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SELECTION, CHARACTERIZATION AND MASS CULTIVATION OF MICROALGAE FOR BIODIESEL PRODUCTION

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Abstract:-Now a days, Global warming, CO_2 discharge, petrofuels price hike and many other problems that lead us to think thoroughly for green future and renewable energy. Biodiesel is the only alternate renewable liquid fuel to petro based fuels. About ten microalgae strains were isolated from twelve fresh and brackish water samples. Potential lipid producing microalgae strains were screened based on Nile red staining method and their species level identification was done by LSU (D1-D2) region sequencing. Lipids from selected microalgae species were characterized by Gas Chromatography and Mass Spectroscopy (GC-MS) and Fourier Transform Infra Red Spectroscopy (FT-IR). The results showed *C. vulgaris* and *A. obliquus* produces more saturated fatty acid, whereas *N. gaditana* and *I. galbana* showed maximum Poly unsaturated fatty acids (PUFA) accumulation. *C. vulgaris* and *N. gaditana* showed higher biomass productivity (1.4 and 1.6 g/l) when growing in open raceway pond than other microalgae species. Therefore *C. vulgaris* and *N. gaditana* are suitable for large scale production of biodiesel.

Keywords: Biodiesel, Fatty acids, Lipids, Microalgae, Mass Cultivation.

INTRODUCTION:-

Biodiesel is a viable alternative to the current use of petroleum-based fuels in the transportation sector because it is a renewable resource and can be used in traditional diesel engines with little or no modifications. The microalgae are the potential feedstock for biodiesel production. Oil yield per area of microalgae culture can exceed the yield of the best oilseed crops. Microalgae can be cultivated easily in seawater, brackish water and on non arable land (Li et al., 2008). Microalgae are highly biodegradable, environmentally sustainable, while reducing emissions of particulate matter, CO_2, hydrocarbons and SOx. Microalgae lipids mainly composed by 90– 98% of triglycerides, and smaller amounts of mono and diglycerides and free fatty acids, and residual amount of compound lipids like phospholipids, phosphatides, carotenes, sulphur compounds (Kong et al., 2007).

In the first process of microalgae biofuel research, collection, Isolation and identification of microalgae for various potent strains are the major tasks to assess their lipid productivity by analyzing physiology and biochemistry. Microalgal collection is mainly influenced by environmental factors (both biotic and abiotic), parameters measured onsite, type of aquatic system and sampling equipment (Brennan and Owende, 2010).

The up scaling of unialgal cultures in outdoor open culture systems is often a challenging process which depend on weather, water chemistry (salinity, turbidity, pH, dissolved oxygen, chemicals), biological contamination and operational factors. One of the main challenges of large-scale cultivation of microalgae in outdoor raceway ponds is that biomass productivities obtained over extended periods of time are low, ranging between 10-37 g (DW).$m^{-2}.d^{-1}$ (Grobbelaar, 2009c; Pulz, 2001) while higher biomass productivities between 30 and 40 g (DW).$m^{-2}.d^{-1}$ have been achieved only on a short-term basis (Goldman, 1980).

In the present study, lipid producing indigenous fresh and marine microalgae strains were isolated, identified and screened. Lipids were extracted and transesterified by using different ratios of solvent mixtures. Tranesterified FAMEs were characterized by FT-IR and GC-MS. Finally, optimization of open raceway pond cultivation system for potential microalgae strains mass production were done.

MATERIALS AND METHODS

Sample Collection, Isolation and Screening

Inland Fresh water algal samples were collected from Pragatheswara Temple tank (13°06'17.29"N, 80°27'25.13"E) Triplicane, Chennai, Parthasarathi Temple tank (13°05'38.53"N, 80°27'79.89"E) Triplicane, Chennai, Nagaraja Temple tank (8°16'79.57"N, 77°56'49.87"E) Nagercoil, Vattakottai pond (8°12'56.89"N, 77°42'08.36"E) Kannyakumari District, Melmaruvathur (12°43'10.19"N, 79°82'77.17"E) kanchipuram District Tindivanam Rock pond (12°23'34.94"N, 79°64'67.64"E), Villupuram District and Brackish water algal samples were collected from Nemmeli (12°72'12.75"N, 80°22'00.26"E) kanchipuram District, Mahabalipuram (12°.58'56.43"N, 80°16'68.97"E) Kanchipuram District, Kulayankarisal (8°70'57.67"N, 78°08'11.23"E) Thuthukudi District , Kovalam (8°09'20.94"N, 77°48'38.33"E) Kanniyakumari District, Muttam (8°07'65.73"N, 77°54'45.96"E), Kanyakumari District and Rameshwaram (9°32'72.84"N, 79°30'67.9"E), Tamil Nadu, India. The samples were collected between May 2010 to June 2010. The algae were subjected to purification by serial dilution followed by plating. The individual colonies were isolated and inoculated into Bold basal media for freshwater microalgae and F2 liquid media (Guillard and Ryther, 1962) for marine water microalgae. The plates were incubated under light intensity at 120 µmol photons/m-2/s-1 on 12:12 h Light/Dark with 25 °C and were regularly checked for any presence of microalgae. High lipid producing microalgae strains were screened based on nile red staining method (Thi Thai Yen Doan et al., 2011).

