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EFFECT OF TEMPERATURE ON THE LIPID CONTENT IN *Nannochloropsis oculata*, *Dunaliella salina* AND *Isochyrsis galbana* FOR BIODIESEL PRODUCTION

V. RAMESH KUMAR[*1] AND G. MELCHIAS[2]

[1] *Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Chennai, India.*
[2] *Department of Botany, St.Joseph's college, Tiruchirappalli, India.*

ABSTRACT

Microalgae, an autotrophic organism has been established as a potential source for biodiesel production. Suitable candidates from marine origin have been selected as viz., *Nannochloropsis oculata*, *Dunaliella salina*, and *Isochrysis galbana*. The microalgae were grown in controlled conditions and an assessment of their growth rate and lipid productivity were made. To analyze the effect of temperature on the growth of different microalga, temperature stress was induced. Biochemical changes in the culture and changes in fatty acid profiles of the culture before and after temperature stress are reported. Temperature stress increased lipid productivity in microalgae. The lipid obtained from microalgae were converted to biodiesel by chemical transesterification. Also the biodiesel thus obtained was mixed with commercial diesel to produce biodiesel blend in different ratios. The biodiesel properties of Biodiesel 20, Biodiesel 50 and Biodiesel 100 (pure biodiesel), were comparable to that of commercial diesel.

KEYWORDS: ***Nannochloropsis oculata, Dunaliella salina, Isochrysis galbana,*** **biodiesel, heat shock and transesterification.**

V. RAMESH KUMAR
Department of Biotechnology, Sathyabama University,
Jeppiaar Nagar, Chennai, India.

***Corresponding author**

INTRODUCTION

The world energy demand is growing day by day. The demand for refined products is forecast to rise at a higher rate[1]. The fossil fuels not only are diminishing but also excessive use of them leads to environmental pollution, global warming and climate change. A renewable, clean fuel as a stable energy alternative is the need of the hour, which will not only meet the world demand but would also mitigate climate change. This has led both the government and private industries in many a country to look for alternative sources of energy. The irony is that as many who work on renewable energy concentrate on electricity generation, but the majority of energy consumption in the world is in the form of liquid fuels. Finding sufficient supplies of clean energy for the future is one of the society's most daunting challenges and is intimately linked with global stability, economic prosperity and quality of life. Biofuels have been around as long as motor vehicles have. Availability of crude oil kept gasoline and diesel cheap and biofuels were forgotten. Rise in oil prices, global warming and environmental concern have driven biofuels popularity of late. Biodiesel fuel has received considerable attention in recent years, as it is made from non-toxic, biodegradable and renewable resources, and provides environmental benefits, since its use leads to a decrease in the harmful emissions[2,3]. Biodiesel is usually produced from oleaginous crops through a chemical trans esterification process of their oils with short chain alcohols, mainly methanol. Biodiesel can be a supplement to crude oil and can significantly address the environmental issues crude oil poses. Large-scale production of biodiesel based on edible oil has led to food shortages in many parts of the world. The use of waste oil as the triglyceride source is one promising approach for biodiesel production[4]. In addition, microalgae with high lipid content have been proposed as feedstock for biodiesel production[5,6,7]. Microalgae are ideal sources for liquid fuel production as they can accumulate a large amount of oil in their cells and have high biomass productivity[8,9,10,11]. More importantly, mass algal cultivation can be performed on unexploited lands using saline water in arid regions, thus avoiding competition for limited arable lands[12]. The transesterified oil from microalgae seems to be the only renewable biofuel with a potential to completely replace the existing transport fuels. Each microalgae produces different ratios of lipids, carbohydrates and proteins. These tiny organisms have the ability to manipulate their metabolism as a response to the chemical composition of the culture medium, which can be utilized to obtain high lipid productivity. Under natural conditions, photosynthesizing microorganisms are subjected to abrupt changes in temperature and irradiance as well as to deficiencies in mineral nutrition[12,13,14,15]. In order to cope with the unfavourable conditions, certain algal species have developed a number of adaptations, including coordinated synthesis of non-membranous lipids such as triacylglycerols[16] and carotenoids. These lipids accumulated by algal cells under stressful conditions are often deposited in cytoplasmic lipid globules (oil bodies)[17].

