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GROWTH KINETICS AND SECONDARY METABOLITE PRODUCTION OF *EURYCOMA LONGIFOLIA* JACK CELL CULTURE ELICITATED BY UV IN FLASK SCALE AND BUBBLE COLUMN BIOREACTOR SCALE

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***Abstract*— *Eurycoma longifolia* Jack or tongkat ali is a medicinal plant that has been long used in Southeast Asia, including Indonesia. The demand of tongkat ali extract is increasing, therefore the bigger production system is needed. The increment of production required a growth kinetics model hence biomass production and secondary metabolite production can be estimated. In order to enhance the production of secondary metabolites, UV elicitation method could be applied.**

In this study, cell suspension cultures were grown in Murashige-Skoog Media with the addition of 1,1 ppm 2,4-D and 1 ppm kinetin for 14 days. Furthermore, the cultures were exposed to UV for 18 hours daily. The capacities of the culture in this study were 25 mL (flask scale) and 750 mL (bubble column bioreactor scale).

The biomass obtained at the end of the 14th day in control culture was higher than in culture that was exposed to UV. The specific growth rate (μ) of cell cultured in control flask culture was 0.0257 day^{-1} while the specific growth rate of UV-exposed culture was 0.0225 day^{-1}. In the UV-exposed bioreactor, the culture specific growth rate was 0.0556 day^{-1}. The result also showed that pattern of sucrose, mineral, and inorganic compounds consumption followed the growth of cell biomass. The production of secondary metabolite, e.g. canthin-6-one and beta-carboline, were higher in UV-exposed flask culture than control culture. In the UV-exposed culture, the canthin-6-one and beta-carboline contents were 5.12% and 25.11%. While, the canthin-6-one and beta-carboline in UV-exposed bioreactor contents were 6.81% and 29.24%.

***Index Terms*—2,4-D, bubble column bioreactor, *Eurycoma longifolia* Jack, kinetin, UV elicitation**

I. INTRODUCTION

*Eurycoma longifoli*a Jack or tongkat ali is one of the medicinal plants that has been very commonly used in Southeast Asia, including Indonesia. Generally, this plant is used as an aphrodisiac, but it also can be used as a medicine to cure malaria, anti-inflammatory, anti-pyretic, anti-microbial, stomachache, and is still being developed as anticancer. Generally, the chemical compounds, which are beneficial for medicines, are alkaloids and terpenoids [1].

Nowadays, herbal medicine industry which use tongkat ali as raw material is only harvest the source from nature, and the number of industries that are interested in developing the plant is low. If this exploitation continues, the number of tongkat ali will decrease. According to [3], the status of this plant is critically endangered and if this situation keeps on, it is feared that these plants will become extinct. Alternative method to get these metabolites in other ways is needed. Tissue culture technique can be used to solve the problem.

The production of secondary metabolites by *in vitro* culture could be improved in several ways, e.g. elicitation. Elicitation is a treatment given to culture, such as by exposing ultra-violet light, to induce the production of specific metabolites. UV elicitation has been proved able to induce the formation of the enzyme tryptophan decarboxylase on *Catharantus roseus* L. The enzyme plays a main role in the formation of secondary metabolites such as terpene indole alkaloids, or in other words to increase the production of alkaloid compounds [4], as well as in callus culture of tongkat ali [5]. However, the effect of UV light on tongkat ali cell suspension cultures is not known so that more research is needed.

Biomass propagation in the bioreactor is a way to increase the biomass and secondary metabolites production in a larger scale. Scale-up process is done in order to increase the scale of production, thus increasing the possibility to meet the demand for a bio-product. There are several factors that must be considered in the scale-up process, such as: profiles of stirring, the solubility of oxygen in the medium, the exact dimensions of the reactor, the size of the cells used, and many more [6]. In this study, the effect of scale-up of cell suspension cultures of *E. longifolia* in bioreactor conditions and treated by UV light on the growth and secondary metabolite production [7] will be evaluated.