Molecular Phylogenetic Characterization

Doyle and Doyle protocol was used to isolate DNA of Screened microalgal species. Large ribosomal (LSU) D1-D2 region primer (5'-AGCGGAGGAAAAGAAACTA-'3 as forward 5'-TACTAGAAGGTTCGATTAGTC-'3 as reverse) were used to characterize the phylogeny of microalgae (Sigma, USA).

PCR were performed in a total reaction mix of 50µl of the isolated genomic DNA of four samples to amplify LSU D1-D2 gene. The PCR mixture contained 1µl of isolated genomic DNA (50 ng), 1 µl primer (10 pmol/µl), 2 µl 10 mM dNTPs, 5 µl 10X PCR buffer containing MgCl2, and 1µl 5 U/µl Taq DNA Polymerase. The PCR reaction was conducted with the initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 45 seconds, annealing at 62°C for 60 seconds and elongation at 72°C for 2 min. Totally 34 cycles were performed, the final elongation step at 72°C for 10 min. The PCR product was electrophoresed in a 1 % (w/v) agarose gel stained with ethidium bromide and observed on an UV transilluminator. DNA was eluted by using Gene Jet Gel extraction kit (Fermentas). Sequencing was done at Scigenom Labs Pvt Ltd, Cochin, using the big dye terminator kit in ABI 3730 XL DNA analyzer. A Phylogenetic tree was constructed using the neighbour – joining (NJ) algorithm using Kimura's two parameter model of sequence evolution, in the MEGA4 software package (Tamura and Dudley, 2007). The high lipid producing screened indigenous native microalgal strains *(Chlorella vulgaris, Acutodesmus obliquus, Isochrysis galbana, Nannochloropsis gaditana)* LSU rRNA D1-D2 regions gene sequence were amplified, sequenced and submitted to GenBank (Table. 1)

Table 1: Microalgae, location and 28S rDNA accession number used in this study

Microalgae	Accession number (NCBI)	Base pair
***C. vulgaris* (SE002)**	JX401408.1	781bp
***A. obliquus* (PRR02)**	JX413595.1	834bp
***I. galbana* (SEISO)**	KC461129	500bp
***N. gaditana* (SE006)**	JX401413.1	407bp

Lipid Extraction and Characterization

Lipids of the selected species were extracted at early stationary phase of growth cycle using Bligh and Dyer method, (1959). The transesterification reagent was prepared by adding 0.30 g NaOH and 2ml of methanol. After adding the reagent the solution was kept for 16 h to settle the biodiesel and sediment layers clearly.

The transesterified lipid extracts from each microalgae species were characterized by FT-IR analysis, the samples containing lipid fractions were analyzed in NaCl pellet. (Resolution: 4 cm^{-1}, Scan Number: 3, Scan range: 450 cm1 to 4000 cm^{-1}) by FT-IR (Perkin Elmer model spectrum-I PC). The spectra of each sample were recorded.

The methyl esters of fatty acids were quantified by a gas chromatograph (Agilant-JEOL GC & MS). The column (HP5) was fused silica 50m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C, 3 min at 235°C for column temperature, 240°C for injector temperature, helium (99.9% purity) was the carrier gas and split ratio was 5:4. Fatty acids

methyl esters were identified by comparison with known standard mixtures (PUFA no.3 cat.47085-U, Supelco, USA) and comparison of their retention times with authentic standards by GC–MS post run analysis by Spectral Data Base Software (SDB Software). The FAME determination was done by calculating area percentage of each fatty acid peaks response from detector.

Optimization of Mass Cultivation

An outdoor algal pond was constructed with the wall thickness of 0.27 m and 4.50 × 2.12 × 0.79 m length, width and depth. A partition wall was constructed in the middle of the pond with a length of 3.68 m and a width of 0.25 m. The floor was constructed with a slight slope on either side of the partition in the opposite direction. The inside of the pond was covered with ceramic tiles lining and provided with a tap water connection for preparation of the medium. The metal framed paddlewheels (5) were fixed at 90° angles used for adequate mixing of the culture in order to increase aeration and minimize the shading effect as culture density increase. The pond was covered by transparent plastic greenhouse material to prevent flooding due to excess rainwater accumulation, prevent dust exposure, maintain temperature and also serve to diminish contamination from airborne sources.

Prior to culture inoculation, the open ponds were cleaned and inoculated with 10 up to 50% of final culture volume. The initial culture depth was kept at 10 cm (100 L) until the culture was dense enough to provide sufficient biomass production. More medium was subsequently added until the desired cell density was reached. *Chlorella vulgaris, Acutodesmus obliquus* were grown in improvised CFTRI medium and *Isochrysis galbana, Nannochloropsis gaditana* were grown in F2 medium with the incubation temperatures ranging from 20°C to 30°C with natural day/night cycle (20000–75000 lux). Cell growth, water level, pH, salinity and water temperature were recorded daily. Evaporative losses were made up with tap water restore the salinity. The biomass was harvested after 30 days.

