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# Culture and biofuel producing efficacy of marine microalgae *Dunaliella salina* and *Nannochloropsis* sp.

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## Abstract

Biodiesel from oil crops, waste cooking oil and animal fat cannot realistically satisfy even a small fraction of the existing demand for transport fuels. As demonstrated here, microalgae appear to be the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels. Marine microalgae *Dunaliella salina* and *Nannochloropsis* sp. was cultured in in-door and out-door systems and harvested biomass was utilized for biodiesel fuel production. The direct transesterification of microalgae biomass shows good biodiesel yield where *Dunaliella salina* yielded 66.6 % and *Nannochloropsis* sp. 68.5% respectively. It is inferred that the fatty acid methyl esters (FAME) such as palmitic acid (16:0), oleic acid (18:1) and linolenic acid (18:3) which found in microalgae are accountable for yielding more biodiesel than other oil crops. It is understood that, the marine microalgae *Nannochloropsis* sp and *Dunaliella salina* were considered as promising feedstock for biodiesel production.

## Introduction

The need of energy consumption is increasing continuously due to increases in industrialization and population. Accompanied by fossil fuel depletion, has led to a search for alternate biofuel sources. The basic sources of fossil fuels are petroleum, natural gas, coal, hydro and nuclear (Kulkarn *et al.*, 2006). However, continuous use of coal base fuel leads to environmental issues including $NO_2$, $SO_2$, $CO_2$, particulate matter and volatile organic compounds (Klass, 1998). Bioenergy is one of the most important components to mitigate greenhouse gas emissions and substitute of fossil fuel. Moreover, biodiesel is a non-toxic and biodegradable with low pollutant. Crops such as soya bean, jatropha, castor, coconut, animal fats and fish oils have been used to produce biodiesel but the price and supply of those sources are unstable. However, the use of microalgae can be a suitable alternative as a potential source of the future renewable bioenergy. Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels, foods, feeds and high-value bioactives (Metzger and Largeau, 2005; Spolaore *et al.*, 2006). The idea of using microalgae as a source of fuel is not new, but it is now being taken seriously because of the increasing price of petroleum fuel, more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Gavrilescu and Chisti, 2005). According to various reports microalgae were one of the best sources of biodiesel which can produce up to 250 times higher the amount of oil per acre as soybeans produces. In fact, producing biodiesel from microalgae may be only the way to produce enough automotive fuel to replace current gasoline usage. Today, the potential value of microalgal biodiesel is widely recognized. The oil productivity of many microalgae exceeds the best producing oil crops. Microalgal bio-refinery approach can be used to reduce the cost of making microalgal bio-diesel. Microalgal-based carbon sequestration technologies cover the cost of carbon capture and sequestration. Therefore, the present attempt was made on the culture and biodiesel production using marine microalgae as feedstock.

## Materials and Methods

*Microalgae culture*

The indoor stock cultures of microalgae, *Dunaliella salina* and *Nannochloropsis* sp. were maintained in air conditioned room. Conway's medium was prepared using autoclaved seawater in 2 L flasks. 10 ml of culture inoculums from the exponential phase was transferred to the culture flasks and incubated at 5000 lux using two fluorescent bulbs for 12:12 hrs light and dark cycle. The maximum exponential phase was obtained after 8-10 days. The temperature and salinity

were maintained in the ranges between 23 and 25ºC and 28 and 30 ‰ respectively for entire culture period. The continuous aeration was provided for culture.

For large scale production of microalgae, 100 liters FRP tanks were used. The fully grown stock culture was used as inoculum for mass culture. For efficient growth of algae commercial fertilizers namely, ammonium sulphate, super phosphate and urea in the ratio of 10:1:1 are added. For 100 litres of seawater 2 litres of inoculum was added to the culture tank. The continuous and vigorous aeration was provided to keep the culture always in suspension besides uniform distribution of nutrient in the medium.

