LIGHT-COLOR-INDUCED CHANGES IN FATTY ACID BIOSYNTHESIS IN *Chlorella* sp. STRAIN KS-MA2 IN EARLY STATIONARY GROWTH PHASE

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ABSTRACT

Optimization of light supply remains a critical issue in microalgae biotechnology. The impacts of light color on fatty acid production and biosynthesis in microalgae are poorly understood. The aim of this study was to determine the effect of light color on growth and fatty acid content in *Chlorella* strain KS-MA2. Cells were cultured on F/2 medium and incubated under blue, green, red or white light. The cells' growth, fatty acid composition and the expression levels of the ketoacyl synthase 1 (*KAS-1*), omega-6 desaturase (*ω-6 FAD*) and omega-3 desaturase (*ω-3 FAD*) genes were measured at the early stationary growth phase. Results of this study indicated that light color affected cell density and fatty acid profile produced by *Chlorella* sp. strain KS-MA2. Cells cultured under blue, red and white light had higher cell density than those cultured under green light. Palmitic acid (38.62 ± 3.29% of biomass dry weight) and linolenic acid (7.96 ± 0.88% of biomass dry weight) were highly accumulated under white light. Stearic acid was dominant under blue light (11.11 ± 0.14% of biomass dry weight), whereas oleic acid was dominant under red light (30.50 ± 0.14% of biomass dry weight). Linoleic acid was highly produced under green and blue light (28.63 ± 1.36% and 26.00 ± 0.81 % of biomass dry weight, respectively). *KAS-1* and *ω-6 FAD* were highly expressed under blue light, whereas *ω-3 FAD* was highly expressed under green light. The production of particular fatty acids of interest from *Chlorella* could be achieved by shifting color of light used during the incubation of the cell cultures. Blue-light is the most suitable light color for producing biomass and stearic acid by *Chlorella* strain KS-MA2.

Keywords: Light spectrum, linoleic acid, linolenic acid, oleic acid, palmitic acid, stearic acid

INTRODUCTION

Demand for microalgae as food, animal feed supplements, feedstock and biofuel is increasing tremendously (Wacker *et al.* 2016). The success of algae-based industrial products depends on high biomass productivity and quality with low production cost (Abu-Ghosh *et al.* 2016). Biomass productivity is closely interrelated with the amount of light energy received, which is processed and stored in chemical compounds (Chen *et al.* 2015; Liu *et al.* 2012). Sunlight is the most cost-effective energy source for microalgae production, but it has drawbacks, including changes in weather, day and night cycles, and seasonal changes, which affect light intensity and spectrum (Abu-Ghosh *et al.* 2016). Optimization of light supply remains a critical issue in microalgae biotechnology. Varying light intensity might reduce the photosynthetic rate, hindering microalgae growth due to shortage of energy (Ge *et al.* 2013). Light intensity also contributes to temperature changes and affects the accumulation of polyunsaturated fatty acids on

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microalgae (Wacker *et al.* 2016; Piepho *et al.* 2011). Cell division is an energy-consuming process and microalgae tend to accumulate sufficient energy before proliferation (Zachleder *et al.* 2016) The compounds of energy storage, such as lipids and starch in particular, tend to accumulate in the algal cells under stress conditions, when cell growth halts (Chen *et al.* 2015). In addition, light intensity can affect microalgae physiological characteristics, including pigment and chemical composition, growth rate, ion transport (Kwon *et al.* 2013) and lipid classes (Wacker *et al.* 2016; Liu *et al.* 2012). Sun *et al.* (2014) showed that the total lipid content of *Neochloris oleoabundans* increases when the light intensity is increased from 50 to 200 μmol/m^2/s, but decreases with light intensity higher than 200 μmol/m^2/s. The rate of photosynthetic activity declines during limited light, but linearly increases with increasing light availability (Choi *et al.* 2013).

Generally, light intensity refers to the number of photons (energy) per unit area and unit time. The energy of each photon is inversely proportional to the wavelength of the associated electromagnetic wave i.e. the shorter the wavelength, the more energetic the photon. For example, the energy of a photon of blue light (λ = 450 nm) is 2.76 eV, while the energy of a photon of red light (λ = 700 nm) is 1.8 eV. White light contains a spectrum of visible light with different wavelengths. Microalgae usually absorb photons in the range of 400 to 700 nm for photosynthesis; the wavelengths absorbed are species-specific (Kim *et al.* 2014). Therefore, light color has been shown to have a significant impact on microalgae productivity. For example, de Mooij *et al.* (2016) showed that the highest productivity of *Chlamydomonas reinhardtii* is obtained under yellow light, whereas the lowest is obtained under blue and red light. The lowest biomass of *Spirulina platensis* culture is obtained with blue LED, and the highest is obtained with pink and red LED (Markou 2014). On the other hand, Ge *et al.* (2013) found that red light enhances biomass production of *Chlorella vulgaris*. However, the impacts of light color on fatty acid production and expression of fatty acid biosynthesis genes, particularly in microalgae, are poorly understood. Previous research has focused on the photoautotrophic production of microalgae oil using white light as energy source. However, no explicit data are available concerning the differences in cell proliferation and energy accumulation with different wavelengths of light.

