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Effect of Salinity and pH on Fatty Acid Profile of The Green Algae *Tetraselmis suecica*

Adel Waslallah Almutairi[1] and Hussein Elsayed Toulibah[1,2]*

[1]*Biological Sciences Department, Rabigh-Faculty of Science and Arts, King Abdulaziz University, P. O. box 344, Rabigh 21911, Saudi Arabia*
[2]*Botany Department, College of Women for Arts, Science and Education – Ain Shams University - 1, Asmaa Fahmy Street - Heliopolis, Post cod No. 11757 - Cairo, Egypt*

Abstract

In this study, the marine alga *Tetraselmis suecica* was cultured in F/2 medium with various rang of salinity and pH. The influence of stressed algal cells to produce biofuels was studied. The neutral lipid (triacylglycerol) assembled by *T. suecica* was determined using Nile Red dye under salinity stress (up to 1 M NaCl) and pH (pH 7 to 9). Salinity showed a stimulating effect on triacylglycerol in the algal cells at the 1 Molars, while the effect of pH was changeable. Then fatty acid profile of *T. suecica* cells was evaluated by gas chromatography–mass spectrometry (GC-MS) after direct trans-esterification with hydrochloric acid in methanol. It was shown that, high salinity was ideal for biodiesel production due to increasing of monounsaturated fatty acids produced by *Tetraselmis suecica*.

Keywords: *Tetraselmis suecica;* Neutral lipid (triacylglycerol); Salinity; pH

Introduction

The majority of the energy request is met from the consuming of non-renewable energy sources (oil, petroleum gas and coal) which are simple to utilize, give high energy and are economy when compared other energy sources. The ability and response of *Tetraselmis suecica* in a batch culture with wide range of light regimes, nutrient concentrations and salinity were studied by Şen, Abmohra [1,2]. Hook and Tang [3] stated that, the continues using of fossil fuels are not recommended due to increase the air pollution as well as increase of rate the gathering of greenhouse gases (GHG) that lead to production of acid rain. In addition to the rapid depletion the fossil fuels resources. Therefore, a large amount of research has been carried out with the aim of finding new renewable energy sources that are sustainable and environmentally friendly. Christi [4], mentioned that using microalgae is more likely to produce higher levels of neutral lipids (triacylglycerol, TAG). The vital point to produce algal biodiesel in commercial scale is to find a strain with high production levels of neutral lipid. Fakhry and El Maghraby [5] studied the stress conditions by the nitrogen Depilation and variation of temperature on the growth of *Nannochloropsis salina*. Monika [6] studied the effect of high salinity, light intensity, photoperiod and pH on the growth and lipid accumulation of *Chlorella sp*. Miranda [7] studied the effect of nitrate, phosphate and salinity to enhance the accumulation of fatty acids *Ankistrodesmus* sp. and *Chlamydomonas sp*. The microalgal growth and metabolism will be affected by the change of some physic-chemical parameters as light, nutrients, temperature, salinity and pH [8]. Miranda [6] discussed the energy transfer and biosynthesis of macromolecules bound in algal membrane were dependent on lipid composition, Hence, at any environmental stress the microalgae, the physiological function of the microalgae membrane tries to readjust to tolerate severe stress. This lead to, the microalgae accumulated the lipds in the form of triacylglycerol (TAG) up to 20% to 50% of their dry cell weight.

Algal lipids can be divided into polar and non-polar lipids. The biodiesel production come mainly from the non-polar lipid fraction, fatty acid attached with glycerol backbone (neutral lipid, triacylglycerol, TAG). Therefore, the suitability of microalgae for the production of biodiesel depends on their TAG content. Consequently, it must be understanding at what growth stage and under which culture conditions that TAG accumulation is maximised. Since TAG is synthesized as a storage compound, it makes sense to stress the algae using environmental factors like temperature, light intensity, pH and salinity to reduce growth and switch on TAG production by altering the activity of metabolic pathways [9]. However, this means that growth is halted to induce TAG synthesis, rather than having TAG synthesized as the cells grow. The latter is more desirable since it would lead to higher productivity. The other aspect of TAG synthesis is the composition of the fatty acid chains. The number of characteristics of the fatty acids can change including length (number of carbons) and presence/absence and number of double bonds (i.e., degree of unsaturation). The properties of the fatty acids affect the properties of the biodiesel produced [10].

