Lipid Productivity and Biosynthesis Genes Response of Indigenous Chlorella sp. T4 Strain under Different Nitrogen and Phosphorus Load

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Research Article

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Abstract

Microalgae can synthesize and accumulate high neutral lipids upon exposure to abiotic stress such as nutrient starvation or limitation. In this study, indigenous microalgae *Chlorella* sp. T4 was cultivated in nitrogen and phosphorus under both limiting and replete conditions. Growth, lipid yield, fatty acid profiles and biosynthetic gene expression levels were determined to ascertain cell's response under these conditions. An impaired cell growth was observed under nitrogen limiting condition, evident by the lowest biomass yield (0.58±0.03 g L\(^{-1}\)) as revealed by low quantum efficiency of photosystem II (Fv/Fm) value and chlorophyll a content. An increase in lipid content yield was observed under nitrogen and phosphorus limiting conditions as compared to the control. Nutrient limiting conditions produced fatty acid methyl ester that is suitable for biodiesel production compared to the control (BG-11). Gene expression analysis using real time q-PCR for photosynthesis (*rbcL*) and lipid biosynthesis (*accD, KAS-1, \(\omega-6\) FAD, \(\omega-3\) FAD) genes revealed different expression levels under both limiting and replete conditions. Under nutrient limiting conditions, increase in the expression of *accD*, *KAS-1*, \(\omega-6\) FAD and \(\omega-3\) FAD genes was observed, whereas a decrease in *rbcL* gene expression level was noted. A significant correlation could be drawn between the expression levels of the biosynthetic genes and growth rate, biomass yield, physiological response, lipid yield and fatty acid composition. These results provide an insight into the physiological response and gene expression level under different nutrient levels, which could be harnessed for future genetic engineering of *Chlorella* sp. T4 for improved lipid production.

1. Introduction

The demand for biodiesel, as a replacement for the conventional fossil fuel, is growing worldwide in recent years [1]. Microalgae have been a favourable feedstock for biodiesel production due to its fast growth rate, high lipid yield, suitable fatty acid composition and adaptability to a wide range of climatic environment [2, 3], but high production cost at commercial scale is still a major drawback [4]. Microalgae tend to accumulate energy storage material in form of lipids and starch under stress conditions, when the cell growth reaches the stationary phase [5]. It has been shown that optimization of microalgal culture condition can result into high lipid productivities in microalgal biomass. Manipulation of several key intrinsic and extrinsic factors such as nutrient stress, light intensity, temperature, and carbon source triggers lipid accumulation pathway [6–8]. Nutrient deprivation is often used by many researchers to improve overall microalgal lipid productivity, due to it's low cost and easy application during the cultivation process [9]. This approach causes decrease in photosynthetic rates, and compromises biomass accumulation, while resulting in enhanced overall lipid storage in form of triacylglycerol [10, 11]. Nitrogen (N) is regarded as important micro-nutrient for microalgal growth, as it is associated with protein synthesis and cell division [11], whereas phosphorus (P) contributes to various metabolic processes such as signaling pathways, energy generation and photosynthesis.

Most studies have been focused on obtaining high lipid productivity yield under nutrient-stress conditions, without a proper understanding of the effect of these conditions on the microalgae photosynthesis activity, physiological response, and gene expression levels under these conditions [12,
The understanding of microalgal response at the molecular level is limiting to few species, such as Chlamydomonas reinhardtii, Thalassiosira psuedonana and Dunaliella salina [14]. The microalgae under study, Chlorella sp. T4 strain, isolated in our laboratory, have demonstrated huge potential to accumulate large amount of fatty acid that can be used for biodiesel production [15]. In this study, different concentrations of N and P were applied to trigger hyper lipid accumulation in this indigenous microalga. Furthermore, the physiological response of the microalgae under different nutrient conditions and expression levels of five key fatty acid biosynthetic genes (rbcL, accD, KAS-1, ω-6 FAD and ω-3 FAD) were investigated. The rbcL gene encodes the catalytic large subunit of the enzyme RuBisCO (ribulose 1.5-bisphosphate carboxylase/oxygenase) which is responsible for carbon fixation, catalyzing the first step in the Calvin cycle [14]. Previous study reported a decrease in rbcL gene expression under N and P deficient conditions in Chlorella sorokiniana [3]. accD encodes for acetyl-coenzyme A carboxylase carboxyl transferase subunit beta which is responsible for fatty acid biosynthesis, and catalyses the conversion of acetyl-CoA to malonyl-CoA during the lipid biosynthesis [6]. An increase in accD expression under nutrient deficient conditions has been reported in Chlorella pyrenoidosa [16]. The expression levels of three fatty acid biosynthetic genes; KAS-1, ω-6 FAD and ω-3 FAD in relation to fatty acid yield under different N and P concentrations have been investigated. The KAS-1 gene encodes for ketoacyl-ACP synthase-1 responsible for the addition of malonyl-CoA to elongate 4-carbon fatty acid to 6-, 12- and 16 carbon fatty acid chains, for the production of palmitic and stearic acid. Furthermore, ω-6 FAD gene which encodes for omega-6 desaturase responsible for catalyzing the conversion of oleic acid into linoleic acid, while ω-3 FAD gene code for omega-3 desaturase which is responsible for the conversion of ω-6 fatty acid into ω-3 fatty acid [17–19]. Therefore, the aim of the present study was to design suitable cultivation conditions for high lipid accumulation that can be used for biodiesel production. To understand the expression of key functional genes (rbcL, accD, KAS-1, ω-6 FAD and ω-3 FAD) by varying N and P concentration in the growth medium.

