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ARTICLE

The effects of nutrient stress on marine microalgae for enhancing the biodiesel production

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ABSTRACT

This study aimed to investigate the effect of nutrient stress such as nitrogen and phosphorus on the growth rate of green algae, *Chlorella* sp. and the mechanism secreted to survive during the depletion of nutrients. The sample was tested with several vital parameters, suppressed Total Nitrogen (TN) and Total Phosphorus (TP). A process known as acclimatization was carried out to ensure that the algae can adapt to a new environment which was repeated 2 times, each taking about 2 weeks. The active compounds in the samples were measured using Gas Chromatography-Mass Spectrophotometry. The experiments showed that the control microalgae secreted hexadecanoic acid and methyl stearate while nitrogen suppressed microalgae secreted Undecanoic acid, 10-methyl- and methyl ester. Phosphorus limited microalgae secreted 1,3-Propanediamine, N-(2-aminoethyl)- due to suppress of nutrients. This experiment should be tested in several study areas in Mexico and outside Mexico to compare the productivity of green algae and the main factors that contribute to the eutrophication problem.

1. Introduction

Chlorella sp. is a single-celled alga that grows in freshwater (Bhuyar et al., 2020a; Nithin et al., 2020). It exists over 2 billion years ago and has a well-defined nucleus that forms the first plant. *Chlorella* sp. is a microscopic organism; it was discovered only in the late 19th century. Derived its name from Greek, "chloros" means green and "ella" means small. *Chlorella* sp. also contains the highest amount of chlorophyll of any known plant. A *Chlorella* sp. has vitamin C and carotenoids, both antioxidants (Ramli et al., 2020a,b). Antioxidants are the compounds that prevent the action of the free radicals where the unstable molecules can damage the cells. It is also reported that *Chlorella* sp. has excellent iron and B-complex vitamins (Moorhead et al., 2006; Bhuyar, 2020b). The high chlorophyll content that is in the algae gives *Chlorella* sp. the green colour. Plants also need chlorophyll to undergo photosynthesis, where plants convert light (photon) into chemical energy (Bhuyar et al., 2020c; Khammee et al., 2020).

Because the photosynthetic efficiency of *Chlorella* sp., in theory, reaches about 8%, comparable with other highly efficient crops such as sugar cane, many people believed that it could act as a potential source of energy and food (Zelitch, 1971; Saengsawang

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et al., 2020; Ahmad et al., 2020). Pregnant women are less likely to get an iron deficiency, a common pregnancy problem when taking Chlorella as a supplement. However, there is no available scientific proof that Chlorella is effective in fighting cancer. Reduce research in cell cultures, and animals recommend that chlorella powder may inhibit the activity of molecules involved in the growth of cancer cells. These results have not been tested in humans, and further testing must be done to determine if these results hold for people and animals. Phycocyanins also synthesize food in these algae under the limited light conditions in which they usually live. The first evident products of photosynthesis are sugars and glycogen. They are converted into glycoproteins (Bhuyar et al., 2018; Bhuyar, 2017).

Algae are the natural food source of these animals, making them essential in aquaculture (Jones et al., 1987; Bhuyar et al., 2020d). These supplements are often taken to support healthy brain functions, detoxification, and building the immune system. Some microalgae are used to treat fibromyalgia, high blood pressure, fungal infections, cholesterol-related conditions, and certain types of cancer (Bhuyar et al., 2019a; Bhuyar et al., 2020d). The identification of nutrient limitation of cyanobacteria growth is essential to understand the aquatic ecology. Specifically, the water quality of freshwater systems is organized by the availability of nutrients limiting the aquatic primary productivity. Green algae cells usually undergo metabolic acclimatization due to nutrient stress, resulting in macromolecules' cellular composition (Bhuyar et al., 2019b; Bhuyar et al., 2020c). Nitrogen limitation frequently results in reduced protein content and relatively enhanced carbohydrate or lipid storage. Phosphate limitation also significantly impacted reducing the number of proteins, lipids and carbohydrates (Radjenovic et al., 2008). Therefore, the biochemical configuration of green algae is linked with the growth rate and reflects the physiological potential of the primary productivity (Bhuyar et al., 2019c). Society and science are usually more fascinated by the conditions of lakes rather than sampling sites.

