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Full Length Research Paper

Towards understanding of physiological changes in cell culture of recalcitrant woody plant, *Eurycoma longifolia,* in response to carbon and nitrogen sources

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Eurycoma longifolia **possesses high medicinal and economical values owing mainly to its aphrodisiac properties claimed by the local communities in Southeast Asia regions. However, the long cultivation period, low successful rate of seed propagation and susceptibility towards pests and diseases have affected the supply of *E. longifolia* to meet the high market demand. Thus, the large scale production of *E. longifolia* using cell suspension technique is tantalizing. In this study, the *E. longifolia* cells cultivated in shake flask system were subjected to different carbon and nitrogen sources treatments. The cells treated with glucose gave the highest increment of fresh weight (0.4386 ± 0.0120 g/mL), with increment of total soluble protein content, 0.71 ± 3.05 mg/g FW and increment of specific activity of peroxidase, 5410.04 ± 1221.43 U/mg. Glucose-treated cells also achieved the highest carbon source utilization rate (2.81 ± 0.31 mg/mL/Day). For the cells treated with different nitrogen sources, the potassium nitrate (KNO_3) treatment gave the highest increment of fresh weight (0.2601 ± 0.0387 g/mL), with increment of total soluble protein content, 0.62 ± 0.00 mg/g and increment of specific activity of peroxidase, -3691.57 ± 2717.18 U/mg. The cells also had the highest sucrose utilization rate (2.92 ± 0.02 mg/mL/Day).**

Key words: Carbon utilization rate, cell suspension culture, *Eurycoma longifolia*, specific activity of peroxidases, total soluble protein content.

INTRODUCTION

Eurycoma longifolia, is one of the well-known folk herbal plants in Southeast Asia region due to its remarkable aphrodisiac, anticancer, antimalarial, antiulcer, antipyretic and restorative properties (Ang et al., 2003). This wide range of curative properties are due to the presence of various bioactive compounds such as quassinoids, squalenes derivatives, biphenylneolignans tirucallanetype triterpenes, canthine-6-one and β-carboline alkaloids (Kuo et al., 2003). These bioactive compounds have tempted many pharmaceutical companies to use *E. longifolia* to produce medicines and health supplements. Consequently, this has increased the market demand for

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Abbreviations: 2, 4-D, 2,4-Dichlorophenoxyacetic acid; **DNS,** dinitrosalicyclic; **H_2O_2,** hydrogen peroxide; **HCl,** hydrochloric acid; **HMF,** 5-(hydroxymethyl)-2-furaldehyde; potassium nitrate; KNO_3, potassium ion; K^+, potassium nitrate; **KOH,** potassium hydroxide; **NaOH,** sodium hydroxide; **NH_4^+,** ammonium ion; **NH_4NO_3,** ammonium nitrate; **ROS,** reactive oxidative species.

E. longifolia and also its commercial value. To satisfy the increased market demand, the domestication of *E. longifolia* is introduced. However, this large scale plantation of *E. longifolia* is performed by using traditional seed propagation method, which is vulnerable to problems, like low successful rate of seed germination (Hussein et al., 2005), infections (caused by pests like the tiger moth) and disease (Sudden death syndrome) (Patahayah et al., 2009). These problems have severely impact the supply of *E. longifolia* to meet the high market demand. Therefore, the urge for a viable alternative in *E. longifolia* domestication arises.

In this regards, plant tissue culture techniques render a good alternative, where, *E. longifolia* is able to be mass produced in form of cell suspension culture in a bioreactor. The cells produced in the bioreactor could then as served as raw materials for the production of medicines or health supplements. Furthermore, plant tissue culture techniques are able to provide consistent supply of *E. longifolia* cells, regardless of the disease and infection of pests. However, the use of plant suspension culture technique for large scale production of biomass requires a detailed study on the plant cells' growth rate as the cell biomass and the production of bioactive compounds are greatly influenced by it. To control the growth rate of plant cells, the determination of the composition of growth of cells are vital. Thus, in this study, the physiological changes of *E. longifolia* cells under the influence of various carbon and nitrogen sources were further explored.

MATERIALS AND METHODS

Medium preparation

The medium used in this study was full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1 mg/L of 2, 4-D and 3% (w/v) sucrose. The pH of the medium was then adjusted to pH 5.7 ± 0.1 by using 0.1 M of HCl or 0.1 M of NaOH. The medium was then distributed into the Erlenmeyer flasks, sealed with aluminum foil and autoclaved at 121°C, 15 psi for 15 min.

Establishment and maintenance of cell suspension culture

The 3 week-old, yellow, friable callus of *E. longifolia* was used for the establishment of suspension culture. The friable callus was cut finely and inoculated into 50 mL MS liquid medium containing 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa, Netherland) and 3% (w/v) sucrose (Fisher Scientific, USA) in 250 mL of Erlenmeyer flask. After the inoculation, the flasks were sealed and placed on the orbital shaker at 100 rpm. The established suspension culture was maintained at 25 ± 1°C under cool fluorescent light with regular photoperiod of 16 h light and 8 h dark. After one week, subculturing of cells was done by inoculating 25 mL of the newly established suspension culture into a 250 mL of Erlenmeyer flask, containing 25 mL of fresh medium. Following that, the suspension culture was subcultured weekly. Only cells with at least three passages were used in this study. For this study, 10 mL of the 9 day-old suspension cells were inoculated into each treatment medium.

