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Exploring biostimulation of plant hormones and nitrate supplement to effectively enhance biomass growth and lutein production with thermo-tolerant *Desmodesmus* sp. F51

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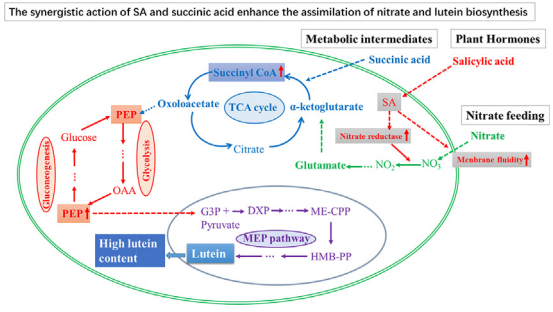
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the interactive effect of plant hormone-salicylic acid and succinic acid on biomass growth, lutein content, and productivity of *Desmodesmus* sp. F51 were investigated. The results demonstrated that the synergistic action of salicylic acid and succinic acid could effectively enhance the assimilation of nitrate and significantly improve lutein production. The maximal lutein content 7.01 mg/g and productivity 5.11 mg/L/d could be obtained with a supplement of 100 μM salicylic acid and 2.5 mM succinic acid in batch culture. Furthermore, operation strategy of nitrate fed-batch coupled with supplementation for succinic acid and salicylic acid resulted in further enhancement of lutein content and productivity by 7.50 mg/g and 5.78 mg/L/d, respectively. The performance is better than most of the previously reported values.

1. Introduction

Microalgal are microorganisms synthesizing biomass abundant in various compounds (e.g., carbohydrate, lipid, protein, pigments, and carotenoids) from light energy and inorganic nutrients (Markou and Nerantzis, 2013). Among microalgal production, carotenoids are lipophilic compounds with 40-carbon backbone structure of polyene chain originated from isoprene units (Croteau et al., 2000), that could be further divided into carotene and xanthophyll. Lutein is one of the most bioactive xanthophyll pigment family present in the phototrophic

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microorganism (e.g., microalgae and plants) due to its essential role in photosynthesis and protection of photodamage (Chen et al., 2017; Fernandez-Sevilla et al., 2010). Lutein could also act as a potential antioxidant scavenging free radicals generated from the environment (Peng et al., 2013). Furthermore, lutein has some excellent characteristics against different types of diseases (e.g., atherosclerosis, cardiovascular diseases, age-related molecular degenerative diseases, cancer, and skin related diseases) (Astorg, 1997; Sun et al., 2016). Lutein could be not only used in human health caring but also used in cosmetics, pharmaceutical products, feed additive in poultry, and aquaculture industries (Johnson and Schroeder, 1996). The most important is that the demand expected to increase from US$ 249.7 million in 2016 to US $ 357.7 million by 2022 with an annual growth rate of 6.3% from 2017 (Hu et al., 2018).

It is known that the petals of the marigold flowers are the main commercial bio-producer of lutein (Cordero et al., 2011; Del Campo et al., 2000; Del Campo et al., 2004; Xie et al., 2013). However, lutein production from marigold petals is still accompanied by some technical obstacles (e.g., low biomass and low lutein content (0.03%) and high labor demand for harvesting and separation of the petals). That was why microalgal has been emerged as a promising alternative marigold as sources of lutein production due to high lutein content (0.2–0.7%). They could be harvested throughout the year under optimal condition, using total biomass for extraction of lutein and other production of the value-added compounds other than lutein (Ho et al., 2015). Some literature has been mentioned to maximize production of microalgal biomass and lutein content using from different cultivation modes (phototrophic, heterotrophic and mixotrophic), different operation strategy (semi-batch, fed-batch, and two-stage) by manipulating environmental and nutrient factors (Xie et al., 2013). For example, phototrophic cultivation of microalgal has been considered as a strategy to optimize lutein production due to the increased mixing efficiency, higher gas retention time and shorter cost-effective light path (Benavente-Valdes et al., 2016; Ho et al., 2012). Recent studies also reported the enhancement of lutein production with appropriate carbon and nitrogen sources in *Desmodesmus* sp. F51 (Xie et al., 2013). Moreover, Chen pointed out optimal nitrate and acetate concentrations to maximize lutein productivity (Chun-Yen et al., 2017). However, combined supplementation of stimulating hormones and other nutrient sources have not been disclosed for optimal production of microalgal. Therefore, it is incredibly vital to seek for more promising stimulants to optimize cell growth and the biosynthesis of lutein significantly improving the utilization and conversion of nitrogen source when the concentration of nitrogen source was sufficient to provide for growth and carotenoids production of *Desmodesmus* sp. F51.