2. Materials And Methods

2.1. Algal strain and seed preparation

The microalgal strain Chlorella sp. T4 used in this study was isolated from freshwater body in KwaZulu-Natal, South Africa [15]. The strain was preserved in BG-11 medium which is composed of (g L⁻¹): NaNO₃, 1.5; K₂HPO₄, 0.04; MgSO₄.7H₂O, 0.75; CaCl₂·2H₂O, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; Na₂CO₃, 0.02 and 1 mL of micronutrient or trace metal solution containing (g L⁻¹): H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.22; NaMoO₄·5H₂O, 0.079; Co(NO₃)₂·6H₂O, 0.04. The medium was adjusted to a pH of 7 and sterilized in an autoclave at 121°C for 15 min.

The culture (10% v/v) was inoculated in 500 mL conical Erlenmeyer flasks containing 200 mL of BG-11 medium. An aliquot of tetracycline (0.5 µl mL⁻¹) was added to the growth medium to prevent any bacterial contamination in the microalgal samples. The culture was incubated at 25°C under cool white fluorescent illumination of 200 µmol m⁻²s⁻¹ with a photoperiod of 12h: 12h, light: dark cycle under
ambient CO\textsubscript{2}. Similar cultivation conditions were maintained for all the subsequent experiments. The cultures were hand-shaken two to three times daily to avoid settling and sticking of the culture onto the bottom of the flask.

### 2.2. Experimental design and physiological parameters analysis

To find the best nitrogen (N) and phosphorus (P) concentration for high lipid productivity yield, \textit{Chlorella} sp. T4 was cultivated in BG-11 medium containing sodium nitrates (0.75 and 2.25 g L\textsuperscript{-1}) and di-potassium-ortho-phosphate (0.02 and 0.06 g L\textsuperscript{-1}). These nutrient concentrations were selected based on the lipid productivity yield obtained from the preliminary study conducted by growing the microalgae strain on BG-11 containing different concentrations of N (0, 0.35, 0.75, 2.25 g L\textsuperscript{-1}) and P (0, 0.02, 0.04, 0.06 g L\textsuperscript{-1}). Optimization was conducted with one factor at a time, and other individual media composition kept constant as in BG-11 to assess the individual effect of the culture treatment on \textit{Chlorella} sp. T4. In addition, control experiment was conducted using BG-11 medium with normal concentration of sodium nitrates (1.5 g L\textsuperscript{-1}) and di-potassium-ortho-phosphate (0.04 g L\textsuperscript{-1}) that is known to support microalgae growth. The algal cell was standardized to the optical density of 0.05 at 680 nm. The cells were harvested by centrifugation at 5000 rpm for 10 min, washed with distilled water, and resuspended into appropriate medium containing different concentrations of sodium nitrate and di-potassium-ortho-phosphate. The culture condition was maintained at 25°C under continuous fluorescent light with light intensity of approximately 100 µmol m\textsuperscript{-2}s\textsuperscript{-1}, and the flasks were hand shaken 2 to 3 times a day.

The Chlorophyll a content of \textit{Chlorella} sp. T4 was measured as described previously [20]. The physiological and photosynthesis efficiency of the microalgal cells were studied as described previously [21]. The maximum quantum efficiency of Photosystem II (PS II) was calculated using the equation: \( \frac{F_v}{F_m} = \frac{(F_m-F_0)}{F_m} \) as previously described [21], Where \( F_m \), \( F_o \), and \( F_v \) represents the maximum, minimum and variable fluorescence, respectively.

### 2.3. Measurement of cell growth, biomass concentration, lipid yield and fatty acid content

Cell growth determination, biomass concentration measurement, and algal lipids extraction and weight determination were carried out as described previously [15]. The harvesting was done by centrifugation at 5000 rpm for 10 min. The fatty acid content were quantified as described previously [15] while the biodiesel properties were estimated using the web version of the Biodiesel Analyzer 2.2 [22].

### 2.4. Gene expression analysis

The expression levels of five key fatty acid biosynthetic genes (\textit{rbcL}, \textit{accD}, \textit{KAS-1}, \( \omega-6 \) \textit{FAD} and \( \omega-3 \) \textit{FAD}) were determined in samples collected at the early log phase (day 7), late log phase (day 14) and stationary phase (day 21) of growth in the different nutritional growth conditions. The total RNA was
extracted from \( \approx 100 \) mg of algal cells using GeneJet RNA purification kit (Thermo Fisher Scientific, MA, USA) followed by synthesis of first-strand cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instruction. The level of gene expression was monitored by Real-time quantitative PCR performed with Universal SYBR Green Supermix (Bio-Rad, CA, USA) in Hard-Shell High-Profile 96-Well Semi-Skirted PCR Plates (Bio-Rad, CA, USA) using 50 ng cDNA as the template and primer pairs listed in Table 1.

| Gene  | Sequence (5'-3') | Tm (°C) | Amplicon size (bp) | Reference |
|-------|------------------|---------|--------------------|-----------|
| accD  | (F) TTTGGTTTGTGCTTCTGGTG  
         (R) CACCACCAGTTGTTGGAGAA | 51.9    | 149                | [3]       |
| rbcL  | (F) CTTTCCAAGGTCTCCTCAC  
         (R) TCTCTCCAACGCATAATGG | 56.4    | 208                | [3]       |
| KAS-1 | (F) CCATGATTGGTCATTGCTTGAGGAC  
         (R) GCTCTTGCTTCATGTTTGGGACCAC | 58      | 151                | [17]      |
| \( \omega \)-6 FAD | (F) CTTCACCACCAAGGCACAGGC  
                     (R) CCTGCACACTGCTGGGAACG | 58.8    | 129                | [17]      |
| \( \omega \)-3 FAD | (F) CATGTTGAGAAGCAGAGGTCTGCTTGAT  
                     (R) GTCAAGTGGGAGCCAGTCTTGC | 59      | 162                | [17]      |
| 18S rRNA | (F) CCTGCGGCTTATTTTGAACCTCAACACG  
              (R) TAGCAGGCTGAGGCTACGTTGC | 60      | 172                | [17]      |