Effects of carbon and nitrogen sources

Three carbon sources tested in this study were sucrose (Fisher Scientific, USA), glucose (Fisher Scientific, USA) and fructose (Fisher Scientific, USA) at the concentration of 3% (w/v). As for nitrogen sources, two different nitrogen sources, ammonium nitrate and potassium nitrate (NH_4NO_3 + KNO_3) were used in this study. These two nitrogen sources were originally present in the composition of MS medium at the concentration of 1.6500 g/L and 1.9000 g/L, respectively, which made up to the total nitrogen concentration of 60 mM. In this study, the concentration of total nitrogen sources were remained the same by modifying the types of nitrogen sources.

For treatment that used NH_4NO_3 as the sole nitrogen source, 2.4013 g/L of NH_4NO_3 was added, while for another treatment, 6.0662 g/L of KNO_3 was added as the sole nitrogen source. The treatment containing both nitrogen sources (NH_4NO_3 + KNO_3) was used as the control. Collection of samples for the analysis of physiological and biomass changes was conducted every 5 days, continuously for 20 days. All the samples collected were subjected to the fresh weight, medium pH, total soluble protein content, specific activity of peroxidase and carbon source utilization rate analysis.

Fresh weight and medium pH

The sample obtained from each treatment was undergoing filtration through the Buchner funnel under vacuum condition. The filtrate was collected and its pH value was measured using the pH meter (Mettler Toledo, USA). After the measurement of pH, the cells collected on the Buchner funnel was rinsed with distilled water to wash away the medium. After that, the fresh weight of the cells was obtained.

Total soluble protein content

Sample extraction

After obtaining the fresh weight, the cells were transferred onto a pre-chilled mortar in an ice bath. The cells were ground with pestle together with the protein extraction buffer with the ratio of 1 g of cells to 3 mL extraction buffer. The crude extract was then subjected to the centrifugation at 12,000 rpm at 4°C for 20 min. The pellet was then discarded, while the supernatant obtained from each sample was subjected to the total soluble protein and specific activity of peroxidase assay.

Total soluble protein assay

The total soluble protein of the suspension cells was determined by using the Bradford (1976) method. A total of 20 µL of supernatant was mixed with 80 µL of protein extraction buffer and 5 mL of protein reagent. The absorbance for this mixture was measured at 595 nm with spectrophotometer (Bio-rad Smartspec Plus, USA). The blank for this mixture consisted of 20 µL of distilled water, 80 µL of protein extraction buffer and 5 mL of protein reagent. The standard calibration curve for total soluble protein was obtained by using bovine serum albumin (BSA) (Sigma Aldrich, USA) as the standard at the concentration of 2.0, 4.0, 6.0 and 8.0 mg/mL. The absorbance of the samples obtained was compared with the

calibration curve and the total soluble protein content was further expressed in milligram per gram fresh weight of suspension cells (mg/g FW).

Specific activity of peroxidase assay

The specific activity of peroxidase was determined by using the Kokkinakis and Brooks (1979) method. A total of 0.5 mL of supernatant was mixed with 1.0 mL of 1% guaicol (Fisher, USA), 1.0 mL of 30% H_2O_2 (Fisher, USA) and 7.5 mL of 0.1 M sodium phosphate buffer (pH 6.0).

Then, the initial and maximum absorbance for this mixture was determined at 420 nm. On the other hand, the blank for this assay consisted of the same composition as the sample, except that the supernatant was replaced by protein extraction buffer. The absorbance obtained from the sample was converted into the total peroxidase activities (U) by using the following equation:

$$\text{Total peroxidase activity, U} = \frac{\Delta \text{Abs} \times \text{dilution factor} \times 1000}{\text{mL of sample extract}}$$

One unit of peroxidase activity was the amount of peroxidase that increased the absorbance by 0.01 in 1 min. The total peroxidase activity (U) obtained was converted into specific enzyme activity (U/mg) by using the following equation:

$$\text{Specific enzyme activity, U/mg} = \frac{\text{Total peroxidase activity (U)}}{\text{Soluble protein content (mg)}}$$

Carbon source utilization rate

Sucrose utilization rate

The sucrose present in the culture medium throughout the 20 days was determined by using modified dinitrosalicyclic (DNS) colorimetric method. The filtrate collected from the suction filtration was used to determine the carbon source utilization. For sucrose detection, 1 mL of filtrate was added to 1 drop of 30% (w/v) HCl (Merck, USA) solution and incubated in 90°C water bath for 5 min. After that, 6 drops of 5 N KOH solution (Merck, USA) were added to neutralize the acidic condition, followed by 3 drops of 10% NaOH and 1 mL of DNS reagent.

The mixture was incubated at 100°C for 5 min. Later, the solution was cooled down under running tap water. Then, a total of 10 mL of distilled water was added and the solution was swirled to obtain a homogenized solution.

After 20 min, the absorbance for solution was measured at 540 nm with spectrophotometer (Bio-rad Smartspec Plus, USA). The blank was prepared by the same steps as mentioned previously except by replacing 1 mL filtrate with 1 mL of distilled water. The standard calibration curve was constructed by using the sucrose (Fisher, USA) as the standard. A concentrated sucrose solution (1 mg/mL) was prepared and was diluted to 0.2, 0.4, 0.6 and 0.8 mg/mL.

The absorbance for 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL of sucrose solution was measured and was used to construct the standard calibration curve. By comparing the absorbance obtained from the sample with standard calibration curve, the concentration of the sucrose (mg/mL) in the culture was determined. The sucrose concentration was further converted to sucrose utilization rate (mg/mL/day).

