

# Indole-3-acetic acid (IAA) induced changes in oil content, fatty acid profiles and expression of four fatty acid biosynthetic genes in *Chlorella vulgaris* at early stationary growth phase



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## ABSTRACT

Microalgae lipids and oils are potential candidates for renewable biodiesel. Many microalgae species accumulate a substantial amount of lipids and oils under environmental stresses. However, low growth rate under these adverse conditions account for the decrease in overall biomass productivity which directly influence the oil yield. This study was undertaken to investigate the effect of exogenously added auxin (indole-3-acetic acid; IAA) on the oil content, fatty acid compositions, and the expression of fatty acid biosynthetic genes in *Chlorella vulgaris* (UMT-M1). Auxin has been shown to regulate growth and metabolite production of several microalgae. Results showed that oil accumulation was highest on days after treatment (DAT)-2 with enriched levels of palmitic (C16:0) and stearic (C18:0) acids, while the linoleic (C18:2) and  $\alpha$ -linolenic (C18:3n3) acids levels were markedly reduced by IAA. The elevated levels of saturated fatty acids (C16:0 and C18:0) were consistent with high expression of the  $\beta$ -ketoacyl ACP synthase I (*KAS I*) gene, while low expression of omega-6 fatty acid desaturase ( $\omega$ -6 *FAD*) gene was consistent with low production of C18:2. However, the increment of stearoyl-ACP desaturase (*SAD*) gene expression upon IAA induction did not coincide with oleic acid (C18:1) production. The expression of omega-3 fatty acid desaturase ( $\omega$ -3 *FAD*) gene showed a positive correlation with the synthesis of PUFA and C18:3n3.

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## 1. Introduction

Microalgae are photosynthetic organisms that are capable of using light and carbon dioxide (CO<sub>2</sub>) to produce energy-rich compounds, such as carbohydrates and lipids that can be converted into fuels. Some microalgae species are reported to contain significant quantities of lipid and oil with compositions similar or far more superior than plant oil (Chisti, 2007). Several factors such

as nitrogen deficiency (Cha et al., 2011; Converti et al., 2009; Msanne et al., 2012; Yeesang and Cheirsilp, 2011), low temperature (Converti et al., 2009), high salinity (Rao et al., 2007) and high irradiance (Yeesang and Cheirsilp, 2011) have been shown to increase the lipid content of microalgae, in particular the triacylglycerols (TAGs) (Hu et al., 2008). Besides these stimuli, microalgae growth was also influenced by the plant hormones as evidenced by the growth promoting effect of auxin, cytokinins, and brassinosteroids throughout the microalgal life cycle (Tarakhovskaya et al., 2007). However, little is known about the effect of these plant hormones on lipid and oil productions in microalgae.

There are three stages in producing microalgae-based oils, namely microalgae cultivation, cells harvesting, and lipid extraction (Harun et al., 2011). Among these stages, cultivation is often considered as the most important step as the growth conditions will determine the quality and quantity of the lipids produced which will directly affect their downstream applications. Therefore, it is necessary to increase the efficiency of cultivation in order

**Abbreviations:** DAT, days after treatment; IAA, indole-3-acetic acid; IAM, indole-3-acetamide; JA, jasmonic acid; *KAS I*,  $\beta$ -ketoacyl ACP synthase I; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;  $\omega$ -3 *FAD*, omega-3 fatty acid desaturase;  $\omega$ -6 *FAD*, omega-6 fatty acid desaturase; *SAD*, stearoyl-ACP desaturase; SD, standard deviation; SFA, saturated fatty acid; wt, weight.

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to maximize the lipid contents (Harun et al., 2011; Park et al., 2013). During microalgae cultivation, a significant inverse relationship between growth rate and lipid content was observed at optimum culture condition (Williams and Laurens, 2010). For most microalgae species, the highest lipid content was recorded at their early stationary growth phase as compared to the exponential growth phase and the late stationary growth phase (Hu et al., 2008). Extensive manipulations of culture conditions through chemical and physical stimuli have been applied in microalgae cultivation to improve lipid yields. Among these stimuli, few studies have reported an effect of hormones on microalgae culture that are particularly focussed on lipid content (Park et al., 2013). Although numerous studies have indicated that microalgae alter their fatty acids and lipids in response to stimuli, the mechanism of gene regulation is still poorly defined.