Regarding plant hormones, salicylic acid (SA) is one of such metabolically essential hormones to stimulate different plant growth stage in response to various hostile environments. Nearly all essential plant hormones are metabolically available in both plants and microalgal, but the mechanisms of those hormones for microalgal seemed to be limited to be entirely deciphered (Tarakhovskaya et al., 2007). Recently, some literature has indicated that significant enhancement of total carotenoids content could be achieved in several kinds of microalga (e.g. *D. salina*, *T. suecica* and *C. vulgaris*) with use of plant hormones (e.g. salicylic acid and methyl jasmonate) (Ahmed et al., 2015; Lin et al., 2018). Some plant hormones could even significantly augment the assimilation of nitrate sources, which led to a significant imbalance of carbon and nitrogen utilization in microalgal cultures. Thus, more technically operation strategies to consider both sources for a balanced lifestyle for maximal lutein production could be implemented for system optimization. Here, fed-batch strategy to maintain nitrate concentration nearly invariant to reflect the effect of salicylic acid was first considered for cultivation. In addition, succinic acid is one of the keystone intermediates of tricarboxylic acid cycle that can enhance carotenoids production by increasing the acetyl-CoA pool of isoprenoid synthesis and forming a carbon skeleton for carotenoid (Bhosale et al., 2004; Certik et al., 2009), which was used as chemical stimulators for inducing carotenoid accumulation in microalgal. To the best of our knowledge, there was no report on the use of succinic acid for lutein production in microalgal culture.

This study provided the first attempt to evaluate the interactive effects of salicylic acid and succinic acid on biomass growth and lutein production of *Desmodesmus* sp. F51. Also, the supplement effects of nitrate fed-batch with and without salicylic acid and succinic acid on *Desmodesmus* sp. F51 were also exhibited.

2. Materials and methods

2.1. Microalgal strain and growth conditions

The thermo-tolerant *Desmodesmus* sp. F51 was kindly provided by prof. Ching-Nen Nathan Chen. The medium used for pre-culture was 3N medium contained (in g/L): $NaNO_3$, 0.750; $MgSO_4{\cdot}7H_2O$, 0.075; $CaCl_2{\cdot}2H_2O$, 0.025; K_2HPO_4, 0.0383; KH_2PO4, 0.088; and $NaCl_2$, 0.025. The vitamins and metal solutions are shown as described elsewhere (Berges et al., 2001). The microalgal were grown at 28 °C for 5 days with continuous illumination at a light intensity of 200 μmol/m^2/s under 2.5% CO_2 at aeration at 0.2 vvm. The production culture in a 1L glass vessel (15.5 cm length and 9.5 cm diameter) was grown in modified Bristol's medium as described in the literature (Del Rio-Chanona et al., 2017) via photobioreactor (PBR). The 5-day pre-cultured cells were inoculated in 1L, PBR cultivation medium with an inoculum size of 0.25 g/L for culture. The PBR culture was operated at 35 °C with a light intensity of 600 μmol/m^2/s using an external light source (21 W T5 white LED lamps, Philips Co., China) fixed at both sides of PBR, with continuous sparging of 2.5% CO_2 air at 0.2 vvm.

2.1.1. Preparation of carbon sources

The salicylic acid and succinic acid substrates were used as supplement nutrients to guarantee the higher performance of cells growth and lutein accumulation. These two substrates were simultaneously prepared by using distilled sterilized water to supplement with modified Bristol's medium. The control of the experiment was implemented in the absence of substrate. To obtain optimal operation performance, salicylic acid and the succinic acid for supplement were designated from 100 to 500 μmol and 2.5–10 mmol, respectively. 1L, determined optimum concentrations of the two substrates, PBR using the modified Bristol's medium with cultures of six days at 35 °C with a light intensity of 600 μmol/m^2/s, 300 rpm. The samples were collected every 24 h to determine the transient dynamics of biomass growth, nitrate concentration, and lutein accumulation.

2.1.2. Fed-batch operation

Nitrogen sources were metabolically essential for cell growth and metabolic production of microalgal cells. For the persistent synthesis of lutein, carbon supply was provided in excess to maintain limited nitrogen conditions to be taken place (Xie et al., 2013). Fed-batch cultivation was initially operated from a batch operation with the same inoculum size, and then supplementing different concentrations of the bio-stimulants at appropriate cell population was carried out. When initial nitrate concentration was depleted, appropriate concentrated nitrate solution was fed by impulse addition by a fed-batch model (Eqs. (1) and (2)) by mass balance as previously mentioned (Manirafasha et al., 2018)

$$C_f \cdot V_f = C_0 \cdot V_0 + C_s \cdot V_f \quad (1)$$

$$V_f = V_0 + V_x \quad (2)$$

where C_f was the final nitrate concentration in the culture broth after nitrate feeding. V_f denoted the final volume of the culture broth after nitrate feeding. C_0 was the nitrate concentration in the culture broth before nitrate feeding. V_0 was the volume of culture broth before nitrate

feeding. *Cs* was the nitrate concentration of stock concentrated nitrate, and V_x was the volume of nitrate feed concentration.

