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# Use of Raman microspectroscopy to detect changes in lipid pools of microalgae

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Raman spectropscoy was used to study the changes in lipid pools adapted by algae subjected to salinity dependent nutrient stress. The changes in the lipid in the algal cells cultured in varying salt concentrations were followed over a course of seven days. The Raman results showed prominent change in lipid pools. Raman spectroscopy demonstrates the potential to be an important technique in monitoring change in lipid pools due to salinity stress.

Keywords: Raman micro spectroscopy, lipid, algae, salinity

## INTRODUCTION

Energy plays a very vital role in the development f a region. Dwindling stock of fossil fuels coupled with the increased demand of conventional energy sources and impending changes in the climate due to enhanced levels of GHG's (Green house gases) have resulted in the fuel crisis, necessitating for sustainable energy replacement. Major research emphasis have been on the development of petroleum, coal, and natural gas based refinery to exploit the cheaply available fossil feedstock. Declining global oil productive capacity, economic vulnerability and global climate change are the prime movers of a clean energy drive across the globe. Currently, the fossil resources are not regarded as sustainable and questionable from the economic; ecology and environmental point of view (Kamm et al., 2006). Cost-effective renewable energy sources and other energy alternatives is the need of the hour (Groom et al., 2008).

## DIATOMS AS A PROMISING CANDIDATE FOR BIOFUEL PRODUCTION

Diatoms (Greek "cut in half") under class Bacillariophyceae are eukaryotic, autotrophic microorganisms with a ubiquitous distribution. They are characterized by a unique siliceous ($SiO_2$ x $nH_2O$) cell wall (Round et al., 1990). Diatoms have large proportion of lipids in their body, about 70% of their dry weight. Diatom lipids have been suggested as a potential diesel with emphasis on the neutral lipids due to their lower degree of unsaturation and their accumulation in algal cells at the end of growth stage. Both polar lipids and non polar (neutral) lipids are found in diatoms.

## STRESS ON DIATOMS

In diatoms stress or unfavourable condition leads to the production of more lipids (Hu et al., 2008). Environmental parameters like pH, temperature, light, nitrogen, carbon, silicon, phosphorous, iron, salt concentration, etc affect the lipid composition of the diatoms. Lipids act as a secondary metabolite for diatoms and these lipids maintain specific membrane functions and cell signaling pathways in microalgae and play a role in responding to changes in the environment (Hu et al., 2008). Stress condition ceases the growth of the species, thereby reducing the biomass content but enhances the lipid composition.

## FACTORS RESPONSIBLE FOR ACCUMULATION OF TRIACYLGLYCEROL AND CHANGES IN FATTY ACID COMPOSITION

The lipid content, lipid class composition and the proportions of the various fatty acids in a microalgae vary according to the environmental or culturing variables such as light intensity, growth phase photoperiod, temperature, salinity, $CO_2$ concentration, nitrogen and phosphorous concentration (Dunstan et al., 1993).

## NUTRIENT STRESS

**NITROGEN:** Most critical nutrient affecting lipid metabolism in microalgae is nitrogen limitation (Kilham et al., 1997). Nitrogen limitation leads to a decrease in protein content in both freshwater algae and diatoms. Cell size for nutrient limited cultures were significantly smaller than the non limited cells size and was more pronounced in N-limited cells (Lynn et al., 2000). Microalgae cells are capable of using organically combined nitrogen forms, especially amino acids, urea and purines, as their sole nitrogen source (Fabregas et al., 1997). Li et al., 2008 investigated the effect of different nitrogen sources sodium nitrate, urea and ammonium bicarbonate on the lipid content of green algae *Neochloris oleoabundance*. It turned out that sodium nitrate is the most favorable nitrogen source for both cell growth and lipid accumulation of N. *oleoabundans*.

**SILICA:** In diatoms, silicon is an equally important nutrient that affects cellular lipid metabolism. For example, silicon-deficient *Cyclotella cryptica* cells had higher levels of neutral lipids (primarily TAG) and higher proportions of saturated and mono-unsaturated fatty acids than silicon-replete cells (Roessler, 1988). Tilman (1977) studied the effect of wide range of Si: P supply ratios on the mixed population of the two diatoms A. *formosa* and *Cyclotella meneghiniana* grown in semicontinuous cultures. When the molar ratio of these elements was smaller than 6:1 by moles, A. *formosa* dominated, while C. *meneghiniana* dominated when the ratio exceeded 90:1 by moles.