MATERIALS AND METHODS

(i) Culture conditions

The marine microalgal cultures for the present study viz., *Nannochloropsis oculata*, *Dunaliella salina*, and *Isochrysis galbana*, were sourced from the culture collection centre at CMFRI, Cochin, India. An economically viable f/2 medium was used to maintain axenic cultures of the microalgae. The cultures were maintained in 1L erylenmeyer flask at a constant temperature of 25□C in the culture room in an aseptic condition with a light ratio of 12:12h and at an intensity of 2000 lux. The cultures were harvested in their stationary phase, which they reached in their 25th day.

(ii) Stress induction

Simultaneous to growing microalgae in control conditions, replicates of microalgal cultures were given temperature stress by keeping them in an incubator at 55□C for 2 hours every day during their growth period of 25 days.

(iii)Productivity determination

The weight of dry biomass of the different microalgae were taken. This weight was used for the subsequent productivity calculations.

(a) Biomass productivity. The biomass productivity (mg L^{-1} d^{-1}) in the batch culture was calculated as follows;

$$P = \frac{(B_f - B_i)}{(t_f - t_i)}$$

where, P – Biomass productivity, B_f – Final biomass, B_i - Initial biomass, t_f – final time and t_i – initial time.

(b) Lipid productivity. The lipid productivity (mg L^{-1} d^{-1}) for the microalgal cultures were calculated as follows;

$$LP = \frac{(B_f.\%FA_f - B_i.\%FA_i)}{[100.(t_f - t_i)]}$$

where, LP – Lipid productivity, B_f – Final biomass, B_i – Initial biomass, $\%FA_f$ – Final fatty acid percentage, $\%FA_i$ – Initial fatty acid percentage, t_f – Final time and t_i – Initial time.

(iv) Biochemical composition

The 25 day control culture and stressed culture were subjected to the following biochemical studies.

(a) Estimation of carbohydrate. Carbohydrates were estimated using the anthrone method[18] with glucose as standard.

(b) Estimation of protein. Proteins were estimated using the Lowry's method[19] with BSA as standard.

(c) Total lipid measurement. To estimate the total lipid content in algal cells, dried algal cells were blended with 0.5mL distilled water and 3mL chloroform/methanol (2:1). The contents were mixed for 20 minutes in a shaker and then centrifuged at 10000rpm for 10 minutes. The chloroform phase was collected. The pellet was washed with chloroform for five times. All the chloroform phases were collected and were subjected to evaporation. The final weight was estimated to get the total lipid content.

(d) Chlorophyll-a estimation. The chlorophyll-a was extracted and its concentration was estimated[20]. The chlorophyll-a content was extracted in 90% acetone and estimated using a spectrophotometer. The concentration of chlorophyll-a was estimated using the following equation;

Chlorophyll-a = 11.93 A_{664} – 1.93 A_{647} [mg chl-a /L]

(v) Extraction of oil

Oil extraction process differs from the oil estimation process explained in 2.4.3. The oil from the microalgal biomass was extracted in a Soxhlet apparatus using n-hexane as solvent. The algal biomass were subjected to sonication with hexane and the resulting algal oil was extracted. Sonication was done at a pulse rate of 0.5sec and at a temperature of 42°C. The resultant extract was kept for evaporation of hexane. The oil settled down after the hexane had evaporated.

(vi) Lipid profiling

About 1.0 g of the algae was taken in a 250 ml round-bottomed flask with a ground-glass neck fitted with a reflux condenser. To this 40 ml of anhydrous methanol and 0.2 ml of a 60 g/l solution of sodium hydroxide in methanol were added. This was attached to the reflux condenser , shaken and heated to boil. When the solution was clear (usually after about 10 min), heating was continued for a further 5 minutes. Methyl orange indicator was added followed by 2% conc. sulphuric acid in methanol to neutralize, and was refluxed further for 30 minutes. The flask was cooled under running water and the contents were separated in a separating funnel. The flask was rinsed with 50ml of a petroleum ether and the organic layer was passed through anhydrous sodium sulphate. The petroleum ether layer was evaporated and sufficient acetone was added. From this 1µl was injected in to the chromatograph. The chromatograph was CHEMITO GC 8610 Flame ionization detector, with nitrogen and hydrogen as carrier gas. Oxygen was used for ignition purpose. The column was BPX – 70 (50% cyanopropyl, 50% methylsiloxane). The data was collected using winchrome software.