Reducing sugar utilization rate

The presence of reducing sugar (glucose and fructose) during multiplication of *E. longifolia* cells were also estimated by using the DNS method. The protocol to perform the DNS assay was the same as the modified DNS method except that the hydrolysis with HCl was not carried out. Firstly, a total of 1 mL of filtrate was added with 3 drops of 10% NaOH and 1 mL of DNS reagent. The mixture was subjected to 100˚C of water bath for 5 min. It was then cooled down to room temperature, followed by the addition of 10 mL distilled water. The swirling of mixture was done to obtain homogenous solution. Then, the absorbance of the mixture at 540 nm was measured after 20 min. The blank was prepared by replacing the filtrate with distilled water.

The standard calibration curve for both the reducing sugar was constructed using glucose (Fisher, USA) and fructose (Fisher, USA) as standards. The concentrated solutions (1 mg/mL) for glucose and fructose were prepared separately and were diluted to 0.2, 0.4, 0.6 and 0.8 mg/mL. The absorbance of glucose and fructose at 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL at 540 nm was obtained and two standard calibration curves for both glucose and fructose were constructed. The absorbance of sample was compared to the standard calibration curve to obtain the concentration of the reducing sugar (mg/mL) as well as the reducing sugar utilization rate (mg/mL/day).

Statistical analysis

The experiments in this study were carried out at three replicates and the experiments were repeated once. All the results in these studies, except the morphological study, were subjected to statistical analysis using SPSS software (version 17.0) (SPSS Inc., USA). The One-way analysis of variance (One-way ANOVA) and Tukey's honestly significant differences (Turkey's HSD) test were conducted to determine the significant differences between the treatments for each parameter at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of carbon sources on the growth of suspension cells

The three different carbon sources (sucrose, glucose and fructose) tested would distinctly affect the metabolism of cells in the medium. These "callogenic" sugars were able to trigger the synthesis and accumulation of starch and differentiation (vacuolation and elongation) in callus (Maataoui et al., 1998). Thus, the phenomenon of elongation and cell compartments differentiation was able to be observed on day 15. For the sucrose treatment, the sucrose would be rapidly hydrolysed first by the cell wall–associated invertase enzyme into direct assimilable sugars (glucose and fructose) before being uptaken into the plant cells (Felker et al., 1989). This hydrolysation process would also cause the acidification of the medium. This phenomenon was proven in the present study, whereby, the medium pH for sucrose treatment on day 5 was 0.44 unit more than glucose (0.595 unit) and fructose (0.625 unit) treatments (Figure 1). In this treatment medium which contains both glucose and fructose, the

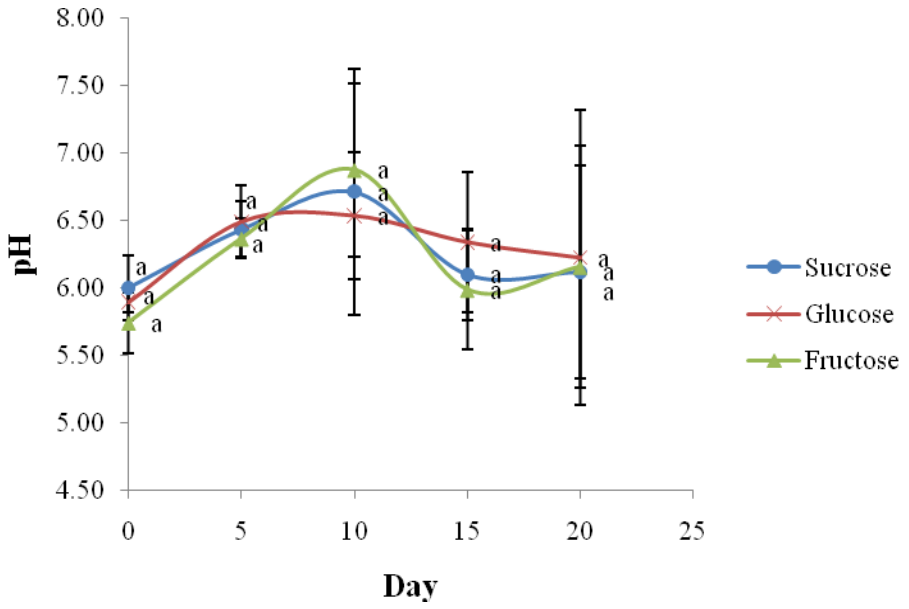

Figure 1. The effect of different carbon sources on the pH of the medium for *E. longifolia* suspension culture. Mean with different letter(s) within the same time period are significantly different between treatments by Turkey's HSD test ($p< 0.05$). The error bars indicate the mean ± standard deviation.