Nutrients such as phosphorus, nitrogen, and silicate as limiting factors of microalgae are vital for eutrophication mitigation and management (Domingues, 2011). This research aims to expand essential issues associated with variability of data, investigate what compound excreted by cyanobacteria when the nutrient for growth is limit, and discuss the effect of nutrient stress on the productivity of cyanobacteria. Nutrient availability is always referred to as a factor regulating microalgae growth, biomass and species composition.

2. Materials and Methods

2.1. Experimental Preparation

The site selected for our study area was a dam nearby in Pedro Escobedo, Queretaro, Mexico (Latitude 20°29'36.7"N, Longitude 100°11'27.1"W) used for irrigation in the crop fields around the town. Therefore, collecting the samples and bringing them back to the laboratory for research was more accessible and affordable. Thus, it was believed that the sample is abundant in that area.

Furthermore, no research about green algae, *Chlorella* sp., has been done at the abovementioned site. Sediment samples were collected from the study area during low tide time using a boat and transferred to the laboratory to isolate and identify green algae by the following method. The green algae were collected by using 0.50µm holes size of plankton net. In brief, the collected green algae were put into the small plastic container and diluted twice with filtered seawater. From that, 1mL was transferred to an S-R Counting chamber. A single green algae cell was picked up from the Petri dish using a micropipette under an inverted microscope.

2.2. Isolation and identification

The collected samples were transferred into a small dissection box. Single-cell algae were picked by taking a 1mL sample and put onto a glass slide. It was observed under an inverted light microscope, Nikon Eclipse E100-LED, with a 40x objective lens. The standard manual by Desikachary (1959) was referred to observe the morphology of the sample. Sampling was ensured that the sample we taken is green algae needed for research.

2.3. Media preparation

10mL from stock solution 1, 80mL from stock solution 2 and 1mL from stock solution 3 was mixed into a 1L Schott bottle. The 40g of sea salt was weighed and mixed with water by using a magnetic stirrer. Then, the sea salt solution was filtered to remove any impurities that may contaminate the media. The 250mg/L of ampicillin was added to the media. Next, water was poured and top up until it reaches one litre.

2.4. Mass cultivation

All of the glassware was autoclaved, and any sample transfer was done in a laminar flow. The laminar flow was clean first with 70% alcohol and let for UV exposure for 5 minutes. The single-cell was transferred to a test tube containing 10mL of culture media for culturing, and sub-cultured species were transferred into a conical flask containing 100mL of BG II media. The culture was let to grow for 7 days. Then, from 100mL culture, it was transferred to 500mL of BGII media. The culture was let to grow for another 7 days. Then, one flask was labelled as control where the adequate nutrients such as nitrogen, phosphorus, carbon, vitamins, silicate and trace elements were added. The other flask is where we reduce the number of nutrients (N and P) stated. During 15 days of the experiment running, the biomass concentration (cell count, chlorophyll-a, pH and temperature) of green algae were measured every day. The response of spectral changes was observed and related to another nutrient stress-induced physiological change. Finally, we verified whether the indicators from laboratory-grown cells might benefit from understanding the nutrient level of phytoplankton cells taken from natural populations (Stehfest,

2005). The conical flasks of samples were put on a shaker to let them grow, and the nutrients and gases were distributed evenly.

2.5. Nutrient stress

Each fractionated *Chlorella* sp. assemblage was inoculated into a filtered seawater sample in a 70mL test tube and incubated for algal assay. Concentrated stock solutions of NH_4NO_3 and KH_2PO_4 were added for nitrogen (10µg at N 1-1) and phosphorus (1.0µg at P 1-1) for spikes, respectively. Both nitrogen and phosphorus were spiked to confirm the growth of *Chlorella* sp. No nutrient was added to the control. The incubation was carried out at 20ºC with fluorescent illumination of 2000 1x after nutrient spikes. The growth of *Chlorella* sp. was monitored by optical density (OD) using a spectrophotometer (Milton Roy Spectronic 21D: 660nm) (Lee, 1996). The pelleted sample that remained after chlorophyll extraction was homogenized in 50 ml distilled water and used to estimate protein employing Lowry's methods (Lowry et al., 1951).

