration can also play a significant role in PHA accumulation as organisms can metabolize carbon sources better for enhanced PHA accumulation. Also, many of these species offer a response towards certain nutrients, mainly nitrates and phosphates (as they are directly or indirectly involved in many of the central cellular metabolic processes). Limiting or supplementing excess of these nutrients can also help microbes accumulate high PHA. Thus, we can provide our candidate species with different combinations of these nutrients (N&P) ranging from their maximum to minimum concentrations once finalizing the best carbon source to see the effect on their biomass production and PHA accumulation.

Furthermore, in the future if this study is scaled up to commercialize bioplastics production, these microbial species could be grown and cultivated in different waste-water steams (which can be a rich source of nitrates, phosphates, heavy metals, volatile fatty acids, salts, etc.) to enhance the economic factor of the overall process. Also, these factors are known to possess different kinds of effects on microbial cells, interfering with their overall metabolic processes in multiple ways. For e.g., heavy metals are known to interfere with minerals uptake via cells,

inhibit several enzymes binding to their SH-groups [51, 52], and activate Rubisco oxygenase activity, thereby decreasing the rate of phosphorylation; also, metals like iron plays a crucial role in altering chlorophyll structure and regulating overall metabolism in living cells [53, 54]. Similarly, salt-induced stress in bacterial and algal cells has been reported to accumulate more lipids inside the cells. Also, salts cause an osmotic imbalance in the cells, thus obstructing the usual pathways involved in cell growth and maintenance, which creates a pool of extra reducing molecules, which can trigger PHA accumulation [55, 56]. The salt-induced stress is also known to activate the enzyme keto-acyl CoA (KCS), which is directly involved in PHB synthesis pathway II [57,13].

Talking about PGRs (plant growth regulators), as they are well known to increase growth and overall cellular metabolism inside plant cells, they can have a positive or negative effect on the PHB synthesis pathway. The plant growth promoters, cytokinin, and auxins are known to have a synergistic effect on intracellular lipid accumulation, and this can also lead to higher PHB accumulation inside microalgal cells [58,59]. Volatile Fatty Acids (VFAs) that are bountiful in industrial wastewater can act as a cheaper C-source in comparison with pure C-sources. VFAs used in this study: Acetate, Propionate or Butyrate can either directly be assimilated in synthesis pathways or can be converted into acetyl co-A pool to kick start the pathway towards either Lipid or PHA accumulation [60,13].

Therefore, these factors and parameters can be selected on the basis of their future implementation on a larger scale as well as on the basis of studies that have already been performed and strongly support the fact that these factors must have some or the other effect on PHB accumulation inside the microalgal. Likewise, these stresses or different conditions (including phosphates presence or absence, harvesting time duration, VFAs effect, etc.) become equally important to be provided to the bacterial cells, as in any of the realistic scenarios of wastewater or natural ponds, these factors would be readily available.

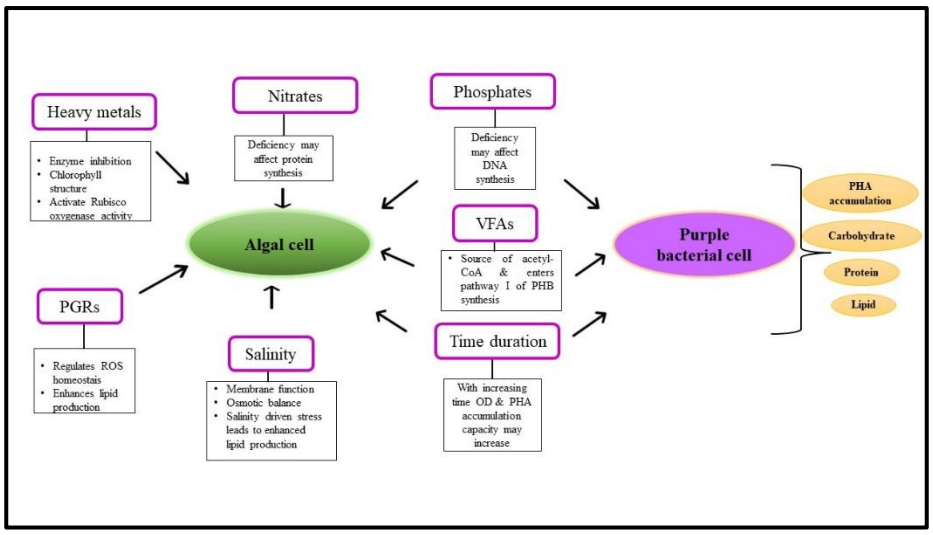


Fig. 3: Representation of hypothesis diagrammatically

3.2. Significance of the study

Since bioplastics are emerging as an attractive alternative to conventional petroleum-derived plastics, they provide a significant area to be further explored. Considering our selected microalgal and bacterial species as a budding candidate for PHA accumulation owes certain facts. Firstly, these species are indigenous, i.e., are native to Indore, M.P., and can quickly be acclimated to outdoor cultivation. Secondly, they possess a high growth rate, enhancing the overall productivity of products such as PHA, lipid, carbohydrate, and protein. In conclusion, these factors could be helpful in the scale-up of the study in the future. The selected species have not been studied for their PHA accumulation potential to date; this provides a broad scope for exploring this field.

Moreover, our study also involves the estimation of carbohydrates, proteins, and lipids accumulation potential of our candidate species and PHAs (bioplastics). We are also studying the effect of heavy metals, salts, volatile fatty acids, nitrates, and phosphates, which comprises the constituents of waste water; in the future, these can replace the costly pure C-

sources (that contribute more than 70% of the total cost involved) in scale up to establish a sustainable biorefinery for PHA production [63].

Chapter 4

Objectives

4.1. Major Objectives

I. Screening best microalgal and purple bacterial species from available seven and four strains, respectively.

II. Amelioration of PHA accumulation competence in microalgae and purple bacteria.

4.2. Minor Objectives:

I. To screen the best microalgal species from total of seven available species in the lab for PHA accumulation.

II. To screen the best purple bacterial strain from a total four available in the lab for PHA accumulation.

III. To determine the growth kinetics of microalgal and purple bacterial species from their respective growth profile study.

IV. To study the effect of harvesting duration: 72 hrs, 7 days, 14 days and 21 days on growth and PHA accumulation in *Ettlia texensis.*

V. To study the effect of N-P variation on PHA accumulation and lipid, protein, and carbohydrate synthesis in microalgae *E. texensis.*

VI. To study the effect of (VFAs) volatile fatty acids (acetic acid, propionic acid, butyric acid) on growth, PHB, and biochemical profile in *E. texensis.*

VII. To study the effect of salinity (NaCl and $MgCl_2$) on growth, PHB, and biochemical profile in *E. texensis.*

VIII. To determine the effect of the two conditions, i.e., stirring and still on PHA accumulation in our candidate purple bacterial strain.

IX. To determine the effect of phosphates, VFAs, and time duration on PHA accumulation, OD (optical density), pH, and DO (dissolved oxygen) in purple bacteria.

Chapter 5

Materials and Method

5.1. Basic methodology and instruments used in experiments

I. BG-11 media is used for growing microalgal cells, and minimal salt media (MSM) is used to grow purple bacterial cells; the composition of these two media is given in section 5.1.1

II. The required amount of media is weighed using weighing balance Fig. 5.1. (a) and is then prepared in a beaker mixing it with distilled water.

III. For proper mixing of media components in the water, a magnetic stirrer Fig. 5.1. (b) is used that ensures a homogenous composition of the media. This is followed by making up the volume and balancing the pH using pH-Meter Fig. 5.1. (c).

IV. Once the media is prepared, it is kept for sterilization in an autoclave Fig. 5.1. (d) at high temperature (120^{o} C) and a pressure of 15 psi for 15 to 20 minutes of the cycle. Along with the media, all the flasks (experimental and for inoculation), measuring cylinders, centrifuge tubes, and some distilled water (used during harvesting for inoculation) are also autoclaved.

V. Since we follow the 2-stage cultivation approach, a mother culture is set up first, for 10 days, keeping an initial OD of 0.2.

VI. This is followed by setting up the experiment, wherein all the other required media (e.g., heavy metals, salts, sugars, etc.) are also weighed and mixed properly in distilled water.

VII. These all are autoclaved, and the experiment is set up for an optimized duration by keeping an initial OD of 0.5 using UV-spectrophotometer Fig. 5.1. (e).

VIII. The microalgal cells are kept for a photoperiod of 12:12 hours in a culture room at 27^{o}- 28^{o} C, and purple bacterial cells are kept in incubators for 24:0 hours photoperiod 30^{o} C at temperature Fig. 5.1. (f).

NOTE-

a. A final media is prepared depending on the number of experimental flasks to be set up for control etc. inside the laminar air flow Fig. 5.1. (g), in order to prevent any bacterial or microbial attack in the media.
b. The inoculation process for purple non-sulfur bacteria is also carried out in the laminar air flow chamber since they are anoxygenic in nature, so it is required to keep them away from external air/O_2 supply.

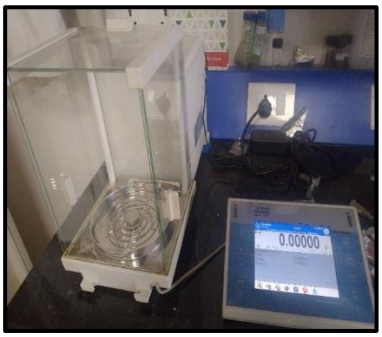
IX. Once the experiment duration is completed, it is followed by the harvesting of the cells using a centrifuge Fig. 5.1. (h). Harvesting is usually done in 50 ml centrifuge tubes.

X. The microalgal cells are also taken for chlorophyll estimation from the culture before harvesting, the protocol of which is described in section 5.4.

XI. Once the cells are harvested, they are kept for freeze-drying in a lyophilizer Fig. 5.1. (i) to obtain dry algal and bacterial biomass. This dried algal biomass is made into a powdered form using mortar-pestle Fig. 5.1 (j) and transferred into MCTs (microcentrifuge tubes).

XII. With these dry biomass samples, further biochemical analysis, i.e., lipid, protein, and carbohydrate estimation, is done in a microplate reader Fig. 5.1 (k) and as shown in and described in section 5.5.

XIII. Also, the most significant part of our study, i.e., estimation of biopolymer (PHA) content, is done with the dry biomass sample using gas chromatography, wherein benzoic acid is used as the internal standard, poly (R)-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid as an external standard, FID (flame ionization detector) as the detector and silica-based capillary column as the column Fig. 5.1. (l). The method described in [64] was used to estimate PHA content in our experiments.



(a)

(b)

(c)

(d)

(e)

(f)

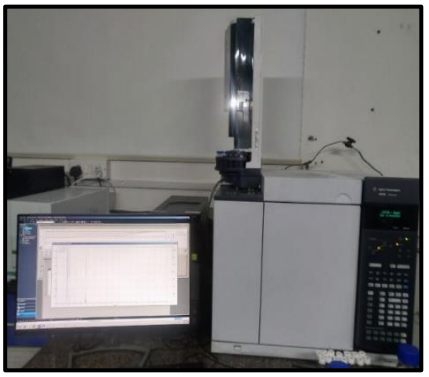
(g)

(h)

(i)

(j)

(k)



(l)

Fig. 5.1: Common instruments used in our lab for carrying out the experiments- (a) Weighing balance, (b) Magnetic stirrer, (c) pH meter, (d) Autoclave, (e) UV-Visble Spectrophotometer, (f) Incubator, (g) Laminar air flow, (h) Centifuge, (i) Lyophilizer, (j) Mortar-pestle, (k) Microplate reader, (l) Gas chromatography

5.1.1. Composition of media used in the experiments:

BG-11 media Composition

	Components	**g/litre**
i)	Sodium nitrate ($NaNO_3$)	1.500
ii)	Dipottasium hydrogen phosphate (K_2HPO_4)	0.031
iii)	Magnesium sulphate ($MgSO_4$)	0.036
iv)	Calcium chloride dihydrate ($CaCl_2. 2H_2O$)	0.036
v)	Sodium carbonate ($NaCO_3$)	0.020
vi)	Disodium magnesium EDTA	0.001

vii)	Citric acid	0.006
viii)	Ferric ammonium citrate	0.006

- Trace elements consisting of (H_3BO_3, Zn^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}) is added as 1 ml/L.
- This media was used to grow microalgae.
- The final pH is maintained at 7.4 ±2.

MSM media Composition

- MSM media consisted of $MgSO_4$, KH_2PO_4, K_2HPO_4, $C_6H_5FeO_7$, CH_3COONa.
- Trace elements consisting of (H_3BO_3, Zn^{2+}, Mn^{2+}, Co^{2+}, and Cu^{2+}) is added as 2 ml/L.
- This media is used to grow purple non-sulfur bacterial cells.
- The final pH is maintained at 6.5 ±0.2.