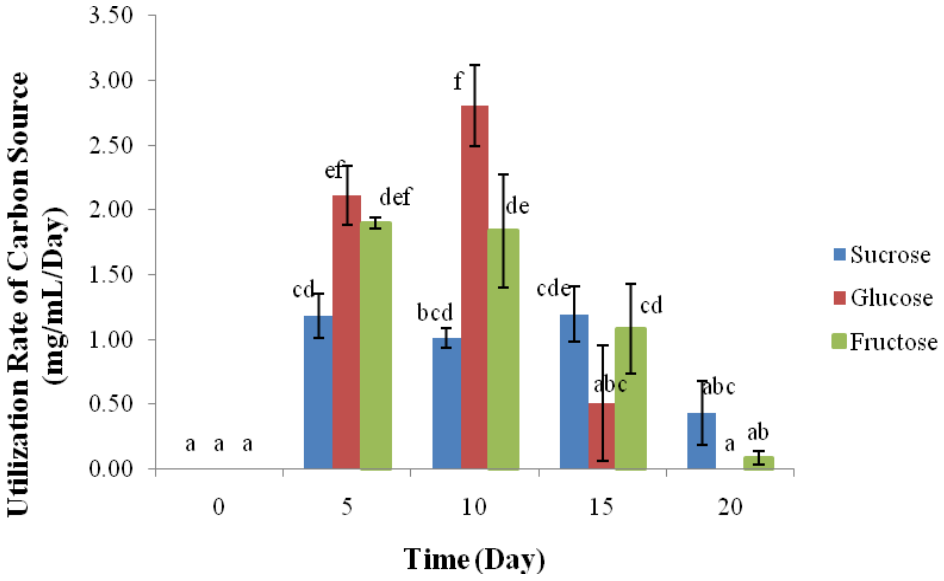

Figure 2. The carbon source utilization rate of the *E. longifolia* suspension culture containing MS medium supplemented with sucrose, glucose or fructose. Mean with different letter(s) within the same period of time are significantly different between treatments by Turkey's HSD test ($p< 0.05$). The error bars indicate the mean ± standard deviation.

plant cells would uptake the glucose preferentially as the glucose had competitively inhibit the uptake of fructose. The preference of cells towards the glucose than fructose was caused by its ability to be catabolized directly through the glycolysis, whereas, fructose has to be converted into glucose and sucrose prior to glycolysis (Dijkema et al., 1988).

In addition, the cells treated with sucrose gave the overall lowest utilization rate due to the presence of extra amount of monosaccharides (Figure 2). Since sucrose is

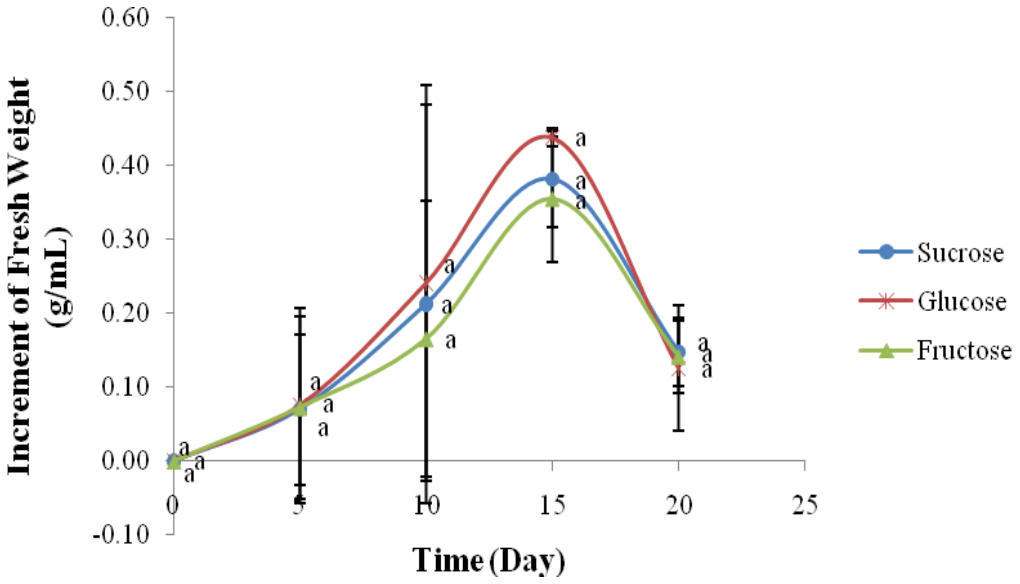

Figure 3. The effect of different carbon sources on the growth of *E. longifolia* suspension culture measured by the increment of fresh weight. Mean with different letter(s) within the same time period are significantly different between treatments by Turkey's HSD test ($p< 0.05$). The error bars indicate the mean ± standard deviation.

hydrolysed extracellularly into equimolar amount of glucose and fructose, the total amount of monosaccharides in the medium is twice the amount of monosaccharide in glucose and fructose treatment medium (Krook et al., 2000). The uptake and utilization of sucrose was done part by part with the hydrolysed components (glucose and fructose) so the rate of depletion of sucrose in the medium was lower than the other treatments, which involved the direct consumption of carbon source. Besides that, being disaccharide, sucrose was able to sustain the growth of the plant cells for a much longer period. This might possibly explain the reason on why sucrose had the highest increment of fresh weight on day 20, which was during the end of the cultivation. The result in this study showed that glucose treatment gave the highest increment of fresh weight on day 15 (0.4386 ± 0.0120 g/mL), followed by sucrose (0.3817 ± 0.0651 g/mL) and fructose (0.3546 ± 0.0840 g/mL) (Figure 3). The cells treated with fructose gave the lowest yield of biomass might be due to the presence of 5-(hydroxymethyl)-2-furaldehyde (HMF). The presence of this toxic compound, HMF was resulted from the autoclaving of the monosaccharide especially fructose due to the hydrolysis (Büter et al., 1993). Büter et al. (1993) also stated that the autoclaved fructose medium contained higher concentration of HMF as compared to the autoclaved glucose medium. In other words, the autoclaved fructose medium possessed greater inhibitory effect as compared to glucose. Thus, this explained why cells treated with fructose gave the lowest biomass. Moreover, the fructose treatment also induced serious aggregation of cells, which was already observable on day 5. This aggregation phenomenon indicated that the culture was not suitable for the growth of the cells.