2.6. Cell Count

The cell count of *Chlorella* sp. was measured by using a hemacytometer (Figure 1). One mL of sample was pipetted onto the hematocytometer and placed on the microscope. The respective *Chlorella* sp. was counted. Three times measurements are done.

2.7. Measurement of temperature and pH

A 50mL of *Chlorella* sp. was taken from the cultured conical flasks and transferred into 3 beakers of 200mL. They are labelled control, N and P, respectively. Using Eutech Instrument pH510, the temperature and pH probe measured each sample's temperature and pH (Figure 3). Three times replication was done.

2.8. Measurement of Chlorophyll a

The 8mL of sample control, N-limit and P-limit were centrifuged at 5000rpm in 5 minutes. The supernatant was separated, and this step was repeated several times to get a concentrated pellet. A sonicator was used to remove the pellet from the bottom of the tubes. A 1mL of 90% aqueous acetone was poured into the tubes. Next, the tubes were covered with aluminium foil and stored at 4ºC chiller in the dark for 2 hours. After 2 hours, the tubes were removed and centrifuged at 5000rpm for 5 minutes again. The supernatant was poured into a glass cuvette accordingly. Lastly, the samples absorbance or optical density was read at 663nm through Genesys 10s UV-Vis Spectrophotometer (Thermo Scientific) to determine the level of chlorophyll a (Figure 2).

2.9. GCMS

The last method used GCMS to detect the composition secreted by *Chlorella* sp. on the 15th day of the experiment. The three samples were centrifuged. The supernatant was removed. A sufficient amount of methanol was poured into the tubes. Next, the pellet mixed with methanol was left for 24 hrs in the chiller at 4ºC. The samples were centrifuged again, and the supernatant was taken. The supernatant was poured into the GCMS glass tube to be analyzed. Separation and identification of active compounds secreted in the microalgae are carried out. They were analyzed in Agilent 7890A Gas Chromatography (GC) system equipped with a DB-1MS (30m×0.25mm ID, 0.25µm film thickness, Agilent 122-0132) and Agilent 5975C inert Mass Spectrometer Detector (MSD) with Triple-Axis Detection using helium as carrier gas at 1.0ml/min. Samples were injected at 1µL volume at these conditions: the column temperature was 160°C during the first 4 minutes, then the thermal gradient went up to 190°C at a rate of 3°C min-1, and maintained for 5 minutes, then the thermal gradient went up to 200°C at the rate of 2°C min-1, and was maintained for 2 minutes, finally, the thermal gradient went up to 230°C at a rate of 5°C min-1 and was maintained for 2 minutes. Injector and flame ionization detector temperatures were 250°C and 280°C, respectively. The solvent was then hexane with an internal standard methyl nonadecanoate (C19) concentration of 1mg/ml.

4. Results and Discussion

4.1. Cell growth measurement

Based on Figure 1, all three samples started at different cell counts/mL. Control started with the lowest at 35 cells/mL, Total Nitrogen (TN) sample with the highest at 62 cells/mL and Total Phosphorus (TP) at 52 cells/mL. Next, all of the samples increasing slightly from day 1 until day 9. During that period, on the 5th day, TP raised to 74 cells/mL. The 11th day showed a very productive or optimum day because all of the samples were increasing significantly. The control sample increased at 90/mL, TN increased at the highest at 151 cells/mL, and TP at 130 cells/mL. The TN sample still has the highest cell count than the control sample and TP sample. On the 12th day, the control sample increased a little bit. But TN and TP samples lowering their cell count. The readings of all three samples keep decreasing until the last day, the 15th day. The control sample has the lowest energy from the beginning until the end compared to TN and TP samples.