*Estimation of algal density and biomass*

Microalgal biomass was harvested by filtering the culture through Millipore filtering equipment using filter paper (0.45cm dia.) and also by centrifugation at 3000 rpm for 10 min. The cell pellet was washed twice with distilled water. The collected cell pellets were dried in an hot air oven at 80ºC for 40 min. and the weight was determined gravimetrically ($g/l^1$). The density of algae in culture system was determined by cell counts using Sedgewick counting chamber under light microscope followed by method of Venugopalan and Paulpandian (1989). The biomass of microalgae was estimated by the standard method of Strickland and Parsons (1972). 10ml of algal culture was filtered by using Millipore filtering system fitted with a 4.5cm diameter GF/C filter paper by applying low suction. Before filtering the sample, a thin bed of magnesium carbonate (2 ml) was made for effective filtration. After the filtration, the filter paper was removed by using clean forceps and ground with 90% acetone using mortar and pestle. The ground samples are transferred to screw cap test tubes and covered by using black cloth and incubated in the refrigerator for 24 hrs. The contents were regrinding with 90% acetone and centrifuged at 3000 rpm for 10 min. The optical density was measured at different wavelengths of 630, 645 and 665nm for chlorophyll ‘a’ estimation.

*Biodiesel Production*

The biodiesel from microalgae was produced by direct transesterification as described by Johnson and Wen (2009). The dried algal biomass (1 g) was placed in a glass test tube and mixed with 3.4ml of methanol, 0.6ml of sulfuric acid and 4.0 ml of chloroform. The reaction mixture was heated in a water bath at 90ºC for 40 minutes. After the reaction was completed, the tubes were allowed to cool at room temperature. 2 ml of distilled water was added to the tubes and mixed for 45 seconds. Then the samples were centrifuged at 3000 rpm for 10 min. to accelerate phase separation. The organic layer that contained biodiesel (FAME) was collected and transferred to a pre-weighed glass vial. The solvent was evaporated using nitrogen gas and the biodiesel was determined gravimetrically.

*Estimation of total lipid*

The lipid concentration of microalgae was estimated by the method of Bligh and Dyer (1959). 10 mg of dried sample was homogenized with 10 ml of chloroform: methanol mixture (2:1v/v). The homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was washed with 0.9% saline solution (KCl) to remove the non-lipid contents and allowed for phase separate. The upper phase was discarded by siphoning. The lower phase was allowed to dry in an oven and the lipid was weighed and calculated using standard formula.

*Estimation of fatty acids*

About 0.45 g of microalgae sample was transferrred into a 10 ml volumetric flask, dissolved in hexane containing 50 ml of butyl hyroxytoluene per litre and made up to 10 ml. 2 ml of the solution was transferred into a quartz tube and the solvent was evaporated with a gentle current of nitrogen. 1.5 ml of sodium hydroxide (20 g/l) in methanol was added to above and covered with nitrogen cap tightly with a polytetrafluoroethylene lined cap mix and solution was kept in a water-bath for 7 min. at 90˚C followed by cooling. The 2 ml of boron trichloride-methanol solution was added and covered with nitrogen cap tightly and mixed and heated in a water-bath for 30 min. at 90˚C and cooled the solution to 40-50 ºC. One ml of trimethylpentane was added to this sample and capped. The sample was vortex vigorously for 30 seconds. The saturated sodium chloride solution was added immediately, covered with nitrogen cap and vortexes the sample for at least 15 seconds. The upper layers was allowed to become clear and transferred to a separate tube. Then the methanol layer was shaken properly once again with 1 ml of trimethylpentane and combine the trimethylpentane extracts. The combined extracts were washed with 2 quantities, each of 1 ml of water and dried over anhydrous sodium sulphate. The combined extracts were washed with 2 quantities, each of 1 ml of water and dried over anhydrous sodium sulphate, two solutions were prepared for each sample. 1µl of sample was injected twice, quality and quantity of fatty acids were identified by Gas Chromatography installed with fused silica column in the size of l = 30 m, Ø = 0.25 mm. The helium was used as carrier gas for chromatography R where oxygen scrubber applied.