Growth rate was measured by Spectrophotometer (Hitachi U 2900) throughout the study periods. Optical wavelength of 680 nm was determined for the cultures and the results range from 677 nm to 684 nm. Growth rate (K) and generation time (G) was calculated by the equations 1 and 2 (Qin, 2005).

$$K = \frac{\log ODf - \log ODi}{T} \times 3.322 \longrightarrow (1)$$

ODf : Final optical density, ODi : Initial optical density, T : time in days

$$G = \frac{0.301}{K} \qquad (2)$$

RESULTS AND DISCUSSION

Identification and Screening

The algae were selected based on their morphologies. Compound microscopic images of the microalgal species isolated in this study are shown in Fig 1a. Microscopic analysis of the samples allowed preliminary identification of isolates as genus Chlorella, Scenedesmus, kirchenerrialla, Haematococcus, *Chlorococcum, Isochrysis, Nannochloropsis, Dunaliella, Nitzchia* and *Navicula* respectively. The identification of microalgae was based on their distinctive characteristics (Butcher, 1959; Carmelo and Grethe, 1997). Totally ten microalgae from different genera, were investigated with Nile red staining method. The fluorescence was read from lipid content in the cells was evaluated during the 20 day. *Chlorella, Scenedesmus, Isochrysis, Nannochloropsis* strains yield very strong fluorescence with great 492a.u intensity. Whereas *Dunaliella, Haematococcus* and *Chlorococcum* showed moderate emissions (39 – 80 a.u) and poor emission were observed in *kirchenerrialla, Nitzchia* and *Navicula* (13 – 28 a.u). Fig. 1b showed fluorescent microscopic images of the ten microalgal strains from Tamil Nadu

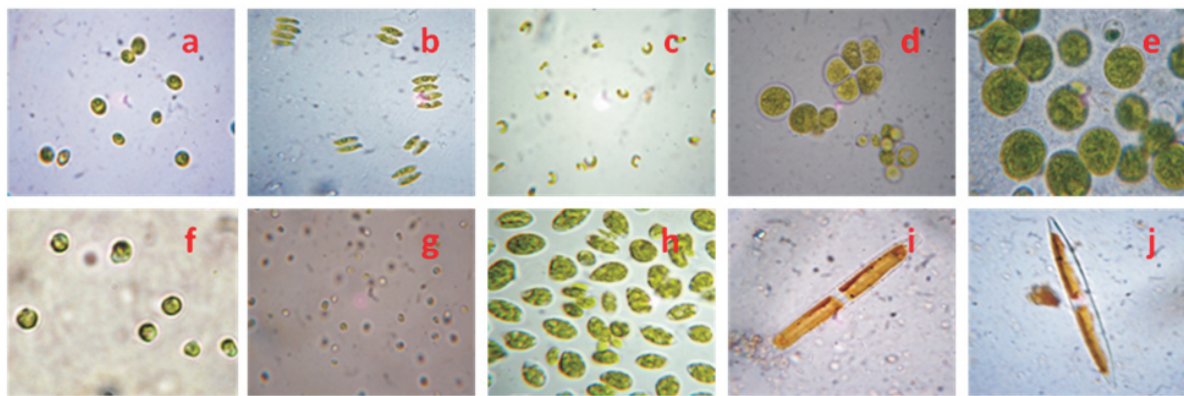

Fig. 1a Microscopic View of isolated Microalgae (100X) ***a. Chlorella vulgaris, b. Scenedesmus obliquus, c. kirchenerrialla lunaris, d. Chlorococcum ellipsoideum, e. Haematococcus pluvialis, f. Isochrysis galbana, g. Nannochloropsis gaditana, h. Dunaliella salina, i. Navicula diverta, j. Nitzchia divergens***

