Bioresource Technology 232 (2017) 44–52

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

The boosted biomass and lipid accumulation in *Chlorella vulgaris* by supplementation of synthetic phytohormone analogs

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HIGHLIGHTS

- Synthetic hormone analog NAA was tested to boost algal biomass and lipid accumulation.
- NAA performed remarkable promoting effect on cell growth and lipid biosynthesis.
- NAA modified proportions of fatty acids which were prone to high-quality biofuels.
- NAA-treatment manipulated endogenous phytohormones metabolism.
- Economic-estimation of NAA indicated possibility in developing lipid for biofuels.

ARTICLE INFO

Article history:
Received 11 January 2017
Received in revised form 1 February 2017
Accepted 2 February 2017
Available online 7 February 2017

Keywords:
Chlorella vulgaris
1-Naphthaleneacetic acid (NAA)
Lipid
Endogenous hormones
Economic and ecological estimation

ABSTRACT

This study attempted at maximizing biomass and lipid accumulation in *Chlorella vulgaris* by supplementation of natural abscisic acid (ABA) or synthetic 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) hormone analogs. Amongst three tested additives, NAA-treatment performed remarkable promoting effect on cell growth and lipid biosynthesis. The favorable lipid productivity (418.6 mg/L/d) of NAA-treated cells showed 1.48 and 2.24 times more than that of 2,4-D and ABA. NAA-treatment also positively modified the proportions of saturated (C16:0 and C18:0) and monounsaturated fatty acids (C18:1) which were prone to high-quality biofuels-making. Further, NAA-treatment manipulated endogenous phytohormones metabolism leading to the elevated levels of indole-3-acetic acid, jasmonic acid, and salicylic acid and such hormone accumulation might be indispensable for signal transduction in regulating cell growth and lipid biosynthesis in microalgae. In addition, the economic-feasibility and eco-friendly estimation of NAA additive indicated the higher possibilities in developing affordable and scalable microalgal lipids for biofuels.

1. Introduction

As part of endeavors to tackle the energy crisis and global warming, oleaginous microalgae were increasingly being recognized as renewable and environment-friendly alternative to fossil energy. This interest toward microalgae is principally due to its fast growth, non-arable cultivation, and potential to modify metabolism pathway for a higher lipid production (Chisti, 2007). However, a series of challenges have to be conquered before commercial application of microalgae-based biodiesel, one of which is how to effectively improve the lipid productivity.

The versatile chemical and physical stressors such as nitrogen-deficiency, high-salinity, and high irradiance have been verified to effectively stimulate lipid biosynthesis in microalgae (Cheng et al., 2016). However, even large promotions in lipid content will not pay off lipid productivity because cell growth is severely impeded under adverse environment conditions. Subsequently, the ample evidence indicated that manipulation of algal growth media could also achieve improvements in lipid biosynthesis (Jusoh et al., 2015a). In this regard, some organic carbon sources (e.g., sweet sorghum juice, corn powder and cassava starch hydrolysates), nitrogen (e.g. urea and amino acid), and other nutrients ingredients (e.g. phosphorus and iron) have been well-documented to exert positive effects on lipid productivity (Salama et al., 2014). However, these achieved lipid productivities was still being a big gap with the theoretical value proposed by Hu et al. (2008). Therefore, the cost-effective and biologically-acceptable cultivation additives for microalgae lipids production were needed to be further explored.

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http://dx.doi.org/10.1016/j.biortech.2017.02.004
0960-8524/

Hormones in microalgae presented new opportunities in developing microalgal lipids for biodiesel production (Lu and Xu, 2015). However, the manipulation of hormones metabolism in microalgae is still in its infancy as well as the concentration of hormones present in microalgae is considerably lower than in plants (Sulochana and Arumugam, 2016). Therefore, considering the evolution relationship between microalgae and plants, the majority of scientific literatures focused on exogenous application of hormones for microalgal lipids. For instance, Cho et al. (2015) reported an increment in lipid productivity of *D. salina* by supplement of myo-inositol; the exogenous applications of diethyl aminoethyl hexanoate boosted the cell growth and fatty acids content of *Scenedesmus obliquus* (Salama et al., 2014). Indole-3-acetic acid, phenylacetic acid, indole-3-butyric acid, and abscisic acid have also been highlighted as potentially valuable culture supplements for microalgae to promote cell growth and lipid metabolite biosynthesis (Piotrowska and Bajguz, 2014; Bajguz and Piotrowska, 2014). However, the precise mechanisms of lipid accumulation by exogenous application of phytohormones remained elusive. The existing evidences suggested that the exogenous phytohormones could effectively regulated fatty acid biosynthesis genes and manipulated endogenous phytohormones metabolism, which might be directly or indirectly related with lipid biosynthesis (Stirk et al., 2014; Jusoh et al., 2015a,b).

Although phytohormones were typically active to regulate various aspects of microalgae growth and lipid accumulation at very low concentrations, the cost impact of natural phytohormones application for microalgae cultivation must be considered from a commercial standpoint before implementation of mass production (Salama et al., 2014). Consequently, exploring economic and high-efficient natural phytohormones alternatives for large-scale lipid production is of increasing interest. Numerous studies suggested that the synthesized hormones analogs (e.g. 2,4-Dichlorophenoxyacetic acid) induce similar physiological responses as natural phytohormones in bioassays (Szechyńska et al., 2007; Piotrowska-Niczyporuk et al., 2012). However, little is known about the roles of exogenous application of synthetic hormones analogs for biomass and lipid accumulation in microalgae. This study aimed to probe the feasibility of using synthetic hormone analogs (NAA) for lipid production by microalga *Chlorella vulgaris*. The biomass, lipid content, fatty acid compositions, endogenous phytohormones, and fatty acid biosynthesis genes would be employed to identify biochemical characteristics that lead to the optimal lipid productivity of *C. vulgaris*. The economic-feasibility analysis and eco-friendly estimation of using synthetic NAA for mass production of microalgae was also evaluated.