The strategy of nitrate feeding was targeted to analyze the effect of nitrate concentration on microalgal biomass and lutein accumulation. The cultivation of *Desmodesmus* sp. F51 has carried out under optimal operation condition in the nitrate fed-batch cultures for six days cultivation. The initial nitrate concentration was 750 mg/L; the pulse feeding strategy was then implemented at two days cultivation after the residual nitrate concentration was less than 300 mg/L.

2.2. Analytical procedures

2.2.1. Estimation of biomass and nitrate concentration

The biomass concentration of *Desmodesmus* sp. F51 was determined spectrophotometrically at an optical density of 685 nm using UV 1780 spectrophotometer (Shimadzu, Japan). The OD_{685} values were converted to biomass concentration (DCW) through following a standard calibration (Eq. (3)) with a linear range at OD_{685nm} = 0.1–0.8 (R^2 = 0.999)

$$DCW(g/L) = 0.329 \times OD_{685nm} \pm 0.0536 \quad (3)$$

The nitrate concentration was estimated by the spectrophotometric method, as mentioned previously (Ho et al., 2013). The linear calibration between the residual nitrate concentration and absorbance was established the following Eq. (4) (R^2 = 0.999)

$$Nitrate\ (mg/L) = 22.478 \times OD_{220nm} \pm 0.1309 \quad (4)$$

2.2.2. Quantitative determination of lutein content

The carotenoid content was determined as described previously (Sánchez Mirón et al., 2002). The microalgal content biomass was harvested by centrifugation at 7000 rpm for 15 min and twice washed with deionized water to remove the residual compositions from the culture broth. A 10 mg of lyophilized biomass added with 1 mL of 60% (w/w) KOH aqueous solution for disruption by using bead beater for 7 min. The mixture was maintained at water bath after cell disruption for 40 min at 40 °C. The carotenoids were extracted until supernatant nearly become clear by mixing of 2 mL diethyl ether. The solvent extracted was evaporated by nitrogen gas, and the residual solution was resuspended in 3 mL of acetone. The lutein content of the extracted carotenoids was analyzed using high-performance liquid chromatography HPLC (Agilaten technologies 1200 series, USA) (Taylor et al., 2006). A YMC 30 column operated at 25 °C with 1 mL/min of mobile phase consisted of (A) 3% deionized H_2O in methanol containing 0.05 M ammonium acetate and (B) 100% TBME (tert-butyl methyl ether). Both of the mobile phases contained 0.01% (w/v) butylated hydroxytoluene (BHT) and 0.05% triethylamine (TEA). The lutein content was then determined spectrophotometrically at 450 nm.

2.3. Statistical analysis

Lutein production from *Desmodesmus* sp. F51 results under substrate supplementation were statistically compared to the control results without addition of substrate supplementation by one-way analysis of variance (ANOVA) (i.e., Fisher LSD test) for comparison (OriginPro 9, OriginLab Corporation, USA). All analyses at $P < 0.05$ were considered as statistically significant. All assays were conducted in triplicate for data reproducibility presented as a mean ± standard deviation.

3. Results and discussion

3.1. Effect of salicylic acid on cell growth and lutein production

Salicylic acid is a well-characterized signaling molecule that affects different biochemical and physiological activities of the plant by manipulating cellular growth and productivity (Raman and Ravi, 2011). Here, the effect of salicylic acid on cell growth and lutein production of *Desmodesmus* sp. F51 was studied under phototrophic cultivation with supplementation of different concentrations of salicylic acid (100–500 µM) in microalgal cultures. After 7 days cultivation, the highest biomass and productivity (2.26 g/L and 0.61 g/L/d) were obtained at 100 µM salicylic acid compared to the control 1.96 g/L and 0.36 g/L/d, respectively (Fig. 1a). Table 1 also shows that 100 µM salicylic acid was thus the optimum concentration to significantly enhanced the biomass concentration and productivity compared to those of other concentrations. However, the salicylic acid concentration increased from 200 to 500 µM could result in biomass concentrations decrease compared to control, while the lower concentration of salicylic acid failed to induce biomass concentration and high lutein accumulation by *Desmodesmus* sp. F51 (data from the preliminary experiment with concentration below 100 µM are not shown in this manuscript because they were very negligible). Such decreases were likely attributed to the high concentration of salicylic acid, reducing the

