Investigating the role of *Arthrospira (Spirulina) platensis* in phosphate bioremediation

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DECLARATION BY THE STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Investigating the role of *Arthospira (Spirulina) platensis* in phosphate bioremediation."** is based on the results of investigations carried out by me in the M.Sc. Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University, under the supervision of Dr. Sangeeta M. Naik and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation **"Investigating the role of *Arthospira (Spirulina) platensis* in phosphate bioremediation."** is a bonafide work carried out by Mast. Harshad Dilip Binnar under my supervision in partial fulfillment of the requirements for the award of the degree of **Masters in Science** in the Discipline **Marine Microbiology** at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Date: 2nd May 2023

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Harshad Dilip Binnar

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1. INTRODUCTION

As an essential nutrient for living things, phosphorus (P) is vital for industry growth and agronomic production. Phosphorus plays an important role in agriculture and industrial systems (Withers et al., 2015). Organic and inorganic compounds are deliberately released into the atmosphere by means of domestic, agricultural and industrial wastewater pollution. However, over a long period, phosphate-rich wastewater discharge from agriculture and industry has caused phosphate enrichment and extreme-level eutrophication of aquatic habitats, endangering both human health and the ecology in the water (Diaz and Rosenberg, 2008; Bacelo et al., 2020; Conley et al., 2009). Eutrophication caused by excessive phosphate has become a global environmental issue that urgently needs to be solved due to its destructive effect on the ecosystem and adverse effect on human health. The process of eutrophication involves the gradual enrichment of a body of water, or portions of it, with minerals and nutrients, especially nitrogen and phosphorus. Eutrophication is normally thought to occur when phosphorus concentrations exceed 0.1 mgL^{-1} (Kumar et al., 2019). Therefore, effluent phosphate from the wastewater must be removed before sewage water is discharged. Phosphorus is widely used in agriculture and is an essential component in fertilizer and feed, but it is a non-renewable resource. Because natural phosphate ores are a non-renewable mineral resource that is slowly depleting, their sustainability is severely constrained (Withers et al., 2015). Phosphate has finite natural reserves and will likely run out in 50 to 100 years (Zhang et al., 2016) . As a result, phosphate removal and recycling from wastewater is receiving more and more attention for various reasons, including the sustainable development of contemporary agriculture and industry and the treatment of eutrophic water (Bacelo et al., 2020).

Several methods have been devised for this aim, including chemical precipitation, biological therapy, and adsorption (Chen et al., 2015; Ye et al., 2017; Egle et al., 2015). However, chemical precipitation and biological treatment have drawbacks, including high costs, significant secondary pollution, and ineffectiveness at a relatively low phosphate concentration (Hu et al., 2020; Ye et al., 2017). Contrarily, adsorption is environment-friendly, easy to use, very economical, and maintains a strong adsorption activity even at low phosphate concentrations (Ma et al., 2017). In order to remove and recycle phosphate from wastewater, it is therefore generally considered to be a viable technique.

A variety of absorbents have been used to remove and recycle phosphate from wastewater, including metal oxides/hydroxides (Yu et al., 2019; Xiang et al., 2019), minerals (Wei et al., 2018), porous silica (Huang et al., 2014), zeolites (Mitrogiannis et al., 2018; Li et al., 2020a), polymer-based absorbent (Liao et al., 2018; Zhang et al., 2016; Hu et al., 2020; Wang et al., 2016a, b). Nature contains a wide variety of phosphate sorbents. The most typical types are those made of metal hydroxides and metal oxides. The difficulty in isolating and recycling metal oxides/hydroxides from the solution prevents their widespread use even if they have a high capacity to adsorb phosphate (Zhang et al., 2019; Liu and Zhang, 2015). Moreover, the limited adsorption activity, high cost, non-renewable raw resources, and unfavorable environmental impact of most minerals, porous silica, zeolites, and polymer-based materials are drawbacks (Xu et al., 2016; Bacelo et al., 2020; Ogata et al., 2011). In contrast, adsorbents derived from biomass waste have gained considerable interest due to their many advantages, including low cost, abundant renewable raw materials, and environmental friendliness (Dai et al., 2020; Trazzi et al., 2016; Liu et al., 2020).

There are two different kinds of biomass-derived adsorbents: biomass-derived carbonbased materials made by carbonizing biomass feedstock and biomass-based composites made by directly functionalising biomass feedstock. In addition to recovering phosphates from wastewater, the development of biomass-derived adsorbents could encourage value-added applications for biomass wastes (Banu et al., 2019; Dodson et al., 2015; Wang et al., 2020). As a result, this is an active area of research, and the number of publications on phosphate adsorption using adsorbents derived from biomass is steadily rising over time.

Organic matter dominates the content of municipal and industrial effluents. Organic material in wastewater is removed using a variety of procedures, and treated water contains just a small amount of removed organic material. The potential of the soil is greatly enhanced by the small amounts of organic matter and nutrients. The plant operation parameters range affects the concentration and composition of organic matter. Advection, sorption, volatility, air stripping, biotransformation, and degradation are the typical removal mechanisms of xenobiotics, which are foreign substances and trace organic substances (Stevens Garmon et al., 2011; Byrns 2001).

1.1. Phosphate in wastewater

In many industries, including contemporary agriculture, phosphorus is regarded as a vital mineral. Wastewater contains three main types of phosphates: orthophosphates, condensed phosphates including pyrophosphates, metaphosphates, and polyphosphates, and organic phosphates (Yousef et al. 2019; Bacelo et al. 2020). Orthophosphate (PO_4), which also goes by the names phosphoric acid (H_3PO_4), dihydrogen phosphate ($H_2PO_4^{-1}$), hydrogen phosphate (HPO_4^{-2}), and phosphate ion (PO_4^{-3}) is present in wastewaters in amounts ranging from 50 to 70 percent (Yin et al.,

2018). In lakes, reservoirs, controlled water systems, and coastal water zones, dissolved phosphates in wastewater are to blame for eutrophication (Zhao and Sengupta, 1998). The water becomes dirty, polluted, and unfit for use for any other purpose at concentrations higher than this one (Awual et al., 2011). The growth of aquatic photosynthetic bacteria is accelerated by phosphates. Eutrophication occurs when the phosphate concentration exceeds 0.02 mg/L. (Yao et al., 2011; Kilpimaa et al., 2014). Water quality declines due to eutrophication, which also lowers the amount of dissolved oxygen in the water, rendering it unfit for further use. As a result, cyanobacteria that produce toxins can develop quickly in an environment that favors them. Waterborne outbreaks are brought on by the presence of soluble phosphates. It affects how much drinking water is produced, outdoor activities, and the environment (Akpor, 2011).

A higher phosphate concentration increases the cost of water treatment so that it can be used for different things. Dead algae and other organisms that easily oxidise in wastewater result in a reduction in water quality as well as its eventual unusability. Enhanced biological phosphate removal (EBPR) is a procedure that lowers the quantity of phosphates discharged into the aquatic environment (Korostynska et al., 2012; Wang et al., 2020; Nassef, 2012). The toxicity of phosphates affects humans and aquatic life, which can lead to tumor lysis syndrome, rhabdomyolysis, and compromised renal function (Kumar et al., 2016).

The utilisation of microalgae in treating wastewater has been an emerging topic focused on finding an economically sustainable and environmentally friendly approach to treating wastewater. Because it has less demanding growth requirements and is simpler to collect due to its filamentous structure, *A. platensis* is a better choice for the tertiary wastewater treatment. However, using this microalga to treat effluent has a

number of advantages, including lower culture costs and the generation of highvalue algal biomass. Almost all types of wastewater, including secondary treated wastewater, sago starch factory effluent, pig wastewater, and anaerobically treated swine wastewater, can be used to cultivate *A. platensis*.