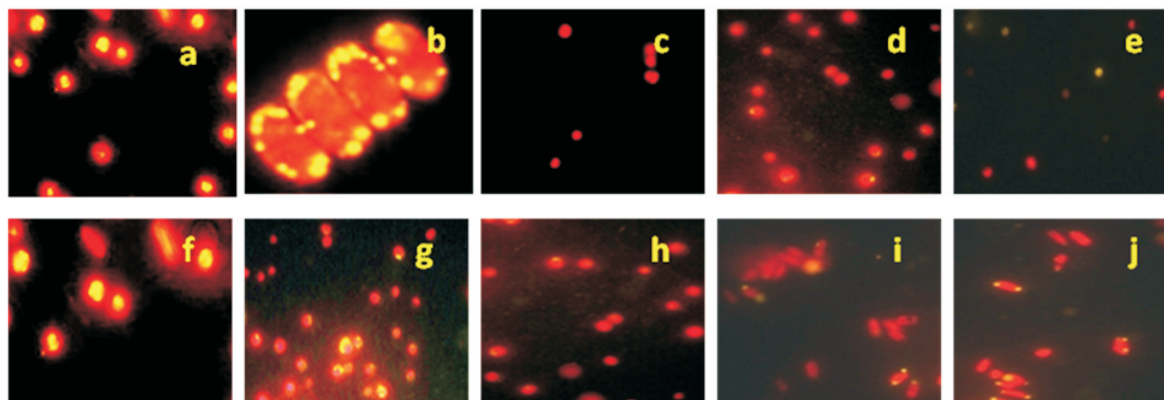

Fig. 1b Nile Red Staining Observations of isolated microalgae (100X) ***a. Chlorella vulgaris, b. Scenedesmus obliquus, c. kirchenerrialla lunaris, d. Chlorococcum ellipsoideum, e. Haematococcus pluvialis, f. Isochrysis galbana, g. Nannochloropsis gaditana, h. Dunaliella salina, i. Navicula diverta, j. Nitzchia divergens***

Molecular Phylogeny

A single band of amplified 28S LSU rDNA product with a size of ~ 850 bp was recorded for all isolates. The LSU rRNA gene has a higher evolutionary rate than the SSU rRNA gene and should therefore allow for better discrimination between closely-related species using short diagnostic sequences. Based on the LSU rDNA (D1- D2) sequences, we concluded that microalgal isolates were closely related to *Chlorella vulgaris, Acutodesmus obliquus, Isochrysis galbana and Nannochloropsis gaditana* based on 98%, 97%, 99% and 95% sequence similarities, respectively.

Identification of the four microalgal strains was also supported by the results from the phylogenetic analysis of the LSU rDNA D1-D2 sequence. The LSU rDNA sequences of isolates, SE002, PRR02, SEISO and SE006 confirmed their identification as *Chlorella vulgaris, Acutodesmus obliquus, Isochrysis galbana* and *Nannochloropsis gaditana*; they had sequence similarities of 98%, 97%, and 99% and 95 to, *C. vulgaris* JX401408.1, *A. Obliquus*, JX413595.1, *I. galbana* KC461129, and *N. gaditana* JX401413.1, respectively (Fig. 2).

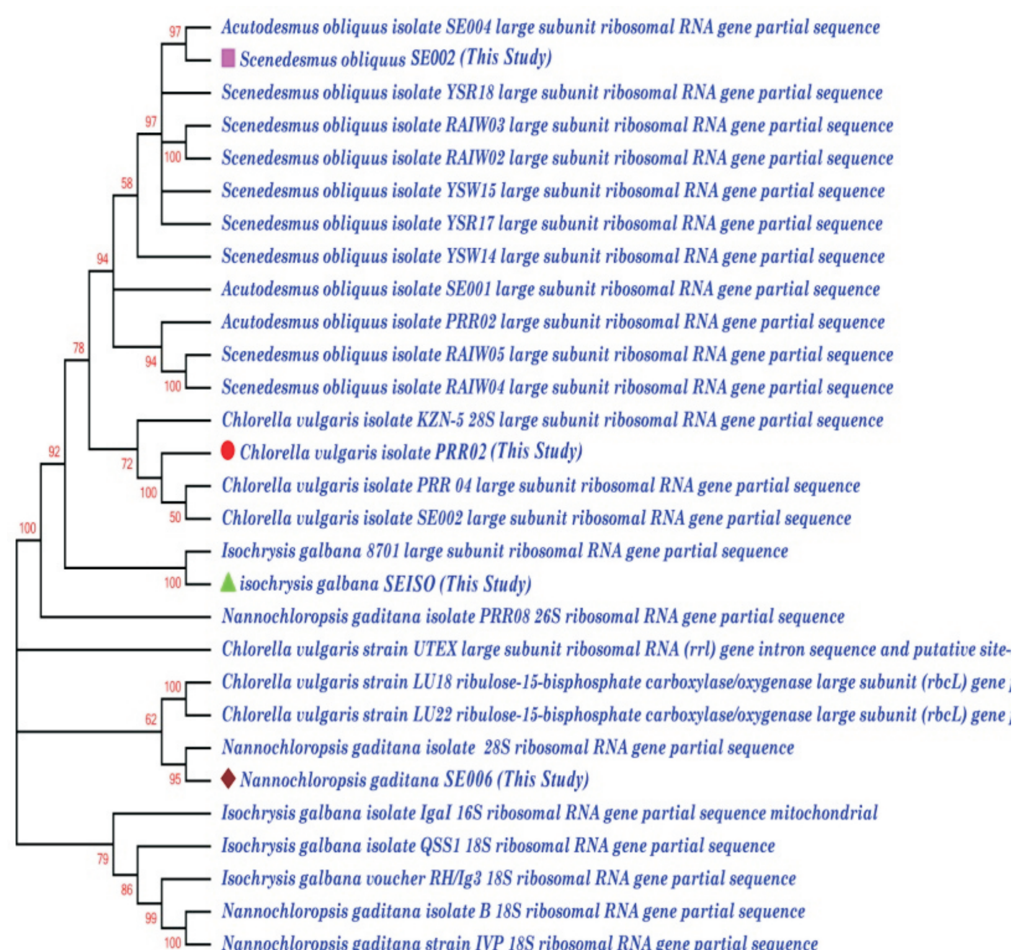

Figure 2: Phylogenetic tree showing the relationships among LSU rDNA D1-D2 sequences of isolates SE002, PRR02, SEISO and SE006.