The objectives of this study were to determine the effects of different-colored light on growth, fatty acid composition, total oil content and expression of genes for fatty acid biosynthesis proteins ketoacyl-ACP synthase-I (*KAS-1*), omega-6 desaturase (*ω-6 FAD*) and omega-3 desaturase (*ω-3 FAD*) in *Chlorella* strain KS-MA2. KAS is required for the addition of malonyl-CoA in the elongation of butyryl-ACP from a 4- to 14-carbon chain (Kainou *et al.* 2006). FAD enzymes (*ω-6* FAD and *ω-3* FAD) are involved in the insertion of double bonds at the *ω-6* and *ω-3* positions of pre-formed fatty acid chains in reactions requiring oxygen, NADH and cytochrome *b5* and reducing equivalent (Zhang *et al.* 2016).

MATERIALS AND METHODS

A stock culture of mangrove-isolated *Chlorella* strain KS-MA2 was obtained from the microalgae stock culture collection of the Universiti Malaysia Terengganu, Malaysia and used in this study. The *Chlorella* cells were maintained on F/2 medium (Guillard & Ryther 1962) agar plates, which were prepared using natural seawater with salinity of 30 mg/L. Medium was solidified with 10 g/L of bacteriological agar and sterilized by autoclaving at 121 °C for 20 minutes. A single colony was streaked onto a sterilized agar plate and subsequently incubated in a growth chamber at 25 °C under 24 hour white-light illumination with 55 - 70 μmol photons/m/s light intensity with constant aeration. This culture was considered the *Chlorella* stock culture and was re-streaked onto a newly prepared agar plate every two months to maintain the cells in the Institute of Marine Biotechnology, Universiti Malaysia Terengganu.

Light Color Treatments

A total of 5×10^6 cells/mL of *Chlorella* strain KS-MA2 was aseptically transferred into 2.5 L freshly prepared F/2 medium. The salinity level was adjusted to 30 mg/L. The cultures were incubated at 25 ± 1 °C, with illumination for 24 hous per day at 55 - 70 μmol photons/m/s light intensity and constant aeration. The light colors used during incubation were white (control; λ = 400 - 750 nm), red (λ = 650 nm), blue (λ = 475 nm)

or green (λ = 510 nm) provided by a fluorescent lamp source covered with colored cellophane. Cell densities were measured every two days until a constant reading was obtained, indicating the cells had attained early stationary phase, and the cells were later harvested. Three replications were prepared for each treatment. At harvest, cell numbers, biomass as dry weight, and total oil and fatty acid content were determined. The cell numbers and absorbance at 600 nm were measured on a 10-fold serial dilution of culture using a haemocytometer (Neubauer) and BioPhotometer (Bio-Rad), respectively, in triplicate. Cell density was calculated based on plotted calibration curve at 600 nm.

Quantification of Oil and Fatty Acid Contents

Oil extraction and esterification of fatty acids to methyl esters (FAMEs) was carried out according to the modified methods of Cha *et al.* (2011). Oil was extracted from a dried sample of *Chlorella* cells. A 500 mg of sample was vortexed in 10 mL of HCl (concentrate) for 3 minutes and subsequently boiled using double-boiling technique for 30 minutes. After cooling to room temperature, the oil were extracted with 25 mL hexane for 1 minute and repeated twice with 15 mL hexane. Extracts containing FAMEs were combined, hexane residue was removed using vaporization with a Rotavapor R-210/215 (Buchi). The extracted oil was incubated at 80 °C until a constant weight was achieved. FAMEs were analyzed using a gas chromatograph (GC-6890, Agilent) equipped with a flame ionization detector fitted with a capillary column (DB-225MS, Supelco). The injector temperature was set at 250 °C, and the flow rate of carrier gas (He) was 2.4 mL/minute. The flow rate for H_2 gas and air were 35 and 350 mL/minute, respectively. The temperatures were programmed as follows: an initial temperature of 35 °C for 0.5 minute; increased to 195 °C at a rate of 25 °C/minute; subsequently increased to 205 °C at a rate of 3 °C/minute; and finally increased to 320 °C/minute at a rate of 8 °C/minute and held for 6.64 minutes. The fatty acid components were identified by comparing their retention time and fragmentation pattern with established standards. The Supelco 37 Component FAME reference standard mix (Sigma-Aldrich) was used to identify and quantify the percentage of *cis*- and *trans*-fatty acid isomers in the samples.