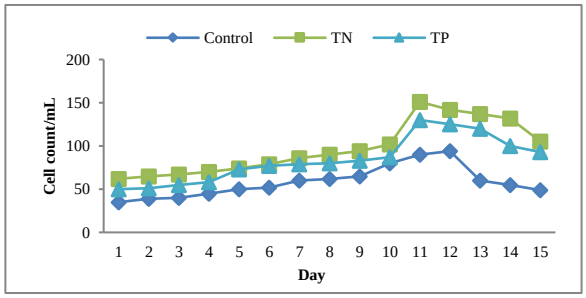

Fig. 1 Cell count of *Chlorella* sp. for control, TN, and TP for 15 days.

4.2. Chlorophyll a content

In the beginning, the chlorophyll-a content in the control sample is the lowest, at 0.178A. The TN and TP are slightly higher at 0.213A and 0.217A. The TP sample increases rapidly on 2nd day. All three samples were increasing until day 5, but from day 6 to 7, only a slight change can be seen. Next, they keep increasing until day 13, where the TP has the peak value at 0.425A and TN at 0.374A. But the control sample is reduced on that day. The control

sample reached its death and decreasing until 0.212A on day 15th. Meanwhile, the TP sample is done at a high value on the 15th day, 0.390A and TN at 0.312A. It showed that the highest value of chlorophyll could be measured on the 13th day of growth, as shown in Figure 2.

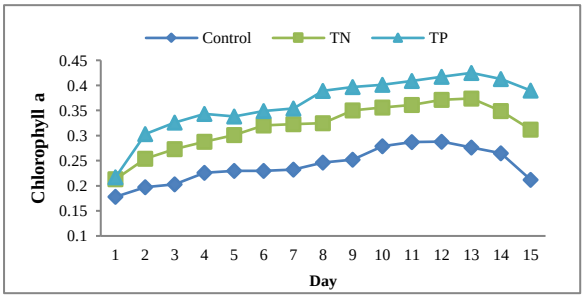

Fig. 2 Chlorophyll content of *Chlorella* sp. at absorbance (663nm) for control, TN and TP for 15 days.

4.3. pH measurement

The pH value for control, TN and TP started at averagely from pH 8.0 to pH 8.5, slightly alkaline. The control sample decreased drastically on 2nd day around pH 6.0. TP sample also decreases and have a fluctuation at day 3. It decreased again on the 4th day. From day 5 until 10, the TP sample pH value is considered stable and fluctuated between pH 7.79 to pH 8.07, and has the highest alkaline value on day 11, which is pH 8.34. Next, its pH value was reduced on days 12 and 13. Day 13 had the lowest pH value for TP and reaching neutral at pH 7.32. TN sample also having fluctuation from day 2 to day 8. From day 8 until 11, only a small increase of pH can be measured due to the adaptation of samples throughout the experiment. The pH value of TN is constant from day 12 until day 15. The control sample has the 2nd highest value on day 8, which is pH 6.73. But keep decreasing and reach its plateau from day 13 until day 15, like TN and TP samples. Based on Figure 3, the pH values were varied between pH 6.22 to pH 8.37. According to Abdel_raouf et al. (2012), pH varied from 1 to 9 depends on the strain being cultured, but the majority in the range of 6 to 8. Some of the disadvantages of long-term microalgae maintenance are the risk of contamination and characteristic change due to mislabeling or loss of cultures. Any severe problems have been avoided by replicating samples and a backup system for the growth flasks so that there is at least 1 viable culture in every 3 samples.

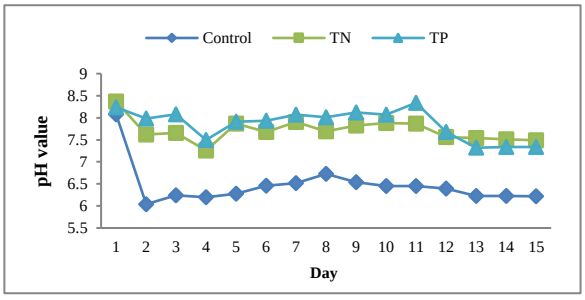

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Fig. 3 pH value of *Chlorella* sp. for control, TN and TP for 15 days.