Auxin, predominantly indole-3-acetic acid (IAA), is a growth hormone that has profound effects on plant growth and development (Zhao, 2010). Auxin influences nearly all stages of plant development and this hormone is required for viability, due to the fact that to date no mutant lacking auxin has been found. Auxin plays a regulatory role in root architecture (Casimiro et al., 2001), apical dominance (Booker et al., 2003), phyllotaxis (Reinhardt et al., 2003), vascular differentiation (Aloni et al., 2006) and fruit development (Gillaspy et al., 1993). Despite their well characterized functions in vascular plants, the role of auxin in algae remains largely unknown. However, it is accepted that the functions of auxin in microalgae are parallel to those in higher plants. Several publications have provided evidence for the presence of auxin in unicellular and multicellular algal species (reviewed in Tarakhovskaya et al., 2007). Currently, only two forms of auxin, IAA and indole-3-acetamide (IAM), have been detected in microalgae, and often IAA occurs in higher concentration as compared to IAM (Jirásková et al., 2009; Stirk et al., 2013). Auxin has been shown to promote cell enlargement in *Chlorella vulgaris* (Yin, 1937) and cell division in *Chlorella pyrenoidosa* (Vance, 1987). It was also reported recently that auxin, together with gibberellic acid, are capable of increasing both *Chlamydomonas reinhardtii* cell size and the number of daughter cells produced during cell division (Park et al., 2013). Furthermore, auxin treatment also increase carotenoid and xanthophyll contents in *C. pyrenoidosa* (Czerpak and Bajguz, 1997). This information is exciting as an increase in size and cell division may influence total oil production in microalgae. In addition, with the advances in genomic techniques, several putative orthologs to enzymes involved in auxin pathway have been detected in some green algae (Blanc et al., 2010).

In this study, exogenous IAA was applied to *C. vulgaris* (UMT-M1) cultures at the early stationary growth phase to investigate its effect on cell growth and total oil accumulation. Five major fatty acids (C16, C18, C18:1, C18:2 and C18:3) and four fatty acid biosynthetic genes (*KAS I*, *SAD*,  $\omega$ -6 and  $\omega$ -3 *FADs*) were analyzed to examine the relationship between gene regulation and fatty acid biosynthesis mediated by IAA. The correlations between gene expression and fatty acids accumulation were performed to better understand the regulatory mechanism of the fatty acid biosynthesis and oil production in *C. vulgaris* (UMT-M1).

## 2. Results and discussion

### 2.1. Effect of IAA on cell growth

In order to examine the growth curve of *C. vulgaris* (UMT-M1), cells were cultured and the cell density was recorded at 2 day intervals until a constant value was achieved. The results showed that *C. vulgaris* (UMT-M1) required 22 days cultivation time to reach the early stationary growth phase (Supplementary Fig. S1).

The cell density of this microalga at an early stationary growth phase was  $9.37 \pm 0.15 \times 10^6$  cells/mL (Table 1). After addition of 100  $\mu$ M IAA at day-22, the cell density remained similar to the control until DAT-4. This result was unexpected, as previous research had shown that auxin and its related substances had a stimulatory effect on *Chlorella* sp. (Czerpak and Bajguz, 1997; Hunt et al., 2010; Tate et al., 2013; Vance, 1987). There were two possibilities for this discrepancy, which were (1) exogenous application of 100  $\mu$ M IAA alone was insufficient to promote cell division and (2) IAA was added to the culture medium during an early stationary growth phase, where cell division was essentially completed. Results from this study seemed to favor the first possibility, because previous literature data indicated that auxin is necessary but not sufficient to stimulate cell division in cultured cells or plant tissues, because the presence of cytokinins is also required (Inzé and De Veylder, 2006; Perrot-Rechenmann, 2010).

On DAT-6, the results showed that IAA significantly ( $p < 0.05$ ) inhibited cell growth by approximately 16% relative to the control (Table 1). It has been reported that a high concentration of auxin (500  $\mu$ M) induced *de novo* synthesis of ethylene which resulted in growth inhibition, leaf epinasty and senescence in cleavers (*Galium aparine*) (Grossmann, 2000). Furthermore, auxin-induced ethylene production also triggers abscisic acid (ABA) biosynthesis (Hansen and Grossmann, 2000). ABA is recognized as plant hormone that affects biomass productivity by reduction of stomatal aperture and consequently  $\text{CO}_2$  assimilation. Together, ABA and ethylene promote leaf senescence and inhibit growth (Grossmann, 2000). The concentration of IAA used in this study was considered high, as endogenous IAA levels reported for *C. vulgaris* are in the range of 0.5–5  $\mu$ M (Jirásková et al., 2009; Stirk et al., 2013). The inhibitory effect of IAA that was recorded only on DAT-6 cells may be a result of a longer acclimatization phase required by the algal cells. On DAT-8 and DAT-10, the densities for both IAA-treated and control cells gradually decreased as the cells entered the late stationary phase. DAT-8 and DAT-10 represented cultivation days of 30 and 32, respectively, which coincided with the decline phase for *C. vulgaris* (UMT-M1) culture.