All PCR reactions consists of 1 µL 50 ng cDNA template, 1.5 µL of 10 mM deoxyribonucleotide triphosphates, 0.4 µM final concentration of each forward (F) and reverse (R) primers (Table 1), 10 µL 2 × iQ SYBR Green Supermix and nuclease-free water to final volume of 20 µL. RT-qPRC amplification protocol for targeted genes consists of initial denaturation for 3 min at 95°C, followed by 40 cycles of three steps consisting of 15 s at 95°C, 20 s at appropriate annealing temperature and 30 s at 72°C. The specificity of all PCR amplifications was verified with melting curve calculation at the completion of each run, set from 55°C to 95°C at 0.5°C increment. The gene expression levels were normalized by the expression level of 18S rRNA gene and data presented as fold increase or decrease of the target gene expression levels in the treated samples relative to the control sample [23, 24, 3].

2.5. Statistical analyses of experimental results
The data were analysed by one-way ANOVA at 95% confidence limit (α = 0.05). All statistical tests were performed using SPSS (v. 20, IBM). Unless otherwise stated, p < 0.05 denotes a statistically significant difference. The values were expressed as the mean ± standard deviation.

3. Results And Discussion

3.1. Cell growth and biomass accumulation

The effects of varying nutrient concentrations on the growth of Chlorella sp. T4 were investigated to ascertain suitable condition for biomass yield and high lipid productivity. Cultivation of microalgae under nutrient limiting condition has been reported to decrease the overall algal biomass, while inducing synthesis of neutral lipid suitable for biodiesel production [12]. In this study, nutrient stress conditions produced adverse effects on the proliferation of Chlorella sp. T4 cells (Fig. 1). As shown in Table 2, low specific growth rates $0.055 \pm 0.004 \text{h}^{-1}$ was observed when Chlorella sp. T4 was cultivated under N-limiting medium, with short generation time of $0.079 \pm 0.005 \text{day}^{-1}$ compared to the control. High specific growth rate of $0.079 \pm 0.004 \text{h}^{-1}$ was observed under N-replete medium which was not significantly higher than that the growth rate in the control medium. This is also reflected by similar growth pattern of Chlorella sp. T4 obtained in N-replete medium and control medium (Fig. 1a). It proves the importance of nitrogen as a macro nutrient required for protein synthesis and cell division in microalgae [11]. The observed growth patterns under N and P-limiting medium (Fig. 1) are consistent with those reported for other Chlorella strains [17, 14, 25].

| Nutrient treatment | Growth rate (h$^{-1}$) | Generation time (day$^{-1}$) |
|--------------------|------------------------|-----------------------------|
| Control            | $0.076 \pm 0.014^{b}$   | $0.109 \pm 0.020^{ab}$      |
| N-0.75             | $0.055 \pm 0.004^{c}$   | $0.079 \pm 0.005^{ab}$      |
| N-2.25             | $0.079 \pm 0.004^{b}$   | $0.114 \pm 0.006^{b}$       |
| P-0.02             | $0.050 \pm 0.011^{c}$   | $0.072 \pm 0.016^{c}$       |
| P-0.06             | $0.098 \pm 0.014^{a}$   | $0.147 \pm 0.020^{a}$       |

N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-$1.5 \text{g L}^{-1}$ + P-$0.04 \text{g L}^{-1}$. Different letters depict significant difference within the group according to one-way ANOVA at p < 0.05. Values show the average of three replicates ±SD.

The microalgae Chlorella sp. T4 showed tolerance to high N concentration as demonstrated by the growth curve similar to that obtained in the control medium. Similarly, higher specific growth rate was
observed in medium with higher concentration of phosphorus. The specific growth rate \((0.050 \pm 0.011 \text{ h}^{-1})\) observed under P-limiting condition is about two-fold less compared to the value obtained in the P-replete medium \((0.098 \pm 0.014 \text{ h}^{-1})\). This was also corroborated by the growth patterns of the strain under P-limiting condition (Fig. 1b). P-replete medium produced higher generation time of \(0.147 \pm 0.020 \text{ day}^{-1}\), which is 1.35- and 2.04-fold higher than the generation times obtained in the control and P-limiting medium. Phosphorus is a constituent element of ATP, essential for photophosphorylation which has significant relevance to the cell growth and metabolism of microalgae. Photosynthetic microalgae require large amounts of proteins (mainly RuBisCO) which is synthesized by phosphorus-rich ribosome [26].