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## Results

*Cell density and biomass of microalgae*

In the present study, marine microalgae *Dunaliella salina* and *Nannochloropsis* sp. were cultured in both in-door and outdoor system successfully with maximum cell density and biomass. The present results on microalgae culture indicated that their production efficiency was economically chief and environment friendly technique. It is well known that the microalgae can grow rapidly when they get favourable environmental condition. The daily growth in terms of cell density and biomass in terms of chlorophyll 'a' concentration of *D. salina* and *Nannochloropsis* sp. were estimated and results were given in Figures 1 to 8. *D. salina* shows maximum growth and biomass on 9$^{th}$ day when the dark green colour bloom obtained with a maximum cell density (Figs. 1-4). The growth in terms of cell density of *D. salina* in indoor system was found in the range between 23, 925 and 2, 14, 300 cells/ml (Fig. 1). The maximum cell density was noticed on 9$^{th}$ day of the culture whereas the minimum was obtained on 1$^{st}$ day. The *D. salina* biomass in terms of chlorophyll 'a' concentration was found in the range between 0.029 and 0.5847mg/10ml (Fig. 2). The maximum biomass was procured on 10$^{th}$ day of culture, while minimum density obtained on the first day. After 9$^{th}$ day the declining phase was started. The cell density of *D. salina* in out-door system was ranged from 34, 900 to 3, 56, 250 cells/ml with maximum density obtained on 9$^{th}$ day whereas minimum on initial day (Fig. 3). In outdoor system, the maximum biomass (0.6158 mg/10ml) was gained on 9$^{th}$ day. However the minimum biomass (0.0311) was acquired at first day (Fig. 4). In in-door system, *Nannochloropsis* sp. was grows to a maximum density of 1, 20, 582cells/ml during the 10$^{th}$ day (Fig. 5) whereas the low cell density was reported (31, 000 cells/l) on 1$^{st}$ day of culture. The biomass of *Nannochloropsis* sp. was noticed in the range between 0.1028 and 0.5954 mg/10ml with maximum at 10$^{th}$ day and the minimum at 1$^{st}$ day (Fig. 6). In out-door culture, the density was found to maximum (1, 42, 000 cells/ml) on 10$^{th}$ day and minimum (25, 825 cells/ml) at 1$^{st}$ day (Fig. 7). The biomass of *Nannochloropsis* sp. was reported maximum at 10$^{th}$ day with 0.6358 mg/10ml whereas the low biomass of 0.0954 mg/10ml was recorded on initial day