**OBJECTIVE:** The objectives of this work were to find out whether different nitrogen sources contribute to the accumulation of lipids.

## MATERIALS AND METHODS

The epiphytic (collected from *Eicchornia* sp. and *Hydrilla* sp.) diatom samples were collected and stored in non-reactive plastic bottles using the procedure followed by (Kelly et al., 2008)

**ISOLATION:** 'The collected samples were then isolated by using modified Pasteur pipette (see Andersen and Kawachi, 2005) under the inverted microscope (40X magnification). The isolated cells were grown in WCg medium (Wright 1964, Guillard and Lorenzen 1972, Guillard 1975; Guillard, unpublished) with 16:8 light/dark photoperiod for a period of 7 days. Diatom identification was carried out by observing the isolated sample under the light microscope (40X magnification). The culture conditions were maintained at 26-27$^0$C with a light intensity of 28-32 µmol $m^{-2}$ $s^{-1}$.

**CULTURE CONDITIONS:** The different nitrogen sources used to check the response of sources on *Nitzschia* sp. cell as well as lipid quantity were sodium nitrate and urea.To study the effect of both the stresses, experiments were carried out in 250 ml Erlenmeyer flasks each containing 100 ml of appropriate medium. Control cultures were also maintained under similar culture conditions. The experiments were set up for 7 days. The cell count and extraction of total lipid for the two microalgae were determined every day. All the experiments were carried out in triplicate.

**CELL COUNT ESTIMATION:** Growth rate was determined using cell counting method throughout the study period. 1 ml of the culture was taken onto a slide and counted for growth rate measurement. Diatom frustules devoid of chloroplasts were not included during the counts. The growth rate calculation is based on the fact that during exponential growth, the rate of increase in cells/unit time is proportional to the number of cells present in the culture (Anderson, 2005). The specific growth rate (µ) is defined as the increase in cell density/unit time (Garcia et al., 2007) and formulated.

**LIPID ANALYSIS:** Lipid was extracted using Bligh and Dyer, 1959 method. 25 ml of the sample was sonicated for 1 hour at room temperature to disrupt the cell wall. This was done with the addition of the chloroform: methanol (2:1 v/v) solvent. Chloroform layer was extracted since the lipid from the algae dissolves in chloroform layer. This layer was further evaporated using rotary evaporator to obtain lipids. The total lipid content was determined gravimetrically.

## RESULTS AND DISCUSSION

### GROWTH AND LIPID CONTENT OF NITZSCHIA SP. IN WCg MEDIA CONTAINING DIFFERENT NITROGEN SOURCES

Nitzschia sp. obtained maximum cell growth in media containing sodium nitrate as the nitrogen source as shown in Table 01. It was observed that nitrogen source had a significant effect on the growth rate of *Nitzschia* sp. The maximum growth rate was high in media containing sodium nitrate as nitrogen source (0.14 ± 0.02) compared to media containing urea (0.067 ± 0.001).

**Table 01: Cell abundance in *Nitzschia* sp. across nitrogen sources**

| Hours | Sodium nitrate ($10^4$ cells/ml) | Urea ($10^4$ cells/ml) |
|---|---|---|
| 0 | 2.60 | 2.60 |
| 24 | 3.07 | 3.01 |
| 48 | 3.19 | 3.05 |
| 72 | 3.38 | 3.12 |
| 96 | 3.87 | 3.33 |
| 120 | 5.30 | 3.71 |
| 144 | 5.31 | 3.89 |
| 168 | 4.91 | 3.53 |

The difference in cell density of *Nitzschia* sp. with relation to nitrogen source is shown in Table 01. As shown in Figure 01 urea can only support poor growth rate of (0.041 ± 0.0018) of *Nitzschia* sp. under the given experimental conditions. On the other hand, sodium nitrate proved to be a better nitrogen source than urea as high cell density (5.30 x $10^4$ cells $ml^{-1}$) in media containing sodium nitrate was observed. The maximum cell density (5.31x$10^4$ cells $ml^{-1}$; Figure 01) occurred 144th hour after inoculation in both the tested sources and thereafter it showed a gradual decline (4.91x$10^4$ cells $ml^{-1}$) in cell density.

