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Lipid Granules Staining (Nile Red and Bodypy) of Different Biofuel Producing Fresh Water Microalgae Growing under Various Stress Conditions

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ABSTRACT: *Chlorella vulgaris* and Diatoms were cultivated and maintained in different stress system like high light intensity (18:6, Light: Dark) and N_2 limited condition. After the stationary phase, the cells were stained by Nile red and Bodipy. The slides were examined under fluorescence in situ hybridization microscopy, it showed the *Chlorella vulgaris* cultivated under N_2 limited system yield high lipid bodies than light induced system and Stressed Diatoms. While Bodipy is a best tool for staining algal lipid bodies than Nile Red stain.

Key words: Bodipy, Chlorella Vulgaris, Diatoms, Light, Lipid Bodies, Nile Red

Introduction

Microalgae are photosynthetic microorganism that is able to use the solar energy to combine water with carbon dioxide to create biomass. Hundreds of microalgal strains capable of producing high content of lipid have been screened and their lipid production metabolism has been characterized and reported (Sheehan *et al.,* 1998). Much research was focused on the lipid trigger which refers to the observation that under environmental stress, many microalgae produce more lipids. It is generally accepted that the depletion of the nitrogen from the medium induces lipid accumulation (Evans and Ratledge, 1984; Yoon and Rhee, 1983). Using CO_2 as carbon source, the strain yielded only about 15% of lipid. Despite under different stressful conditions like nutrient deprivation and light stress the microalgae can accumulate large amount of lipid bodies.

Nile red, a lipid - soluble fluorescent dye, has been frequently employed to evaluate the lipid content of animal cells and microorganisms, such as mammalian cells (Genicot *et al.,* 2005), bacteria (Izardand Limberger, 2003), yeasts (Evans *et al.,* 1985), zooplankton (Kamisaka *et al.,* 1999), and microalgae (McGinnis *et al.,* 1997; Eltgroth *et al.,* 2005; Elsey *et al.,* 2007). Bodypy a green lipophilic fluorescent dye serves as an excellent vital stain for the oil-containing lipid bodies of live algal cells. It can be used in combination with fluorescent activated cell sorting to detect and isolate algal cells possessing high lipid content (Mark Scott Cooper *et al.,* 2010).

In this present study aimed to study Nile red and Bodypy staining method with various fresh water microalgae growing under nutrient limited and light stress condition. Under such stress conditions the lipid accumulation in microalgae can be changed. The lipid granules of these microalgae can be studied and evaluated.

Material and Methods

Microalgal Strains

Chlorella vulgaris, Diatoms, and some of the Blue Green algae were collected from Culture collection centre, Plant Biotechnology Department, Presidency College and Water and Waste water Technology Centre, Durban University, Durban, South Africa.

Cultivation of microalgae

The media composition were $NaNO_3$, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, NaCl (each 10ml), Alkaline EDTA Solution 1mL, Acidified Iron Solution 1mL, Boron Solution 1mL, Trace Metals Solution 1mL. It consist of $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, MoO_3, $CuSO_4 \cdot 5H_2O$, Co $(NO_3)_2 \cdot 6H_2O$. The microalgae were cultivated and growing under nutrient limitation (N_2 deprivation) , Light stress (18:6 day [100 μmol photons $m^{-2} s^{-1}$] and night).

Preparation of Nile Red Solution

The 0.5g of Nile Red powder was suspended in one ml of acetone it used as stock solution. From this 0.05ml was mixed with 50ml of glycerol mixture (75:25, Glycerol and water). This solution was directly used for staining the lipid bodies of algal cells.

Nile red staining

Nile red (9-(Diethylamino) -5H benzo [α] phenoxazin- 5-one) staining was conducted to detect intracellular lipid droplets (Greenspan *et al.* 1985). 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 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 (Tadashi Matsunaga *et al.* 2009).

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 this 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. (Mark Scott Cooper *et al.* 2010)

Result and Discussion

Nile Red Staining

Cells were stained with Nile Red are agent that yields brilliant yellow fluorescence in a neutral lipid environment (Cooksey, K. E *et al.* 1987) and selectively stains lipid bodies in *C.vulgaris.* Nile red staining of *chlorella vulgaris* grown under light stress condition showed the higher neutral lipid accumulation inside the cell than control (Fig.1). The stained lipid granules are yellow in colour but the chloroplast were stained as red (Fig. 2).