II. MATERIALS AND METHODS

A. Preparation of Callus E. longifolia

Suspension cells used in this study were prepared in advance from callus cultures grown on solid medium. Callus was initiated from *E. longifolia* leaves on solid MS medium with 2.2 ppm 2,4-D 2 ppm and 2 ppm kinetin as growth hormone [5].

B. Propagation and Initiation of Callus Cells

Multiplication of callus was conducted in solid MS medium with sucrose as much as 20 g /L. Growth regulators used in solid culture were 2,2 ppm 2,4-D and 2 ppm kinetin. These cultures were incubated for 30 days at room temperature (25° C).

Cell culture was obtained by transferring the callus from the solid culture into a liquid medium with a composition of MS half-strength and sucrose 25 g / L. Growth regulators used in liquid culture were 1.1 ppm 2,4-D and 1 ppm kinetin. The cultures were incubated at room temperature with stirring or shaking speed of 100 rpm for 14 days, and subcultured into fresh liquid for three times.

C. Secondary Metabolite Production by Suspension Culture Cells in Flask Scale and Bioreactor Scale

Cell suspension culture was subcultured three times and separated by centrifugation at the rate of 250 rpm for 10 minutes. Cells obtained were dried using Whatman filter paper and weighed. Cells were subcultured into fresh liquid medium containing the same composition as the previous media. Inoculum cells in the culture were expected 5 g fresh weight / L. Control cells in cultures grown in dark conditions with agitation rate of 100 rpm. On a bioreactors scale, cell inoculum used was also 5 g wet weight / L liquid medium with the same composition used in liquid culture in flask scale. Aeration rate used was 0.3 vvm. Cell culture, both in the flask and bioreactor cultures were incubated for 14 days. Treated cells were grown in environments with exposure to UV light for 18 hours a day.

D. Harvesting Process

The biomass was separated from the liquid medium by using vacuum filtration. Biomass obtained were weighed and dried at a temperature of 50ºC-60ºC for 24 hours. Then, dried biomass was weighed and extracted using 95% ethanol and incubated for 24 hours. This method was adopted from previous studies [5].

E. Medium Analysis and Secondary Metabolites

Fresh medium and remaining medium were analysed for the content of residual sucrose and salt. The content of sucrose could be analysed using a refractometer. Salt content was analysed by using Eutech Instruments Con-110 conductivity meter. Secondary metabolites were extracted from cells by using 95% ethanol. The content of the extract was analysed using gas chromatography - mass spectra (GC-MS) method.

III. Results and Discussion

Figure 1 showed the specific growth rate of the culture that had been grown for 14 days. Based on calculations, the specific growth rate of control cells in flask was 0.0257 per day and specific growth rate of treated cells in flask was 0.0225 per day. In the bubble column bioreactor system exposed to UV light, cell growth had faster growth rate, with specific growth rate of 0.0553 per day.

Stress caused by UV light due to the excitation of the compounds that were unstable in the cell might form free radicals. Free radicals will produce ROS (reactive oxygen species) such as peroxide (H_2O_2), which is toxic to cells. If the peroxide levels continue to rise, then the cells will die [8]. In addition to generating peroxidase, plant cells also produce metabolites such as alkaloids, terpenoids, and flavonoids in order to reduce the damaging effects of UV [4]. As a result of this mechanism, the cell growth might decrease because the flux of metabolites (displacement compounds involved in a metabolic pathway) involved in the formation of biomass was reduced [9]. This also might be the reason why the culture of cells on UV had a slower growth rate compared to that in control culture.