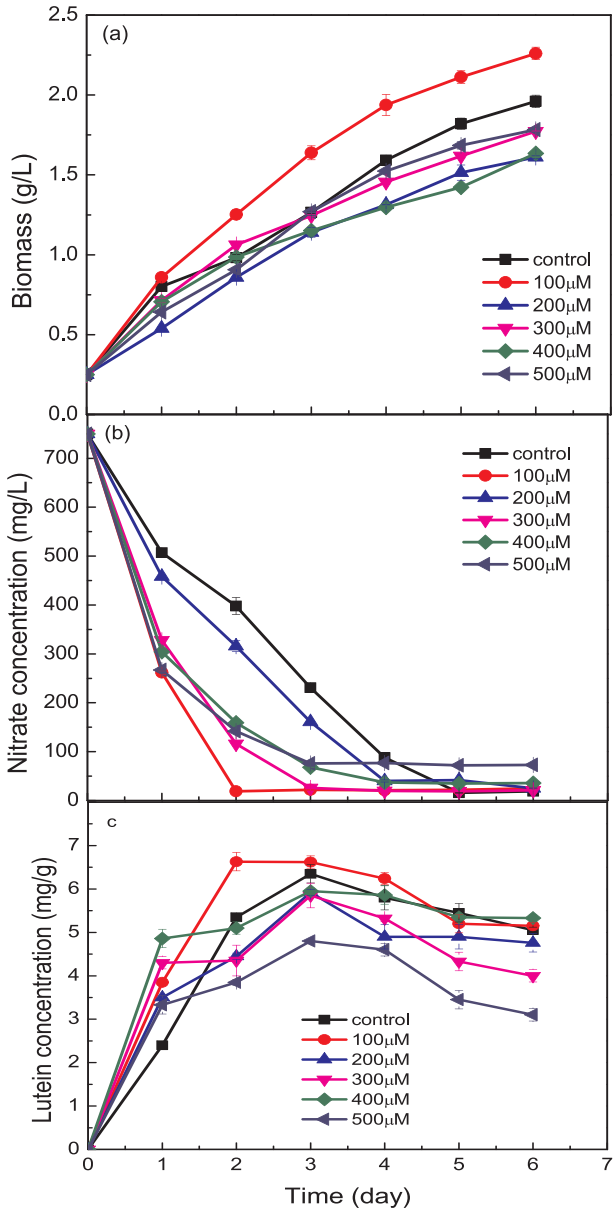

Fig. 1. The effect of the different concentration of salicylic acid on cell growth and lutein production from *Desmodesmus* sp. F51. (a): Biomass growth, (b): Nitrate consumption, (c): Lutein accumulation.

Table 1
Comparative lists of the cell growth and lutein production performance of the different concentration salicylic acid on the batch culture of *Desmodesmus* sp. F15. (Operating conditions: CO_2 concentration, 2.5%; CO_2 flow rate, 0.2 vvm; light intensity, 150 mol/m^2/s; temperature, 35 °C.)

Salicylic acid concentration (μM)	Maximum biomass (g/L)	Maximum biomass productivity (mg/L/d)	Lutein content (mg/g)	Lutein productivity (mg/L/d)
Control (0)	1.82 ± 0.46	540 ± 0.14	6.35 ± 0.79	3.43 ± 0.034
100	2.26 ± 0.57	700 ± 0.18	6.65 ± 0.83	4.66 ± 0.037
200	1.61 ± 0.40	320 ± 0.08	5.92 ± 0.77	1.89 ± 0.031
300	1.77 ± 0.44	460 ± 0.12	5.85 ± 0.73	2.69 ± 0.032
400	1.63 ± 0.41	460 ± 0.12	5.95 ± 0.74	2.74 ± 0.048
500	1.78 ± 0.45	390 ± 0.10	4.80 ± 0.60	1.87 ± 0.026

photosynthetic activities in *Desmodesmus* sp. F51. As some literature reported, salicylic acid above the optimum concentration would decrease the expression of photosynthetic enzymes in some plant (Pancheva et al., 1996). Ahmed et al. also mentioned a decrease in the biomass concentration with augmentation salicylic acid and methyl jasmonate in *Chlorella* sp. (Ahmed et al., 2015). The result might suggest that 100 μM salicylic acid should be the optimal concentration for biomass growth in *Desmodesmus* sp. F51 and biomass growth was inhibited as salicylic acid *above* the optimum concentration. Therefore, the optimum concentration of salicylic acid seems to be promising in terms of biomass production.

As shown in Fig. 1c, the maximal performance of lutein content and productivity were obtained by supplementing with 100 μM salicylic acid after 2 days cultivation. The lutein content of 6.65 mg/g and productivity of 4.66 mg/L/d were higher than those of the other concentration of salicylic acid and control. Moreover, this content was and also higher than most of the reported value (in the range of 0.5–7.2 mg/g) obtained in related studies (Ahmed et al., 2015; Del Campo et al., 2004; Raman and Ravi, 2011; Xie et al., 2017). The result pointed out the potential for adding phytohormones salicylic acid to increase lutein production. However, the optimal supplementing concentrations of salicylic acid may be likely different due to the different metabolic expression and mechanism for lutein production. This difference would depend upon the microalgal species and culture conditions. This study showed the nearly consistent result with previous studies in which enhancement of carotenoid biosynthesis in different microalgal species (e.g., *Dunaliella salina, Tetraselmis suecica,* and *Chlorella vulgaris)* after supplementations of different concentrations of salicylic acid and methyl jasmonate (Ahmed et al., 2015; Lin et al., 2018). The related studies also indicated that the application of salicylic acid to cultures in *H. Pluviallis* led to the enhancement of *β*-carotene and astaxanthin (Gao et al., 2012; Raman and Ravi, 2011). Compared to lutein productions in prior studies, this study showed higher lutein content after supplementation of salicylic acid (Table 1). This result is attributed to the response of the salicylic acid strongly dependent upon microbial characteristics and the microalgal species in response to different concentrations of the phytohormones. The result suggested that 100 μM salicylic acid was optimum for microalgal growth and lutein accumulation of *Desmodesmus* sp. F51.