2. Materials and methods

2.1. Materials and chemicals

The green *Chlorella vulgaris* was purchased from the Culture Collection of Algae at University of Texas at Austin (UTEX), USA. The natural indole-3-acetic acid, jasmonic acid, and salicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synthetic 2,4-Dichlorophenoxyacetic acid and 1-Naphthaleneacetic acid were supplied by Hefei Bo Mei Biotechnology. CO., Ltd., (Anhui province). GF-1 Total RNA Extraction Kit (Vivantis, USA) was purchased from Solarbio Biotechnology. CO., Ltd., (Shanghai). All other chemicals were of the highest purity available.

2.2. Microalgae cultivation

The pre-culture of *Chlorella vulgaris* was initiated from a single colony taken from the stock agar plate and cultured in the improved liquid Basal medium as described in Mu et al. (2015). Subsequently, 5 mL of the pre-cultured liquid cells was inoculated into 250-mL flask containing 100 mL Basal media and cultured on a reciprocating shaker (160 rpm/min) at 28 °C and light intensity of 100 μmol/m^2/s. The natural abscisic acid (ABA), synthetic 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1-Naphthaleneacetic acid (NAA) were incorporated into Basal medium from the beginning of culture at different concentrations: 0.5, 1.0, 1.5, and 2.5 mg/L. All treatments carried three biological duplicates. The algal cells cultured with Basal medium (without hormones) was treated as control group. The cultivation conditions (temperature, light intensity, and agitation) of the control cells were consistent with hormones-treated cells. The biomass and lipid content were measured at 12 ~ 24 h interval.

2.3. Analytical methods

2.3.1. Biomass measurement

The algal cultures were collected by centrifugation at 4000 rpm for 20 min. The supernatant was carefully removed and pellet washed twice with di-ionized water. Subsequently, the pellet was dried in an oven at 50 °C for 12 ~ 24 h until constant weights were achieved.

2.3.2. Total lipid content

Lyophilized algae powder was mixed with distilled water and chloroform/methanol (2:1, v/v). The mixtures were shaken for 15 min using a vortex mixing apparatus and then centrifuged at 5000 rpm for 15 min. All the chloroform phases were collected and evaporated to constant weight by nitrogen gas. The total lipid contents were determined by biomass dry weight.

2.3.3. Fatty acids compositions

The fatty acid methyl esters (FAMEs) profile of *C. vulgaris* was measured by transesterification. The algae powder was performed saponification at 75 °C in a thermostated water bath for 20 min by introducing NaOH-CH_3OH solution. The boron trifluoride-methanol (1:2, V/V) was then added to the saponified samples and shaken for 30 min. The esterified oil samples were mixed with hexane and the upper-layer FAMEs profile was characterized by an Agilent 6890 gas chromatograph (Agilent Technologies, USA) fitted with a HP-88 capillary column (0.25 mm inner diameter × 30 cm length) and a flame ionization detector. 2 μL of FAMEs solubilized in chloroform were injected at chromatograph by splitless mode using helium at 1.5 mL/min as carrier gas. The ion source and quadruple temperatures were 230 °C and 150 °C respectively. And the flame ionization detector (FID) temperature was 270 °C. The initial oven temperature was set at 130 °C for 1 min, increased to 200 °C at a rate of 5 °C/min, and then held at this temperature for 7 min. Detection was done using full scan mode between 35 and 500 m/s and EMV mode gain factor 5 and identification was performed using the NIST08.LIB mass spectral database.

2.3.4. Fatty acids biosynthesis gene expression analysis

Total RNA of *C. vulgaris* cells was extracted with GF-1 Total RNA Extraction Kit according to the manufacturer's protocol. 100 mg of algal cells treated with hormone analogs was grinded in liquid nitrogen to fine powder before adding 400 μL Buffer TR and centrifuged at 8000 rpm for 3 min. The clear lysate was then centrifuged to collect the flow-through. 350 μL of 80% ethanol was subsequently added to the flow-through before transferring the mixture into a RNA binding column and centrifuged at 8000 rpm for 1 min. The final RNA was treated with DNase I to remove contaminating DNA and then performed reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's instructions. The generated cDNA was directly used

for RT-PCR, which 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 consisted of 50 ng cDNA (1 μL), 0.4 μM final concentration of each forward and reverse primers, 10 μL 2 × iQSYBRGreen Supermix and nuclease free water to the final volume of 20 μL. After heating at 95 °C for 15 s, the real-time PCR amplification was programmed for 40 cycles of 95 °C for 35 s, 64.2 °C for 35 s and 72 °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 °C to 95 °C at 0.5 °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 $2^{-\Delta\Delta Ct}$ method. Fold changes (up-regulation or down-regulation) of the target gene in hormone-treated sample relative to the control sample were normalized by the expression of 18 srRNA reference gene.

2.3.5. Endogenous phytohormones

The algal cell samples were grinded by liquid nitrogen and the grinded algal powder was mixed with n-propanol/water/hydrochloric acid (200:100:0.2, v/v/v). The mixtures were shaken for 30 min on a table concentrator apparatus without light-exposure. Dichloromethane was then introduced into the mixtures and continuously shaken for 30 min on table concentrator. Subsequently, the mixtures were centrifugation at 4500 rpm for 10 min and the supernatant was collected into centrifuge tubes. Finally, the supernatant was dried by nitrogen and re-suspended with methanol/water (4:5, v/v) to purify endogenous phytohormones. The phytohormones standards (i.e. IAA, JA, SA) were used to draw calibration curve. The regression equation of IAA, SA, and JA were $Y = 1.15e^3X - 4.34e^3$ ($R^2 = 0.9990$), $Y = 1.75e^3X + 218$ ($R^2 = 0.9992$), and $Y = 5.25e^3X - 340$ ($R^2 = 0.9984$), respectively. The purified endogenous phytohormones in algal samples were quantified and analyzed by ultra high performance liquid chromatography UPLC (Acquity UPLC™ System, Waters, Milford, MA, USA) coupled to a triple-stage quadrupole mass spectrometer MS/MS (Xevo® TQ MS, Waters MS Technologies, Manchester, UK) equipped with an electrospray (ESI) interface. The quantitative data analysis was carried out by Analyst Software 1.6 and ACD/Spectrus Processor 2015. The content of endogenous phytohormone was calculated in terms of the equation ($X = C \times V/M$), where C was the phytohormone concentration (ng/mL), V and M represented the concentrated sample volume (mL) and sample weight (mg).