Fatty acid Characterization

FT-IR Analysis

In *Chlorella vulgaris* C-H absorption bands characteristic of the vibrations of C-H bonds, corresponds to aromatics compounds. C-C stretching vibrations produce a strong band of aromatic compounds in the 1452 cm^{-1} region. The C-O corresponding to ethers, alcohol, carboxylic acids, esters C-N bonds with aliphatic amines stretching vibrations produce a strong band in the 1000 – 1320 cm^{-1}.

In *Acutodesmus obliquus*, C-H bonds correspond to aromatic compounds and N-H bonds with 713 cm^{-1} of 1'2' amines as functional group. The C-H bonds stretching absorption band in the region of 2922 cm^{-1} and 1456 cm^{-1} with alkanes. The O-H stretching band and carboxylic acids give bands at 2958 cm^{-1} and 2851 cm^{-1}.

In Isochrysis galbana N-H, C-H and C-Cl they produce strong bands in 729 cm^{-1} region of the IR spectra region of 1°2' amines aromatics and alkyl halides. C-O stretching absorption band in the region of 1080 cm^{-1} produce a strong band with compounds alcohol, carboxylic acid, ester and ethers between 1320 – 1000 cm^{-1} O- H stretching absorption band in the region of 2923, 2955 and 2852 cm^{-1} of the IR spectra corresponding to carboxylic acid. The C-H bond stretching absorption band in the region 1456 cm^{-1} with alkanes.

In Nannochloropsis gaditan, the OH stretching band with alcohol, phenols gives bands at 3369 cm^{-1} region with N-H corresponding to 1'amines. The C-H bond stretching absorption band in the region of 2922 cm^{-1} with alkanes. The O-H stretching band and carboxylic acid gives band at 2853 cm^{-1} region. The main characteristics of the IR spectra of C-O stretching vibration correspond to alcohols, carboxylic acids, esters, and ethers (Table. 2).

Table 2: FT-IR of Functional Groups of Constituents from Microalgae

Microalgae	Peaks	Bond	Functional group
C. vulgaris	736 cm^{-1}	N-H, C-H, C-Cl	1°2'amimes, aromatics , alkyl, halides
	1085 cm^{-1}	C-O, C-N	alcohol, carboxylic acid, ester, ethers
	1317 cm^{-1}	N-O	nitro compounds
	1452 cm^{-1}	C-C	Aromatics
	1644 cm^{-1}	-C=C-	Alkenes
	2136 cm^{-1}	-C-C-	Alkynes
	3402 cm^{-1}	O-H	Alcohol or Phenol
S.acutodesmus	3395 cm^{-1}	N-H, O-H	1°2'amimes, amides, Alcohol or Phenol
	1456, 2922 cm^{-1}	C-H	Alknes
	1642 cm^{-1}	- C=C-	Alkenes
	2162 cm^{-1}	- C=C-	Alkynes
	2851, 2958 cm^{-1}	O-H	Carboxylic acids
	713 cm^{-1}	C-H	Aromatics
I. galbana	729 cm^{-1}	N-H, C-H, C-Cl	1°2'amimes, aromatics, alkyl halides
	1080 cm^{-1}	C-O	alcohols, ester, ethers,
	1213 cm^{-1}	C-H, C-N	alkylhalides, aliphatic amines
	1456 cm^{-1}	C-H	Alkanes
	1642 cm^{-1}	- C=C-, N-H	Alkenes,1' amines
	2852, 2955, 2923 cm^{-1}	O-H	Carboxylic acids
	3399 cm^{-1}	O-H, N-H	Alcohol, Phenol, 1°2'amimes, amides
N. gaditana	724 cm^{-1}	C-H, C-Cl	aromatics,alkyl halides
	1058 cm^{-1}	C-N, C-O	aliphatic amines, alcohols, carboxylic acid, ester, ethers
	1462, 2922 cm^{-1}	C-H	Alkanes
	1630 cm^{-1}	N-H	1°2'amimes
	2853 cm^{-1}	O-H	Carboxylic acids
	3369 cm^{-1}	O-H	Alcohol, Phenol,