Due to the high levels of nitrogen and phosphorus needed for their growth, microalgae are known to have a remarkable capacity to consume nutrients from wastewaters. Microalgae biomass is a potential source of valuable chemicals and other goods, as well as enabling efficient wastewater treatment, and has recently attracted a lot of interest (Vuppaladadiyam et al., 2018). It has been shown that a number of microalgae strains can efficiently lower the amount of phosphorus in different kinds of wastewater (T. Cai et al., 2013; Whitton et al., 2015). Moreover, when accessible, algae have the capacity to absorb substantial amounts of phosphorous in the form of specific organic compounds and inorganic orthophosphate

(P_i).

There are two mechanisms used by microalgae to remove phosphorus. The most frequent method is direct remediation, which involves the intake of nutritional components into the biomass for the creation of nucleic acids and proteins through biochemical pathways. Furthermore, phosphate can be taken up by microalgae via a different absorption mechanism and stored as an acid-insoluble polyphosphate granule. The increase of biomass and metabolite activity are typically linked to the remediation of phosphate. According to the literature, environmental factors that affect the cultivation of microalgae, such as temperature, irradiation, mixing, and aeration, are related to remediation (Whitton et al., 2015).

1.2. Arthrospira platensis

Arthrospira (Spirulina) platensis is Blue-green algae (cyanobacteria), among the most primitive life forms on Earth. Their cellular structure is a simple prokaryote. They share features with plants, as they are photosynthetic microalgae. They also share features with primitive bacteria because they lack a plant cell wall and characteristics of the animal kingdom as they contain on their cellular membrane complex sugars similar to glycogen. Among blue-green algae, both edible and toxic species adapted to almost any of the most extreme habitats on Earth. Moreover, these can be consumed by humans and animals. Edible blue-green algae, including *Nostoc*, *Spirulina*, and *Aphanizomenon* species, have been used for food for thousands of years. (Saranraj et al., 2014). One of the trends in biotechnology is associated with Blue green microalgae *A. platensis,* which has been widely employed as a food and feed additive in agriculture, the food industry, pharmaceuticals, perfume making, medicine, and science (Sivasakthi et al., 2014).

A. platensis has been used as food for centuries by different populations and has only been rediscovered recently. The Aztecs and other Mesoamericans, until the 16th century, used Spirulina as a food source. Once classified as the - blue-green algae, it does not strictly belong to the algae, even though it continues to be referred to in that way for convenience. It grows naturally in the alkaline waters of lakes in warm regions. It generally takes the form of tiny green filaments coiled in spirals of varying tightness and number, depending on the strain. Its impressive protein content and its rapid growth in entirely mineral environments have attracted the attention of both researchers and industrialists alike. *A. platensis* is a unicellular, filamentous bluegreen alga that has grown significantly in popularity as a protein and vitamin supplement to aquaculture diets and in the health food sector. Those who live close to the alkaline lakes where it is naturally found have long utilised it as a food supplement. *A. platensis* has been

employed as an additional dietary component in fish, prawns and poultry feed. Among the various species of *Arthrospira*, the bluegreen alga *A. platensis* has drawn more attention because it shows a high nutritional content characterised by a 70% protein content and by the presence of minerals, vitamins, amino acids, essential fatty acids, etc (Campanella et al., 1999).

Arthrospira platensis is commonly found in aquatic ecosystems like lakes, ponds, and tanks. It is one of nature's first photosynthetic organisms capable of converting light directly for complex metabolic processes. *Arthrospira platensis* has been used for food from time immemorial by tribes living around Chad Lake in Africa and as a nutritional supplement known as "dihe".The predominant species of phytoplankton in the lake is *A. platensis*. The algae *A. platensis* was eaten in Mexico under the name 'Tecuitlatl' (Farrar, 1996). *Arthrospira platensis* grows optimally in the pH range of 9 - 11, and there is the slightest chance of contamination by other microbes.

1.2.1. Morphology of Arthrospira platensis

A. platensis is symbiotic, multicellular, and filamentous blue-green microalgae with symbiotic bacteria which fix the nitrogen from the air. The shape of *A. platensis* can be like a rod or disk. The main morphological feature of *A. platensis* genes would be the arrangement of the multicellular cylindrical trichome. The photosynthetic pigment of *A. platensis* is phycocyanin, and its colour is blue. This type of bacteria contains chlorophyll and carotenoids. Some bacteria contain pigments like phycoerythrin, which is red and pink. *A. platensis* is photosynthetic, so, therefore, it is autotrophic. The reproduction of *A. platensis* is due to binary fission. *Arthrospira platensis* has a screw like trichome with a generally closed, uniform, and narrow diameter from 0.5-3 μm. Cells with cross walls are visible under a light microscope,

without gas vacuoles, and with prominent granules. The trichomes are up to 4 µm wide and range from 50 to 500 µm. Gram-negative bacteria share peptidoglycan and lysozyme-sensitive heteropolymer in their cell walls with cyanobacteria. Environmental factors that affect helix geometry are temperature and physical and chemical conditions. One of the drastic alterations in the geometry is the reversible transition from helix to spiral shape while transferring the filaments from liquid to solid media (Ahsan et al., 2008).

1.2.2. Scientific classification (Raghuraman et al., 2021)

Domain: Bacteria

Phylum: Cyanobacteria

Class: Cyanophyceae

Order: Oscillatoriales

Family: Microcoleacea

Genus: *Arthrospira*

Species: *A. platensis*

Figure 1. Light microscopic images of *A. platensis*

1.2.3. Natural habitat

Arthrospira platensis can be found in soil, marshes, freshwater, brackish water, ocean, and thermal springs. It grows well in alkaline, saline water (>30 g/l) with a high pH (8.5-11.0), especially in tropical regions with high levels of solar radiation at altitude. *Arthrospira platensis* and *A. maxima* flourish in highly alkaline lakes in Africa and Mexico, where the cyanobacteria population is almost monospecific. The likelihood of *Arthrospira spp*. predominating increases with water conductivity and pH. This is evident in the lakes of the Rift Valley in Eastern Africa, where sodium carbonate is common, and pH values can reach 11. *Arthrospira platensis* was isolated from waters containing 85 to 270 g of salt per litre, with optimal growth between 20 and 70 g. This microorganism's ability to utilise ammonia as a source of nitrogen at high alkaline pH values may be explained by a cytoplasmic pH that is relatively high (4.2 to 8.5) (Sason, 1997).

Like most cyanobacteria, *A. platensis* is an obligate photoautotroph, meaning it cannot thrive on a substrate containing organic carbon molecules in the dark. In the presence of light, it absorbs primarily nitrates while reducing carbon dioxide. Glycogen is the primary assimilation by product of spirulina photosynthesis. In a lab setting, *A. platensis* exhibits optimal development between 35 and 37 °C. A brief rise in temperature of up to 39 °C outside has no adverse effects on the blue-green alga's capacity to photosynthesise. *Arthrospira platensis* is a thermophilic or thermotolerant strains and can be grown at temperatures between 35 and 40 °C. The advantage of such a property is that it eliminates mesophilic microbial contaminants. *Arthrospira platensis* can develop at a minimum temperature of roughly 15 °C during the day. It can endure reasonably low temperatures at night. Certain species of spirulina are known to have high U.V. resistance (Richmond, 1986).

A fundamental aspect of *A. platensis* biology is its life cycle due to the taxonomic, physiologic, and cultivation implications (Vonshak, 1987). This period is summarised in three fundamental stages: trichomes fragmentation, hormogonia cell enlargement, maturation processes, and trichome elongation. The mature trichomes are divided into several tiny filaments or hormogonia through the previous formation of specialised nephridium cells, in which the cell material is reabsorbed, allowing fragmentation. The number of cells in the cosmogonies is increased by binary fission. In this process, the trichomes grow lengthwise and take their helical form (Balloni, et al., 1980).

1.2.4. Life cycle of A. platensis

The life cycle of *A. platensis* is crucial to understanding its biology because of the consequences it has for taxonomy, physiology, and production (Ciferri, 1983; Richmond, 1984). Trichomes fragmentation, hormogonia cell enlargement, maturation processes, and trichome elongation are the three main phases that best describe this time period. By first forming specialised cells called nephridium cells, in which the cell substance is reabsorbed to allow fragmentation, the mature trichomes are split into many tiny filaments or hormogonia—binary fission results in an increase in the number of cells in the cosmogonies. The trichomes grow longitudinally and assume their helical shape during this procedure (Balloni, et al., 1980).

