



# EFFECT OF NITRATE, SILICATE, AND, PHOSPHATE ON LIPID CONTENT UNDER STARVATIONS FOR IDENTIFICATION OF FATTY ACIDS OF TWO MARINE PLANKTONIC DIATOM SPECIES FOR BIODIESEL PRODUCTION BY USING GC-MS

<sup>1</sup>Rajesh Gandhi. Gunti, <sup>2</sup>Paramageetham.Chinthala, <sup>3</sup>Prasada Babu. Gundala, <sup>4</sup>Udhaya Nandhini.M,  
<sup>1</sup>Research scholar, <sup>2</sup>Professor, <sup>3</sup>Postdoc, <sup>4</sup>Student  
Department of Microbiology,  
Sri Venkateswara University, Tirupathi.  
E-mail: gandhi\_fsl@yahoo.co.in

**Abstract:** An everlasting consumption of fossil fuels and the effect of greenhouse-gas emissions on global climate change, global warming, and exhaustion of fossil fuels are major environmental and economic issues the world is facing today. In order to offset the massive influx of CO<sub>2</sub> into the atmosphere, the utilization of renewable biofuels (e.g., ethanol, butanol, H<sub>2</sub>, CH<sub>4</sub>, and biodiesel) is needed so that research has begun to focus on alternative biomass-derived fuels. Bacillariophyceae (diatoms) are eukaryotic photoautotroph's that can utilize inorganic carbon (e.g.CO<sub>2</sub>) as a carbon source and sunlight as an energy source can store carbon and energy in the form of neutral lipids [e.g., triacylglycerides (TAGs)]. It has an ecologically significant role in nitrate, phosphate, and silicate biogeochemical cycling under starvations. *Amphora Coffeiformis* and *Thalaseosera Pseudonana* commonly contain fatty acids ranging from C<sub>12</sub> to C<sub>24</sub> often with C<sub>16</sub> and C<sub>18</sub> majorly 1-Docosene, Hexadecanoic acid, methyl ester, and the total lipid obtained from the above diatoms was converted into biodiesel through transesterification and its fatty acid composition was analyzed with GC-MS.

**Index Terms-** Diatom lipids, Biodiesel, Nutrient starvation, *Amphora Coffeaeformis*, and *Thalassiosera Pseudonana*, GC-MS.

## INTRODUCTION:

Diatoms play a prominent role in the production of biofuels, in current research characteristically accumulates valuable storage lipids throughout their entire life which can further contribute to reduced CO<sub>2</sub> percentage in the atmosphere and hydrocarbon emissions there through the utilization of atmospheric CO<sub>2</sub>, and a large number of studies have shown that biodiesel is environment-friendly and therefore are appeal choices once to be used after stock for commercial proud products biodiesel. For above all these reasons,

eukaryotic photoautotrophs especially diatoms have been studied in the context of lipid accumulation. Can grow rapidly, and synthesize and accumulate more amounts of neutral lipid (20-50% of dry weight), mainly in the form of triacylglycerol, (TAG) and which were stored in cytosolic lipid bodies [1] [2].

Some species of diatoms such as *Amphora coffeaeformis* and *Thalassiosera pseudonana* are been considered to be an ideal source of neutral lipids suitable for biodiesel production. Biodiesel is one of the most promising renewable fuels that have achieved remarkable success worldwide according to the World Bank report (2008) [3]. Oil-rich microalgae have been demonstrated to be a promising alternative source of lipids for biodiesel production [4] [5] [6] [7] [8]. It has better properties than that petroleum diesel because biodiesel is renewable, biodegradable, non-toxic essentially free of sulfur and aromatic compounds.

Recent investigation has indicated that the use of biodiesel can reduce 90% pollution [9]. Microalgae lipids are mostly neutral lipids due to their lower degree of unsaturation and their accumulation in the microalgae at the early or late end of the growth phase. This makes a microalga potential diesel fuel substitute [10] [11]. Diatom lipids are classified according to the number of carbon atoms in the chain and the number of double bonds, e.g. mono saturated (one double bond) or polyunsaturated (many double bonds) and saturated (no, double bonds). It contains large amount contains eicosapentaenoic acid, cycloecosane, etc. [12].

In this present study marine diatoms, *Amphora coffeaeformis* and *Thalassiosera pseudonana* are isolated from the Visakhapatnam coast in Andhra Pradesh, were chosen, and experiments conducted to enhance lipids by using f/2 media [13] containing with and without nitrate, silicate, and phosphate, and lipids can be extracted through blight and dyer method [14] and converted into crude oil through transesterification method and resulted biodiesel can estimated through GC-MS for different fatty acids in the laboratory conditions.

## 2. RESEARCH METHODOLOGY

### 2.1. Isolation and growth condition of Diatoms

The samples are collected from Visakhapatnam costal area in Andhra Pradesh, India, using a phytoplankton net of 25µm. The samples contained diatoms were transferred to f/2 medium and kept at 24±1°C in a thermostatically controlled room, illuminated with cool white florescence lamps at an intensity 2000-2500lux with 16:8 hours light: dark period. After 5 days the samples were serially diluted up to 1:10 and 0.1mL spread on 2% f/2 agar medium. The Cyanobacterial contaminants were eliminated by treating them with 3000 ppm of the antibiotic, Streptomycin sulphate for 30 min under 30µEm -2s -1 light intensity and transferred to antibiotic free media containing the cultures of diatoms *Amphiprora Coffeaeformis* and *Thalassiosera Pseudonana* was isolated and identified based on its morphological characters. The composition of f/2 medium was as follows: NaNO<sub>3</sub> –75 mg/L, NaH<sub>2</sub>PO<sub>4</sub> – 5 mg/L, Na<sub>2</sub>SiO<sub>3</sub> – 30 mg/L, FeCl<sub>3</sub>.6H<sub>2</sub>O – 3.15 mg/L, Na<sub>2</sub>EDTA.2H<sub>2</sub>O – 4.36 mg/L, CuSO<sub>4</sub> - 0.0098 mg/L, Na<sub>2</sub>MoO<sub>4</sub> – 0.0063 mg/L, ZnSO<sub>4</sub>.7H<sub>2</sub>O – 0.022 mg/L, CoCl<sub>2</sub>.6H<sub>2</sub>O – 0.010 mg/L, MnCl<sub>2</sub>.4H<sub>2</sub>O – 0.180 mg/L and trace amounts of VitaminB<sub>12</sub>, Biotin, Thiamine HCl and 1000 ml filtered Sea water. The diatom cultures were identified morphologically with light microscope and purified by spread plate method. The individual colonies were isolated and inoculated in to liquid medium f/2medium and incubated at 24±2°C under 2000-2500lux light intensity with 16:8 hours light: dark period. The purity of the cultures was monitored by regular microscopic observation.