It is also observed in Fig. 1 that nitrate consumption rates were higher than that of the control after adding salicylic acid in culture media. After 2 day cultivation, the lutein content and productivity increased simultaneously along with in parallel with nitrate consumption, and the maximal values (6.65 mg/g and 4.66 mg/L/d) were obtained at the beginning of nitrate depletion (more precisely, nearly 95% nitrogen consumption). Under nitrate depletion conditions, both of them decreased significantly with prolonged cultivation time. These findings are quite similar to the results of Xie et al. (2013). Lutein belongs to a family of primary carotenoid required for the structure and function of the light-harvesting complexes in photo-synthesis (Cordero et al., 2011). It explained that the intracellular proteins were degraded as a source of nitrogen to maintain the metabolic functions of microalgal under nitrate depletion condition. Therefore, to attain the maximal lutein content and productivity, the initial time of nitrate depletion should be the most appropriate time for nitrate feeding during the batch cultivation with supplementing of salicylic acid.

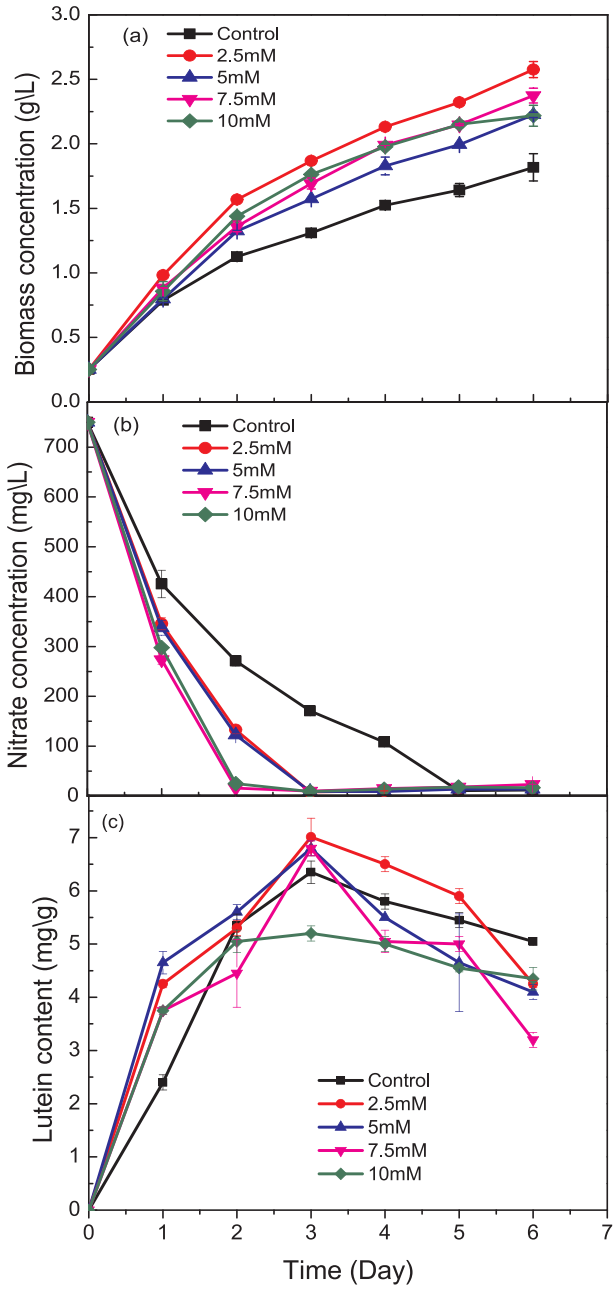

Fig. 2. The effect of the different concentration of succinic acid on cell growth and lutein production of *Desmodesmus* sp. F51 with 100 μM salicylic acid. (a): Biomass growth, (b): Nitrate consumption, (c): Lutein accumulation.