3. Results and discussion

3.1. Effect of natural phytohormone and synthetic hormone analogs on biomass and lipid content of C. vulgaris

Microalgae cultivation was generally considered as vitally important and technically-difficult procedure as the growth conditions will determine biomass productivity as well as the quality and quantity of the lipids (Arumugam et al., 2013). It was well-documented that phytohormone or hormone analogs have been highlighted as potentially valuable cultivation additives in promoting algal growth and lipid biosynthesis (Jusoh et al., 2015a). Therefore, this study attempted at maximizing biomass and lipid accumulation in *C. vulgaris* by supplementation of natural abscisic acid (ABA) or synthetic 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) hormone analogs and aimed to explore new possibilities in developing affordable and scalable algal lipids for biofuels.

As indicated in Fig. 1, the supplementation (0.5 to 2.5 mg/L) of synthetic NAA and 2,4-D significantly contributed to the growth of *C. vulgaris* cells as well as the application (0.5–2.5 mg/L) of ABA showed a slight increase in biomass in comparison with control cells. Interestingly, NAA and 2,4-D-treated cells exhibited faster growth and the cultivation time for reaching the stationary growth phase was 96 h while ABA-treated cells spend 120 h under all concentrations conditions. Actually, cell cycle composed of growth phase 1 (G_1), DNA synthetic phase (S), growth phase 2 (G_2) and mitosis (M) with two main check points-the G_1/S and G_2/M transitions was prone to influenced by environmental factors. Stirk et al. (2014) suggested that auxins are the most influential cell cycle hormones as auxins affect the re-entry into the cell cycle as well as most other phases, such as G_1, S, G_2, or M. Many hormone (e.g. NAA) signaling pathways converge at the G_1/S and G_2/M transitions to promote the normal progression of the cell cycle while ABA inhibits DNA replication and blocks the G_1/S transition (Hunt et al., 2011; Stirk et al., 2014). Therefore, such intriguing discrepancy in time for arriving stationary growth phase might be due to the supplementation of NAA and 2,4-D shortened the cell cycle and natural ABA was not appointed this functionality for *C. vulgaris*. The biomasses showed an increasing trend in different degree within 24 ~ 96 h of cultivation response to all NAA and 2,4-D concentrations treatment and subsequent 48 h of cultivation presented slight decrease (Fig. 1). The biomass of ABA-treated cells showed also increasing trend within 24 ~ 120 h and then decreased during subsequent 24 h of cultivation. Although all tested phytohormone and hormones analogs at 1.5 ~ 2.5 mg/L also supported the cell growth, the biomass accumulation effect was less than the stimulation of low concentration (0.5–1.0 mg/L). The results agreed with the report of Jusoh et al. (2015a), where the higher JA concentration (10^{-5} to 10^{-4} M) give an inhibitory on cell growth while addition of low concentration of JA (10^{-8} to 10^{-6} M) exerted positive effect in *C. vulgaris*. Actually, the maximum biomass (3.5 g/L) was achieved by 1.0 mg/L NAA-treatment at 96 h, which represented 1.24-fold and 1.92-fold more than that of the favorable biomasses of 2,4-D and ABA, respectively. And also the maximum biomass of NAA-treated cells was 2.57 times more than that of control cells. This indicated that the synthetic NAA performed the most remarkable promoting effect on cell growth amongst all tested phytohormone and hormone analogs. Additionally, the biomass productivity by supplementation of synthetic NAA in this study was comparable with natural IAA in the report of Piotrowska and Bajguz (2014). Collectively, the synthetic hormone analogs (NAA) was characterized by the potential superiority in improving biomass productivity.

Exogenous application of phytohormone could not only stimulate microalgae growth but also trigger intracellular lipid biosynthesis at different levels (Cho et al., 2015; Lu and Xu, 2015; Jusoh et al., 2015b). This study catered to this conclusion by the results that all tested additives (NAA, 2,4-D, and ABA) ranging from 0.5 to 1.0 mg/L induced lipid biosynthesis in a concentration-dependent manner within 96 h or 120 h, respectively. While the lipid content were not particularly prominent in algal cells subjected to high phytohormone and hormone analogs concentration induction (1.5–2.5 mg/L) in comparison with control. The highest lipid content of 50% was achieved by 1.0 mg/L of ABA-treatment at 120 h, which was almost equal to the highest lipid content (47.84%) of NAA-treated cells and represented 1.26-fold more than the favorable lipid content of 2,4-D-treated cells. The highest lipid content (20.16%) of control cells was only around two-fifths to one half of the lipid content in ABA, 2,4-D, and NAA-treated cells. These results indicated that the natural ABA exhibited the highest biological activity (almost equivalent to NAA) in terms of inducing lipid biosynthesis, followed by 2,4-D and then control cells. The superiority of ABA in promoting lipid biosynthesis was also reported by Park et al. (2013), where ABA-treatment favor energy storage in the form of either starch or lipid, though it does not have any sig-

nificant effect on algal growth. However, lipid productivity is really important indicator in economically-feasible alga-based biodiesel processes because it takes into account both biomass production and lipid accumulation. The lipid productivity (418.6 mg/L/d) of NAA-treated cells was the highest amongst three tested phytohormone and hormone analogs, which was 1.48 and 2.24 times more than that of 2,4-D and ABA. NAA-treatment was also preponderant in aspect of maximizing lipid productivity of *C. vulgaris* than other reported phytohormone additives (Piotrowska and Bajguz, 2014; Jusoh et al., 2015a,b). Therefore, considering both of biomass and lipid content, the synthetic hormone analogs (NAA) might be the most suitable supplementation in developing algal lipids for biodiesel production.