(vii) Transesterification

The extracted oil was evaporated to release the organic solvents in it. Also to 0.25g of sodium hydroxide (catalyst), 24ml of methanol was added and stirred for 20 minutes. The catalyst and methanol were taken in a conical flask and the microalgal oil was added to it. The conical flask containing the mixture was shaken for 3 hours at 300rpm. After allowing time for transesterification to occur the solution was kept for 16 hours to settle down in a separating funnel. When the biodiesel and glycerol layers separated clearly, the two layers were segregated. The biodiesel was washed with 5% water until it was clean. The

biodiesel was dried at room temperature for 12 hours.

(viii) Assessment of Biodiesel properties

The final biodiesel that was obtained from the microalgal lipids was subjected to a series of tests for standardization. The biodiesel as such was kept as Biodiesel 100. Since 100% biodiesel is not approved commercially by many countries, properties of biodiesel blends were studied. Biodiesel blends were prepared by mixing commercial diesel with biodiesel in the ratio of 1:1 and labeled as Biodiesel 50. Also a Biodiesel to commercial diesel blend was prepared in a 1:5 ratio and labeled as Biodiesel 20. The tests were done according to the ASTM standards viz., Density (ASTM D1298), Viscosity (ASTM D445), Flash point (ASTM D93), Acid value (ASTM D664), Sulphur (ASTM D5453) and Cetane number (ASTM D613).

RESULTS AND DISCUSSION

The cultured microalgae acclimatized easily to the laboratory conditions as is expected of marine organisms which are known for its stress tolerance. The microalgae, also survived the temperature stress that they were subjected to daily. They responded to stress through physiological changes in them, which can be seen from the different biochemical parameters analysed. Biomass productivity is a key parameter for the economic evaluation of biodiesel production from algae. The amount of reduction in biomass productivity is tabulated in Table 1 for all the microalgae under study. Under stress the biomass productivity, reduced for all the microalgae. The maximum amount of reduction was in *Isochrysis galbana* indicating that it was more susceptible to stress. *Nannochloropsis oculata* was tolerating stress very much as the biomass productivity was least affected and it resisted stress in that the biomass reduction was less pronounced compared to the other microalgae under study.

Table 1

Biomass productivity in control and stressed cultures

	Biomass productivity (DW in mg L^{-1} d^{-1})		Percentage reduction in biomass productivity (%)
	Control	Stressed	
Nannochloropsis oculata	202	174	13.86
Isochrysis galbana	138	109	21.05
Dunaliella salina	184	150	18.47

Lipid productivity would be a measure of the yield of lipid from a culture. This is tabulated in Table 2 for both control and stressed microalgal cultures. The lipid productivity has increased upon stress throughout all the microalgae used for the present study. Lipid productivity was maximum in *Nannochloropsis oculata* which is considered to be the model organism with respect to high lipid content. In *Dunaliella salina* and *Isochrysis galbana*, lipid productivity was very minimally increased due to stress.

Table 2

Lipid productivity in control and stressed microalgal cultures

Name of the microalgae	Lipid productivity (mg L^{-1} d^{-1})	
	Control	Stressed
Nannochloropsis oculata	36.45	45.29
Isochrysis galbana	29.15	30.55
Dunaliella salina	27.68	28.58

The effect of temperature on the levels of different biochemical parameters or biomolecules like proteins, carbohydrates and lipids is tabulated in Table 3 as milligrams per litre microalgae culture. The protein levels decreased quantitatively when stress was induced in the microalgae across all microalgae under the present study. Percentage wise there was an increased

protein level across all the microalgae under study.