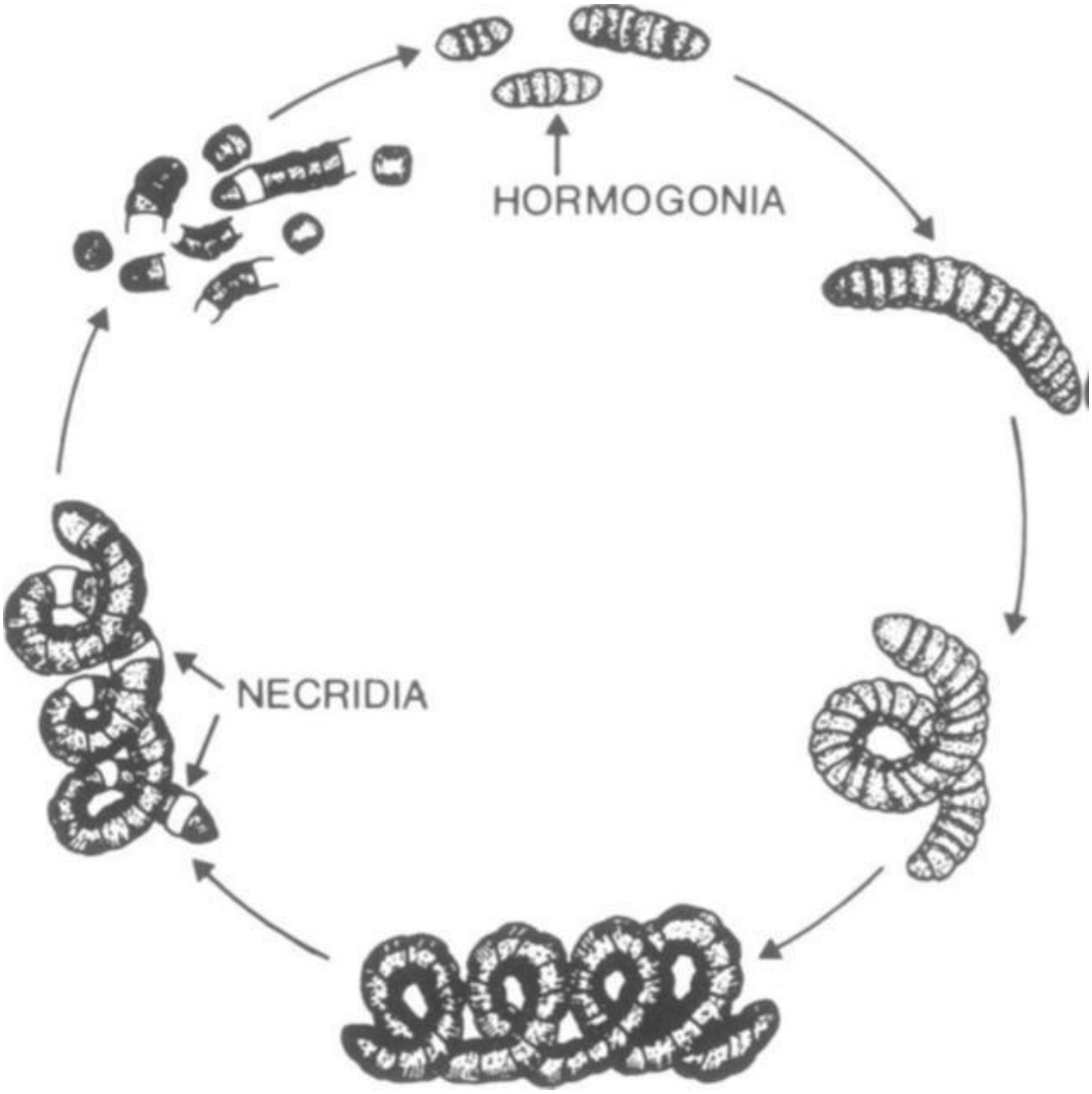

Figure 2. Life cycle of *A. platensis* (Kashani, 2015).

Accurately determining when man first used microalgae is unknown. Three things have come before the current usage of these resources: custom, advancements in science and technology, and the so-called "green trend" (Henrikson,1994). A soldier under Hernán Cortez named Bernal Díaz del Castillo wrote in 1521 that *A. maxima* were taken from Lake Texcoco, dried, and sold for human consumption in a market in Tenochtitlán (modern-day Mexico City). According to one author, "..Natives took out of the lake small cakes made of a mud-like alga that has a cheeselike flavour and was used to make bread" (Ciferri, 1983). Years later, the Franciscan priest Bernardino de

Sahagn wrote: “... extremely soft things are harvested from Mexican lakes during specific times of the year. They have a curd-like appearance and a clear blue tint and are used to produce baked bread. Tecuitlalt is the indigenous people's name given to this dish, meaning "excrement of stones" in their language”.

The dihé cake was mentioned by the French phycologist P. Dangeard in 1940. It was eaten by members of the Kanembu tribe in the sub-desert region of Kanem, close to the African Lake Chad. Dihé is a blue-green algal cake that has been dried in the sun after being collected from the shores of little ponds surrounding the lake. After analysing the dihé samples, Dangeard concluded that the substance was a purée of springtime blue algae, which is the primary component of the phytoplankton in many of the lakes in the African Valley (Ciferri, 1983). At that time, *Arthrospira* (*A. maxima*) samples from Lake Texcoco, close to Mexico City, were analysed by a team of French researchers (Ciferri, 1983; Richmond, 1992).

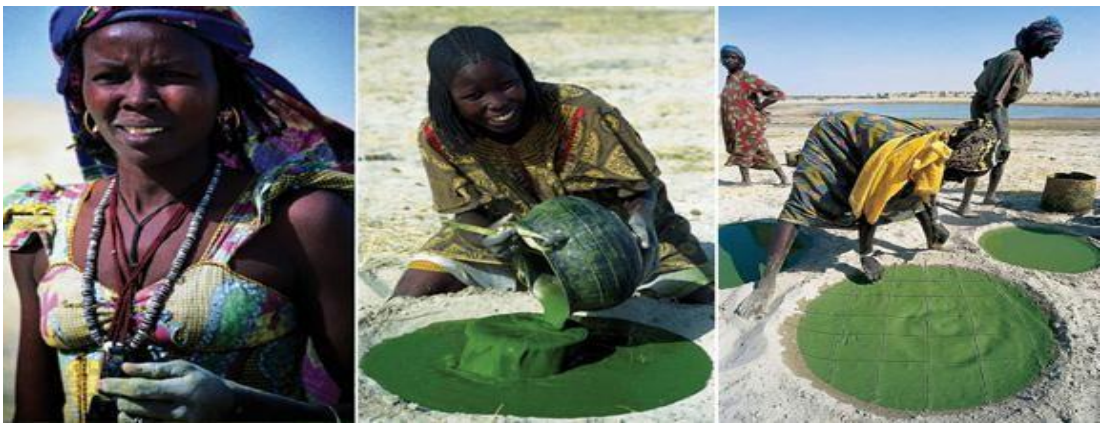

Figure 3: *Arthrospira* harvested and processed manually by women of the Kanembu tribe, Chad, West Africa (Koru, 2012) .

Figure 4: Kanembu women harvesting *Arthospira* from Lake Boudou Andja in Chad, West Africa (Hamed, 2016).

Figure 5: Harvesting of *Arthrospira* from lake (Hamed, 2016).

1.3. Objective of work

The storage and exchange of information and energy in all cells, including those of microalgae, depends on phosphorus (P). The vast majority of microalgae are adapted to low P acclimated naturally; as a result, they are able to absorb and store huge amounts of P whenever it becomes available. The capacity to consume more phosphorus than required for rapid growth is known as luxury uptake. The majority of P obtained during luxury absorption is stored as inorganic polyphosphate, a substance that is almost always present in cells and serves a variety of purposes (Solovchenko et al., 2019). By taking the nutrients into their cells, certain microalgae have been shown to simultaneously remove phosphorus from domestic wastewater down to very low concentrations of 0.15 mg L^{-1} (Boelee et al., 2011). The use of microalgae in wastewater treatment (Oswald and Gotaas, 1957) and has received much attention in recent decades (Olguín et al., 2003).