The composition of diesel fuels is covered by strict standards throughout the world (e.g. EN 14214 is the fuel standard used in Europe). This standard ensures that the ignition properties of the fuel, its viscosity and oxidative stability all fall within acceptable limits [10]. Fully saturated fatty acids tend to add stability to the fuel, but can cause problems in cold weather [11]. Fatty acids rich in monounsaturated fatty acids are likely to be suitable and should make up a high proportion of the biodiesel. Polyunsaturated fatty acids are detrimental to fuel due to their susceptibility to oxidation [10]. The chain length is also important with C16 and C18 fatty acids being most suitable. Prakasam discussed the algal biomass, lipid and carbohydrate production of *Nannochloropsis salina*, *Dunaliella tertiolecta* and *Tetraselmis suecica* as a result of stressing the F2/medium by different carbon sources. Abomohra [2] discussed the efficiency of *Tetraselmis elliptica* isolated from Bardawil lagoon (hypersaline water) as a hopeful species for biodiesel feedstock. The maximum production of monounsaturated fatty acid profile of *T. elliptica* was recorded at the late exponential phase of the growth curve.

***Corresponding author:** Toulibah HE, Biological Sciences Department, Rabigh-Faculty of Science and Arts, King Abdulaziz University, P. O. box 344, Rabigh 21911, Saudi Arabia, Tel: +966555878142; E-mail: touliabah2@hotmail.com

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Current study focused the enhancement correlation between increased salinity in the medium as well as pH and the production of neutral lipids (TAG) of the *T. suecica*.

Materials and Methods

Microalgae and growth medium

Tetraselmis suecica (CCAP 66/4) was obtained from the Culture Collection of Algae and Protozoa, Oban, UK [12]. A primary stock culture (in a 100ml flask) was prepared using the liquid *T. suecica* samples received from the CCAP and allowed to grow in the culture room at 25 ± 1°C with continuous light (50 – 70 µmol m^{-2} s^{-1}) supplied by daylight fluorescent lights. When the culture reached the stationary phase after around 2 weeks incubation, F/2 was inoculated from the primary stock culture and allowed to grow to allow for adaptation to the medium before starting experiment.

F/2 medium

F/2 Medium is an artificial seawater medium and was prepared as described in Guillard and Ryther [13].

Lipid determination by Nile red fluorescence

A number of experiments have been done to measure neutral lipid concentration in *T. suecica*. Firstly, droplets of neutral lipids were visualised using a fluorescence microscope. Secondly, optimisation of Nile Red Fluorescence emission using the 96 well microplate methods involved optimisation of Nile Red concentration, cell concentration and time of staining.

Lipid body visualisation using a fluorescence microscope: Qualitative analyses of microalgae accumulate and store natural lipids in lipid bodies which appear as oil droplets inside their body. For visualisation of those lipid bodies Nile Red lipo-philic dye was used for staining and a Fluorescence Microscope with a Nikon Digital camera attached was used to capture the images. This approach is based on the work by Cooksey [14].

Nile red sample measurement test: Nile Red measurements on algal cells grown under different environmental conditions were done after performing optimisation test for Nile Red peak fluorescence and Nile Red concentration test based on the work done by Alonzo, Chen and Bertozzini [15-17]. Also, to find the optimise time for harvesting cells during the induction of lipid accumulation, measurements were performed on cells grown for 1 week, 2 weeks, 3 weeks and 4 weeks.

Lipid determination by direct trans-esterification methods: This work was done based on methods demonstrated by Griffiths [18]. The extracted FAMEs from the transesterification reactions were then identified by gas chromatography mass spectroscopy (GC-MS) using a Perkin Elmer - Auto System XL Gas Chromatograph (CHM-100-790) and Perkin Elmer - Turbo Mass Mass-Spectrometer (13657). The resultant peaks were identified and integrated using a Perkin Elmer's Turbo mass software linked to a NIST database.