Table 3
Measures of proteins, carbohydrates ,lipids and chlorophyll-a. (Values given as Mean ± SD of replicates)

Organism	mg/L	Total Protein	Total Carbohydrates	Total Lipids	Chlorophyll-a content (mg/L)
Nannochloropsis oculata	Control	141.36 ± 0.94	31.92 ± 0.5	72.82 ± 0.31	3.56±0.32
	Stressed	132.04 ± 0.62	20.7 ± 0.57	90.84 ± 0.75	2.88±0.24
Isochrysis galbana	Control	72.30 ± 0.83	24.48 ± 0.9	56.52 ± 0.46	2.66±0.23
	Stressed	67.70 ± 0.67	17.68 ± 0.46	61.48 ± 0.37	2.04±0.25
Dunaliella salina	Control	100.98 ± 0.48	42.54 ± 0.45	52.60 ± 0.41	2.56±0.28
	Stressed	99.4 ± 0.54	28.7± 0.44	57.60 ±0.54	1.78±0.16

There was a significant decrease in carbohydrate levels upon stress induction in all the microalgal cultures. Lipid concentration increased drastically after stress in all the marine microalgal cultures with *Nannochloropsis oculata* showing maximum increase. Temperature stress has a visible effect on reducing the chlorophyll-a content in the microalgal culture as shown in Table 3. The chlorophyll-a content in all the microalgal cultures reduced drastically as chlorophyll was in direct correlation with growth parameters for a cell. The reduction of chlorophyll-a content was higher in *Dunaliella salina* The chlorophyll-a content reduction was minimal in *Nannochloropsis oculata* Cumulatively all the microalgal cultures responded to stress as a result of which the chlorophyll-a content was reduced in correlation to growth measured with respect to biomass. The fatty acid profile in *Nannochloropsis oculata* is given in Table 4 for both the control and stress culture. The fatty acid is given as an % Total Lipid from the GC analysis. The overall saturated fatty acid concentration has increased significantly in *Nannochloropsis oculata.* after stress. Lauric acid, a small chain fatty acid, decreased to 25% when stress was induced. The medium chain fatty acids viz., Myristic acid, Palmitic acid and Stearic acid level increased phenomenally when temperature stress was induced in the microalga. The amount of oleic acid after stress increased noteably. However the level of unsaturated linoleic acid decreased to a bare minimal. Also the long chain fatty acid level (Arachidic acid) decreased by a huge percentage.

Table 4
Fatty acid profile in Nannochloropsis oculata

Fatty acid Nomenclature	Fatty acid	Control culture (% Total Lipid)	Stressed culture (% Total Lipid)
12:0	Lauric acid	0.04	0.02
14:0	Myristic acid	0.25	3.70
16:0	Palmitic acid	16.82	17.97
18:0	Stearic acid	19.25	26.45
18:1	Oleic acid	42.36	51.36
18:2	Linoleic acid	15.07	0.21
20:0	Arachidic acid	4.91	0.38

The fatty acid profile in *Isochrysis galbana* is given in Table 5 for both the control and stress culture. The fatty acid is given as an % Total Lipid from the GC analysis. The levels of fatty acids in *Isochrysis galbana* when subjected to temperature stress did not change much as compared to that of control cultures. There was an appreciable increase in the small chain fatty acid levels (Lauric acid and Myristic acid). Lauric acid levels were identified only upon stress. The quantity of medium chain fatty acids viz., palmitic acid and stearic acid decreased upon stress but only marginally. There was a slight increase in fatty acid levels in the case of Linoleic acid and arachidic acid. Significant amount of increase in levels of oleic acid and docosahexaenoic acid were estimated upon temperature stress.

Table 5
Fatty acid profile in Isochrysis galbana.

Fatty acid Nomenclature	Fatty acid	Control culture (% Total Lipid)	Stressed culture (% Total Lipid)
12:0	Lauric acid	-	0.03
14:0	Myristic acid	1.78	3.06
16:0	Palmitic acid	16.71	15.99
18:0	Stearic acid	4.90	3.76
18:1	Oleic acid	31.95	46.74
18:2	Linoleic acid	22.19	22.65
20:0	Arachidic acid	0.21	0.47
22:6	DHA	3.37	6.92

The fatty acid profile in *Dunaliella salina* is given in Table 6 for both the control and stress culture. The fatty acid is given as an % Total Lipid from the GC analysis. The level of small chain fatty acids in *Dunaliella salina* reduced dramatically upon temperature stress. Especially the small concentration of lauric acid found in the control cultures were totally absent after stress. The level of myristic acid came down by 50% whereas palmitic acid concentration was reduced by 25% after stress. This was true also in the long chain fatty acid (Arachidic acid) concentration, as it also reduced drastically to less that 50% upon stress. The concentration of stearic acid, oleic acid and linoleic acid increased after temperature stress. Especially the levels of stearic acid and oleic acid increased two fold and more upon stress.

