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EFFECT OF PLANT GROWTH REGULATORS ON GROWTH AND LIPID ACCUMULATION OF MICROALGAL *HAEMATOCOCCUS PLUVIALIS* FLOTOW IN TWO-STAGE CULTURE

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SUMMARY

Haematococcus pluvialis cells were cultured in aerated liquid Bold's Basal medium in two-stage (initial stage during in 7 weeks for increased biomass growth and second stage during in 3 weeks for increased lipid accumulation) with different volumes 250 mL, 10 L, and 1,000 L. With a volume of 250 mL, the medium was supplied with benzyl adenine (BA), indole-3-acetic acid (IAA) or gibberellic acid (GA_3) at concentration from 0.1 - 0.2 mg/L in initial stage and IAA or GA_3 at concentration from 0.1 - 0.2 mg/L in second stage. After 10 weeks of culture, results showed that supplement of 0.1 mg/L BA in initial stage and 0.125 mg/L IAA in second stage increased cell density, and microalgal cells had green color with a spherical shape. On the contrary, supplement of 0.15 mg/L IAA in initial stage and 0.175 mg/L GA_3 in second stage increased lipid accumulation, and microalgal cells had red color with a spherical shape. With a volume of 10 L, the medium was supplied with 0.1 mg/L BA in initial stage, and treated with separation or combination from 2 - 3 of these factors (nitrogen starvation, 0.5% NaCl, 4.98 mg/L $FeSO_4$) were applied in second stage. The result showed that the cultures was treated with nitrogen starvation increased dry biomass and biofuel, but treated with 4.98 mg/L $FeSO_4$ only increased biofuel. With a volume of 1,000 L, microalgal cells were cultured in BB liquid medium in initial stage, and treated with 4.98 mg/L $FeSO_4$ increased fresh 78.67 mg/mL and dry biomass 2.05 mg/L and total lipid content 28.24 %/ DW.

Keywords: *Biofuel, Haematococcus pluvialis* Flotow*, nitrogen starvation, plant growth regulators, two-stage culture*

INTRODUCTION

Nowadays, biofuel, which was considered as renewable, environment-friendly, safe to use, will eventually alternate from fossil non-renewable resources. The development of 4th generation biofuel production (algae-to-biofuels) based on metabolic engineering of algae is still in its infancy, due to the lacking of understanding of microalgal growth, metabolism and biofuel production processes. *Haematococcus pluvialis* is green microalgal, which is considered as a potential biodiesel feedstock (Lei *et al.*, 2012). Growth of *H. pluvialis* significantly increased under different growth regulators, such as auxin or combined with cytokinin (Raposo *et al.*, 2006). Concerning to taxanomy of algae, several different references indicated the essentially all known phytohormones detected in concentrations comparable with their contents in higher plants (Tarakhovskaya *et al.*, 2007). Plant growth regulators are not only increasing the growth but also increasing quantity and quality of fatty acid of microalgal, which is necessary to biofuel production (Salama *et al.*, 2014). Previous study indicated that two-stage culture were used to increase microalgal biomass in the initial stage and improve the biomass concentration as well as lipid production in second stage (Cui *et al.*, 2017).

In our previous study, we successfully identified the condition of *H. pluvialis* culture by identification of different concentration of BA, IAA and GA, which impacted on growth and lipid accumulation in cells (Nguyen *et al.*, 2015). In addition, we also established the molecular method to evaluate the presence of biotin carboxylase gene (*BC*) and fatty acyl-acyl carrier protein thioesterase gene (*FATA*) on *H.*

pluvialis (Nguyen *et al.*, 2016). Therefore, continuously, the aims of current study are to estimate the effects of different plant growth regulators on the growth and lipid accumulation of microalgal *H. pluvialis* in two-stage culture with three different volumes as 250 mL, 10 L and 1,000 L.

MATERIAL AND METHODS

Material

H. pluvialis Flotow was supplied from Algatechnologies, Institute of Biotechnology, Vietnam Academy of Science and Technology.

