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Effects of Medium Constituents on Growth and Canthinone Accumulation in Cell Suspension Cultures of *Eurycoma longifolia* Jack

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The effect of various macronutrients, micronutrients and sucrose on growth and canthinone alkaloid production in cell suspension cultures of Pasak Bumi (*Eurycoma longifolia* Jack) was investigated. The optimum macronutrients and micronutrients content for the high alkaloid production of *E. longifolia* Jack was different to that found in the Murashige and Skoog (MS) medium. The highest amount of alkaloids, 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one, could be obtained from *E. longifolia* Jack cells cultured in modified MS liquid medium that containing macronutrients: 21.50 mM NH_4NO_3, 14.25 mM KNO_3, 7.50 mM $CaCl_2 \cdot 2H_2O$, 2.50 mM $MgSO_4 \cdot 7H_2O$, 1.45 mM KH_2PO_4, while content of micronutrients was 0.233 mM FeNa-EDTA, 0.215 mM $MnSO_4 \cdot 4H_2O$ and without $CuSO_4 \cdot 5H_2O$. Increased sucrose concentration to 4.00% (w/v) in modified MS liquid medium could increase total of two-alkaloid. The modification of macronutrients and micronutrients concentration based the optimum production of biomass was obtained MSBs medium that producing high biomass but also increasing the production of 9-hydroxycanthin-6-one. The modification of macronutrients or macronutrients and micronutrients based the optimum total of two-alkaloid was obtained MSC and MSD medium that producing low fresh weight but producing the high 9-hydroxycanthin-6-one.

Key words: *Pasak Bumi*, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one, macronutrients, micronutrients, sucrose

INTRODUCTION

The roots of *Eurycoma longifolia* Jack from the Simaroubaceae family, known locally as *Pasak Bumi* in Indonesia or *Tongkat Ali* in Malaysia, growing wildly in the jungle slopes of Indonesia and Malaysia, are popularly sought after as an essential ingredient in Malay herbal medicine for intermittent fever (malaria) (Nooteboom 1972). The plant contains a series of quassinoids, which are mainly responsible for its bitter taste and some have been shown to posses aphrodisiac activity (Ang *et al*. 1997) and antimalaria (Chan *et al*. 1986; Kardono *et al*. 1991). The plant also contains a series of alkaloids, such as 9-methoxycanthin-6-one, 9-hydroxycanthine-6-one, 9-methoxycanthine-6-one-N-oxide and 9-hydroxycanthine-6-one-N-oxide, which have been shown to possess cytotoxic activity (Kardono *et al*. 1991). Chloroform extract of *E. longifolia* Jack, especially 9-methoxycanthin-6-one, showed as antimicrobial agent of *Bacillus cereus* (Choo *et al*. 2000). Besides, alkaloid 9-methoxycanthine-6-one exhibited higher potency than chloroquine diphosphate against the chloroquine-resistant isolate strain of *Plasmodium falciparum* parasites (Chan *et al*. 2004).

Plant cells have the potential to produce a wide variety of secondary metabolites which can not be obtained by chemical means. Many studies have been conducted on the production of useful compounds by culture cells or organs. The use of plant cell culture as an alternative supply of high value phytochemicals will only be accepted if it can compete in terms of cost with the normal plantation supply. Several plant cell culture media widely used to support plant tissue growth are normally not optimizing for product formation. This has been illustrated by the successful use of a production medium to optimize the yields of desired product (Fujita *et al*. 1981). Trejo-Tapia *et al*. (2003) also reported that hormonal and nutritional factors in the medium can exert an influence on the expression of secondary metabolism. The manipulation of the components of the culture medium (e.g. carbon source, nitrogen, and phosphate) has been used successfully to increase the production of secondary metabolites by cell suspension culture of *Anchusa officinalis* (De-Eknamkul & Ellis 1985). The effects of nitrogen, phosphate, and other inorganic salts in culture medium on indole alkaloid production by *Catharanthus roseus* cell cultures have been extensively studied (Ganapathi & Kargi 1990). Therefore, media optimizations for maximum biomass and secondary metabolites production via cell suspension culture system require knowledge of the components requirement and the effect of components concentration.

In previous report, we reported that friable callus was obtained using leaf explant of *E. longifolia* Jack. Friable callus cultured on MS liquid medium + 2.69 µM NAA + 1.13 µM 2,4-D produced the best *E. longifolia* Jack suspension cells (Siregar 2000). Cell selection and modified MS inorganic macronutrients medium in the cell suspension cultures have been used to increase *E. longifolia* Jack cell biomass (Siregar *et al*. 2003). We have identified alkaloid production in nine of callus and cell lines (Siregar *et al*. 2004).

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In this report, biomass and alkaloid production as affected by different concentration of macronutrients (NH_4NO_3, KNO_3, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, and KH_2PO_4), micronutrients concentration (FeNa-EDTA, $MnSO_4 \cdot 4H_2O$ and $CuSO_4 \cdot 5H_2O$) and sucrose were elaborated. New five medium compositions were formulated to determine the best medium for biomass and alkaloid production. The objective of this study was to establish *Eurycoma longifolia* cell suspension culture for maximum cell biomass and alkaloids production by modified macronutrients, micronutrients and sucrose concentrations in MS media composition.

MATERIALS AND METHODS

Preparation of Cell Cultures. The cell suspension culture of *E. longifolia* Jack, Eu-9 line (the best cell line on biomass production and moderate on alkaloid production), initially derived from leaves callus (Siregar *et al*. 2003, 2004), was prepared by culturing 1.0 g fresh weight of 12 days old cells in 100 ml Erlenmeyer flask containing 20 ml of MS (Murashige & Skoog 1962) liquid medium + 2.69 µM NAA (α-naphtaleneacetic acid) and 1.13 µM 2,4-D (2,4-dichlorophenoxyacetic acid). The pH of culture medium was adjusted to 5.75 before autoclaving at 121 °C for 13 minutes. The cultures were placed on an orbital shaker operating continuously at 130 rpm. Continuous light was provided at an intensity of about 18 µE m^{-2} $s^{-}1$. The culture room temperature was set at 25 ± 2 °C.