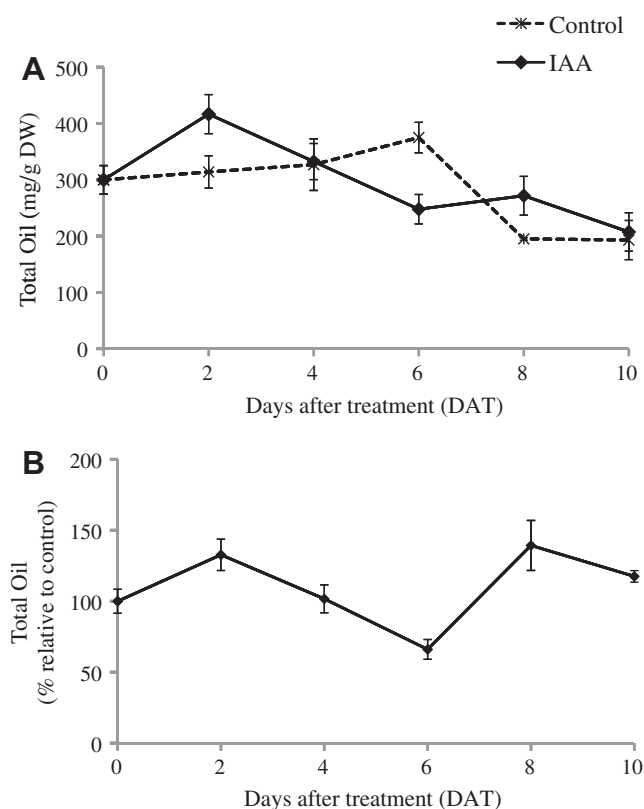
### 2.2. Effect of IAA on total oil content

To study the effect of IAA on total oil accumulation, the cells were subjected to oil extraction and the oil produced (Fig. 1A) was measured as mg/g dry weight (dry wt). For the untreated culture, the total oil content recorded on DAT-0 was approximately 300 mg/g dry wt of *C. vulgaris* (UMT-M1) cells (Supplementary Table S2). The stimulatory effect of IAA on the total oil content was recorded on DAT-2 and DAT-8 with approximately 32% and 39% increases, respectively in total oil accumulation as compared to the control (Fig. 1B). This observation was interesting, because treated and controlled cells had the same cell density (Table 1) and this indicated that each of the IAA-treated cells produced more

**Table 1**  
Effect of IAA treatment on cell growth of *C. vulgaris* (UMT-M1).

Cultivation days	Days after treatment (DAT)	Cell density ( $10^6$ cells/mL)		Cell density* (% relative to control)
		Control	IAA	
22	0	9.37 $\pm$ 0.15	9.37 $\pm$ 0.15	100.00 $\pm$ 1.61 <sup>b</sup>
24	2	9.35 $\pm$ 0.23	9.63 $\pm$ 0.28	103.03 $\pm$ 3.04 <sup>b</sup>
26	4	9.42 $\pm$ 0.16	9.58 $\pm$ 0.16	101.77 $\pm$ 1.71 <sup>b</sup>
28	6	9.30 $\pm$ 0.27	7.78 $\pm$ 0.13	83.69 $\pm$ 1.35 <sup>a</sup>
30	8	7.43 $\pm$ 0.20	7.50 $\pm$ 0.22	100.90 $\pm$ 2.93 <sup>b</sup>
32	10	6.90 $\pm$ 0.23	7.32 $\pm$ 0.55	106.04 $\pm$ 8.01 <sup>b</sup>

\* Means followed by the same letter (a, b) within the same column are not significantly different according to Tukey's HSD test at  $p < 0.05$ .



**Fig. 1.** Effect of IAA treatment on total oil content of *C. vulgaris* (UMT-M1). (A) Total oil content in control (star) and IAA (triangle) media as presented by dry weight basis, (B) Percentage of total oil in IAA media as relative to control media. Vertical bars represent the means  $\pm$  SD ( $n = 3$ ).

oil than control cells. The results from DAT-2 and DAT-8 suggested that IAA supplemented in this study was only sufficient to promote cell enlargement but not cell division. Cell enlargement due to accumulation of oil bodies and starch granules was observed in the green microalga UTEX 2219-4 (*Scenedesma* sp.) 3 days after nitrogen starvation (Wang et al., 2011). However, the increment at DAT-8 was not practically significant as the total oil content for IAA-treated cells was only 272 mg/g dry wt (control 195 mg/g dry wt) which was lower than the total oil content of 300 mg/g dry wt on DAT-0 (see Fig. 1A and Table S2, Supplementary data).

Total oil production in IAA-treated cells was comparable on DAT-0, 4 and 6 (Fig. 1), although significant ( $p < 0.05$ ) reduction in cell growth was recorded at DAT-6 (Table 1). This suggested that although IAA inhibits cell growth through an unknown mechanism, the oil production in algal cells is maintained. One possible hypothesis is that auxin-induced ethylene and ABA production form an adverse environment for algal cells, and that this adverse environment, similar to nitrogen starvation and osmotic stress, induced lipid biosynthesis. Lipid biosynthesis could help microalgae quench electrons from the thylakoid membrane, as well as increase cell buoyancy thereby enhancing survivability of the microalgae by drifting away from the adverse environment, especially those microalgae without flagella (Wang et al., 2011).