### 2.2. Measurement of Growth Rate

The experiments were carried out in Erlenmeyer flasks of 500ml capacity containing 200ml f/2 medium for a period of twenty to thirty days. At every three days of interval dry biomass and total lipid [15] were estimated and recorded, using f/2 media having with and without nitrate, silicate, and phosphate, culture flasks were inoculated and incubated at 24±1°C under 2000-2500lux intensity with 16:8 hours light and dark cycle. The growth rate of isolated diatoms was measured by optical density of an aliquot of the culture at 750nm using Evolution 201 UV-visible Spectrophotometer and Growth was calculated as doublings per day by using the following formulae [16]

$$\text{Doubling/Day} = \text{LogOD2} - \text{LogOD1} / \text{T2} - \text{T1 (h)} \times 34.632$$

Where OD = Optical density; T = Time; h = Hour.

### 2.3. Dry biomass

After the incubation period, diatom cultures were settled in bottom of the flask cultures were harvested by centrifugation at 5000 rpm for 10 minutes. The pellet was washed three times with ammonium formate 0.65M [17] in order to remove the salt content and Once with sterile distilled water then it was transferred to pre dried GF/C glass fiber filter paper and keep at 60°C for overnight in a hot air oven and cooled in a desiccators before weighing dry biomass.

### 2.4. Stress Induced by using without nitrate, without silicate and without phosphate Starvations

The cultures were harvested by centrifugation at 5000rpm for 15 minutes during their early stationary phase and washed with sterile distilled water. The experiment was done in three different flasks. First harvested cells were measured and inoculated in sterilized nitrate free f/2 medium. Secondly, the harvested cells were measured and inoculated in sterilized silicate free f/2 medium, finally, the harvested cells were measured and inoculated in sterilized phosphate free f/2 medium and some pure diatom cultures were inoculated in nutrient sufficient f/2 medium as control. All the flasks were incubated for 21 to 30 days at 24±2 °C under 2000-2500lux light intensity with 16:8hours light and dark cycle.

### 2.5. Extraction and purification of total lipids

Lipid extractions were performed to all the experiments at their stationary phases and were extracted by the modified procedure as described by Bligh and Dyer (1959). The cells were homogenized with chloroform: methanol: water in the ratio of (2:1:0.8, v/v/v) for 2 min. An equal volume of chloroform and distilled water was then added, to bring the final ratio of the mixture to 2:2:1.6 v/v and the mixture were then homogenized for 1 min. The chloroform layer containing lipid fraction was separated, and the alcoholic layer, contained the residues, were re-extracted twice with methanol and chloroform (1:2, v/v). The chloroform layers were pooled and subjected to a “folch wash” to remove all non-lipid contaminants [18]. The mixture was washed with one-fourth of the volume of 0.88% (wt/v) potassium chloride, followed by methanol/saline solution (1:1, v/v). The purified chloroform extract was then evaporated under room temperature and total lipid content was estimated, lipids are converted in to biodiesel through transesterification (FAMES), then the biodiesel was further determined using gas chromatography and mass spectroscopy for different fatty acids (GC 2014, Shimadzu, Japan)

## 3. RESULTS AND DISCUSSION:

### 3.1. Micro algal Growth Rate (Biomass and lipid production)

The diatoms grown at with and without sodium nitrate, sodium silicate and sodium phosphate, shows a typical growth curve i.e. day 0 to day 3 lag phase, day 6 to day 15 exponential phase and after day 15th stationary then decline phase the isolated diatoms were identified as *Amphora Coffeformis*, and *Thalassiosera Pseudonana*, growth of *Amphora Coffeformis* was greater when compared to that of *Thalassiosera Pseudonana* in f/2 medium. *Amphora Coffeformis* grew exponential Phase with first 14 days and by day 22<sup>nd</sup>, growths were reached stationary phase then the growth phase declines, whereas in *Thalassiosera Pseudonana* reached exponential phase by 19<sup>th</sup> day and by day 29 growths and reached stationary phase declines. In our studies, the growth rate of *Amphora Coffeformis* and *Thalassiosera Pseudonana* were greatly reduced but growth did not completely ceased in nitrate, silicate, and phosphate starved cultures.

**3.2. Nitrate starvation:** Nitrate limitation was reported to significantly influence micro algal lipid storage that to in positive aspect by increasing the total lipid content, nitrate deficiency leads to an increase in production of triacylglycerols in diatoms [19-20]. Under nitrate deficiency conditions, the protein

biosynthesis has been ceased, due to the cell division has been stopped which results in cessation of micro algal growth and animates a metabolic pathway favorable to the accumulation of reserve lipids [21] so that it indicates that the media containing nitrate shows more growth and less lipids and media containing without nitrate shows less growth and more lipid, due to cessation of diatom growth.

