# Stress Induced Lipids Accumulation in Naviculoid Marine Diatoms for Bioenergy Application

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**Abstract**: Microalgae are expected to play promising role in the production of biofuel in current research. Two of marine diatoms, *Navicula* sp. and *Amphora* sp. were isolated and their growth rate was also studied. Total lipid content was analyzed in stationary growth state under normal conditions. By the two stage process, both the diatoms were subjected to nitrogen and silicon undersupplied for five days and the total lipid accumulation in the diatoms were found to be increased during nutrient deficiency period. The nutrient deficit conditions prone to increased total lipid content and also altered the fatty acid profile in diatom. The total lipid content of *Navicula* sp. and *Amphora* sp. were found to be 34.93% DCW and 41.10% DCW under normal conditions and in nitrogen deficiency conditions it has been increased to 60.71% DCW and 64.72% DCW respectively. The major fatty acids were found to be cis-10-Heptadecanoic acid (27.54%) and stearic acid (24.57%). The level of saturated and monounsaturated fatty acids were found to be high in both the diatoms. The presence of low level of polyunsaturated fatty acids indicated that these two organisms could find future application in bioenergy production.

**Keywords:** Biodiesel, Fatty acid, *Navicula* sp., *Amphora* sp., Nitrogen and silicon starvation.

## 1. INTRODUCTION

The world is entering an era of declining non-renewable energy resources, popularly known as 'Peak oil', while energy demand is increasing day-by-day and the world's oil production is expected to decline in between one and ten decades [1]. As supply dwindles and costs rise, the nations will be forced to utilize alternative energy sources especially biodiesel. Biodiesel is characterized as a renewable, biodegradable and eco-friendly fuel which has attracted wide attention. Most recently, research effort has been aimed at identifying suitable biomass species which can provide high energy outputs to replace the fossil fuels [2]. Many of the researchers have attempted to produce biodiesel from non-edible sources like frying oil, greases, tallow, jatropha, mahua and soy bean oils [3-9]. Nevertheless, the cost of biodiesel production is still a major obstacle for large scale commercial exploitation, mainly due to the high feed cost of vegetable oils [10]. So we are in need to depend on other existing sources.

In recent years, microalgae are emerging as one of the most promising source of biodiesel. Obviously, they are higher photosynthetic efficiency and faster growth rate as compared to any other energy crop [11]. Nowadays, the potential value of microbial and particularly microalgal photosynthesis to produce biofuels is, however widely recognized [12-16]. However, the lipid content in the microalgae required to be high, otherwise the economic performance would be very hard to achieve [17].

Each species of microalga produces different ratios of lipids, carbohydrates and proteins. Nevertheless, these tiny organisms have the ability to manipulate their metabolism through simple modifications of the chemical composition of culture medium [18]. For example, it was demonstrated that significant increase in lipid content occurred in several species of unicellular algae after subjecting them to nitrogen deficient environment [19-21]. Microalgae in particular, diatoms and some green algae were considered as useful neutral lipid sources.

Many microalgae can accumulate lipids due to excessive photosynthesis and some species can accumulate certain amount of lipids under heterotrophic conditions or environmental stress, such as nutrient deficiency [22]. The medium which supports the growth does not favour the increased lipid accumulation. In the current research, a two stage process has been carried out for cultivation of diatoms (*Navicula* sp. and *Amphora* sp.) In this study, we have

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isolated, investigated the growth rate and the total lipid content of two diatoms. The total fatty acid content and fatty acid profiles of these strains were analyzed, under normal and nutrient deficiency (nitrate and silicate) conditions.

## 2. METHODS

### 2.1. Isolation and Growth Condition of Microalgae

The samples were collected from Rameshwaram coastal area (Latitude 9°7´N. Longitude 79°18´E Altitude 8m) and cultured in F/2 medium. The composition of F/2 medium was as follows: $NaNO_3$ – 75 mg, $NaH_2PO_4$ – 5 mg, $Na_2SiO_3$ – 30 mg, $FeCl_3.6H_2O$ – 3.15 mg, $Na_2EDTA.2H_2O$ – 4.36 mg, $CuSO_4$ - 0.0098 mg, $Na_2MoO_4$ – 0.0063 mg, $ZnSO_4.7H_2O$ – 0.022 mg, $CoCl_2.6H_2O$ – 0.010 mg, $MnCl_2.4H_2O$ – 0.180 mg and trace amounts of Vitamin $B_{12}$, Biotin, Thiamine HCl and 1000 ml distilled water [23]. 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/2 medium) and incubated at 24±2℃ under 37.5 $\mu mol^{-1}m^2s^{-1}$ 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 250ml capacity containing 100ml F/2 medium for a period of two weeks. The culture flasks were inoculated and incubated at 24±2℃ under 2000-2500 lux 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 UV-Vis Spectrophotometer and was calculated as doublings per day by using the following formulae [24]

$$\text{Doubling/Day} = \text{Log}OD_2 - \text{Log}OD_1 / T_2 - T_1(h) \times 34.632$$