3.2. Effect of succinic acid on cell growth and lutein production

Furthermore, succinic acid is one of the intermediates of the tricarboxylic acid product with significant potentials for industrial applications (e.g., food and pharmaceutical sciences) (Kamm, 2009). Meanwhile, intermediates of the TCA cycle would play as chemical stimulants for the accumulation of zeaxanthin from Flavobacterium (Bhosale et al., 2004). Here, based on the optimization as mentioned

above of SA, the effects of succinic acid on cell growth and lutein production of *Desmodesmus* sp. F51 were investigated under phototrophic cultivation with supplementation of succinic acid (0–10 mM) in microalgal cultures. As shown in Fig. 2a, the highest biomass of 2.37 g/L and productivity of 0.73 g/L/d were obtained for 2.5 mM succinic acid compared to the control (i.e.1.82 g/L and 0.54 g/L/d) on the 6th-day cultivation. Also, the maximal lutein content and microalgal productivity of 7.5 mg/g and 5.51 mg/L/d were obtained with the same 2.5 mM concentration of succinic acid compared to control (Fig. 2c). Therefore, The result indicated that 2.5 mM succinic acid was optimum for microalgal growth and lutein production of *Desmodesmus* sp. F51. Bhosale et al. have reported the enhancement of zeaxanthin production by succinic acid from *Flavobacterium multivorum* (Bhosale et al., 2004). Succinic acid could also be used as the growth promoter to enhance cell growth and phycocyanin accumulation in *Arthrospira platensis* (Manirafasha et al., 2018). These results confirmed that the effect of intermediate of TCA cycle would still strongly depended upon types of microorganisms (e.g., bacteria, yeast, and microalgae), the applied concentration of intermediate (e.g., 10, 7.5 and 2.5 mM) and target products (e.g., zeaxanthin, phycocyanin, and lutein).

3.3. Effect of salicylic acid and succinic acid coupled with nitrate fed-batch

Results above showed that salicylic acid (SA) could simultaneously trigger effective assimilation of nitrate and improve lutein production. The depletion of nitrate was progressed for 2 days after adding 100 μM salicylic acid in batch cultivation. The related reports have proved that maintaining the optimum amount of residual nitrogen for the growth and lutein biosynthesis was inevitably required (Chun-Yen et al., 2017; Ho et al., 2014; Ratushnyak et al., 2010). Thus, nitrate fed-batch strategy was correctly applied after nitrate concentration depleted to the lowest level at 2 days of the batch cultivation, and the feeding nitrate concentration was then manipulated after measuring residual nitrate in a medium according to the rate of nitrate depletion. To achieve maximal lutein production, the effect of SA accompanied by feeding nitrate on microalgal growth and lutein accumulation of *Desmodesmus* sp. F51 was implemented. Based on the optimal scheme of salicylic acid (SA) in batch culture, the strategy of nitrate fed-batch coupled with an augmentation of 100 μM salicylic acid should result in further enhancement the cell growth and lutein production of *Desmodesmus* sp. F51. As shown in Fig. 3, The highest lutein content of 7.25 mg/g and lutein productivity of 5.29 ± 0.040 mg/L/d were obtained at 4 days cultivation. The highest biomass of 2.5 g/L and productivity of 0.70 ± 0.001 g/L/d were also obtained after 7 days of cultivation period.

Furthermore, operation strategy of nitrate fed-batch combined with the simultaneous supplement of 100 μM salicylic acid and 2.5 mM succinic acid resulted in the highest lutein content of 7.5 mg/g and lutein productivity of 5.78 ± 0.041 mg/L/day after 4 days cultivation (Table 2 and Fig. 4c). The highest biomass of 2.52 g/L and productivity of 0.77 ± 0.001 g/L/day were obtained after 7 days cultivation (Fig. 4a and Table 2). The result showed that the addition of SA and precursors, coupled with nitrate feeding strategy were higher than those of sole adding SA in batch cultivation. This result also indicated that nitrogen availability is one of the sufficient conditions for lutein accumulation. Both of microalgal biomass and the maximum specific growth of fed-batch cultures significantly increased compared to those of prior batch cultures (refer to Section 3.1), all of which were higher than the reported result for *Desmodesmus* sp. F51 strains in the literature (García-Cañedo et al., 2016; Xie et al., 2013).

The lutein content and productivity reached to the maximum value from day two to day four cultivation; then both dynamic profiles started to decline (Figs. 3c and 4c). This change tendency could be attributed to light limitation and nutrient deficiency with the prolonged cultivation time, which is consistent with the literature findings (Xie et al., 2013). With nitrate fed-batch combined with precursors operation strategy for