2. MATERIALS AND METHODOLOGY

2.1. Preparation of standard Zarrouk's media (Zarrouk's, 1966)

Materials required

Glassware – conical flasks, measuring cylinder, Glass rod

Chemicals – Sodium chloride (NaCl), Calcium chloride dihydrate ($CaCl_2.2H_2O$), Sodium nitrate ($NaNO_3$), Ferous sulfate heptahydrate ($FeSO_4.7H_2O$), Ethylenediaminetetraacetic acid sodium salt (EDTA-Na), Potassium sulfate (K_2SO_4), Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), Sodium hydrogen carbonate ($NaHCO_3$), Potassium hydrogen phosphate (K_2HPO_4), Boric acid (H_3BO_3), Manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$), Sodium Molybdate (Na_2MoO_4),

Copper(II) sulfate pentahydrate ($CuSO_4.5H_2O$), Distilled water.

Other requirements – Butter paper, spatula

Instruments – Weighing balance, autoclave, laminar air flow

Method

Zarrouk's media was prepared using the composition given by Zarrouk's, 1966. To prepare 1 L of Zarrouk's media all given chemicals as shown Table 1.a. has to be taken in 1 L distilled water in a 1 L flask and autoclaved. Then micronutrient solution were prepared as shown in Table 1.b. After the autoclaving media add 1 mL L^{-1} of micronutrient solution to it.

Table 1.a: Standard Zarrouk's media (1 L)

Components	Amount (g)
NaCl	1
$CaCl_2.2H_2O$	0.04
$NaNO_3$	2.5
$FeSO_4.7H_2O$	0.01
EDTA (Na)	0.08
K_2SO_4	1
$MgSO_4.7H_2O$	0.2
$NaHCO_3$	16.8
K_2HPO_4	0.5

Table 1.b: Micro nutrients solution (1 L)	
Micronutrient	Amount (g)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.810
$ZnSO_4.4H_2O$	0.222
Na_2MoO_4	0.0177
$CuSO_4.5H_2O$	0.079

2.2. Preliminary experiments

Preliminary experiments were done to check the growth conditions of the given culture samples.

2.2.1. Biomass

Using a UV-visible spectrophotometer, sample cultures were scanned between 550 and 800 nm to check the maximum absorbance for each microalgae species under study (Jasco V-630, USA). The maximum absorbance value for each microalga was employed for the growth curve using optical density (OD) (Ribeiro-Rodrigues et al., 2011).

These results are similar to other research in which the wavelengths used for some microalgae species' cell growth ranged from 664 to 678 nm (Padovan,1992), 680 nm (Geis et al.,2000), 684 nm (Ribeiro-Rodrigues et al.,2011), and 687 nm (Valer and Glock, 1998). These results are similar to other research in which the wavelengths used for some microalgae species' cell growth ranged from 664 to 678 nm (Padovan,1992),

680 nm (Geis et al.,2000), 684 nm (Ribeiro-Rodrigues et al.,2011), and 687 nm (Valer and Glock, 1998). The biomass concentration was determined by spectrophotometry.

Using a standard curve of *Spirulina sp*. LEB 18. This curve was obtained by measuring the optical density of the *Spirulina* inoculum in a spectrophotometer (QUIMIS Q798DRM, Diadema – SP –Brazil), at 670nm, by relating the optical density and dry weight biomass, as performed by Costa et al. (2002).

2.2.2. Standard plot for carbohydrate

Carbohydrate estimation was done by using the phenol-sulphuric acid method as described by Dubois et al. (1956).

Material required

Glassware - Beakers, Stoppered test tubes, Glass pipettes, Measuring cylinders, 1 cm quartz cuvettes

Chemicals - 5% Phenol, Concentrated Sulphuric acid (H_2SO_4), Glucose, Sterile distilled water

Other equipment - Spatula, Micropipette, Pipette tips, Test tube stand

Instruments - Weighing balance, Spectrophotometer

Method

Preparation of standards and blank

A Glucose standard stock solution was prepared by adding 25 grams of Glucose in 25 mL distilled water to make a final concentration of 1000 µg/mL and this was used for the preparation of standards.

2 mL standard solutions of 20 µg/mL, 40 µg/mL, 80 µg/mL, 150 µg/mL, 250 µg/mL and 500 µg/mL concentrations were prepared in duplicates in stoppered tubes labelled accordingly.

2 mL distilled water was used as blank.

Spectrophotometric analysis of carbohydrate standards

To the stoppered tube containing 2 mL of standard solution/blank, 0.5 mL 5% Phenol and 5 mL of concentrated Sulphuric acid was added.

Then the absorbance was measured at 490 nm wavelength.

Average was taken and a standard curve of concentration v/s absorbance at 490 nm was plotted to obtain an equation which would be used to determine the unknown concentration

2.2.3. Standard plot for phosphate

Phosphate analysis

Method used to determine the inorganic phosphate in seawater are based on the reaction of the ions with acidified molybdate reagent to produce a phosphomolybdate heteropoly acid, which is then reduced to a highly coloured blue compound

Material required

Glassware – Volumetric flasks, beakers, pipettes and bulbs, stoppered tubes, glass cuvettes, 1 cm quartz

Chemicals – Sulphuric acid, ascorbic acid, potassium dihydrogen phosphate, ammonium heptamolybdate tetrahydrate, potassium antimony tartrate and distilled water

Other equipment - Spatula, Micropipette, Pipette tips, Test tube stand

Instruments - Weighing balance, Spectrophotometer

Reagent Preparation

Sulphuric acid 25% (Reagent 1) – Add 250 mL of concentrated sulphuric acid to 750 mL of distilled water. Allow to cool and dilute to 1 L. Store in a polyethylene bottle.

Ascorbic acid solution – Dissolve 10 g of ascorbic acid, $C_6H_8O_6$, in 50 mL of distilled water, then add 50 mL of sulphuric acid (Reagent 1). Stored in the dark in a brown bottle at < 8 °C.

Mixed reagent – Dissolve 12.5 g of ammonium heptamolybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$, in 125 mL of distilled water. Also, dissolve 5 g of potassium antimony tartrate, $K(SbO)C_4H_4O_6$, in 20 mL of distilled water. Add molybdate solution to 350 mL of sulphuric acid (reagent 1), mix it well and store it in a glass bottle.

Preparation of standards and blank

A Phosphate standard stock solution was prepared by adding 139.09 mg of Potassium hydrogen phosphate (K_2HPO_4) in distilled water and 0.2 mL sulfuric acid (Reagent 1) was added, volume was make upto 100 mL in volumetric flask to make a final concentration of 10000 µM and this was used for the preparation of standards.

25 mL standard solutions of 1 µM, 2.5 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM concentrations were prepared in duplicates in stoppered tubes labelled accordingly.

25 mL distilled water was used as blank.

Spectrophotometric analysis of carbohydrate standards

To the stoppered tube containing 25 mL of standard solution/blank, 1 mL Ascorbic acid solution and 1 mL of mixed reagent was added.

Then the absorbance was measured at 880 nm wavelength.

Average was taken and a standard curve of concentration v/s absorbance at 880 nm was plotted to obtain an equation which would be used to determine the unknown concentration.

2.3. Experimental analysis

2.3.1. Biomass

Requirements – quartz cuvvetes, distilled water, tissue paper, spectrophotometer, beaker

Biomass of *A. platensis* culture were estimated at 670 nm using spectrophotometer on the same day of sampling

2.3.2. Estimation of carbohydrate content

Carbohydrate estimation was done by using the phenol-sulphuric acid method as described by Dubois et al. (1956).