Preparations of biomass and lipid accumulation with two-stage culture in the volume of 250 mL

H. pluvialis were cultured in aerated liquid Bold's Basal (BB) (Barsanti, Gualtieri, 2006) medium of 50 mL, pH 7. The initial cell densities $4.3.10^3$ cell/mL were used for experiment of effect of plant growth regulators on growth and lipid accumulation in two-stage. Initial stage, *H. pluvialis* was cultured in BB medium during 7 weeks, supplied with 0.15 mg/L indole-3-acetic acid (IAA - Merck), 0.1 mg/L benzyl adenine (BA - Merck), or 0.2 mg/L gibberellic acid (GA_3 - Merck). Second stage, microalgal from BB medium supplied with IAA 0.15 mg/L in initial stage were moved to fresh BB medium and supplied with GA_3 in range of concentration from 0.15 mg/L, 0.175 mg/L or 0.2 mg/L, and cultured during 3 weeks. Similarly, microalgal from BB supplied with 0.1 mg/L BA or 0.2 mg/L GA_3 in initial stage were moved to fresh BB medium and supplied with IAA in range of concentration from 0.1 mg/L, 0.125 mg/L or 0.15 mg/L.

Different conditions, including temperature, light intensity, light period were remained at 25 ± 3°C, 50 µmol photons m^{-2} s^{-1}, 12 h/day, respectively. The morphology, color, fresh biomass, dry biomass and biofuel concentrations of microalgal were observed and quantified at 8 a. m on next morning.

Preparations of biomass and lipid accumulation with two-stage culture in the volume of 10 L

H. pluvialis was cultured in aerated 10 L liquid BB medium in 15 L white plastic boxes, pH 7. The initial cell densities $8.6.10^3$ cell/mL were used to estimate effect of plant growth regulators on growth and lipid accumulation in two stages. Initial stage was cultured in BB medium during 7 weeks supplied with 0.1 mg/L BA. Second stage, microalgal were cultured in initial stage moved to fresh BB medium supplied with 0.5% NaCl (Na^+), or 4.98 mg/L $FeSO_4$ (Fe^{2+}), or nitrogen starvation (NS), or combined with 2 - 3 of these factors as Na^+Fe^{2+}, Na^+NS, Fe^{2+}NS, Na^+Fe^{2+}NS.

Preparations of biomass and lipid accumulation with two-stage culture in the volume of 1,000 L

H. pluvialis was cultured in aerated 1,000 L liquid BB medium in 1,500 L containers, pH 7. The initial cell clusters of $8.6.10^3$ cell/mL were used for salinity treatment, temperature stress or heavy metal on growth and lipid accumulation in two-stage. Initial stage, microalgal was cultured in liquid BB medium during 7 weeks. Second stage, microalgal was cultured in BB medium in 3 weeks supplied with 0.5% NaCl (Na^+) or 4.98 mg/L $FeSO_4$ (Fe^{2+}). On temperature stress (TS), biomass of 10-week-old microalgal was collected and put on freezer at 7 ± 3 °C for 2 hours.

The quantitation of biofuel

Biofuel of microalgal *H. pluvialis* was transesterification and collected according to method of Johnson and Wen (2009).

The analysis of fatty acid content and total lipid

Briefly, 20 mg of *H. pluvialis* cells were kept in the microtube, supplied with 2 M NaOH-CH_3OH and shaken at 80 rpm at room temperature for 60 min. After cooled down, the mixture was spiked with 1 mL 4 M HCl-CH_3OH and pH was adjusted to below 2.0 with HCl, followed by incubation at 75°C for 15 min. Then, fatty acid methyl esters (FAMEs) were extracted with 1 mL hexane, shaking by hand for 30 s and then centrifuged at 4,000 g for 2 min. The hexane phase was collected and stored at -20°C for further Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Qualification and quantification of FAMEs were performed on a GC-MS (GC Agilent 6890 MS 5973 inert, column HP5-MS, He 9.3 psi) with initial temperature 100°C, increasing 10°C/min to 200°C and kept on 5 min, continued increasing 10°C/min to 300°C and kept in 5 min (Lu *et al.*, 2012). Besides, total lipid of microalgal was quantified by AOCS Aa-38 method. Fatty acid content and total lipid were analyzed at Research Institute of Oil and Oil Plants.

