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Ultra Structural Analysis and Lipid Staining of Biodiesel Producing Microalgae - *Chlorella vulgaris* Collected from Various Ponds in Tamil Nadu, India

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Abstract

The present research paper describes to evaluate 10 different Microalgal lipid contents by Nile red and Bodipy staining. The ultra structural studies carried out by Scanning Electron Microscope (SEM). The micro algal samples were collected from province of Tamil Nadu, located in Southern India. Compare to Nile Red stain Bodipy is a specific stain for to detect lipid content present in the living algal cells. In this study clearly describe *Chlorella vulgaris* stained by Bodipy have shown more lipid content than other microalgae collected from various location.

Key Words: Biodiesel, *Chlorella vulgaris*, Lipids, Microalgae, Nile Red

Introduction

Micro algae, which are one of the most abundant organisms in the world, sunlight driven cell factories that convert carbon dioxide to potential biofuel, foods, feeds and high value bio-active compounds, received attention because of its high capacity to produce biofuel as compared to the other plants. As a matter of fact, average biodiesel production yield from microalgae can be 10 to 20 times higher than the yield obtained from oleaginous seeds and/or vegetable oils [1]. In the world, over 50000 microalgae species are present in not only aquatic but also terrestrial environments, implying their widespread availability [2]. Among these, Diatoms and green algae are relatively abundant [3]. Most common microalgae (*Botryococcus*, *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Neochloris*, *Chaetoceros*, *Scenedemus*, *Chlorococcum sp*, etc.) have oil levels between 20 and 75% by weight of dry biomass. The above mentioned microalgae are potential sources for biodiesel production.

Biodiesel consists of fatty acid methyl esters, which typically are derived from triacylglycerols (TAGs) by transesterification with short chain alcohols such as methanol, with glycerol as a byproduct [4].

Key technical challenges include identifying the strains with the highest growth rates, oil content with adequate composition and ultra structural morphology, which were the aim of the present work.

Material and Methods

Sample collection and identification

In the present study, survey was conducted to examine the population and Nile Red and Bodipy method was used for 10 microalgal genus of various classes. Various physical and chemical treatments were applied to the existing Nile Red method to improve the effectiveness and efficiency. Species

and Genus of microalgae were obtained from the cultures isolated from different locations of Tamil Nadu having different environmental conditions like temple tanks, rock ponds, forest lagoons and inland lakes. The microalgal cultures were isolated and identified by standard procedures.

Simple microscopy

The 10 samples were subjected to light microscopic studies by simple wet mount method. The aliquots of each sample was placed in centre of clean glass slide and covering the drops with thin cover slips, it was observed under light microscope by 100 X objective lens and photographs were taken.

Nile red staining

Nile red (9-(Diethylamino)-5H benzo [a] phenoxazin-5-one) staining was conducted to detect intracellular lipid droplets. Microalgal cells (0.5 ml) were collected by centrifugation at 1,500 rpm (Rotation per minute) for 10 min and washed with physiological saline solution (0.5 ml) several times. After the collected cells were re-suspended in the same solution (0.5 ml), the Nile red solution (0.1 mg/ml in acetone) was added to cell suspensions (1:100 v/v) and incubated for 10 min. After washing once, stained microalgal cells were observed by fluorescent microscopy [5].

Microscopic photographs were taken with a Nikon E600 microscope (Nikon, Tokyo, Japan) equipped with a color CCD digital camera (DP12, Olympus, Tokyo, Japan) using a 450–490-nm excitation filter, a 505-nm dichroic mirror and a 520-nm barrier filter with 40x or 60x objective lens. The digital color pictures taken were corrected to grey scale figures by PC software.

Bodipy lipid staining

The intensely fluorescent Bodipy 4, 4-difluoro-3a, 4 adiaza-s-indacene) fluorophore is intrinsically lipophilic, unlike most other long-wavelength dyes. Consequently, probes incorporating the fluorophore are more likely to mimic the properties of natural lipids. Molecular Probes prepares bodipy fatty acid, phospholipid, cholesteryl ester and sphingolipid analogs that undergo native-like transport and metabolism in cells, they are therefore effective tracers of lipid trafficking, as well as being useful general-purpose membrane probes. [6]

Scanning electron microscopy

The sample was screened by SEM (Scanning Electron Microscopy) for their absolute morphological studies. The basic steps for SEM sample preparations are fixing it with buffered

aldehyde, post fixing it in Osmium tetroxide, dehydrating it in ethanol, drying it with air dryer, mounting it on a specimen stub, coating it with Carbon and examining under the HRSEM (Quanta FEG 200). The topography of green water algae analyzed (Figure. 3a and b).