Examination of Gene Expression Levels by Real-time PCR

Total RNA was isolated with GF-1 Total RNA Extraction Kit (Vivantis) according to the manufacturer's instructions and treated with DNase I (Fermentas) to remove contaminating DNA. DNA-free RNA was confirmed by PCR amplification of the 18S rDNA gene using the RNA as templates. Subsequently, 1 µg of the RNA was reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad) in accordance with the manufacturer's instructions. The cDNA generated was directly used for quantitative real-time PCR. The real-time PCR was performed in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) using SYBR Green real-time PCR master mix (Bio-Rad) according to the manufacturer's instructions. The PCR mixture consisted of cDNA (50 ng, 1 µL), 0.4 µM final concentration of each forward and reverse primer (Table 1), 10 µL 2× iQ SYBR Green Supermix and nuclease-free water to the final volume of 20 µL. After heating at 95 °C for 15 seconds, the real

Table 1 Forward and reverse primers for quantitative Real-rime PCR

Primers name	Primers sequence (5' - 3'')	Size of amplicon (bp)
18S F1	5'-CCT GCG GCT TAA TTT GAC TCA ACA CG -3'	172
18S R2	5'-TAG CAG GCT GAG GTC ACG TTC G -3'	
KAS1 F5	5'-CCA TGA TTG GTC ATT GCT TGG GAG C -3'	151
KAS1 R5	5'-GCT CTT GCT TCA TGT TTG GGA CCA C-3'	
O6D F3	5'-CTT CAC CCA CGA AGG CAC AGG C -3'	129
O6D R3	5'-CCT GCA CAC TGC TGG GAA CG-3'	
O3D F2	5'-CAT GTT GAG AAC GAC GAG TCC TGG TAT C -3'	162
O3D R2	5'-GTC AAA GTG GGA GCC AGT CTT GC-3'	

time PCR amplification was programmed for 40 cycles of 95 °C for 35 seconds, 64.2 °C for 35 seconds and 72 °C for 30 seconds. Specificity of all PCR amplifications was verified by a melting curve at the completion of each run from 55 to 95 °C at 0.5 °C increment. The gene expression data were analysed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008; Livak & Schmittgen 2001). The data obtained were represented as the fold change in the target gene in the treatments relative to the control and normalized to the expression level of the 18s rRNA reference gene.

Statistical Analysis

Differences in total oil and fatty acid content among the light-color treatments of the *Chlorella* strain were statistically analysed by one-way ANOVA using SPSS Version 16.0. Significant differences among means were identified by Tukey's test at $p = 0.05$. (www.ibm.com/software/analytic/spss).

RESULTS AND DISCUSSION

Growth of Chlorella

Results of this study showed that light color did not affect the cell growth of *Chlorella* strain KS-MA2. Nevertheless, light color changed fatty acid composition. Increased cell proliferation led to the increase of cell densities and biomass obtained. Cell densities varied among light treatments (Fig. 1). Cells cultivated under blue light reached early stationary phase after 16 days of culture. Cells cultivated under green, white and red light reached early stationary phase after 18, 20 and 22 days of culture, respectively (Fig. 1a). Comparatively, cells incubated under red light took longer to attain stationary phase compared to those under other light colors. Cell densities in red-, white- and blue-light treatments did not differ significantly ($p > 0.05$). Cell numbers recorded were fluctuated from 2.08 10^8 cells/mL to 1.72 10^8 cells/mL. Biomass dry weight