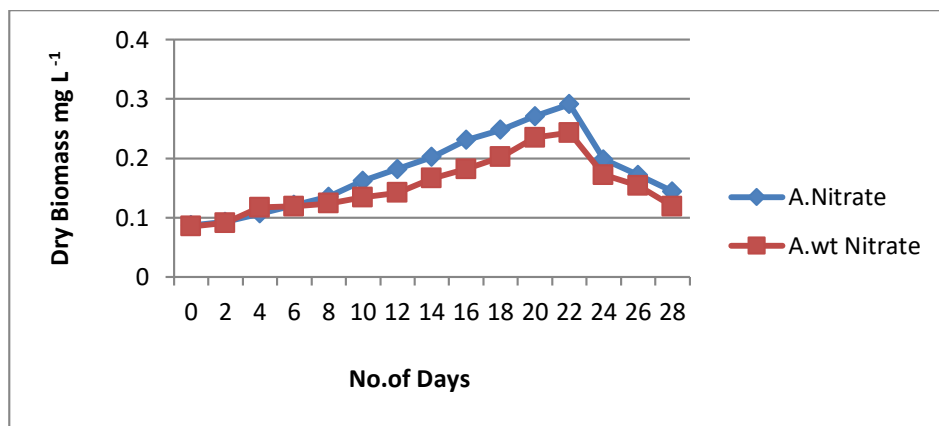


Figure 1: Effect of with nitrate and without nitrate on biomass production of *Amphora coffeaeformis*

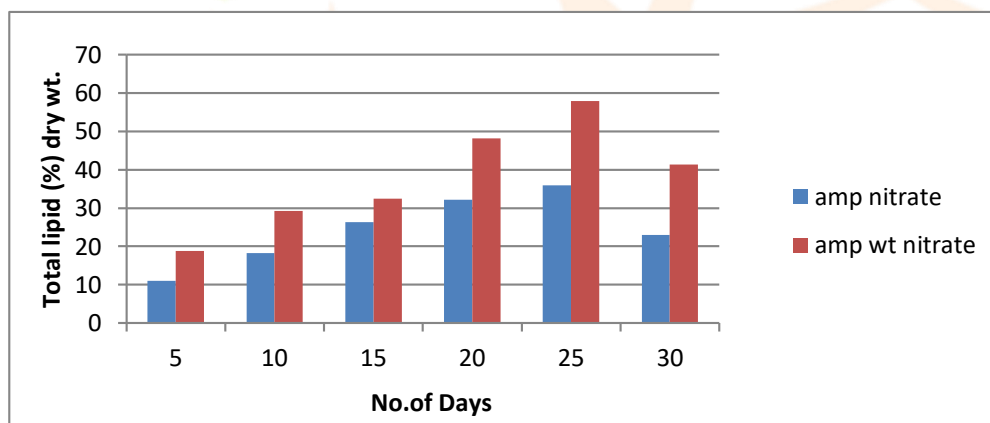


Figure 2: Effect of with nitrate and without nitrate on total lipid production of *Amphora coffeaeformis*.

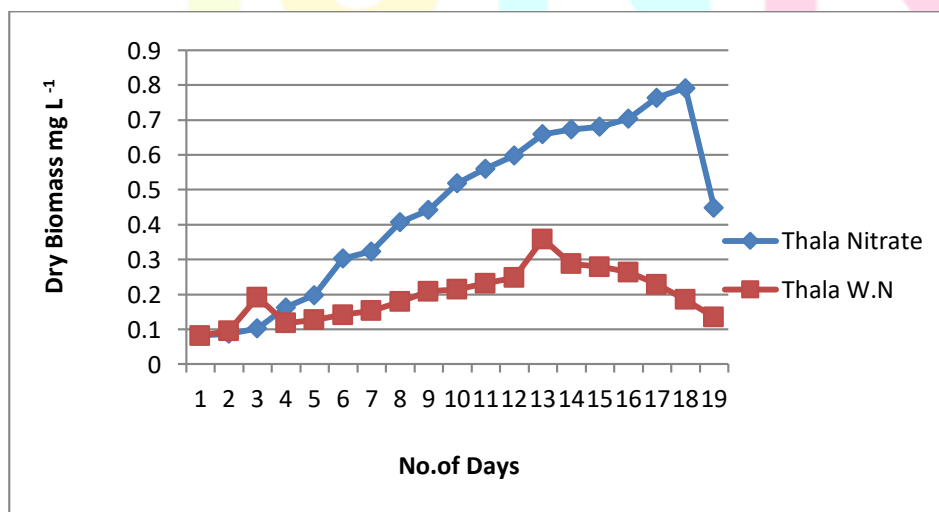


Figure 3: Effect of with nitrate and without nitrate on biomass of *Thalassiosera pseudonana*

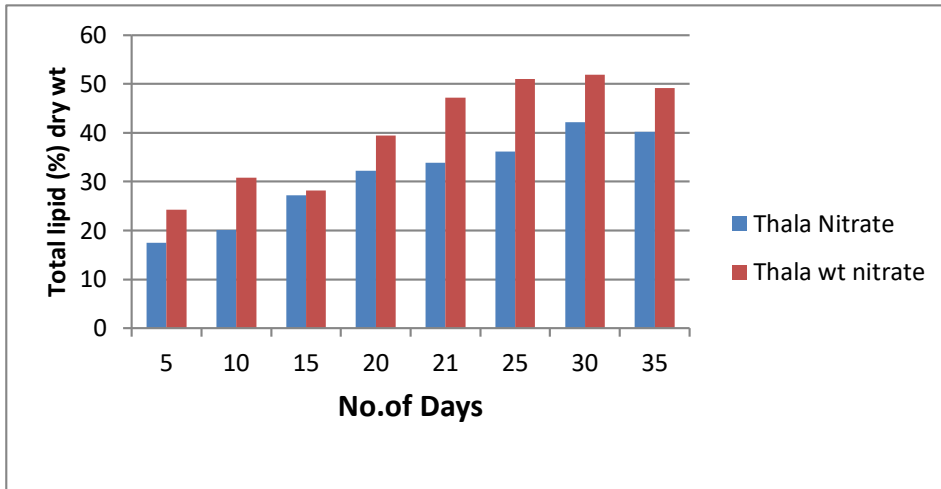


Figure 4: Effect of with nitrate and without nitrate on total lipid production of *Thalassiosera pseudonana*

