The Ecoscan 10(3&4): 461-466, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF ENVIRONMENTAL SCIENCES
www.theecoscan.com

ISOLATION AND CHARACTERIZATION OF A NEW MARINE *CHLORELLA* SP. JD-2016 FROM CHILKA LAKE

DASH JAYALAXMI AND SAMANTARAY SAUBHAGYA MANJARI
Department of Microbiology,
Orissa University of Agriculture and Technology, Siripur Square,Bhubaneswar - 751 003, Odisha, INDIA
e-mail:dash.jayalaxmi@gmail.com

ABSTRACT

An attempt was made to isolate and identify green algae from Chilka Lake suitable for neutral lipid accumulation. Out of all isolated microalgae a green algae was screened using Nile Red dye showing high lipid content. Morphological study using scanning electron microscopy, phase contrast microscopy and molecular identification by 18 S r-RNA gene sequencing revealed that the organism belongs to genus *Chlorella* (KU497645). In laboratory flask culture the growth followed sigmoid nature of curve with a specific growth rate of 0.133 day $^{-1}$. The photosynthetic pigments (Chl-a, Chl-b, carotenoids) increased during exponential growth and declined in their respective ratios. Spectrofluorometric quantifications revealed accumulation of large amount of neutral lipids suggesting it could be a better strain with regards to its lipid accumulating efficiency for biofuel and other purposes.

KEY WORDS
Green algae
SEM analysis
NR
Neutral lipid

Received : 16.06.2016
Revised : 25.08.2016
Accepted : 13.10.2016

***Corresponding author**

INTRODUCTION

Algae are a group of photosynthetic micro-organisms that are able to capture solar energy and convert them to useful biomass. They have high growth rate and photosynthetic activity as compared to terrestrial plants. Micro-algal biomass could be used as an alternative to the traditional fossil fuel derived from plant biomass due to its high lipid and carbohydrate content (Chisti, 2007; John *et al*., 2011). Other than biofuel, bio-ethanol and bio-hydrogen, they also produce large amount of unsaturated fatty acids like MUFA (mono unsaturated fatty acid) and PUFA (poly unsaturated fatty acids) having additive value in food, nutraceuticals and cosmetic industries (Brennan and Owende, 2010; Mata *et al*., 2010). An altered cellular physiology including the metabolite profile is encountered in algal cell upon alteration in their growth parameters like light, pH, temperature and nutrients. Such changes also influence their biomass production (Procházková *et al*., 2014). One of the important metabolite that undergoes severe alteration is the lipid. Microalgae are reported to accumulate large amount of lipids, when cultivated under stress conditions (Guschina and Harwood, 2006; Hu *et al*., 2008). While cyanobacterial group of algae under stress condition could produce large amount of polar lipids like, diacyl glycerols (DAG), in eukaryotic algae these are stored as triacylglycerols (TAG). Further, cyanobacterial lipids are more polar as against lipid from green algae. Many green algae are reported to be efficient in TAG (neutral) accumulation and also in producing large quantity of bioactive compounds. Therefore, there is a continuous search for new indigenous green algal species(s) that could be capable of producing both TAG and other bioactive compounds.

Indigenous microalgae have the advantage of growing efficiently adjusting with the environment and thereby efficiently producing large amount of lipids in their cell. There are continuous attempt to screen algae from natural environment and exploit them for their lipid accumulating capacity. Chilka, the largest brackish water lagoon in Asia, is very rich in its biodiversity. Though different species of marine and freshwater microalgae are abundant in Chilka environment, still these are under explored regarding their lipid production efficiency. Hence NR probe has been used to screen the microalgae from Chilka and an attempt has been made to find out native marine green algal strain having high lipid accumulating capacity.

MATERIALS AND METHODS

Study Area

Chilka is the largest brackish water lagoon of Asia situated at the east coast of India (19º282 and 19º542 N latitude and 85º062 and 85º352 E longitude). Samples were collected from different study sites viz. Kalupadaghat, Balugaon, Rambha near Chilka Lake.

Isolation and Screening of the algae using NR probe

Algal water samples were collected from different study sites and brought to the laboratory. Then samples were serially diluted and cultured using BG-11 and f/2 medium following methods of Stein, 1973. Repeated culture and subculture of algae were practiced to obtain the pure cultures. The algal cultures were maintained at 27 ± 2ºC temperature and at a light of intensity 250 μmol $m^{-2}s^{-1}$ under laboratory conditions. Cultures were shaken thrice daily manually for aeration and examined regularly under microscope for the purity.