5.2. 2-Stage cultivation approach

- The microalgal cells are used for our experiment in a 2-stage cultivation method approach.
- This means, that first these cells are grown in normal BG-11 media, keeping the initial OD at 0.2.
- The cells are likewise allowed to grow for 10 days to let them enter the log phase.
- Once they have attained the log phase, they are used for the experiment, providing them with different kinds of conditions and stresses, maintaining the initial OD for an experiment at 0.5.
- This approach is useful as the cells in the log phase would be capable of bearing the stress being provided to them in the experimental set-up. Otherwise, had the cells been provided with stress during the initial period itself, they would not have been able to survive.
- The microalgal cell flasks are grown inside the culture room, providing 12:12 hours of photoperiod (light and dark period,

respectively) at about 27±2 ^{o}C and keeping the light intensity around 3500 lux.

(a) (b)

Fig. 5.2: Flasks kept at culture room showing 2-stage cultivation-
a) Mother culture flasks b) Experimental flasks

5.3. Optical density measurement

OD measurement is done mainly to determine the growth profile of our candidate microalgal or purple bacterial cells using a UV-Spectrophotometer.

- For microalgae, we measure the OD at 680 nm, and for purple bacteria, we take OD at 600 nm using a spectrophotometer.

5.4. Chlorophyll estimation

To estimate chlorophyll content in microalgal cells, we used the method described by Arnon et al. (1949) [65];

I. 5 ml microalgal sample (from the experimental flask) is taken in a 15 ml falcon tube.
II. The falcon tube is then centrifuged for 5 minutes at 7500 rpm and 28^{o} C.

III. The supernatant is carefully discarded, and the remaining pellet is rinsed properly by adding 5 ml of distilled water and vortexing it.

IV. OD is then measured at 680 nm, taking distilled water as blank, and the cells are not thrown after taking OD. Rather, they are again centrifuged for 5 minutes at 7500 rpm and 28^{o} C.

V. After discarding the supernatant, 5 ml (95%) ethanol is added to the tube (pellets), followed by vortexing the tube to dissolve the ethanol properly.

VI. The tube is then kept in a hot water bath at 100^{o} C for 15 minutes.

VII. It is then kept for cooling at room temperature for 5-10 minutes.

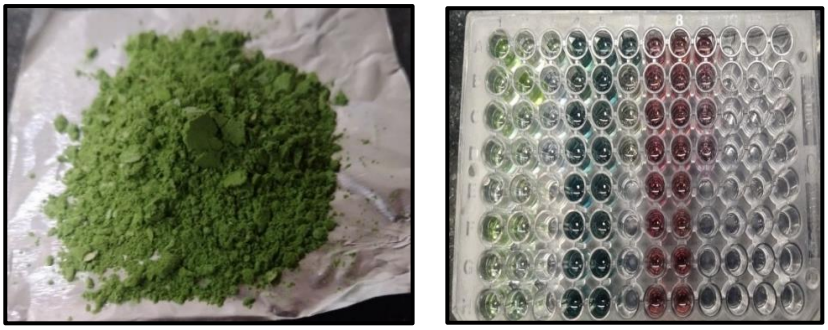
VIII. Since the ethanol present in the tube, kept in a hot water bath might undergo some evaporation, so volume is make-up to 5 ml again (with 95% ethanol).

IX. The content present in the falcon tube is again centrifuged for 5 min at 7500 rpm at 28^{o} C.

X. Without disturbing the pellet, carefully sample is taken from the supernatant for chlorophyll (OD) measurement at 645 nm, and 663 nm, 95% ethanol was used as blank.

5.5. Caliberation Curves Carbohydrate, Protein and Lipid Estimation

From the obtained dry algal biomass Fig. 5.3 (a), the biochemical characterization is done . For lipids, carbohydrates and proteins, we use microplates to put in the samples (200µl each) as shown in Fig. 5.3 (b), followed by their concentration analysis using microplate readers.



(a) (b)

Fig. 5.3: (a) Dry algal biomass, (b) Microplate (having carbohydrate, protein and lipid samples)

A) Determining carbohydrates – Anthrone method [66]

Principle of Anthrone test:

- Anthrone reagent first hydrolyzes the carbohydrate (present as either poly or mono saccharide, bound as component in glycolipid or glycoprotein) into a monosaccharide component.
- The conc. Sulfuric acid then causes the catalysis of that monosaccharide component formed forming a furfural or hydroxyl furfural (from pentoses and hexoses respectively).
- The furfural ring formed then undergoes condensation with 2 naphthol molecules from the Anthrone reagent, thereby giving a dark green-blue colour complex.
- The complex formed can be further quantified via absorbance measurement at 620 nm using a spectrophotometer or a red filter colorimeter.

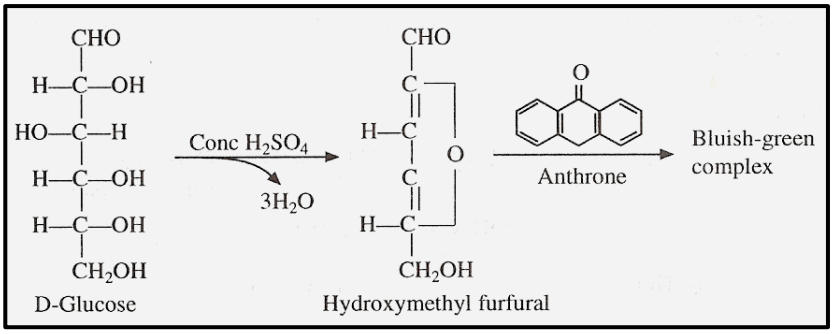


Fig. 5.4: Mechanism showing Anthrone reaction [67]

Reagent preparation:

I. To prepare 100 ml of Anthrone reagent, 100 mg of Anthrone is added to 75% of conc. H_2SO_4.

II. Further to this, 1g of thiourea is added, giving a yellowish colour Anthrone reagent.

Procedure:

I. Standard was prepared with glucose as a standard. Five different concentrations i.e., 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml and 250 µg/ml was taken in triplicate in a 15 ml falcon tubes each and the volume was make up to 1 ml using DW.

II. To the above tubes 4 ml of Anthrone reagent was added.

III. It was followed by vortexing the tubes.

IV. The tubes were then kept for incubation at 100 ^oC for 15 minutes.

V. Tubes were then placed in ice for 5 minutes.

VI. From this, 200 µl of the sample was taken in a microplate having dark green to bluish appearance and along with the experimental tube, a blank was also prepared having every component of Anthrone test (1 ml DW+ 4 ml Anthrone reagent) except for the sample.

VII. Reading (OD) was then taken at 620 nm.

Observation and Result:

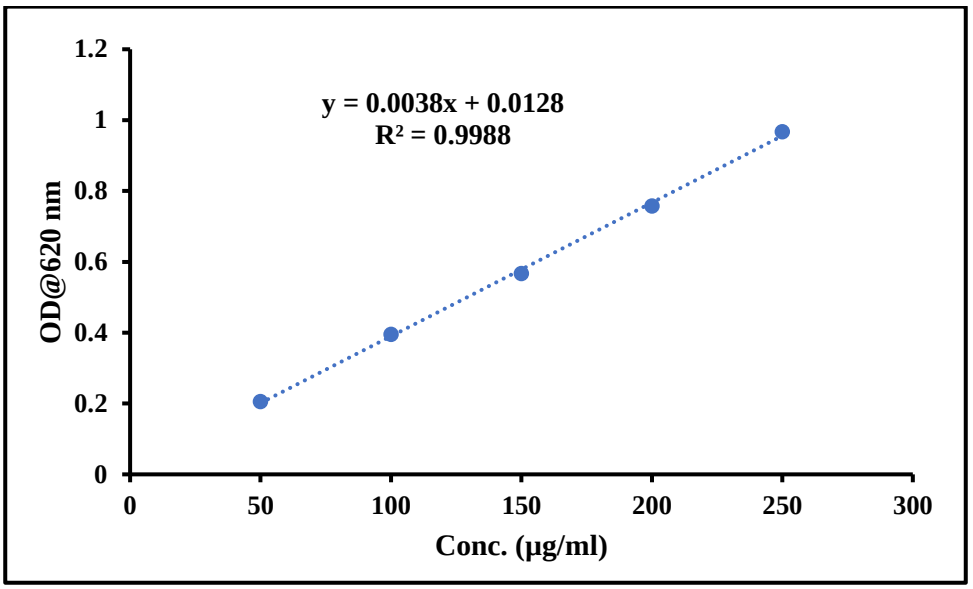


Fig. 5.5: Graph showing relation between the concentration of carbohydrate (x-axis) and the corresponding OD (y-axis)

Conclusion:

A linear curve was found with an increasing concentration of standard. The R^2 value was 0.99.

The equation obtained *via* the graph was used to calculate the concentration of carbohydrates in unknown samples.

$$\mathbf{y = 0.0038x + 0.0128} \qquad \mathbf{(1)}$$

Here, y = OD (absorbance) at 620 nm.
x = Concentration of carbohydrate (in microgram)

B) Determining proteins- Biuret method [68]

Principle:

- A Biuret test is performed to check the presence of peptide bonds in a compound. The compound biuret is prepared by heating up urea at 180° C.

- Treating biuret with dilute $CuSO_4$ in a basic medium gives violet-purple colored compound, forming the basis for proteins detection in biuret test (as shown in figure 5.5.3).
- Compounds having two or more peptide bonds can show a positive result for this biuret test.
- So, copper sulfate in the basic medium reacts with the ($CO\text{-}NH_2$), i.e., two or more peptide bonds in the compound, giving a purple colour compound (product), which is the result of a reaction involving co-ordination complex formation by cupric ions (Cu^{2+}) with a pair of unshared electrons from peptide (nitrogen) and (oxygen) from water.

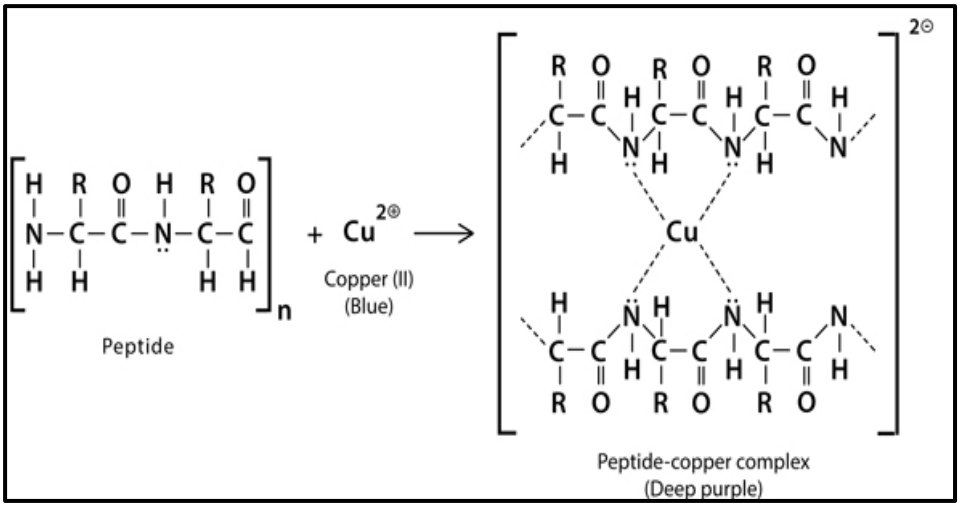


Fig. 5.6: Mechanism showing Biuret reaction [69]

Reagent preparation:

Reagent 1- 1N NaOH is added in 25% methanol.

Reagent 2 (Biurette reagent)- 30% NaOH is added in 0.21% $CuSO_4$.

Procedure:

I. BSA (bovine serum albumin) was used as standard. Five concentrations were used to prepare a linear curve, i.e., 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml, as protein standard is taken in triplicates. Volume was made up to 1 ml with DW.

II. To the above tubes, 500 µl of Biuret reagent was added, i.e., in a 2:1 ratio.

III. The tubes were then kept for incubation at room temperature for 15 minutes in the dark.

IV. 200 µl of each of these samples were taken in a microplate to measure absorbance at 310 nm.

Result:

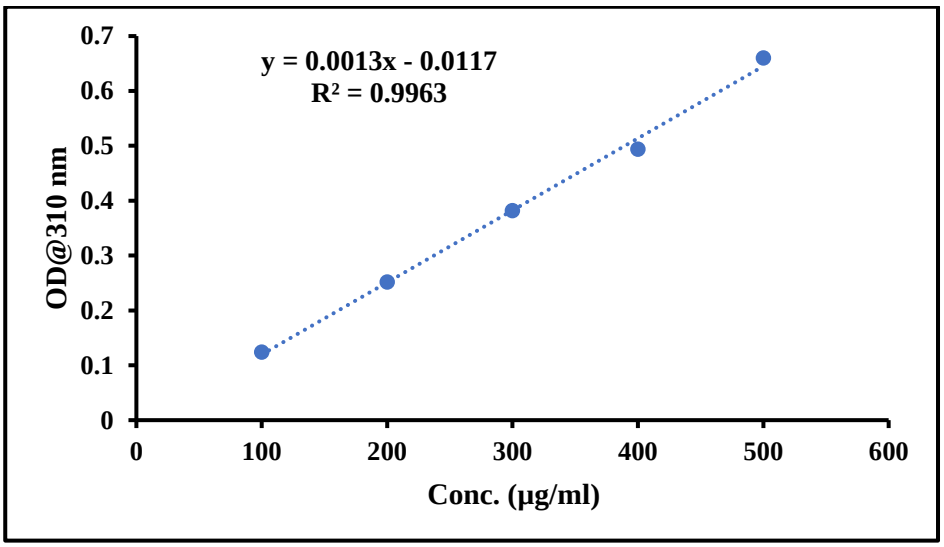


Fig. 5.7: Graph showing a relation between the concentration of protein (x-axis) and the corresponding OD (y-axis)

Conclusion:

With increasing protein concentration, the OD (absorbance) also increases.