Effect of Macronutrients. The effect of the five macronutrient was tested by transferring one-gram fresh weight of line Eu-9 into 100 ml Erlenmeyer flask containing 20 ml MS liquid medium + 2.69 µM NAA + 1.13 µM 2,4-D + various concentration of the tested macronutrients. A completely randomized design was conducted to test the single effect of the each macronutrient. The concentration of the tested macronutrients were NH_4NO_3 (0.00, 5.25, 10.50, 15.75, 21.00, 31.50, 42.00, and 52.50 mM), KNO_3 (0. 00, 4.75, 9.50, 14.25, 19.00, 28.50, and 38.00 mM), $CaCl_2 \cdot 2H_2O$ (0, 0.75, 1.50, 2.25, 3.00, 4.50, 6.00, and 7.50 mM), $MgSO_4 \cdot 7H_2O$ (0.00, 0.38, 0.75, 1.13, 1.50, 2.25, 3.00, and 3.75 mM) and KH_2PO_4 (0.00, 0.31, 0.63, 0.94, 1.25, 1.88, 2.50, and 3.13 mM). Six replicate were used for each modified medium.

Effect of Three Micronutrients. Cell line of Eu-9 was prepared by subculturing 1.0 g fresh weight into 100 ml Erlenmeyer flask containing 25 ml of TAM liquid medium based on previous experiment (Siregar *et al.* 2003) plus 2.69 µM NAA and 1.13 µM 2,4-D. The effect of the three micronutrient was tested by transferring 1.0 g fresh weight of line Eu-9 and subcultured into 100 ml Erlenmeyer flask containing 25 ml TAM liquid medium + 2.69 µM NAA + 1.13 µM 2,4-D + various concentration of the tested micronutrients. A block randomized design was conducted to test the single effect of the each micronutrient. Concentrations of the tested micronutrients were FeNa-EDTA (0.0, 0.025, 0.050, 0.075, 0.100, 0.150, 0.200, and 0.25 mM), $MnSO_4 \cdot 4H_2O$ (0.0, 0.025, 0.050, 0.075, 0.100, 0.150, 0.200, and 0.250 mM) and $CuSO_4 \cdot 5H_2O$ (0.0, 0.025, 0.050, 0.075, 0.10, 0.15 0.20, 0.25 µM). Three experiment units were used for each modified medium and the experiment for each medium was repeated three times.

Effect of Sucrose Concentrations. The effect of sucrose was tested by transferring one-gram fresh weight of Eu-9 cell line of into 100 ml Erlenmeyer flask containing 25 ml TAM liquid medium + 2.69 µM NAA + 1.13 µM 2,4-D + various concentration of sucrose. Concentrations of the tested sucrose were 0.00, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, and 7.00% (w/v). Three experimental units were used for each modified medium and the experiment for each medium was repeated three times.

Effect of Modified MS Medium. One-gram of 12-days old of *E. longifolia* Jack cells were cultured into 100 ml Erlenmeyer flask containing 25 ml TAM and the new modified MS medium (MSB, MSBs, MSC, MSD). The detailed constituents of the new formulated medium were shown in Table 1. Three experimental units were used for each modified medium and the experiment for each medium was repeated three times.

Table 1. Modified composition of macronutrients and micronutrients in TAM medium

Medium components modified	Medium				
	TAM	MSB	MSBs	MSC	MSD
Macronutrient (mM)					
NH_4NO_3	21.50	21.50	21.50	21.50	21.50
KNO_3	12.25	12.25	12.25	14.25	14.25
$CaCl_2 \cdot 2H_2O$	3.10	3.10	3.10	7.50	7.50
$MgSO_4 \cdot 7H_2O$	0.58	0.58	0.58	2.50	2.50
KH_2PO_4	1.83	1.83	1.83	1.45	1.45
Micronutrient (mM)					
Fe-EDTA	0.100	0.110	0.110	0.110	0.233
$MnSO_4 \cdot 4H_2O$	0.100	0.110	0.110	0.110	0.215
$ZnSO_4 \cdot 4H_2O$	0.030	0.030	0.030	0.030	0.030
H_3BO_3	0.13×10^{-1}	0.13×10^{-1}	0.13×10^{-1}	0.13×10^{-1}	0.13×10^{-1}
KI	0.05×10^{-1}	0.05×10^{-1}	0.05×10^{-1}	0.05×10^{-1}	0.05×10^{-1}
$Na_2MoO_4 \cdot 2H_2O$	0.01×10^{-1}	0.01×10^{-1}	0.01×10^{-1}	0.01×10^{-1}	0.01×10^{-1}
$CuSO_4 \cdot 5H_2O$	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	1.00×10^{-4}	-
$CoCl_2 \cdot 6H_2O$	1.10×10^{-4}	1.10×10^{-4}	1.10×10^{-4}	1.10×10^{-4}	1.10×10^{-4}
Myoinositol (mM)	0.56	0.56	0.56	0.56	0.56
Vitamins	Vitamins of MS Medium	Vitamins of MS Medium	Vitamins of MS Medium	Vitamins of MS Medium	Vitamins of MS Medium
Sucrose (%)	3.00	3.00	4.00	4.00	4.00
Hormone (µM)					
NAA	2.69	2.69	2.69	2.69	2.69
2,4-D	1.13	1.13	1.13	1.13	1.13

Harvesting of Cell Biomass. In all study, the cells were harvested by suction filtration after a 14-day incubation period in the test medium. The cells were dried at room temperature until constant weight and this was considered as the dried weight of the cells. The contents of alkaloids, 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one, were determined from the air-dried cells. The curve fitting and optimum concentration of macronutrients, micronutrients and sucrose for the optimum cell growth and alkaloids production was determined and drawn using Sigmastat® (Jandel Scientific Software) and Microsoft® Excel.

Analysis of Alkaloid. A given weight (0.50 g) of air-dried cell biomass was extracted three times overnight by percolation in methanol at room temperature. The combined extract was filtered and evaporated to dryness at 45 °C using a rotary evaporator. The dried residue was reconstituted in methanol and filtered through a micro-filter (Whatman) (0.45 µm pore size) prior to high performance liquid chromatography (HPLC) analysis. The authentic standards, 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one, were obtained previously from Dr. Tripetch Kanchanapoom, Thailand (Kanchanapoom *et al*. 2001, 2002). The standards were confirmed with GC-MS by comparison of its spectroscopic data with literature previously reported (Kardono *et al*. 1991; Mitsunaga *et al*. 1994). For the quantitative analysis of 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one, the alkaloid extracts were loaded into a 20 µl injector loop connected to a reversed-phase 5 µm Hypersil® ODS column 250 x 4.6 mm and controlled by using a HPLC system Shimadzu Class *Vp* software program, with LC-10 AD*Vp* Shimadzu Liquid Chromatograph pump and SPD-10 A*Vp* Shimadzu UV-VIS detector. The mobile phase was consisted of acetonitrile and 0.2% acetic acid (42:58) and the flow rate was maintained at 2.0 ml/min. Alkaloid elution was routinely monitored at 280 nm, and the alkaloids were identified on the basis of retention time. Quantitative determinations of the alkaloids concentration were calculated from calibration curve, putting in relation the relevant peak area with standard concentration. Eight standard solutions of the alkaloid (0.75-500 mg/l) were analyzed for the calibration curve in triplicates. A separate curve was plotted on each day of analysis and its linearity was determined by regression line analysis.