For all the experiments, biomass yield and productivity together with lipid content and productivity were calculated after 21 days cultivation period. Nitrogen replete medium (N-2.25) produced the highest biomass yield of \(0.82 \pm 0.06 \text{ g L}^{-1}\) which is 41.4% significantly \((p < 0.05)\) higher than the biomass yield in the nitrogen deficiency (N-0.75) medium but not significantly different from that obtained in the control medium (Table 3). Similarly, 23.4% significantly \((p < 0.05)\) higher biomass yield was obtained in P-replete (P-0.06) medium compared to the P-deficiency (P-0.02) medium. This is further correlated by the high biomass productivity \(38.95 \pm 0.84 \text{ mg L}^{-1} \text{ d}^{-1}\) obtained under N-replete medium \(38.95 \pm 0.84 \text{ mg L}^{-1} \text{ d}^{-1}\) and P-replete medium \(37.52 \pm 0.53 \text{ mg L}^{-1} \text{ d}^{-1}\) due to high nutrient availability to utilize for cell division (Table 3). Reduction in nutrients concentration in media has been shown to slow down the metabolic activity and cell division in most microalgae, while triggering lipid accumulation [27]. It is therefore not surprising that a significantly 1.6-fold and 1.2-fold increases in lipid productivity and lipid content were obtained in nitrogen limiting and phosphorus limiting medium, respectively, relative to the nutrient replete medium.
High biomass productivity by this microalgae strain under both nutrient rich and nutrient stressed conditions is very promising, since biomass productivity is one of the major traits that makes microalgae attractive feedstock for biofuel applications over plant-based feedstocks [28]. Similarly, [29] found that an increase in N concentration resulted high biomass yield of 1.56 and 1.78 g L\(^{-1}\) for *Chlorella sorokiniana* (PCH02) and *Chlorella vulgaris* (PCH05), respectively. The observed overall increase in biomass yield and biomass productivity in N and P rich medium of this strain may be due to luxurious uptake of phosphorus which gets deposited in the cell as polyphosphate involved in metabolic pathway and storage for further use during phosphorus starvation/ limitation [30]. Similarly, [25] reported an increase in biomass concentration of *Chlorella minutissima* MCC as the phosphate concentration increase from 0 to 3 mM.

### 3.2. Chlorophyll content and physiological response of Chlorella sp. T4 under different nutrient conditions

Chloroplast is an important unit component for most photosynthetic plants and algae. Hence, the chlorophyll content and the viability of the photosynthetic process are critical physiological indicator for monitoring microalgae adaptability to different culture conditions [14]. The chlorophyll a content was measured during the cultivation period as an indicator of the physiological response of *Chlorella* sp. T4 in different N and P conditions. An increase in chlorophyll a content was observed as the concentration of N and P increased in media. Highest chlorophyll a content (in mg/g dcw) of 27.11 ± 0.01 (Fig. 2a) and 26.50 ± 0.67 (Fig. 2b) was observed after 21 days in N and P-replete medium, respectively. A significantly

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| Experiments (N, P) | Biomass (g L\(^{-1}\)) | Biomass productivity (mg L\(^{-1}\) d\(^{-1}\)) | Lipid content (%dcw) | Lipid productivity (mg L\(^{-1}\) d\(^{-1}\)) |
|--------------------|------------------------|-----------------------------------------------|----------------------|---------------------------------|
| Control            | 0.77 ± 0.01\(^{ab}\)   | 36.48 ± 0.64\(^{a}\)                        | 25.87 ± 1.03\(^{c}\)   | 12.93 ± 0.51\(^{b}\)            |
| N-0.75             | 0.58 ± 0.03\(^{ab}\)   | 27.58 ± 1.23\(^{a}\)                        | 31.07 ± 0.53\(^{a}\)   | 15.54 ± 0.27\(^{a}\)            |
| N-2.25             | 0.82 ± 0.06\(^{ab}\)   | 38.99 ± 0.85\(^{b}\)                        | 19.99 ± 0.01\(^{e}\)   | 10 ± 0.15\(^{b}\)               |
| P-0.02             | 0.64 ± 0.01\(^{b}\)    | 30.28 ± 0.68\(^{a}\)                        | 28.33 ± 1.35\(^{b}\)   | 14.17 ± 0.67\(^{b}\)            |
| P-0.0.6            | 0.79 ± 0.50\(^{a}\)    | 37.52 ± 0.53\(^{a}\)                        | 23.80 ± 0.26\(^{d}\)   | 11.90 ± 0.13\(^{b}\)            |

N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L\(^{-1}\) + P-0.04 g L\(^{-1}\). Different letters depict significance difference within the group according to one-way ANOVA at \(p < 0.05\). Values show the average of three replicates ±SD.
A (p < 0.05) decrease in chlorophyll a content by 1.2-fold and 1.4-fold under N and P-deficient conditions compare to the control, respectively, were recorded. It suggests that the decrease of N and P concentration in the medium results in lower cell chlorophyll accumulation due to scarcity of intracellular nitrogen and phosphorus pool to synthesize chlorophyll for further cell reproduction. Nitrogen and phosphorus are most important elements contributing to the growth of microalgae cell, it limitation significantly changes the biosynthesis of cell pigment [31]. The results clearly show the influence of nutrient limitation on the growth physiology of Chlorella sp. T4 as the growth rate and biomass yield also decreased under these conditions.

The widely used fluorescence parameter Fv/Fm, an index reflecting irradiance acclimation status [32] was investigated under nutrient deficient and replete medium. It represents the measure of photosystem II (PSII) quantum yield and could be used to evaluate the photo induced damage to protein complex [33]. The positive influence of N and P treatment conditions was observed on the growth of Chlorella sp. T4 (Fig. 2c, d). A significantly (p < 0.05) decrease in Fv/Fm by 1.21-fold and 1.12-fold was observed under N and P-limiting condition compared to the control, respectively. The decrease in Fv/Fm is an indicative of the photoinhibition of PSII by Chlorella sp. T4. Microalgae tend to redirect their energy from photosynthetic process under nutrient starved conditions toward maximizing nutrient uptake upon nutrient addition, leading to a net decrease in the capacity of cells to dissipate energy photochemically [34]. Chlorella sp. T4 exhibited a steady decline in the photosynthetic parameter Fv/Fm under N and P-replete medium by 1.13-fold and 1.12-fold compared to the control, respectively. It has been shown that the Fv/Fm ratio in microalgae increase markedly when cultured under nutrient sufficient condition [3].