The equation obtained via the graph, i.e.,

$$\mathbf{y = 0.0013x - 0.0117} \qquad \mathbf{(2)}$$

Here, y = OD (absorbance) at 310 nm.

x = Concentration of protein in the sample (in microgram).

So, knowing the OD obtained from the experiment, the concentration of protein in the sample could be determined.

C) **Determining lipids- Sulpho-vanillin method (SPV)** [70]

Principle:

- Conc. H_2SO_4 reacts with C-C double bond (unsaturated fatty acid), which leads to the formation of a carbonium ion.
- Conc. orthophosphoric acid produces a phosphate ester by reacting with vanillin; this results in increasing the reactivity of the carbonyl group.
- The carbonium ion further reacts with the carbonyl group (of sulpho-phosphovanillin) that leads to the formation of a reddish-orange coloured compound that can be quantified via absorbance measurement at 530 nm using a spectrophotometer.

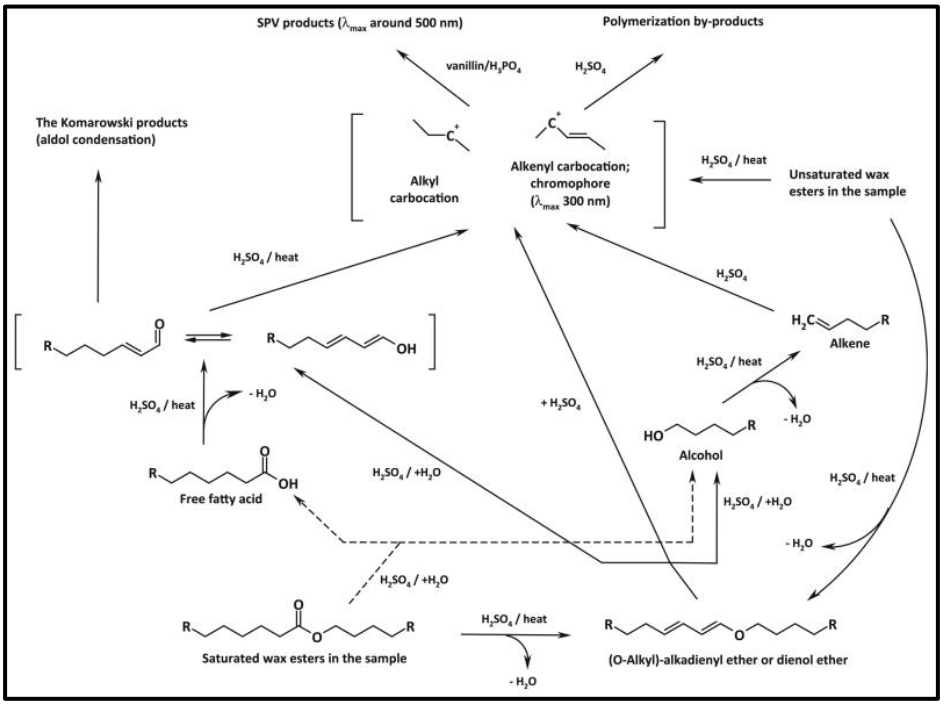


Fig. 5.8: Detailed mechanism showing SPV reaction [71]

Reagent preparation:

I. To prepare 100 ml of SPV reagent, 0.12 g of Vanillin is added to 2 ml of absolute ethanol.

II. To this, 18 ml of DW is added, followed by the addition of 80 ml of conc. Orthophosphoric acid.

Procedure:

I. Five different concentrations of triolein (lipid standard), i.e., 20 µg, 40 µg, 60 µg, 80 µg, and 100 µg, were taken in 15 ml falcon tubes each in triplicates along with a triplicate of the blank.

II. To each of these tubes, 100 µl of distilled water, followed by 2 ml of conc. H_2SO_4 was added.

III. The tubes were then kept at incubation at 100 ^oC, followed by transferring them in ice for 5 minutes.

IV. To each of these tubes 5ml of SPV reagent was added.

V. These tubes were then kept on a shaker incubator at 37 ^OC for 15 minutes at 200 rpm.

VI. 200 µl from each of these samples were taken in a microplate for OD measurement at 530 nm.

Result:

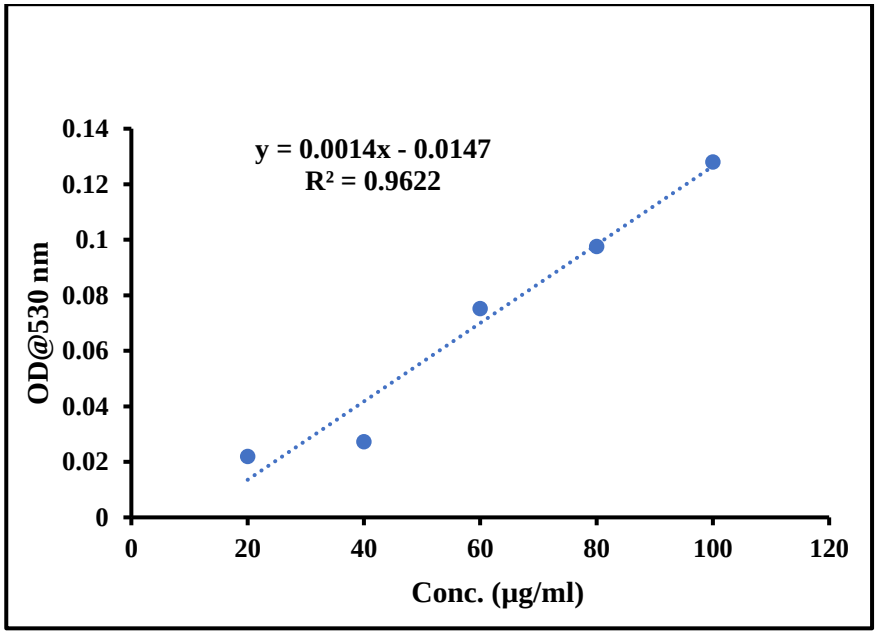


Fig. 5.9: Graph showing a relation between the concentration of lipid (x-axis) and the corresponding OD (y-axis)

Conclusion:

With increasing lipid concentration, the OD (absorbance) also increases.

The equation obtained via the graph, i.e.,

$$y = 0.0014x - 0.0147 \quad (3)$$

Here, y = OD (absorbance) at 530 nm.

x = Concentration of lipid in the sample (in microgram).

So, knowing the OD obtained from the experiment, the concentration of lipid in the

5.6. Growth Kinetics: Specific Growth Rate, Biomass Productivity and Division Time

Specific Growth Rate- It can be described as the time taken to the overall increase in biomass of a cell group (population) per unit concentration of biomass. A sigmoidal growth profile attains the region lying between the lag and the stationary phase. [72]

Biomass Productivity- This can be simply defined as the rate of biomass produced by the algal culture at a particular time interval. [73]

Division Time- As the name suggests, division time is the time taken by a microbial cell to double in number. [74]

These three above mentioned parameters can be determined by the formulas mentioned below, taken from methods described in [75, 76]

$$P = (X_2 – X_1)/ (t_2 - t_1) \quad (4)$$

P = biomass productivity

X_2 and X_1 = Final OD and initial OD, respectively

t_2 and t_1 = Final and initial time duration, respectively

$$\mu = \ln N_t/N_0 /T_t – T_0 \quad (5)$$

Where, μ = Specific growth rate (per day)

N_t and N_0 = Cell densities at time final and initial time duration, respectively

T_t and T_0 = Time duration (final and initial, respectively)

Division time = 0.69/μ **(6)**

Where μ is the specific growth rate

5.7. Workplan

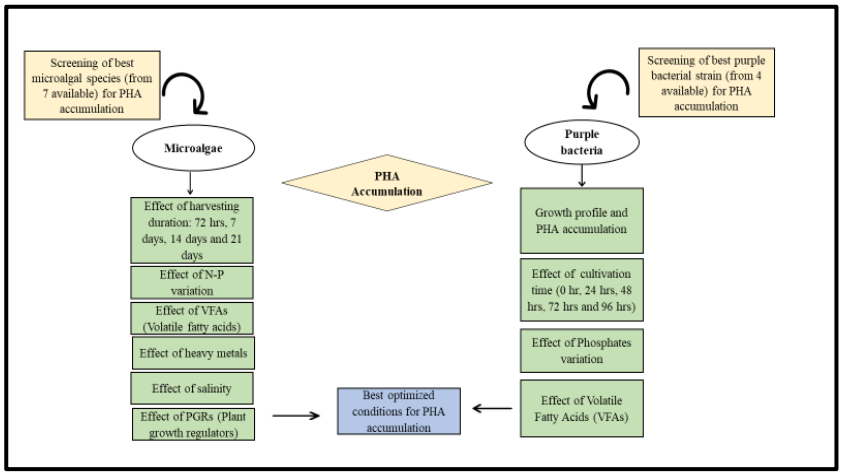
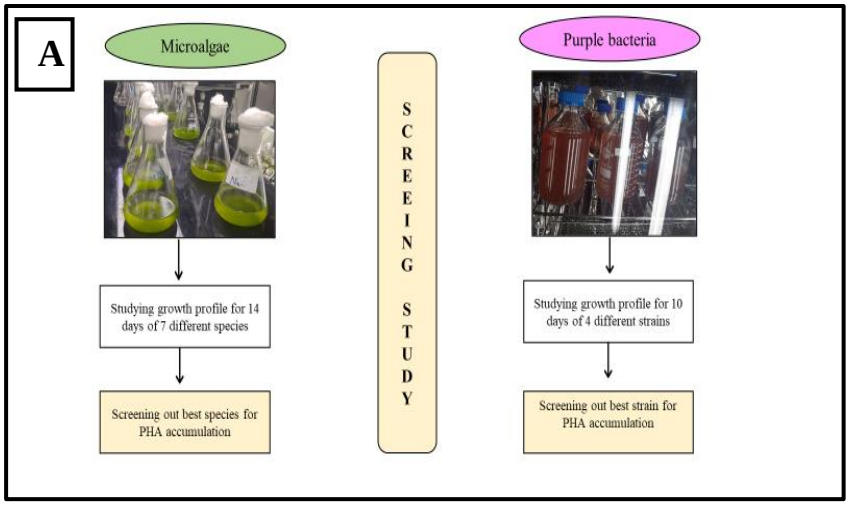


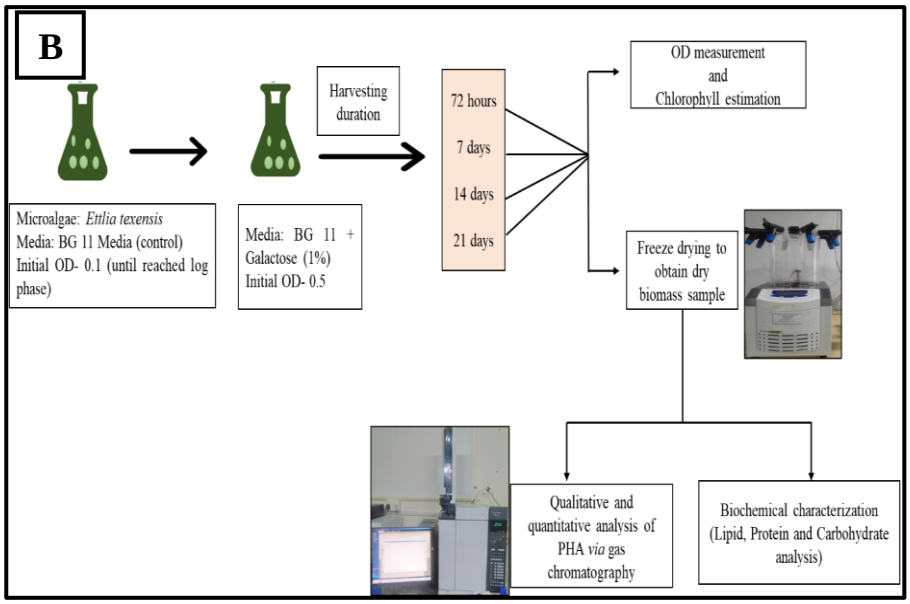
Fig. 5.10: Flow diagram representing work plan for objectives of this study