RESULTS

The optimum concentration of NH_4NO_3 in liquid MS medium for increased fresh and dry weight was obtained at 21.5 mM, and this was almost similar to the concentration of NH_4NO_3 found in MS medium (Figure 1a). Based on the total alkaloid production from each of the 20 ml cell culture per flask, the optimum concentration of NH_4NO_3 for the optimum production of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one were 15.5 and 24 mM, respectively. Total of 9-methoxycanthin-6-one was lower with increasing concentration of NH_4NO_3 up to 31.5-52.5 mM. The total amount of two alkaloids (9-hydroxycanthin-6-one and 9-methoxycanthin-6-one) was the maximum at 21.5 mM, and it was lower when the concentration of NH_4NO_3 increased to more than 30 mM (Figure 1b).

The optimum concentration of KNO_3 in liquid MS medium for production of fresh weight was 12.25 mM (Figure 2a). KNO_3 concentration for optimum 9-hydroxycanthin-6-one production was 21 mM. For the optimum of 9-methoxycanthin-6-one production was 9.5 mM KNO_3 in culture medium. While the optimum of KNO_3 concentration for maximum of two alkaloids production on cell biomass was 14.25 mM. This indicated that optimum cell biomass and alkaloid production require on different concentration of KNO_3 (Figure 2b).

The high production of biomass was obtained in MS medium containing 3.1 mM $CaCl_2 \cdot 2H_2O$, and these also resemble to its concentration in MS medium (3.00 mM) (Figure 3a). The addition of different concentration of $CaCl_2 \cdot 2H_2O$ showed no significant effect on production of 9-hydroxycanthin-6-one. On the other hand, the production of 9-methoxycanthin-6-one and production of two alkaloids increased when cells were cultured in medium containing 7.5 mM $CaCl_2 \cdot 2H_2O$ (Figure 3b).

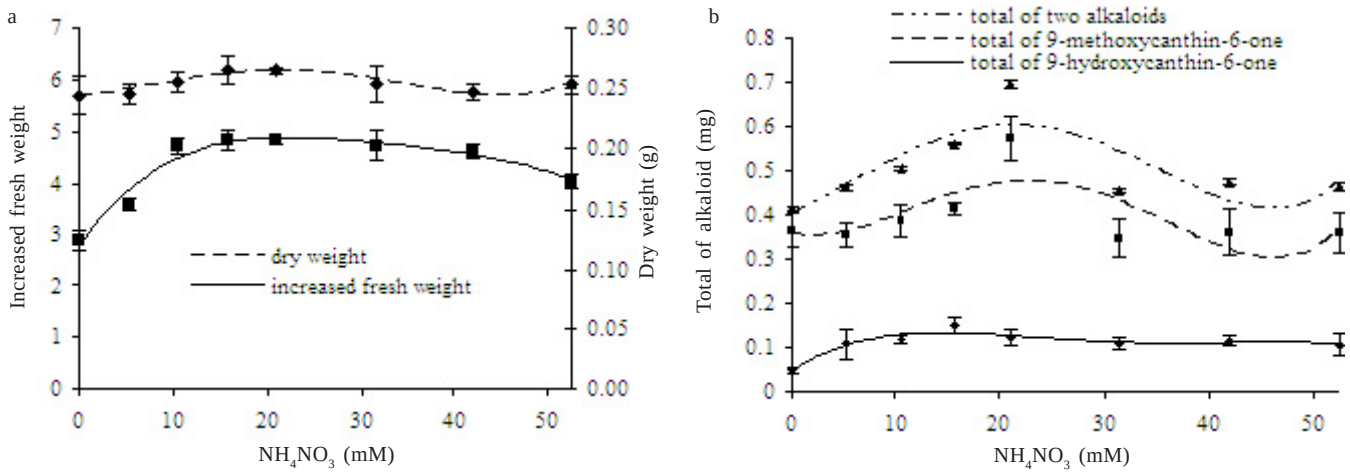

Figure 1. Effect of NH_4NO_3 concentration on a. cell biomass and b. total alkaloid content of *E. longifolia* Jack cell suspension culture in 20 ml liquid MS medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

The optimum concentration of $MgSO_4 \cdot 7H_2O$ for high production of cell biomass was 0.575 mM (Figure 4a). This optimum concentration was only two-fifth of MS medium concentration (1.5 mM). However, for the optimum production of 9-hydroxycanthin-6-one was 3 mM $MgSO_4 \cdot 7H_2O$. While total of 9-methoxycanthin-6-one was required only 2.25 mM $MgSO_4 \cdot 7H_2O$. To obtain higher of two alkaloids production in cell biomass, culture medium required supplementation of 2.5 mM $MgSO_4 \cdot 7H_2O$ (Figure 4b).

The optimum concentration of KH_2PO_4 for high production of cell biomass was 1.83 mM (Figure 5a). For optimum of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one were required 1.40 mM and 1.55 mM KH_2PO_4, respectively. To obtain higher of two alkaloids production in cell biomass, culture