3.2. Effect of natural phytohormone and synthetic hormone analogs on fatty acid compositions

Fatty acids were pivotal component of lipids as well as the fatty acid profiles were directly related to the biofuels properties such as cold flow, viscosity, and oxidative stability (Salama et al., 2014). Therefore, the fatty acid compositions in *C. vulgaris* cells treated by supplementation of phytohormone and hormone analogs (i.e. ABA, 2,4-D, and NAA) needed to be investigated. As illustrated in Table 1, no significant difference in fatty acid profiles was detected in ABA, 2,4-D, and NAA-treated cells and control cells, being mainly composed of C16-C18. Such fatty acids profiles were coincident with the reports of Jusoh et al. (2015a,b), where JA-treated and IAA-treated *C. vulgaris* cells produced a high proportion of C16-C18 fatty

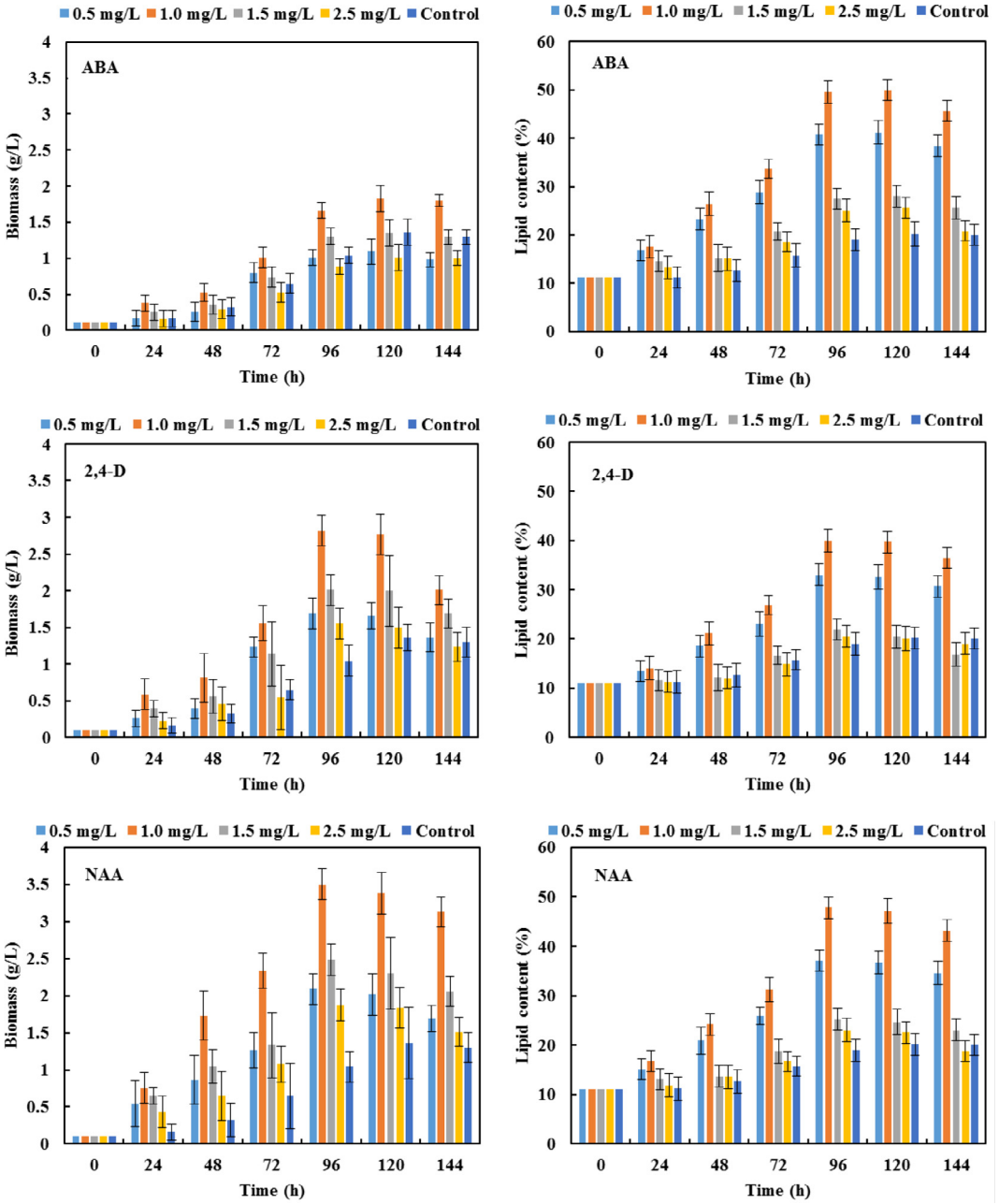

Fig. 1. The effect of phytohormone and hormone analogs (ABA, 2,4-D, and NAA) on biomass and lipid content of *Chlorella vulgaris*. *Chlorella vulgaris* was cultured in Basal medium supplemented with 0.5 ~ 2.5 mg/L of ABA, 2,4-D, and NAA, respectively. The algal cells cultured with Basal medium (without hormone) was regarded as control. The biomass and lipid content were measured at 24 h interval. Error bars represent mean standard deviation of triplicate experiments.

Table 1
Fatty acid profiles of *C. vulgaris* cells treated with the supplementation of ABA, 2,4-D, and NAA.

Fatty acids	Relative content (%)			
	ABA	2,4-D	NAA	Control
Palmitic acid (C16:0)	20.07 ± 0.09	19.94 ± 0.08	21.03 ± 0.12	19.98 ± 0.16
Hexadecadienoic acid (C16:2)	3.43 ± 0.11	3.88 ± 0.06	3.10 ± 0.08	4.21 ± 0.04
Hexadecatrienoic acid (C16:3)	5.69 ± 0.06	3.32 ± 0.11	1.99 ± 0.06	6.44 ± 0.07
Octadecanoic acid (C18:0)	7.14 ± 0.12	2.77 ± 0.12	6.19 ± 0.11	5.13 ± 0.11
Oleic acid (C18:1)	38.86 ± 0.11	42.28 ± 0.14	49.82 ± 0.07	26.43 ± 0.07
Linoleic acid (C18:2)	11.48 ± 0.05	20.06 ± 0.07	11.54 ± 0.08	20.74 ± 0.13
Linolenic acid (C18:3)	3.21 ± 0.10	2.65 ± 0.11	3.73 ± 0.10	2.96 ± 0.08
SFAs	27.21 ± 0.11	22.71 ± 0.03	27.22 ± 0.08	24.11 ± 0.09
MUFAs	38.86 ± 0.11	42.28 ± 0.14	49.82 ± 0.07	26.43 ± 0.07
PUFAs	23.81 ± 0.03	29.91 ± 0.12	19.36 ± 0.05	33.35 ± 0.12
SFA + PUFAs	51.02 ± 0.08	52.62 ± 0.13	46.58 ± 0.17	59.46 ± 0.14