Nile Red fluorescence parameter was used to screen the neutral lipid accumulation under fluorescent microscope following Chen *et al*., 2009 with some modifications. In brief, 5 ml of cells were harvested during late exponential phase of growth and centrifuged at 10,000 rpm for 10 min following three steps of washing with distilled water. Cells were then mixed with 20% DMSO in 1:20 ratio. Cells with DMSO were placed in a microwave oven for 30 sec and to it 20 μl of Nile red dye (200 μg/ ml acetone) was added. The mixture was pre-treated for about 30 sec in the microwave oven (100ºC) and incubated in dark about 30 min at room temperature. Cells were then visualized under 40X objective using blue filter in a fluorescent microscope (Leica DM LED 500). Presence of neutral lipid was confirmed from the golden-yellow fluorescence while red fluorescence signifies non-lipid bodies.

Maintenance of the screened algae

The pure green algal species, screened based on NR fluorescence and neutral lipid content, was maintained in 250ml Erlenmeyer flask with 100ml f/2 medium (Guillard , 1973) under 16:8 L: D photoperiod, 100 μmol $m^{-2}s^{-1}$ light intensity (white fluorescent bars) and 24 ± 2 ºC.

Identification of the algae

The morphology and size of the algae, was examined using 40X objective and photographs were taken using phase contrast microscope. For scanning electron microscope (SEM) analysis, 10 ml culture was harvested, washed and fixed in glutaraldehyde (final volume 1-2%) over night. Sample was dehydrated passing through a series of alcohols in increasing concentrations. The dried material is then coated with gold in a sputter system and finally examined using Carl Zeiss (EVO 18) scanning electron microscope.

The molecular identification of the screened alga was performed associated with 'Genotypic' (Bengaluru). The protocol prescribed by them is as follows. DNA was isolated from the culture by the standard CTAB cell lysis method. It was purified by phenol chloroform method and its quality was evaluated on 1.0% agarose gel. Fragment of 18S r DNA was amplified by PCR. The PCR amplicon was column purified to remove contaminants. Known purified amount of amplicon was sequenced by 18S forward and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 18S r RNA gene was generated from forward and reverse sequence data using aligner software.

Sequence alignment and analysis was done using BLAST tool of NCBI. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.78121251 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown below the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 44 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 482 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Measurement of growth

Culture turbidity, cell count and dry mass measurements (4, 8 and 12days) were made to record the growth of the algae. The turbidity was recorded at 750 nm (scattering). The algal cell density was numbered using Neubauer hemocytometer. An X-Y curve of absorbance vs. cell number yielded a straight-line suggesting that abs_{750} refers to relative cell number.

For dry weight determination of the algal biomass, 10ml algal culture were harvested, washed and filtered through pre-weighed Whatman GF/C filters (0.45 μm) and dried at 80ºC till constant weight was obtained. Specific growth rate and biomass productivity was calculated from the data according to the equation; $K' = \text{Ln}\,(N_2/N_1)/(t_2-t_1)$ where N_1 and N_2 refers to biomass at time (t_1) and (t_2) respectively (Levasseur, 1993).

Determination of photosynthetic pigment concentration

The pigment concentration (Chl-*a* and –*b and* carotenoids) of the alga in methanolic extract was determined following Lichtenthaler, 1987. The methanolic extract of the pigments were achieved by percolation method. In order to percolate the pigments 1ml algal culture was centrifuged at 8000 g for 10 min and the pellet was washed thrice with distilled water and incubated with 2ml of absolute methanol and kept at -4º C for 48-h in dark, by which time the extraction of the pigment is completed leaving behind a colourless pellet.The respective absorption peak values for carotenoids (470 nm), Chl-b (652 nm) and Chl-a (665 nm) in methanolic extract were corrected for turbidity, if any, by subtracting the values obtained at 750 nm.