RESULTS

Effect of plant growth regulators on growth and lipid accumulation of *H. pluvialis* with two-stage culture in volume 250 mL

Based on the observation of biofuel accumulation, biofuel from microalgal cultured in BB medium supplied with 0.15 mg/L IAA (initial stage, 7 weeks) and 0.175 mg/L GA_3 (second stage, 3 weeks) was significantly higher than others treatments. However, dry biomass was as same as control (Table 1). Some treatments supplied GA_3 in initial stage changed color of microalgal from green to red phase. On the contrary, microalgal in others treatments were still remained in the green phase (Figure 1).

Table 1. Growth of *H. pluvialis* 10 weeks in aerated liquid BB medium in two-stage (7 weeks in initial stage and 3 weeks in second stage) with volume 250 mL.

Plant growth regulators	Cell densities ($x10^3$ cell/mL)	Fresh biomass (mg/mL)	Dry biomass (mg/mL)	Biofuel (mg/mL)
Control	52.00^{f}	7.10^{d}	3.13^{b}	0.062^{cd}
0.1 mg/L BA - 0.1 mg/L IAA	606.67^{d}	15.03^{cd}	4.50^{a}	0.070^{bcd}
0.1 mg/L BA - 0.125 mg/L IAA	3096.67^{a}	24.46^{bc}	1.83^{b}	0.076^{abc}
0.1 mg/L BA - 0.15 mg/L IAA	1200.00^{b}	26.43^{bc}	0.80^{b}	0.080^{ab}
0.15 mg/L IAA - 0.175 mg/L GA_3	283.33^{e}	30.07^{bc}	1.40^{b}	0.085^{a}
0.15 mg/L IAA - 0.2 mg/L GA_3	643.33^{d}	28.10^{bc}	3.67^{b}	0.069^{bcd}
0.2 mg/L GA_3 - 0.1 mg/L IAA	980.00^{c}	36.90^{b}	1.77^{b}	0.050^{bcd}
0.2 mg/L GA_3 - 0.125 mg/L IAA	493.33^{d}	55.00^{a}	3.33^{b}	0.066^{bcd}
0.2 mg/L GA_3 - 0.15 mg/L IAA	556.67^{d}	26.47^{bc}	1.67^{b}	0.057^{d}