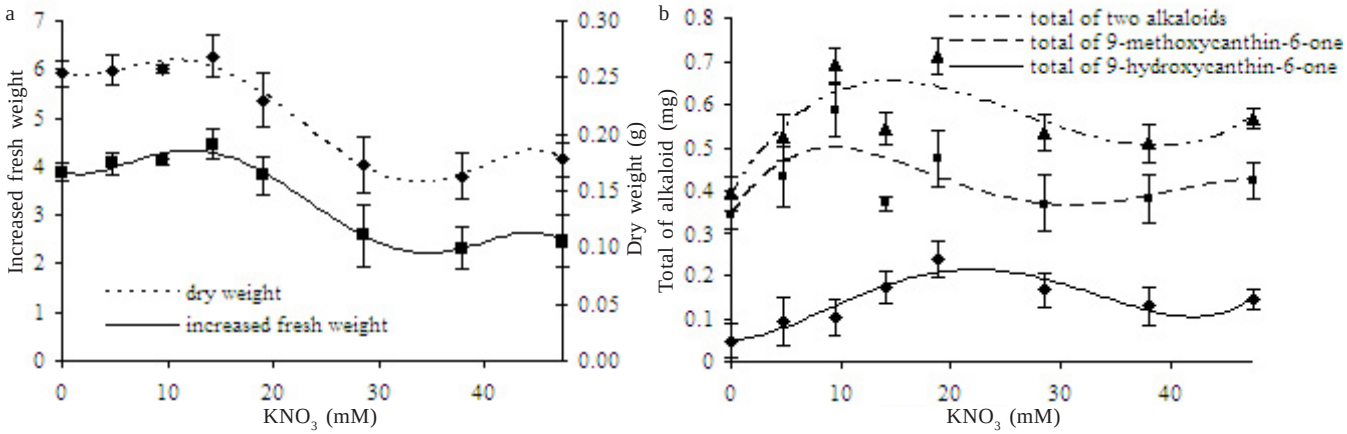

Figure 2. Effect of KNO_3 concentration a. on cell biomass b. and total alkaloid content of *E. longifolia* Jack cell suspension culture in 20 ml liquid MS medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

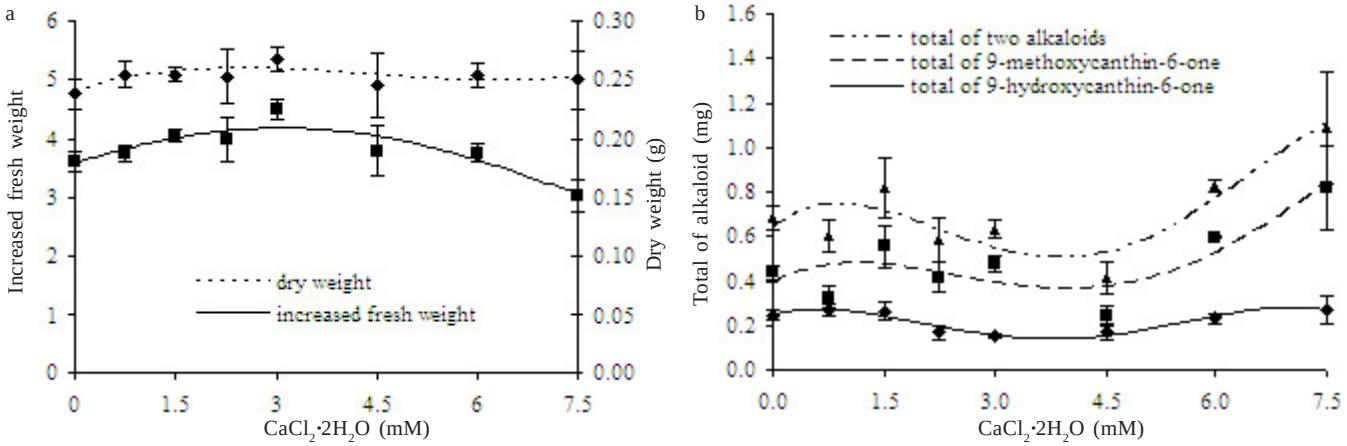

Figure 3. Effect of $CaCl_2 \cdot 2H_2O$ concentration on a. cell biomass b. and total alkaloid content of *E. longifolia* Jack cell suspension culture in 20 ml liquid MS medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

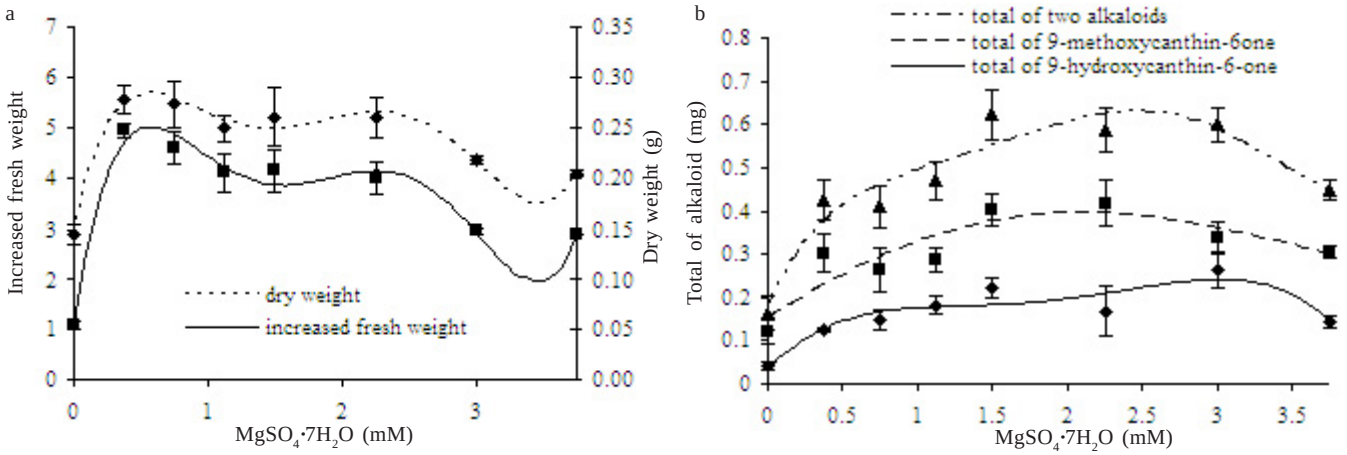

Figure 4. Effect of $MgSO_4 \cdot 7H_2O$ concentration on a. cell biomass b. and total alkaloid content of *E. longifolia* Jack cell suspension culture in 20 ml liquid MS medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

medium required supplementation of 1.45 mM $MgSO_4 \cdot 7H_2O$. Total of alkaloid decreased when KH_2PO_4 concentration was increased more than 1.86 mM (Figure 5b).

The optimum concentration of FeNa-EDTA for high production of cell biomass was 0.11 mM, and this was almost similar to the concentration of FeNa-EDTA found in MS medium (0.10 mM) (Figure 6a). For optimum of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one were required 0.03 and 0.24 mM FeNA-EDTA, respectively. To obtain higher of two alkaloids production in cell biomass, culture medium required supplementation of 0.23 mM FeNa-EDTA (Figure 6b).

The optimum concentration of $MnSO_4 \cdot 4H_2O$ for high production of cell biomass was 0.11 mM, and this was almost similar to the concentration of $MnSO_4 \cdot 4H_2O$ found in MS medium (0.10 mM) (Figure 7a). For optimum of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one were required 0.03 and 0.24 mM FeNA-EDTA, respectively. To obtain higher of two alkaloids production in cell biomass, culture medium required supplementation of 0.23 mM FeNa-EDTA (Figure 7b).