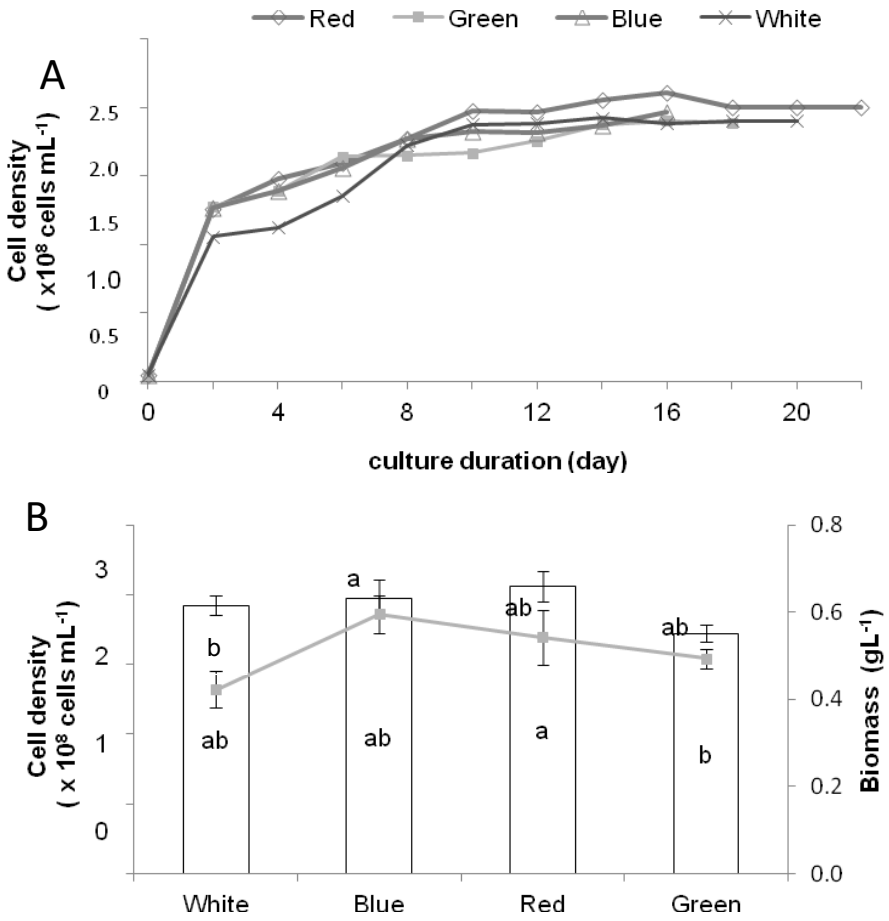

Figure 1 Effect of light colors on: (A) cell density and biomass at the early stationary phase and (B) growth of *Chlorella* strain KS-MA2 on F/2 medium
(Note: Bars represent cell densities and lines represent biomass dry weight (mean ± SD; n=3); values followed by the same letter were not significantly different according to Tukey's test with $p = 0.05$ significance level)

obtained also did not significantly differ ($p > 0.05$; Fig. 1b).

Cell proliferation is dependent on the available energy in forming ATP or NADPH. The available energy is derived from photosynthetic activity (Zachleder *et al.* 2016). In addition, photosynthetic efficiency is associated with photon energy, which is inversely proportional to light wavelength (Kim *et al.* 2014), i.e. the shorter the wavelength, the more energetic the photon (Choi *et al.* 2013). Thus, blue light is more efficient for photosynthesis and lipid biosynthesis (Markou 2014). However, blue light appeared to cause photo-inhibition, inducing cell damage and reduced the time to attain early stationary phase. Blue light has been reported to up-regulate the LHcx1 protein and zeaxanthin epoxidase, a proteins involved in photoprotection in *Phaeodactylum tricornutum* (Costa *et al.* 2013). On the other hand, red light has longer wavelength than blue light and thus, might prevent photoinhibition (George *et al.* 2014; Xu *et al.* 2013). Therefore, the highest cell density and biomass were obtained in the red-light treatment (Fig. 1b). Similar findings have been reported for *Scenedesmus* sp. (Kim *et al.* 2013) and *C. vulgaris* (Xu *et al.* 2013; Yan *et al.* 2013). However, different algal classes have different light requirements for growth and photosynthesis (Kwon *et al.* 2013; Cheirsilp & Torpee 2012).

Total Oil Content and Fatty Acid Composition

Total oil content (Fig. 2) and fatty acid composition (Fig. 3 & 4) varied among the light color treatments ($p < 0.05$). Cells under blue-light and white light treatments contained the highest percentage of total oil, up to 22.6% of total dry weight (Fig. 2). This was also 3.4- and 3.7-fold higher than that of cells under red- and green-light treatments, respectively. Saturated fatty acid accumulation did not differ significantly ($p > 0.05$) in the treatments of white-, blue- and green-light (Fig. 3a), but it was higher than the red-light. Meanwhile, monounsaturated fatty acids were highly accumulated under the red-light treatment (30.9%) (Fig. 3b). This accumulation was 1.19-, 1.44- and 1.47-fold higher than under white, blue and green light, respectively. In contrast, cells cultured under green light produced higher proportions of polyunsaturated fatty acids (PUFAs: 34.5%) compared to those under white (25.7%) or red (30.3 %) light (Fig. 3c). However, this value did not significantly differ ($p > 0.05$) from the blue-light treatment (32.2%).