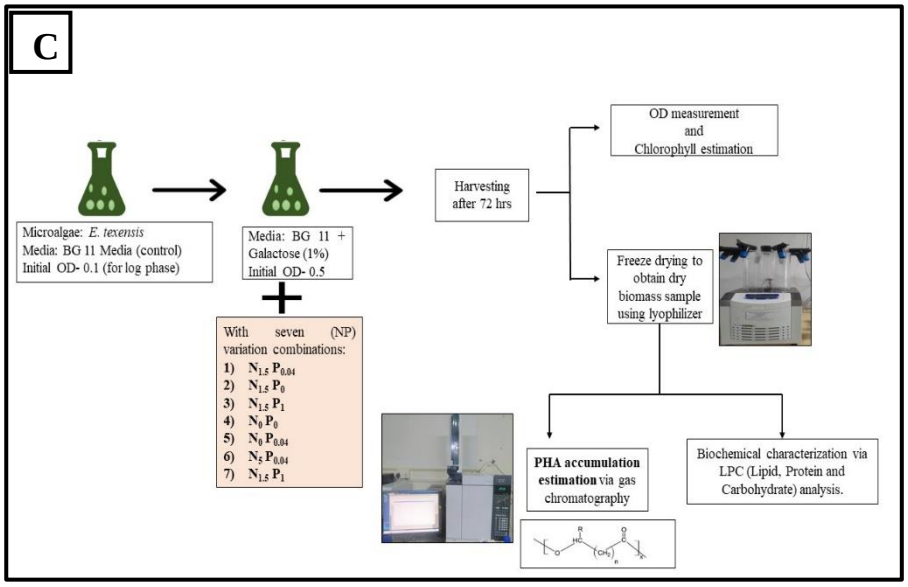
To proceed with the work plan as per our hypothesis, first of all, we will screen the best species available in our lab of microalgae and purple bacteria in terms of PHA accumulation. We will do this by studying the growth profile of all the seven microalgal species and four purple bacterial strains under normal conditions of BG-11 media. Once finalizing with the candidate species, we will then proceed with our further experimental plan. So, we will check on the effect of the duration at which they attain maximum growth by looking for the harvesting duration intervals for (3 days, 7 days, 14 days, and 21 days) as after 21 days; they are expected to reach a decline phase in a controlled condition. Once determining this period of their log phase for attaining the highest growth rate, we will try to optimize and screen the

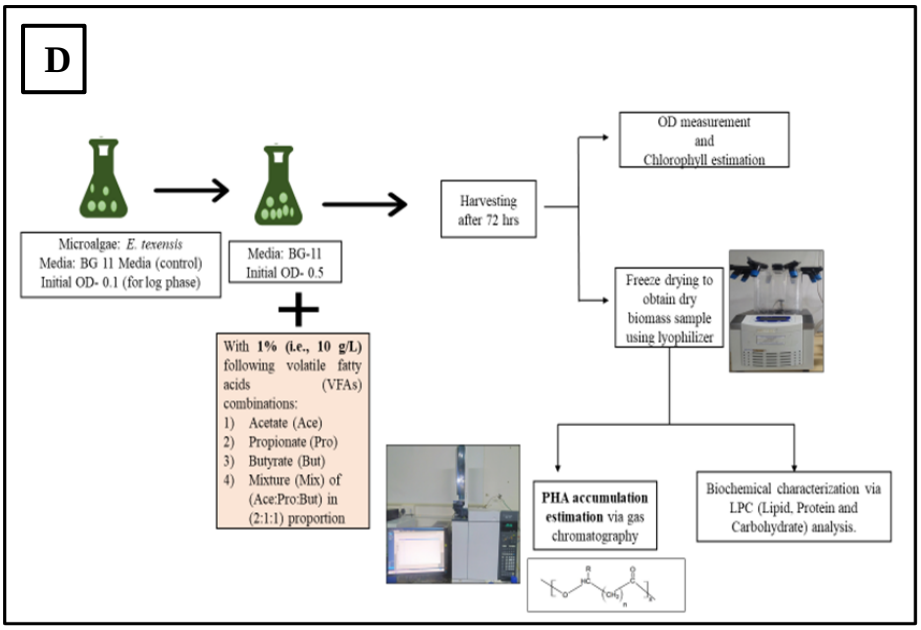
optimum nitrate and phosphate concentration which would respond to a higher rate of metabolism and hence would lead to maximum PHA accumulation. We will then try to optimize the best possible conditions for our microalgal species (*E. texensis*) where highest PHA accumulation is found and similarly best possible conditions for purple bacteria (Isolate 1) would be determined with prime focus on highest PHA accumulation. The different parameters that we would check upon for microalgae include volatile fatty acids (VFAs) that comprise (butyric acid, propanoic acid, acetic acid, etc.), which is an inexpensive C-source that can be provided to the microalgae as well as purple bacteria; heavy metals exposure (e.g., Fe, Cu, Co, Cr, Ag, etc.) that could be easily availed from different waste-water sources and industrial effluents; salinity effect ($MgCl_2$, NaCl), and impact of some plant growth hormones (e.g., gibberellic acid, auxin, cytokinin, etc.).We will similarly evaluate the effect of phosphates, harvesting time duration, and VFAs on purple bacteria to determine their effect on PHA accumulation.

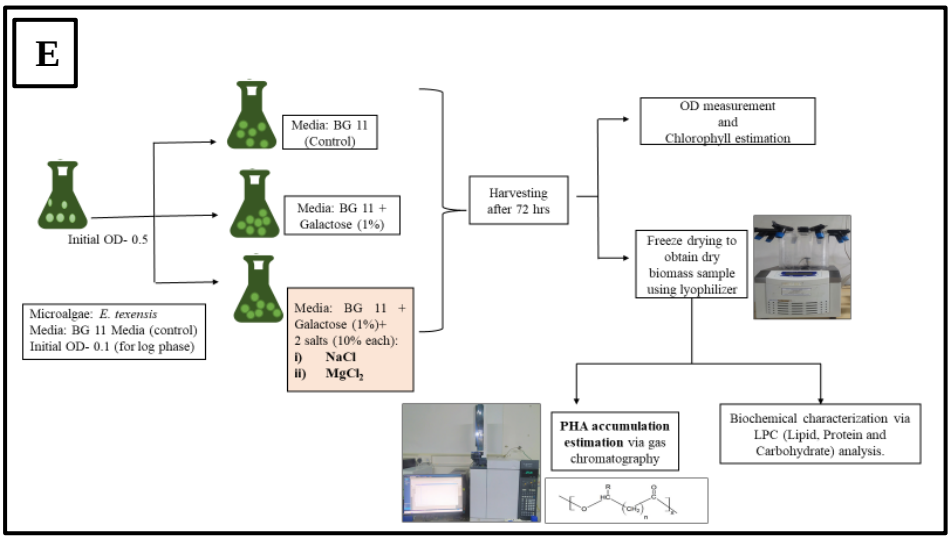
5.8. Experimental Set-up for Workplan

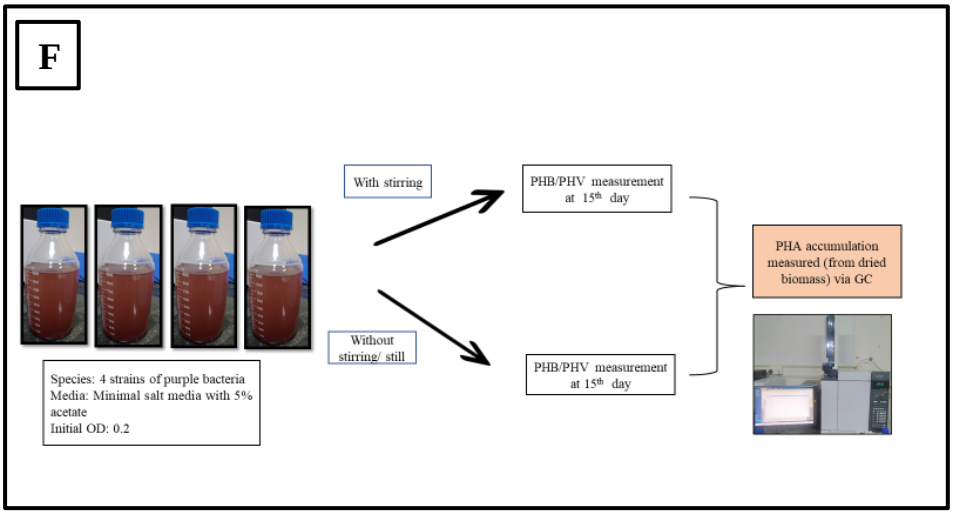



B
Harvesting duration
72 hours
7 days
14 days
21 days
OD measurement and Chlorophyll estimation
Freeze drying to obtain dry biomass sample
Microalgae: Ettlia texensis
Media: BG 11 Media (control)
Initial OD- 0.1 (until reached log phase)
Media: BG 11 + Galactose (1%)
Initial OD- 0.5
Qualitative and quantitative analysis of PHA via gas chromatography
Biochemical characterization (Lipid, Protein and Carbohydrate analysis)


C
Microalgae: E. texensis
Media: BG 11 Media (control)
Initial OD- 0.1 (for log phase)
Media: BG 11 + Galactose (1%)
Initial OD- 0.5
With seven (NP) variation combinations:
1) $N_{1.5}$ $P_{0.04}$
2) $N_{1.5}$ P_0
3) $N_{1.5}$ P_1
4) N_0 P_0
5) N_0 $P_{0.04}$
6) N_5 $P_{0.04}$
7) $N_{1.5}$ P_1
Harvesting after 72 hrs
OD measurement and Chlorophyll estimation
Freeze drying to obtain dry biomass sample using lyophilizer
PHA accumulation estimation via gas chromatography
Biochemical characterization via LPC (Lipid, Protein and Carbohydrate) analysis.


D
Microalgae: E. texensis
Media: BG 11 Media (control)
Initial OD- 0.1 (for log phase)
Media: BG-11
Initial OD- 0.5
With 1% (i.e., 10 g/L) following volatile fatty acids (VFAs) combinations:
1) Acetate (Ace)
2) Propionate (Pro)
3) Butyrate (But)
4) Mixture (Mix) of (Ace:Pro:But) in (2:1:1) proportion
Harvesting after 72 hrs
OD measurement and Chlorophyll estimation
Freeze drying to obtain dry biomass sample using lyophilizer
PHA accumulation estimation via gas chromatography
Biochemical characterization via LPC (Lipid, Protein and Carbohydrate) analysis.


E
Initial OD- 0.5
Microalgae: E. texensis
Media: BG 11 Media (control)
Initial OD- 0.1 (for log phase)
Media: BG 11 (Control)
Media: BG 11 + Galactose (1%)
Media: BG 11 + Galactose (1%)+ 2 salts (10% each):
i) NaCl
ii) MgCl2
Harvesting after 72 hrs
OD measurement and Chlorophyll estimation
Freeze drying to obtain dry biomass sample using lyophilizer
PHA accumulation estimation via gas chromatography
Biochemical characterization via LPC (Lipid, Protein and Carbohydrate) analysis.


F
Species: 4 strains of purple bacteria
Media: Minimal salt media with 5% acetate
Initial OD: 0.2
With stirring
Without stirring/ still
PHB/PHV measurement at 15th day
PHB/PHV measurement at 15th day
PHA accumulation measured (from dried biomass) via GC

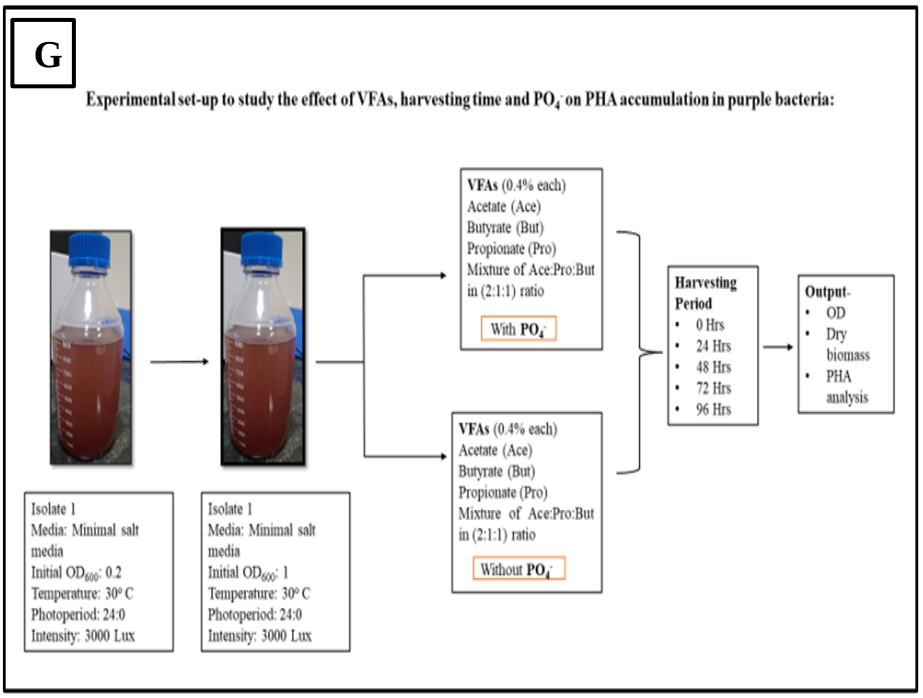


Fig. 5.11: Experimental plan for **A)** screening of microalgal and purple bacteria species by studying their growth profile for PHA accumulation **B)** effect of cultivation time, **C)** effect of N & P variation, **D)** effect of VFAs, **E)** effect of salinity on PHA accumulation and biochemical profiling in *E. texensis* **F)** PHA accumulation potential of 4 purple bacterial strain in stir and still condition **G)** effect of VFAs, phosphates, harvesting time duration on PHA accumulation in purple bacteria (Isolate 1)