Gas Chromatographic and Mass Spectroscopic observation

Fatty acids in the four microalgae *Chlorella vulgaris, Acutodesmus obliquus, Isochrysis galbana and Nannochloropsis gaditana* were primarily esterified and the major fatty acid composition of each isolates was determined using GC analysis (Table 3). The fatty acids profiles of the Chlorella vulgaris isolates indicated the presences of tridecanoate acid (C13:0), pentadecanoate acid (C15:0), 9-tetradecenoate acid (C14:1), 11-hexadecenoate acid (C16:1), 6,11-octadecadienoate acid (C18:2), 6,9,12-hexadecatrienoate acid (C16:3), 9,12,15-hexadecatrienoate acid (C16:3), 15,18-tetracosadienoate acid (C24:2), 5,9,17-tetracosatrienoate acid (C24:3). In *Acutodesmus obliquus* fatty acids profiles of the isolates, showed the presence of tetradecanoate acid (C14:0), pentadecanoate acid (C15:0), hexadecanoate acid (C16:0), 11-hexadecenoate acid (C16:1), 9-heptadecenoate acid (C17:1), 7,10-hexadecadienoate acid (C16:2), 6,11-octadecadienoate acid (C18:2).

Table 3: Fatty acid composition of the four micro algal species

Fatty Acid	*C. vulgaris*	*A. obliquus*	*I. galbana*	*N. gaditana*
Saturates				
C12:0	nd	nd	7.39	nd
C13:0	15.58	nd	nd	15.24
C14:0	nd	21.98	nd	10.32
C15:0	17.61	5.17	nd	nd
C16:0	nd	6.38	19.27	nd
MUFA				
9-C14:1	28.81	nd	nd	nd
11-C16:1	3.19	24.20	nd	nd
9-C17:1	nd	2.71	nd	6.70
9-C18:1	nd	nd	12.19	3.47
PUFA				
7,10-C16:2	nd	26.63	39.99	17.03
6,11-C18:2	2.18	12.96	nd	19.39
6,9,12-C16:3	25.20	nd	nd	nd
9,12,15-C16:3	1.89	nd	nd	nd
3,9,12-C18:3	nd	nd	6.80	nd
15,18-C24:2	3.45	nd	nd	nd
5,9,17-C24:3	2.03	nd	nd	nd
5,9,19-C26:3	nd	nd	14.33	10.68
Saturates	33.19	33.53	26.66	25.56
MUFA	32	26.91	12.19	10.17
PUFA	34.75	39.59	61.12	47.1

The *Isochrysis galbana* isolates of fatty acid (Table 3), indicated the presence of dodecanoate acid (C12:0), hexadecanoate acid (C16:0), 9-octadecenoate acid (C18:1), 7,10-hexadecadienoate acid (C16:2), 3,9,12-octadecatrienoate acid(C18:3), 5,9,19- octadecosatrienoate acid (C26:3) (Fig. 42). The fatty acids profiles of the *Nannochloropsis gaditana* isolates, showed the presence of triecanoate acid (C13:0), tetradecanoate acid (C14:0), 9-heptadecenoate acid (C17:1), 9-octadecenoate acid (C18:1), 7,10-hexadecadienoate acid (C16:2), 6,11-octadecadienoate acid (C18:2), 5,9,19-octadecosatrienoate acid (C26:3). All the four microalgae showed PUFAs high in percentage compared with saturates and Monoenes. I. galbana showed the rich PUFA among the microalgae (Fig. 3).

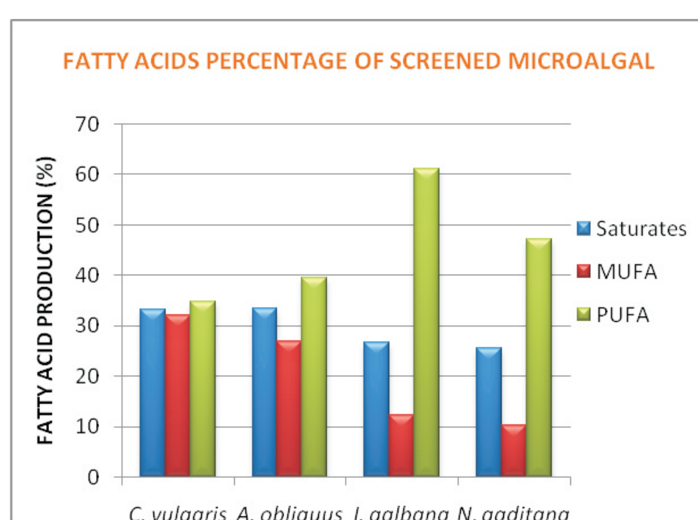

Figure 3: Statistical graph of fatty acid production from four microalgal strains

Outdoor Cultivation

Four kilolitre of CFTRI medium and four kilolitre of F2 media were prepared and poured equally into four separate open ponds. Five litre of actively grown culture of *Chlorella vulgaris Acutodesmus obliquus, Isochrysis galbana*, and *Nannochloropsis gaditana* were inoculated in individual pond. The four cultures were harvested after 30 days (Fig. 4).