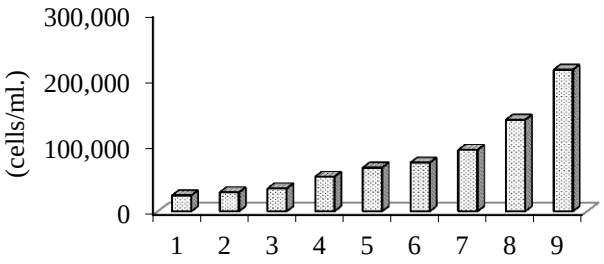


**Fig.1 Daily growth (cells/ml) of *Dunaliella salina* at indoor culture system**

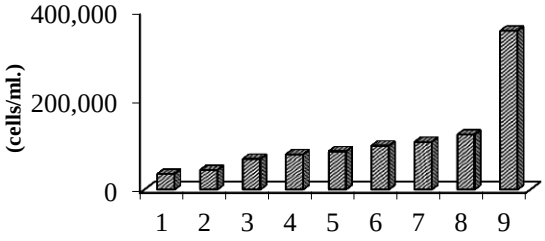


**Fig.3 Daily growth (cells/ml) of *Dunaliella salina* at out-door culture system**

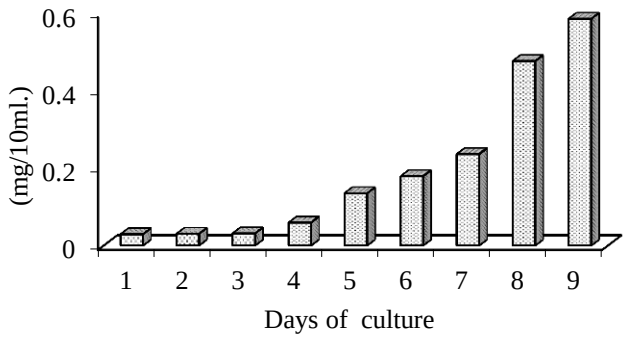


**Fig.2 Daily biomass (mg/10ml) of *Dunaliella salina* at indoor culture system**

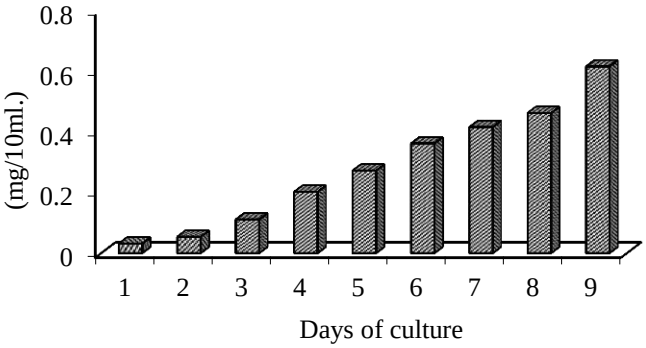


**Fig.4 Daily biomass (mg/10ml) of *Dunaliella salina* at out-door culture system**

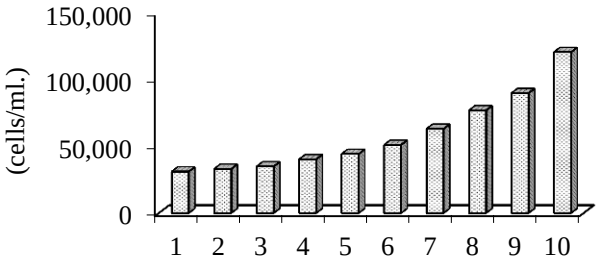


Fig.5 Daily growth (cells/ml) of *Nannochloropsis* sp. at indoor culture

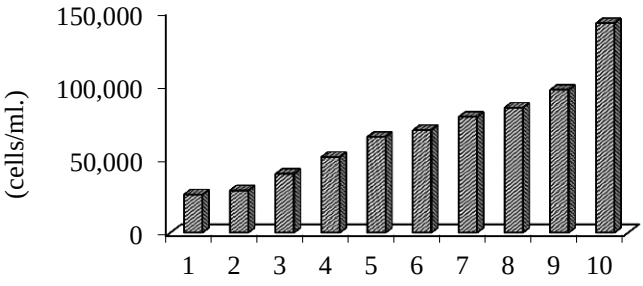


Fig.7 Daily growth (cells/ml) of *Nannochloropsis* sp. at out-door culture

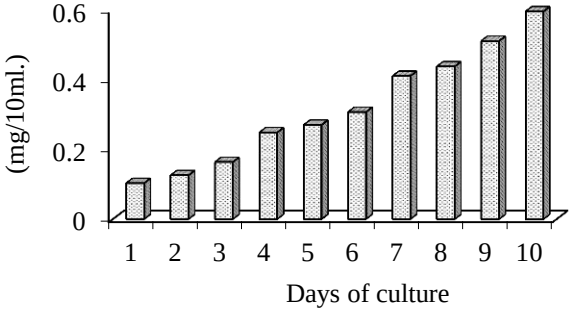


Fig.6 Daily biomass (mg/10ml) of *Nannochloropsis* sp. at indoor culture

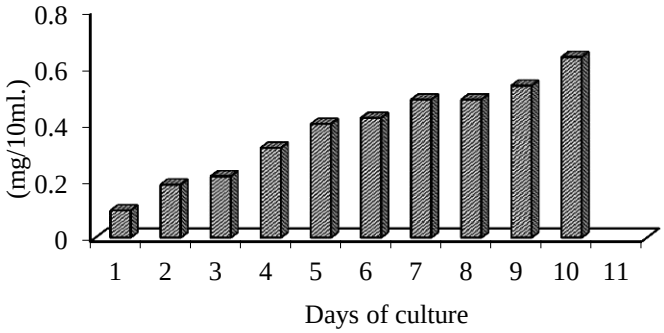


Fig.8 Daily biomass (mg/10ml) of *Nannochloropsis* sp. at out-door culture

*Biodiesel Yield*

There are different methods are available for biodiesel production from microalgae. Among these, direct transesterification is the efficient one. Hence, in the present study**,** the biodiesel was extracted through direct transesterification. The results on biodiesel yield of marine microalgae *D. salina* and *Nannochloropsis* sp. was compared with biofuel yield of oil crops. The direct transesterification method was resulted the maximum biodiesel yield of 68.5% in *Nannochloropsis s*p. followed by *Dunaliella salina* where the biodiesel yield reported as 66.6% (Table 1). However, the biodiesel yield of oil crops such as Jatropha, coconut, groundnut, white soy bean, castor and red soy bean were 62.2, 57.4, 51.2, 37.8, 33.4 and 9.4 % respectively. The gravimetric biodiesel yield of *Nannochloropsis* sp. and *D. salina* was 1.028 and 1.0 (g/1.5g DW of biomass) respectively. However, gravimetric biodiesel yield of Jatropha, coconut, groundnut, white soy bean, castor and red soy bean were 0.933; 0.862; 0.768; 0.568; 0.501 and 0.141 respectively (Table 1).