4.4. Temperature

Based on Figure 4, it looked like a U shaped. All of the three samples start at a high temperature, on averagely, 29ºC on day 1. The control kept decreasing until day 7 at the lowest value, 26.4ºC. TN and TP samples increasing from day 1 until day 3 at 29.3ºC and 29.0ºC, respectively. Both temperatures of the samples also reduced until day 9 for TN and day 7 for TP. From day 7 to 10, all three samples showed unstably, and they are at their lowest temperature during the experiment's period. The rapid increase for the control sample is at day 12 from 27.0ºC to 28.4ºC. The rapid increase for TP sample also at day 12 from 27.4ºC to 29.2ºC. TN samples increase from 27.2 ºC to 29.1 ºC from day 11 to day 12. Control and TP samples are increasing a little after day 12. However, the TN sample decreased to 29.0ºC on day 14 and increased back at day 15.

All operations were conducted in the biohazard safety cabinet to prevent contamination. The microalgae were aerated with filtered air and cultivated at the conditions specified in the factorial design for 15 days, consisting of cycles of 24 hours of light. The strategies in producing cultures of Chlorella sp. need much patience due to knowledge of physiology, ecology and taxonomy of the algae and suitable techniques.

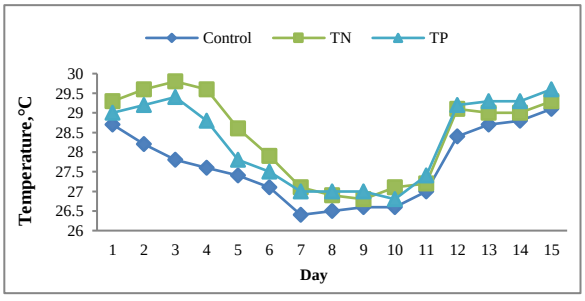

Fig. 4 Temperature, ºC of *Chlorella* sp. for control, TN and TP for 15 days.

4.5. GCMS analysis

Many oceans deplete nutritionally in nitrogen, and marine algae can withstand and adapt their metabolism to environmental stress. They may have mechanisms that block interaction with stress factors or counteract the stress-induced damages (Saha, 2003). Many photosynthetic microorganisms rely on ammonium and nitrate as their only source of energy. The change between initial and final chlorophyll-a concentration is needed to measure the nutrient enrichment effect. Additions of phosphorus are involved in the highest increase in chlorophyll a. Nitrogen starvation usually lowers protein content and enhances carbohydrate or lipid storage, plus phosphorus can also change the amount of protein, lipid and carbohydrate (Bhuyar et al., 2020c). The experiments showed that the control microalgae secreted

hexadecanoic acid and methyl stearate (fatty acids) while nitrogen suppresses microalgae secreted Undecanoic acid, 10-methyl-, methyl ester, and phosphorus limited secreted 1,3-Propanediamine, N-(2-aminoethyl)- due to suppress of nutrient.

Figures 4, 5 and 6 are representing the GCMS analysis. Hexadecanoic acid is palmitic acid or fatty acid. Essential fatty acids or good fats are needed because fatty acids support the cardiovascular, reproductive, immune, and nervous systems. The human body needs the essential fatty acids to manufacture and repair cell membranes, enabling the cells to obtain optimum nutrition and expel harmful waste products (Bajpai et al., 2009). The primary objective of good fats is the production of prostaglandins, which regulate body functions such as heart rate, blood pressure, blood clotting, fertility, conception and play a role in immune function by regulating inflammation and encouraging the body to fight infection. Essential Fatty Acids are also needed for proper growth in children, particularly for neural development and maturation of sensory systems, with male children having higher needs than females. Fatty Acids are aliphatic carboxylic acid with varying hydrocarbon lengths at one end of the chain joined to the terminal carboxyl (-COOH) group at the other end. The general formula is R-(CH_2)n-COOH.