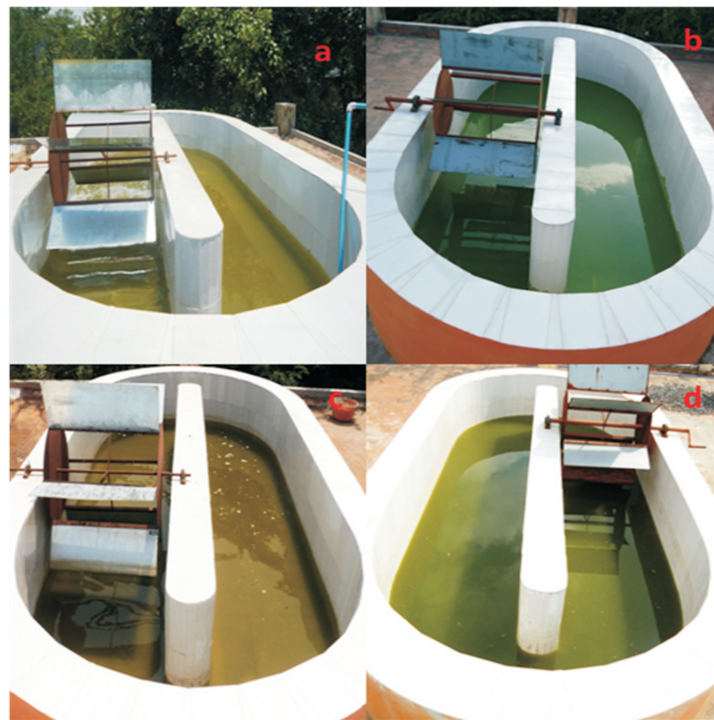

Fig. 4 Open Raceway Ponds containing *a. Chlorella vulgaris, b. Acutodesmus obliquus, c. Nannochloropsis gaditana, d. Isochrysis galbana* culture

The cultures were sampled once in every 2 days and the cell growths were measured using a Spectrophotometer. The cultures entered the logarithmic phase in which the cell growth increased to on the 22-26 day. The cells were in the stationary phase for 2–3 days before coming down to 30th day (Fig. 5).

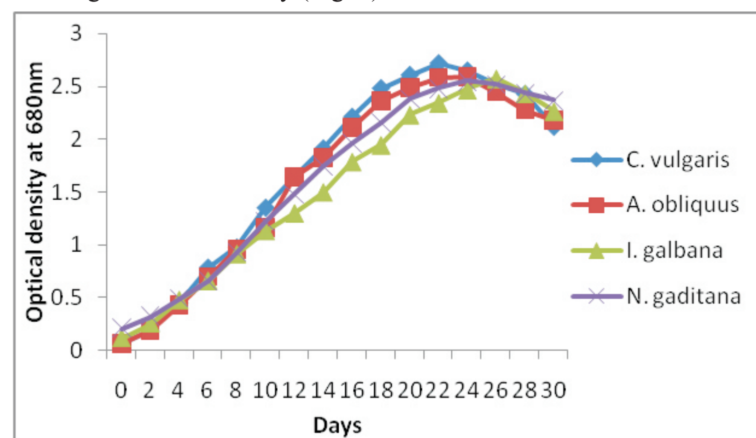

Figure 5: Growth pattern of four different strains from open pond culture

Samples were drawn from the *Chlorella vulgaris, Acutodesmus obliquus, Isochrysis galbana and Nannochloropsis gaditana* culture tank on a routine basis. Both pH and conductivity were measured daily and the results indicate that the pH level rise from 8.01 on day 0 to 9.62 on day 25. For all the 25 days, the increase in pH was gradual and after the 25 day, it around 9.65. Regarding the electrical conductivity, it was around 4.5 mm hos/cm throughout the study (Fig. 6a)

Water temperatures were ranged between levels of 28°C to 36°C. Biomass showed an increase with a decrease in temperature during days 16 to 22 and decrease in days 24 to 30 that correlated with an average increase in temperature for said

period (Fig. 6b).

At regular intervals, the samples were withdrawn from the open pond for studying bacterial counts. The bacterial cell numbers were measured by standard plate count method (pour plate technique) after serially diluting it with saline. The bacterial numbers showed a gradual increase up to 15 days and thereafter it reduced with the increase in algal cell numbers. Contamination by other microalgae, yeast and anaerobic bacteria were commonly observed.