Chapter 6

Results and discussion

6.1. Growth Profile Study of Seven Microalgal Species

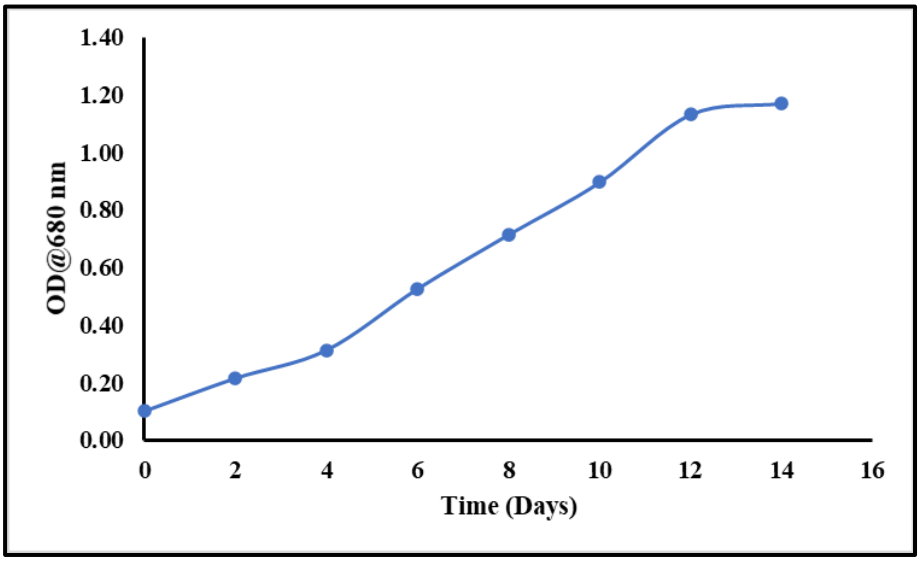


Fig. 6.1: Growth profile of *Desmodesmus psuedocommunis*

The *Desmodesmus psuedocommunis* species, whose growth curve was studied for 14 days, showed an exponential growth till the 12th day, after which it attained a stationary phase. The lag phase was just for a while as it acquired the log phase immediately as their growth cycle started.

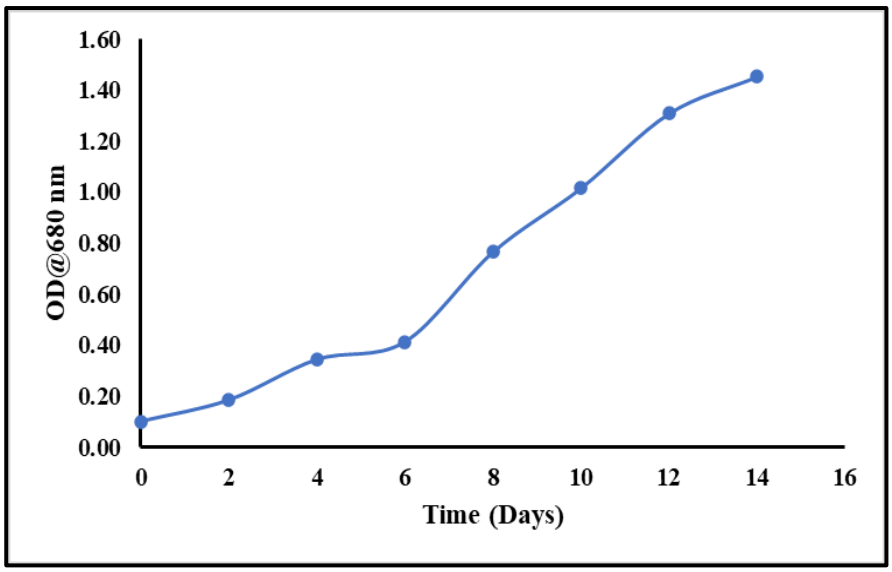


Fig. 6.2: Growth profile of *Asterarcys quadricellulare*

The growth curve of *Asterarcys quadricellulare*, which was studied *for 14 days*, shows that after a quick lag phase for about an initial two days period, it attained the log phase that continued till 14th day.

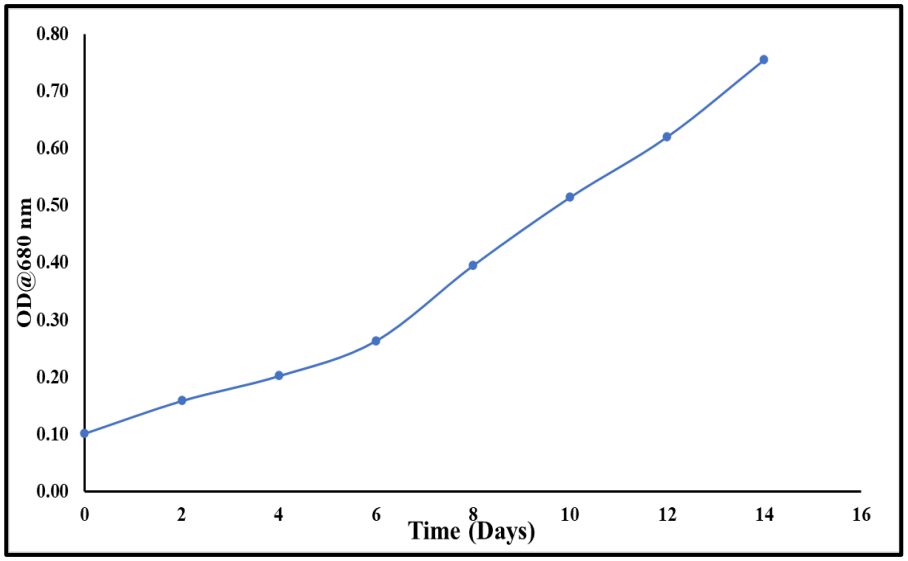


Fig. 6.3: Growth profile of *Pediludieta daitoensis*

The growth curve of *Pediludieta daitoensis* performed for 14 days demonstrates an exponential phase from the very beginning of their growth profile study till 14th day. The log phase had a steep rise after 6th day of their growth.

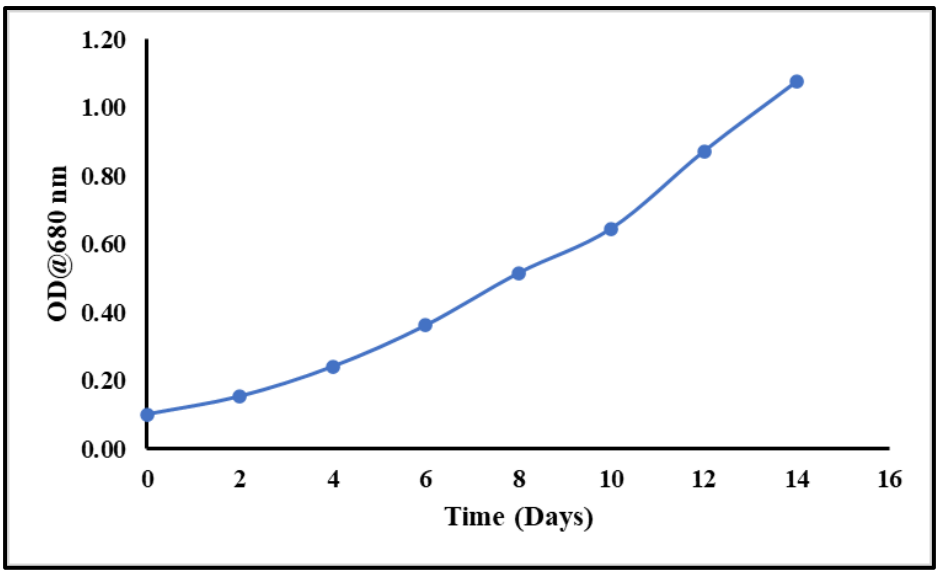


Fig. 6.4: Growth profile of *Coelastrum proboscideum*

The growth profile study of *Coelastrum proboscideum* demonstrates that this species had an initial lag phase till around 2nd day of their growth, followed by their log phase that continued till 14th day of their growth curve study.

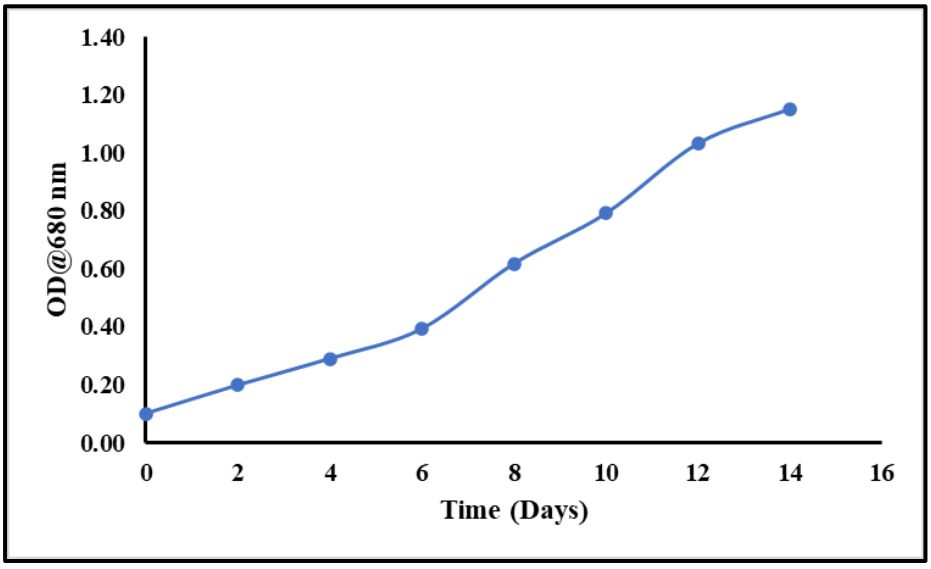


Fig. 6.5: Growth profile of *Ettlia texensis*

A growth curve study of of *Ettlia texensis*, done for 14 days, was also quite convincing as it had a negligible lag phase followed by their exponential phase continuing for 14 days.

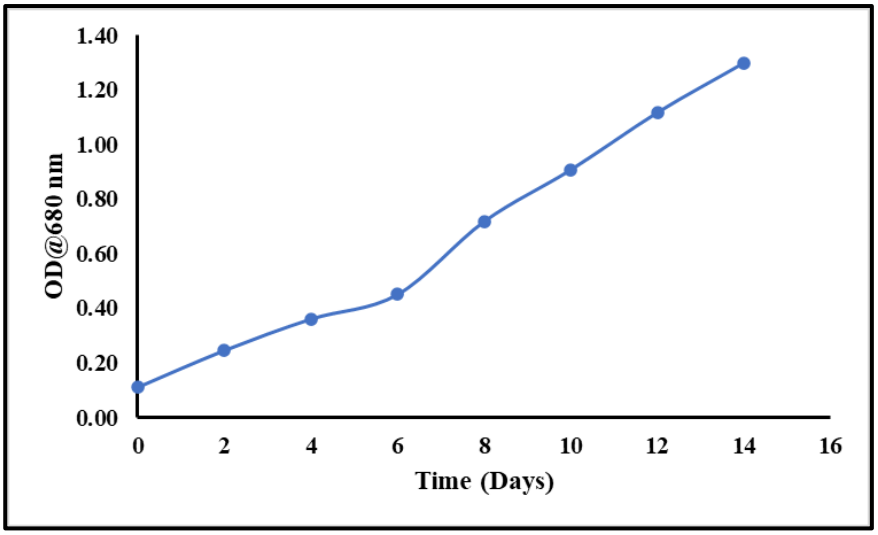


Fig. 6.6: Growth profile of *Pectinodesmus sp.*

The growth curve of *Pectinodesmus sp.* studied for 14 days shows that the species had a log phase from the very beginning with a steep rise followed after the 6th day of their growth.

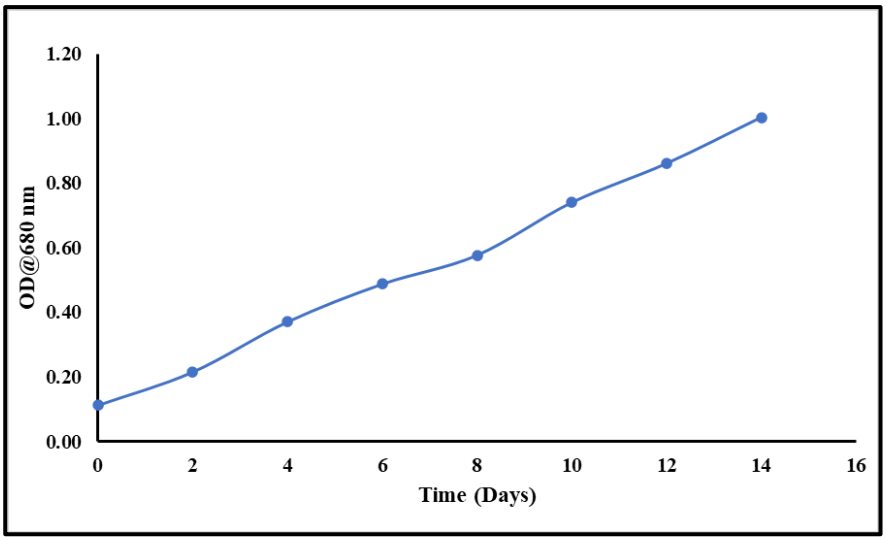


Fig. 6.7: Growth profile of *Coelastrella sp.*

The growth profile study of *Coelastrella sp.* performed for 14 days demonstrates a gradual rise as their log phase, which continued for 14 days. Therefore, a conclusion that can be drawn from Figures 6.1 to 6.7 represents the growth profile of seven different microalgal species, where the growth of all of these species was studied for 14 days, and each of them had attained their best exponential phases till the 14th day of their growth.