Fatty acids are predominantly unbranched, and those with even numbers of carbon atoms between 12 and 22 carbons long react with glycerol to form lipids (fat-soluble components of living cells) in plants, animals, and microorganisms. Fatty acids all have familiar names, respectively link lauric (C12), myristic (C14), palmitic (C16), stearic (C18), oleic (C18, unsaturated), and linoleic (C18, polyunsaturated) acids. The saturated fatty acids have no double bonds, while oleic acid is unsaturated with one double bond (also described as olefinic). Polyunsaturated fatty acids like linolenic acid contain polyunsaturated fatty acids with two or more double bonds. Eutrophication is a complex problem with severe effects on the state and health of the ecosystem. Therefore, nutrient over-enrichment had increased since the 20th century, and the problem awareness had ranged from marine science to some research, management and the whole human society (Lundberg 2013; Malek et al., 2020).

Since the last few decades, discharges of nutrients had caused many changes in the algal community along the European continental coast. The food web also changes in areas that cause various phenomena such as oxygen depletion, mortality of some organisms, foams from beaches, high productivity of benthic communities, and some commercial fish species. The existence of Phaeocystis summer blooms concurred with a modification from P-limitation towards N-limitation in the Dutch coastal area because of a more substantial increase in P-discharge comparative to the increase in N-discharge. This microzooplankton species mainly nourish cyanobacteria, prochlorophytes and some nano-algal species. Based on food web building and the carbon fluxes in marine food webs, eutrophication leads to the control of poorly edible algal species. Eutrophication helps the descending transport of carbon and nutrients towards the sediments because of higher algal biomasses and concern of a shift towards larger algal species with higher sedimentation characteristics (Riegman, 1995).

Coastal eutrophication from the early 1970s became the primary danger to the marine ecosystem of the Archipelago Sea (the Åland Islands and the SW Finnish archipelago) in the northern Baltic Sea. Nutrient levels (N, P) have risen both in coastal areas and basin-wide, which has directed to increased primary production (both pelagic and benthic), decreased transparency and increasing amounts of oxygen-consuming. Prospects for the archipelago and coastal ecosystem are unfortunate, except local and regional measures to radically lessen nutrient levels of the archipelago are commenced. Even then, positive effects are unlikely to show immediately (Bonsdorff, 1997).

All sewage inputs into the lake ended before 1968. After the sewage diversion, total phosphorus decreased significantly, being 0.07 mg/L, 0.03 mg/L and 0.02 mg/L in 1963, 1968 and 1979, respectively. There was less disparity of nitrogen. Nitrate concentrations were 0.44 mg/L, 0.37 mg/L and 0.30 mg/L respectively. Therefore, phosphorus reduction is the main reason to explain the recovery of Lake Washington from eutrophication (Wang and Wang, 2009).

Soil erosion is caused due to natural processes and human activities. For example, forestry and construction. Agriculture has the most significant risk for soil erosion and the highest potential for reducing P loads related to erosion. Water protection measures must be introduced to prevent soil detachment and transport from cultivated fields to reduce eutrophication. Farmers globally are advised to favour reduced tillage, contour cropping, and cultivation of cover crops, establish buffer strips and riparian zones, and construct settling ponds and wetlands, partly at least to reduce P losses.

Aquatic microbiology showed that P cycling is related to the availability of common electron acceptors, Fe oxides and SO_4, by the anaerobic mineralization reactions in sediments. More research is needed on aquatic P cycling concerning the processes occurring in the water phase (desorption) and those taking place after the soil particles have settled on the sediment surface (Ekholm and Lehtoranta, 2012). Bio-treatment with microalgae is mainly great due to photosynthetic capabilities, converting solar energy into valuable biomasses and incorporating nutrients such as nitrogen and phosphorus, causing eutrophication (Abdel-Raouf et al., 2012).