Figure 4 shows the relative amount of the five major fatty acids i.e. palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid, in *Chlorella* strain KS-MA2 cultured under different light colors. Palmitic acid (38.6%) and linolenic acid (7.9%) were highly accumulated ($p < 0.05$) under white-light conditions (Fig. 4a & 4e) compared to the other treatments. Meanwhile, the accumulation of stearic acid (11.1%) was the highest under blue-light treatment, 2.3-fold higher than that under white light (Fig. 4b). Conversely, oleic acid (30.5%) was highly accumulated under red light (Fig. 4c), and linoleic acid (28.6%) was highly accumulated under green light (Fig. 4d).

Results of this study are in line with previous reports stating that different light colors change the total oil accumulated (Markou 2014; Liu *et al.* 2012; Marchetti *et al.* 2012) and fatty acid profile (Wacker *et al.* 2016; Piepho *et al.* 2011). Light with higher

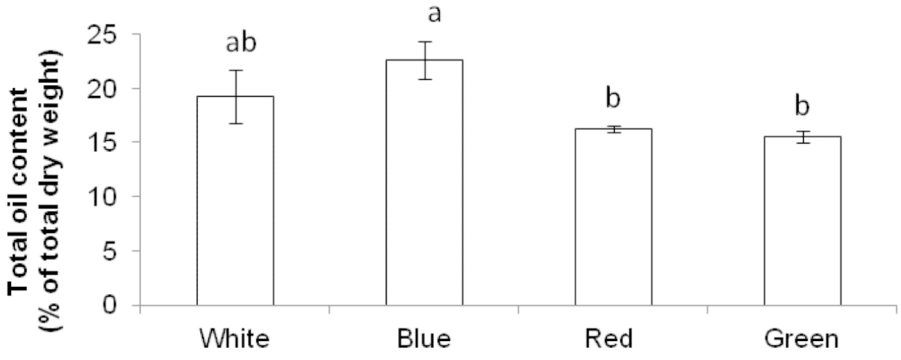

Figure 2 Effect of light color on total oil (mean ± SD)
(Note: Values followed by the same letter were not significantly different according to Tukey's test with p = 0.05 significance level)