Table 6.1: Growth kinetics (biomass productivity, specific growth rate, and division time) from their growth study for seven microalgal species on 7th and 14th days.

Species	Biomass Productivity (mg/L/D)		Specific Growth Rate (per day)		Division time (Day)	
	7th Day	14th Day	7th Day	14th Day	7th Day	14th Day
Desmodesmus psuedocommunis	**27.5**	31.62	**0.23**	0.17	**3.01**	4.17
Asterarcys quadricellulare	22.35	**45.48**	0.21	**0.19**	3.35	**3.65**
Pediludieta daitoensis	18.31	24.14	0.18	0.15	3.8	4.69
Coelastrum proboscideum	19.47	33.34	0.19	0.17	3.61	4.09
Ettlia texensis	20.02	39.37	0.2	0.18	3.52	3.82
Pectinodesmus sp.	17.7	29.45	0.18	0.16	3.86	4.32
Coelastrella sp.	24.16	43.83	0.21	**0.19**	3.22	3.7

- The growth kinetics study of these seven different microbial species calculated for the 7th and 14th day (from their growth profile study based on their respective OD) demonstrates that the highest biomass productivity for the 7th day was obtained for *D. psuedocommunis* (27.5 mg/L/D) and for 14th day it was observed for *Asterarcys quadricellulare* (45.48 mg/L/D).
- For specific growth rate, the highest was obtained for *D. psuedocommunis* on 7th day (0.23 per day), whereas on 14th day, two species gave a maximum specific growth rate (0.19 per day). This also shows that with increasing days, the specific growth rate is declining.
- Lastly, for the third and very significant parameter, i.e., the division time, for 7th day lowest time was taken by the species *D. psuedocommunis* (3.01 days), and for 14th day, the shortest division time was found to be for the species *A. quadricellulare* (3.65 days).

6.2. Growth Profile Study of Purple Bacterial Strain

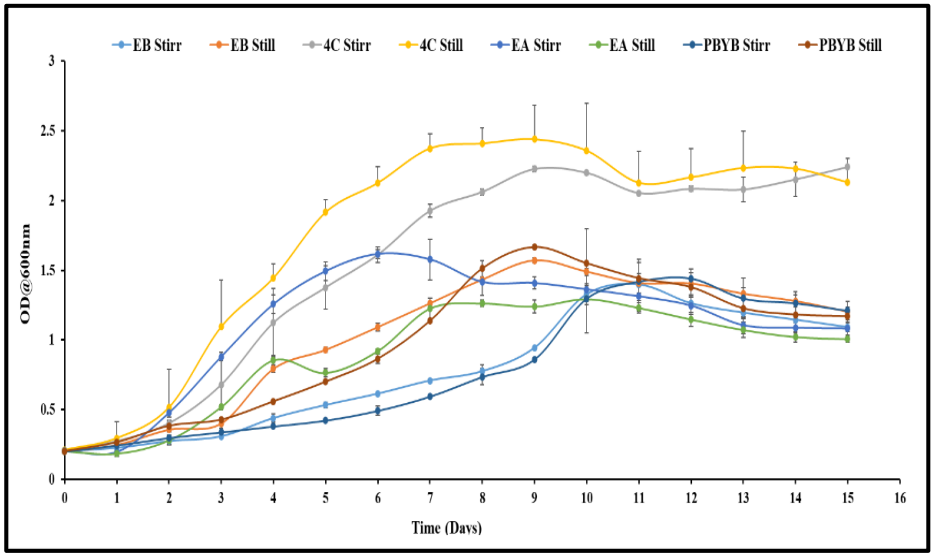


Fig. 6.8: Growth curve for four purple bacterial strains studied for 15 days in both stir and still condition

Here, all the four strains of purple bacteria were grown for 15 days following 2 conditions, i.e., stir and still needs. The bacterial strains had the following growth profile;

I. The 4C strain under still conditions grew better and attained a stationary phase after the 10^{th} day. While, this strain had lower growth when grown under stirring conditions (though attained stationary phase nearly at same duration, i.e., after 10^{th} day). This is possible as stirring ensures aeration and these purple non-sulfur bacteria are anoxygenic in nature, so they would have got stress in this condition hampering their growth.

II. The EB strain had a much better growth in still condition as compared to ones growing in stirring condition. But in still condition, the bacterial population attained stationary phase earlier at around 9^{th} day, whileas, with stir condition they continued to be in log phase for about 11 days followed by their stationary phase.

III. For the EA strain, the bacterial population growing in stirring condition had a higher growth rate though, but they had their decline phase much earlier at about 7^{th} day, while the ones

growing in still condition continued to be at their exponential phase for 11th day as well, after which they had their stationary phase.

IV. For the PBYB strain, again, they possessed a much better growth profile while growing in still condition than ones in stir condition. But the decline phase was attained earlier for the bacterial population ibn still condition, i.e., after the 9th day. The ones in the stirring phase attained their stationary phase after the 10th day, followed by their decline phase after the 12th day.

Table 6.2: Growth kinetics (biomass productivity, specific growth rate, and division time) for four different purple bacterial strains from the 15th day

	EB		**4C**		**EA**		**PBYB**	
	Stir	**Still**	**Stir**	**Still**	**Stir**	**Still**	**Stir**	**Still**
Biomass productivity (mg/L/D)	0.06	0.07	**0.14**	0.13	0.06	0.05	0.07	0.06
Specific growth rate (per day)	0.11	0.12	**0.16**	0.15	0.11	0.11	0.12	0.12
Division time (day)	6.14	5.79	**4.36**	4.48	6.24	6.48	5.77	5.89

- The growth kinetics was calculated for all these four strains of purple bacteria based on their growth curve (OD) for the 15th day. And here from this above-given table, we can refer that, out of these four species of purple bacteria, maximum biomass productivity is obtained for EB strain in still condition while as for stirring condition maximum was found in stirring condition, the values for both being the same, i.e., 0.07 mg/L/D.
- For specific growth rate, maximum was found for 4C strain in both stir and still condition (a slightly higher being in stirring condition), ie., 0.16 per day in stir and 0.15 per day in still condition.

- For division time, lowest division time was found for 4C strain in both stir and still conditions, a slight higher being for still condition, i.e., 4.36 days being for stir and 4.48 days being for still condition (since it is a more feasible condition for purple bacteria being anoxygenic in nature).
- So we may infer from table 6.2 that strain 4C had the best growth kinetics when compared to all other purple bacterial strains.

6.3. Effect of Harvesting duration: 72 Hrs, 7 days, 14 Days, and 21 Days on growth and PHA accumulation in *Ettlia texensis* under Galactose Supplementation

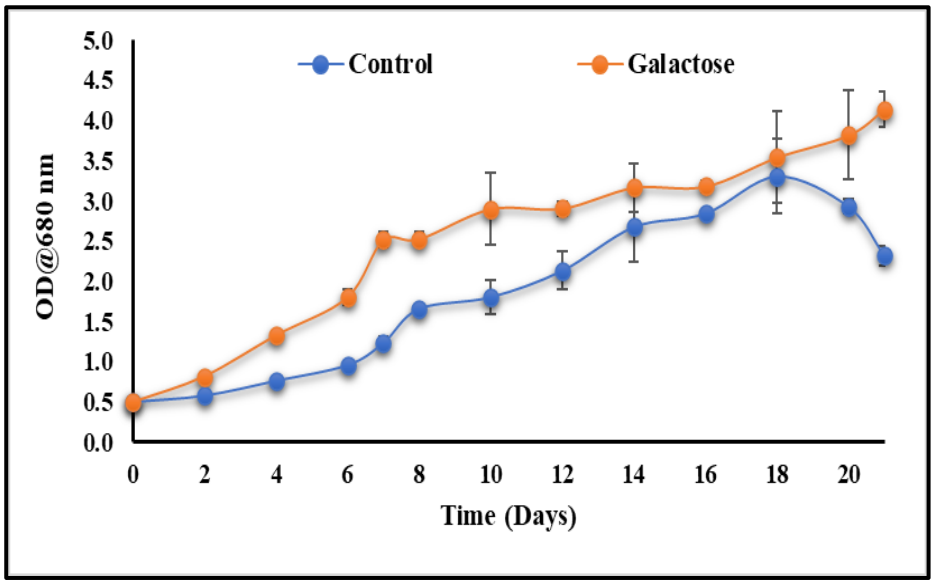


Fig. 6.9: Representation of growth curve for 21 days under mixotrophy and autotrophy

- It was observed that microalgae showed better growth in the mixotrophic mode rather than the photoautotrophic mode.
- In photoautotrophic mode, cells attained declined phase after 18th day. But for, those growing in media with external carbon supply as they switch to a mixotrophic mode of nutrition remained in the log phase till 21 days.
- The exponential phase for *E. texensis* continued for 21 days in autotrophic (without carbon source), while under mixotrophic

condition (with external C-source present in the media), cells started entering the decline phase following 20 days of cultivation.

Table 6.3: Lipid, Carbohydrate, Protein, and PHB concentration (µg/mg) on 21st day under galactose supplementation

		PHB	Lipid	Carbohydrate	Protein
21 day	**Control**	0.13±0.02	105.06±16.49	200.14±17.14	**286.39±21.50**
	Gal	**39.06±7.13**	**150.37±3.16**	**226.45±9.46**	239.72±16.67

- Here, while comparing the biochemical data for *E.texensis* that was done obtained from the biomass after harvesting for 21 days under external C-source supplementation (galactose), we see that higher lipid (150.37±3.16 µg/mg) and carbohydrate (226.45±9.46 µg/mg) got accumulated in the cells in comparison to control (no carbon source in media).
- For proteins, it was still higher in autotrophic conditions (286.39±21.50 µg/mg) as compared to the mixotrophic media having galactose.
- Also, maximum PHB got accumulated in the mixotrophic condition (39.06±7.13 µg/mg) with having an external C-source, hence favoring PHA synthesis pathway.

6.4. Effect of N-P variation on Growth and PHA Accumulation in *E. texensis*

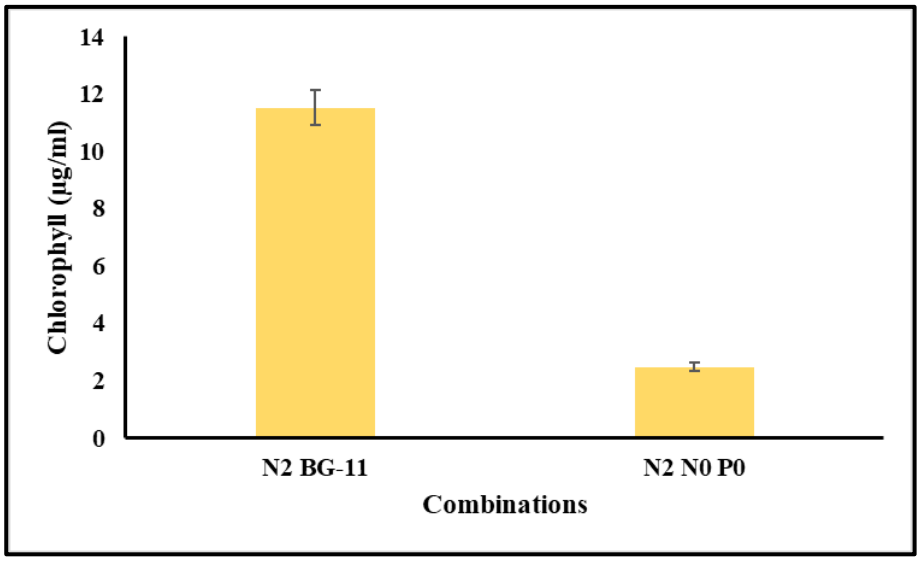


Fig. 6.10: Representation of growth profile via photosynthetic pigment analysis with the absence of nitrates and phosphates at 72 Hrs

The growth profile of *E. texensis* was studied in terms of photosynthetic pigment analysis (i.e., chlorophyll estimation). However, there was a decline in chlorophyll content in the absence of nitrates and phosphates from the media in comparison with the BG-11. It was assumed that other feature analyses of the microalgal cells (biochemical characterization, PHA content analysis) could have a significant contribution as certain times the microalgal cells are known to switch on to different metabolic pathways than performing normal photosynthesis.