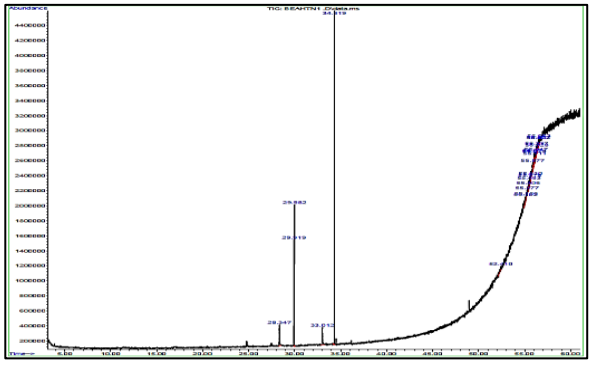

Fig. 5 Gas Chromatography-Mass Spectrometry of a control sample of microalgae.

Nitrogen is a dire nutrient needed in the growth of all organisms. Organic nitrogen can be found in biological substances, such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP), and genetic materials (RNA, DNA). Organic nitrogen derived from inorganic sources includes nitrate

(NO_3^-), nitrite (NO_2^-), nitric acid (HNO_3), ammonium (NH_4^+), ammonia (NH3), and nitrogen gas (N_2). Microalgae play a crucial role in converting inorganic nitrogen to its organic form through a process called assimilation. Plus, cyanobacteria are proficient in converting atmospheric nitrogen into ammonia by fixation. Assimilation, performed by all eukaryotic algae, requires inorganic nitrogen to be only in the forms of nitrate, nitrite, and ammonium. Phosphorus is also essential in the energy metabolism of microalgae and is found in nucleic acids, lipids, proteins, and the intermediates of carbohydrate metabolism. Inorganic phosphates are significant algae cell growth and metabolism. Similar to the removal of nitrogen, phosphorus removal in wastewater is administered by the uptake into the cell and by external conditions such as pH and dissolved oxygen. Phosphorus cannot exist in a gaseous state. Thus, phosphate precipitates from the medium because of elevated pH and high dissolved oxygen concentration.

Table 1 Active compounds in a control sample of *Chlorella* sp.

Retention Time (min)	Compound	Percentage (%)
28.351	Hexadecanoic acid, methyl ester	47.15
33.017	Methyl stearate	42.56
48.184	No matches found	10.29

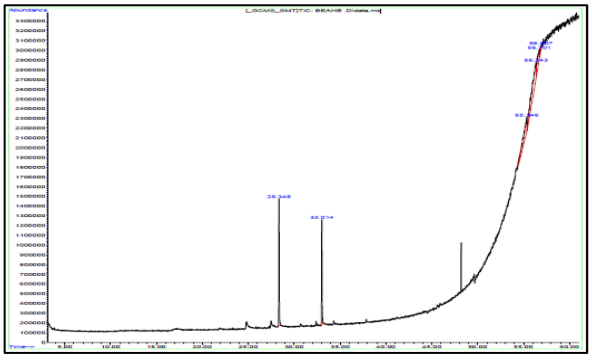

Fig. 6 Gas Chromatography-Mass Spectrometry of nitrogen stress *Chlorella* sp.

Table 2 Active compounds in nitrogen stress of *Chlorella* sp.

Retention Time (min)	Compound	Percentage (%)
28.351	Hexadecenoic acid, methyl ester	7.48
29.914	No matches found	6.46
29.977	No matches found	10.50
33.007	Methyl stearate	6.67
34.325	3-Methoxyamphetamine	25.36
52.415	Thymol	2.33

Table 3 Active compounds in phosphorus stress *Chlorella* sp.

Retention Time (min)	Compound	Percentage (%)
3.873	Tetratriacontane	3.44
24.780	No matches found	6.25
27.501	No matches found	4.62
28.351	Hexadecanoic acid, methyl ester	42.20
33.007	Heptadecanoic acid, methyl stearate	38.57
45.591	No matches found	4.91

Although nitrogen and phosphorous are the two core nutrients of concern in eutrophication, being limiting factors in most growth scenarios, other micronutrients, including silicon and iron, can affect the wealth of phytoplankton communities. But many of the micronutrients are toxic to most algae species at high concentrations. Some of them also form precipitates with other essential elements and lessen their availability. However, some algae strains are tolerant to heavy metals and their potential to absorb metals has been demonstrated (Cai et al., 2013; Sundararaju et al., 2020).