**3.3 Silicate starvation:** silica are also known to be strongly related to growth and cell metabolism in number of microalgae especially in diatoms as it is the major component of diatom cell walls, culture containing sodium silicate shows more growth than the culture containing without sodium silicate similar to nitrate starvation due to lipid trigger effect produced by silica deficiency, silica depletion also results in a decrease in cell growth and often is accompanied by an accumulation of lipid within the cells of all the nutrients evaluated, silica limitation are the most critical nutrient which affect lipid metabolism in diatoms thus increase in the total lipid content, lower concentrations of sodium silicate induced high lipid content.

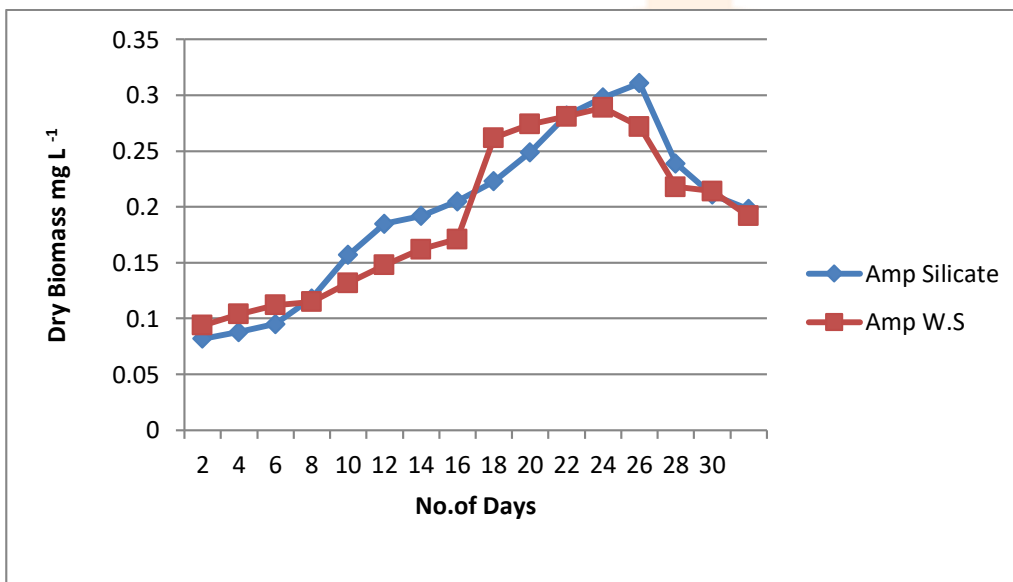


Figure 5: Effect of with silicate and without silicate on biomass Production of *Amphora coffeiformis*

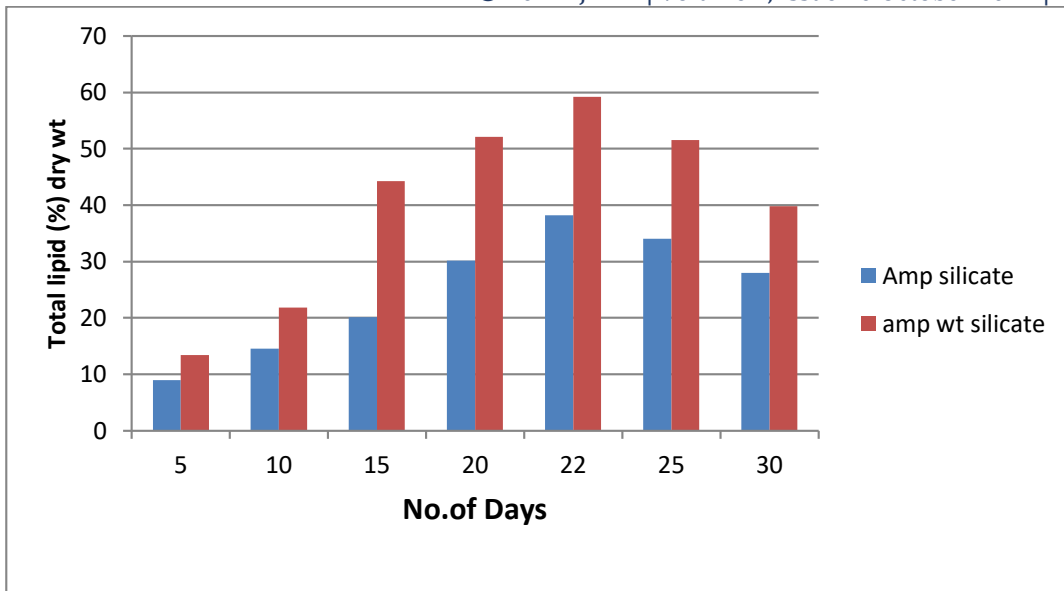


Figure 6: Effect of with silicate and without silicate on lipid production of *Amphora coffeaformis*