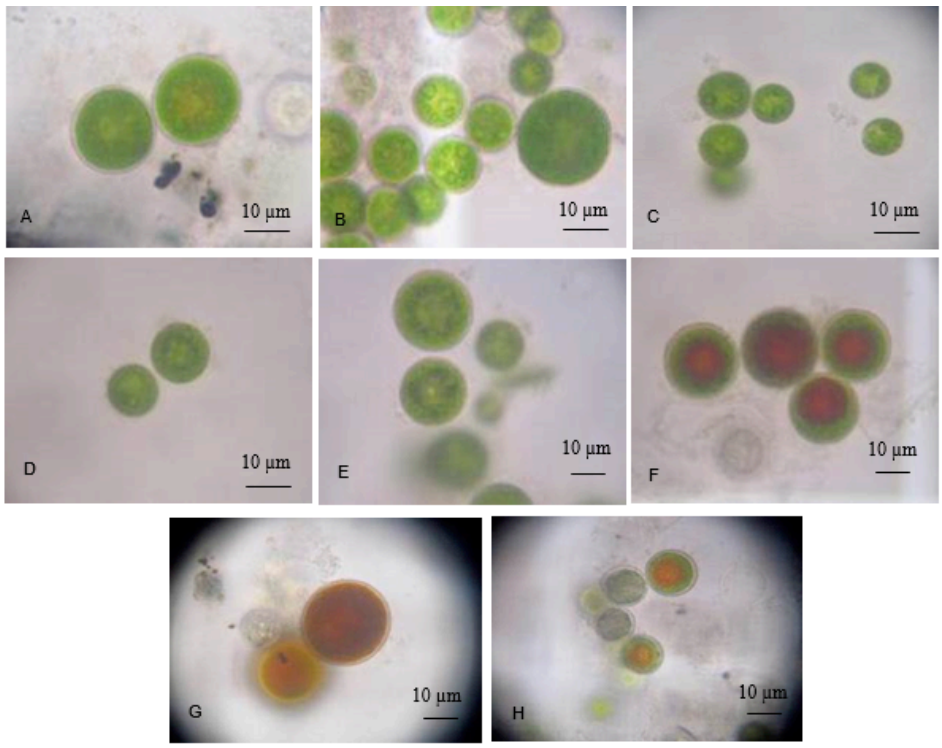

Figure 1. Cell color changed of 10-week-old *H. pluvialis* in aerated liquid BB medium in two-stage. (7 weeks in initial stage and 3 weeks in second stage) with volume 250 mL. (A) 0.1 mg/L BA - 0.1 mg/L IAA: green ; (B) 0.1 mg/L BA - 0.125 mg/L IAA: green; (C) 0.1 mg/L BA - 0.15 mg/L IAA: green; (D) 0.15 mg/L IAA - 0.175 mg/L GA_3: green; (E) 0.15 mg/L IAA - 0.2 mg/L GA_3: green; (F) 0.2 mg/L GA_3 - 0.1 mg/L IAA: red; (G) 0.2 mg/L GA_3 - 0.125 mg/L IAA: red-orange (H) 0.2 mg/L GA_3 - 0.15 mg/L IAA: red with some cells destroyed.

Effect of plant growth regulators on growth and lipid accumulation of *H. pluvialis* with two-stage culture in the volume of 10 L

After 7 weeks, the microalgal was cultured in liquid BB medium supplied with 0.1 mg/L BA (initial stage) and then cultured with supplement of Na^+, Fe^{2+}, NS or combined with 2 - 3 factors in 3 weeks (second stage), the results showed that treatment with 0.1 mg/L BA in two-stage increased the fresh and dry biomass of microalgal, but the biofuel were not increased in the comparison to the control (BB medium for two-stage). Treatment with Fe^{2+} or NS increased biofuel. Treatment with nitrogen starvation increased fresh and dry biomass better than control (BB - BB) and (BA - BA). The others treatments (BA - Na^+, BA - Na^+Fe^{2+}, BA - Na^+NS, BA - Fe^{2+}NS, BA - Na^+Fe^{2+}NS) were not changed as not decreased biofuel less than control (BB - BB) and (BA - BA) (Table 2).

Microalgal was green phase with thin cell wall in both BB medium and BB supplied with 0.1 mg/L BA. The combination with three factors Na^+, Fe^{2+} and nitrogen starvation (BA - Na^+Fe^{2+}NS) was made microalgal color changed from green to red-orange phase with thick wall and loss cytoplasm (Figure 2).

Table 2. Growth of *H. pluvialis* 10 weeks in aerated liquid BB medium in two-stage (7 weeks in initial stage and 3 weeks in second stage) with 10 L volume.

Two-stage culture	Fresh biomass (mg/mL)	Dry biomass (mg/mL)	Biofuel (mg/mL)
BB - BB	29.42^{g}	0.93^{e}	0.360^{de}
BA - BA	91.48^{d}	9.62^{cd}	0.424^{cde}
BA - Na^+	47.03^{f}	2.13^{e}	0.589^{bc}
BA - Fe^{2+}	73.15^{e}	5.92^{cde}	0.848^{a}
BA - NS	136.95^{a}	38.95^{a}	0.783^{ab}
BA - Na^+ Fe^{2+}	102.62^{cd}	11.50^{c}	0.582^{bcd}
BA - Na^+NS	63.50^{e}	4.10^{de}	0.321^{e}
BA - Fe^{2+} NS	120.07^{b}	25.52^{b}	0.460^{cde}
BA - Na^+ Fe^{2+} NS	113.75^{bc}	22.40^{b}	0.387^{cde}