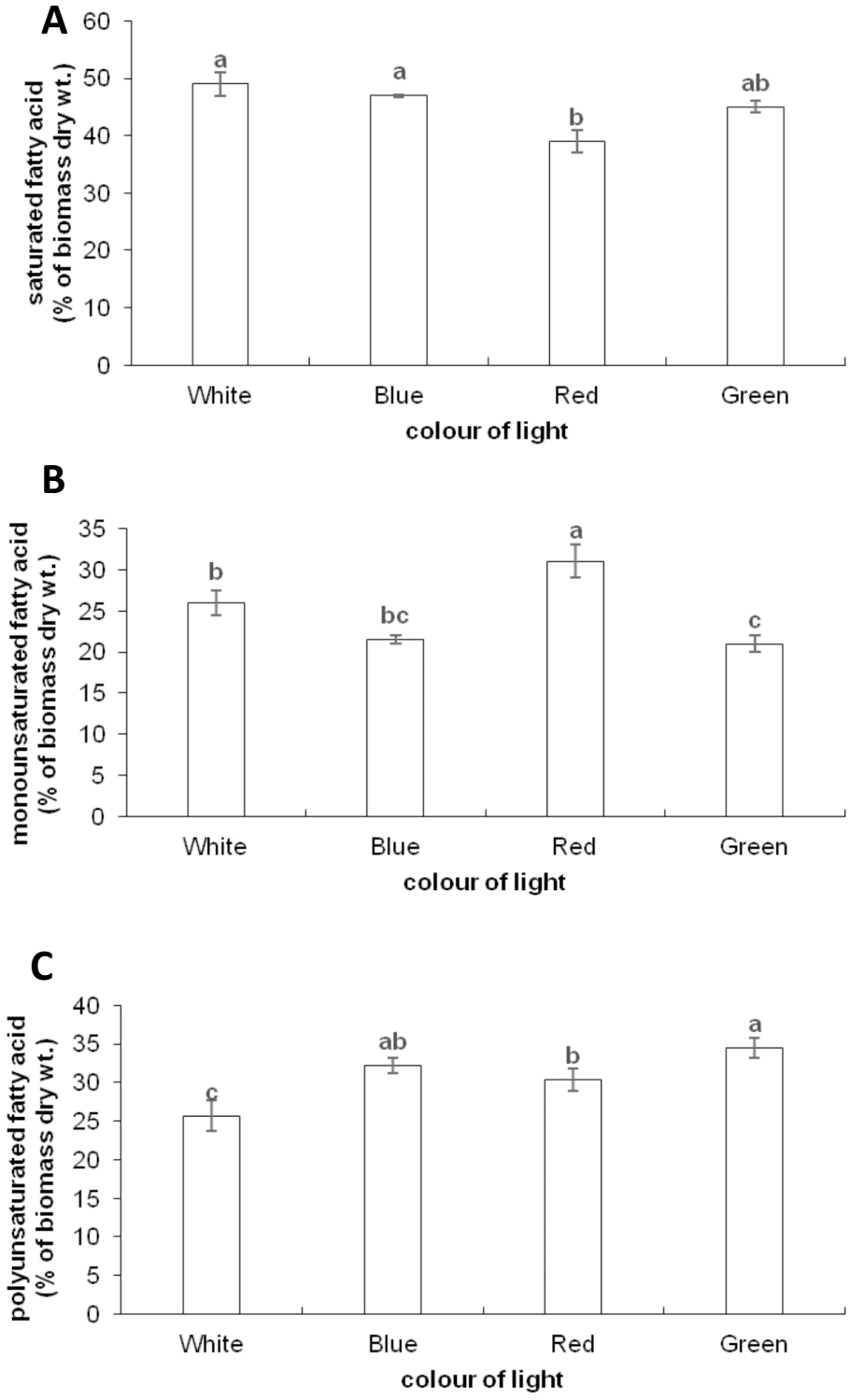

Figure 3 Effect of light color on: (A) saturated fatty acid; (B) monounsaturated fatty acid; and (C) PUFA content at the early stationary phase of *Chlorella* strain KS-MA2 cultured on F/2 medium (mean ± SD; n=3)
(Note: Values followed by the same letter were not significantly different by Tukey's test with $p = 0.05$ significance level)

energy, such as blue or white light, might increase the ATP and NADPH available for triacylglycerol synthesis (Fig. 2). Addition of carbon in the elongation of fatty acid chains requires NADPH, derived from photosynthesis (Chen *et al.* 2015). Moreover, blue light is used for enzyme activation and regulation of gene transcription (Das *et al.* 2011). High transcription of *KAS-1* under blue-light treatment (Fig. 5a) might be due to the increased photosynthetic activity.

Therefore, more carbon flux generated from photosynthesis is channelled to lipid biosynthesis (Liu *et al.* 2012), involving the elongation of fatty acid chains; thus, resulting in higher accumulation of unsaturated fatty acids (Fig. 3a), the palmitic acid (Fig. 4a) and stearic acid (Fig. 4b) under high energy light. In addition, higher total oil production is important for light-stress adaptation (Choi *et al.* 2013). The lipid layers function as a light filter to reduce irradiation on the cell components, to prevent photo oxidative damage and to reduce water loss (Liu *et al.* 2012).