The presence or absence of $CuSO_4 \cdot 5H_2O$ on the TAM medium showed no significant influence on the production of *E. longifolia* Jack cell biomass (Figure 8a). For optimum total of 9-hydroxycanthin-6-one was obtained on TAM medium without $CuSO_4 \cdot 5H_2O$. Besides, the addition of $CuSO_4 \cdot 5H_2O$ (0.00-0.25 x 10^{-3} mM) on TAM medium showed no significant effect on the total amount of 9-methoxycanthin-6-one in cell suspension. To obtain higher of two alkaloids production in cell biomass, culture medium did not required supplementation of $CuSO_4 \cdot 5H_2O$ (Figure 8b).

The optimum concentration of sucrose for high production of cell biomass was 4.24% (Figure 9a). To obtain higher of 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one and two alkaloids production in cell biomass, culture medium required supplementation of 4% sucrose (Figure 9b).

Modified MS medium based on the optimum concentrations of macronutrients for biomass production was obtained in TAM medium (Siregar *et al*. 2003). While modified of macronutrients and micronutrients based the optimum production of biomass was formed in MSB medium. The addition of 4% sugar into MSB medium became MSBs

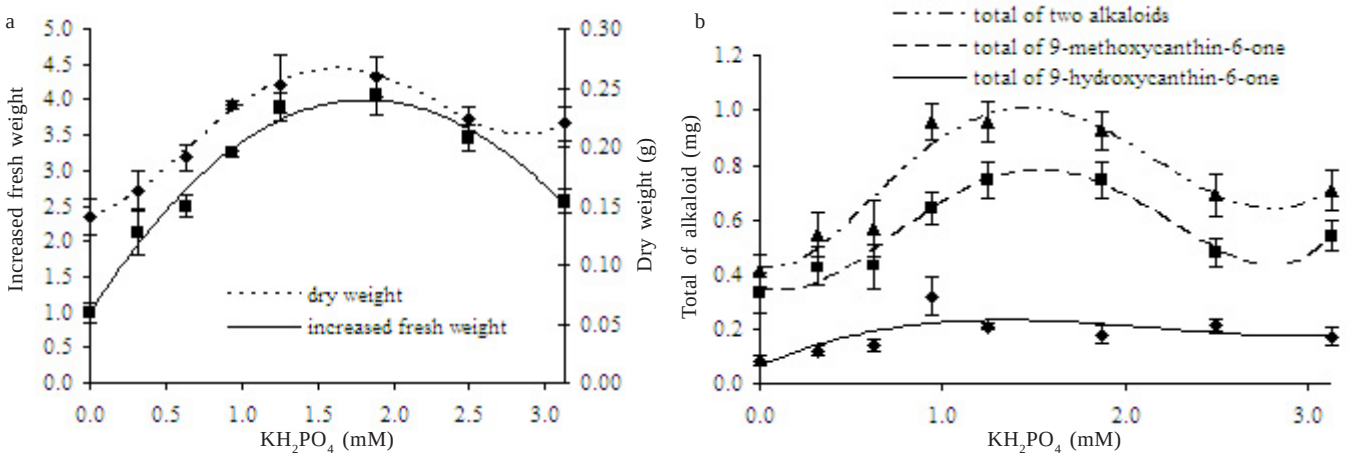

Figure 5. Effect of KH_2PO_4 concentration on a. cell biomass and b. total alkaloid content of *E. longifolia* Jack cell suspension culture in 20 ml liquid MS medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

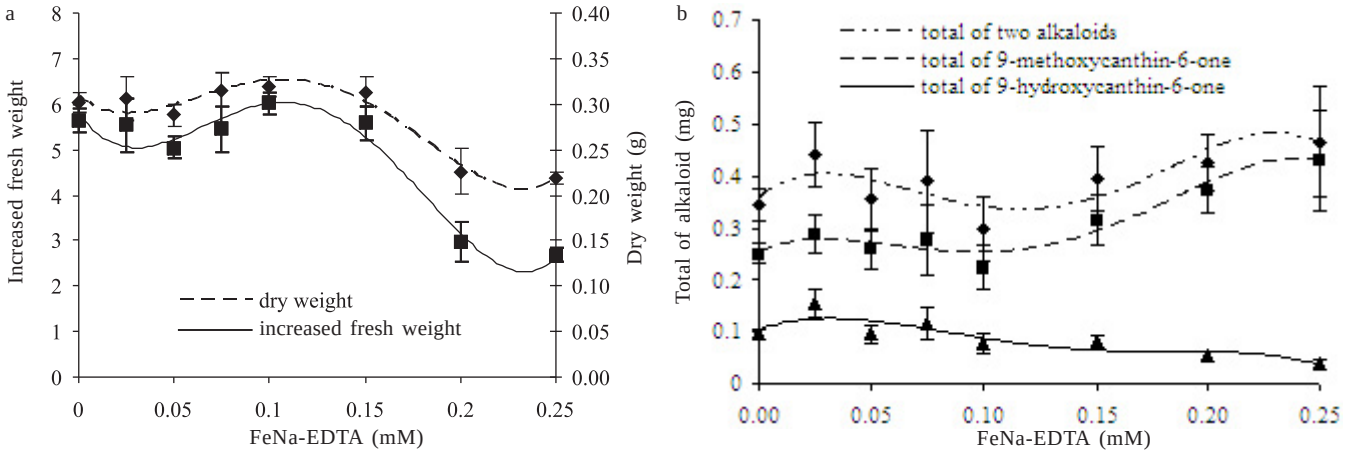

Figure 6. Effect of FeNa-EDTA concentration on a. cell biomass production and b. total alkaloid contentof *E. longifolia* Jack cell suspension culture in 25 ml liquid TAM medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