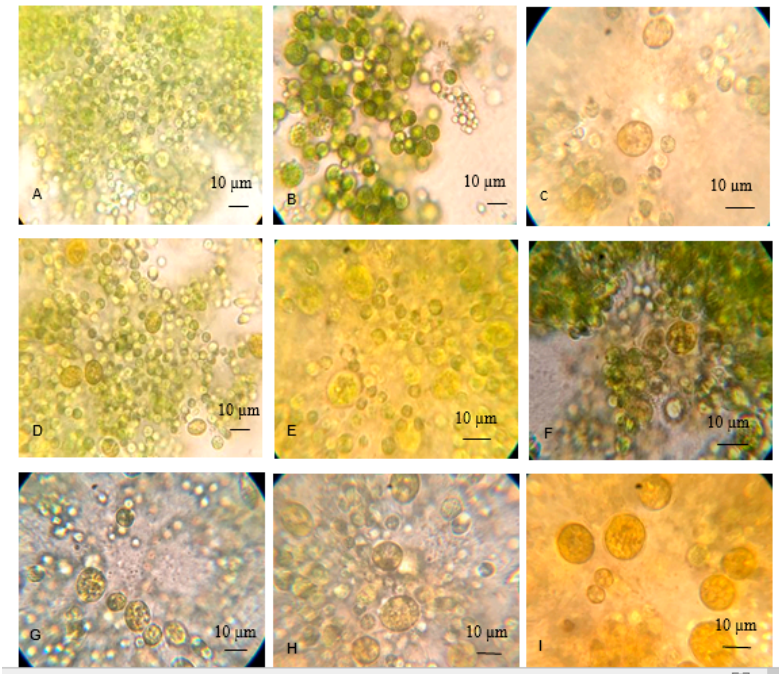

Figure 2. Cell color changed of 10-week-old *H. pluvialis* in aerated liquid BB medium in two-stage. (7 weeks in initial stage and 3 weeks in second stage) with 10 L volume. (A) BB - BB: green; (B) BA - BA: green; (C) BA - Na^+: red-yellow; (D) BA - Fe^{2+}: red-yellow; (E) BA - NS: light green; (F) BA - Na^+Fe^{2+}: green; (G) BA - Na^+NS: green and some cells destroyed; (H) BA - Fe^{2+}NS: red-yellow; (I) BA - Na^+Fe^{2+}NS: red-orange.

The growth and lipid accumulation of *H. pluvialis* with two-stage culture in the volume of 1,000 L

The increasing biofuel in the treatment TS in 2 hours and Na^+ during 3 weeks was observed. Treatment with Na^+ increased dry biomass and not decreased fresh biomass as control (microalgal was cultured in BB medium with volume 1,500 L containers on 10 weeks). Treatment with Fe^{2+} increased total lipid, fresh and dry biomass but not changed biofuel as control (Table 3 and 4).

Fatty acid content after 10 weeks cultured in treatment TS in 2 hours or Na^+ or supplied with Fe^{2+} in 3 weeks showed that oleic acid was absent, whereas palmitic acid in was twice higher than control (Table 4, Figure 3).

Microalgal in control was spherical shape with dark green phase. Microalgal in treatment with TS in 2 hours or Na^+ or supplied with Fe^{2+} in 3 weeks was spherical shape with red phase and thick wall (Figure 4).

Table 3. Growth of 10-week-old *H. pluvialis* in aerated liquid BB medium (7 weeks in initial stage and 3 weeks in second stage) with volume 1,000 L.

Treatment	Fresh biomass (mg/mL)	Dry biomass (mg/mL)	Biofuel (mg/mL)
Control	33.00^{b}	0.987^{c}	0.073^{c}
TS, 2 hours	38.63^{b}	0.953^{c}	0.100^{ab}
Na^+, 3 weeks	37.86^{b}	1.760^{b}	0.114^{a}
Fe^{2+}, 3 weeks	78.67^{a}	2.050^{a}	0.085^{bc}

Table 4. Fatty acid of 10-week-old *H. pluvialis* in aerated liquid BB medium (7 weeks in initial stage and 3 weeks in second stage) with volume 1,000 L.