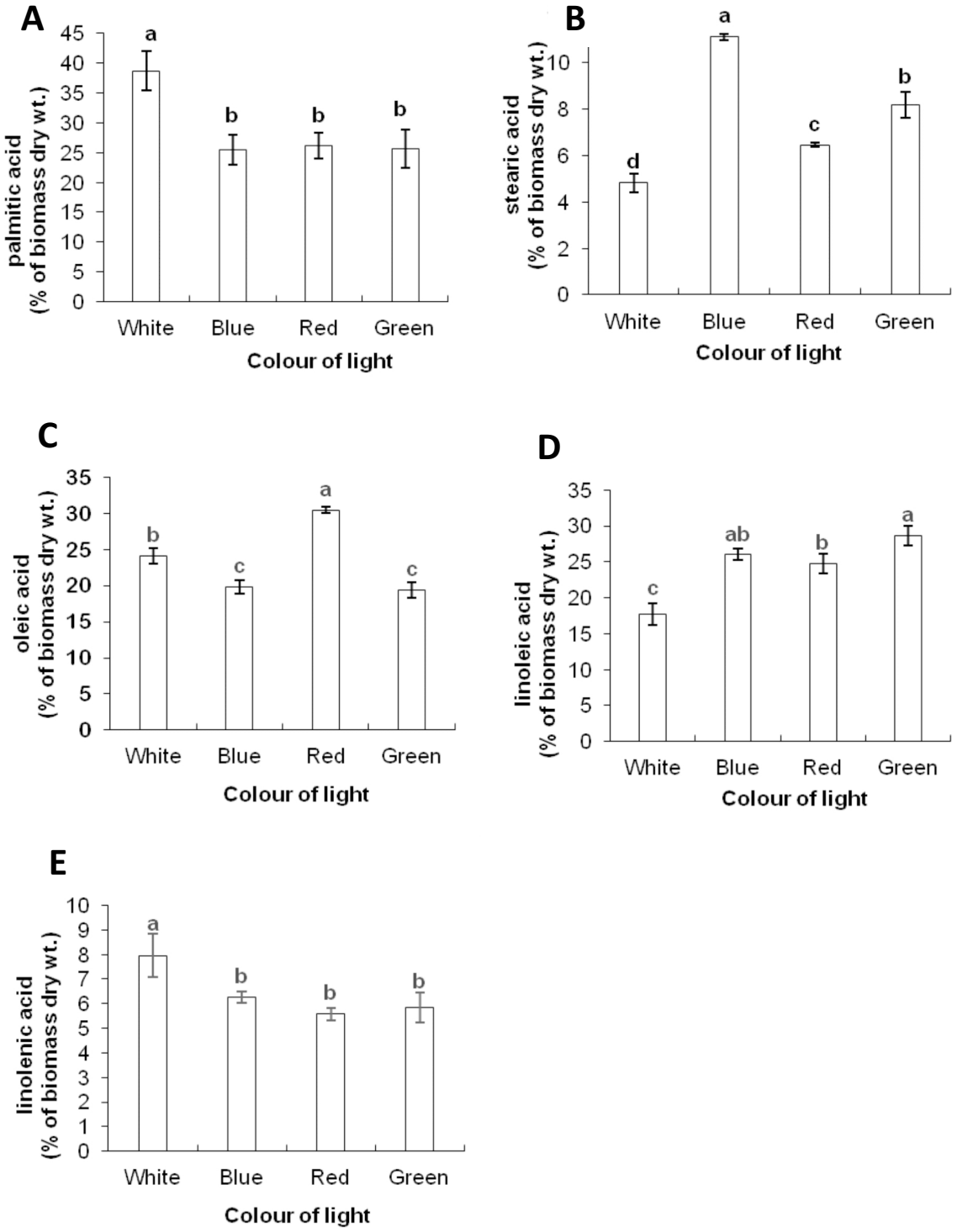

Figure 4 Effect of light color on: (A) Palmitic acid; (B) stearic acid; (C) Oleic acid; (D) Linoleic acid; and (E) Linolenic acid content at early stationary phase of *Chlorella* strain KS-MA2 cultured on F/2 medium (mean ± SD; n=3)
(Note: Values followed by the same letter were not significantly different according to Tukey's test with $p = 0.05$ significance level)

Expression Levels of KAS-1, ω-6 FAD and ω-3 FAD

Results of this study showed that *KAS-1* (Fig. 5a) and ω-*6 FAD* (Fig. 5b) were highly transcribed under blue light. It was 5.3- and 2.2-fold of the reference gene, respectively. The *ω-3 FAD* was more highly transcribed under green light (2.8-fold of the reference gene; Fig. 5c). This was 3.8-, 1.8- and 2.3-fold higher than that under white light, respectively. These genes exhibited the lowest transcription levels under red-light treatment.

Fatty acid desaturation is important for microalgae tolerance toward strong light (Choi *et al.* 2013; Solovchenko *et al.* 2008). PUFAs are