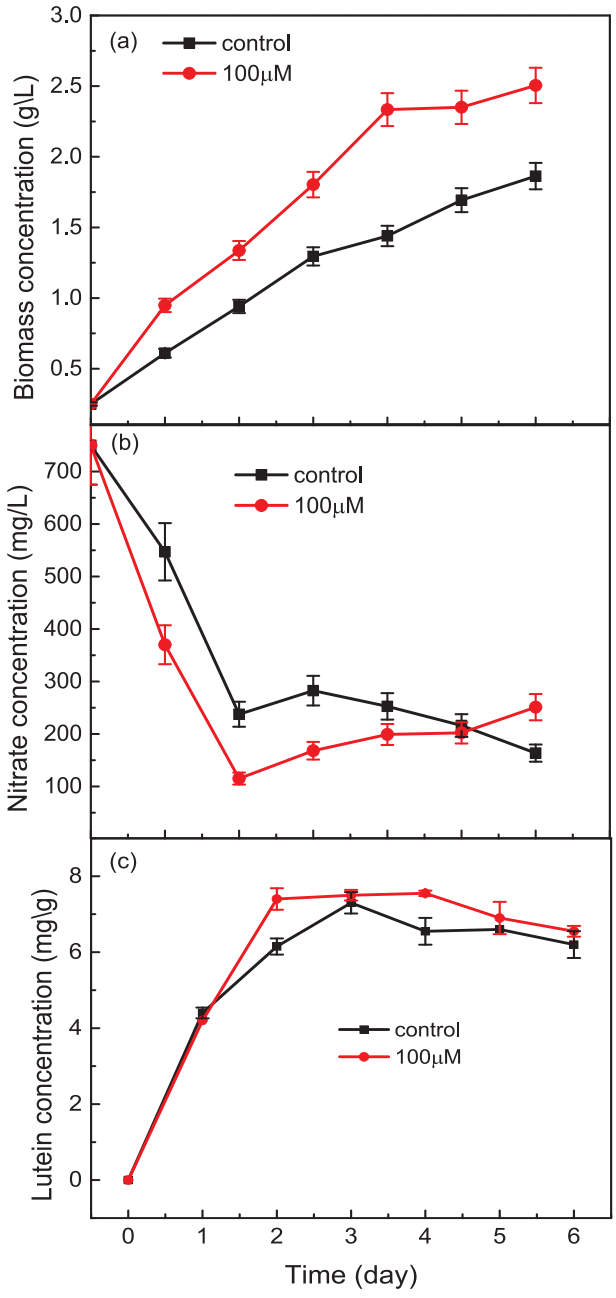

Fig. 3. The effect of 100 μM salicylic acid combined with nitrate fed-batch on cell growth and lutein production of *Desmodesmus* sp. F51. (a): Biomass growth, (b): Nitrate consumption, (c): Lutein accumulation.

cultivation increased in not only biomass growth but also lutein content and productivity. The highest lutein content (7.55 mg/g) was obtained in the culture supplemented with 3 mM nitrate, which was 20% higher than those of the other batch culture, and also higher than most of the reported values obtained in the related studies (Table 3). These results indicated that nitrate fed-batch, coupled with adding precursors cultivation seems to be a feasible strategy for more promising lutein production likely due to synergistic enhancement of nitrate and hormone.

The reason behind this enhancement was likely due to the synergistic interactions of two factors (i.e., the precursor substrate addition in conjunction with the feeding of the optimum nitrate). This results also indicated that succinic acid and salicylic acid were both factors for enhancement of biomass growth and lutein biosynthesis. Here, we proposed a metabolic mechanism of the synergistic action of salicylic acid and succinic acid for improvement of the lutein

Table 2
Comparative lists of biomass growth and lutein production performance at different concentration of succinic acid on the batch culture of *Desmodesmus* sp. F51. (Operating conditions: salicylic acid 100 μM; CO_2 concentration, 2.5%; CO_2 flow rate, 0.2 vvm; light intensity, 150 mol/m^2/s; temperature, 35 °C.)

Succinic acid (mM)	Maximum biomass (g/L)	Maximum biomass productivity (mg/L/d)	Lutein content (mg/g)	Lutein productivity (mg/L/d)
Control	1.82 ± 0.46	540 ± 0.14	6.35 ± 0.71	3.43 ± 0.034
2.5	2.37 ± 0.01	730 ± 0.18	7.01 ± 0.94	5.11 ± 0.041
5.0	2.32 ± 0.01	550 ± 0.14	6.46 ± 0.81	3.55 ± 0.035
7.5	2.07 ± 0.52	630 ± 0.16	5.85 ± 0.73	3.69 ± 0.032
10	2.37 ± 0.59	610 ± 0.15	6.00 ± 0.75	3.66 ± 0.033