Table 6
Fatty acid profile in Dunaliella salina

Fatty acid Nomenclature	Fatty acid	Control culture (% Total Lipid)	Stressed culture (% Total Lipid)
12:0	Lauric acid	0.59	-
14:0	Myristic acid	2.50	1.16
16:0	Palmitic acid	21.76	17.87
18:0	Stearic acid	2.40	4.59
18:1	Oleic acid	31.38	41.10
18:2	Linoleic acid	18.17	26.65
20:0	Arachidic acid	14.78	5.97

The lipid that is obtained from microalgae is converted to biodiesel through the process of transesterification. Transesterification is a process that can be done with a wide range of catalyst and different types of alcohol like methanol and ethanol. Different transesterification processes are validated by the biodiesel yield. The biodiesel yield from different microalgae are to be compared for identifying the one that yield's maximum amount of biodiesel. The biodiesel that is produced from different sources are to possess certain standards as devised by ASTM standards and Indian standards. Key parameters include density, viscosity, flash point, acid number, sulphur content and cetane number. The total biodiesel yield from a liter of microalgal culture (both control and stressed) was calculated and tabulated in Table 7 for the different microalgae under study. The biodiesel yield in all the microalgae before and after temperature stress ranged from 0.7-0.85. Of these the *Nannochloropsis oculata* and *Isochrysis galbana* cultures had a maximum increase in the yield percentage followed by *Dunaliella salina*.

Table 7
Biodiesel yield (ml/L) in control and stressed microalgal culture

Parameters	*Nannochloropsis oculata*	*Isochrysis galbana*	*Dunaliella salina*
Control	49	43	40
Stressed	57	50	44
Percentage increase	14	14	9

Important biodiesel parameters were analyzed in 100%, 50% and 20% biodiesel that were obtained by transesterification of microalgal lipids and were tabulated in Table 8. Also for comparative analysis of these with diesel fuel, ASTM standards and Indian standards for biodiesel are presented in Table 9.

Table 8
Fuel characteristics of Biodiesel and different blends

S.No.	Biodiesel Parameters	Biodiesel blend					
		Biodiesel 100		Biodiesel 50		Biodiesel 20	
		Control	Stressed	Control	Stressed	Control	Stressed
1	Density (Kg/L)	0.874-0.876	0.861-0.863	0.865-0.868	0.857-0.859	0.856-0.858	0.851-0.855
2	Viscosity (mm^2 /s) at 40°C	5.4-5.6	5.0-5.1	4.1-4.3	3.7-3.9	3.2-3.4	2.7-3.0
3	Flash point (°C)	114-117	110-112	101-102	98-100	92-94	88-91
4	Acid value (mg KOH/g)	0.352-0.355	0.347-0.350	0.193-0.195	0.186-0.191	0.127-0.129	0.122-0.124
5	Sulphur (mg/kg)	1.6 – 1.8	1.7 – 1.9	24.6 – 25.8	25.1 – 27.3	52.8 – 53.4	50.3 -52.5
6	Cetane number (min)	51-53	49-50	54-57	49-52	56 -58	53-55

Table 9
Fuel standard characteristics

S.No.	Biodiesel Parameters	Diesel Fuel	ASTM standards (D-6751)	Indian standards
1	Density (Kg/L)	0.838	0.86 – 0.9	0.86 – 0.9
2	Viscosity (mm^2 /s) at 40°C	1.9 - 4.1	1.9 – 6.0	2.5 – 6.0
3	Flash point (°C)	75	130	120
4	Acid value (mg KOH/g)	Max 0.5	Max 0.8	Max 0.5
5	Sulphur	63.1	50 mg / Kg Max	50 mg / Kg Max
6	Cetane number (min)	55.60	47	51

CONCLUSION

The results indicate that microalgae respond to temperature stress by increasing their lipid concentration. This process can be optimized for biodiesel production. The only hurdle being impaired growth which cannot be overlooked. However, for mass scale energy production, genetic engineering of the stress responsive genes can be a boon to satisfy energy demand in the future.

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