Semi-quantitative estimation of neutral lipid

The accumulation of neutral lipid was monitored through measuring the relative intensity of Nile red fluorescence emission spectroscopy at room temperature (Chen *et al*., 2009**)**. Culture (1 ml) in late exponential growth phase was taken out and centrifuged at 10000 g for 20 min. Cells were then washed thoroughly and then mixed with 20% DMSO in 1:20 ratio. Cells with DMSO were placed in a microwave oven for 30 sec and to it 20 μl of Nile red dye (200 μg/ ml acetone) was added. The mixture was pre-treated for about 30 sec in the oven (100ºC) and incubated in dark about 30 min at room temperature.

The above sample was excited at wavelength 488 nm and scanned for emission spectra between wavelengths of 500-750 nm using a fluorescence spectrophotometer. Water, DMSO and Nile red mixture treated in the above steps without taking algal cells were used as control. Final corrected spectrum was calculated by subtracting the emission spectra of Nile red from that of cells treated with Nile red. Only water, DMSO and acetone mixture was taken to obtain the baseline.

RESULTS

Isolation and screening of alga

Samples from different sites along Chilka Lake were collected from water samples. Under laboratory conditions of growth, different cyanobacteria and algae were isolated following preliminary microscopic examinations. The isolates were grown in flask culture till early stationary phase. In stationary phase of growth the cells were treated with Nile red in microwave assisted procedure. The presence of neutral lipid accumulated in the cells was confirmed from golden-yellow fluorescence due to its interaction with Nile red dye (Figure. 1). Cells without neutral lipids on the other hand show red fluorescence indicating absence of these neutral lipid bodies.

Identification

The phase contrast microscopic examination revealed that the selected isolate (on the basis of lipids accumulation) was a green alga, round /oval in structure, and unicellular (Fig. 2A). Detailed surface morphology of alga was further obtained from electron microscopic scanning (EMS). As was found under phase contrast microscopy, the SEM results further indicated that the alga was unicellular, clearly oval shaped and non-flagellated with a size of approximately 2μm (Fig. 2B).

The identity of the isolate was made from 18S r-DNA sequencing (genotypic, Bengaluru).The 18S r-DNA sequence of the isolate was found to be a novel sequence (NCBI accession no. KU497645).The phylogenetic tree construct suggest that this organism belongs to genus *Chlorell* that remain out-rooted among the taxa considered for phylogenetic tree analysis (Fig. 3). Therefore it is assumed that this organism probably represents yet another different species of genus *Chlorella* (referred as *Chlorella* sp. JD-2016 as released by NCBI).

Growth characteristics of the *Chlorella* sp.

The isolate, *Chlorella* sp. was cultured in the laboratory under optimum growth conditions. The growth profile was monitored as the function of days by taking the absorbance at 750 nm. The absorption profile increased by increasing the days of culture till 12-days following which the growth slows down (Fig. 4). The growth curve, as expected, appears to be sigmoid to some extent. The determination of cell number against respective absorbance was also linear (Figure. 4A also see 4B). There exist a linear relationship between absorbance value and the dry matter accumulation (Fig. 4C). The experiments were conducted on 4, 8 and 12-d of growth, since they fall in linear growth curve of the organism. The specific growth rate of the algae was approximately 0.133 day^{-1}, where as the biomass productivity and generation time was found to be 0.04 g/L/day and 4.5/ day respectively.

Photosynthetic Pigments concentration

The accumulation of various photosynthetic pigments during growth of the alga is presented in Table-1. As expected, the total chlorophyll content increased during the exponential phase and as the culture reaches early stationary phase it showed a decline in its concentration. Opposed to chlorophyll, a steady increase in carotenoids was marked. The increase and decrease in various pigment concentrations has also been reflected in their respective ratios.

Estimation of neutral lipids

Nile Red was used to quantify the neutral lipids. It was observed that when the cells in late exponential phase were scanned for emission spectra it exhibited a peak at 575 nm which is due to presence of neutral lipids. Hence it was confirmed that when the cells were scanned for its emission spectra by exciting the Nile red stained cells at 488 nm, due to binding of the neutral lipids to the stain a very strong peak of high intensity appeared at 575 nm (Fig. 5).