Results and Discussions

Microscopic observations

Algae sample collected from Tiruveteeshwarar temple tank shows that vegetative cells are unicellular, spherical about 5-10µm in size and surrounded by a cell wall which consists of two regions (Figure. 1b), an outer thin layer and an inner bulky microfibrillar layer [7]. Other microalgae identified from samples were *Nostoc*, *Anabena*, *Senedesmis*, *Spirulina*, *Volvox*, *Ocellularia*, *Chlorococcus*, *Euglena*, *Diatoms*

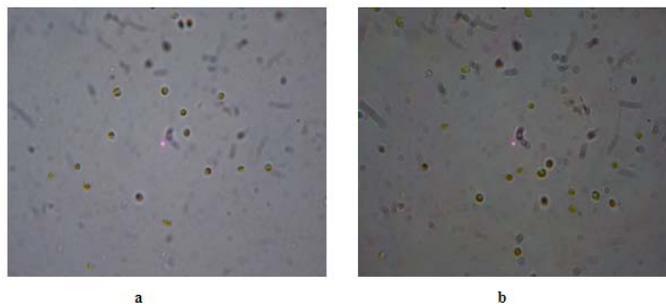


Figure 1 a & b. Light microscopy of *Chlorella vulgaris* under oil immersion (100 X)

Fluorescence microscopic observations

Nile red staining observations

Intracellular lipid droplets of microalgae observed by Nile Red staining under fluorescence microscopy, lipids including hydrocarbons and triglycerides were stained in yellow, while chlorophyll were stained in red (Figure. 2a) [8].

Bodipy staining observations

Oil-containing lipid bodies can be vitally stained and visualized in live oleaginous (oil-containing) algal cells using Bodipy stain. In the figure 2b, vitally stained lipid granules (yellow) are easily distinguished from chloroplasts in *C. vulgaris* freshwater algal cell using a Nikon E600 microscope (Nikon, Tokyo, Japan). Lipid granules appear yellow when they overlap spatially with chloroplasts. Excitation was at 450–490 nm. Emission wave lengths were imaged through a 515 – nm long-pass filter.

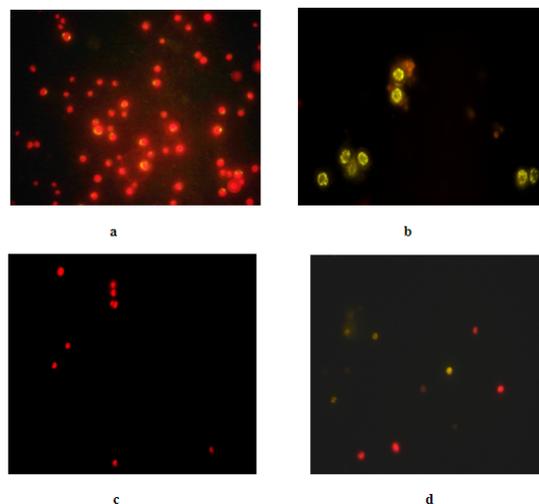


Figure 2a. Nile Red stain of *C. vulgaris*: Chlorophyll emits red fluorescence, lipid granules appeared as yellow; b. Bodipy Stained *C. vulgaris* cells appeared as yellow fluorescence, c and d: Nile red stained cells of *Chlorococcus* and *Scenedesmus* species shows few lipid droplets.

Electron microscopic observations

Scanning electron micrographs of the vegetative cells of algae sample suspended in a hypertonic medium (0.6 M-

sorbitol/mannitol, pH 6.0) showed that outer region had been irregular and the surface of cell wall was roughly folded and vein like furrows extended over the wall (Figure. 3b and c).

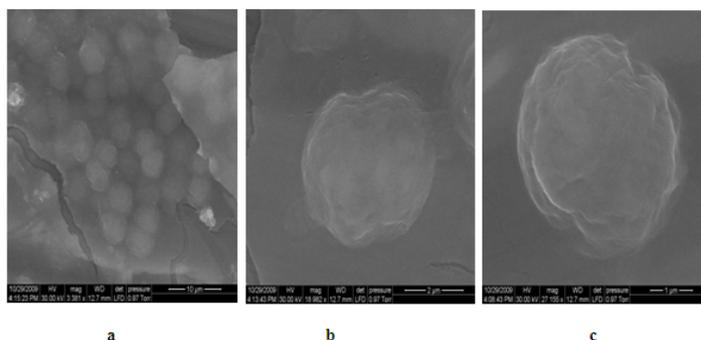


Figure 3. Scanning electron microscopic images of *Chlorella vulgaris* under Morphology of under 3381x (a), 18982x(b), 27155x(c), magnifications.

Conclusions

Based on the survey results, *C. vulgaris* is found predominant microalgae in many samples. The lipid staining confirms that the more quantity of lipids is present in *C. vulgaris* than any other genus. The *Chlorococcus* species and *Scenedesmus* species showed one or two lipid droplets in cytoplasm after stained by Nile Red (Figure. 2c and d) but the *C. vulgaris* have cluster of lipid droplets (Figure. 2b).

Nile red is a promising fluorescence dye in rapid quantification of cellular lipid microalgae. The lipid bodies (yellow fluorescence) can be easily located by Nile Red in the cell and it is a conventional, qualitative method of lipid identification. Usually Bodipy is the dye that has been used to stain lipid-containing yolk platelets in living zebra fish embryos and lipid containing vesicles in immortalized human hepatocytes. A similar dye, Bodipy 493/503, has been used to vital stain lipid droplets in mammalian cells. More importantly the present findings revealed that algal cells remain viable after staining with Bodipy and can be successfully subculture.

The ultra structural morphology of *Chlorella vulgaris* were studied by SEM. There are no much morphological variations observed between *Chlorella* and other microalgae. The lipid droplets present inside the cytoplasm of *chlorella* cannot be observed in SEM.

By capturing and converting CO₂ to commercially useable oil, algae are helpful for both humans and the biosphere (CO₂ imbalance in global ecosystem). Visualizing the biological dynamics of fluorescent 'green oil' in live algal cells using Nile Red and Bodipy, may provide an excellent tool for screening more lipid producing microalgae for biofuel production.

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