The nitrogen deprived culture of *Chlorella vulgaris* showed that the marked increase of lipid production. Each cells contains about 6 to 9 granules, they are spherical shape and vary in size (Fig.3). The cells produce abundant cytoplasmic lipid bodies, as well as abundant starch, via a pathway that accompanies a regulated autophagy program.

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Fig 1: Nile Red staining-*Chlorella vulgaris* Control

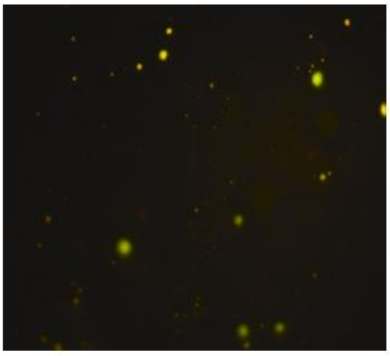


Fig 2: Nile Red staining of *Chlorella vulgaris* under light stress condition

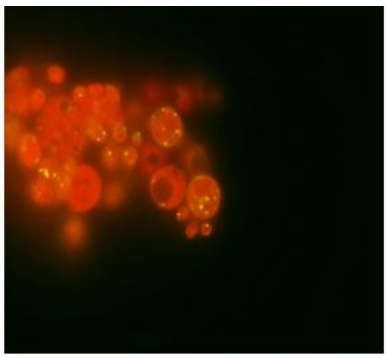
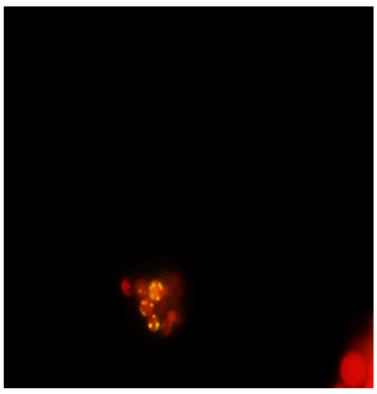


Fig 3: Nile Red staining of *Chlorella vulgaris* under N_2 limitation stress condition



Diatoms store carbon in the form of natural oils with a lipid content of 70% dry weight. The most nutritionally relevant bio molecules produced by diatoms are unsaturated fatty acids like eicosapentaenoic acid (EPA, 3.9–5 % of dry weight in *Phaeodactylum tricornutum* (Lebeauand Robert 2003). Fig 4 and 5 proved the lipid granules (yellow coloured) of Diatoms which grown under light and nitrogen stress condition which yield high lipids due to the shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipids (Klyachko Gurvich, 1974).

Fig 4: Nile red staining of *Diatoms* under Light stress condition

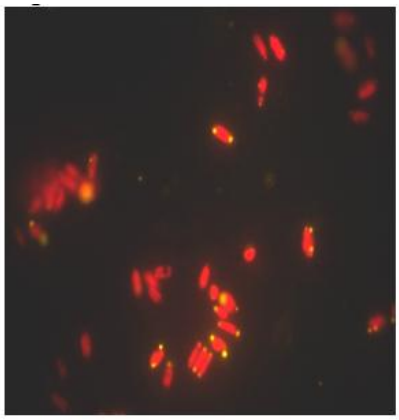
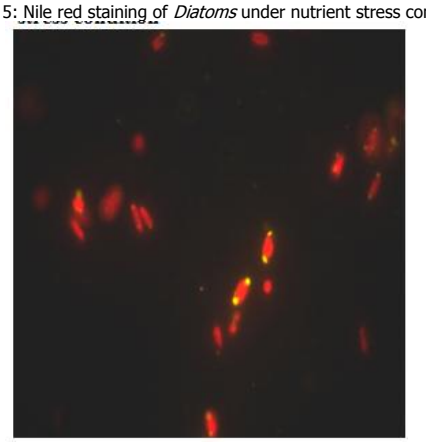


Fig 5: Nile red staining of *Diatoms* under nutrient stress condition



Bodypy Staining

Bodipy has a narrower emission spectrum than Nile Red, making this dye potentially more useful for certain technical applications, such as confocal imaging, where fluorescence contrast enhancement of lipid bodies is critical. Unlike Nile Red, Bodipy has the advantage that it does not localize strongly in cytoplasmic compartments other than lipid bodies. In this staining technique the algal lipid bodies are stained as golden yellow colour. *Chlorella vulgaris* which grown under 18:6 hours light and dark condition emitted yellow fluorescence, where the lipid bodies observe the stain (Fig.7). The microalgae nourished under nitrogen limited condition were produce considerably high amount of lipid (Fig. 8) than light stressed.