Table 6.4: Lipid, Carbohydrate, Protein and PHB concentration (µg/mg) under nitrate and phosphate depletion at 72 Hrs

Combinations	PHB	Protein	Lipid	Carbohydrate
$N_{1.5}P_{0.04}$	0.47±0.09	335.28±31.32	107.04±11.15	168.89±3.61
$Ga_{10}N_0P_0$	**1.41±0.07**	**357.50±16.65**	**156.79±13.10**	**303.54±11.14**

The study was carried out to see the effect of nitrates and phosphates on microalgae in terms of their biochemical profiling. So for this, along with control having a definite proportion of nitrates and phosphates

present in normal BG-11 media, i.e., ($N_{1.5}P_{0.04}$) respectively, a combination was prepared to have nitrates and phosphates absent from BG-11 along with the presence of galactose. And surprisingly, a positive impact on all the three factors that is, proteins (357.50 ± 16.65 µg/mg), lipids (156.79 ± 13.10 µg/mg), and carbohydrates (303.54 ± 11.14 µg/ml), respectively, were found as they got significantly increased.

6.5. Effect of VFAs (Volatile Fatty Acids) on Growth and PHB Acumulation in *E. texensis*

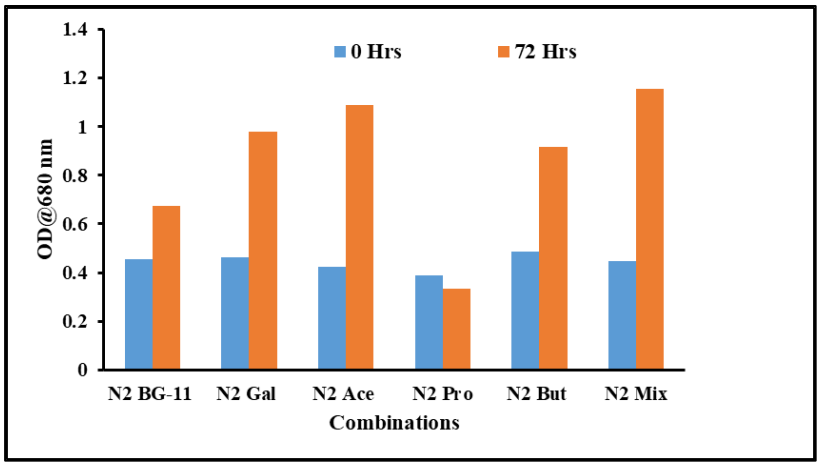


Fig. 6.11: Effect of VFAs on growth on 0 Hrs and 72 Hrs

Since maximum growth was observed with a mixture of all the VFAs present in a definite proportion of (2:1:1 ratio) of acetic acid, propionic acid, and butyric acid, respectively, following study of chlorophyll estimation, biochemical characterization and PHB accumulation were proceeded with this combination.

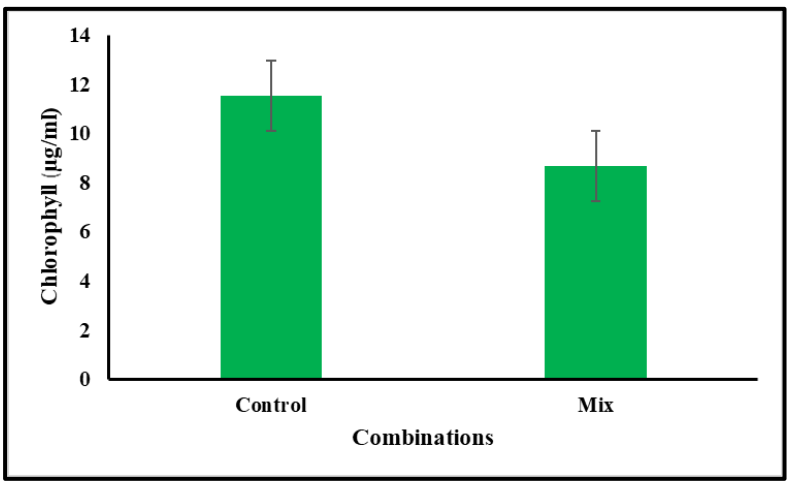


Fig. 6.12: Representation of growth profile of *E. texensis* via photosynthetic pigment (µg/ml) analysis with mixure of VFAs at 72 Hrs

Photopigment analysis was done for 72 Hrs with a mixture of VFAs (having acetate, propionate, and butyrate in a 2:1:1 ratio) since maximum growth (OD) was found with this very combination (Fig. 6.11). But for chlorophyll analysis with this combination, there was a negative response shown by *E. texensis*.

Table 6.5: Lipid, Carbohydrate, Protein, and PHB concentration **(µg/mg)** for a mixture of VFAs at 72 Hrs in microalgae *E. texensis*.

Combinations	PHB	Carbohydrates	Proteins	Lipids
BG-11	3.5±0.05	**80.47±4.05**	**463.32±6.49**	150.77±0.74
Mix	**23.2±6.1**	58.66±14.75	408.69±18.46	**217.86±11.84**

- Here, for biochemical profiling, since maximum growth was observed in the combination having a mixture of all the 3 VFAs (acetate, propionate, and butyrate in a 2:1:1 ratio, respectively), based on their growth profile and OD data, this combination was selected for further studies and analysis.
- As a convincing result, PHB (which is our prime focus), as well as the lipids, were found to be in high concentrations when compared to the control BG-11.
- For instance, PHB (23.2±6.1 µg/mg), i.e., nearly eight times it got enhanced, and lipids (217.86±1.84 µg/mg) got elevated.

6.6. Effect of Salinity on Growth and PHB Accumulation in *E. texensis*

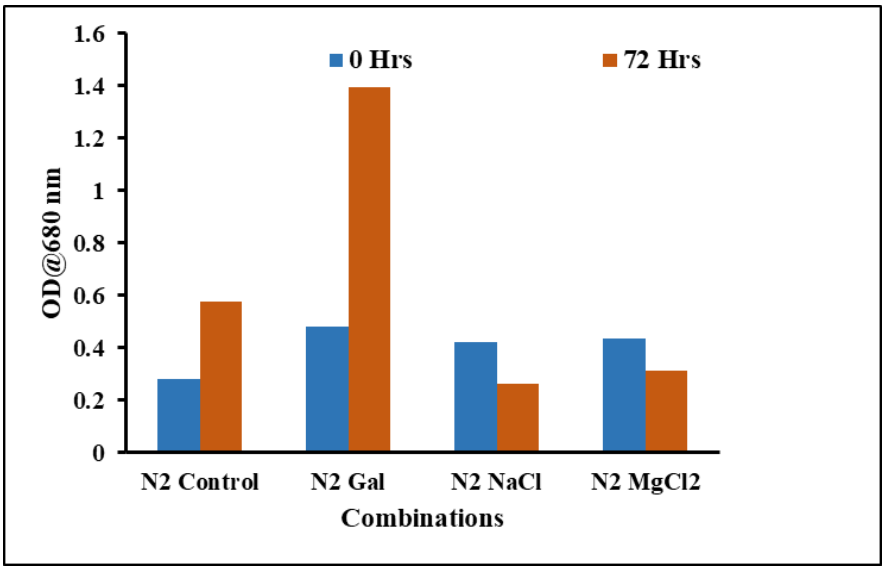


Fig. 6.13: Effect of salinity on growth on 0 Hrs and 72 Hrs.

Though salts had a negative impact on the growth of microalgal cells in comparison with the control, we thought of testing the other following study of chlorophyll estimation, biochemical characterization, and PHA accumulation with both of these salts, in consideration of the fact that the microbial cells are profoundly known to accumulate PHA molecules in response to stress.

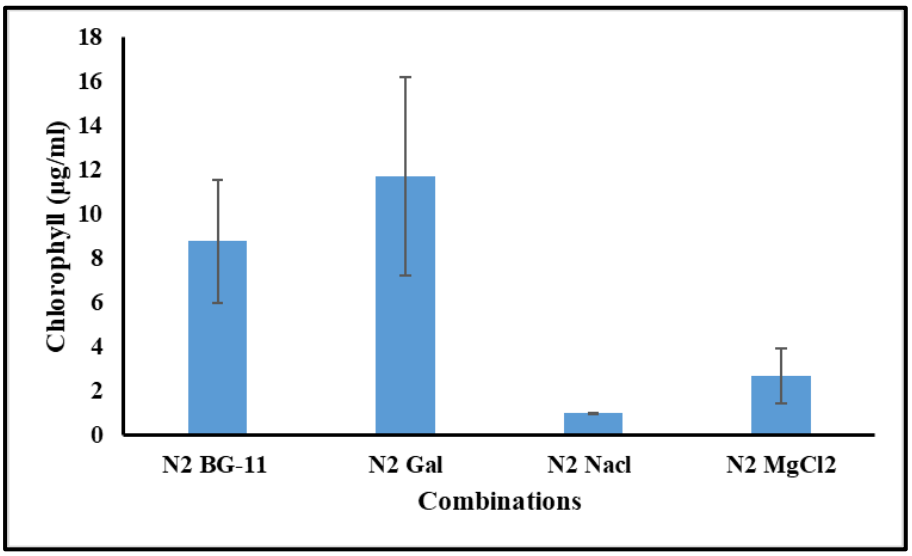


Fig. 6.14: Representation of growth profile of *E. texensis* via photosynthetic pigment (µg/ml) analysis with different salt conditions *E. texensis* at 72 hrs

The maximum photosynthetic pigment was synthesized in the presence of **galactose** (9.38 ± 2.85 µg/ml); with salts in the media, it had a significant decline. This is possible because these salts (used in high concentrations) might be responsible for creating acute stress on the cells, and so they are not able to perform photosynthesis effectively.

Table 6.6: Lipid, Carbohydrate, Protein, and PHB concentration **(µg/mg)** under salinity stress in *E. texensis* at 72 Hrs

Combinations	PHB	Carbohydrates	Proteins	Lipids
BG-11	2.77±0.27	99.17±13.93	**463.32±6.49**	**116.27±6.92**
Galactose (Gal)	**129.11±6**	76.95±11.20	436.73±57.3	107.97±126
Gal+ $MgCl_2$	7.90±2.86	**135.77±17.63**	405.77±50.6	112.21±188
Gal + NaCl	33.20±0.0	74.61±7.58	314.72±16.2	97.21±11.4

- Carbohydrate was maximum accumulated in (135.77±17.63 µg/mg) with $MgCl_2$ salts in the media, while maximum proteins and lipids were accumulated in controlled condition.
- The highest PHB accumulation was found with galactose in the media (129.11±3.66 µg/mg).
- This can be attributed to the fact as, Mg^{2+} ions act as an important co-factor for the enzyme phospho-fructo-kinase, used in glycolysis pathway of carbohydrate metabolism, thereby promoting cells to synthesize more carbohydrates in presence of these Mg salts [77].

6.7. PHA Accumulation Capacity for Isolated Purple Bacterial Strain for 15th Day

Table 6.7: PHA (PHB and PHV) accumulation in 4 purple bacterial strains at 10th and 15th day in stir and still conditions

Species	Condition	10th Day		15th Day	
		PHB	PHV	PHB	PHV
EA	Still	1.5±0.04	0.01±0.00	0.25±0.00	0.01±0.00
	Stir	0.3±0.01	0.00±0.00	0.10±0.01	0.00±00
EB	Still	0.07±0.01	0.00±0.00	0.90±0.02	0.11±0.01
	Stir	0.80±0.01	0.01±0.00	0.10±0.02	0.02±0.01
4C	Still	0.09±0.00	0.00±0.00	0.06±0.00	0.03±0.00
	Stir	0.11±0.00	0.02±0.00	0.11±0.12	0.03±0.00
PBYB	Still	**1.52±0.07**	**0.04±0.00**	**1.50±0.03**	**0.04±0.00**
	Stir	0.12±0.02	0.00±0.00	0.21±0.04	0.03±0.00

- From the above, it can be inferred that PBYB is the best PHA (both PHB and PHV) accumulating purple bacterial strain among the four.
- Also, for both the 10th day and 15th day highest PHB as well as PHV accumulation is seen in still condition.
- It is possible that in still conditions, the bacterial cells being anoxygenic in nature, are more comfortable in synthesizing and accumulating PHA molecules (as stirring ensures aeration to the system, providing stress to the bacterial cell population), hence having a better growth and higher metabolic efficiency.

6.8. Effect of VFAs on DO, OD, pH and PHA Accumulation on PBYB Strain

Since, based on our previous study, the PBYB strain responded best (among the four purple bacterial strains) towards the highest PHB/PHV accumulation, we chose this particular strain for our further studies. So,

for studying the effect of VFAs, we selected specifically acetate (being a direct precursor in the PHA biosynthesis mechanism) to check on various parameters in purple bacteria, including OD, DO, pH, and PHA accumulation potential for five different time intervals of alternate days being 0 Hrs, 24 Hrs, 48 Hrs, 72 Hrs, and 96 Hrs respectively.