Time	Fatty acid content	Treatment			
		Control	TS	Na^+	Fe^{2+}
13.33	Palmitic acid (C16:0)	26.11	44.46	44.00	39.42
18.47	Stearic acid (C18:0)	-	-	-	5.32
Total lipid (% dry biomass)		10.68	8.67	6.63	28.24

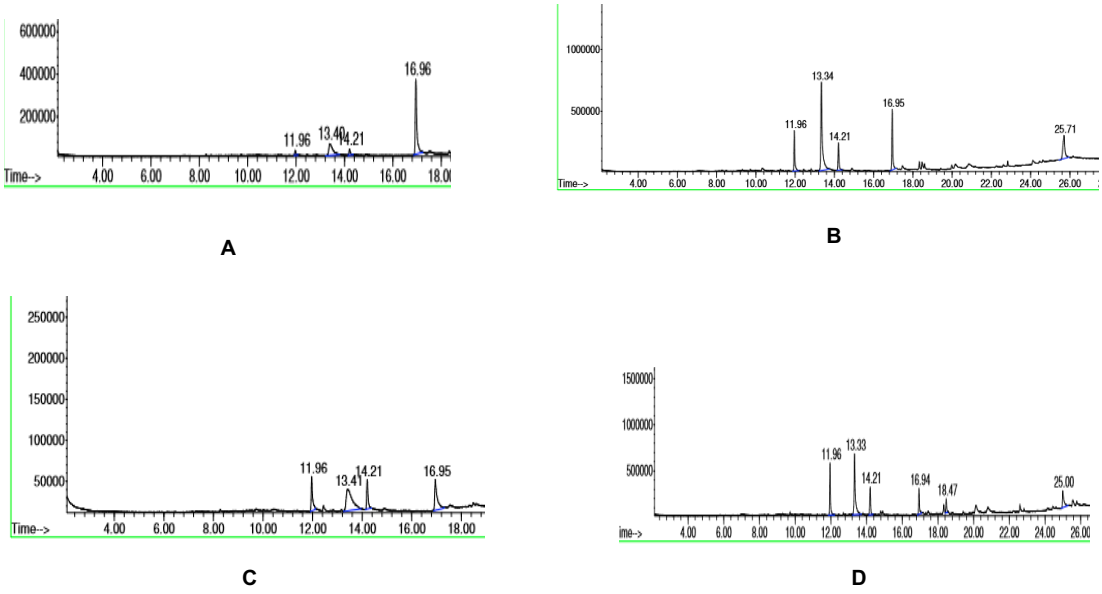

Figure 3. Peak of fatty acid content from *H. pluvialis* with treatment: (A) control, (B) TS, 2 hours, (C) Na^+, (D) Fe^{2+}.