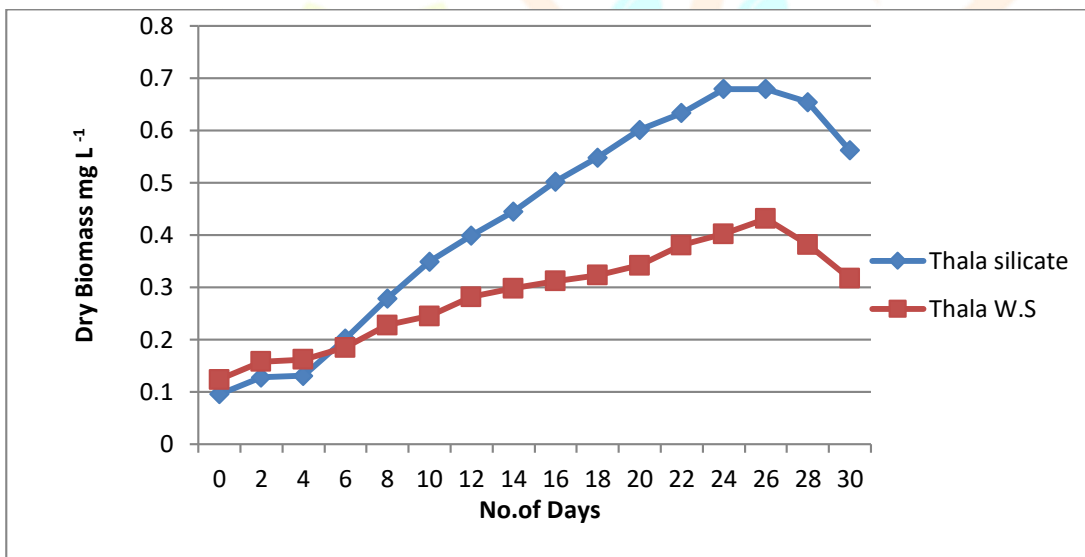


Figure 7: Effect of with silica and without silica on biomass production of *Thalassiosera pseudonana*

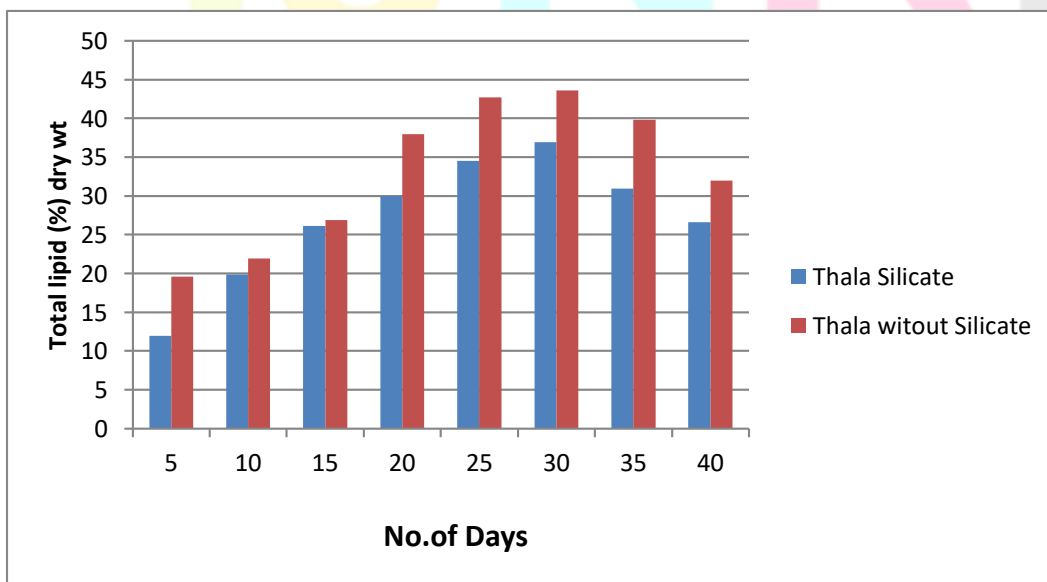


Figure 8: Effect of with silica and without silica on lipid Production of *Thalassiosera pseudonana*

**3.4 Phosphate starvation:** Like same as nitrate and silicate starvation it also shows decreasing the cell biomass and increasing the accumulation of reserve lipids in starvations. The diatom cultures had accumulate high lipids due to the deficiency of phosphate have been reported as being able to cause the cessation of cell growth, and channel metabolic flux generated in photo biosynthesis to lipid biosynthesis.

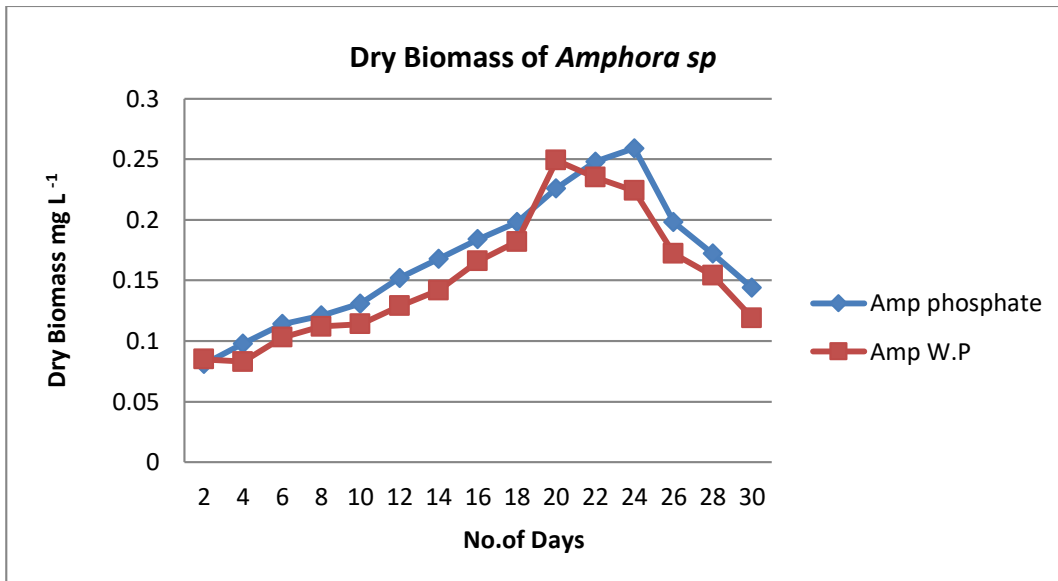


Figure 9: Effect of with phosphate and without phosphate on biomass production at *Amphora coffeaeformis*