DISCUSSION

An attempt was made to isolate and identify microalgae from Chilka Lake. Further, they were screened to select potent oleaginous alga able to accumulate neutral lipids in its stationary phase of growth. Indigenous microalgae have the advantage of growing efficiently adjusting with the environment and thereby efficiently producing different metabolites. According to Hu *et al*., (2008) continuous attempt should be made for isolation and characterization of new algae from unique aquatic environments. The proportion of algal strains

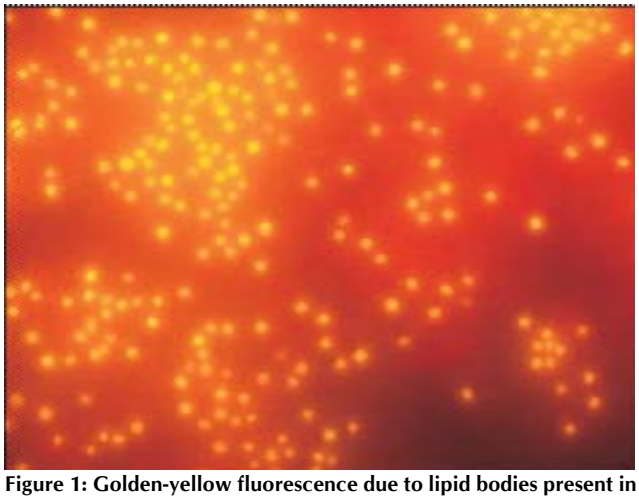

Figure 1: Golden-yellow fluorescence due to lipid bodies present in cells of the green alga

Table 1: Pigments profile of the alga on different days of growth

Days	OD_{750nm}	Chl a (μg/ml)	Chl b (μg/ml)	Total Chl (Chl a + Chl b) (μg/ml)	Chl a/ Chl b	Car(μg/ml)	Chl/Car
6	0.44	0.582	0.203	0.785	2.86	0.325	2.415
12	0.64	1.587	0.637	2.224	2.2491	0.836	2.66
18	0.75	1.33	0.469	1.799	2.835	1.078	1.668
24	0.775	1.02	0.395	1.415	2.58	1.071	1.321