Fig 6: Bodypy staining – *Chlorella vulgaris* control

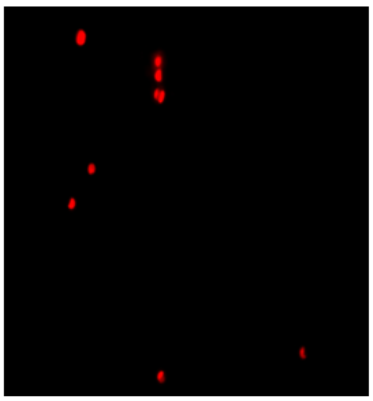


Fig 7: Bodypy staining of *Chlorella vulgaris* under light stress

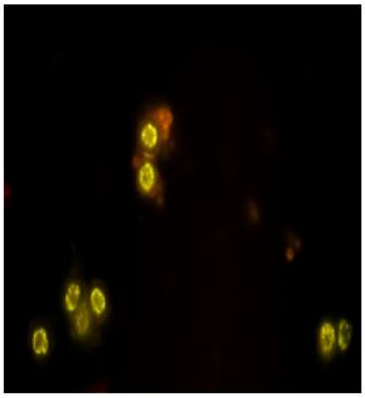
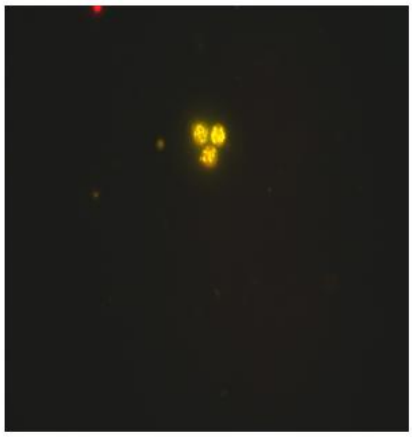


Fig 8: Bodypy Staining of *Chlorella vulgaris* under nutrient stress condition



Conclusion

The key step in Fatty Acid biosynthesis is the irreversible conversion of acetyl coenzymeA (acetyl CoA) to malonyl - CoA, catalyzed by the complex enzyme acetyl-CoA carboxylase. Acetyl CoA is generated via a number of biochemical pathways, one of which utilizes the primary product of CO_2 fixation, 3 – phosphoglycerate (3-PG) ; 3-PG is also the precursor for the glycerol back bone of TAG. Since 3-PG feeds directly into the starch biosynthesis pathway, blocking this pathway would, in theory, free up more carbon skeletons to flow in to both acetyl CoA production and TAG biosynthesis.

The content of storage and structural lipids was significantly affected by the light intensity. Exposure of microalgae to low light conditions induced an increase in the abundance of structural components of the cell membranes, while growth of algae at high light intensity resulted in a 1.5-folder increase in the level of storage lipids, e.g. triacylglycerols. It was due to one of the adaptive responses of the algal cells to the varying growth conditions. The structural lipids are membrane of chloroplast so the different light intensity influences the efficiency photosynthesis and lipid ratio.

Lipid bodies accumulation occur with N_2 starvation, indicating the existence of a nitrogen trigger or, perhaps more generally, a stress trigger. N_2 starvation is accompanied by autophagy, meaning that the nitrogen trigger may act directly to stimulated own stream events and/or it may act in directly by stimulating the formation of stimulatory products (lipid bodies) generated via autophagy.

In our results, suggest *Chlorella vulgaris* with N_2 stress is suitable for production of high fatty acids, it produce more lipid granules compared to light stressed condition. Diatoms produce considerable amount of lipid granules at two corners of the individual cells. But it synthesis low level of lipid bodies compared to *Chlorella vulgaris.* Using Bodiypy stain, may provide a best tool for improving algae oil as a sustainable green technology, for the production of both energy.

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