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Enhanced lipid and fatty acid content under photoheterotrophic condition in the mass cultures of *Tetraselmis gracilis* and *Platymonas convolutae*

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ABSTRACT

Biomass, lipid, fatty acid methyl ester (FAME) profiles and concentrations in two green marine microalgae *Tetraselmis gracilis* (*T. gracilis*) and *Platymonas convolutae* (*P. convolutae*) in culture media with different nitrate and carbohydrate supplement concentrations under natural photoperiod were measured under indoor and outdoor conditions; *T. gracilis* and *P. convolutae* were cultured in a transparent vertical fiber tank and rectangular white fiber-reinforced plastic (FRP) tank, respectively.

The total lipid and FAME concentrations increased under nitrate limitation in both microalgae. The results reflected the dynamic changes in the syntheses of fatty acid (FA) under stress in the studied algae. Between the two algae studied, *T. gracilis* had the highest percentage of eicosapentaenoic acid (EPA: 14.0%) and eicosatetraenoic acid (ETA: 3.1%) at 0.05 g l^{-1} nitrate concentration. The maximum EPA (4.4%) and ETA (2.0%) concentrations in *P. convolutae* were observed in the total polyunsaturated fatty acids (PUFAs) grown at a nitrate concentration of 0.1 g l^{-1} in the culture media under outdoor conditions. The major PUFAs detected under different carbon supplements were C20:4 (ω3), C20:5 (ω3) and C20:4 (ω6). *T. gracilis* had the highest amount of EPA (14.7%) under photoheterotrophic growth with glucose (2 g l^{-1}) as the carbon source. The optimal condition for EPA production by *P. convolutae* (8%) was 1 g l^{-1} glucose at 12-h light photoperiod under indoor mass cultivation and photoheterotrophic growth. These two microalgae had high lipid contents and showed a higher PUFA level under nitrate limitation. This result shows that biomass, lipids, and fatty acids as well as PUFA accumulate and reach elevated levels in these two microalgae species under photoheterotrophic growth conditions.

1. Introduction

Algae of the genus *Tetraselmis* and *Platymonas* have been used as a source of nutrition for invertebrates in aquaculture for decades [1–4] due to their richness of important polyunsaturated fatty acids (PUFAs) and ability to grow under a wide range of physical and chemical environmental conditions [5].

Eicosapentaenoic acid (EPA, C20:5 ω3) and docosahexaenoic acid (DHA, C22:6 ω3) are important ω3 polyunsaturated fatty acids (PUFAs), while arachidonic acid (AA, C20:4 ω6), is a vital ω6-PUFA. They have numerous nutraceutical and pharmaceutical applications [6,7]. EPA and DHA are important in the treatment of atherosclerosis, cancer, rheumatoid arthritis, psoriasis and diseases of old age, such as Alzheimer's and age-related muscular degeneration [8,9]. AA and DHA are of special importance for being essential for blood vessels, pre and post-natal brain and retina development [10]. The eicosanoids, such as prostaglandins, prostacyclins and leukotrienes derived from ω3-PUFAs are important for infant growth, modulatory vascular resistance and wound healing [11].

Nitrogen deprivation can improve the lipid content in microalgae [12–14]. Concentration of nitrogenous compounds in the culture medium can regulate intracellular lipid accumulations [15–18]. In general, microalgae maintain lipid synthesis under nutrient starvation or stress conditions when the production/functioning of other types of cellular constituents are curtailed [19]. Heterotrophic cultures made it possible to reach higher lipid content rather than photoautotrophic cultures [20].

Understanding the importance of these algae in the area of aquaculture, as an important feed for aquatic organism, and in human pharmaceutical applications, as major producers of PUFA, was the motivation for selecting these two algae. The aim of this study was to elucidate the effect of nitrate and carbohydrate nutrients on biomass, lipid and fatty acids of the unicellular green microalgae *Tetraselmis gracilis* and *Platymonas convolutae*.

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2. Materials and methods

2.1. Strains and culture conditions

Two green unicellular marine microalgal species *T. gracilis* and *P. convolutae* were used in this study; stocks were procured from the Central Marine Fisheries Research Institute, Tuticorin, Tamil Nadu, India. Stocks of each culture were maintained in Walne medium [21] at salinity 34 ppt and at 23 ± 2 °C temperature under 12:12 light–dark cycle with light intensity of 180 μE photons m^{-2} s^{-1}.

2.2. Mass cultivation

For all the experiments, both species of microalgae were grown in Walne medium. The indoor mass culture was started using 20 l polyethylene terephthalate (PET) jars (transparent) under 81 μE photons m^{-2} s^{-1} for 12:12 (light:dark) photoperiod with constant aeration at 23 ± 2 °C. Exponential phase of 20 l culture was used as seed culture and inoculated into 180 l culture media in 250 l capacity of the vertical fiber tank (transparent) for indoor and horizontal fiber-reinforced plastic (FRP) tank for outdoor condition.

In outdoor experiments, the following conditions were maintained during the culture period: air temperature 28 °C to 34 °C, salinity 34 to 36 ppt, 12:12 h photoperiod and constant aeration. All experiments were performed with three replications and a control.