**Table 1 Biodiesel yield of marine microalgae and oil crops.**

| Crops | Gravimetric Biofuel yield (g/1.5g dw) | Percentage of Biofuel yield |
|---|---|---|
| Castor | 0.501 | 33.4 |
| Jatropha | 0.933 | 62.2 |
| White soy bean | 0.568 | 37.8 |
| Red Soy bean | 0.141 | 9.4 |
| Coconut | 0.862 | 57.4 |
| Groundnut | 0.768 | 51.2 |
| *Dunaliella salina* | 1.0 | 66.6 |
| *Nannochloropsis* sp. | 1.028 | 68.5 |

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The total lipid content of algae *D. salina* and *Nannochloropsis* sp. were 21.2 and 27.5 % respectively. However, the lipid content of oil crops such as coconut, groundnut, white soy bean, red soy bean, castor and jatropha were 47.8, 42.33, 3.99, 3.33, 43.5 and 50.83 % respectively (Table 2). Totally 9 fatty acids were reported in *D. salina* that including 14:0, 16:0, 16:1, 16:2, 16:3, 16:4, 18:1, 18:2 and 18:3 (Table 3). In general, *D. salina* contains highest percentage of poly unsaturated fatty acids viz., 18:3 (30.26%) and 18:2 (13.24%) followed by mono unsaturated fatty acids like 16:4 (11.24%), 16:2 (3.0%), 16:3(1.16%) and saturated fatty acid such as 16:0 (18.20%), 14:0 (0.51%). As in case of *Nannochloropsis* sp. mono unsaturated fatty acid such as 16:1 was observed in maximum level (26.18%) followed by saturated fatty acid (16:0) with 24.85%. However, the fatty acids like 20:5, 20:4, 18:1 and 18:2 were noticed in the level of 16.53, 3.45, 14.65 and 2.89% respectively.

Table 2 Total lipid content (%) of marine microalgae and oil crops.

| Crops | Lipid (%) |
|---|---|
| Castor | 43.5 |
| Jatropha | 50.83 |
| White soy bean | 4.0 |
| Red Soy bean | 3.33 |
| Coconut | 47.8 |
| Groundnut | 42.33 |
| *Dunaliella salina* | 21.2 |
| *Nannochloropsis* sp. | 27.5 |

Table 3 Fatty acid composition (%) of *Dunaliella salina* and *Nannochloropsis* sp.

| Fatty acids | *Dunaliella salina* | *Nannochloropsis* sp. |
|---|---|---|
| 14:0 | 0.51 | 7.24 |
| 16:0 | 18.20 | 24.85 |
| 16:1 | 0.98 | 26.18 |
| 16:2 | 3.0 | 0.76 |
| 16:3 | 1.16 | 0.68 |
| 16:4 | 11.24 | - |
| 18:0 | - | 0.92 |
| 18:1 | 4.56 | 14.65 |
| 18:2 | 13.24 | 2.89 |
| 18:3 | 30.26 | - |
| 20:4 | - | 3.45 |
| 20:5 | - | 16.53 |

**Discussion**

In the present study, marine microalgae *D. salina* and *Nannochloropsis* sp. were produced the maximum cells and biomass in both in-door and out-door culture systems. The optimization of culture condition was of confronting complexity with many interrelated factors that can each be limiting the growth of algae. The temperature, salinity, pH, dissolved oxygen and nutrients were contributing for good algal production as agreed by Rao *et al.* (2007). The maximum cell density were observed on 9$^{th}$ and 10$^{th}$ day of culture period for *D. salina* and *Nannochloropsis* sp. respectively could be attributed to the provision of favourable illumination and continuous aeration as reported by Srinivasakumar and Rajashekhar (2009) and Rekha *et al.* (2012). In our experiment, the algae were cultured using Conway's medium which was found more suitable for culture of *D. salina* and *Nannochloropsis* sp. so that the maximum cell density and biomass was possibly achieved (Santhanam *et al.*, 2000; Rekha *et al.*, 2012). In the present experiment, the outdoor algal culture was maintained at the temperature of 29-30$^{o}$C by providing partial shading might be the reason for good growth and biomass obtained as agreed by Santhanam *et al.* (2000) who obtained the maximum density of *S. costatum* in the temperature of 29$^{o}$C in out-door culture system. Presently, *D. salina* and *Nannochloropsis* sp. was resulted maximum biomass which is several folds higher than that of previous report (Claudio *et al.* 2009). Both the algae are grown more rapidly and besides greatest density might be owing to short life span and physiological condition of the species and the biomass of both species doubles every 24 hours during the exponential phase, thus reaching high density. It is clear that, the presently obtained algal biomass is more enough to produce the significant amount of biodiesel.