Material required

Glassware - Beakers, Stoppered test tubes, Glass pipettes, Measuring cylinders, 1 cm quartz cuvettes

Chemicals - 5% Phenol, Concentrated Sulphuric acid (H_2SO_4), Sterile distilled water

Other equipment - Spatula, Micropipette, Pipette tips, Test tube stand

Instruments - Weighing balance, Spectrophotometer

Samples - 2 mL samples were taken in vials from the triplicate flasks of 0.5, 0.75, 1.0, and 1.5 phosphate concentration on Day 0, 3 6, 10, 12, 15, 17 and 20 and stored at 4°C until further analysis.

Method

Spectrophotometric analysis of carbohydrate standards.

To the stoppered tube containing 2 mL of standard solution/blank, 0.5 mL 5%

Phenol and 5 mL of concentrated Sulphuric acid was added.

Then the absorbance was measured at 490 nm wavelength.

The absorbance values and the equation obtained from the standard plot was used to determine the unknown concentration.

The obtained values were averaged and plotted to derive results and conclusion.

2.3.3. Estimation of chlorophyll pigments

Method for chlorophyll pigment estimation was adapted from Lesley Clementson CSIRO (April, 2002). Phytoplankton pigment extraction uses a wide range of solvents. The most effective extraction method is dimethyl formamide, although it is extremely poisonous and challenging to handle. Another extremely effective extractor is methanol, particularly for species that are difficult to extract, like certain cyanobacteria. Unfortunately, reliable equations for calculating chlorophyll a, b, and c in methanol pigments are not accessible. Because of this, acetone (either 90% or 100%) is thought to be the best solvent for a variety of marine phytoplankton species. It is frequently used for both field samples and cultured (Jeffrey et al., 1997).

Material required

Glassware - Beakers, Conical flask, 1 cm quartz cuvettes

Chemicals - 90% acetone, Distilled water

Other equipment - 22 mm GF/F filter paper, Forceps, Small filtration unit,

Syringe, 1.5 mL Eppendorf tubes, Centrifuge tubes, Micropipette, Pipette tips,

Test tube stand, Aluminium foil, Parafilm

Instruments - Vortex, Centrifuging machine, Spectrophotometer

Samples - Appropriate amount of samples were taken from the triplicate flasks of 0.5, 0.75, 1.0 and 1.5 phosphate concentration on Day 0, 10, and 20, filtered through GF/F filter paper in dim light condition at low pressure and stored at -20° C in Appropriately labelled Eppendorf tubes until further analysis.

Method

The filter paper with the filtered sample was taken in a centrifuge tube and 5 mL of 90% acetone was added to it.

It was then vortexed for 30 seconds, covered with foil, sealed with parafilm and stored in the refrigerator at 4° C overnight.

Next day, the tubes containing the sample with acetone were centrifuged at 5000 rpm for 5 minutes.

Absorbance was then taken at wavelengths 630 nm, 647 nm, 664 nm and 750 nm.

The absorbance at 750 nm was subtracted from the absorbance at each of the other three wavelengths to obtain the corrected wavelengths and these were then substituted into the following equations:

[chl. a] extract = (11.85 A_{664}/ l) – (1.54 A_{647}/ l) – (0.08 A_{630}/ l)

[chl. b] extract = (21.03 A_{647}/ l) – (5.43A_{664}/ l) – (2.66 A_{630}/ l)

[chl. c] extract = (24.52 A_{630}/ l) – (1.67 A_{664}/ l) – (7.60 A_{647}/ l)

Where,

A = Corrected absorbance.

l = Path length in cm.

The concentration of each chlorophyll in the sample in µg/L was then obtained by the following equation:

[chl.x] sample = [chl.x] extract * (v/V)

Where, v = Volume of extract in mL.

V = Volume of seawater filtered in L.

The total concentration of chlorophyll in the sample in µg/L was obtained by the following equation:

[chl.] total = [chl.a] sample + [chl.b] sample + [chl.c] sample

The obtained values were averaged and plotted to derive results and conclusion

2.3.4 Phosphate analysis

The determination of dissolve inorganic phosphate given by Greenfield and Kalber (1954) but present method follows the procedure given by Murphy and Riley.

Methods used to determine the inorganic phosphate in seawater are based on the reaction of the ions with acidified molybdate reagent to produce a phosphomolybdate heteropoly acid, which is then reduced to a highly coloured blue compound.

Material required

Glassware – Volumetric flasks, beakers, pipettes and bulbs, stoppered tubes, glass cuvettes, 1 cm quarts

Chemicals – Sulphuric acid, ascorbic acid, potassium dihydrogen phosphate, ammonium heptamolybdate tetrahydrate, potassium antimony tartrate and distilled water

Other equipment - Spatula, Micropipette, Pipette tips, Test tube stand

Instruments - Weighing balance, Spectrophotometer

Reagent Preparation

Sulphuric acid 25% (Reagent 1) – Add 250 mL of concentrated sulphuric acid to 750 mL of distilled water. Allow to cool and dilute to 1 L. Store in a polyethylene bottle.

Ascorbic acid solution – Dissolve 10 g of ascorbic acid, $C_6H_8O_6$, in 50 mL of distilled water, then add 50 mL of sulphuric acid (Reagent 1). Stored in the dark in a brown bottle at <8 °C.

Mixed reagent – Dissolve 12.5 g of ammonium heptamolybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$, in 125 mL of distilled water. Also, dissolve 5 g of potassium antimony tartrate, $K(SbO)C_4H_4O_6$, in 20 mL of distilled water. Add molybdate solution to 350 mL of sulphuric acid (reagent 1), mix it well and store it in a glass bottle.

Samples analysis

25 mL of sample were filtered from the triplicate flasks of 0.5, 0.75, 1.0 and 1.5 phosphate concentration on Day 0, 10, 20 and stored in a vials at 4 °C until further analysis of inorganic phosphate.

To 25 mL sample 1 mL Ascorbic acid solution and 1 mL of mixed reagent was added.

Then the absorbance was measured at 880 nm wavelength.

The absorbance values and the equation obtained from the standard plot was used to determine the unknown concentration.

The obtained values were averaged and plotted to derive results and conclusion.

3. RESULTS AND DISCUSSION

3.1. Growth of Spirulina platensis in different Phosphate concentrations

The data obtained in the preliminary experiment is depicted in Figure 6.a. and Table 2.

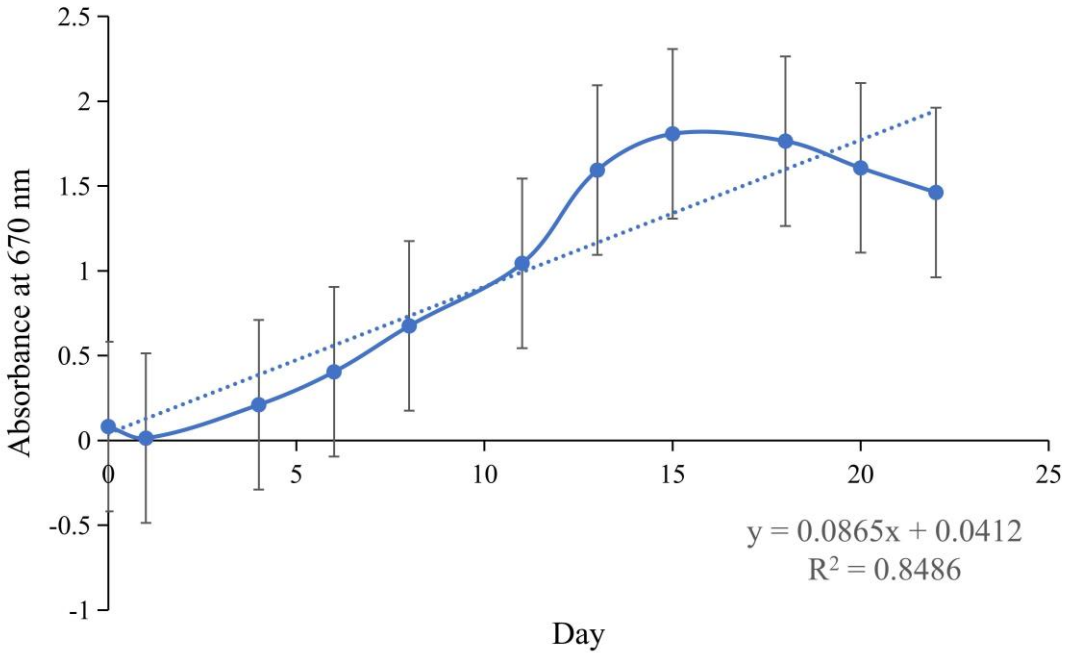

Figure 6.a. *Spirulina* biomass standard calibration curve.