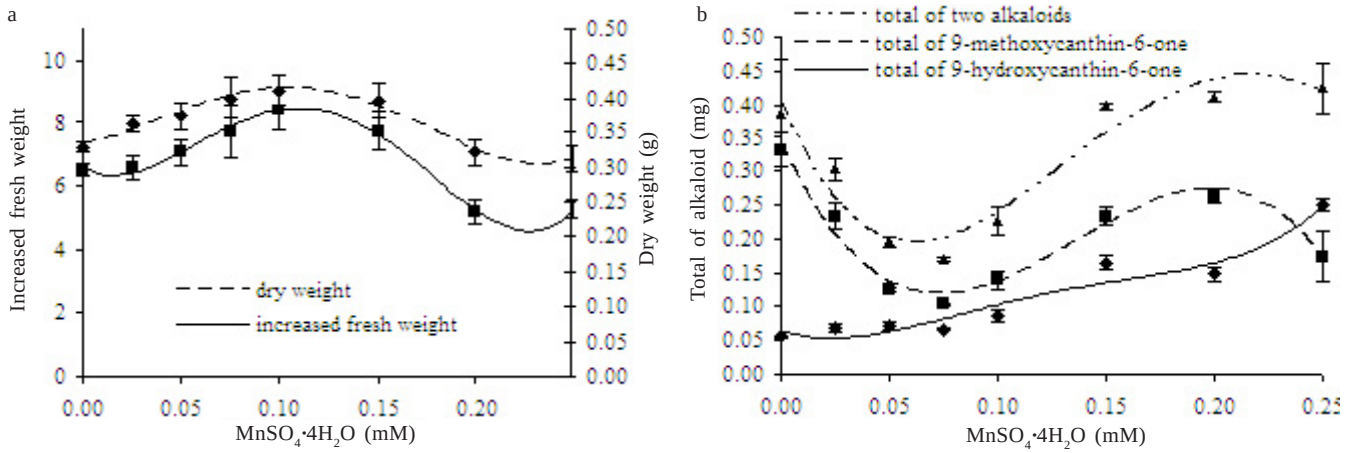

Figure 7. Effect of $MnSO_4 \cdot 4H_2O$ concentration on a. cell biomass production and b. total alkaloid content of *E. longifolia* Jack cell suspension culture in 25 ml liquid TAM medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

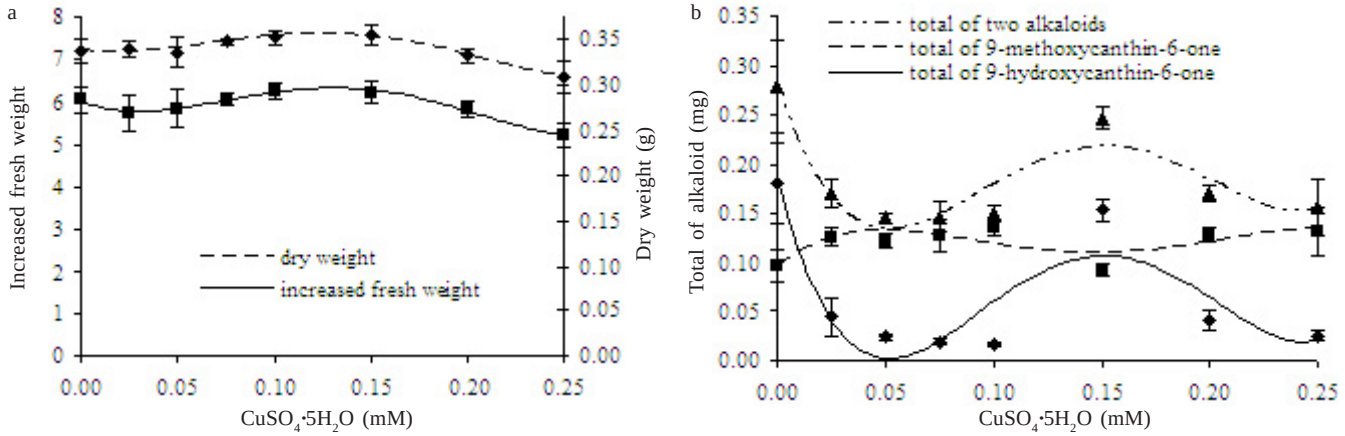

Figure 8. Effect of $CuSO_4 \cdot 5H_2O$ concentration on a. cell biomass production and b. total alkaloid content of *E. longifolia* Jack cell suspension culture in 25 ml liquid TAM medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

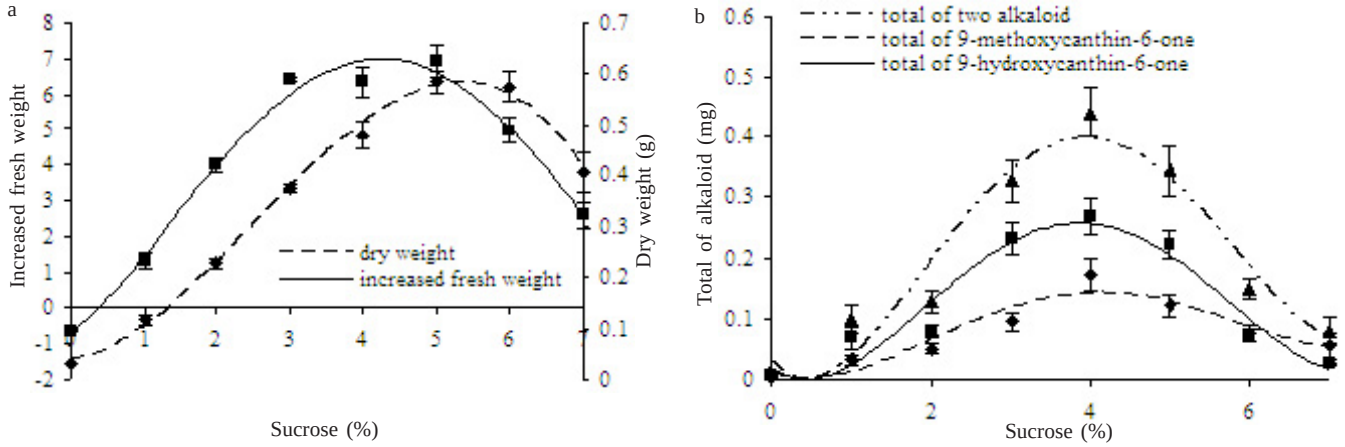

Figure 9. Effect of sucrose concentration on a. cell biomass production and b. total alkaloid content of *E. longifolia* Jack cell suspension culture in 25 ml liquid TAM medium with 2.69 µM NAA + 1.13 µM 2,4-D after 14 days of culture.

medium. Medium MSBs produced the highest production of biomass compare to TAM, MSB, MSC, and MSD medium (Figure 10a). MSC medium was formulated by modified concentration of macronutrients based on the maximum amount of two-alkaloid with 4% sucrose. While MSD medium was formed by modification concentration of macronutrients and micronutrients based on the optimum total of two alkaloids with addition 4% sucrose. Production of cell biomass in MSC and MSD medium were lower compared to TAM, MSB, and MSBs medium.