In our study, we have followed the direct transesterification method using chloroform as solvent for biodiesel production which results the maximum biodiesel yield of 68.5 and 66.6 % for *Nannochloropsis* sp. and *D. salina* respectively as reported earlier by Johnson and Wen (2009) and Rekha *et al.* (2012) who depicted that the direct transesterification resulted the high biodiesel yield in microalga *Schizochytrium limacinum* compared to indirect transesterification. Due to the inherent nature of single stage reaction, direct transesterification was much less time consuming one than the oil extraction and transesterification process. It also avoided the potential

lipid loss during the extraction stage, as a result the direct methylation led to a higher crude biodiesel yield. In our study, we used acid as a catalyst for the transesterification because of the high free fatty acid value and thus soap formation in the alkali-transesterification. Furthermore, in the present study, we have used chloroform solvent for FAME extraction which yields more biodiesel. It is proved that the low biodiesel yield in microalgae when no solvent was used in the direct transesterification, indicating that the solvent is essential for reaction (Nimcevic *et al.*, 2000; Jhonson and Wen, 2009).

In our study, both algal species shows considerable level of fatty acids (Table 3). *D. salina* and *Nannochloropsis* sp. showed C14:0, C16:0, C16:1, C16:2, C18:0, C18:1, C18:2 and C18:3 fatty acids. It is known that the recorded fatty acids in the algae are responsible for reporting rich biodiesel yield than crops which has low fatty acid methyl esters as supported by Thomas *et al.* (1984) and Rekha *et al.* (2012). The high fatty acid content in microalgae might be due to various nutritional and environmental factors maintained during their cultivation (Gouveia and Oliveira, 2009). Although the microalgae oil yield is strain-dependent it is generally much greater than other vegetable oil crops, as shown in Table 1 that compares the biodiesel production efficiencies and other crops, including the amount of oil content in a dry weight basis and the oil yield in percentage as agreed by Hossain and Salleh (2008). The present study indicated that the lipid content of presently studied crop plants was comparatively higher than that of microalgae. Even though, the microalgae produced more biodiesel it could be due to the presence of more FAME in microalgae than oil crops. Furthermore, microalgae have higher conversion efficiency of algal oil into biodiesel and this biodiesel productivity with a clear advantage for microalgae (Huang *et al.*, 2010; Singh and Singh, 2010).

The both microalgae possess a favourable fatty acids profile that can be utilized for biodiesel production with high oxidation stability. The lipids are produced in algal cells as they utilize both inorganic ($CO_2$ from atmosphere) and organic form of carbon (energy substrate from growing ambience). Various classes of lipids (both polar and neutral) such as triglycerides, phospholipids, cholesterol, etc, are produced among which triglycerides are the major raw lipid precursor for biodiesel production. Triglycerides are found have more in presently studied microalgae so that they can deliver the high oil yield than crop seeds as agreed by Huang *et al.* (2010). Presently, the fatty acid such as 20:5, 18:1, 16:1 and 16:0 was found to be maximum in *Nannochloropsis* sp. it might be a reason for enhancing the biodiesel yield of the species. Similarly the fatty acids such as 18:3, 18:4, 16:4 and 16:0 was observed in high percentage which ascribed to increased biodiesel yield in *D. salina* (Rekha *et al.* 2012).

It is very clear that the marine microalgae are able to grow extremely rapidly, generally doubling their biomass within a day. Moreover, they can grow on saltwater found in the coastal belt which is not suitable for domestic and agriculture purpose. The commercial production of biodiesel from microalgae practically successful in developed countries. Moreover the microalgae are attractive is that they can assimilate carbon dioxide as the carbon source for growth which contributes to atmospheric $CO_2$ reduction. In addition, microalgal biofuel is similar to those produced by fossil and crops and it can be used directly to run existing diesel engines or as a mixture with crude oil diesel (Jhonson and Wen, 2009). So, due to the immeasurable advantages behind marine microalgae, it is understood that presently studied marine microalgae *Nannochloropsis* sp. and *D. salina* can be considered as potential feed stock for biodiesel production to compete the future energy crisis.

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