ABA, 2,4-D, and NAA were the abbreviations of abscisic acid, 2,4-Dichlorophenoxyacetic acid, and 1-Naphthaleneacetic acid, respectively. SFAs, MUFAs, and PUFAs indicated the saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, respectively. The fatty acid compositions and contents were calculated when the algal biomass achieved the highest lipid productivity (after 96 h in NAA and 2,4-D-treated cells; after 120 h in ABA-treated and control cells). Data are reported as mean ± standard deviation of three independent biological replicates.

acids. These fatty acids compositions (C16-C18) were also indispensable to endow excellent cold filter plugging point for fuels produced by algal biomass (Mu et al., 2015). In general, the percentage of C18 fatty acids (mainly C18:0, C18:1, C18:2, and C18:3; with total of ~72%) in NAA-treated cells was higher than in 2,4-D-treated cells (~68%) and ABA-treated cells (~61%); nevertheless the content of C16 (mainly C16:0, C16:2, and C16:3) in ABA-treated cells (29.19%) was more than in 2,4-D-treated (27.14%) and NAA-treated cells (26.12%). Amongst these C16-C18 fatty acid compositions, oleic acid (C18:1) was found to be preponderant component in all hormone-treated cells and control cells, followed by linoleic acid (C18:2) and then palmitic acid (C16:0). And also the percentage of linolenic acid (C18:3) present in all hormones-treated cells easily complied with the specifications (C18:3 < 12%) established by ASTM D-6751 and DINV 14214 for biodiesel quality.

Actually, the high percentage of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in lipid are preferred for increasing biofuel yield and superior oxidative stability. However, the lipid components dominated by SFAs and MUFAs are prone to solidify in low temperature environment. While lipids rich in polyunsaturated fatty acids (PUFAs) have excellent performance on the cold-flow property of biofuels, but the abundant highly-unsaturated fatty acids are more susceptible to oxidation. In order to address this contradiction, the percentage of the MUFAs over the summation of SFAs and PUFAs could concurrently provide superior oxidation stability and cold-flow property for alga-based biofuels (Knothe, 2014). In this study, the most interesting result was that the content of MUFA (49.82%) was just higher than the summation of SFAs and PUFAs (46.58%) in NAA-treated cells. Whereas the proportions of MUFA (42.28% and 38.86%) in 2,4-D-treated and ABA-treated cells were lower than the content of SFAs and PUFAs (52.62% and 51.02%) and also the MUFA content (26.43%) in control cells was far lesser than the contents of SFAs and PUFAs (59.46%). Even though the same species of *C. vulgaris* was used, the content of the MUFA content in JA-treated and IAA-treated cells was also a big gap with the summation of SFAs and PUFAs (Jusoh et al., 2015a,b). The above elaborations strongly implied that NAA-treatment performed large potential in modifying the proportions of saturated and monounsaturated fatty acid which were prone to high-quality biofuels-making.

3.3. Regulation of fatty acid biosynthesis genes in response to NAA-treatment and correlation analysis between gene expression and fatty acid compositions

In order to further investigate the regulation of fatty acids compositions in response to NAA-treatment at gene level, four *KAS I*, *SAD*, *ω-6 FAD* and *ω-3 FAD* responsible for fatty acid biosynthesis genes were quantified by real-time PCR (Fig. 2). The positive or negative correlation between gene expression and individual fatty acid compositions was also cleared by Pearson's Correlation analysis under NAA-treatment (Table 2). The up-regulation expression (2.0-fold relative to control) of *KAS I* was detected in NAA-treated cells at 96 h. According to the descriptions of Jusoh et al. (2015a) concerning the function of *KAS I* in saturated fatty acid (i.e. C16:0 and C18:0) biosynthesis, it could be concluded that the increment in SFA in NAA-treated cells might be directly related to the elevated level of this gene. Based on the substrate-enzyme relationship in fatty acid *de novo* biosynthesis pathway, the high percentage of SFAs in this study should correspond to high levels of C18:1 accumulation under NAA-treatment. Such hypothesis was tenable to a certain degree as the high proportion of C18:1 fatty acid (account for 49.82%) was detected in NAA-treated cells. Moreover, both of the evidence of 1) the up-regulation expression (1.6-fold relative to control) of SAD responsible for conversion of 18:0-ACP to 18:1-ACP and 2) the positive correlation between SAD gene and individual C18:1 fatty acid ($p < 0.05$) also strengthened this hypothesis (Fig. 2 and Table 2). Lei et al. (2012) also reported the consistency between the up-regulation of SAD gene and increment of C18:1 fatty acid response to high-salinity and high-temperature treatment. All these results suggested that NAA-treatment could effectively induce *KAS I* and *SAD* gene expression which further regulated fatty acids biosynthesis in microalgae. Whereas the up-regulation of *ω-6 FAD* gene (1.45-fold relative to control) was contradictory with the low accumulation of C18:2 fatty acid (only account for 11.54%) in NAA-treated cells. This contradiction might be due to the transcript level of *ω-6 FAD* was insufficient to convert C18:1 to C18:2 or the transcriptional and post-transcriptional levels of C18:1 receiving the special regulation of NAA (Kachroo et al., 2007). As for *ω-3 FAD* gene associated with PUFA biosynthesis (i.e. C18:3), no appreciable changes (relative to control) was observed in NAA-treated cells (Fig. 2). However, it meant not that the functions of *ω-3 FAD* in regulating PUFA biosynthesis was dispensable as *ω-3 FAD* expression shared a positive correlation with PUFA biosynthesis (C18:2 and C18:3, $p < 0.01$ and $p < 0.05$) (Table 2). The direct data supporting the functions of this gene in fatty acid biosynthesis in microalgae would be further explored.