Table 2 : Biomass estimation at absorbance 670.			
Day	Replicate 1	Reolicate 2	Average
0	0.08	0.083	0.0815
1	0.01	0.016	0.013
4	0.22	0.2	0.21
6	0.406	0.402	0.404
8	0.73	0.62	0.675
11	1.048	1.04	1.044
13	1.512	1.675	1.5935
15	1.93	1.685	1.8075
18	1.806	1.723	1.7645
20	1.706	1.507	1.6065
22	1.678	1.246	1.462
		SD	0.711640125

The data obtained in the main experiment is depicted in Figure 6.b. and Table 3.

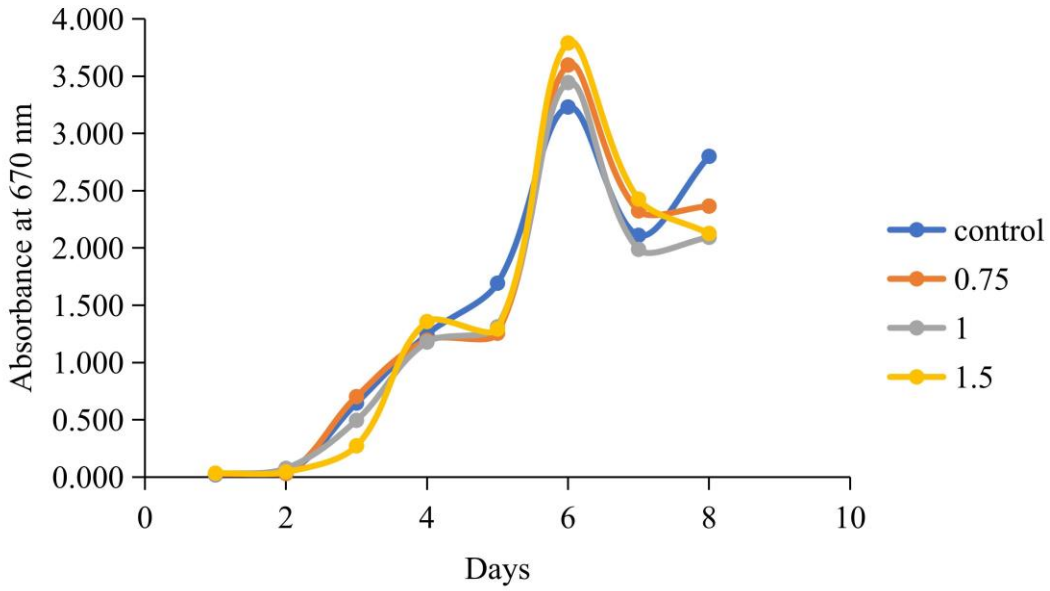

Figure 6.b. Graph absorbance obtained for biomass.

Table 3: Growth rate, doubling time and generation time obtained from main experiment.

Phosphate	Growth rate (d^{-1})	Doubling time	Generation time
Control	0.246	2.802	4.061
0.75	0.226	3.059	4.434
1	0.217	3.181	4.610
1.5	0.207	3.329	4.824

From the growth plot *A. platensis* shows a sigmoidal growth curve. With an exponential phase peaking at day 6 and then showing decline.

3.2. Total carbohydrate content variation of A. platensis at different phosphate concentration

The carbohydrate content were estimated by using an equation obtained from the standard plot of the commonly used standardizing agent for carbohydrate i.e. Glucose. The concentrations used were 20 µg/mL, 40 µg/mL, 80 µg/mL, 150 µg/mL, 250 µg/mL and 500 µg/mL and the standard curve and equation were obtained as shown in Figure 7.a.

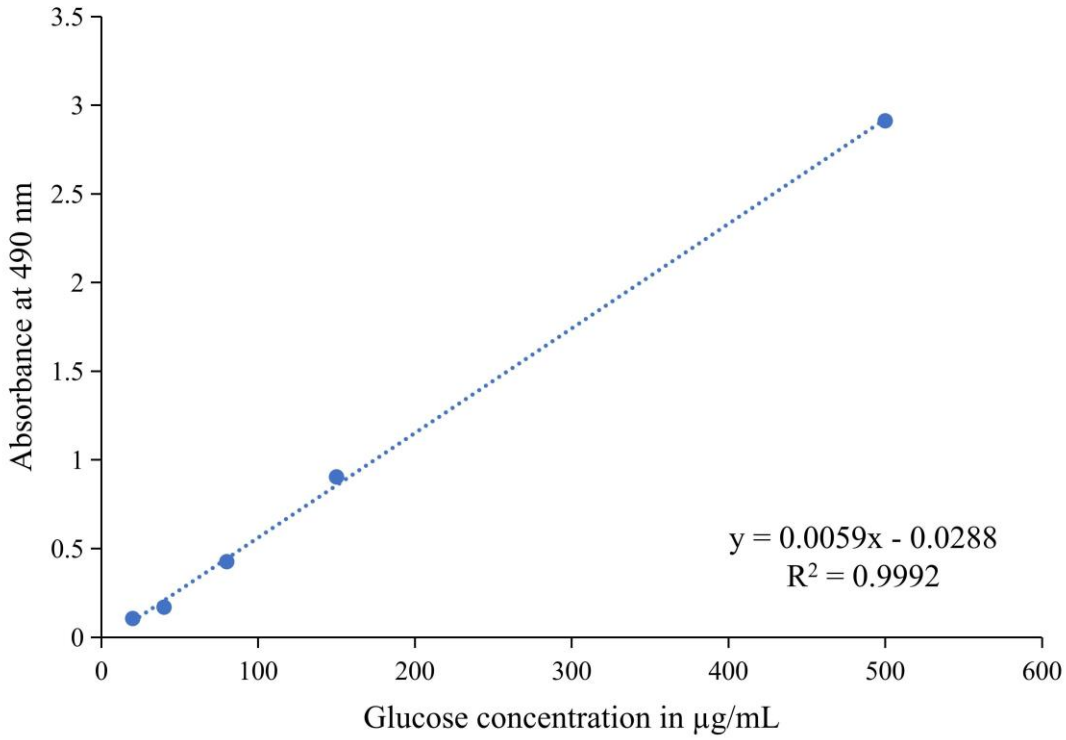

Figure 7.a. Standard plot for carbohydrates.

In Figure. 7.a. the carbohydrate content of *A. platensis* cultivated under the four phosphate concentrations is demonstrated. The carbohydrate content was estimated for phosphate concentration 0.5, 0.75, 1.0 and 1.5 for Day 0, 3, 6, 10, 12, 15, 17 and 20. It was observed upon plotting the graph that S. platensis showed an overall increase in carbohydrate content, as shown in Chart. Initially, i.e. upto day 10 the carbohydrate content of 1.5 phosphate concentration was highest. Simultaneously the carbohydrate

content of control (0.5) phosphate concentration shows steady increase and 0.75 phosphate concentration shows the highest at day 12 and day 15.