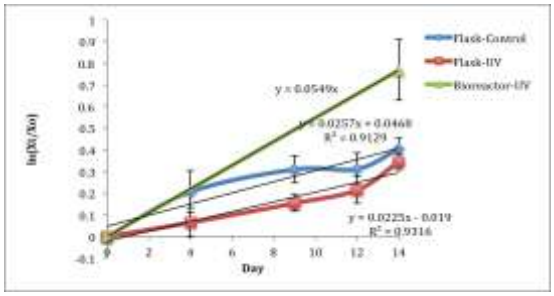

Fig. 1 *E. longifolia* cell culture specific growth rate

Cell cultures grown in the aerated treatment had a higher specific growth rate when compared to the flask culture, it was estimated that the dissolved oxygen concentration in the bioreactor was higher than the levels of dissolved oxygen in the flask. Dissolved oxygen levels in the culture will greatly affect the rate of cell growth. Oxygen is an important substrate in respiration [10]. Respiration is a metabolic process that generates energy for living things including cells. The energy produced from the metabolic processes is also used by living organisms to produce metabolites, which are needed by the cell in order to survive. In addition to survive, this energy is also used to form cell biomass [11].

Figure 2 showed the pattern of mineral uptake of cultures during 14 days of incubation time. All treated culture consumed minerals contained in the medium, therefore the conductivity of three treatments generally decreased. Fluctuation in the flask culture scale occurred at day 12 in the control culture and on day 9 in culture exposed to UV light. Final conductivity was 4.39 mS in control cultures and end conductivity of culture exposed to UV light was 4.43 mS. Consumption of minerals on the bioreactor scale culture was relatively higher compared to culture on flask scale. Medium conductivity was 5.61 mS at the beginning and at the end of the medium conductivity was 4.14 mS.

The decrease of conductivity in the medium showed a consumption of mineral compounds and inorganic compounds which were carried out by the cells. Inorganic compounds, which generally consumed by the cells, include NH^{4+}, NO^{3-} K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, SO_4^{2-}, and PO_4^{3-} [12]. Consumption of mineral compounds and inorganic compounds will be directly proportional to the addition of biomass. The higher increase of biomass, the higher the consumption of mineral compounds and inorganic compounds [13]. However, increase of conductivity occurred on day 12 on the control culture and day-9 on culture exposed to UV light. Not yet known exactly what factors lead to an increase in the conductivity of the medium. However, based on several studies conductivity increase occurs in the stationary phase or when cultures are in a state of stress. Thus, the increase of conductivity in the medium was suspected because of the presence of cell death [12, 13, 14, 15].

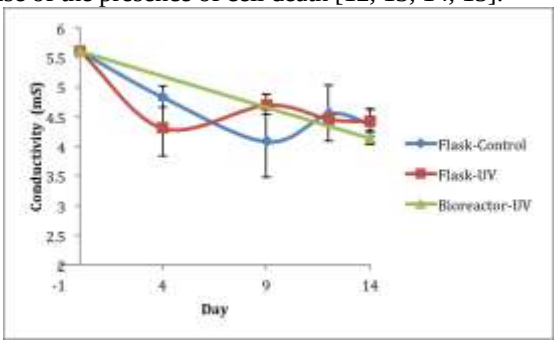

Fig. 2 Medium conductivity of *E. longifolia* cell culture

Sucrose consumption patterns in control culture, UV light exposed culture, and bioreactor scale culture of *E. longifolia* cell suspensions were shown in Figure 3. Sucrose consumption rate of control cell suspension culture was higher when compared to cell suspension culture exposed to UV. Control cell suspension culture had sucrose consumption rate of 0.56 g / (L.day) while the cell suspension culture exposed to UV light had the sucrose consumption rate of 0.43 g / (L.day). The rate of sucrose consumption in bioreactor scale was 0.18 g / (L.day).

The consumption rate of sucrose in the control culture was higher when compared to the treated culture, but this difference did not show a statistically significant difference. The consumption rate of sugar may correlate with the rate of biomass formation. Sucrose is used as carbon source for cell biomass so by increased the weight of biomass, the sugar in the medium would decrease [10]. The sucrose consumption rate in the culture grown in bioreactor treatment was lower when compared to the consumption rate of sucrose in the flask culture. In the bioreactor scale culture, the formation rate of dry biomass was lower when compared with the flask scale culture. The lower the addition of biomass, hence the lower the substrate consumption [10].