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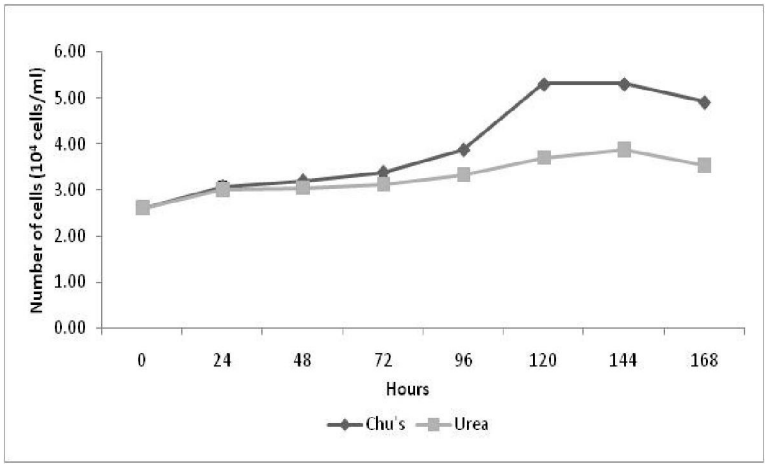


**Figure 01: Maximum cell density and specific growth rate of *Nitzschia* sp. in media containing sodium nitrate and urea**

**LIPID ANALYSIS:**

The total lipid content of *Nitzschia* sp. cultivated in media consisting of sodium nitrate was observed to be higher than that of urea except on the 96th hour (0.58 g $L^{-1}$ in sodium nitrate and 0.45 g $L^{-1}$ in urea) of culture (Table 2). The difference in lipid content of *Nitzschia* sp. grown in both urea and sodium nitrate are shown in Table 2 and Figure 2. The lipid content was high (1.29 $gL^{-1}$ for sodium nitrate and 1.09 $gL^{-1}$ for urea) in the stationary phase of culture in both the cases. The change in lipid content was high in media containing sodium nitrate than urea with a minimum difference. de la Pena (2007) found that the lipid content ranged from 26.4 to 81.5 % DW in Amphora sp. due to change in media composition. These values are several orders of magnitude higher than those reported (0.404 $gL^{-1}$ – 1.29 $gL^{-1}$) in the present study. This is a good indication that lipid content may be enhanced by nutrient stress rather than change in nutrient source.

The results showed that sodium nitrate was the most favourable nitrogen source for the cell growth and lipid production of *Nitzschia* sp. Sodium nitrate was selected to represent nitrate because it is less costly than potassium nitrate, forms the component of almost all media compositions and it would be an advantage in industrial purpose (Li et al, 2008). In a direct comparison of nitrogen sources, sodium nitrate was found to be superior to urea. This observation is in agreement with the work of Li et al., (2008) they observed that sodium nitrate was the best nitrogen source for *Neochloris oleoabundance* for both cell growth and lipid accumulation.

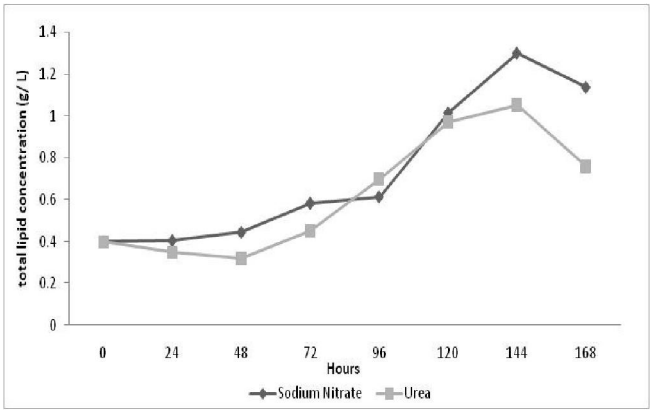


**Figure 02 Change in total lipid content of *Nitzschia* sp. grown in media consisting of sodium nitrate or urea**

| Hours | Sodium nitrate ($gL^{-1}$) | Urea ($gL^{-1}$) |
|---|---|---|
| 0.00 | 0.39 | 0.39 |
| 24.00 | 0.40 | 0.34 |
| 48.00 | 0.44 | 0.32 |
| 72.00 | 0.58 | 0.45 |
| 96.00 | 0.61 | 0.69 |
| 120.00 | 1.01 | 0.97 |
| 144.00 | 1.29 | 1.05 |
| 168.00 | 1.13 | 0.76 |

**Table 03: Total lipid content of *Nitzschia* sp. cultivated in media consisting of sodium nitrate or urea**

**RAMAN MICROSCPECTROSCOPY**

Figure 04 represents the change in the lipid component in the nitrogen source Urea. As seen in the figure, 3000 $cm^{-1}$ shows the peak which is very distinctive from the rest of the peaks. The lipid bands from ~1000 $cm^{-1}$ - ~500 $cm^{-1}$ show a marked variation. Table 04 shows the raman band assignments that were followed.