3.3. Analysis of lipid content and composition

Studies have shown that cultivation of microalgae under nutrients deficiency conditions stimulates lipid biosynthesis in many microalgae species [3, 8]. In this study, Chlorella sp. T4 showed a significant (p < 0.05) increase in total lipid yield under N and P-limiting conditions, accounting for 31.07 ± 0.53% and 28.33 ± 1.35% of dry cell weight, respectively (Table 3). [35], cultivated C. zofingiensis under N and P-deficient medium and reported higher lipid contents as compared to the nutrient sufficient medium which is similar to the present study. [3] also observed high lipid accumulation by Chlorella sorokiniana under N and P-deficient medium compared to the control medium. Contrary, [12] cultivated Chlorella sp. in medium containing different N concentration and observed an increase in lipid accumulation under N-replete medium compared to N-deficient conditions.

Lipid productivity is one of particular importance microalgal lipid production process as it considers both lipid content and biomass production rate. High lipid productivity of 15.54 ± 0.7 mg L⁻¹ d⁻¹ was obtained under N-limiting condition which was 1.37-fold higher than P-limiting condition after 21 days (Table 3). Nonetheless, nutrient limiting conditions repressed the growth of Chlorella sp. T4 and the overall productivity caused by nutrient deficiency was not offset by biomass loss. Similarly, [14] reported high lipid productivity of 47.05 mg L⁻¹ d⁻¹ under N-deficiency in Chlorella pyrenoidosa after 5 days of cultivation. Also, [36] investigated the effects of phosphorus on lipid accumulation of Chlorella vulgaris.
and reported a lipid productivity of 19.40 mg L⁻¹d⁻¹ in phosphorus deficient medium. Low lipid productivity was observed under N-replete medium (10 ± 0.15 mg L⁻¹ d⁻¹) which was 2.32-fold lower than the control medium. These findings clearly show that high lipid productivity yield can be obtained by cultivating microalgae under nutrient deficiency conditions that has just enough nutrients to support the growth.

Microalgae biomass contains significant quantities of lipids in the form of triacylglycerol, which can be converted to biodiesel via transesterification process. This has attracted huge commercial interest of using microalgae as feedstock for biodiesel production [28]. The lipid composition of *Chlorella* sp. T4 varied according to the nutrient concentration of the growth medium (Table 4). Previous studies have also shown that the concentration of nitrogen and phosphorus in microalgae culture alter total fatty acid content and composition [17, 37]. Palmitic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) constitute the major fatty acids in algal oil. Fatty acids of this chain length are reported to be suitable for high quality biodiesel production [1]. Further analysis reveals that saturated fatty acid (SFA) ranged from 32–43.3%, monounsaturated fatty acid (MUFA) ranged from 21.4–37.1%, whereas polyunsaturated fatty acid (PUFAs) ranged from 27.1–37.3%. Polyunsaturated fatty acids are important with nutritionally benefits for infant development and with estimated market value of over 11 billion US dollars [28].

| Fatty acids          | Control | N-0.75 | N-2.25 | P-0.02 | P-0.06 |
|----------------------|---------|--------|--------|--------|--------|
| Palmitic acid (C16:0)| 29.7 ± 1.3a | 31.1 ± 0.1a | 30.8 ± 0.7c | 25.5 ± 0.5b | 30.4 ± 0.9a |
| Stearic acid (C18:0) | 2.8 ± 0.2d | 8.3 ± 0.2b | 6.8 ± 0.8c | 9.5 ± 0.1a | 12.9 ± 0.2e |
| Oleic acid (18:1)    | 37.1 ± 0.1a | 35 ± 1.7b | 30.2 ± 0.3c | 27.4 ± 0.7d | 29.6 ± 1.7c |
| Linoleic acid (C18:2)| 27.6 ± 0.4b | 21.4 ± 0.0a | 28.8 ± 0.6a | 29.9 ± 1.5a | 24.5 ± 0.0a |
| α-Linoleic acid (C18:3n3)| 2.58 ± 0.8a | 4.2 ± 0.5a | 3.3 ± 0.5a | 7.4 ± 0.4a | 2.6 ± 0.1a |

N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L⁻¹ + P-0.04 g L⁻¹. Different letters depict significance difference among the group according to one-way ANOVA at *p* < 0.05. Mean value shown is the average of three replicates ±SD.

There was high level of saturated fatty acid observed under P-replete (49.9%) medium, which is 1.3-fold higher than the control medium (Table 4). High level oleic acid (37.1 ± 0.1%) was obtained in the control medium, but not significantly (*p* ≥ 0.05) different from the level obtained in both the N and P-limiting medium. High content of oleic acid is beneficiary for excellent oxidative stability, increases biodiesel’s flow properties and reduces it solidification temperature [38, 39]. Furthermore, high content of PUFAs was found in P-limiting condition which could cause decline of cetane number and oxidation stability, making
biodiesel prone to oxidation-dependent degradation [38, 40]. This finding was well with the results reported by [17] who reported that high accumulation of PUFAs was obtained under P-limiting condition.