One of the remarkable features of auxin action was demonstrated by the rapidity of auxin-induced growth observed within 5–15 min after auxin exposure (Schenck et al., 2010). This rapid induction was true for higher plants, where auxin was applied in the shoot, root or cotyledon. The cell walls of microalgae consisted of a polysaccharide and glycoprotein matrix (Gerken et al., 2013), which could hinder rapid absorption of IAA and acclimatization of the algal cells. Thus, the action of auxin within days in this study

was considered rapid. Furthermore, most previous studies on microalgae oil production were focused on oil accumulation during the exponential growth phase, rather than in the stationary growth phase (Czerpak and Bajguz, 1997; Hunt et al., 2010; Vance, 1987). However, several studies have reported that during the stationary growth phase, microalgae accumulate more oil as fixed carbon is converted into storage lipids (Feng et al., 2011; Hu et al., 2008; Mansour et al., 2003). Therefore, introduction of IAA during the early stationary growth phase of this study may stimulate algal cells to produce more oil than the maximum that they could produce.

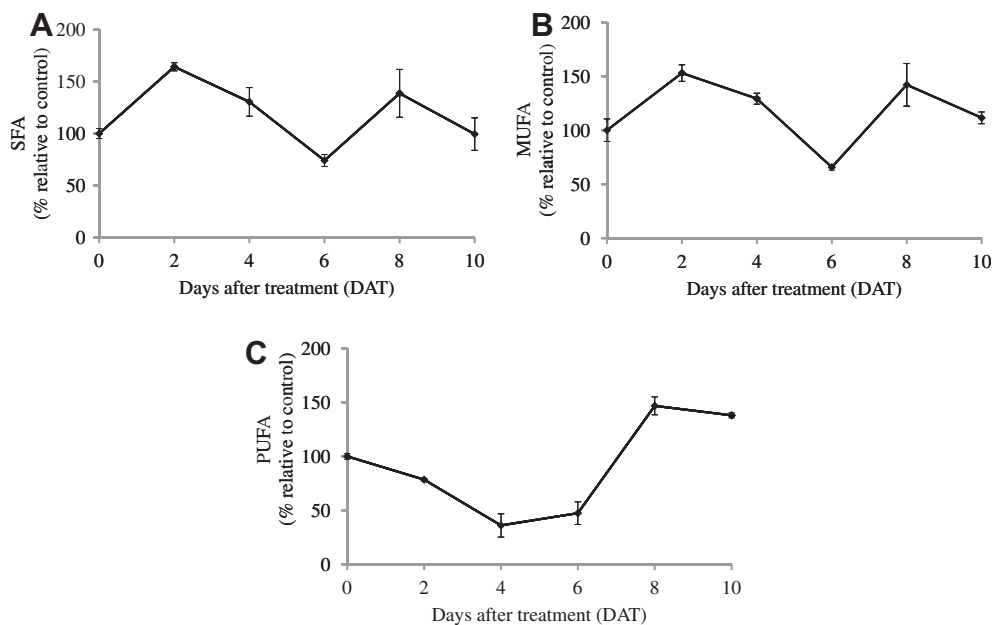
### 2.3. Effect of IAA on fatty acid compositions at early stationary growth phase

To further investigate oil compositions affected by IAA, the total oil was divided into three classes consisting of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). In the culture supplemented with IAA, a stimulatory ( $p < 0.05$ ) effect on accumulation of SFA (164%) and MUFA (153%) was recorded for the first two-DAT (Figs. 2A and B, 3A and C). No further increment, however, was observed in subsequent days of the experiment for SFA production, and a significant decrease in MUFA level was evident at DAT-6. These data were in agreement with the study reported by Stearns and Morton (1975), where while auxin increased the proportion of C16:0 and C18:0 fatty acids, the proportion of C18:2 and C18:3 fatty acids decreased in soybean cell culture. However, in another study by Liu et al. (1995), auxin decreased the C18:2 content, while increasing the C18:3 content in soybean zygotic embryo cotyledons. Conversely, in this study, accumulation of C18:1, C18:2 and C18:3n3 increased at DAT-8 (Fig. 3C–E). One possible explanation for these conflicting data is that lipid biosynthesis is developmentally regulated (Post-Beittenmiller et al., 1992) and in these two studies (Liu et al., 1995; Stearns and Morton, 1975), various plant materials have been used under different conditions for analysis of fatty acids.