Results

Visualisation of lipid bodies using fluorescence microscopy

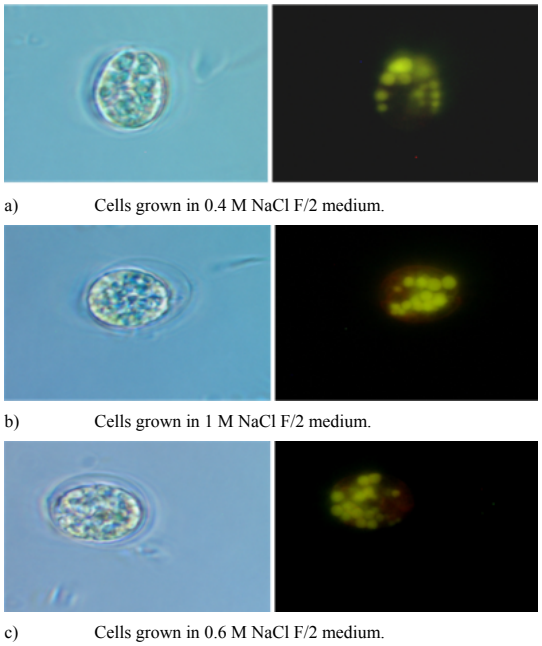
The accumulation of neutral lipids in *T. Suecica* cells was first monitored by examining NR fluorescence under the fluorescence microscope to check that the NR dye dissolved in acetone was able to penetrate into the lipid droplets within the *T. Suecica* cells. The microscope images in Figure 1 clearly show the yellow fluorescence of NR when it is staining neutral lipids (TAG). The staining shows that the neutral lipids are present as discrete droplets within the cells. There may be some indication that the amount of neutral lipid increased with increasing salinity, middle. There may be some indication that the amount of neutral lipid increased with increasing salinity, B) middle images being from highest salinity (Figure 1), but this is not a quantitative method. The key finding is that NR in acetone successfully entered the *T. Suecica* cells and that the stained neutral lipid droplets were obviously visualised under the fluorescence microscope.

Neutral lipid content of *T. suecica* cells grown at different salinities

This experiment focused to determine if there is any correlation between the increasing of salinity and the production of neutral lipids (TAG) in *T. suecica*. The accumulation of lipids in *T. suecica* was assessed using NR dye staining as optimised using 3 µmol/ml of NR dye concentration with the concentration of *T. suecica* cells set at OD_{595}=0.818 (87.5% cell concentration) and the measurement of fluorescence intensities 20 min. after staining the algal cells with NR dye. The test was performed every week for four weeks to investigate the effect of salt stress over a significant time period. Figure 2 shows that the highest levels of neutral lipids were observed with the highest NaCl concentration (1 M) after 4 weeks culture. The relationship between increasing the salt concentration and TAG accumulation was not straight forward. After two weeks growth, the lowest levels of TAG were found in the 1 M NaCl grown cells (Figure 2). It is clear that, age of culture is also important when considering the optimum conditions for TAG accumulation.

Neutral lipid content of *T. suecica* grown at different ph values

The influence of pH on the neutral lipid content of *T. suecica* was examined using NR staining dye as optimised using 3 µmol/ml



a) Cells grown in 0.4 M NaCl F/2 medium.

b) Cells grown in 1 M NaCl F/2 medium.

c) Cells grown in 0.6 M NaCl F/2 medium.

Note: The images on the left are taken under normal light and the images on the right show the same cell under fluorescent light conditions. The key finding is that NR in acetone successfully entered the *T. Suecica* cells and that the stained neutral lipid droplets were clearly visualised under the fluorescence microscope.

Figure 1: Fluorescence microscopy images of *T. suecica* cells stained with NR at a final concentration of 1 µmol/ml.

concentration of NR dye with the concentration of *T. suecica* cells ($OD_{595=}$0.818) and the measurement of fluorescence intensities at 20 min. after staining the algal cells with NR dye. The test was performed each week from 1 to 4 weeks after inoculation to examine the effect of pH stress over a long period. Figure 3 shows that, interestingly, the highest neutral lipid levels were observed in *T. suecica* cells grown at pH 9 for 2 weeks.

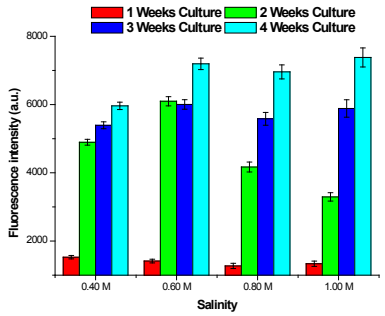


Figure 2: Neutral lipid content measured by NR fluorescence of *T. suecica* in F/2 medium with different concentrations of NaCl.

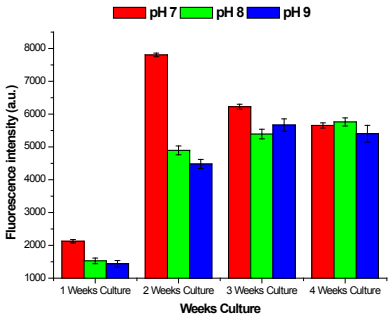


Figure 3: Effect of pH on Neutral lipid content measured by NR fluorescence of *T. suecica* cells grown in F/2 medium at different pH values.