In addition, the hydrolysation of monosaccharide (especially fructose) through autoclaving would acidify the medium (Owen et al., 1991). This explained the medium pH on day 0, where, the fructose treatment had the lowest pH, followed by glucose and sucrose treatment. However, these three treatments had higher pH than the expected pH 5.7 on day 0, which normal show a drop of 0.2 to 0.3 unit after autoclaving of the medium. Thus, the result of more alkaline medium might due to the inoculation of the cells into the media since the inoculum contained medium that was more alkaline. As a result, the addition of cells together with its culture medium (alkaline) into the new medium would generate a rise in medium pH. The similar phenomenon was also reported by Felker et al. (1989).

Effect of carbon sources on total soluble protein content and specific activity of peroxidase

The sucrose treated *E. longifolia* cells gained the highest total soluble protein content, followed by glucose and lastly fructose (Table 1). To be exact, the total soluble protein content for the glucose treatment was close approximate to the sucrose treatment, whereas, the total soluble protein content of fructose treatment was far

Table 1. The increment of total soluble protein content of *E. longifolia* suspension culture treated with different carbon sources.

Carbon source	Time (Day)				
	0	5	10	15	20
Sucrose	0.00 ±0.00[a]	0.89 ± 2.30 [a]	-0.70 ± 1.90 [a]	-0.89 ± 2.01 [a]	-1.58 ± 1.23 [a]
Glucose	0.00 ±0.00 [a]	0.71 ± 3.05 [a]	0.01 ± 3.37 [a]	-1.46 ± 1.65 [a]	-1.48 ± 1.72 [a]
Fructose	0.00 ±0.00 [a]	0.15 ± 2.34 [a]	0.07 ± 1.49 [a]	-0.78 ± 2.11 [a]	-1.42 ± 0.92 [a]

Mean with standard deviation, followed by same letter within the same column are not significantly different from each other by Turkey's HSD test ($p < 0.05$).

Table 2. The increment of specific activity of peroxidase of *E. longifolia* suspension culture treated with different carbon sources.

Carbon source	Time (Day)				
	0	5	10	15	20
Sucrose	0.00 ± 0.00[a]	-1299.87 ± 1648.32 [a]	4335.98 ± 5473.57 [a]	-123.45 ± 4198.96 [a]	174.98 ± 251.63 [a]
Glucose	0.00 ± 0.00 [a]	732.11 ± 1080.40 [a]	2520.36 ± 1531.85 [a]	5410.04 ± 1221.43 [a]	1941.84 ± 1674.59 [a]
Fructose	0.00 ± 0.00 [a]	-64.43 ± 5048.09 [a]	2033.19 ± 5244.82 [a]	-320.43 ± 1133.68 [a]	-656.52 ± 1734.53 [a]

Mean with standard deviation, followed by same letter within the same column are not significantly different from each other by Turkey's HSD test ($p < 0.05$).

below them. This indicated that both sucrose and glucose were able to induce the protein synthesis.

According to Hattori et al. (1991), sucrose could induce the storage of the protein in the leaves cells. Besides that, sucrose was also capable of inducing the nitrogen assimilation, which would eventually lead to the induction in protein synthesis.

In specific, sucrose was able to induce the expression of nitrate reductase gene, which produced the nitrate reductase that enabled the reduction of NO_3^+ to NO_2^- (Cheng et al., 1992). On the other hand, glucose could up-regulate the activity of nitrate reduction in the nitrogen assimilation (Aslam and Oaks, 1975). By up-regulating the nitrate reduction, the rate of nitrogen assimilation would be increased and eventually increased the protein synthesis.

Therefore, this might explain why the total soluble protein content in the *E. longifolia* cells treated with glucose was close approximate to the total soluble protein of the sucrose treatment.

The presence of HMF in the media after the autoclaving might contribute to the high specific activity of peroxidase in the glucose and fructose treatments (Table 2). As mentioned earlier, HMF was the toxic compound generated during the hydrolysis of monosaccharide and this toxic compound would increase the oxidative stress of plant cells and lead to high peroxidase activity in the cells.

This was proven by Sokolnik et al. (2009), in which the toxic compounds (heavy metals) used to induce the oxidative stress of tobacco cells led to the production of hydrogen peroxide (ROS).

Effect of nitrogen sources on the growth of suspension cells

By using KNO_3 as the sole nitrogen source, the *E. longifolia* cells achieved the highest yield of biomass on day 15, followed by (KNO_3 + NH_4NO_3) mixture treatment and lastly NH_4NO_3 treatment (Figure 4). Similar result was obtained by Mizukami et al. (1991), where, the callus of *Hibiscus sabdariffa* L. treated solely with NO_3^- had the highest cell growth, as compared to the others which is treated with different ratio of KNO_3 and NH_4NO_3. Furthermore, the usage of NO_3^- as the sole nitrogen source had caused the cells to have the highest carbon source utilization rate from day 10 to day 15 (Figure 5). This large quantity of carbon source was used for the high biomass generation as carbon source was the major source of energy for the multiplication of cells.

Apart from the KNO_3 treatment, NO_3^- was also present in the other treatments. The NH_4NO_3 treatment and (KNO_3 + NH_4NO_3) mixture treatment contained both NO_3^- and NH_4^+ but in different ratio, which were 1:1 and 2:1 respectively. According to few studies done, the higher ratio of NO_3^- to NH_4^+ in the culture medium would help to increase the biomass obtained in the cell cultures (Mizukami et al., 1991; Luciani et al., 2000). The similar result was also observed in this study, whereby, (KNO_3 + NH_4NO_3) mixture treatment had higher increment of fresh weight than NH_4NO_3 treatment. In addition, this result also suggested that the concentration of NO_3^- in the medium would positively affect the growth of the plant cells. Contrary, the increase of NH_4^+ in the medium would negatively affect the growth of the cells. This was further