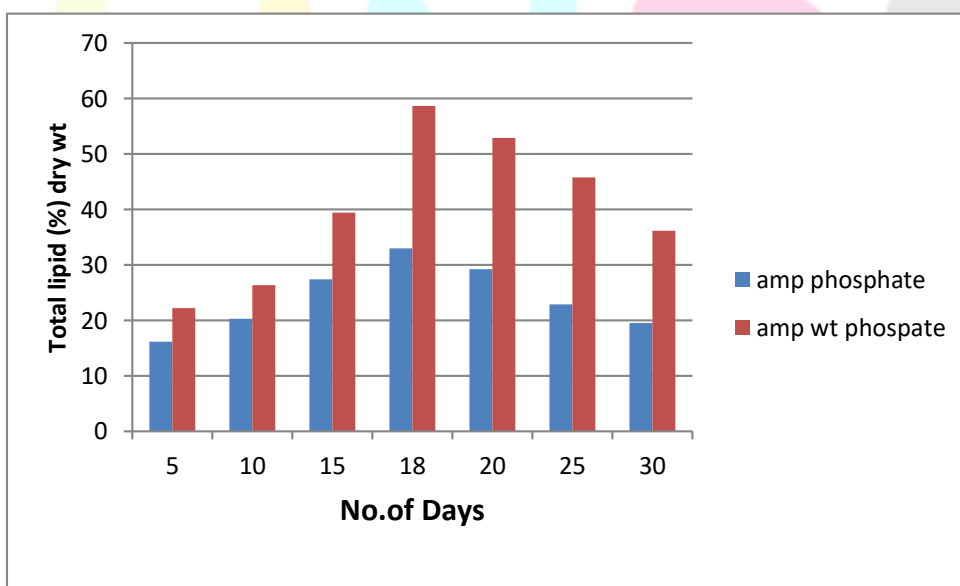


Figure 10: Effect of with phosphate and without phosphate on lipid production of *Amphora coffeaeformis*

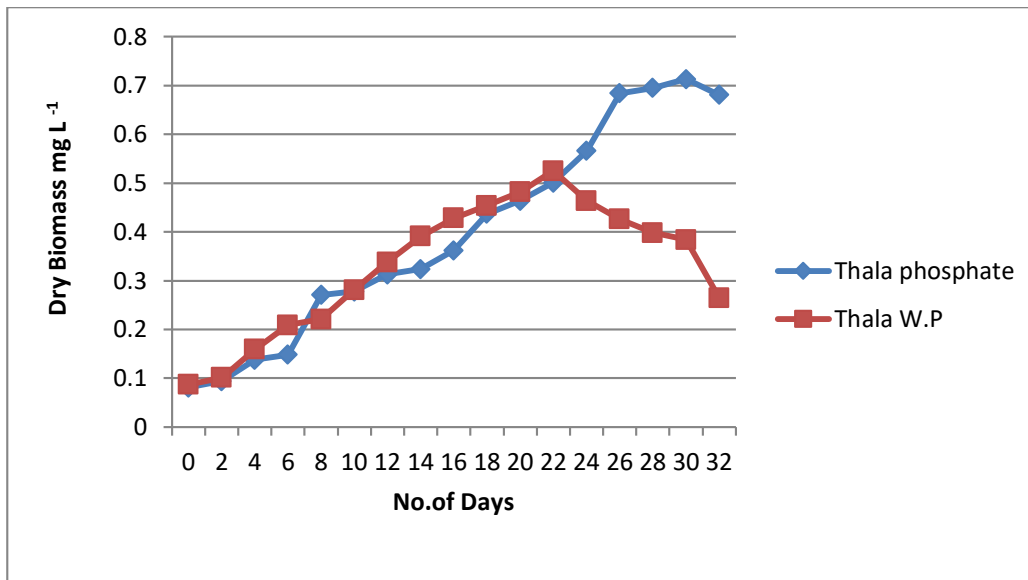


Figure 11: Effect of with phosphate and without phosphate on biomass production of *Thalassiosera pseudonana*