Where,

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

### 2.3. Harvesting and Dry Cell Weight (DCW) Determination

Of the isolated strains growth rate and physiology were studied, based on that cells were harvested by floatation and sedimentation technique for nitrogen and silica starvation. Briefly, the cells were grown in 1000ml Erlenmeyer flask containing F/2 medium. After the incubation period, one diatom culture was settled in bottom of the flask another diatom was free floating in the flask. Both the cultures were harvested by centrifugation for 10mins at 4000 rpm. A known volume of algal culture was centrifuged at 5,000 rpm for 10 minutes. The harvested biomass was washed once with sterile distilled water and dried at 60°C till it reaches constant weight.

### 2.4. Stress Induced by Nitrogen and Silica Starvation

The cultures were harvested by centrifugation at 4000rpm for 15 minutes during their early stationary phase and washed with sterile distilled water. The experimentation was done in two different flasks. First, the harvested cells were measured and inoculated in sterilized nitrogen free F/2 medium. Secondly, the harvested cells were measured and inoculated in sterilized silica free F/2 medium. The diatom cultures were inoculated in nutrient sufficient F/2 medium as control. All the flasks were incubated for five days at 24±2 °C under 37.5$\mu mol^{-1}m^2s^{-1}$ light intensity with 16:8 hours light and dark cycle.

### 2.5. Lipid Extraction and Preparation of Fatty Acid Methyl Ester (FAME)

Lipid extractions were performed to all the experiments at their stationary phases and after an additional five days for both nitrogen and silica starved cultures. Cells were harvested and lyophilized before lipid extraction. The lyophilized cultures were weighed separately before initiating lipid extraction. The lipid was extracted from the algal biomass in its stationary phases [12, 25]. The extracted lipid was dried in rotary evaporator, weighed and stored for FAME preparation. The entire process of FAME preparation [26] is shown in Figure **1**. Then, the oily substance was further determined using gas chromatography (GC 2014, Shimadzu, Japan).

### 2.6. Fatty Acids Analysis Using Gas Chromatography (GC)

The fatty acid compositions were determined by Gas Chromatographic analysis. The prepared FAME was analyzed by Gas chromatographic system equipped with FAME WAX column (RESTEK column, USA). The column details were 30m x 0.3mm ID x0.25µm thickness. The injection port temperature was 250℃. Here, Flame Ionization Detector (FID) was used

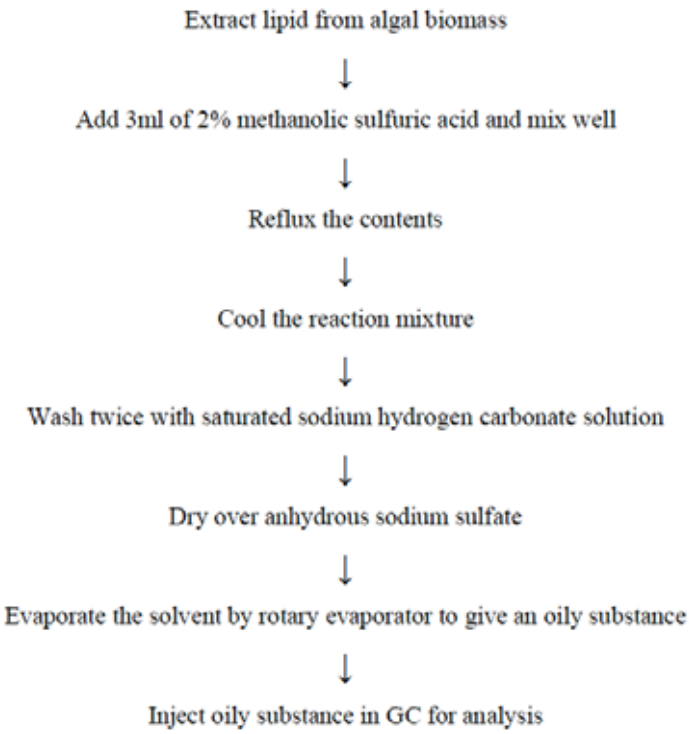


**Figure 1:** Process of FAME preparation.

with temperature of 260℃. Nitrogen was used as a carrier gas kept at a constant rate of 22.2 ml/min. Fatty acid composition was calculated as percentage of the total fatty acids present in the sample determined from the peak areas.