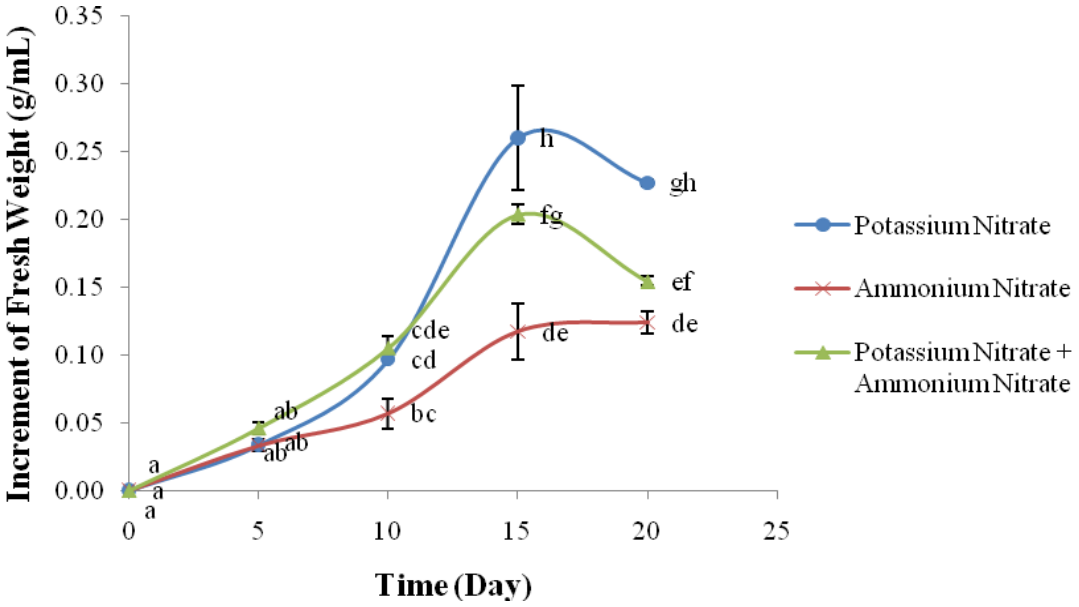

Figure 4. The effect of different nitrogen sources on the growth of the *E. longifolia* suspension culture measured by the increment of fresh weight. Mean with different letter(s) within the same time period are significantly different between treatments by Turkey's HSD test ($p< 0.05$). The error bars indicate the mean ± standard deviation.

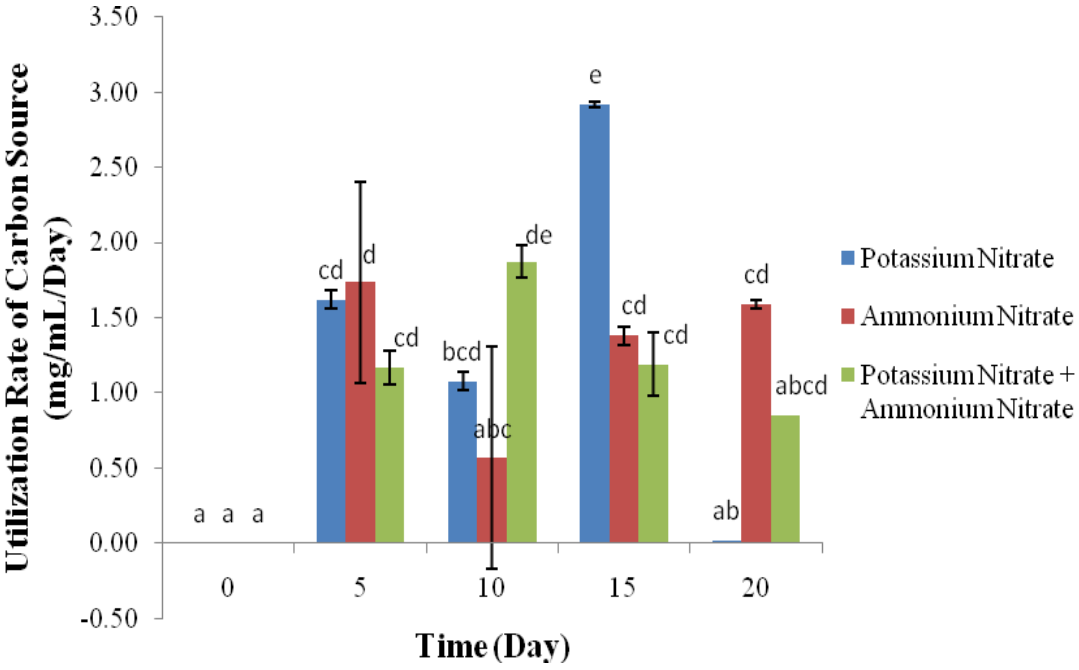

Figure 5. The carbon source utilization rate of the *E. longifolia* suspension culture containing different nitrogen sources. Mean with different letter(s) within the same time period are significantly different between treatments by Turkey's HSD test ($p< 0.05$). The error bars indicate the mean ± standard deviation.

proven by Liu and Zhong (1997), which obtained a similar result by using *Panax ginseng* suspension culture. According to Kaul and Hoffman (1993), NH_4^+ possessed toxic effect toward the plant cells and its accumulation in cells would cause toxicity and inhibit the cell growth. Conversely, NO_3^- was not toxic and could be