The quality of microalgae biodiesel is measured by the important thermophysical properties of biodiesel and comparing those with the international standards such as ASTM D675 or EN14214 (Table 5). Previous studies have demonstrated that fatty acid profile significantly affected the quality of biodiesel [22, 1]. The oxidative stability of the biodiesel produced in this study ranged between 5.75 to 7.20 h which is favourable for biodiesel production due to saturated fatty acid [41]. This microalgae strain showed low cold filter plugging properties (-2.75 ºC) under N-limiting condition which is preferable for biodiesel production. This was caused by a good balance between the saturated fatty acid and monounsaturated fatty acid observed under the N-limiting condition.
| Biodiesel properties (Units) | Standard fuel parameters | Transesterification |
|-----------------------------|--------------------------|---------------------|
|                             | ASTM D6751 | EN 14214 | Control | N-0.75 | N-2.25 | P-0.02 | P-0.06 |
| Iodine value (gI₂/100 g)    | -         | 120 (max) | 82      | 90.54  | 88.36  | 99.05  | 78.11  |
| Saponification value (mg KOH) | -         | -        | 205.42  | 211.12 | 204.67 | 210.51 | 204.62 |
| Cetane number               | 47 (min)  | 51.0 (min) | 54.45  | 52.61  | 50.9   | 49.94  | 55.40  |
| Degree of unsaturation (% wt) | -         | -        | 86.50  | 97.60  | 94.40  | 102.00 | 83.80  |
| Long-chain saturation factor (% wt) | -         | -        | 7.26   | 4.37   | 6.48   | 7.63   | 9.49   |
| High heating value (MJ kg⁻¹) | -         | -        | 39.56  | 39.51  | 39.38  | 40.60  | 39.45  |
| Cold filter plugging properties (ºC) | -         | -        | 6.33   | -2.75  | 3.88   | 7.49   | 13.34  |
| Kinematic viscosity (mm² s⁻¹) | 1.9-6.0  | 3.5-5.0  | 1.35   | 1.33   | 1.33   | 1.36   | 1.35   |
| Density (g cm⁻³)            | -         | 0.86-0.90 | 0.87   | 0.87   | 0.87   | 0.90   | 0.87   |
| Oxidative stability (h)     | 3 (min)   | ≥ 6      | 7.20   | 6.50   | 6.26   | 5.75   | 6.94   |
| Linoleic acid (%)           | -         | 12       | 27.6   | 21.4   | 28.8   | 29.9   | 24.5   |

ASTM D-6751-American Society for Testing and Materials, EN 14214-European standard for biodiesel, N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L⁻¹ + P-0.04 g L⁻¹.

High saturated fatty acid content may reduce the cold filter plugging point properties of biodiesel because saturated fatty acid has higher melting points than unsaturated fatty acid [42]. Furthermore, kinematic viscosity (mm² s⁻¹) produced by *Chlorella* sp. T4 under all N and P conditions was outside the range recommended by ASTM D675 and EN14214. This property may result into biodiesel produced with high viscosity affecting the fuel atomization and lead to deposits forming inside the engine, due to high PUFAs contain by microalgae compare to the other seed oils. Linoleic acid was above 12 recommended by EN14214 for all the conditions, an indication of poor oxidative stability with good cold flow properties.
In this study, the best biodiesel was produced under N-limiting condition, with high ignition quality, good oxidative stability, cetane number value and saponification value (Table 5).

3.4. Effect of culture conditions on the expression of rbcL and accD genes of Chlorella sp. T4

During photosynthesis, the RuBisCo enzyme is involved in carbon fixation process. A large subunit of this enzyme encoded by gene *rbcL* which harbour binding site [43, 44]. The present study evaluates the effect of nutrient conditions on the expression levels of some functional and fatty acids biosynthetic genes from *Chlorella* sp. T4. A significant ($p<0.05$) decrease of 2.09-fold in the *rbcL* gene expression was observed under N-limiting condition after 21 days compare to the control (Fig. 3a). There was no significant difference in the expression level of *rbcL* under N-replete compared to the control medium (Fig. 3a). Similarly, the *rbcL* gene was significantly ($p<0.05$) decreased by 1.59-fold under P-limiting condition after 21 days compare to the control (Fig. 3b). Under nutrient stress, the cell protein synthesis and photosynthetic rates is affected as chlorophyll a is utilized as an intracellular nitrogen to support the growth of microalgae [45]. [14] cultivated *Chlorella pyrenoidosa* under N and P-deficient conditions and reported high expression of *rbcL* gene in the nutrient condition which was two to five times higher than N-deficient condition. [3] also reported 78% and 56% down regulation of *rbcL* gene in N and P stress conditions on *Chlorella sorokiniana*, respectively. The decrease in the expression of *rbcL* gene was also translated by low specific growth rate (Table 2) and decrease of maximum quantum efficiency of PSII (Fig. 2) under N-limiting condition in this study. The expression of *rbcL* gene was significantly ($p<0.05$) increased by 1.12-fold under P-limiting condition compared to the control medium just after 7-day incubation periods. (Fig. 3b). Microalgae utilizes phosphorus for the transfer and signal transduction during photosynthesis [46]. Microalgae under nutrient sufficient medium tend to require more fixed carbon cell construction, which then demand for more RuBisCO to sequester the CO$_2$ in the air.