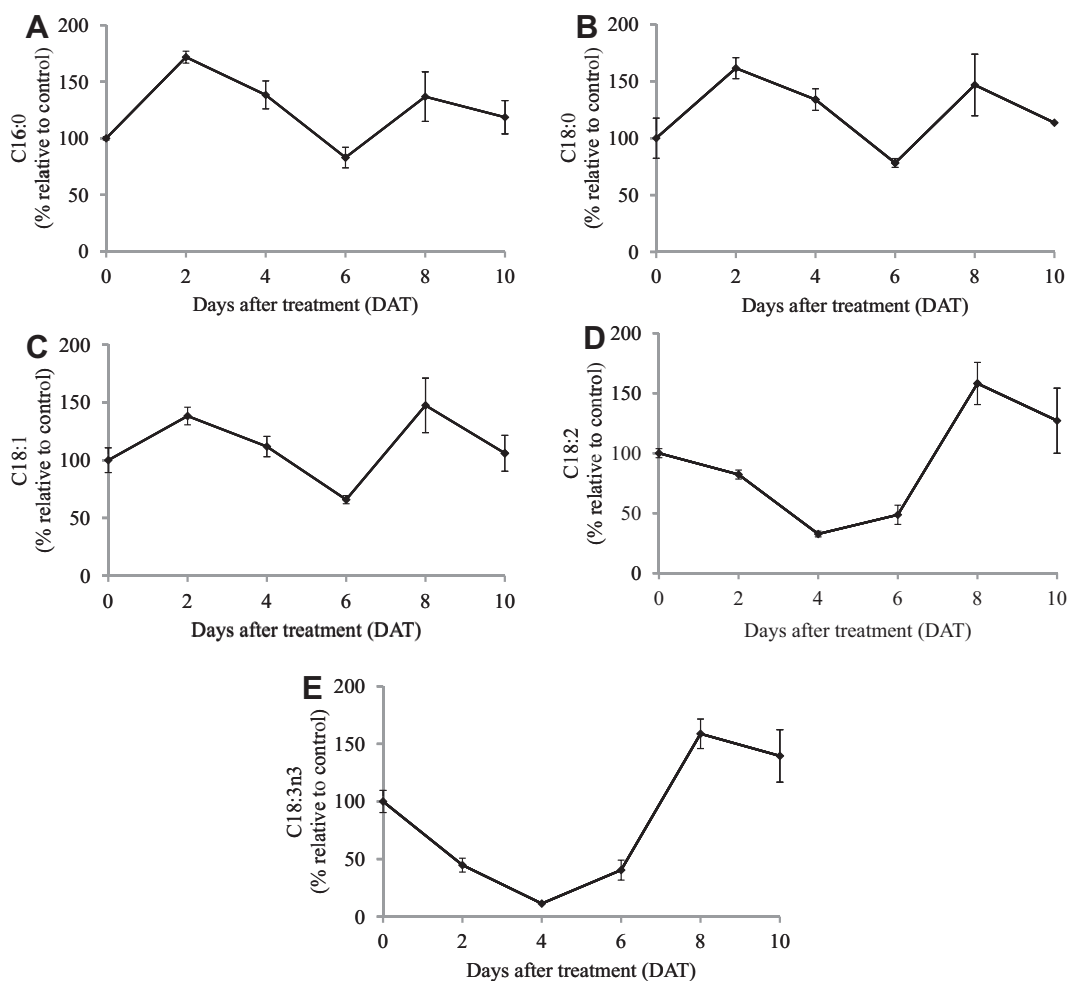
In contrast, IAA treatment significantly ( $p < 0.05$ ) reduced the production of PUFA to 36.00% at DAT-4 and 47.37% at DAT-6 (Fig. 2C). However, PUFA production was increased ( $p < 0.05$ ) to 146.81% 2 days later reaching a plateau at DAT-10 (Fig. 2C). Interestingly, the levels of C18:2 and C18:3n3 significantly increased at DAT-8 (Fig. 3D and E), which suggested that IAA promotes PUFA production in the late stationary growth phase. At this stage, algal cells start to senesce and consequently alter fatty acid composition to produce more C18:3, a precursor needed for jasmonic acid (JA) biosynthesis. JA is well documented as a plant hormone that plays a major role in senescence and defence responses (He et al., 2002; Howe and Jander, 2008). Furthermore, PUFAs may constitute the main fatty acids in storage molecules of TAGs for *C. vulgaris* (UMT-M1) during IAA treatment. Although in most microalgae, TAGs contain mostly SFA and MUFA (reviewed in Hu et al., 2008), some exceptions have been reported. For example, in *Tetraselmis suecica*, TAGs contain significant levels of PUFAs (Danielewicz et al., 2011; Hu et al., 2008).