3.4. Elucidating the role of NAA-treatment in manipulating lipid accumulation in C. vulgaris

Few microalgae strains in nature have a sufficient level of biomass productivity, lipid content, and environmental tolerance to

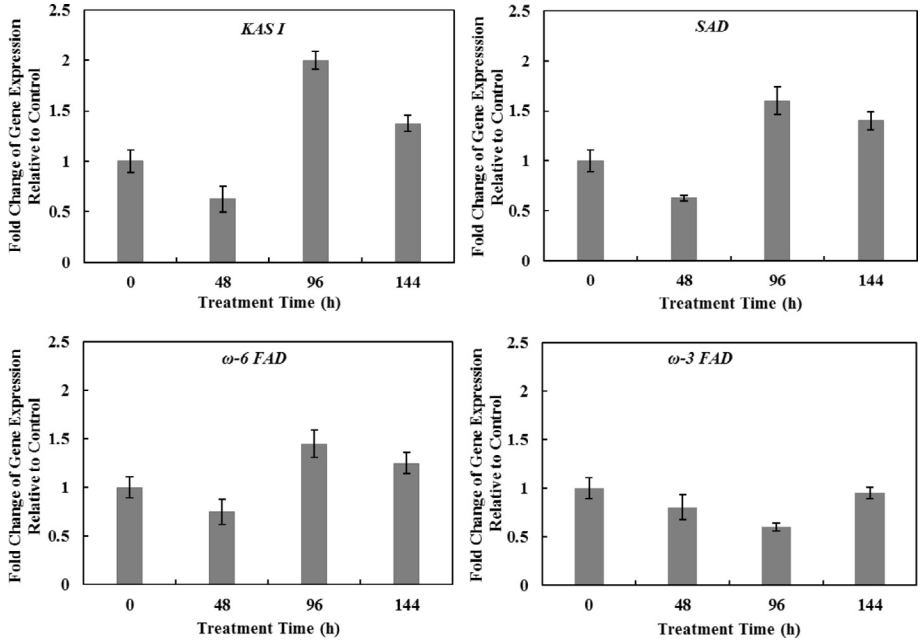

Fig. 2. The variations in expression of fatty acids biosynthesis genes (*KAS I*, *SAD*, *ω-6 FAD*, and *ω-3 FAD*) in response to IAA- treatment. Error bars represent the means ± standard deviation of triplicate experiments.

Table 2
Pearson's correlation coefficient between fatty acids biosynthesis gene and fatty acid compositions in NAA-treated cells.

Fatty acids	Fatty acids biosynthesis genes			
	KAS I	*SAD*	*ω-6 FAD*	*ω-3 FAD*
C16:0	0.348	−0.169	−0.174	−0.772**
C18:0	−0.118	−0.025	0.199	−0.322
C18:1	−0.523*	0.544*	0.604*	−0.013
C18:2	−0.602*	0.402	0.395	0.837**
C18:3	−0.398	0.401	0.299	0.513*
SFA	0.302	0.078	0.085	−0.687**
MUFA	−0.496	0.477	0.479	0.182
PUFA	−0.528*	0.449	0.444	0.825**
Lipid	−0.221	0.392	0.372	−0.408

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

sustain cost-competitive biofuels production. Given the important regulatory roles of phytohormones in higher plants and evolution relationship between microalgae and plants, the discovery and dissection of hormone-related components in microalgae could help researchers to devise rational strategies or serve as potential gene engineering targets for microalgae-based biofuels. The past investigations have largely focused on exogenous application of phytohormones that was because the regulatory functions of endogenous phytohormones in microalgae could be deduced via the supplementation experiments of exogenous phytohormone for microalga cultures (Park et al., 2013; Piotrowska and Bajguz, 2014; Jusoh et al., 2015a,b). However, little is known about the functional role of exogenous phytohormones in regulating lipid accumulation in microalgae, especially the functions of the synthetic hormones analogs. This study was only a preliminary exploration for the functions of the synthetic NAA in manipulating lipid accumulation in microalgae. It was expected to be an available for probing the functions of the endogenous phytohormones in microalgae, which in turn should open new doors for genetically or chemically engineering microalgal lipid production.

Three phytohormones composed of indole-3-acetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA) were detected in *C. vulgaris* cells subjected to NAA-treatment. Interestingly, three detected phytohormones firstly increased and then decreased in NAA-treated and control cells within 72 ~ 120 h and 96 ~ 144 h, respectively, and also presented the most significant accumulation when algal cells achieved the highest lipid content (120 h for control and 96 h for NAA) (Table 3). Compared with control cells, the contents of IAA showed a moderate increase as well as JA and SA exhibited at least 2-fold increment response to NAA-treatment. The results suggested that endogenous phytohormones were vulnerable to be manipulated by growth environment as reported by Stirk et al. (2014) where IAA content in *C. minutissima* were significantly affected by light exposure. Generally, faster growing oleaginous microalgae strains had a higher IAA concentration relative to slower growing strains (Stirk et al., 2013). Therefore, it could be inferred that the elevated levels of IAA response to NAA-treatment in this study might be one of the contributors to support biomass yield and lipid biosynthesis. Such hypothesis also received the supports by some research where exogenous IAA had profound effects on the growth rate and lipid biosynthesis in *C. reinhardtii*, *C. vulgaris*, and *C. sorokiniana* (Park et al., 2013; Piotrowska and Bajguz, 2014). Therefore, based on available data, the boosted lipid accumulation in *C. vulgaris* by NAA-treatment implied two possibilities, which were 1) NAA-treatment stimulated the increment in endogenous IAA concentration that lead to biomass accumula-

Table 3
The endogenous hormones quantified in *C. vulgaris* cells by NAA-treatment.