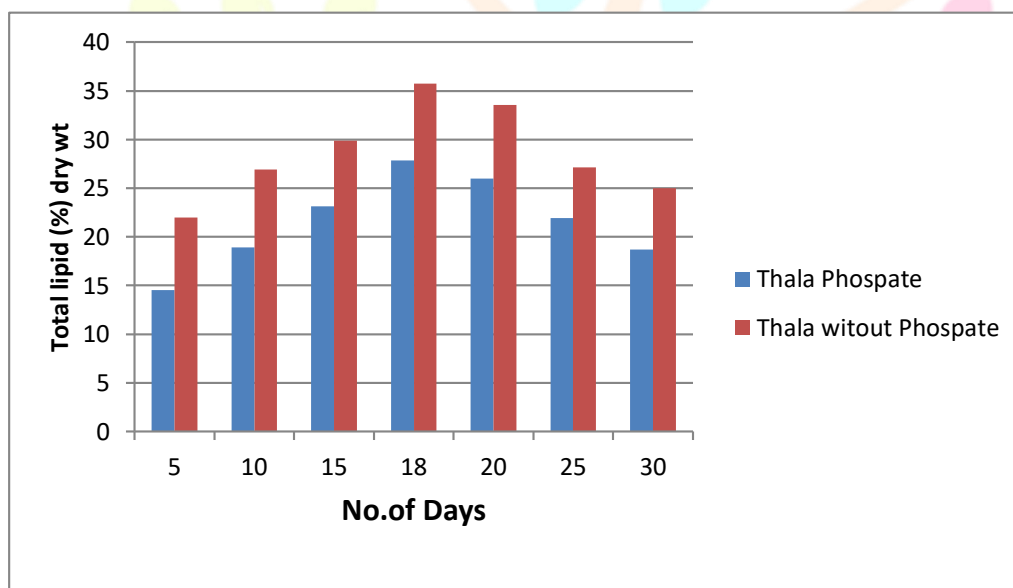
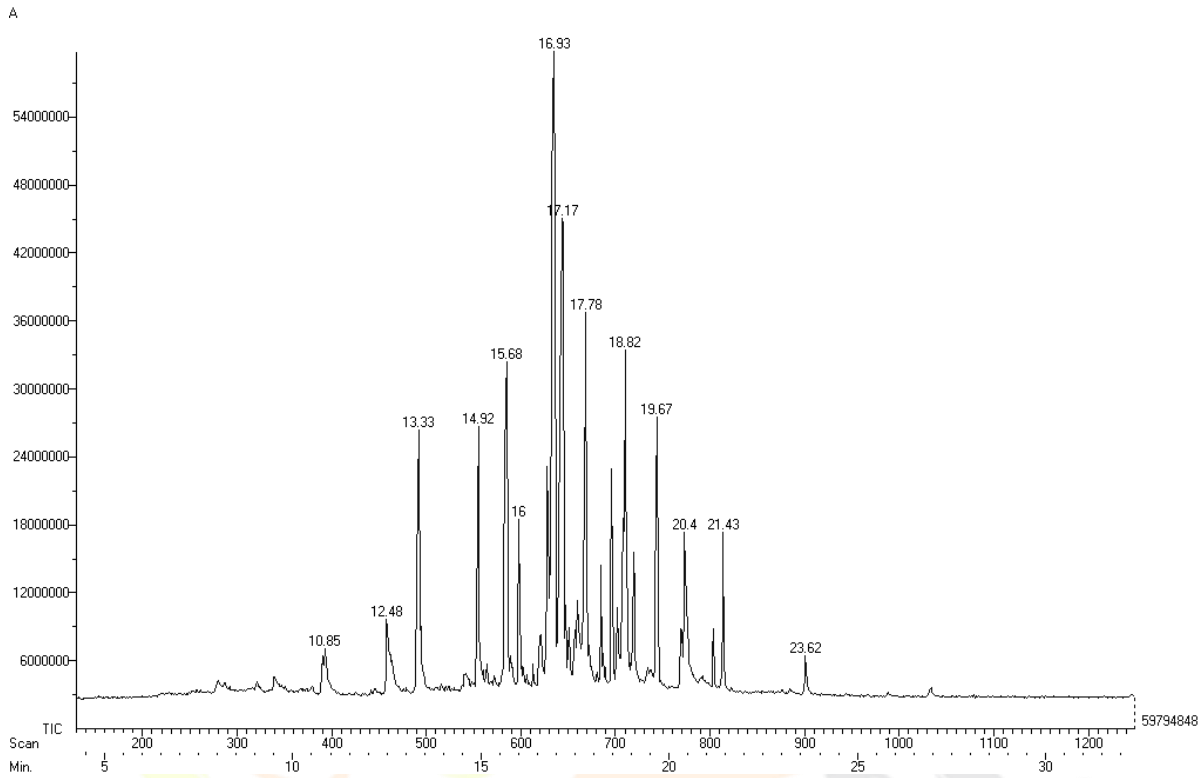


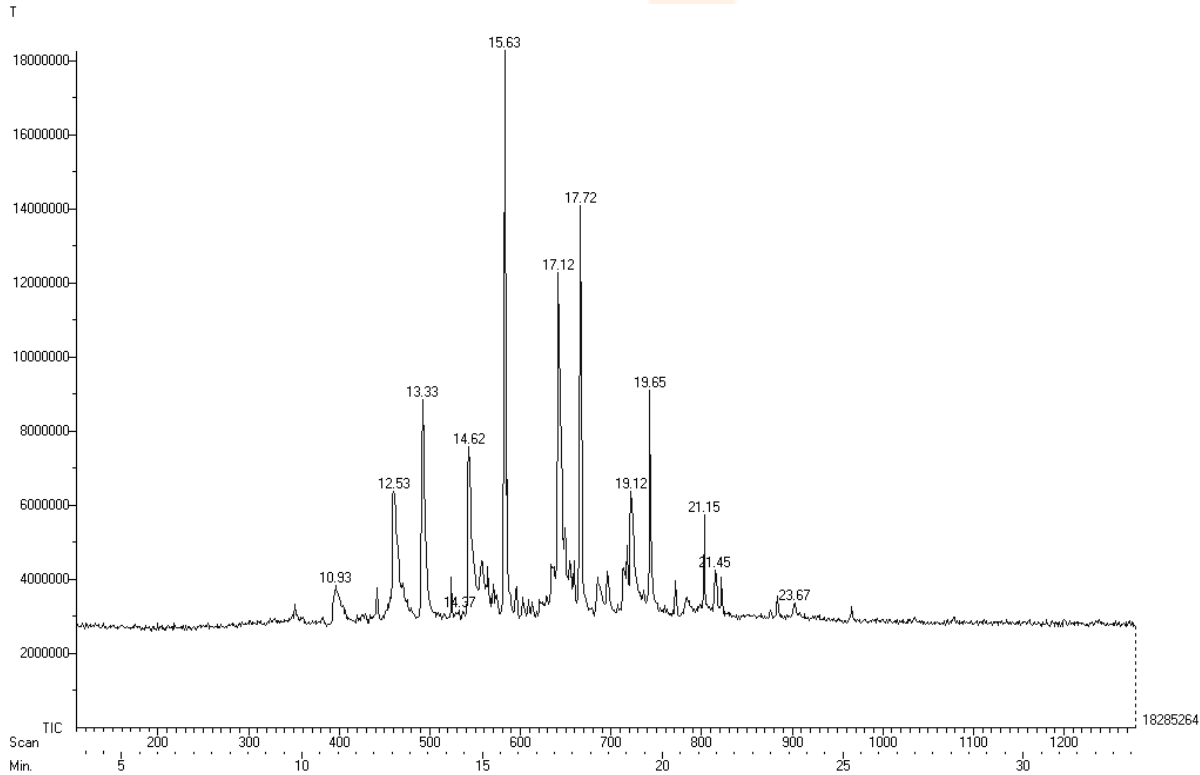
Figure 12: Effect of with phosphate and without phosphate on lipid production of *Thalassiosera pseudonana*