### 2.4. Regulation of fatty acid biosynthesis genes in response to IAA at early stationary growth phase

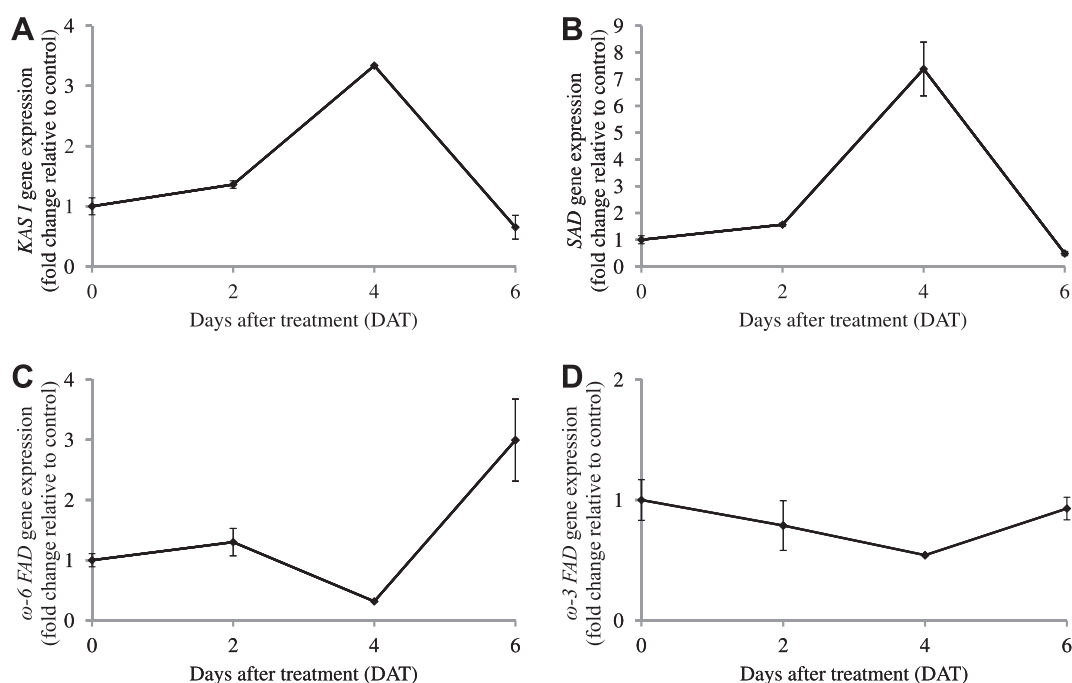
In order to examine regulation of fatty acid compositions at the gene level, the expression of four fatty acid biosynthesis genes: *KAS I*, *SAD*,  $\omega$ -6 *FAD* and  $\omega$ -3 *FAD* genes were studied using real-time PCR (Fig. 4). The increase in SFA production by IAA treatment is in agreement with *KAS I* gene expression where an elevated level of expression was observed at DAT-4 (Fig. 4A). It is also hypothesized that a high percentage of C16:0 and C18:0 is expected to lead



**Fig. 2.** Effect of IAA treatment on fatty acid classes of *C. vulgaris* (UMT-M1). Percentage of SFA (A), MUFA (B) and PUFA (C) in IAA media relative to control media. Vertical bars represent the means  $\pm$  SD ( $n = 3$ ).



**Fig. 3.** Effect of IAA treatment on fatty acid compositions of *C. vulgaris* (UMT-M1). Quantification of five major fatty acid compositions of C16:0 (A), C18:0 (B), C18:1 (C), C18:2 (D) and C18:3n3 (E) during 10 days treatment with IAA. Percentages are relative to the control media. Vertical bars represent the means  $\pm$  SD ( $n = 3$ ).



**Fig. 4.** Changes in expression of the *KAS I* (A), *SAD* (B),  $\omega$ -6 *FAD* (C) and  $\omega$ -3 *FAD* (D) genes in the media supplemented with IAA in relative to control media. Vertical bars represent the means  $\pm$  SD ( $n = 3$ ).

to high levels of C18:1, and subsequently all the other fatty acids along the *de novo* biosynthesis pathway as illustrated by the substrate-enzyme relationship where high concentration of substrate will lead to high concentration of product. This hypothesis is true to some extent for C18:1, as the accumulation of this fatty acid increased at DAT-2. This is consistent with up-regulation of *SAD*, a gene responsible for conversion of 18:0-ACP to 18:1-ACP that increased 1.57-fold (Fig. 4B). However, further increment of *SAD* gene expression by 7.38-fold at DAT-4 did not induce a further increment in C18:1 accumulation level. This elevated level of gene expression, which is inconsistent with the low accumulation of C18:1 level, could be explained by two possibilities, (1) C18:1 levels were regulated at transcriptional and post-transcriptional levels (Kachroo et al., 2007) or (2) thioesterases, presumably oleoyl-thioesterase (OTE), which catalyze cleavage of acyl-ACP from 18:1-ACP could be the limiting enzyme for the production of C18:1 (Salas and Ohlrogge, 2002).