Endogenous hormones concentration (pg/mg DW)	Treatment					
	Control (without NAA)			NAA (1.0 mg/L)		
	96 h	120 h	144 h	72 h	96 h	120 h
IAA	10.09 ± 0.6	19.070 ± 0.9	14.83 ± 1.0	9.0800 ± 0.5	20.790 ± 1.8	13.350 ± 0.7
JA	0.183 ± 0.0	0.2790 ± 0.0	0.125 ± 0.0	0.2990 ± 0.0	0.7380 ± 0.0	0.3420 ± 0.0
SA	11.230 ± 1.2	14.190 ± 0.7	12.26 ± 0.6	18.300 ± 1.1	30.120 ± 2.2	11.300 ± 0.9

The phytohormone concentrations were quantified at three representative time points: the highest lipid productivity point (120 h for control cells and 96 h for NAA-treatment cells) and before and after 24 h of the highest lipid productivity (96 h and 144 h for control cells; 72 h and 120 h for NAA-treatment cells). IAA, JA, and SA indicated indole-3-acetic acid, jasmonic acid, and salicylic acid, respectively. The data are presented as mean ± standard deviation of three independent biological replicates.

tion and lipid biosynthesis in microalgae or 2) exogenous NAA might function as precursors into IAA biosynthesis pathway and the elevated levels of IAA further regulated cell growth and lipid biosynthesis by specific signal transduction in microalgae. Results from the current investigation seemed to favor the second possibility because NAA concentration was significantly decreased from 1.0 mg/L to 0.061 mg/L at the end of the cultivation.

Although extensive investigations and findings on the key players in JA signaling in higher plants, little is known about the precise functions of this hormone in microalgae. Recently, exogenous application of JA was found to act as positive regulator for algal biomass and lipid accumulation. For instance, Jusoh et al. (2015a) reported that a high concentration of exogenous JA accelerated *C. vulgaris* growth with increment of up to 51% of cell density and transiently increased total lipid by 54% relative to the control. In addition, some other investigations have implied that algal cells upon exogenous JA treatment favored energy storage in the form of either lipid or secondary metabolites including terpenoids, flavonoids, and alkaloids (Piotrowska-Niczyporuk et al., 2012). In this study, the endogenous JA concentration was significantly increased from 0.279 to 0.738 pg/mg DW (approximate 2.65-fold) response to NAA-treatment (Table 3). The increased JA might be due to NAA-treatment directly activated JA signal pathway or indirectly caused by the accumulation of IAA because of the collaborative relationship between IAA and JA (Sun et al., 2009). Therefore, in addition to the above two elaborations, another possibility was that exogenous NAA in conjunction with endogenous phytohormones (IAA and JA) to promote cell growth and lipid accumulation in *C. vulgaris*.

Another detected phytohormone molecule (SA) showed 2.12-fold increase in NAA-treated cells relative to control (Table 3). SA participated in regulating defenses in higher plants and was predominantly associated with resistance against biotrophic and hemibiotrophic pathogens (Lu and Xu, 2015). A typical example was that SA modulated lipid metabolism to develop effective defenses during the induction of resistance in wheat against powdery mildew (Tayeh et al., 2013) as well as exogenous SA effectively protected phospholipids against cadmium stress in flax (Belkadhi et al., 2015). So far, this hormone was detected in few algae strains or present in relatively low concentration in microalgae. Therefore, no direct evidence indicated that NAA could effectively regulate growth and lipid biosynthesis in microalgae. However, a recent report suggested that *Chlamydomonas* cells with SA-treatment modified nitrogen and amino acid metabolism which were highly related with lipid biosynthesis (Lee et al., 2016). Likewise, it has been demonstrated that proteins enriched in lipid metabolism were differentially expressed in SA-treated cells, which was consistent with genetic transcriptional expressions involved in fatty acid biosynthesis in *H. pluvialis* (Gao et al., 2016). Therefore, the accumulation of SA (from 14.19 to 30.12 pg/mg DW) response to NAA-treatment might be partly related with lipid biosynthesis in *C. vulgaris* in this study (Table 3).

The aforementioned elaborations was exciting as the boosted biomass and lipid accumulation in microalgae could be positively regulated by the elevated levels of endogenous phytohormones. Therefore, a strategy of perturbation was that some genes like *iaaM*, OPDA reductase (OPR), and multifunctional protein (MFP) involved in phytohormone pathway could be utilized to enrich endogenous phytohormone concentrations by gene overexpression or silencing which in turn positively regulated cell growth and lipid biosynthesis.

3.5. Economic-feasibility and eco-friendly estimation of NAA supplementation for algal biomass production

The cost-impact of NAA application for microalgae biomass production must be considered from a commercial standpoint before implementation of industrial production (Salama et al., 2014). In this study, the economic-feasibility analysis was mainly focused on the cost of culture media, the amount of biomass and lipid production and the expenses of additives (Table 4). As indicated in Table 4, the supplementation of NAA, 2,4-D, and ABA significantly reduced the expenses of biomass and lipid production in comparison with the control group. The synthetic NAA performed the most remarkable superiority in the aspects of production cost for biomass and lipid. The production cost was only 0.069 USD/g Biomass and 0.144 USD/g Lipid by NAA-treatment, which were far less than that of the control (0.176 USD/g Biomass and 0.876 USD/g Lipid) and natural ABA (0.412 USD/g Biomass and 0.824 USD/g Lipid). The production cost for biomass and lipid by NAA-treatment was quite impressive in comparison with the cost by carbon source substrate (e.g. glucose). Even though glucose substrate was much better than the control, substantial inputs of glucose increased the susceptibility to contamination, making this reagent become a significant limitation for further biotechnological applications. Actually, these results showed consistency with some reports, where hormones supplementation (e.g. IAA, DAH, and MI) performed better than other additives (e.g. acetate) and control in terms of production cost for biomass and lipid (Park et al., 2013; Salama et al., 2014; Cho et al., 2015).

Meanwhile, concern is increasing with reference to the expenditure of hormones additives, particularly the cost of synthetic NAA as excellent performance in production cost for biomass and lipid. However, these concerns might be eliminated by the following elaborations: (1) NAA is now commercially produced as an inexpensive chemical reagent. The synthetic NAA currently trades at about 2.65×10^{-5} USD per milligram (CAS No. AN0640, Hefei Bo Mei Biotechnology. CO., Ltd., Anhui province), which is much lower than that of natural ABA (5.10×10^{-1} USD per milligram) and other reported phytohormones ($1.0 \sim 2.0 \times 10^{-3}$ USD per milligram IAA); although the price of NAA is slightly higher than DAH (2.0×10^{-5} USD per milligram), the work concentration and efficiency was really important indicators in economic feasibility analysis. For instance, 2.15 mg/L of DAF was supplied in 1 L of basal

Table 4
Economic-feasibility analysis of using NAA for biomass and lipid production of *C. vulgaris*.