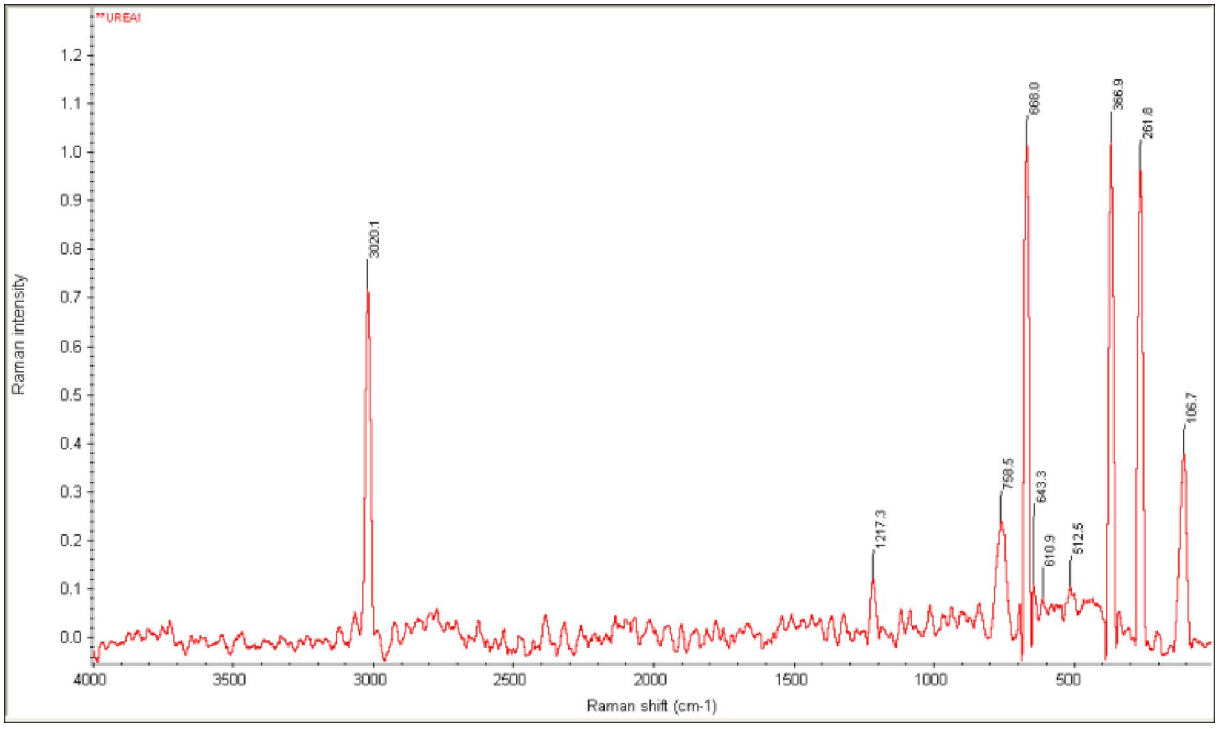


**Figure 04: Raman spectral acquisition of lipid sample**

**Table 04: RAMAN band assignments**

| Wave number values | Assignments |
|---|---|
| ~1670 | C=O of chla, amide 1 of protein |
| ~1605 | CC chla |
| ~1525 | C=C beta carotene |
| ~1495 | C-C, CH3 chla |
| ~1327 | -CN, -CH chla |
| ~1187 | -CH, -N-C chla, -CH beta carotene |
| ~1157 | -CC, CH beta carotene |
| ~915 | -N-C-C chla, -C-C-C chla |
| ~744 | -H-C-O chla, -C-C-C- chla |

## CONCLUSION

Growth of many species of diatoms is limited by the availability of nitrogen and salinity (Lapointe 1989, Russ and McCook 1999). Lipid act as a secondary metabolite for diatoms, and stress or unfavourable condition such as nutrients, growth phase, temperature, light, etc., leads to the production of more lipids in diatoms (Hu et al., 2008). In the present study, maximum total lipid was observed during growth phase in WCg media containing sodium nitrate as a nitrogen source which is also the original source of nitrogen in the media for *Nitzschia* sp. Even though high lipid yield was found in urea source, it accounted for low cell density, which makes it less promising.

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