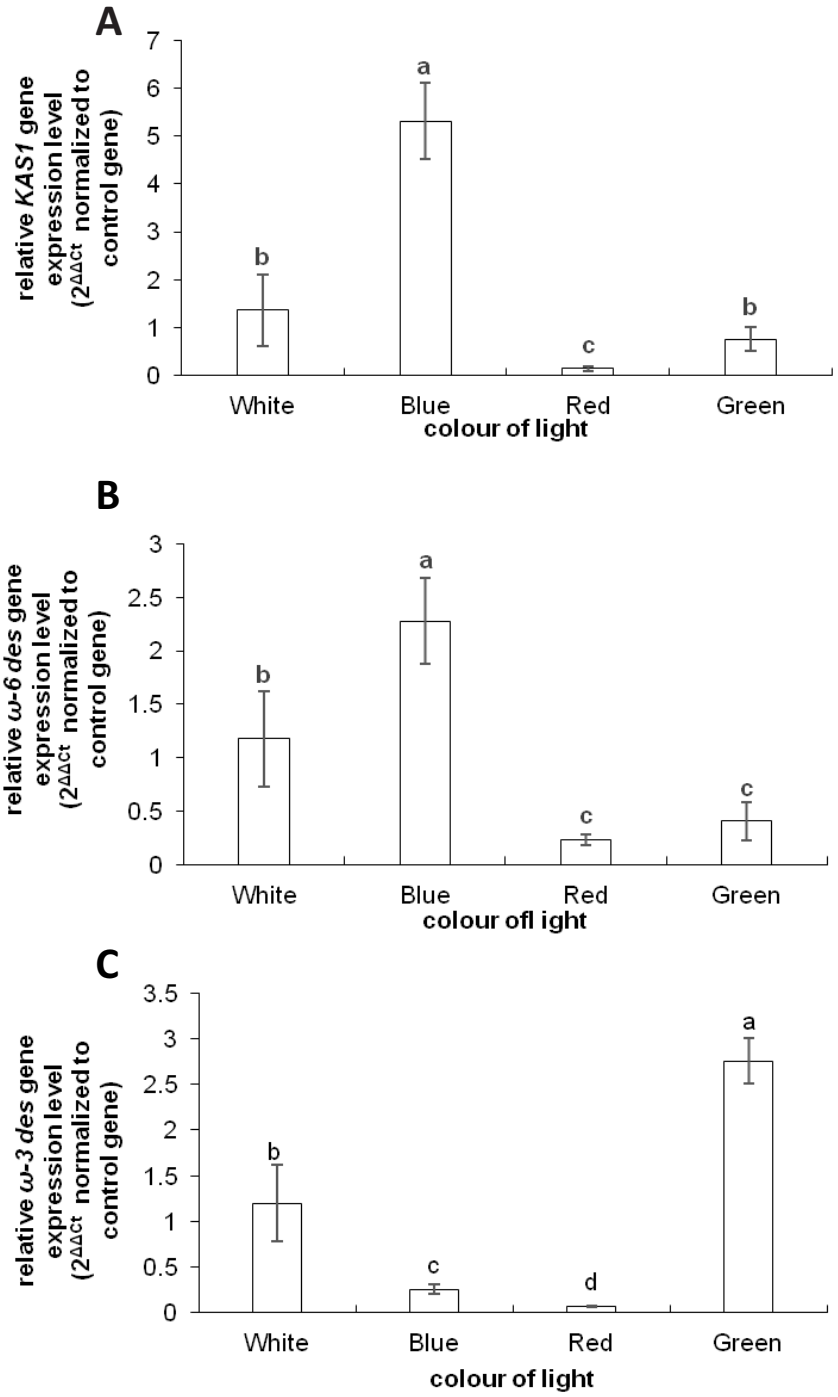

Figure 5 Effect of light color on mean fold change in relative expression of: (A) *KAS-1*; (B) *ω-6 FAD*; and (C) *ω-3 FAD* of *Chlorella* strain KS-MA2 (mean ± SD; n=3)
(Note: Values followed by the same letter were not significantly different according to Tukey's test with $p = 0.05$ significance level)

necessary for the maintenance of photosynthetic membrane function. PUFAs also play an important role in acclimation to low light conditions (Solovchenko *et al.* 2008). High PUFA content obtained in blue- and green-light was mainly contributed by linoleic acid (Fig 4d). The α-linolenic (Fig. 4e) produced in white-light was the least i.e. 8 % of biomass dry weight. Blue light also induced the expression of acyl-lipid desaturase (Kis *et al.* 1998), which might be responsible in higher accumulation of α-linolenic (Fig. 4e) and transcription levels of ω-*6 FAD* (Fig. 5b). On the other hand, higher transcription levels of ω-*3 FAD* under green-light (Fig. 5c) did not contribute to the accumulation of α-linolenic acid. The increase of *KAS-1* and ω-*6 FAD* transcription levels might be associated with photo-protection against higher light intensity (Yoshioka *et al.* 2012). Nonetheless, knowledge on the post translation of these genes is remained unknown. This study showed that there was inconsistent requirement of light in PUFAs production among microalgae species (Wacker *et al.* 2016). This species-specific light acclimation strategy reflected to photosynthetic factors and differences in light spectra.

CONCLUSIONS

Light color played a major role in the proliferation of *Chlorella* strain KS-MA2 and its fatty acids profile. Mass production of *Chlorella* biomass could be obtained under blue-light conditions, whereas total oil was better obtained under white- or red-light conditions. Higher production of specific fatty acids could be achieved using different light color during the cultivation of cells i.e. white light for palmitic acid and linolenic acid, blue light for stearic acid and red light for oleic acid. Linoleic acid accumulation was not influenced by light color. The transcription level of *KAS-1* and ω-*6 FAD* were highly activated by blue-light, while the transcription level of ω-*3 FAD* was highly activated by green-light.

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