In *Amphora coffeaeformis*, and *Thalassiosera pseudonana*, shows maximum biomass production of 38.22%, and 34.29%, DCW under normal conditions and in stress conditions it has been increased to 59.19%, and 47.16% DCW respectively. *Amphora coffeaeformis*, is considered a possible source for biodiesel production due to its high lipid content by dry weight (59.19 %,) DCW. The following major fatty acids were found in above marine diatoms are Cycloecosane, 3-Eicosene, 9-Hexadecanoic acid methyl ester, Penta decanoic acid, Methyl tetra deconate.

### 3.5: Fatty acids of *Amphora coffeaeformis* by using GC-MS



### Fatty acids of *Thalassiosera pseudonana* by using GC-MS



### 3.6.Types of Fatty acids

**Table No. 1: Fatty acid profile of *Ampora coffeaeformis* and *Thalassiosera pseudonana***

S.No	Fatty acids of	R.T.value	<i>Ampora coffeaeformis</i>	<i>Thalassiosera pseudonana</i>
1	3 - Octadecanoate	15.78	-	+
2	1-Eicosene	15.87	+	-
3	1-Docosene	17.8	+	+
4	10-Octadecenoic acid, Methyl ester	17.0	+	-
5	Heptadecanoic acid,16 methyl-methyl ester	19.2	+	+
6	9 - Tricosene	19.8	+	-
7	Hexadecanoic acid, methyl ester	17.03	+	+
8	Phenol 3,5-Bis(1,1-dimethylethyl)	10.77	+	-
9	Cycloecosane	17.88	+	+
10	9-Hexacosane	21.25	+	-
11	Methyleicosa 5,8,11,14,17-pentaenoate	20.57	-	+
12	3-Eicosene	17.87	+	+
13	9-Hexadecanoic acid methyl ester	17.05	+	+
14	Penta decanoic acid, 14 methyl, methyl ester	17.23	+	+
15	1-Hexadecene	13.47	+	+
16	1-Octadecene	13.78	+	+
17	E-7-Octadecene	12.72	+	-
18	1-Heptadecene	13.27	+	-
19	1,3,Pentadiene 2,14 di-t-butyl	18.8	+	-
20	13,Octadecen-1-yl acetate	18.8	+	-
21	Elaidic acid, iso propylester	20.48	+	-
22	Tetradecene 2- decyl	21.4	+	-
23	Methyl tetra deconate	15.02	+	+
24	1- Tricosene	19.67	+	-
25	Dodeconoic acid	12.68	+	-
26	3- Octadecene	15.78	+	-
27	E-6 Tetradecen 1-0l-acetate	16.35	+	-
28	5-Decanedioic acid, 5,6 dimethyl- dimethyl ester	16.98	+	-
29	9 – Octadecene 12 – ynoic acid, methyl ester	18.95	+	-
30	Dodeconoic acid 10 – OXO	12.72	-	+

31	Bicyclo (10.8.0) eicosane cis	16.37	-	+
32	1 – Tetradecene 2 – decyl	21.62	-	+
33	3 –Deconone1 ( 4 – hydroxyl – 3- methoxy phenyl )	16.6	-	+
34	9 – Octadecanoic acid methyl ester	17.23	-	+
35	Octadecanoic acid methyl ester	19.22	-	+
36	Methyl eicosa, 5,8,11,14,17 pentaenoate	20.55	+	+
37	7 - Pentadecen 5yne	12.77	-	+
38	2 – Hexadecanoic acid, 2,3 – di methyl ester	19.0	-	+
39	5, 17 Octadecadein 1 – 01 acetate	19.8	-	+

#### 4. CONCLUSION:

This study revealed that diatoms are fast growing and effective organism for biodiesel production, two diatom species were cultured, biomass is harvested, and lipids were extracted and converted to biodiesel through transesterification using sodium methoxide as a catalyst. Biomass and total lipid content of *Amphora coffeaeformis* and *Thalassiosera Pseudonana* were evaluated at various concentrations of with and without sodium silicate, sodium nitrate, and sodium phosphate in f/2 medium in the laboratory conditions. Resultant biodiesel was analyzed, fatty acids were noted, and it was reported that among the two different diatoms chosen *Amphora coffeaeformis* could play a vital role in biomass and lipid production, more at without nitrate, without silicate and without phosphate Starvations, compare to with nitrate, with silicate and with phosphate Starvations than *Thalassiosera Pseudonana*, and it was observed that among the three different nutrients chosen sodium nitrate and sodium silicate could play a vital role in biomass and lipid production. These characteristics indicate that *Amphora coffeaeformis* might be suitable candidates for future exploitation as an alternative renewable fuel source (or) as a significant source of lipids for other applications.

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