## 3. RESULTS AND DISCUSSION

### 3.1. Microalgal Growth Rate

The isolated diatoms were identified as *Navicula* sp. and *Amphora* sp. Growth of *Navicula* sp. was greater when compared to that of *Amphora* sp. in F/2 medium. *Navicula* sp. grew exponentially with little or no lag phases with first four days and by day seven growths reached stationary phase whereas in *Amphora* sp. it reached stationary phase by day nine. In our investigation, the growth rate of *Navicula* sp. and *Amphora* sp. were greatly reduced but did not completely ceased in nitrogen and silica starved cultures. The doubling time of *Amphora* sp. and *Navicula* sp. were found to be 3.15 and 3.6 respectively. This agrees with the previous studies [27].

### 3.2. Quantitative and Qualitative Analysis of Lipid Content Under Normal Conditions

These two diatoms are differ in their total lipid content and also in their profile under normal nutrient conditions. The total fatty acid content of the isolates *Navicula* sp. and *Amphora* sp. were found to be 34.93% DCW and 41.10% DCW respectively under normal conditions. This agrees with the existing reports regarding microalgal oil content [28]. Previous reports showed that algal oil contains more amount of saturated and monounsaturated fatty acids rather than that of polyunsaturated fatty acids [29].The detected fatty acids of under normal conditions were listed on the Table **1**. The major fatty acids were found to be cis-10-Heptadecanoic acid (27.54%) and stearic acid (24.57%). This is similar to our former works on diatom lipids (data not shown). In our investigation, the amount of saturated and monounsaturated fatty acids are predominant in both the diatoms and this agrees with previous observations in algae [30] (Table **2**). In both the studied diatoms, the linolenic acid proportion was below 12%, which meets the requirements of the European Standard EN 14214 [31] for biodiesel production.

### 3.3. Effect on Total Lipid Content in Nitrogen Starvation

Earlier research indicated that many microalgae can be induced to accumulate lipids under nutrient starving conditions, but those are not suitable to increase the biomass. Therefore, here a two stage process has been implemented for cultivating *Navicula* sp. and *Amphora* sp. with biomass production in the first stage

**Table 1: Total Lipid Content of *Amphora* sp. and *Navicula* sp. under Normal and Nutrient Starved Conditions**

| Type of Culture | Culture conditions | Total lipid content (%) |
|---|---|---|
| *Amphora* sp. | Normal medium | 34.93 ± 4.32 |
| | Nitrogen Deficient (ND) | 51.77 ± 7.1 |
| | Silica Deficient (SD) | 60.71 ± 5.24 |
| *Navicula* sp. | Normal medium | 41.12 ± 1.56 |
| | Nitrogen Deficient (ND) | 60.28 ± 4.92 |
| | Silica Deficient (SD) | 64.72 ± 5.86 |

**Table 2: Level of Saturated, Monounsaturated and Polyunsaturated Fatty Acid in *Amphora* sp. and *Navicula* sp.**

| Fatty acid type | *Amphora* sp. | *Amphora* sp. | | *Navicula* sp. | *Navicula* sp. | |
|---|---|---|---|---|---|---|
| | | N-deficient | Si-deficient | | N-deficient | Si-deficient |
| Saturated (%) | 34.6 | 32.5 | 30.55 | 35.3 | 34.15 | 34.75 |
| Monounsaturated (%) | 33 | 47.9 | 52.2 | 43.5 | 42.46 | 43.05 |
| Polyunsaturated (%) | 8.94 | 2.86 | 5.53 | 11.2 | 8.56 | 5.80 |

and lipid accumulation in the second stage. This two stage culture strategy has proved effective for increased lipid production in case of the selected diatoms. The total lipid content of *Navicula* sp. and *Amphora* sp. were found to be 34.93% DCW and 41.10% DCW under normal conditions and in nitrogen deficiency conditions it has been increased to 60.71% DCW and 64.72% DCW respectively on the basis of their dry cell weight (DCW).