Table 6.8: Effect of acetate on DO, OD, pH, and PHA accumulation on PBYB strain

	DO	**OD**	**pH**	**PHA**
PBYB Control 0 Hrs	**5.29**	0.775	8.76	0
PBYB Acetate 0 Hrs	5.23	1.306	**7.05**	1.84±0.08
PBYB Control 24 Hrs	2.92	0.721	7.79	0
PBYB Acetate 24 Hrs	2.34	1.307	7.67	5.53±0.07
PBYB Control 48 Hrs	3.8	0.683	7.52	1.21±0.08
PBYB Acetate 48 hrs	0.63	1.302	8.07	**6.56±0.07**
PBYB Control 72 Hrs	4.48	0.676	7.26	0.73±0.01
PBYB Acetate 72 Hrs	1.32	1.38	8.74	4.87±0.11
PBYB Control 96 Hrs	5.19	0.636	7.11	0.65±0.01
PBYB Acetate 96 Hrs	0.54	**1.386**	**9.21**	4.52±0.03

- From this table, it can be inferred that acetate (a volatile fatty acid) had quite a significant contribution to the enhancement of PHA accumulation in comparison to the control. Moreover, the highest PHA was accumulated at 48 Hrs.
- In addition to PHA, other parameters were also checked. For instance, the DO (dissolved oxygen), OD (optical density), and pH.
- Though the results for DO for the 2 cases (control and presence of acetate) varied a lot, the highest DO was found at 0 Hrs in controlled condition, followed by the presence of acetate at 0^{th} Hr.

- For OD, the highest was found at 96th Hr in the presence of acetate. One more significant was found in this aspect that, in controlled conditions, the OD gradually decreased with an increase in time duration. While, for the case of acetate in the media, the growth (OD) gradually increased with an increase in time duration (it would be possible because of the presence of external C-source in the media, the bacterial cells would have preferably switched to the mixotrophic mode of nutrition).
- For pH as well, except for the 0th Hr, at all the other time duration, pH was higher for acetate in the media, being maximum at 96th Hr with acetate presence.

6.9. Future Studies: Techno-Economic Analysis (TEA)

Considering the techno-economic analysis for the biosynthesis of bioplastics (PHA) from these microorganisms and comparing it with their petrochemical-derived counterparts, it is evident that certain factors need to be addressed to make the overall process economically more feasible. Some of the crucial factors determining the relative increase in PHA production cost from cradle-to-gate are external carbon-source supplementation, fermentation process, sterilization and production of pure bacterial culture, energy, and cost involved in extraction down-stream processing of final product (PHA) [63,78]. When comparing it with synthetic petroleum-derived plastics, currently, bioplastics (mainly PHAs) are found to be costlier by 20% - 80%, which sets the main bottleneck for their commercialization at a larger scale [79]. However, in one of the studies performed by Choi et al. [80] to evaluate techno-economic analysis for the detailed PHA accumulation by a purple non-sulfur bacteria *Rhodospirillum rubrum* (including all direct expenses, e.g., energy, labour, raw materials, etc. to indirect costs like tax, administrative costs, insurance, etc.) using syngas fermentation, they found the overall process to be economically more viable and technically feasible when compared with sugar fermentation. The PHA production involved a cost of $1.65/kg, by a daily output of 12Mg, while

PHA produced from the fermentation of sugar ranged from $4-$6/kg. Moreover, the hydrogen gas produced from the syn-gas fermentation process as a by-product leads the process to be economically much more attractive. Hence, this could be targeted as an effective strategy of utilizing the by-products synthesized while PHA production adds an advantage to the economic status of PHA production.

Also, one of the efficient strategies explored nowadays is growing the microbial culture in different wastewater streams instead of using a traditional approach for pure bacterial culture production, enhancing PHA production to about 77% of dry weight [81].

Another study was performed to determine cost-benefit analysis for PHB production using activated sludge in which they estimated production cost for a plant of 100m^3/day capacity to be US$ 11.8/kg at 44% PHB yield [82]. Furthermore, the price was found to be reduced to 9.28/kg and US$ 8.56/kg by increasing the plant capacity to 500 m^3/day and 1000 m^3/day, respectively. Also, they assessed that O & P costs significantly contributed to around 50% of the total cost involved in the overall process. Hence approaches need to be made in developing more competitive and economical methods for PHB extraction other than solvent-based extraction. Then we could select wastewater having carbon as a major source so that external carbon supplementation costs could further be reduced. Developing these strategies and involving R & D further in them could help lower the cost involved in chemicals utilization and make the PHA production process economically feasible and more attractive.

We also can proceed with the estimation of a round figure involved in the overall process of bioplastic (PHA) production, involving all direct and indirect costs, capital, and operational expenses to get a fair idea about the economic feasibility of our process in order to commercialize it. To perform this, we could use the following general methodology for our TEA [83].

Talking about the market value, according to many of the studies that have been performed to check on the economic feasibility of

bioplastics, they have classified these biopolymers under medium-value products, their costs usually lying above €2000/tonne [84]. So, our major objective should be to bring these PHAs to a lower market value product.

Methodology to perform TEA (Techno-economic analysis):

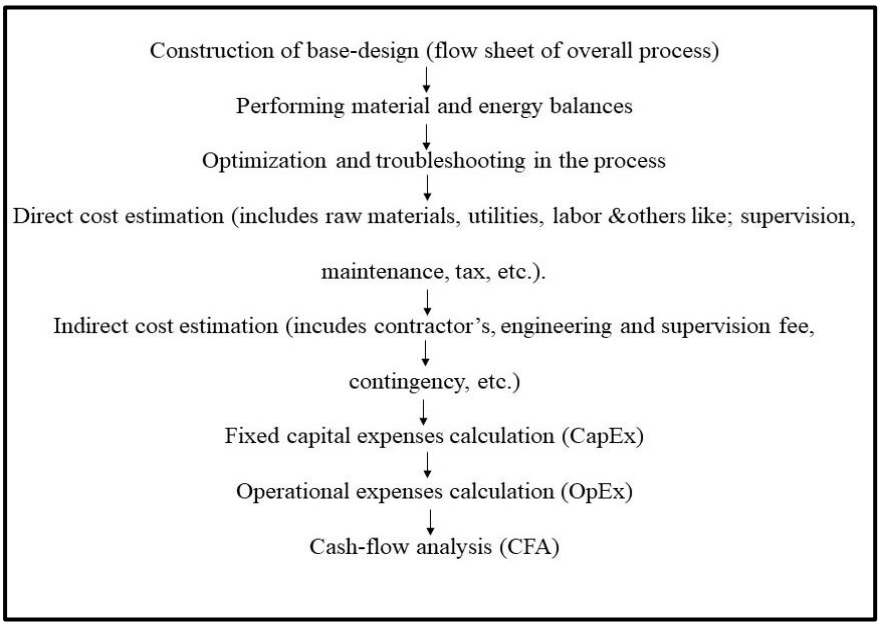


Fig. 6.15: Methodology for perfoming TEA

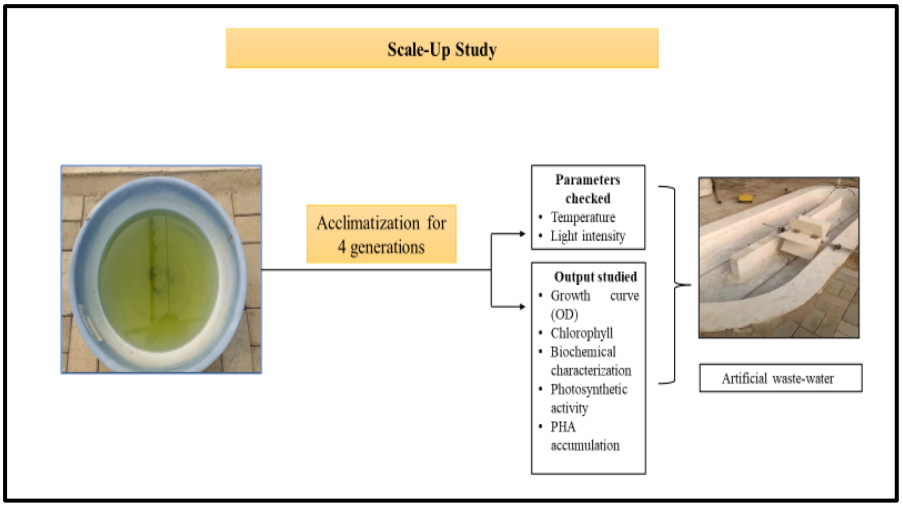


Fig. 6.16: Scale-up studies in raceway ponds for TEA of overall process

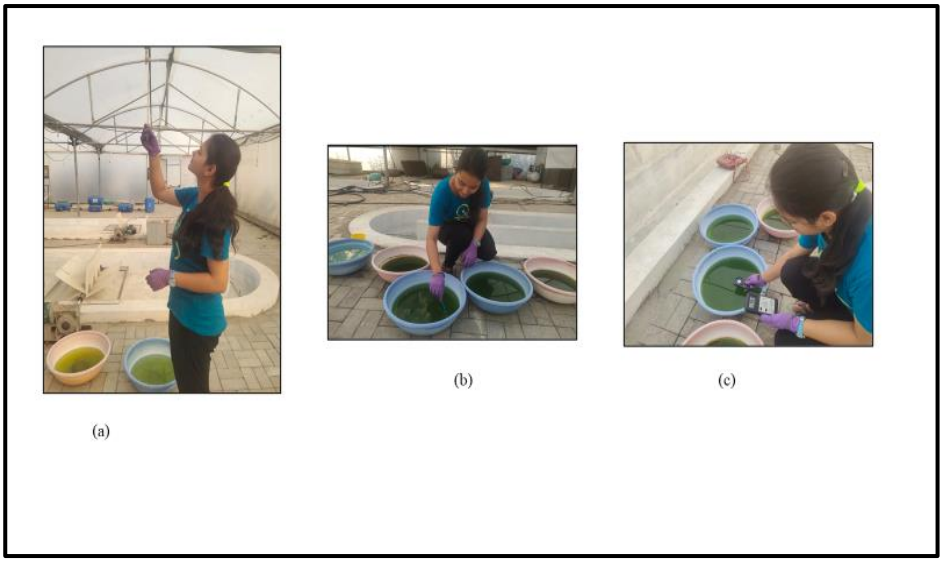


Fig. 6.17: Parameters being checked at polyhouse - (a) temperature measurement (b) mixing of microalgal culture (c) light intensity measurement

Chapter 7

Conclusions and future prospects

7.1 Conclusions

- A screening study for 7 different microalgal species and 4 different purple bacterial species was done to find out their growth profile and growth kinetics of individual species for further experiments by growing them for 14 days and 15 days, respectively.
- *E. texensis* was selected, and PBYB strains were selected as the candidate species showing a convincing growth profile and PHA accumulating potential.
- Since the highest growth was observed on the 21st day in *E. texensis*, further analysis of chlorophyll estimation, biochemical characterization, and PHA accumulation was studied for this very time duration. Also, this study was compiled by comparing the autotrophic and mixotrophic modes of nutrition for the microalgal cells, where it was found that the cells grew better (continued to be in log phase even after 21 days) in mixotrophic mode by feeding on external C-source (galactose). Whereas, under normal controlled conditions, the cells entered the decline phase after 20 days.
- Further, studying the effect of nitrates and phosphates, where the chlorophyll content got reduced in the absence of nitrates and phosphates (N_0P_0) in comparison to control ($N_{1.5}P_{0.04}$). Other factors analysis showed convincing results that lipids, carbohydrates as well as protein content got elevated. Moreover, the PHA accumulation capacity of the microalgal cells also got enhanced in the nitrate/phosphate deficient media in comparison to controlled BG-11.
- This was followed by checking on the effect of VFAs (including acetate, propionate, butyrate, and a mixture of all these three VFAs in a 2:1:1 ratio, respectively). Since the highest growth was observed

for a mixture of these VFAs, further analysis was carried out from the microalgal growth profile with this combination.

- But these VFAs had no significant effect on PHB accumulation.
- For the experiments done to check on the effect of salts on these microalgal cells with NaCl and $MgCl_2$ (10% of these salts in the media along with BG-11 and 1% galactose). The highest PHB accumulation was found with galactose in the media (129.11±3.66 µg/mg), with salts (in high concentrations) having no significant result on PHB accumulation.
- The PHA accumulation potential of the four purple bacterial strains was studied for the 10^{th} day and 15^{th} day in both stir and still conditions, where it was found that in both the conditions (stir and still) and for both the days (10^{th} and 15^{th}), highest PHA accumulation was found for PBYB strain. PHB is the major PHA molecule accumulated in the bacterial cell.
- The effect of acetate (VFA) was also checked on these PBYB strains for (0Hr, 24 Hrs, 48 Hrs, 72 Hrs, and 96 Hrs) on parameters like DO, OD, pH, and PHA accumulation. It was found that other than DO, the rest of all other factors were highest with acetate in the media. OD and pH were highest at 96 Hrs in mixotrophic condition (acetate in the surrounding), and PHA was maximum accumulated at 48 Hrs in the presence of external C-source acetate.

7.2. Future prospects

- We would further try to enhance PHA accumulation in these organisms by providing various conditions and try to perform the techno-economic analysis that could help extract PHA economically on a large scale, bringing evolution to the plastic industry.
- Heavy metals, VFAs, salts, etc., can be utilized from wastewater for PHA synthesis from microalgae and bacterial cells.
- Also, instead of going for sterilizing and producing pure bacterial cultures, which itself is a high cost and energy-demanding process, we can go for testing and exploring PHA production potential from mixed bacterial cultures.

  Along with PHA (bioplastics), other high and low-value-added products too can be synthesized using microalgal and bacterial strains, making the overall process much more economically feasible.

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