Acetyl-CoA carboxylase (ACCase) is regarded as rate-limiting enzyme for fatty acid synthesis and it has been overexpressed in different organism to enhance lipid production [6]. A study by [14] suggested a strong involvement of *accD* in triggering lipid accumulation by the cell under nutrient deficient conditions. The present study evaluated the expression of heteromeric ACCase unit (*accD* gene) as a function of different N and P concentrations on lipid synthesis. A significant increase in the expression of *accD* gene was observed under nutrient limiting conditions during the cultivation period as compared to the cells grown in standard BG-11 medium (Fig. 3c & d). In N-limiting condition, 3.11-fold increase of *accD* gene expression was observed after 21 days cultivation compared to the control (Fig. 3c). Likewise, a significant ($p<0.05$) 1.89-fold increase in the expression *accD* gene by was observed under P-limiting condition after 21 days of cultivation compared to the control (Fig. 3d). Usually, lower photosynthetic rates cause NADH accumulation inhibiting enzyme citrate synthase so that the acetyl-CoA is blocked from entering TCA cycle. By increasing the acetyl-CoA concentration, ACCase is activated resulting in the enhancement of lipid content in microalgae [6]. This was evidently shown by an increase lipid yield by *Chlorella* sp. T4 under N and P-limiting conditions compare to the control medium (Table 3). During nutrient starvation, cell tends to synthesis lipids as a protective mechanism against stressful condition [47].
The expression of \textit{accD} gene under N-replete medium was significantly lower compared to the control and N-deficient medium, with 1.3-fold and 2.6-fold increase obtained, respectively at day 21 (Fig. 3c). Similarly, the expression of \textit{accD} gene under P-replete medium was significantly lower by 2.95-fold compare to the control at day 21 (Fig. 3d). [3], cultivated \textit{Chlorella sorokiniana} under N and P-limiting conditions along with metal stress. They recorded a 3.24-fold and 2.93-fold increase in the expression of \textit{accD} gene at the late log phase compared to the control medium (BG-11). Also, a significant correlation was found between the expression of \textit{accD} gene, growth rate, photosynthetic efficiency, and lipid accumulation. Based on the results obtained in the present study, the expression of \textit{accD} was observed to be higher under nutrient limiting medium. This was attributed by higher amount of lipid content under nutrient limiting medium despite lower biomass yield compared to N-replete medium.

3.5. Effect of culture conditions on the expression of KAS-1, \(\omega\)-6 and \(\omega\)-3 desaturase gene of \textit{Chlorella} sp. T4

Another key gene in the process for fatty acid biosynthesis is \textit{KAS-1} which is required for the addition of malonyl-CoA to elongate a 4-carbon fatty acid to 6-, 12- and 16 carbon fatty acid chains [18]. There was no significant difference in the expression level of \textit{KAS-1} gene under N-deficiency and control medium after 21 days as observed in this study (Fig. 4a). The expression of \textit{KAS-1} gene was significantly \((p < 0.05)\) increased under N and P-replete medium by 1.12-fold (Fig. 4a) and 1.19-fold (Fig. 4d) after 21 days compare to the control, respectively. Usually, microalgae under normal growth condition consume ATP and NADPH produced by the cell though photosynthesis resulting in the formation of ADP and NADP\(^+\) that are being available again as acceptor molecules in photosynthesis [48]. This was translated by high biomass (Table 3) and abundance of saturated fatty acid (Table 4) observed in N and P-replete medium which can be attributed to the high expression level of \textit{KAS-1} gene under these conditions.

Aziz et al. [17] cultivated \textit{Chlorella} strain KS-MAS under different P concentration and observed high expression of \textit{KAS-1} gene under P-replete condition, which is about 3.7 and 4.3-fold higher than the control. They found a strong correlation between the expression of \textit{KAS-1} and saturated fatty acid and biomass yield. The \textit{KAS-1} gene is known to catalyze the production of 18-carbon fatty acid from 16-carbon fatty acid in which palmitic and steric acid are the final product of the fatty acid synthesis [49]. The expression of \textit{KAS-1} gene significantly decreased under N and P-limiting conditions by 1.13- and 1.23-fold after 21 days compare to the control, respectively. This showed a strong correlation with the expression of \textit{KAS-1} gene with high biomass yield and saturated fatty acid accumulation by \textit{Chlorella} sp. T4 under nutrient replete medium. Microalgae structural lipid is known to have a high content of polyunsaturated fatty acid. Under nutrient starvation, the carbon metabolism is affected and results in cell physiology regulation which increases the cellular demand for the synthesis of membrane phospholipids [50, 48].

Omega-6 desaturase encoded by gene \(\omega\)-6 \textit{FAD} catalyzes the conversion of oleic acid to linoleic acid. The expression level of \(\omega\)-6 \textit{FAD} by \textit{Chlorella} sp. T4 was affected by nutrient conditions. The expression of \(\omega\)-6 \textit{FAD} was significantly \((p < 0.05)\) increased under N-limiting condition by 2.09-fold at day 21 compared
to the control (Fig. 4b). The increase in the expression of \( \omega-6 FAD \) under N-limiting condition was corroborated by high level of linoleic acid ascertain under N-limiting condition. Microalgae requires sufficient \( \omega-6 FAD \) gene expression to convert oleic acid substrate to linoleic acid [51]. The expression of \( \omega-6 FAD \) gene was significantly decreased in N-replete medium by 1.83-fold compare to the control medium (Fig. 4b). [52] reported high expression of \( \omega-6 FAD \) gene by *Nannochloropsis oceanica* under N-starvation condition which led to an increase in linoleic acid content.

The expression of \( \omega-6 FAD \) was significantly increased under P-limiting condition by 1.97-fold after 21 days compare to the control (Fig. 4e). The increase in the expression of \( \omega-6 FAD \) under P-limiting condition was corroborated by high level of oleic acid ascertain under N and P-limiting condition (Table 4). Omega-6 desaturase is activated by the availability of oleic acid and \( \alpha \)-linoleic acid. This suggests that the function of desaturase enzyme was satisfaction to the demand of membrane phospholipids for synthesis of PUFAs. The present study demonstrated that nutrient limiting conditions had a significant impact on the expression of \( \omega-6 FAD \) and monounsaturated fatty acids.