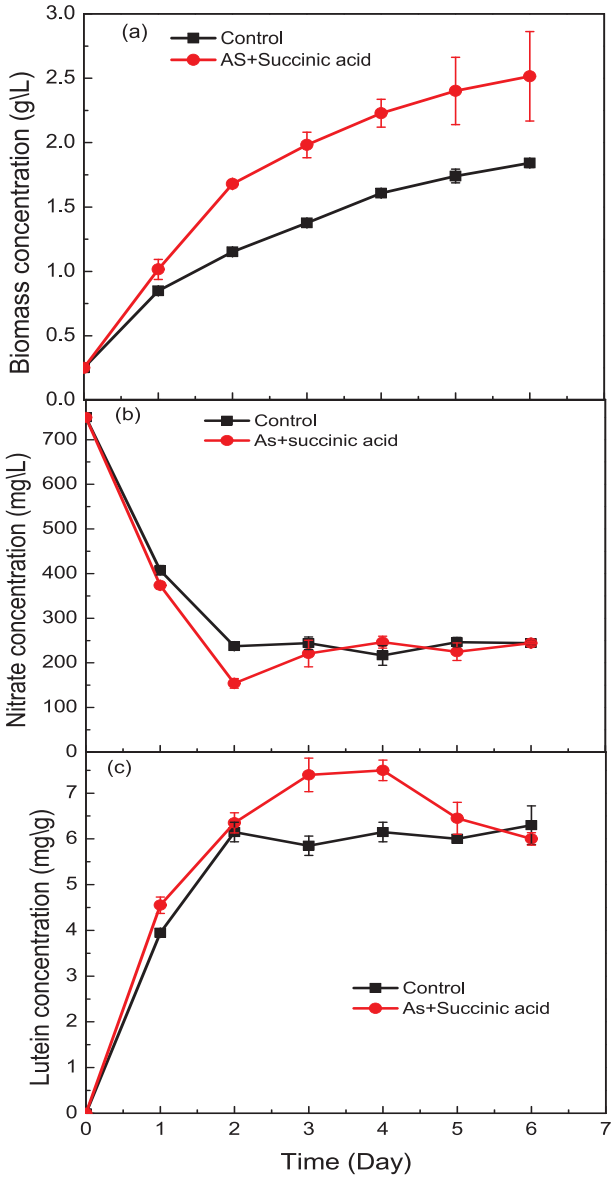

Fig. 4. The effect of 100 μM salicylic acid and 2.5 mM succinic acid combined with nitrate fed-batch on cell growth and lutein production of *Desmodesmus* sp. F51. (a): Biomass growth, (b): Nitrate consumption, (c): Lutein accumulation.

accumulation. Salicylic acid enhanced nitrate assimilation by elevation the nitrate reductase activity and stabilizing the membrane structure and fluidity that may have facilitated increased nutrient uptake, including nitrate (Hayat et al., 2014). Furthermore, nitrate reductase (NR) catalyzes nitrate reduction to nitrite which is then transported to chloroplast where the nitrite reductase (NiR) catalyzes nitrite conversion to ammonium and finally ammonium is converted to glutamate by glutamate synthase or glutamine oxoglutarate aminotransferase (Sanz-Luque et al., 2015). Glutamate was converted to α-ketoglutarate when the succinic acid was converted to succinyl CoA through serial metabolic reactions. Both of the α-ketoglutarate and succinyl CoA interactively augmented the efficiency of TCA cycle converting to oxaloacetate acid. The oxaloacetate acid is converted into phosphoenolpyruvate (PEP) catalyzed by the phosphoenolpyruvate carboxykinase (PEPCK) through gluconeogenesis. PEP was transported from mitochondria to the plastids in green algae and plant for carotenoids biosynthesis through methylerythritol phosphate pathway (MEP) using pyruvate and glyceraldehyde-3-phosphate (G3P) as precursors for lutein biosynthesis in microalgae (Cunningham Francis, 2002; Delbaere et al., 2004; Streatfield et al., 1999). Therefore, with synergistic action of salicylic acid and succinic acid, the lutein content, and productivity of *Desmodesmus* sp. F51 could be dramatically increased by nitrate fed-batch. The present work shows that due to the relatively high lutein content and productivity with the synergistic enhancement of nitrate and hormone seem to be a feasible strategy for commercial lutein production. Nevertheless, more accurate control of nitrate feeding to maintain nitrogen balance should be conducted to further improve the lutein productivity with adding plant hormone cultivation, in order to make it more economically feasible.

4. Conclusions

This study demonstrated that plant hormone-salicylic acid and succinic acid could synergistically enhance the biomass growth and lutein biosynthesis in the *Desmodesmus* sp. F51. The supplementation of succinic acid and salicylic acid under optimum concentrations resulted to the maximal lutein content of 7.01 mg/g and productivity 5.11 mg/L/d while nitrate fed-batch operation strategy couple with the salicylic acid and succinic acid supplementation in culture medium resulted in further enhancement of lutein content and productivity in the *Desmodesmus* sp. F51 by 7.50 mg/g and 5.78 mg/L/d, respectively, which are higher than most of the previously reported values.

Table 3
The interactive effects of 100 μM salicylic acid and 2.5 mM succinic acid on biomass production and lutein accumulation from *Desmodesmus* sp. F51 with nitrate fed-batch strategy.

Substrates	Maximum biomass (g/L)	Maximum biomass productivity (mg/L/d)	Lutein content (mg/g)	Lutein productivity (mg/L/d)
SA	2.50 ± 0.01	610 ± 0.01	7.25 ± 0.02	4.42 ± 0.039
SA + Succinic acid	2.52 ± 0.01	770 ± 0.01	7.50 ± 0.04	5.78 ± 0.041
Control	1.92 ± 0.01	550 ± 0.01	6.58 ± 0.01	3.62 ± 0.010

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