Nitrogen limitation was reported to significantly influence microalgal lipid storage that too in positive aspect by increasing the total lipid content. Under nitrogen deficiency conditions, the protein biosynthesis has been ceased. Due to that the cell division has been stopped which results in cessation of microalgal growth and animates a metabolic pathway favourable to the accumulation of reserve lipids [32]. It was reported that under nitrogen stress the diatom *Chaetoceros mulleri* and *Navicula saprophila* were known to increase their total lipid content respectively [33]. Previous studies showed that the average lipid content of oleaginous diatoms of marine origin grown under normal conditions were found to be 27.1% and it was increased upto 44.6% DCW in nutrient deprived cultures [34]. The present work agreed with those reports. Few studies reported that the two stage process is effective in both biomass production and lipid accumulation. It was demonstrated that in *Nannochloropsis oculata* the two stage process has proved effective in both biomass production and lipid accumulation [35].

The nitrogen deficiency condition not only affects the quantity of the lipid but also its quality. The nitrogen deficiency conditions profoundly affected the fatty acid profile of both the diatom *Navicula* sp. and *Amphora* sp. The content of saturated and monounsaturated fatty acids like cis-10-heptadecanoic acid, stearic acid, palmitic acid, palmitoleic acid and cis-10-pentadecanoic acid were found to be increased whereas the content of polyunsaturated fatty acids like linolenic acid and arachidonic acids were decreased in both the diatoms (Table **3**).

### 3.4. Effect on Total Lipid Content in Silica Starvation

Like nitrogen, 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. It was described that the lipid content of the diatom *Navicula pelliculosa* increased by about 60% during a 14 hours silica starvation period [36]. Specifically, it appears that nitrogen deficiency leads to an increase in production of triacylglycerols in diatoms [37-38]. The present study also indicated the similar results that under silica deficient conditions, the total fatty acid content was increased to 51.77 % DCW and 60.28 %DCW in *Navicula* sp. and *Amphora* sp. respectively (Table **3**). Similar to the lipid trigger effect produced by nitrogen 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

**Table 3: Fatty Acid Profile of *Amphora* sp. and *Navicula* sp. under Normal and Nutrient Starved Condition (Percentage of Fatty Acids)**

| Fatty acid | *Amphora* sp. | *Amphora* sp. | | *Navicula* sp. | *Navicula* sp. | |
|---|---|---|---|---|---|---|
| | | N-deficient | Si-deficient | | N-deficient | Si deficient |
| Caprylic acid 8:0 | 0.14 | 0.17 | 1.34 | 0.18 | 0.15 | 0.13 |
| Capric acid 10:0 | 1.2 | 1.05 | 0.53 | 0.86 | 1.06 | 0.72 |
| Undecanoic acid 11:0 | 0.35 | 0.27 | Nd | 0.22 | 0.26 | 0.1 |
| Lauric acid 12:0 | 0.59 | 0.41 | Nd | 0.36 | 0.4 | 0.33 |
| Tridecanoic acid 13:0 | 0.35 | Nd* | Nd | Nd | Nd | Nd |
| Myristic acid 14:0 | 0.64 | 0.47 | Nd | 0.3 | 0.41 | 0.21 |
| cis-10-Pentadecanoic acid 15:1 | 2.34 | 3.76 | 3.32 | 4.52 | 4.92 | 4.43 |
| Palmitic acid 16:0 | 0.35 | 2.26 | 1.27 | 2.24 | 2.49 | 1.55 |
| Palmitoleic acid 16:1 | 0.41 | 1.14 | 1.06 | 2.37 | 2.99 | 3.25 |
| Heptadecanoic acid 17:0 | 1.2 | 0.9 | 0.57 | 0.85 | 1.08 | 0.61 |
| cis-10-Heptadecanoic acid 17:1 | 27.54 | 39.34 | 46.04 | 29.36 | 28.07 | 28.7 |
| Stearic acid 18:0 | 25.82 | 25.91 | 24.57 | 26.48 | 25.41 | 25.19 |
| Elaidic acid 18:1 | 0.96 | 0.84 | 1.01 | 1.09 | 0.33 | 0.54 |
| Oleic acid 18:1 | 0.41 | 0.91 | 1 | Nd | 0.3 | Nd |
| Linolelaidic acid 18:2 | Nd | Nd | 1.92 | Nd | 0.66 | Nd |
| Linoleic acid 18:2 | 0.45 | 0.84 | 1.13 | 0.86 | 0.92 | 0.96 |
| Linolenic acid 18:3 | 0.53 | 0.37 | Nd | Nd | Nd | Nd |
| Arachidic acid 20:0 | 1.39 | 0.56 | 0.49 | 2 | 1.85 | 1.84 |
| cis-11-Eicosenoic acid 20:1 | Nd | Nd | Nd | 1.49 | 0.9 | 0.89 |
| cis-5, 8,11,14,17-Eicosapenatenoic acid 20:1 | 2.26 | 0.46 | Nd | 1.06 | 0.25 | 2.5 |
| cis-11,14-Eicosadienoic acid 20:2 | Nd | Nd | Nd | Nd | 0.15 | Nd |
| Behenic acid 22:0 | 0.27 | 0.38 | Nd | Nd | Nd | 0.55 |
| cis-4,7,10,13,16,19-Docosahexaenoic acid 22:6 | 0.34 | 0.26 | 0.84 | 0.48 | 0.35 | 1.59 |
| Tricosanoic acid 23:0 | Nd | Nd | Nd | Nd | 0.21 | Nd |
| Lignoceric acid 24:0 | 2.68 | 1.08 | 1.75 | 1.76 | 0.79 | 3.4 |
| Arachidonic acid | 5.34 | 2.9 | 2.37 | 8.66 | 6.19 | 7.83 |
| Nervonic acid | 0.78 | 0.5 | | 0.54 | 0.68 | 0.58 |
| Erucic acid | 0.53 | 2.15 | 0.8 | 4.58 | 4.25 | 4.67 |
| cis-13,16, Docosadienoic acid | 0.4 | Nd | Nd | Nd | 0.27 | Nd |
| Others | 21.95 | 13.07 | 9.9 | 9.74 | 14.66 | 9.43 |