From our result, by increasing pH values the neutral lipid values showed a remarkable increase till the fourth week of the experiments. Then the increase in pH values was not effective on the neutral lipid values anymore. The effects of pH are presented in Figure 3.

Fatty acid profiles of *T. Suecica* cells grown at different salinities

Figures 4-6 show the spectra of fatty acids found in *T. suecica* cells exposed to increasing salinity. In each figure, the top panel shows the fatty acid spectrum for cells grown in normal salinity (0.4 M NaCl) and two saturated fatty acids dominate the spectrum – hexadecanoic acid (palmitic acid, C16:0) and octadecanoic acid (stearic acid, C18:0). In cells grown in 0.6 M NaCl, several other fatty acids are detected in addition to the two-major saturated fatty acids i.e., pentadecanoic acid (C15:0), cis-10-heptadecanoic acid (C17:1), elaidic acid (C18:1) and linolelaidic acid (C18:2). At higher salinities (0.8 and 1 M NaCl), the same spectrum of additional fatty acids were found (Figures 5 and 6) It seems clear that with increased salinity above 0.4 M NaCl, more unsaturated fatty acids are produced in response to the salt stress.

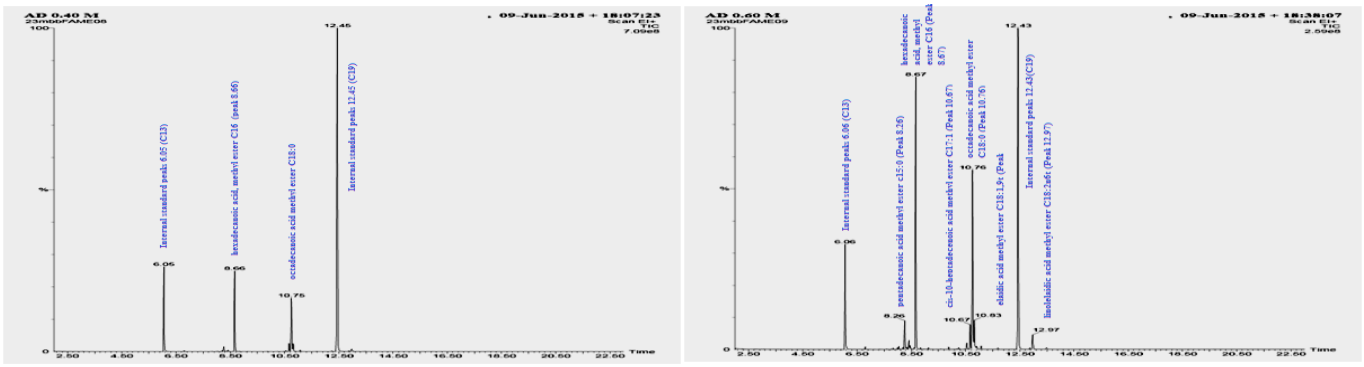
Fatty acid profiles of *T. suecica* grown at different pH values

Figures 7 and 8 shows that stressing the *T. suecica* cells, through changing the external pH to 7 or 9, had a very similar effect on the fatty acid profile as increasing the salinity. Again, a number of unsaturated fatty acids were produced in response to the change in pH including linolelaidic acid, which contains two double bonds and is a polyunsaturated fatty acid.

Discussion

The work aimed to identify the best conditions for neutral lipid accumulation in *Tetraselmis suecica*. This alga has been identified as a good candidate for the production of biodiesel and other fine chemicals on the basis of high lipid content and the fact that *T. suecica* is tolerant to a range of environmental extremes including high salinity and pH [19].