Table 1 showed the secondary metabolites produced by cell in flask scale culture, while Table 2 showed the secondary metabolites produced by cell in bioreactor scale culture on day 14. In flask scale, treatment with UV light proved to be able to induce the production of terpenoid metabolites (squalene and quasinoid) since the beginning of culture incubation. However, the production of alkaloid compounds in UV treatment cells (beta-carboline and Canthin-6-one) was only detected on day 14 with a higher number when compared to control cultures. Increased production of alkaloid compounds might be due to the effect of induced of genes expression that produce secondary metabolites containing N such as alkaloids. Elicitation by UV light induces the gene producing strictosidine synthase enzyme (STR) and tryptophan decarboxylase enzyme (TDC) in *C. roseus* [4] which are key enzymes in the production of secondary metabolites containing N such as Canthin-6-one and beta-carboline. Through UV elicitation, enzyme production is enhanced resulting in the higher production of secondary metabolites when compared to control cultures [4].

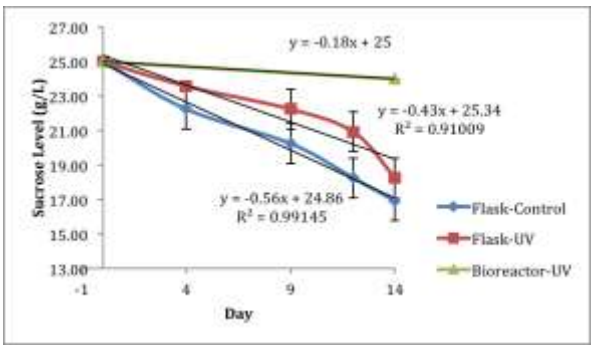

Fig. 3 Sucroce level in *E. longifolia* culture medium

TABLE 1 Secondary Metabolites of *E. longifolia* Culture in the Flask Scale

Day	Metabolites in Control Culture	Amount
9	Eurylactone	3.75%
14	Canthin-6-one	2.67%
14	Squalene	3.50%
Day	**Metabolites in UV Exposed Culture**	**Amount**
4	Squalene	4.38%
9	Squalene	8.04%
12	Quasinoid	4.03%
14	Canthin-6-one	5.12%
14	Beta-carboline	25.11%

TABLE 2 Secondary Metabolites of *E. longifolia* Culture in Bioreactor Scale on Day 14

Metabolites in Bioreactor Scale Culture	Amount
Canthin-6-one	6.81%
Beta-carboline	29.24%

Culture in the bioreactor scale produced metabolites similar to metabolites produced in flask scale but in higher number. This was presumably because the flux metabolites of the culture grown in the bioreactor was higher when compared with the flux metabolites of the flask scale. Increased flux metabolites were apparently due to the energy produced from the respiration by the culture in bioreactor scale was higher than the culture in flask. Increased respiration energy was due to the intake of oxygen in the bioreactor [10]. At the beginning of the culture period, metabolite flux was directed to the formation of biomass. However, after day 14, most of the flux of metabolites might allegedly be diverted to the production of secondary metabolites such as beta-carboline alkaloids and Canthin-6-one. The higher flux of metabolites were directed to metabolite production pathways, which then related to the higher the production of metabolites [9]. This might explain the production of secondary metabolites of alkaloids in the bioreactor, which was higher when compared with the culture in flask.

IV. Conclusions

E. longifolia cell cultures that were grown in bioreactor had a faster growth rate than in the flask culture. UV light inhibits the growth of *E. longifolia* cell culture. Nevertheless, the growth rate between control cultures and treated cultures showed no statistically difference. Substrate consumption either sucrose or mineral salts follows the pattern of biomass formation. The faster the rate of biomass formation, the faster the rate of sucrose and mineral salts consumption. In addition, elicitation with UV light increases the production of secondary metabolites in both the flask scale culture and bioreactor scale culture.