Omega-3 desaturase encoded by gene \( \omega-3 FAD \) plays important role in the conversion of linoleic acid to form trienoic fatty acid known as \( \alpha \)-linoleic acid [53]. The expression of \( \omega-3 FAD \) gene was significantly \( (p < 0.05) \) increased by 1.93-fold and 1.65-fold under N and P-limiting condition after 21 days compare to the control, respectively (Fig. 5c, f). The expression levels of \( \omega-3 FAD \) gene is strongly associated with the increase in \( \alpha \)-linoleic acid level [51], which was corroborated by high levels of \( \alpha \)-linoleic acids content that was ascertain under N and P-limiting conditions by *Chlorella* sp. T4 (Table 4). [54], reported overexpression of \( \omega-3 FAD \) gene by *Chlorella vulgaris* in transgenic tobacco plant which resulted to an increase in \( \alpha \)-linoleic acids content. The expression of \( \omega-3 FAD \) gene was significantly \( (p < 0.05) \) decreased under N and P-replete medium by 2-fold and 1.89-fold after 21 days compare to the control, respectively (Fig. 5c, f). Nevertheless, the accumulation of \( \alpha \)-linoleic acid by *Chlorella* sp. T4 was relatively low to compare to other fatty acid. Omega-3 desaturase gene have been successfully overexpressed to increase the \( \alpha \)-linoleic acids content [52, 51]. Polyunsaturated fatty acid are major constituents of biological membrane which plays important role in maintaining the membrane fluidity and are essential for cell growth at low temperatures [53].

4. Conclusion

The cultivation of *Chlorella* so. T4 in nutrient replete medium has resulted in increase in the cell growth rate which was attributed by high chlorophyll content and quantum efficiency of photosystem II (Fv/Fm) value. The biomass was significantly decreased under nutrient-stress condition, as corroborated by significantly decrease in the expression of \( rbcL \) gene. The correlation between the upregulation of \( accD \) gene and enhanced lipid productivity by N and P limitation was observed indicating a clear impact of nutrient stress in *Chlorella* sp. T4. The level of \( KAS-1 \) gene was upregulated under nutrient replete medium, translated by high level of saturated fatty acid under non-nutrient stress conditions. Furthermore, an increase in the expression level of \( \omega-6 FAD \) and \( \omega-3 FAD \) genes under N and P-limiting medium was observed which corresponded to high levels of monounsaturated and polyunsaturated fatty
acid. This provides a clue for future prospective metabolic engineering to make microalgal biodiesel economically viable. FAMEs produced under nutrient limiting condition were suitable for production of high-quality biodiesel with better oxidative stability and cold flow properties. Future research may focus on the overexpression of these key biosynthetic genes through metabolic engineering for higher yield of neutral lipid with good biodiesel properties.

### Abbreviations

ASTM D-6751  
American Society for Testing and Materials  
DWC  
dry cell weight  
EN 14214  
European standard for biodiesel  
fatty acid methyl ester  
FAME  
fatty acid methyl ester  
Fv/Fm  
Low maximum quantum efficiency  
GC-MS  
gas chromatography mass spectrometry  
P  
phosphorus  
PUFA  
polyunsaturated fatty acid  
MUFA  
monounsaturated fatty acid  
N  
nitrogen  
SFA  
Saturated fatty acid.

### Declarations

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#### Conflicts of interest and statement of informed consent, human/animal rights
Authors declare that they have no conflicts of interest. No conflicts, informed consent, human or animal and no financial or other interest that could influence the outcome of this research applicable.

Author contributions

All authors have contributed to this research and agree to authorship and submit this manuscript for its revision and publication. S.G., A.K and A.O conception and design of the paper. S.G and A.K analysis and interpretation of the data. S.G drafting of the manuscripts. A.K and A.O critical revision of the article for important intellectual content and final approval of the manuscripts.

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**Figures**

**Figure 1**

Growth curve of Chlorella sp. T4 under different nutrient conditions (a) N-treatment (g L$^{-1}$), (b) P-treatment (g L$^{-1}$). N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L$^{-1}$ + P-0.04 g L$^{-1}$. Values show the average of three replicates ±SD.
Figure 2

Photosynthetic activity of Chlorella sp. T4 under different nutrient conditions (a) Chlorophyll a under N treatment, (b) Chlorophyll a under P treatment, (c) Fv/Fm value under N treatment and (d) Fv/Fm value under P treatment. N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L-1 + P-0.04 g L-1. Values show the average of three replicates ±SD.
Figure 3

Mean fold of relative gene expression of rbcL gene of Chlorella sp. T4 cultured in different N and P concentration. Fold change was relative to the control treatment. (a) rbcL- N-treatment, (b) rbcL- P-treatment, (c) accD- N-treatment and (d) accD-P treatment. N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L-1 + P-0.04 g L-1. Different letters depict significance difference among the gene expression according to one-way ANOVA at p < 0.05. Values show the average of three replicates ± SD.
Figure 4

Mean fold of relative gene expression of rbcL gene of Chlorella sp. T4 cultured in different N and P concentration. Fold change was relative to the control treatment. (a) KAS-1-N-treatment, (b) ω-6 FAD- P-treatment, (c) ω-3 FAD- N-treatment and (d) KAS-1- P-treatment, (E) ω-6 FAD- P-treatment and (f) ω-3 FAD- P-treatment. N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L-1 + P-0.04 g L-1. Different letters depict significance difference among the gene expression according to one-way ANOVA at p < 0.05. Values show the average of three replicates ±SD.