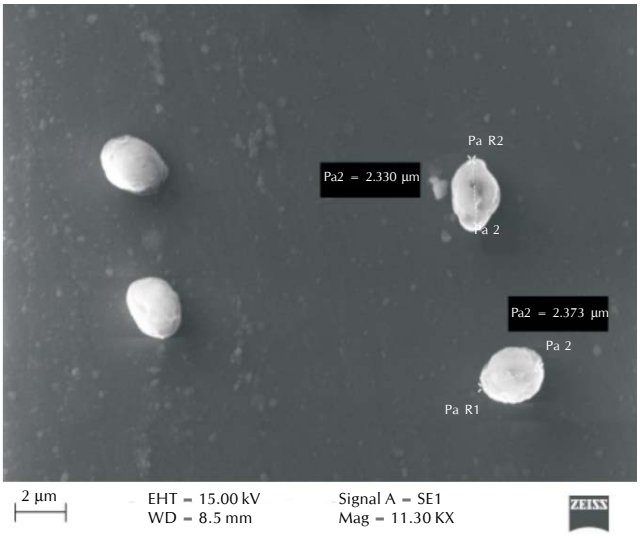

Figure 2B: Scanning electron micrograph of the selected alga

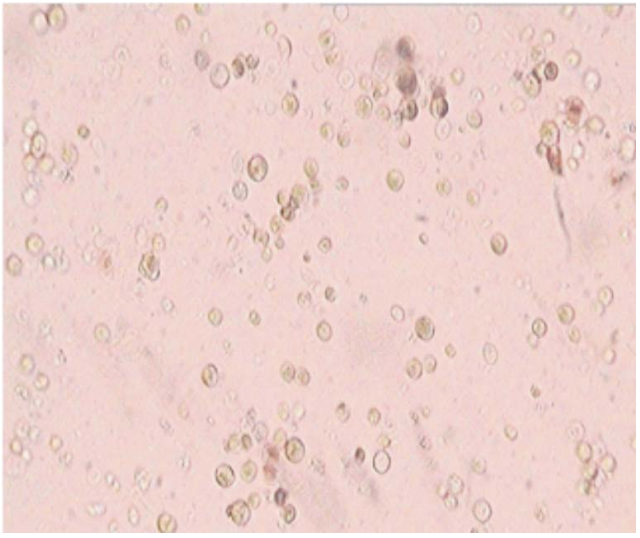

Figure 2A: Phase contrast micrograph of the selected alga

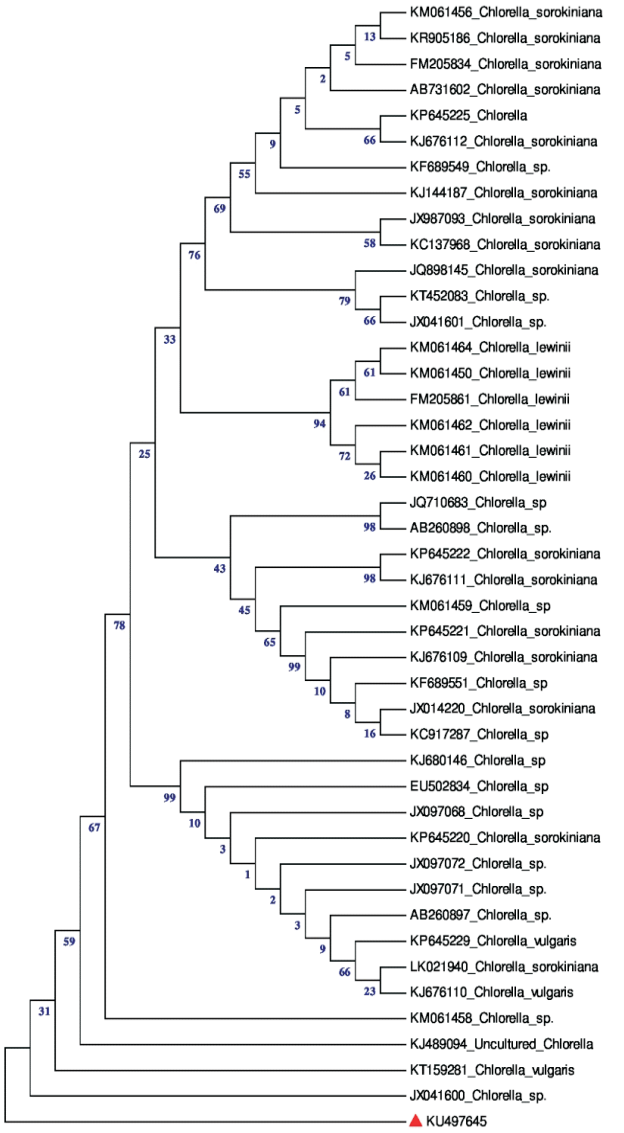

Figure 3: Phylogenetic tree showing relationship of *Chlorella* sp. JD-2016 to other sp. of genus *Chlorella*

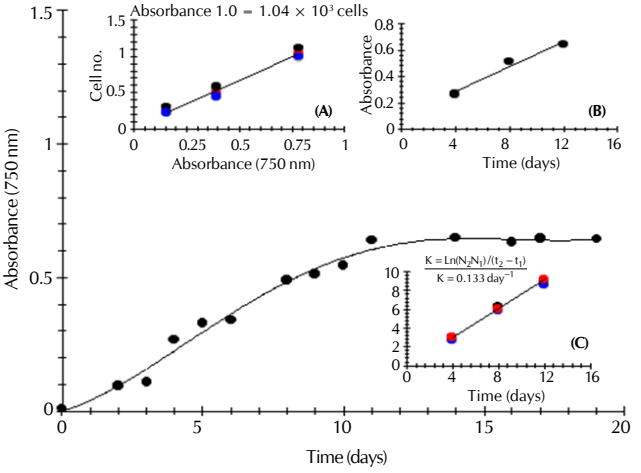

Figure 4: Growth characteristics of the *Chlorella* sp.