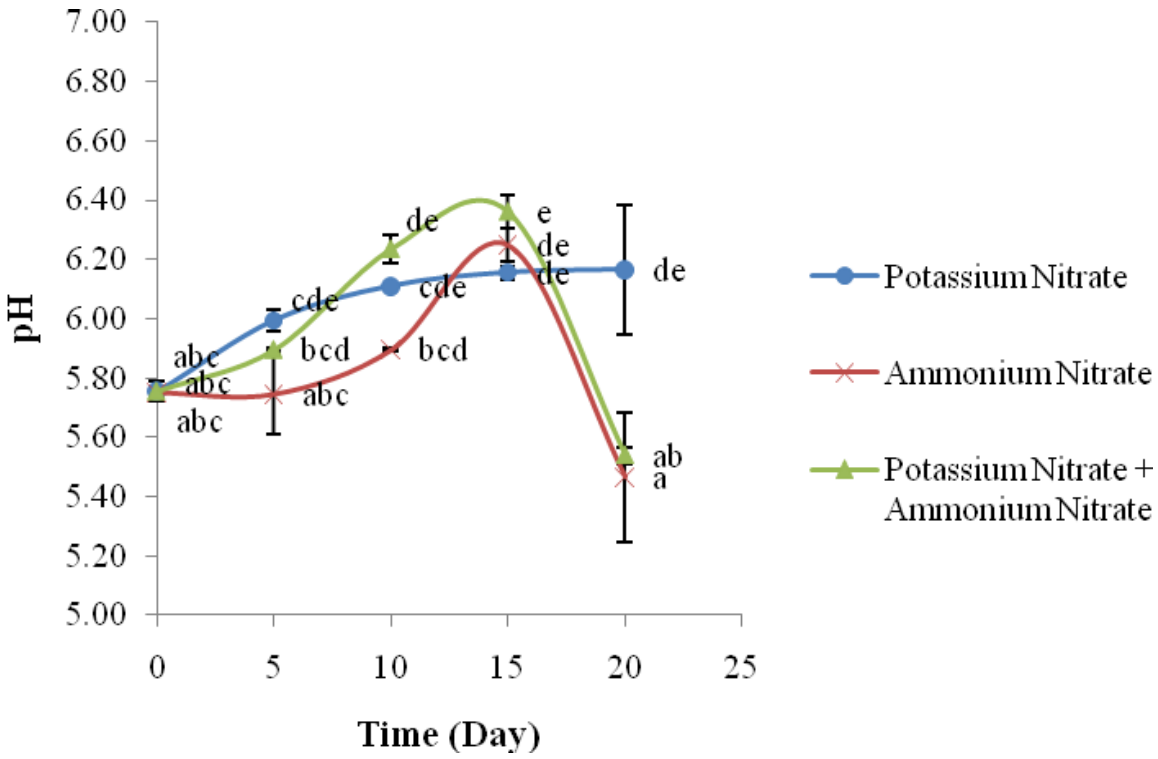

Figure 6. The effect of different nitrogen sources on the pH of the medium for *E. longifolia* suspension culture. Mean with different letter(s) within the same time period are significantly different between treatments by Turkey's HSD test ($p < 0.05$). The error bars indicate the mean ± standard deviation.

accumulated in the cells before the reduction (Franco, 1982).

Even though NH_4^+ would reduce the cell biomass, it was still added into the medium as to counteract the high pH generated during the uptake of NO_3^-. In specific, the uptaken NH_4^+ and NO_3^- would undergo assimilation and generate H^+ and OH^- respectively. The H^+ or OH^- generated would be excreted into the medium in order to maintain the cytoplasmic pH (Kwa et al., 1995). Thus, without the presence of NH_4^+ in the medium, the OH^- (generated from the uptake of NO_3^-) released into the medium would not be able to neutralized and thus led to the subsequent increase in the medium pH, resulting in an alkaline medium (Liu and Zhong, 1997). This phenomenon was obviously observed in the KNO_3 treatment medium, in which, the medium pH was increasing throughout the cultivation period (Figure 6). For the medium containing both NH_4^+ and NO_3^-, the uptake of NO_3^- would begin in the early of cultivation, followed by NH_4^+ when the NO_3^- in the medium was depleted.

Apart from different types of nitrogen sources (NO_3^- and NH_4^+), the presence of K^+ in the medium might also be crucial in contributing to the biomass generation as K^+ was responsible for the plant cell's metabolism by serving as the enzyme system activator (Marschner, 1995). Normally, cell multiplication requires the activation of a large number of enzymes for synthesis of cell wall, proteins and DNA replication, etc. Thus, without the activation of these enzymes, it would negatively affect the growth of the cells and the biomass generation. In specific, the deficiency of K^+ would inhibit the glycolysis and eventually lead to depletion of pyruvate and accumulation of hexoses in the cells (Armengaud et al., 2009). When the glycolysis of the cells was inhibited, the respiration of the cells was also negatively affected. Thus, this might also be one of the reasons why NH_4NO_3 treatment had the lowest increment of fresh weight in this study. In addition, Ben-Hayyim et al. (1987) also stated that any stress that cause the lost of K^+ from the cells would result in decrement of cell growth.

For the K^+ containing treatments, both the KNO_3 treatment (6066.18 mg/L of KNO_3) and (KNO_3 + NH_4NO_3) mixture treatment (263.10 mg/L of KNO_3) had different concentration of K^+. By relating the concentration of K^+ to the increment of fresh weight obtained, it was found that the higher the concentration of K^+, the higher the increment in fresh weight of *E. longifolia* cells. This was further proven by Liu and Zhong (1996), where, they stated that the high concentration of K^+ would positively affect the dry weight of *Panax ginseng* cells until a certain concentration, where, the dry weight would be leveled off.

Table 3. The increment of total soluble protein content of *E. longifolia* suspension culture treated with different nitrogen sources.