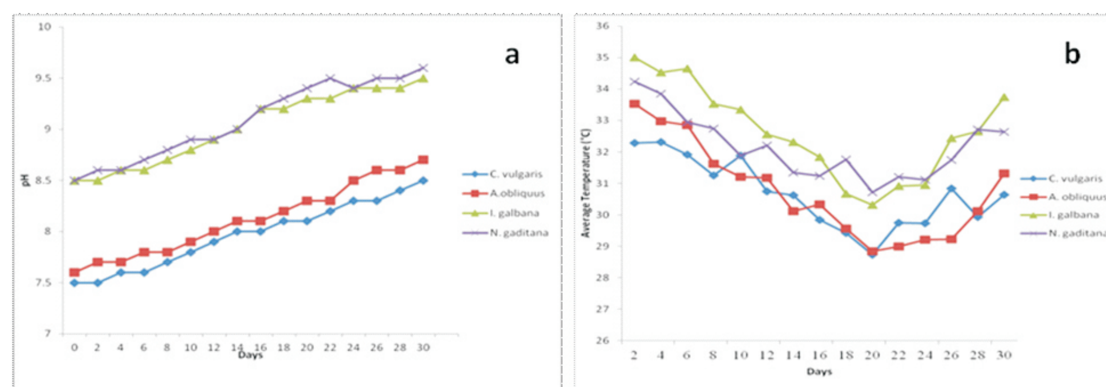

Figure 6a: pH graphs of different microalgae, 6b: Water temperature graphs for open pond

DISCUSSION

The isolated microalgal cultures such as *C. vulgaris, A. obliquus, I. galbana* and *N. gaditana* were successfully identified and the best lipid produced algal strain was confirmed by Nile Red fluorescence staining method. The 28S ribosomal DNA (D1-D2) region sequences were used for the identification of algal species. The fatty acid contents were determined by FT-IR and GC-MS. Various algal biomass were collected and analysed for various parameter from open raceway system. The microalgal strains were screened based on Nile red staining method for biofuel production. Kalacheva *et al,* (2002) reported *Botyrococcus* produce storage lipids and Triacylglycerides in fewer amounts during initial stage of inoculation and it was reported 2% of lipid accumulation had occurred after 13th of cultivation. Therefore in our present study we took culture after 13th for staining and measured the lipid content of the cells. In this period the microalgal cells can accumulate substantial amount of lipids such as TAGs, which serve as storage compound and act as important source for biofuel production (Illman et al., 2000).

Chlorella species was known best lipid producing candidate (provost *et al*., 2011). Chlorella species is generally had high amount of C16:0 fatty acids, the fatty acids are suitable for conversion to biodiesel. Cheng *et al*, (2009) reported *Chlorella* sp produces certain fatty acids. *Scenedesmus* sp is reported to accumulate 21.1% lipid content per gram of dry cell weight (Chen *et al*., 2009). *Chlorella* sp has been found to accumulate between 28% and 36% lipid content per gram of dry cell weight (Chiti, 2007; Chen et al., 2009). The aim of the study is isolation of high lipid yielding algal species for the production of biodiesel. Being a low value product, Logical choice for industrial production is that of an open pond system. The high oil producing microalgae are generally slower growing microalgae, the culture of choice should therefore be of an extremophilic and indigenous to enable a competitive advantage in open pond culturing by the adaptation for culture conditions to favor the culture of choice over contaminant strains (Liu *et al*., 2011)

The ability to cultivate species at increased pH is highly beneficial for open pond systems as these systems requires highly selective environments (Brennan and Owende, 2010). Maintenance of high pH (10 -11) is possible to achieve growth of single microalgal species as a dominant monoculture however requires an extreme culture environment that support only a small number of strains (Singh et al., 2011). Elevated pH serves to chemically alter nutrient compositions by volatilization of ammonia and precipitation of phosphorus with unheated metals (Park et al., 2011). Oswald, (1988) eluded that the toxicity brought about by high pH. Thus the most appropriate method to control zooplankton and bacteria is increase of pH to 11 (Park et al., 2011), this is the range of optimal pH for many microalgal species. Our experiment showed as gradually increase in pH reaching to around 7.5 to 8.5 for *C. vulgaris,* 7.6 to 8.7 for *A. obliquus,* 8.5 to 9.5 for *I. galbana* and in *N. gaditana* 8.5 to 9.6 (Table 19). Temperature was measured in the open pond culturing increase in cell volume with a decrease in temperature.

Chlorella sp has such an advantage in that they are a high nutrient requiring genus of microalgae (Borowitzka, 1999). In our study we found, *C. vulgaris, A. obliquus, I. galbana* and *N. gaditana* can be maintained in open pond under natural climatic condition as the dominant culture in the system. Among that, *C. vulgaris* and *N. gaditana* yield 1.4 and 1.6 g/l of biomass compared than other microalgae.

CONCLUSION

Pre-screening and growth rate calculations enabled selection of four strains, *C. vulgaris, A. obliquus I. galbana* and *N*

gaditana that showed potential to meet the some important of the criterion for selection. *C. vulgaris* and *A. obliquus* produced 33.19% and 33.53% of saturated fatty acids that is more suitable for biodiesel production than PUFA. Microalgal biodiesel is technically feasible and to be economically competitive with petrodiesel. The lipid content in algal oil has to be high to achieve sustainable economic performance. Successful cultivation in open system become more viable thus makes the process more economical. *C. vulgaris* and *N. gaditana* can be sustainably grown using open pond system for biomass and biodiesel production.

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