As for  $\omega$ -6 *FAD* gene expression, significant down-regulation of this gene expression on DAT-4 is consistent with low accumulation of C18:2 at DAT-4 (Fig. 4C) and significant up-regulation of expression at DAT-6 was again consistent with a high accumulation of C18:2 at DAT-8. There was a 2 day delay in the accumulation of C18:2 in comparison with  $\omega$ -6 *FAD* gene expression. This result was unexpected, as auxin triggered rapid changes in gene expression within 5–10 min of application (Abel et al., 1994), whereas a similar auxin-induced cell expansion and growth was also reported within that short period of time (Schenck et al., 2010). There are three possible explanations for the delay in IAA-induced up-regulation of  $\omega$ -6 *FAD* (1) IAA treatment alone is not sufficient to trigger this gene expression as reported by Matsuda et al. (2001), where auxin requires cytokinin to help alter expression of Arabidopsis ER-type  $\omega$ -3-*FAD* (*FAD3*), (2) these genes are not directly regulated by auxin or (3) *C. vulgaris* (UMT-M1) like many other microalgae possesses a distinct mode of auxin action for gene expression in comparison to higher plants as this microalgae does not contain the auxin transport inhibitor response/auxin signaling F-box (TIR/AFB) gene family (De Smet et al., 2011). The AFB protein is required for auxin induced rapid changes in global gene expression as an *afb*

mutant shows great attenuation of gene expression, but normal auxin-induced growth, as compared to wild-type (Schenck et al., 2010).

For  $\omega$ -3 *FAD* gene expression, there was no appreciable change in its gene expression during the experimental time points (Fig. 4D), although significantly decreased C18:3n3 production was recorded until DAT-6 (Fig. 3E). This could be due to substrate C18:2 limitations rather than  $\omega$ -3 *FAD* activity as shown previously by overexpression of soybean *FAD* cDNA in Arabidopsis roots that did not lead to higher PUFA contents (Heppard et al., 1996). In this study, gene expression analysis was carried out until DAT-6 as the cell number and total oil production of *C. vulgaris* (UMT-M1) at DAT-8 and 10 were even lower than at DAT-0. Low production of total oil was not practically significant for mass production of microalgae-based oils. Furthermore, gene expression at an early stationary growth phase was of interest where exogenous application of IAA transiently modified fatty acid compositions of *C. vulgaris* (UMT-M1).

## 2.5. Correlation analysis between gene expression and fatty acid compositions

In order to study the relationship between gene expression and fatty acid profiles, Pearson's Correlation analysis was carried out with results summarized in Table 2. To the best of our knowledge, this is the first report describing a direct relationship between gene expression and fatty acid profiles in microalgae under hormone treatment (Table 2). Correlation between genes and individual fatty acid levels showed that the *KAS I* gene expression was positively correlated with C18:0 and negatively correlated with PUFA (C18:2 and C18:3n3) levels, which verified the function of this gene in the fatty acid biosynthesis pathway (Ohlrogge and Browse, 1995; Shimakata and Stumpf, 1982). As for *SAD* gene expression, however, a positive correlation with C18:0 levels was contradicted by Lei et al. (2012), where they reported that *SAD* gene expression had a negative correlation with C18:0 and a positive correlation with C18:1 levels. This contradiction could be due to substrate preferences of *C. vulgaris* (UMT-M1) *SAD* to desaturate shorter

**Table 2**

Pearson's correlation coefficient between gene expression (*KAS I*, *SAD*,  $\omega$ -6 *FAD* and  $\omega$ -3 *FAD*), total oil content (oil), fatty acid classes (SFA, MUFA and PUFA) and fatty acid compositions (C16:0, C18:0, C18:1, C18:2 and C18:3n3).

	Gene			
	<i>KAS I</i>	<i>SAD</i>	$\omega$ -6 <i>FAD</i>	$\omega$ -3 <i>FAD</i>
Oil	0.164	0.151	-0.263	-0.196
SFA	0.415	0.465	-0.259	-0.441
MUFA	0.420	0.399	-0.298	-0.303
PUFA	-0.559*	-0.586*	-0.129	0.588*
C16:0	0.379	0.457	-0.188	-0.373
C18:0	0.466*	0.470*	-0.059	-0.575**
C18:1	0.298	0.317	-0.317	-0.212
C18:2	-0.623**	-0.598*	-0.088	0.403
C18:3n3	-0.639**	-0.574*	-0.209	0.513*

Single asterisk (\*) denote statistically significant correlation coefficients ( $p < 0.05$ ) while double asterisks (\*\*) denote statistically significant correlation coefficients ( $p < 0.01$ ).

length fatty acids (Haas and Fox, 2002) although herein there is no experimental data to support this. Similarly, the expression of the  $\omega$ -3 *FAD* gene, which was shown to have a positive correlation with the synthesis of PUFA and C18:3n3 supported the role of this gene in PUFA biosynthesis (Ohlrogge and Jaworski, 1997).

### 3. Conclusions

IAA treatment significantly affected oil accumulation, fatty acid compositions and gene expression in *C. vulgaris* (UMT-M1). Transient increases of total oil and modification of fatty acid compositions under IAA treatment were correlated with the genes expression. Therefore, a potential application of this finding that now seems possible is to manipulate the regulatory mechanisms of fatty acid biosynthesis in microalgae. The great advantage of using exogenous plant hormones to transiently increase and modify total oil content and composition in microalgae may hold the possibility to produce affordable, scalable and sustainable microalgae-based biodiesel.