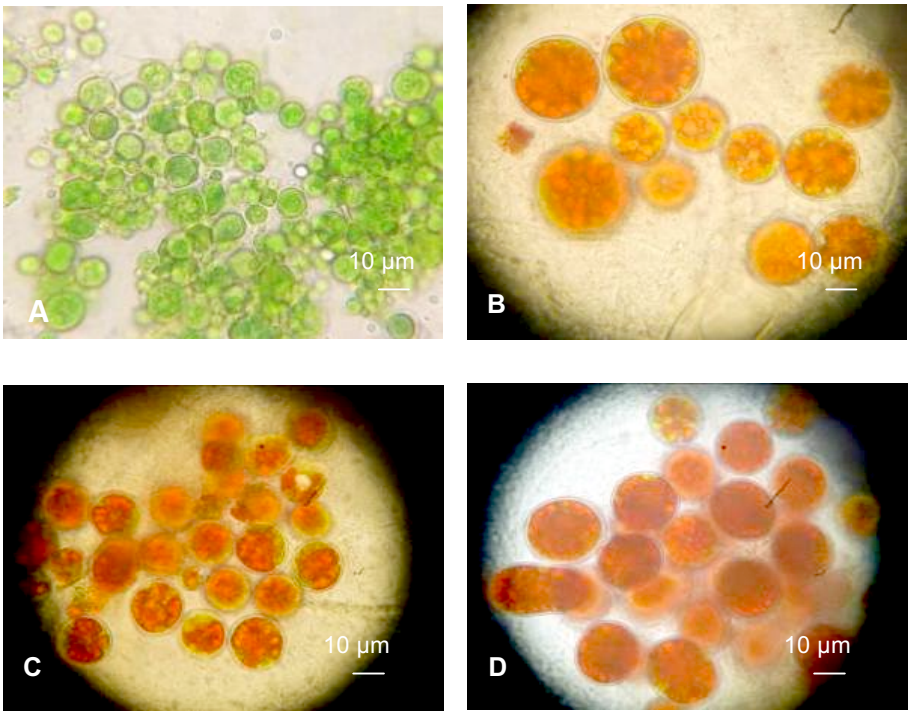

Figure 4. Cell color changed of 10-week-old *H. pluvialis* in aerated liquid BB medium in two-stage. (7 weeks in initial stage and 3 weeks in second stage) with volume 1,000 L. (A) Control: green; (B) TS, 2 hours: red; (C) Na^{+}, 3 weeks: red; (D) Fe^{2+}, 3 weeks: red.

DISCUSSION

In the culture of volume 250 mL, treatments with plant growth regulators (0.1 mg/L BA in initial stage - 0.1 mg/L IAA in second stage) have increased the cell density and dry biomass. This result was similar to Raposo *et al.,* (2006), Czerpak *et al.,* (1994) reported the effects of natural and synthetic auxins on the growth of algal *Chlorella pyrenoidosa* Chick, their metabolic activity was significantly higher than compared with control cultures. An increase in the number of cells was also reported by Prasad (1982) *Skeletonema*, *Chlorella*, *Scenedesmus*, and other microalgal under IAA and NAA (1-naphtalenic acetic acid) treatment. Treatment with 0.15 mg/L IAA in initial stage and 0.175 mg/L GA_3 in second stage made increasing lipid in microalgal. Auxin and gibberellin on concentration will increase lipid accumulation in microalgal. Treatment with 0.2 mg/L GA_3 in initial stage made microalgal changed cyst phase with red color. Gao *et al.,* (2013) showed that GA_3 has increased astaxanthin accumulation of *H. pluvialis*.

In the volume of 10 L, BB medium supplement with 0.1 mg/L BA in initial stage increased fresh and dry biomass indicating that BA has affected to increase accumulation biomass in *H. pluvialis*. 0.1 mg/L BA combined with treatments Fe^{2+} or nitrogen starvation upregulated growth in initial stage and lipid accumulation in second stage of *H. pluvialis*.

In the volume of 1,000 L, *H. pluvialis* after 10-week culture and treatments low temperature or Na^{+} or Fe^{2+}, fatty acid in microalgal was palmitic acid (C16:0) which was more than twice higher than control. It could be explained that treatment effected the change of the flux of carbon to palmitic acid accumulation. Total lipid of microalgal in treatment with Fe^{2+} was higher than that treated with low temperature or Na^{+}. Biofuel in microalgal treated with Fe^{2+} was less than that treated with low temperature or Na^{+}.