Nitrogen source	Time (Day)				
	0	5	10	15	20
Potassium Nitrate	0.00 ± 0.00^{a}	0.12 ± 0.00^{a}	0.00 ± 0.08^{a}	0.12 ± 0.04^{a}	0.62 ± 0.00^{cd}
Ammonium Nitrate	0.00 ± 0.00^{a}	0.95 ± 0.04^{e}	0.77 ± 0.04^{de}	0.38 ± 0.04^{b}	-0.01 ± 0.06^{a}
Potassium Nitrate + Ammonium Nitrate	0.00 ± 0.00^{a}	0.38 ± 0.00^{b}	0.71 ± 0.08^{cd}	0.74 ± 0.13^{de}	0.52 ± 0.06^{bc}

Mean with standard deviation, followed by same letter within the same column are not significantly different from each other by Turkey's HSD test ($p< 0.05$).

Table 4. The increment of specific activity of peroxidase of *E. longifolia* suspension culture treated with different nitrogen sources.

Nitrogen source	Time (Day)				
	0	5	10	15	20
Potassium Nitrate	0.00 ± 0.00 ^{a}	-3691.57 ± 2717.18 ^{a}	-3992.04 ± 2900.39 ^{a}	-4027.41 ± 2903.47 ^{a}	-4031.42 ± 2874.12 ^{a}
Ammonium Nitrate	0.00 ± 0.00 ^{a}	-1567.65 ± 1463.63 ^{a}	-1647.46 ± 1874.12 ^{a}	-1625.91 ± 1625.76 ^{a}	-496.97 ± 1137.49 ^{a}
Potassium Nitrate + Ammonium Nitrate	0.00 ± 0.00 ^{a}	-2560.69 ± 187.53 ^{a}	-2876.48 ± 186.52 ^{a}	-2977.63 ± 259.28 ^{a}	-2918.03 ± 177.49 ^{a}

Mean with standard deviation, followed by same letter within the same column are not significantly different from each other by Turkey's HSD test ($p< 0.05$).

Effect of nitrogen sources on total soluble protein content and specific activity of peroxidase

Nitrogen sources are vital components for the synthesis of protein in plant cells. The uptaken NO_3^- (highly oxidized form of nitrogen) would undergo several reductions to form NH_4^+ (highly reduced form of nitrogen) before being incorporated into amino acid to produce protein (Wiren et al., 2000). On the other hand, the uptaken NH_4^+ would be used for the synthesis of amino acid directly (Cao et al., 2008). Even though the presence of NH_4^+ in the medium would reduce the biomass generated, NH_4^+ was found to assist in the reduction and assimilation of NO_3^- and thus increased the protein accumulation in the plant cells (Bradford and Fletcher, 1982). This phenomenon was able to be observed in this study, in which, the NH_4NO_3 treatment gave the highest total soluble protein content followed by the (KNO_3 + NH_4NO_3) mixture treatment and lastly the KNO_3 treatment (Table 3).

Since NH_4^+ is toxic in nature, its presence in the cells would trigger the oxidative stress in the cells, leading to the increase in production of hydrogen peroxide in the cells (Sokolnik et al., 2009). Thus, this explained the high specific activity of peroxidase in cells treated with NH_4NO_3 and (KNO_3 + NH_4NO_3) mixture treatments (Table 4). In order to avoid the toxicity caused by NH_4^+, the uptaken NH_4^+ had to be incorporated immediately into the amino acids through assimilation (Bradford and Fletcher, 1982). As a result, the cell treated with higher concentration of NH_4^+ would have higher amount of total soluble protein content since the NH_4^+ uptaken was also increased. This could be observed in this study, where, NH_4NO_3 treatment had the highest concentration of NH_4^+ among the treatments also possessed the highest total soluble protein content. In addition, the assimilation of NH_4^+ requires high consumption of carbon source in the cells and the rate of carbon source consumption would determine the rate of assimilation (Raab and Terry, 1994). Thus, the high total soluble protein content obtained on day 5 for the NH_4NO_3 treatment would possibly explain the high utilization rate of carbon source from day 0 to day 5.

Even though the NH_4NO_3 treatment had the highest total soluble protein on day 5, a steady fall occurred for the following days. This might be due to the K^+ present inside the *E. longifolia* cells fell below the critical concentration after five days. Since there was no K^+ present in the NH_4NO_3 treatment medium, there would be no uptake of K^+ and the plant cells had to depend solely on the K^+ originally present in the cell to synthesize protein (Bush and Jacobson, 1986). The protein synthesis caused the K^+ concentration in the cell to fall below the critical concentration, which led to a fall of total soluble protein content for the following days. According to Koch and Mengel (1974), the absence of K^+ in the medium would reduce the protein synthesis over time and cause the amino acid accumulation. In other words,

the low concentration of K^+ in the cells would cause the accumulation of amino acids due to the failure of incorporating the amino acids into the proteins (Leigh and Jones, 1984).

Conclusions

This study gave a general view on the cultivation conditions required in order to maximize the production of biomass in *E. longifolia* cells. The data obtained showed that *E. longifolia* cells possess a high potential for large scale production in bioreactors. As such, its production in several types of bioreactors such as stirred tank bioreactor, airlift bioreactor, bubble column bioreactor and helical-ribbon impeller bioreactor can be further studied and optimized. Furthermore, the protein extracted from the *E. longifolia* cells can be further subjected to two-dimensional gel electrophoresis (2-D electrophoresis) to obtain the protein profile that will provide a better idea on the effect of different treatments towards the protein synthesis in the *E. longifolia* cells.

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