### 4. Experimental

#### 4.1. Microalgal cell culture conditions

The microalgal sample, *C. vulgaris* (strain UMT-M1), was obtained from the microalgae stock culture at Universiti Malaysia Terengganu, Malaysia. The algal culture was initiated from a single colony taken from the stock agar plate and cultured in F/2 medium (Guillard and Ryther, 1962) prepared with natural sea water (30 ppt). Illumination was provided by continuous cool-white fluorescent lamps at a photon flux density of  $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ , with cultures aerated with  $0.22 \mu\text{m}$  filter sterilized air. Cell density was determined by counting cells on a haemocytometer (Neubauer) pre-affixed with a cover slip under a microscope with  $400\times$  magnification. The initial cell density of each experiment was standardized at  $6 \times 10^5$  cells  $\text{mL}^{-1}$  in 2 L culture medium prepared in 3 L conical flasks. All cultures were maintained at  $24^\circ\text{C}$  ( $\pm 2^\circ\text{C}$ ). Cultures growth was monitored by recording cell density values at 2 day intervals until a constant reading was obtained. This constant reading (three readings) represented the early stationary growth phase. IAA at a final concentration of  $100 \mu\text{M}$  was added to the culture at the early stationary growth phase, with the microalgae and harvested at 2 day intervals. Samples for "days after treatment (DAT)-0" were taken immediately after the culture reached the early stationary growth phase before being subjected to IAA treatment. For control (without IAA), EtOH-H<sub>2</sub>O (95:5, v/v) was added to the culture to a final concentration of 0.038% (v/v)

to compensate for the solvent used to dilute IAA. All treatments were conducted in three replicates.

#### 4.2. Determination of oil content and fatty acid analyses

Microalgal cells were harvested by centrifugation at  $5000\times g$  for 10 min and dried at  $80^\circ\text{C}$  until constant weights were obtained. Total oil contents were determined according to the methods of Cha et al. (2011). Esterification of fatty acids into methyl esters were carried out using a modification of the method described by Cha et al. (2011). Esterified oil samples ( $1 \mu\text{l}$ ) were analyzed using an Agilent 6890 gas chromatograph (Agilent Technologies, USA) fitted with a HP-88 capillary column (0.25 mm inner diameter  $\times$  30 cm length) and a flame ionization detector. The oven temperature was programmed from  $175^\circ\text{C}$  (10 min hold) to  $220^\circ\text{C}$  (15 min hold) at a rate of  $3^\circ\text{C min}^{-1}$  with He as carrier gas at a constant flow rate of  $2 \text{ mL min}^{-1}$ . Identification of fatty acids was accomplished by comparing peak and retention time of the reference standard, Supelco 37 Component FAME Mix (Sigma-Aldrich). The data were presented as percentage relative to the control.

#### 4.3. Total RNA extraction and cDNA synthesis

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. Removal of genomic DNA contamination was confirmed by amplification of 18S rDNA gene using RNA as PCR templates. Next, RNA ( $1 \mu\text{g}$ ) was reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad), in accordance with manufacturer's instructions. The cDNA generated was used directly for real-time (RT) PCR.

#### 4.4. Real-time PCR for gene expression

Real-time PCR was performed in a MyiQ Single Colour 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 consists of cDNA ( $50 \text{ ng}$ ,  $1 \mu\text{l}$ ),  $0.4 \mu\text{M}$  final concentration of each forward and reverse primer,  $10 \mu\text{l}$   $2\times$  iQ SYBR Green Supermix and nuclease free water to the final volume of  $20 \mu\text{l}$ . After heating at  $95^\circ\text{C}$  for 15 s, the RT-PCR amplification was programmed for 40 cycles of  $95^\circ\text{C}$  for 35 s,  $64.2^\circ\text{C}$  for 35 s and  $72^\circ\text{C}$  for 30 s. Specificity of all PCR amplification was verified by melting curve at the completion of each run which was set from  $55^\circ\text{C}$  to  $95^\circ\text{C}$  at  $0.5^\circ\text{C}$  increment. The nucleotide sequences of all primer pairs and amplicon sizes are presented in Supplementary Table S1. The gene expression data was analyzed using the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The data obtained represented the fold change (increase or decrease) of the target gene in the treated sample relative to the control sample and was normalized to the expression of 18S rRNA reference gene.

#### 4.5. Statistical analysis

The effect of IAA treatment on oil content and fatty acid profiles of *C. vulgaris* (UMT-M1) was determined in three replicates, with data checked for normality and equal variances before being analyzed statistically by one-way analysis of variance (ANOVA). Significant differences were identified by post-hoc Tukey's honestly significant difference (HSD) test at  $p < 0.05$ . Correlation analysis was done using Pearson's Correlation with significant two-tailed at  $p < 0.05$  and  $p < 0.01$ . All statistical analyses were carried out using SPSS 16.0 ([www.ibm.com/software/analytic/spss](http://www.ibm.com/software/analytic/spss)).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2014.12.022>.

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