CONCLUSION

In the volume of 250 mL, two-stage culture with 0.1 mg/L BA in initial phase and 0.125 mg/L IAA in

second phase increased cell density 3,096.67 x 10^3 cell/mL, and microalga cells had green color with a spherical shape. The supplement of 0.15 mg/L IAA in initial phase and 0.175 mg/L GA_3 in second phase increased 0.085 mg/mL of biofuel, and microalgal cells had red color with a spherical shape. In volume 10 L, the medium was supplied with 0.1 mg/L BA in initial phase (7 weeks), and nitrogen starvation in second phase increased 38.95 mg/mL of dry biomass and 0.783 mg/mL of biofuel, or supplied 4.98 mg/L $FeSO_4$ increased 0.848 mg/mL of biofuel. In volume 1,000 L, microalgal cells were cultured in BB liquid medium in initial phase (7 weeks) and supplied 4.98 mg/L $FeSO_4$ in second phase (3 weeks) increased 78.67 mg/mL of fresh biomass, 2.050 mg/mL of dry biomass and total lipid 28.24 % dry biomass.

Acknowledgement: *Haematococcus pluvialis Flotow was supplied from Algatechnologies, Institute of Biotechnology, Vietnam Academy of Science and Technology*.

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ẢNH HƯỞNG CỦA CÁC CHẤT ĐIỀU HÒA TĂNG TRƯỞNG THỰC VẬT LÊN SỰ TĂNG TRƯỞNG VÀ TÍCH LŨY LIPID CỦA VI TẢO (*HAEMATOCOCCUS PLUVIALIS* FLOTOW) TRONG NUÔI CẤY HAI GIAI ĐOẠN

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TÓM TẮT

Vi tảo *Haematococcus pluvialis* Flotow được nuôi cấy trong môi trường lỏng Bold's Basal được sục khí ở các thể tích khác nhau (250 mL, 10 L và 1.000 L) theo hai giai đoạn (giai đoạn 1 trong 7 tuần nhằm gia tăng sinh khối và giai đoạn 2 trong 3 tuần nhằm gia tăng lipid). Với thể tích 250 mL, trong giai đoạn 1 môi trường BB được bổ sung benzyl adenine (BA), indole-3-acetic acid (IAA) hoặc gibberellic acid (GA_3) ở các nồng độ từ 0,1 - 0,2 mg/L và bổ sung IAA hoặc GA_3 trong giai đoạn 2 ở các nồng độ 0,1 - 0,2 mg/L. Kết quả cho thấy, môi trường bổ sung BA 0,1 mg/L ở giai đoạn 1 và IAA 0,125 mg/L ở giai đoạn 2 kích thích gia tăng mật độ tế bào và vi tảo có dạng hình cầu, màu lục. Môi trường có bổ sung IAA 0,15 mg/L ở giai đoạn 1 và GA_3 0,175 mg/L ở giai đoạn 2 kích thích gia tăng hàm lượng dầu sinh học và vi tảo có hình cầu, màu đỏ. Với thể tích 10 L, giai đoạn 1 vi tảo được nuôi trong môi trường lỏng BB có bổ sung BA 0,1 mg/L, giai đoạn 2 vi tảo được chuyển sang môi trường BB mới được xử lý riêng lẻ hoặc kết hợp hai đến ba yếu tố (đói đạm, NaCl 0,5 %, $FeSO_4$ 4,98 mg/L). Môi trường bổ sung BA 0,1 mg/L và xử lý đói đạm kích thích gia tăng trọng lượng khô và hàm lượng dầu sinh học. Môi trường bổ sung BA 0,1 mg/mL và xử lý $FeSO_4$ 4,98 mg/L kích thích gia tăng hàm lượng dầu sinh học. Với thể tích 1.000 lít, giai đoạn 1 vi tảo được nuôi trong môi trường lỏng BB, giai đoạn 2 môi trường nuôi vi tảo được bổ sung $FeSO_4$ 4,98 mg/L hoặc NaCl 0,5 % hoặc xử lý nhiệt 7 ± 3 °C. Trong các xử lý này, $FeSO_4$ 4,98 mg/L kích thích gia tăng trọng lượng tươi 78,67 mg/mL và trọng lượng khô 2,05 mg/mL và gia tăng tích lũy lipid tổng số 28,24 %/TLK.

Từ khóa*: Chất điều hòa tăng trưởng thực vật, dầu sinh học, đói đạm, Haematococcus pluvialis, nuôi cấy hai giai đoạn*