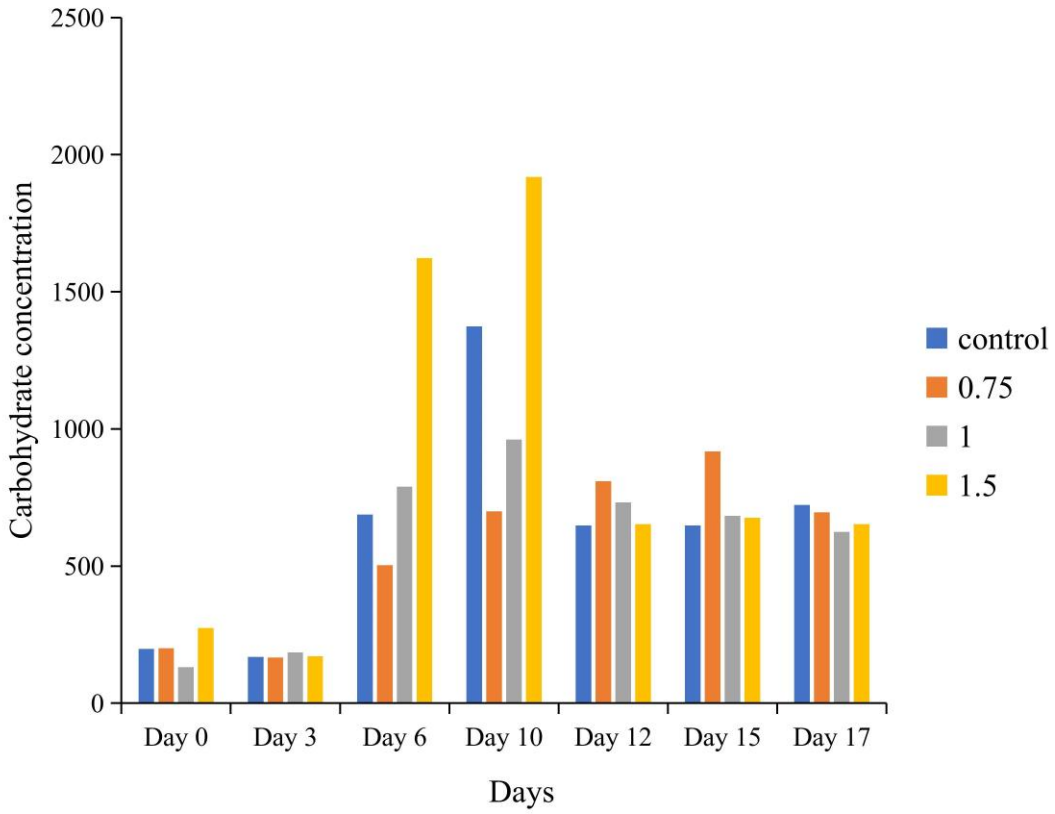

Figure 7.b. Total carbohydrate concentration of *A. platensis* at different phosphate concentration

3.3. Production of pigment by A. platensis at different phosphate concentrations

The production of pigments (Chlorophyll a, b and c) by *A. platensis* was estimated for phosphate concentration 0.5 (control), 0.75, 1.0 and 1.5 g/L for Day 0, 10, and 20. Figure a. to d. shows the total concentration of chlorophyll and the concentration of different chlorophyll pigments (a,b and c) produced by *S. platensis.*

It can be observed that there is an increasing trend in the amount of chlorophyll at different phosphate concentrations 0.5 (control) and 1.0 and it shows the highest

chlorophyll content on day 20. There is very low chlorophyll production by different phosphate content, while only 0.5 (control) phosphate concentration shows the highest chlorophyll production. Chlorophyll b and c shows an increasing trend with increasing phosphate concentration.

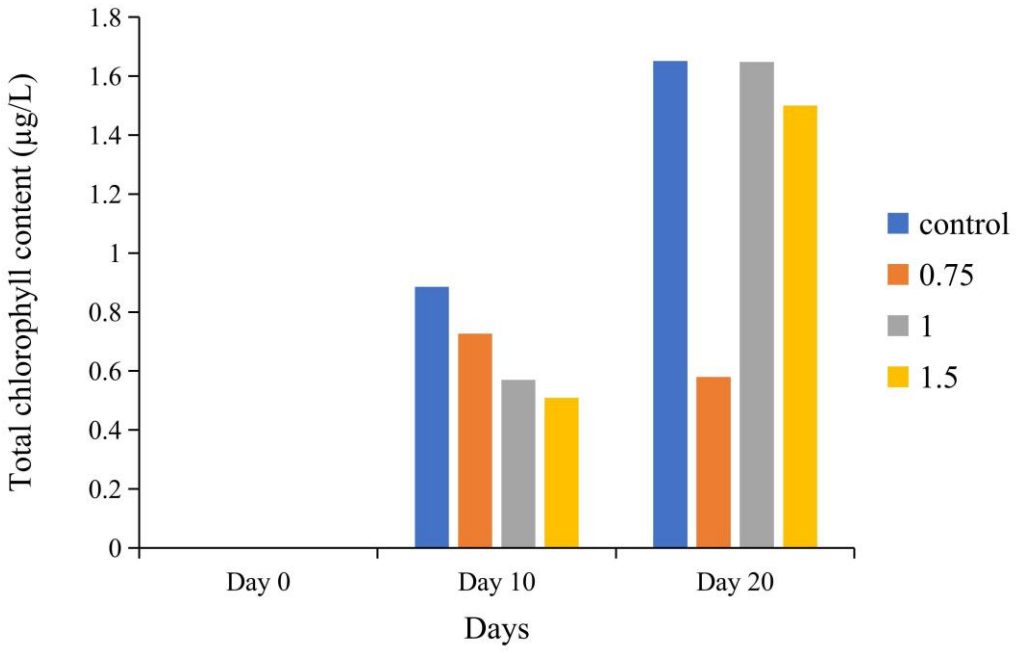

Figure 8.a. Concentration of total chlorophyll of *A. platensis* at different phosphate concentration

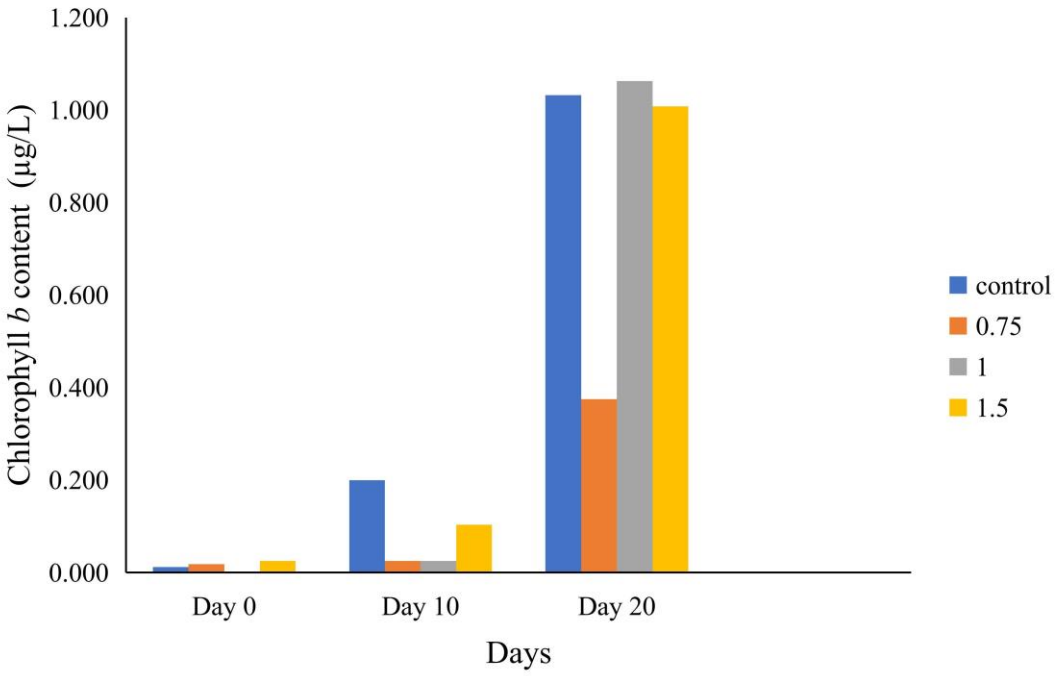

Figure 8.b. Concentration of chlorophyll *b* of *A. platensis* at different phosphate concentration