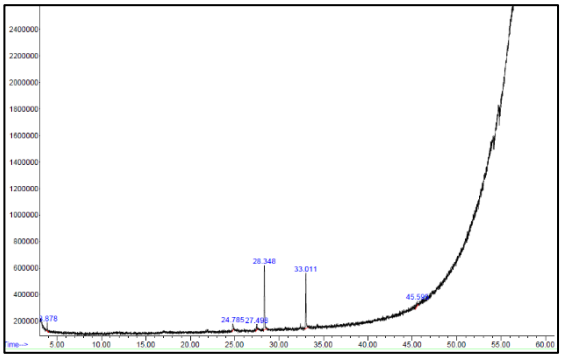

Fig. 7 Gas Chromatography-Mass Spectrometry of phosphorus stress *Chlorella* sp.

The active compounds visualized in GC analysis are displayed in Tables 1 to 3. Physiological parameters used as nutrient status depend on cell function changes during nutrient starvation or perturbations in energy metabolism, which occurred during a limiting nutrient is resupplied to cells. N deprivation usually affects the chlorophyll-a more than carotenoids, enhancing the carotenoid to chlorophyll ratios feature of N-stressed cells. Under stressful conditions, microalgae slow down their cell growth and remobilize carbon to produce energy storage products such as lipid or starch to acclimate change in nutrient existence. The environment can affect the cellular chemical composition of phytoplankton, and nutrient stress causes a reduction in intracellular nutrients. As they are vital elements for cell composition, nitrogen and phosphorus limitation likely affect normal cell functions (Bhuyar et al., 2019a).

There are some methodological problems and challenges when interpreting assay of nutrient limitation. For example, extrapolating from lab scale to understand nutrient limitations on a larger scale. Plus, we need to address the nutrient patchiness and heterogeneity in microalgae species composition and nutrients within a single sample. Flow cytometry can examine individual cells, but only a limited number of volumes can be investigated.

4. Conclusion

Many factors affect algal growth: abiotic factors, for example, light (quality, quantity), temperature, nutrient concentration, dissolved O_2 concentration, dissolved CO_2 concentration, pH, salinity, and toxic chemicals. Biotic factors such as pathogens (bacteria, fungi, viruses) and competition by other algae; operational factors such as shear produced by mixing, dilution rate,

depth, harvest frequency, and addition of bicarbonate (Mata et al., 2010). It is proved that the Kuantan study area has marine green algae of *Chlorella* sp. The nutrient stress study is significant in identifying the problem of eutrophication due to algal bloom. In addition, different ponds or oceans have different chemical compositions in the body of the same species of marine algae. The nitrogen limitation somehow secretes fatty acids and change the chemical composition in its body. In this research, the variables nitrogen and phosphorus concentrations were investigated in flask cultures of microalgae, and the optimum levels of these variables were determined. Although several decades had passed assisting in experimenting with nutrient limitation in phytoplankton, there are still problems common to the methods discussed in this thesis. However, each method is characterized by specific advantages and limitations. Therefore, the best solution is not to depend only on one technique but to employ appropriate steps and parameters to detect nutrient limitation in phytoplankton. It could significantly strengthen results interpretation. The experiment precautions must be followed. During transferring the culture, it must be done in a laminar flow to avoid contamination. When measuring the NH_4NO_3 and KH_2PO_4, it is essential to be careful and slowly assist the weighing balance. This is because a slight change of mass can contribute to the alteration of the whole experiment.

Next, all the glassware must be washed and autoclaved before using them to prevent contamination due to impurities. The practical approach to increasing microalgae content is to obtain genetically modified species through genetic engineering, which is to produce a high-yielding transgenic microalgae strain with a selective advantage that would allow it to grow in highly selective environments so that it can be grown in open culture systems while remaining relatively free of contamination by other algae and bacteria. To continue improving the existing method, identifying new beneficial assessing nutrient deprivation of phytoplankton growth needs input from physiologists, limnologists, and oceanographers alike. The biosynthetic response of microalgae thus proved that nutrient limitation could cause lipid accumulation in algae communities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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