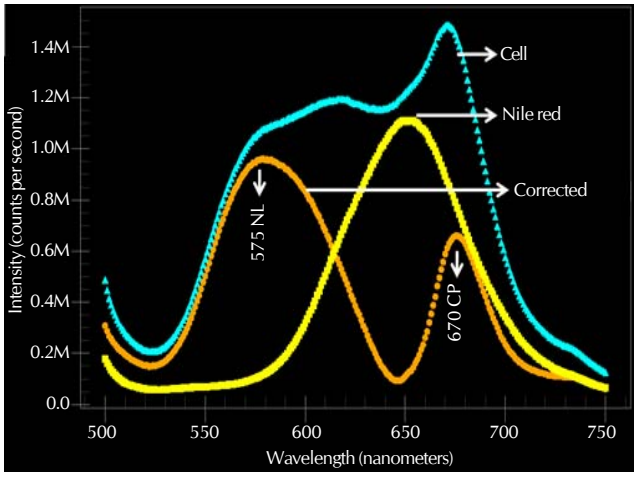

Figure 5: Spectrofluorometric quantification of neutral lipid

examined for lipids/oils is very less comparable to the total identified species so far available in nature. The variation in their fatty acids composition and content promotes more research on mechanisms that are involved in efficient lipid production. Hence it is expected that search for a new strain of algae from the natural environment is desirable and these are further exploited for their multiple uses. The preliminary phase contrast microscopic studies revealed that the selected isolate was a unicellular spherical shaped alga, which was further established by taking its scanning electron microscope picture. To get complete identity 18 S r-RNA gene sequencing was done. The phylogenetic tree constructed with other similar sequences in NCBI database suggested it was a *Chlorella* sp. that was out rooted among the taxa.

Chlorella is a genus of single-celled green algae, belonging to the family Chlorellaceae within the class Trebouxiophyceae. The cells are solitary, 2–10 μm in diameter and spherical, globular or ellipsoidal in shape. The cells are devoid of flagella, having a parietal and cup-shaped chloroplast with a single pyrenoid surrounded by a thin cellulose wall. Some other workers have also isolated *Chlorella* and established its identification through sequencing of the gene. Ruiz-Domínguez *et al*. (2016) isolated a microalgal strain from Cepsa's refinery wastewater treatment plant in Huelva (south-west of Spain). Genetic analysis of the chloroplastic rbcL gene encoding for the large subunit of the ribulose bisphosphate carboxylase enzyme (Rubisco) showed the strain had high homology with other known rbcL sequences of the genus *Chlorella*.

Similarly, Tale *et al*. (2014) isolated and characterised five microalgae from effluents of Nisargruna biogas plants. Preliminary morphological identification of microalgal cultures by microscopic analysis showed that three isolates (KMN1, KMN2, KMN3) belonged to the genus *Chlorella* .This identification was further confirmed based on morphology and partial 18S and 23S r-RNA gene sequences.

The growth rates of different organisms vary depending on the culture conditions, culture mediums and to a much extent differ among species. The photobiorector which is more controlled condition of growth produces more biomass (Mahmoud *et al.,* 2016). In this study the *Chlorella* sp. was grown in flask culture and no external CO_2 was supplied. So the specific growth observed was less as compared to previous data for other *Chlorella* sp., but they were grown in completely different set of growth conditions. So comparison of the data with others is not appropriate in this case.

This alga was screened for neutral lipid accumulation under a fluorescent microscope using Nile red (NR) dye. The cells possessed golden-yellow fluorescence upon exciting with a blue filter under fluorescent microscope indicating the presence of neutral lipids. Traditional methods for lipid analysis require large amounts of algal samples, demand complete extraction avoiding decomposition and/or oxidation of the lipid constituents, time-consuming and difficult to screen large numbers of algal strains rapidly. As a result, screening methods of cellular lipid content using NR is appreciable (Cooksey *et al*., 1987).It is important to note that Nile red in acetone stains the whole cell but only the non-polar regions fluoresce at the yellow wavelengths. Nile red fluorescence accompanies a spectral blue shift and reflects its presence in a hydrophobic lipid environment, so account for the selective detection of neutral lipid in the cell. Nile red thus serves as an excellent fluorescent lipid probe (Greenspan and Fowler, 1985). By proper selection of spectral conditions (Excitation and emission), Nile red could be used to monitor neutral lipid in a cell. Also, it is used in a microwave- assisted method where the otherwise impermeable NR through the rigid cell wall of some microalgae can penetrate into the cell and bind to cytosolic lipid (Chen *et al.,* 2009).

Hence it is suggested to further grow the *Chlorella* sp. under various growth conditions to exploit its ability to accumulate neutral lipids under normal growth conditions.

ACKNOWLEDGEMENT

Special thanks are from the authors to the Institute of Life Sciences, Bhubaneswar for providing its laboratory facilities during the work.

Conflict of interest

The authors declare that they don't have any conflict of interest.

REFERENCES

Brennan, L. and Owende, P. 2010. Biofuels from microalgae - A review of technologies for production, processing and extractions of biofuels and co-products. *Renewable Sustainable Energy Rev*. **14:** 557-577.