*Nd = Not detected.

evaluated, nitrogen and silica limitation are the most critical nutrients which affect lipid metabolism in algae and diatoms thus increase in the total lipid content.

Earlier research indicated that silicon deficient *Cyclotella cryptica* cells had higher proportions of saturated and monounsaturated fatty acids than silicon replete cells [39]. In our study, the level of saturated and monounsaturated fatty acids were found to be higher in silica deplete cultures than in silica replete cultures. In *Navicula* sp. the level of saturated fatty acids were lower than that of control whereas the level of monounsaturated fatty acid was increased than control. In *Amphora* sp. the level of polyunsaturated fatty acid was lower compared to that of control, at the same time the level of saturated and monounsaturated fatty acids were increased compared to that of the control.

With respect to biodiesel application, the fatty acid characterization is important because it helps in rating the hydrocarbons as substitute liquid fuels. The fatty acid compositions of microalgal oil may vary with individual species / strains and their environmental conditions. The physical characteristics of fatty acids can be determined by chain length, number of double

bonds and the amount of each fatty ester component. The high proportion of saturated and monounsaturated fatty acids in the algae is considered to be optimal from a fuel quality standard point, in that fuel polymerization during combustion would be considerably less than the polyunsaturated fatty acid derived fuel. The two diatom isolates have high level of saturated and monounsaturated fatty acids.

According to biodiesel standard EN14214 methods, the concentration of linolenic acid and acid containing four double bonds in FAME should not exceed the limit of 12% and 1% respectively. Here, both the isolates specify these criteria by having trace amount of linolenic acid. The algal oil also contains some amount of polyunsaturated fatty acids that may lead to oxidation. In order to overcome the major problem of its stability and to guarantee a specific quality, it's vital to use antioxidant additives for biodiesel to improve its stability. It was reported that antioxidants like tert-butylhydroquinone (TBHQ), propyl gallate (PG), and pyrogallol (PA) proved efficiently in preventing the oxidation of biodiesel thereby reduced the stability problem [40].

## CONCLUSION

In conclusion, both the naviculoid diatoms i.e. *Navicula* sp. and *Amphora* sp. exhibit high lipid content and its GC analysis revealed the presence of higher proportions of saturated and monounsaturated fatty acids which is preferable for biodiesel application. Moreover, both the diatoms meet the requirements of suitable feedstock for biodiesel in the aspect of lipid productivity. While comparing these two isolates it was revealed that *Amphora* sp. is considered to be efficient lipid producer than *Navicula* sp. Being, a marine organism it can be cultivated in wide range of saline areas and also yielded more quantity of lipid by simple modifications in their culture medium. These characteristics indicate that these species 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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