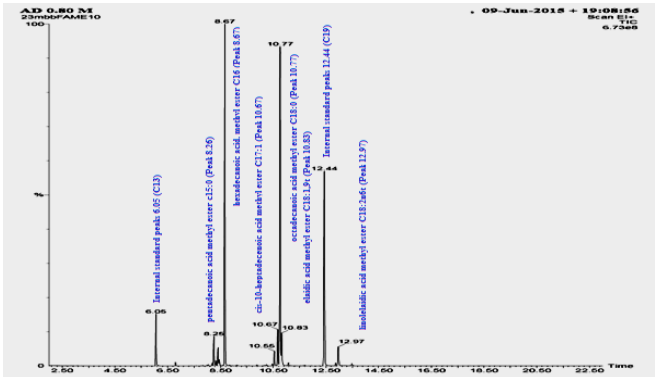
Nile Red (NR) fluorescence was shown to be a right method for



4a: *T. suecica* grown in normal conditions with 0.4 M salt concentration and pH 8.
The fatty acids accumulated were identified as:
(1) - hexadecanoic acid, methyl ester C16 (peak 8.66)
(2) - octadecanoic acid methyl ester C18:0 (Peak 10.75) and the other two peaks were internal standard peaks 6.05 (C13) and peak 12.45 (C19).

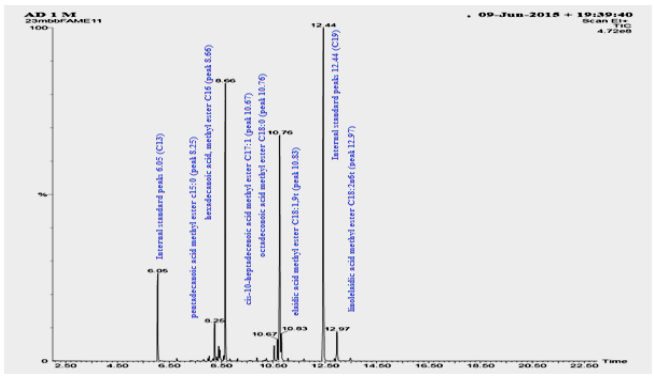
4b: T. Suecica cells grown under stress conditions with 0.6 M salt concentration and the fatty acids accumulated were:
(1) Pentadecanoic acid methyl ester c15:0 (Peak 8.26),
(2) Hexadecanoic acid, methyl ester C16 (Peak 8.67),
(3) Cis-10-heptadecenoic acid methyl ester C17:1 (Peak 10.67),
(4) Octadecanoic acid methyl ester C18:0 (Peak 10.76),
(5) Elaidic acid methyl ester C18:1,9t (Peak 10.83),
(6) Linolelaidic acid methyl ester C18:2n6t (Peak 12.97).

Figure 4: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. Suecica*.



T. suecica cells grown under stress conditions with 0.8 M salt concentration and the fatty acids accumulated were:
(1) Pentadecanoic acid methyl ester c15:0 (Peak 8.26),
(2) Hexadecanoic acid, methyl ester C16 (Peak 8.67),
(3) Cis-10-heptadecenoic acid methyl ester C17:1 (Peak 10.67),
(4) Octadecanoic acid methyl ester C18:0 (Peak 10.77),
(5) Elaidic acid methyl ester C18:1,9t (Peak 10.83),
(6) Linolelaidic acid methyl ester C18:2n6t (Peak 12.97).
The other two peaks were internal standard peaks 6.05 (C13) and 12.44 (C19).

Figure 5: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. suecica*.



T. suecica grown under stress conditions with 1 M NaCl concentration and the fatty acids accumulated were:
(1) Pentadecanoic acid methyl ester c15:0 (peak 8.25),
(2) Hexadecanoic acid, methyl ester C16 (peak 8.66),
(3) Cis-10-heptadecenoic acid methyl ester C17:1 (peak 10.67),
(4) Octadeconoic acid methyl ester C18:0 (peak 10.76),
(5) Elaidic acid methyl ester C18:1,9t (peak 10.83),
(6) Linolelaidic acid methyl ester C18:2n6t (peak 12.97).
The other two peaks were internal standard peaks 6.05 (C13) and 12.44 (C19).

Figure 6: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. Suecica*.

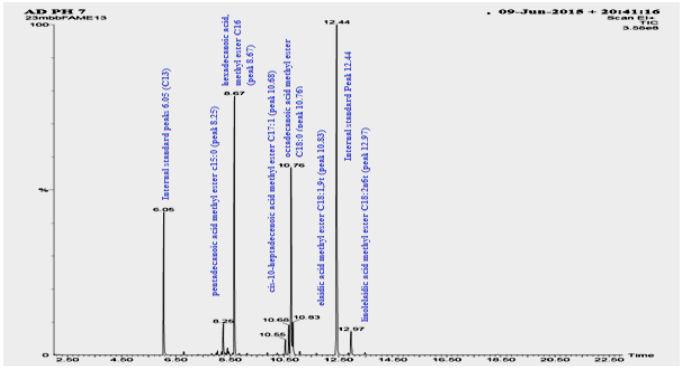
visualising lipid droplets inside *T. suecica* cells (Figure 1). The NR fluorescence method was then optimised for a quantitative assay in a 96 well plate reader format by setting the time required for peak fluorescence and the concentration of both cells and NR dye that are optimal. Using these parameters, the amount of neutral lipid (TAG) accumulated by *T. suecica* cells under salinity and pH stress was measured (Figure 2 and 3).