The content of 9-hydroxycanthin-6-one alkaloid in cell biomass that cultured in MSC (0.518%) medium was highest

compare with MSBs (0.484%), MSD (0.464%), MSB (0.385%), and TAM (0.317%) medium. While MSD medium produced highest content of 9-methoxycanthine-one namely 0.877%, and TAM medium showed lowest content of 9-methoxycanthin-6-one namely 0.020 % produced (Figure 10b).

Based on total of alkaloid, MSBs medium produced highest total of 9-hydroxycanthin-6-one (1.654 mg/flask) compare to MSC (1.499 mg/flask), MSD (1.405 mg/flask), MSB (1.224 mg/flask), and TAM (1.018 mg/flask) medium. The total of 9-methoxycanthin-6-one was produced highest in MSD medium (0.265 mg/flask). While the lowest total of 9-methoxycanhin-6-one was produced in the cell biomass that cultured on TAM medium (0.064 mg/flask) (Figure 10c).

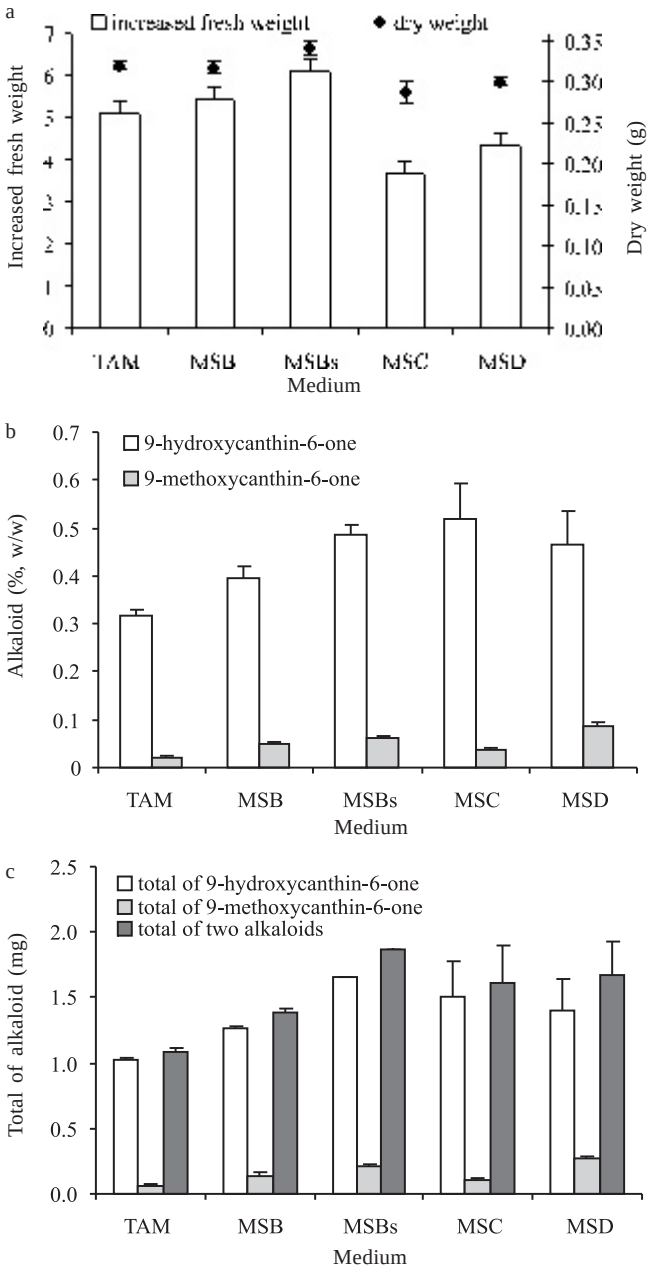

Figure 10. Production of a. cell biomass, b. alkaloid content, and c. total alkaloid content of *E. longifolia* Jack cell suspension culture in TAM, MSB, MSBs, MSC, and MSD medium after 14 days of culture.

DISCUSSION

The optimum concentration of NH_4NO_3 to produce the high *E. longifolia* Jack cell biomass and total of two alkaloids were resemble with NH_4NO_3 concentration in MS medium (21 mM). High cell biomass and alkaloid production could be obtained at 12.25 and 14.25 mM KNO_3, respectively. These concentrations were lower compared to KNO_3 concentration in MS medium (19 mM). De-Eknamkul and Ellis (1985) reported that the optimum cell biomass in cell suspension culture of *Anchusa officinalis* was the same in NO_3^- concentration (15 mM) as to produce high anthocyanin. Jung *et al*. (1994) reported that addition of KNO_3 up to 12.5 mM increased total of catharantine content in hairy root cultures of *Catharanthus roseus*. However, the addition of higher KNO_3 concentration did not increase catharantine content, even though growth of hairy roots biomass was optimum at 25 mM KNO_3. Marero *et al*. (1997) reported that cell suspension cultures of *Polygonum tinctorium* produced high indirubin content in SH liquid medium containing only KNO_3 as source of nitrogen compared to the addition of NH_4NO_3 or combination of NH_4NO_3 and KNO_3.

The production of the highest *E. longifolia* Jack cells biomass was produced in MS medium containing 3.1 mM $CaCl_2 \cdot 2H_2O$. While, for the optimum total of two alkaloids and 9-methoxycanthin-6-one production in *E. longifolia* Jack cells biomass was produced with increased calcium become 2.5 fold from its concentration in MS medium. Marero *et al*. (1997) reported a same phenomena compare to our study, that the increased calcium concentration become two fold from its concentration in SH liquid medium could increase indirubine content of 140% in cell suspension culture of *Polygonum tinctorium*. Margaritha *et al*. (1995) reported that the growth of *Digitalis thapsi* cell suspension culture was obtained limited in liquid MS medium without calcium, but carotenoid content produced was high.

The addition of 0.58 mM $MgSO_4 \cdot 7H_2O$ or only 2/5 from its concentration in MS Medium (1.5 mM) could produce the optimum *E. longifolia* Jack cell biomass. To obtain higher two-alkaloids production in cell biomass, culture medium needed the addition of 2.50 mM $MgSO_4 \cdot 7H_2O$. The different condition from above was found in cell suspension culture of *Lithospermum erythrorhizon* (Fujita *et al*. 1981). They reported that Mg^{2+} concentration did not showed changes in shikonin production. While Marero *et al*. (1997) reported that indirubin content was produced high in cell suspension culture of *Polygonum tinctorium* when cells were cultured in SH liquid medium containing 1.62 mM Mg^{2+}, and this concentration was the same with SH basic medium.