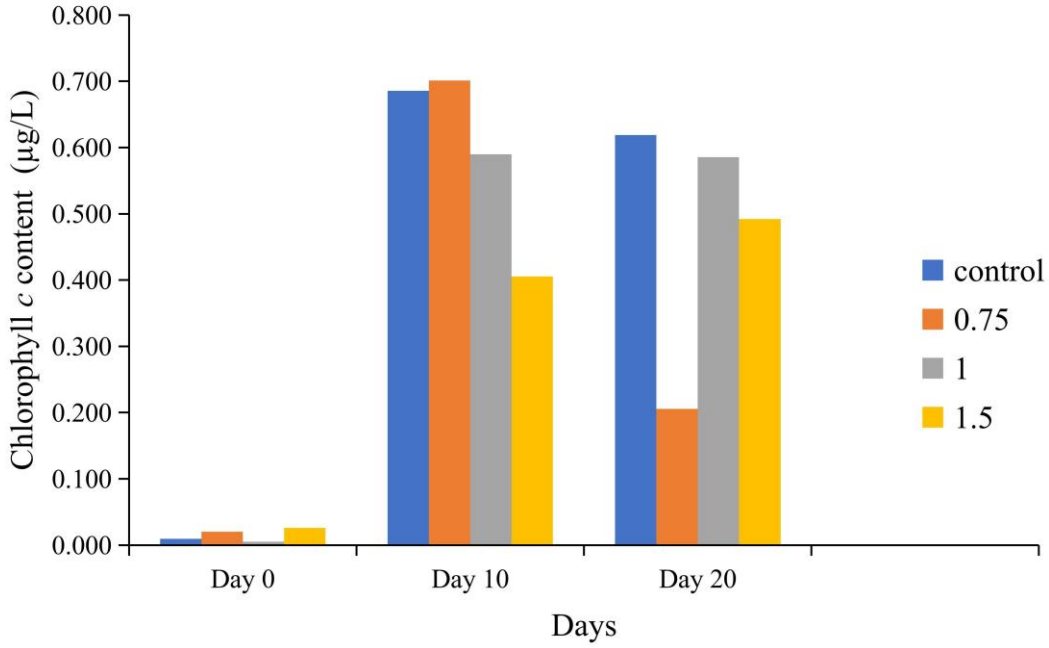

Figure 8.c. Concentration of chlorophyll *c* of *A. platensis* at different phosphate concentration.

3.4. Utilisation of phosphate by A. platensis at different phosphate concentrations.

The data obtained in the preliminary experiment is depicted in Figure 9.a. and Table 4.

Table 4: Phosphate calibration curve	
Concentration	OD at 880 nm
1	0.0135
2.5	0.024
5	0.0615
10	0.105
25	0.28
50	0.578
100	1.155

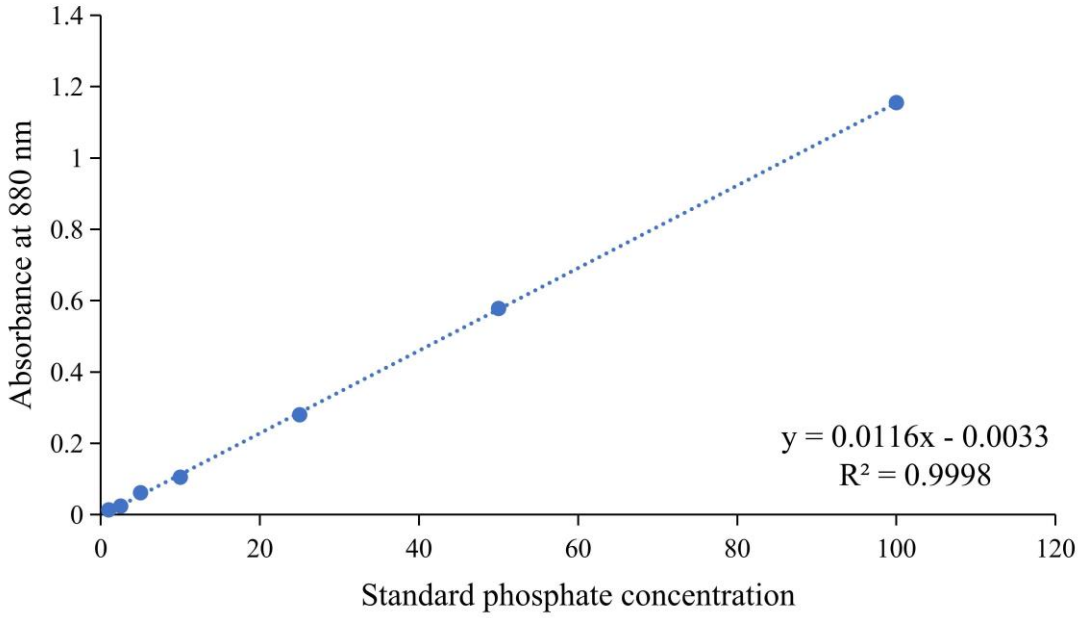

Figure 9.a. standard phosphate calibration curve

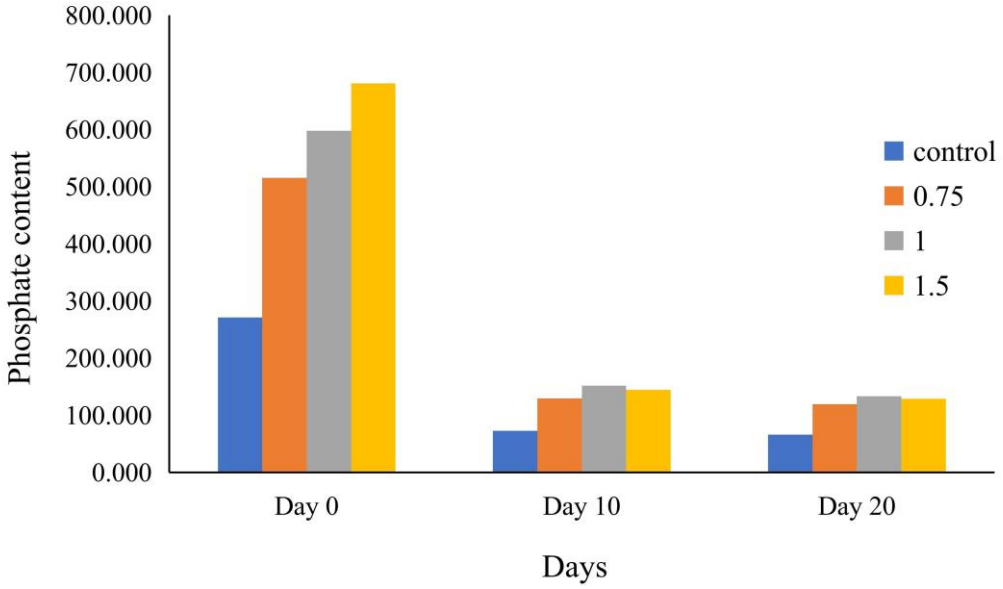

Figure 9.b. Experimental data obtained of phosphate

The variations of the phosphorus concentrations in the experimental cultures over time are shown in Figure 9.b. Phosphorus concentration decreased over time, on day 10 it was showing 3/4th concentration of phosphorous was utilised. Phosphate concentration decreases with increase in the biomass of the *A. platensis* and there is steady decreasing in phosphate concentration.

4. CONCLUSION

Marine microalgae primarily consist of pigments, proteins, lipids, and carbohydrates. Each of these chemicals has a varied composition in different microalgal species. Even rare, species-exclusive metabolites can be produced by some marine microalgae. Ma et al. (2020) state that this capacity for multi-compound coproduction is species-specific. This study examined total carbohydrate, pigment (Chlorophyll a, b, and c) content, and phosphate utilisation to determine the effects of phosphate concentrations of 0.5 (control: used to grow *S. platensis*), 0.75, 1.0, and 1.5 g/L on *A. platensis*. The phosphate utilisation happened at all the concentrations of the

experimental setup, indicating that *A. platensis* could uptake phosphate and participate in bioremediation. However, more research is necessary to support it.

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