At the normal pH of 8, it was found that four weeks incubation at 1 M NaCl gave rise to the highest amount of TAG (Figure 2). In contrast at the normal salinity of 0.4 M NaCl, it was found that 2 weeks culture at pH 9 produced the most TAG. This established that environmental stresses can turn on TAG synthesis as has been shown previously for N-limited *Tetraselmis* cultures [20].

Conclusion

The conclusion to be drawn is that a large increase in salinity can induce increased TAG accumulation and high salinity may be the best condition to use for TAG synthesis in a commercial biofuel process based on *T. suecica* as the feedstock. This has the additional advantage that contamination of *T. suecica* cultures will be more easily controlled at elevated salinities [21-24].

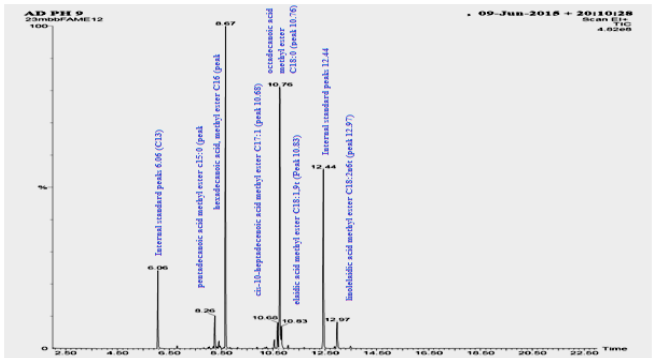
The fatty acid profiles of the TAG accumulated under salinity and pH stresses were measured using GC-MS. Any increase in salinity above 0.4 M NaCl induced the production of monounsaturated fatty acids and one polyunsaturated fatty acid. The mixture of saturated, unsaturated and polyunsaturated fatty acids looks very promising as a base for biodiesel production and should meet the requirements for cold-flow, ignition properties, viscosity and oxidative stability [10]. The same behaviour of *Nannochloropsis saline* was be obtained by Fakhry and El Maghraby [5]. They attributed the maximum production of the lipids as biodiesel feedstock was obtained as a result of depletion of



T. suecica grown under stress conditions at pH 7 and the fatty acids accumulated were:
(1) Pentadecanoic acid methyl ester c15:0 (peak 8.25).
(2) Hexadecanoic acid, methyl ester C16 (peak 8.67),
(3) Cis-10-heptadecenoic acid methyl ester C17:1 (peak 10.68),
(4) Octadecanoic acid methyl ester C18:0 (peak 10.76),
(5) Elaidic acid methyl ester C18:1,9t (peak 10.83),
(6) Linolelaidic acid methyl ester C18:2n6t (peak 12.97).

The other two peaks were internal standard peaks 6.05 (C13) and Peak 12.44 (C19).

Figure 7: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. Suecica*.



T. suecica grown under stress conditions at pH 9 and the fatty acids accumulated were:
(1) Pentadecanoic acid methyl ester c15:0 (peak 8.26),
(2) Hexadecanoic acid, methyl ester C16 (peak 8.67).
(3) cis-10-heptadecenoic acid methyl ester C17:1 (peak 10.68).
(4) Octadecanoic acid methyl ester C18:0 (peak 10.76),
(5) Elaidic acid methyl ester C18:1,9t (Peak 10.83),
(6) Linolelaidic acid methyl ester C18:2n6t (peak 12.97).
The other two peaks were internal standard peaks 6.06 (C13) and 12.44 (C19).

Figure 8: Comparison of GC-MS chromatographs for fatty acid accumulation in *T. Suecica*.

nitrogen and high levels of temperature from *Nannochloropsis saline* [25-30].

The fatty acid profiles were also determined using GC-MS and increasing salinity induced the synthesis of fatty acids with one double bond (monounsaturated), which are ideal for biodiesel production [10,11]. It appears likely that growing *T. suecica* at high salinity will result in a favourable mix of fatty acids for biodiesel.

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