2.3. Effect of nitrate concentration on lipid and FAME composition in nutrient medium under outdoor culture conditions

This experiment was carried out in individual 250 l rectangular FRP tanks, filtered seawater enriched with Walne medium. Four different concentrations of solution 'A' (50 g l^{-1}, 75 g l^{-1}, 100 g l^{-1} and 125 g l^{-1}) of the Walne medium were prepared with potassium nitrate.

2.4. Effect of different carbon sources on lipid and FAME composition in a culture medium under indoor and outdoor conditions

Seed culture grown under photoautotrophic condition with 12:12 h photoperiod was used for outdoor mass culture. In the outdoor experiments, four different carbohydrates such as glucose, fructose, sucrose and maltose were supplied as carbon sources at a concentration of 2 g l^{-1} in the culture medium, under photoheterotrophic condition. Indoor experiments were conducted with two concentrations of glucose (1 g l^{-1} and 2 g l^{-1}) as a carbon source in culture medium under photoheterotrophic conditions. Growth was monitored in terms of cell concentration until the culture entered into stationary phase. In all experiments, dry biomass from cultures in late exponential was used for lipid extraction.

2.5. Harvesting

Cells were harvested from late exponential phase cultures by adding 0.1 g l^{-1} aluminum sulfate. Wet biomass was dried under direct sunlight until it formed a slurry. The slurry was thoroughly rinsed with distilled water to remove the salt and excess alum, followed by drying in a precision hot air oven (Thermo Scientific) at 60 °C for 24 h to obtain the dry biomass. To ensure moisture free biomass, cell dry weight was checked every 2 h after the 18th hour. The cell dry weight was constant after 24 h drying period.

2.6. Determination of lipid content

The total lipid was extracted following the Bligh and Dyer [22] method for algal lipids. Crude lipid was extracted from 5 g of algal dry biomass using chloroform:methanol (2:1, v/v; 75 ml) in two stages (8 h) in an automatic Soxhlet extractor (Velp/Soxtherm, Gerhardt) in the presence of BHT (butylated hydroxytoluene) added as an antioxidant. After extraction, the combined organic solvent was evaporated using a rotovap apparatus.

2.7. Determination of fatty acid composition

The direct trans-esterification method of Lepage and Roy [23] was adopted in this study. The dry biomass (5 g) was mixed in 25 ml of methanol with 2% H_2SO_4 and the resulting reaction mixture was refluxed for 4 h at 90 °C. Methanol was removed after completion of the reaction by using a rotovap system and the methylated product extracted with ethyl acetate and washed with water until neutralization. The organic phase was dried over anhydrous Na_2SO_4, concentrated in the rotovap, dried under vacuum and weighed as FAME content. Thin-layer chromatography (TLC) was applied to monitor the progress of methyl esterification on the extracted fatty acids. The TLC was performed using silica gel 60 F_{254} plates (20 × 20 cm, 250 μm layer thickness, Merck, Darmstadt, Germany) and pure chloroform as the solvent system. The reaction products were analyzed by gas chromatography–mass spectrometry (GC–MS) and also by gas chromatography (GC).

2.8. GC–MS/GC analysis protocol

The fatty acid composition of algal oil was analyzed qualitatively using GC–MS and quantitatively using GC. The GC/GC–MS analyses were carried out using an Agilent 6890N gas chromatography connected to an Agilent 5973 mass selective detector at 70 EV (m/z 50–550; sources at 230 °C and quadrupole at 150 °C) in the electron impact mode with a HP-5 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The oven temperature was programmed for 2 min at 160 °C and raised to 300 °C at 5 °C min^{-1} and maintained for 20 min at 300 °C. The carrier gas, helium, was used at a flow rate of 1.0 ml min^{-1}. The inlet temperature was maintained at 300 °C with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation patterns of standard compounds.

GC analysis was performed on a HP 6850 series gas chromatography equipped with an FID detector and DB-225 capillary columns (30 mm × 0.25 mm i.d. × 0.25 μm film thickness). The injector and detector temperatures were maintained at 300 and 325 °C, respectively. The oven temperature was programmed for 2 min at 160 °C and raised to 300 °C at 5 °C min^{-1} and maintained for 20 min at 300 °C. The carrier gas, nitrogen, was used at a flow rate of 1.5 ml min^{-1}. The injection volume was 1 μl, with a split ratio of 50:1. The identification of individual fatty acids was based on retention time of fatty acid standards.

2.9. Statistical analysis

The statistical significance of the differences observed between treatments was assessed using one way ANOVA. The normality and homoscedasticity of the data were checked through Kolmogorov–Smirnov and Cochran's tests, respectively prior to conducting ANOVA. The Tukey's HSD test was used after ANOVA to identify significant differences between mean values, with a probability level of 5% ($p < 0.05$) indicating significance. All statistical analyses were conducted with SPSS version 17.0 software for PCs. All treatments were carried out in triplicate and the results were shown as the means of three replicates ± standard deviation (SD).

3. Results and discussion

3.1. Biomass and lipid concentration under different nitrate concentrations under outdoor mass cultivation

The observed biotic production parameters (biomass concentration in terms of dry weight and lipid concentration) of *T. gracilis* and

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