Chen, W., Zhang, C. W., Song, L. R., Sommerfeld, M. and Hu, Q. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J. Microbiological Methods*. **77(1):** 41-47.

Chen, W., Zhang, C. W., Song, L. R., Sommerfeld, M. and Hu, Q. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J. Microbiol. Methods*. **77:** 41-47.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol Adv*. **25:** 294-306.

Cooksey, K. E., Guckert, J. B., Williams, S. A. and Callis, P. R. 1987. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. *J. Microbiological Methods*. **6:** 333-345.

Elsie, B. H., Sudha, P. N. and Dhanarajan, M. S. 2010. In Vitro Studies on Antimicrobial Activity and Phytochemical Analysis of Marine Algae (Seaweeds) *Sargassum witti* and *Gracilaria edulis* Using Three Different Solvents Extracts. *The Ecoscan*. **4(4):** 343-345.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*. **39:** 783-791.

Greenspan, P. and Fowler, S. D. 1985. Spectrofluorometric studies of the lipid probe, Nile red. *J. Lipid Res*. **26:** 781-789.

Guillard, R. R. L. 1973. Division rates. In: Stein, J. R. (Ed), Culture Methods and Growth Measurements, Handbook of Phycological Methods. *Cambridge University Press, Cambridge,* pp. 289-312.

Guschina, I. A., Harwood, J. L. 2006. Lipids and lipid metabolism in eukaryotic algae. *Prog. Lipid Res*. **45:** 160 -186.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *Plant J*. **54(4):** 621-639.

Hu, C., Li, M., Li, J., Zhu, Q. and Liu, Z. 2008. Variation of lipid and fatty acid compositions of the Marine microalga *Pavlova viridis*

(Prymnesiophyceae) under laboratory and outdoor culture conditions. *World J. Microbiol. Biotechnol*. **24:** 1209-1214.

John, R. P., Anisha, G. S., Nampoothiri, K. M., Pandey, A., 2011. Micro and macroalgal biomass:a renewable source for bioethanol. *Bioresour. Technol*. **102:** 186-93.

Levasseur, M., Thompson, P. A. and Harrison, P. J. 1993. Physiological acclimation of marine phytoplankton to different nitrogen sources. *J. Phycol*. **29:** 87-595.

Lichtenthaler, H. K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol*. **148:** 350-382.

Mahmoud, R., Ibrahim, M. and Ali, G. 2016. Closed photobioreactor for microalgale biomass production under indoor growth conditions. *J. Algal. Biomass. Utilization*. **7(1):** 86-92.

Mata, T. M., Martins, A. A. and Caetano, N. S. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable Sustainable Energy Rev*. **14:** 217-232.

Procházková, G., Brányiková, I., Zachleder, V., Brányik, T. 2014. Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. *J. Appl. Phycol*. **26:** 1359-1377.

Ruiz-Domínguez, M. C., Vaquero, I., Obregón, V., Morena, B., Vílchez, C. and Vega, J. M. 2014. Lipid accumulation and antioxidant activity in the eukaryotic acidophilic microalga Coccomyxa sp. (strain onubensis) under nutrient starvation. *J. Applied Phycology*. **27:** 1099-1108.

Sahoo, N. K., Azeez, P. A., Khillare, P. S. and Naik, S. N. 2013. Secondary nutrient limitation in phytoplankton: implication for biofuel feedstock production. *The Ecoscan*. Special issue. **III:** 01-08.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4:** 406-425.

Stein, J. 1973. Handbook of Phycological Methods: Culture Methods and Growth Measurements. *Cambridge University Press, London,* p. 448.

Tale, M., Ghosh, S., Kapadnis, B. and Kale, S. 2014. Isolation and characterization of microalgae for biodiesel production from Nisargruna biogas plant effluent. *Biosource Technology*. **169:** 328-335.

Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (USA). **101:** 11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. *Biol. Evol*. **30:** 2725-2729.

Tang, H., Chen, M., Ng, K. Y., Salley, S. O. 012. Continuous microalgae cultivation in a photobioreactor. *Biotechnol. Bioeng*. DOI: 10.1002/bit.24516.

Wu, Q. and Miao, X. 2006. Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol*. **97:** 841-846.