The concentration of KH_2PO_4 increased from 1.25 mM (MS medium) to 1.83 mM could produce the optimum cell biomass. While increased to 1.45 mM KH_2PO_4 could produce the optimum of total of two-alkaloids. It showed that phosphate was a part of essential macronutrient that to be limited factor for increased secondary metabolite production. Takeda (1988) reported that decreased concentration of PO_4^{3-} became 1/10 of its concentration in Lin and Staba medium (Lin & Staba 1961) was obtained an increased the anthocyanin content in

cell suspension cultures of *Daucus carota*. Ilieva and Pavlov (1996) were also found production of rosmarinic acid in cell suspension cultures of *Lavandula vera* was stimulated with increasing PO_4^{3-} in medium. Trejo-Tapia *et al*. (2003) achieved that PO_4^{3-} showed the most important effect compare with nutrient of NO_3^- and Fe^{2+} component and sucrose for blue pigment production in cell suspension cultures of *Lavandula spica*.

Result of the study showed that increased concentration of FeNa-EDTA by 2.3-fold of that found MS medium was obtained the optimum total of two-alkaloids. The various phenomena were found to strongly repress secondary metabolism. Nakao *et al*. (1999) reported that the increased concentration of Fe ($FeSO_4$) by five-fold of that found in the MS medium result a decreased cell growth and the amounts of total flavanols and catechin derivatives in the cell cultures of *Polygonum hydropiper*. Trejo-Tapia *et al*. (2003) obtained that Fe^{2+} in the MS medium modified was a component of nutrient needed for production of blue pigment in cell suspension culture of *Lavandula spica*. When Fe^{2+} was not present in medium culture, cells only produced yellow pigment else. However, Fe^{2+} occasionally did not indicate the effect on production of secondary metabolite. Jung *et al*. (1994) found that $FeSO_4$ showed no significant influence on catharanthine production in hairy root culture of *Catharanthus roseus* within the range of concentration of $FeSO_4$ tested in 1/3 SH liquid medium.

The optimum concentration of $MnSO_4 \cdot 4H_2O$ for biomass production was nearly equal to that contained in basic MS medium (0.1 mM). Beside increased concentration of $MnSO_4 \cdot 4H_2O$ by twice (0.215 mM) of that present in the basic MS medium enabled the *E. longifolia* Jack cells to produce the optimum total of two-alkaloids. The different phenomena were found in cell suspension culture of other plants. Fujita *et al*. (1981) found that the presence of manganese in White's liquid medium for the cell suspension culture of *Lithospermum erythrorizon* decreased the production of the shikonin derivatives, therefore manganese was removed from White's medium for the optimum production of shikonin. Nakao *et al*. (1999) reported that increased concentration of manganese by fivefold that present in MS medium caused a reduction in cell growth and the amounts of total flavanols and catechin derivatives in the cell suspension cultures of *Polygonum hydropiper*.

The optimum production of two-alkaloids and cell biomass could be obtained in TAM medium without $CuSO_4 \cdot 4H_2O$. Fujita *et al*. (1981) also found that copper element of White's liquid medium in cell suspension culture of *Lithospermum erythrorhizon* had little effect on cell growth, but the concentration of 1.20×10^{-3} mM copper or equal to 30 times higher than that in White's medium, had significantly effect on the production of shikonin derivatives. Morimoto *et al*. (1988) reported that when the concentration of copper in the LS medium was increased to 10 times (1.00×10^{-3} mM) that of the basic LS medium, the berberine yield was increased by 20-30% but the increased in copper concentration had no effect on cell growth. Bligny and Douce (1977) suggested that the amount of copper in the medium could affect the cell mitochondrial respiration via copper incorporation into cytochromes in suspension culture of sycamore cells. Therefore, the effect of copper on secondary metabolite production may be based on its participation in the establishment of mitochondrial electron transport system.

The effects of sucrose concentration on the yield of secondary products have been examined in a number of plant cell cultures. The optimum concentration seems to vary according to the plant species. Result of the study showed that addition of 4% sucrose into liquid TAM medium increasing total of alkaloids in *E. longifolia* Jack cell biomass. Karam *et al*. (2003) reported that the optimum of rosmarinic acid production in hairy root culture of *Salvia fruticosa* was obtained in B5 liquid medium with 4% sucrose. The growth and rosmarinic acid content on cell suspension cultures of *Anchusa officinalis* was found high level in B5 liquid medium + 4.52 µM 2,4-D + 0.47 µM kinetin with 3% sucrose, while sucrose concentration more and less than 3% produced growth and rosmarinic acid production become lower (De-Eknamkul & Ellis 1985).

From this study, it have been formulated a MSBs medium that could produce the highest cell biomass and total of two-alkaloids medium with modifying concentration of MS macronutrient and micronutrient based the optimum production of cell biomass and including 4% sucrose. Trejo-Tapia *et al.* (2003) reported that production of blue pigment in cell suspension culture of *Lavandula spica* could be increase to sevenfold and produced the best cell growth rate in modified MS medium (5.71 ìM IAA + 9.29 ìM kinetin + 1.12 mM myoinositol) with containing 2.50 mM PO_4^{3-}, 14.10 mM, NO_3^-, 1.00 mM Fe^{2+} and 10 g/l cell inoculums compare with cultured cell in modified MS medium with containing 1.25 mM PO_4^3, 39.40 mM NO_3^-, 1.00 mM Fe^{2+} and 5 g/l cell inoculums.

The modification of MS macronutrients and sucrose concentration based the optimum total of two-alkaloids were achieved MSC medium, even though the modification of MS macronutrients, micronutrients and sucrose concentration were achieved MSD medium. Both of these medium did not produced high cell biomass, besides total of two-alkaloid rate produced were lower compare to MSBs medium. De-Eknamkul and Ellis (1985) reported that a combination of various nutritional factors at optimum concentrations determined from individual component manipulation was not necessarily capable of supporting maximum product formation of the *Anchusa officinalis* cell suspension culture.

Based of the study, the modified MS medium on macronutrients, micronutrients, and sucrose concentration based the production of cell biomass were obtained the MSBs medium that producing high cell biomass and total of two-alkaloids. It would be important to relate the observed effects with metabolic pathway involved in the synthesis of the alkaloid of *E. longifolia* Jack. However, there is no report to discuss in this case. Therefore, further research is needed to elaborate this matter. It is also necessary to determine a medium for highest increase 9-methoxycanthin-6-one in cell suspension culture of *E. longifolia* Jack.

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