References

[1] Bhat, R., & Karim, A. (2010). "Tongkat Ali (*Eurycoma longifolia* Jack): A Review on Its Ethnobotany and Pharmacological Importance." *Fitoterapia*. 669–679.

[2] Pribadi, E. (2009). "Pasokan dan Permintaan Tanaman Obat Indonesia Serta Arah Penelitian dan Pengembangannya." *Perspektif* , 8, 52-64.

[3] Sudiarto, Pribadi, E. R., Rahardjo, M., Nurhayati, H., Rosida, S. M., & Yusron, M. (2002). "Strengthening Farmer-industry Linkage for Sustainable Utilization of Medicinal Plant Resources." *International Conference on The Modernization of Traditional Chinese Medicine*. Chengdu, China.

[4] Ouwerkerk, P. B., Hallard, D., Verpoorte, R., & Memelink, J. (1999). "Identification of UV-B light-responsive Regions in the Promoter of the Tryptophan Decarboxylase Gene from *Catharanthus roseus*." *Plant Molecular Biology*, 41 (4), 491-503

[5] Parikrama, R., & Esyanti, R. (2014). "Effect of UV Elicitation on Callus Growth, Alkaloid and Terpenoid Contents in *Eurycoma longifolia* Jack." *Int'l Journal of Advances in Chemical Eng. & Biological Sciences*, 12-15

[6] Taticek, R. A., Young, M. M., & Legge, R. L. (1991). "The Scale-up of Plant Cell Culture: Engineering Considerations." *Plant Cell, Tissue and Organ Culture*, 24, 139- 158.

[7] Rahmalia, A. (2011). "Evaluasi Pengaruh Berat Inokulum Agregat Sel *Eurycoma longifolia* Jack. pada Bioreaktor Tipe

Bubble Column untuk Biosintesis Alkaloid dan Terpenoid." Bandung: Institut Teknologi Bandung.

[8] Ramani, S., & Chelliah, J. (2007). "UV-B-induced Signaling Events Leading to Enhanced-production of Catharanthine in *Catharanthus roseus* Cell Suspension Cultures." *BMC Plant Biology*, 1-17.

[9] Stephanopoulus, G., Aristidou, A., & Nielsen, J. (1998). *Metabolic Engineering: Principles and Methodologies*. Massachusetts, United States of America: Academic Press.

[10] Shuler, K. L., dan Kargi, F. (2002). *Bioprocess Engineering Basic Concepts Second Edition*. New Jersey, USA: Prentice Hall PTR.

[11] Schlatmann, J., Moreno, P., Vinke, J., ten Hoopen, H., Verpoorte, R., & Heijnen, J. (1994). "Effect of Oxygen and Nutrient Limitation on Ajmalicine Production and Related Enzyme Activities in High Density Cultures of *Catharanthus roseus*." *Biotechnology and Bioengineering*, 44, 461-468.

[12] Taya, M., Hegglin, M., Prenosil, J., & Bourne, J. (1989). "On-line monitoring of cell growth in plant tissue cultures by conductometry." *Enzyme Microb. Technol.*, 11, 170-176.

[13] Hahlbrock, K. (1975). "Further Studies on the Relationship Between Rates of Nitrate Uptake, Growth and Conductivity Changes in the Medium of Plant Cell Suspension Cultures." *Planta*, 311-318.

[14] McDonald, K., & Jackman, A. (1989). "Bioreactor Studies of Growth and Nutrient Utilization in Alfalfa Suspension Cultures." *Plant Cell Reports*, 455-458.

[15] Chen, H., & Chen, F. (2000). "Effect of Yeast Elicitor on the growth and Secondary Metabolism of High-Tanshinone-Producing Line of the Ti Transformed Salvia miltiorrhiza Cells in Suspension Culture." *Process Biochemistry*, 837-840.