Chemicals	Additives	Price			Biomass (g/L)	Lipid yield (g/L)	Cost (US $/g Biomass)[g]	Cost (US $/g Lipid)[g]	References
	Hormone (mg/L)/ Substrate (g/L)	Hormone (US $/mg)/ Substrate (US $/g)	Basal medium (US $/L)	Total (US $/L)					
Control	0	–	0.240[c]	0.24000	1.36	0.274	0.176	0.876	This study
NAA 0.144	(>95%)[a]	1.0	2.65×10^{-5}	0.240	0.24027	3.5	1.674	0.069	
2,4-D	(>98%)[a]	1.0	1.03×10^{-4}	0.240	0.24010	2.8	1.120	0.086	0.214
ABA	(>98%)[a]	1.0	5.10×10^{-1}	0.240	0.75000	1.82	0.910	0.412	0.824
Mu et al. (2015)	Glucose	10	6.20×10^{-3}[b]	0.240	0.30200	2.16	0.605	0.140	0.499
Control	0	–	0.471[d]	0.47100	1.05	0.100	0.450	4.690	Park et al. (2013)
IAA	3	2.0×10^{-3}	0.471	0.47700	1.69	0.180	0.280	2.720	
Acetate	10	1.08×10^{-1}	0.471	1.55100	2.00	0.780	0.780	4.560	
Control	0	–	0.250[e]	0.25000	0.32	0.032	0.780	7.810	Salama et al. (2014)
IAA	1.75	1.0×10^{-3}	0.250	0.25175	0.64	0.064	0.390	3.910	
DAH	2.15	2.0×10^{-5}	0.250	0.25004	0.83	0.081	0.300	3.090	
Control	0	–	0.440[f]	0.44000	0.65	0.017	0.680	25.88	Cho et al. (2015)
MI	500	8.5×10^{-6}	0.440	0.44425	0.96	0.024	0.460	18.33	

[a] The purity of NAA (1-Naphthaleneacetic acid), 2,4-D (2,4-Dichlorophenoxyacetic acid), and ABA (abscisic acid) were >95%, >98%, and >98%, respectively.
[b] The price of glucose substrate was from the recent report of Mu et al. (2015).
[c] The basal medium in this study was the modified Basal medium and the price was from http://www.alibaba.com.
[d] The basal medium was Bold's Basal Medium from the report of Park et al. (2013).
[e] The basal medium was Tris-Acetate-Phosphate from the report of Salama et al. (2014).
[f] The basal medium was Erd-Schreiber's modified (ESM) medium from the report of Cho et al. (2015).
[g] The cost of the microalgal biomass or lipid by supplementation of additives was calculated using the following: Cost (US$) = (g/biomass or lipid) = (A × B) + C/D, where A was the amount of each additive added to a unit of culture medium (mg/L), B is the price of the substrate (US $/mg), C is the price of the unit medium (US $/L), and D is the biomass or lipid yield (g/L).

medium, the total cost was 0.25 USD/L, which resulted in 0.3 USD/g Biomass and 3.09 USD/g Lipid; While 1.0 mg of NAA was added into 1 L of basal medium to contribute the total cost was 0.240 USD/L, which only spend 0.069 USD/g Biomass and 0.144 USD/g Lipid; (2) The commercial price of available phytohormones or hormone analogs depend on the purity. Although the purity of synthetic NAA (>95%) was generally less than natural phytohormones (>98%), the use of phytohormone or hormone analogs with different purities had no different effects on microalgae responses by Park et al. (2013). And also the synthetic NAA performed better than natural phytohormones in promoting microalga growth and metabolite accumulation (Piotrowska and Bajguz, 2014). Given the findings of this study, it could be further extended on large-scale and low-cost microalgae cultivation by substituting basal media with various wastewaters enriched in N, P and C in the presence of NAA.

Certainly, another major concern was the contamination of the extensive use of synthetic hormone analogs on the environment since underused hormones residue into soil or water body might be toxic to human and animals (Boivin et al., 2005). However, the initial concentrations of NAA and 2,4-D used for alga cultivation was 1.0 mg/mL and the residual concentration was 0.004 mg/L and 0.12 mg/L when individual highest lipid productivity was achieved in this study. The degradation rate of NAA and 2,4-D were 99.6% and 88.0% across 96 h of cultivation. Therefore, such trace amounts of NAA and 2,4-D hormone (particularly NAA) left in culture media might be negligible for soil and water pollution. Liu et al. (2001) also implied that the considerably low NAA hormone concentration could be beneficial for plant growth and would not cause harm to the environment. In addition, even trace amounts of 2,4-D and NAA hormone into soil and water body, the natural microflora in soil able to mineralize 2,4-D and the ultraviolet light was also more effective in promoting degradation NAA chemical in water body (Boivin et al., 2005; Liu et al., 2001). Such natural hormones degradation countermeasures were encouraging in developing economically-feasible and eco-friendly NAA for alga-based biodiesel.

4. Conclusion

Collectively, using exogenous NAA to increase lipid accumulation and modify the proportions of fatty acid compositions in microalgae could present new opportunities in developing affordable, high-quality and scalable microalgae-based biodiesel. Due to limited phytohormones information on microalgae, the precise roles of NAA in manipulating lipid biosynthesis could not be well elucidated but most of the data provided deep insights to the regulated response of endogenous phytohormone in *C. vulgaris*. These results also formed a basis for manipulating phytohormone metabolism to target enhanced lipid biosynthesis in microalgae.

Acknowledgements

This work was supported by National Natural Science Foundation of China (21676228) and Scientific Research Fund of Hunan Provincial Education Department (16B254), and Collaborative Innovation Center of New Chemical Technologies for Environmental Benignity and Efficient Resource Utilization.

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.biortech.2017.02.004.

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