

Effect of Immobilization on Cell Growth and Alkaloid Contents in Cell-Aggregate Culture of *Eurycoma longifolia* Jack

Putri Reno Galih, and Rizkita Rachmi Esyanti

Abstract—Cell immobilization techniques by entrapping cell-aggregate in alginate polymer is commonly used to increase secondary metabolites content in cell culture. This study was carried out to evaluate the effect of immobilization on growth of cell-aggregate and to increase the content of alkaloid in cell-aggregate culture of *Eurycoma longifolia*. Cell-aggregate of *Eurycoma longifolia* was immobilized in 2.5% of alginate polymer for 20 days. The results showed that growth of immobilized cell-aggregate was lower. GCMS analysis showed that immobilized cell-aggregate produced canthin-6-one alkaloid 2.09 times higher and 4H-imidazol-4-one alkaloid 1.82 times higher compared to unimmobilized culture. The results of protein analysis with SDS-PAGE showed that a protein weighted ± 40 kDa was detected on gel, which was probably a putative strictosydine-synthase, an enzyme which has a role in alkaloid synthesis.

Keywords—alginate polymer, *Eurycoma longifolia* Jack, immobilization, secondary metabolites.

I. INTRODUCTION

INDONESIA is known as a megabiodiversity country, that includes a variety of medicinal plants. Exploitation of the plant was caused by its secondary metabolite content, forming a bioactive compound with medical effects. These secondary metabolites can be used as an alternative for drug development since it is more easily accepted by the body than synthetic drugs [1]. Research in the field of herbal medicine is still focused on the identification and isolation of the bioactive components that responsible for generating medical effects, but not much devoted to the production of secondary metabolites itself.

One of the herbs that have many medical properties is *Eurycoma longifolia* Jack, commonly known as Pasak Bumi in Indonesia. *Eurycoma longifolia* is known for its properties as an aphrodisiac agent [2], anti-malaria [3], and anti-cancer [4]. Various medical properties of *E. longifolia*, make this plant heavily exploited for their secondary metabolites used in the pharmaceutical industry. Harvesting root of *E. longifolia* in a large-scale can result in a decline of the wild population [5], while the distribution of this plant in Indonesia is limited only in Kalimantan and Sumatra [6].

One alternative method in secondary metabolite production

is using *in vitro* culture techniques. This method can be used to produce a high commercial value of secondary metabolites that are difficult to obtained conventionally. Controlled environmental condition in *in vitro* system can increase the cell proliferation rate. Some of secondary metabolites were also reported to accumulate higher in *in vitro* condition [7]. *In vitro* methods can also be used to increase the desired secondary metabolites production, as an example by adding a precursors, elicitation, medium optimization, and immobilization. Cell immobilization itself is a physical entrapment method or restrictions on cell movement in a certain part of the culture to obtain a desired cell activity [8], in this case is secondary metabolites. Podophyllotoxin production in immobilized *Juniperus chinensis* cells using alginate polymer increased up to 96-98% compared to suspension culture without immobilization treatment [9]. Immobilization in *Catharanthus roseus* cell-aggregate with alginate polymer could also increase the content of Catharantine up to 248% [10]. From these studies, it was also observed inhibition of cell growth in immobilized culture.

This study used alginate polymer to trap the cell-aggregate of *E. longifolia*. Alginate polymer can inhibit cell growth and nutrients flow rate which become an abiotic stress that causes the cell to form a defense mechanism in the form of secondary metabolite production. This study was carried out to evaluate the effect of immobilization on growth of cell-aggregate and to increase the content of alkaloid in cell-aggregate culture of *Eurycoma longifolia*.

II. MATERIALS AND METHODS

A. Callus Preparation

This study used *E. longifolia* callus culture which has been grown, maintained, and adapted into *in vitro* condition. Callus was maintained in solid Zenk medium added with 0.5 ppm NAA and 0.5 ppm kinetin. Calli were then subcultured three times into liquid Zenk medium added with 0.5 ppm NAA and 0.5 ppm kinetin to obtain cell-aggregate culture.

B. Immobilization Treatment

Cell-aggregate were separated from the medium and treated/ trapped in 2.5% Na-alginat medium. The cells were then transferred into a solution of 50 mM CaCl₂ medium to produce small particles like beads, hereinafter referred to as immobilized cells. Immobilized cells in 50 mM CaCl₂ medium was allowed to stand for 20 minutes, then the solution was discarded. The 25 mM CaCl₂ medium was then added couple of times to wash the immobilized cells. Immobilized

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cells were incubated in liquid Zenk medium with the addition of 0.5 ppm NAA and 0.5 ppm kinetin at room temperature, in 120 rpm agitation. Immobilization treatment was conducted for 20 days. The control group was prepared for comparison. Immobilized cells culture as the treatment group and aggregate culture as control group were harvested every 4 days by sampling the cell-aggregate, medium, and alginate polymer.

C. Biomass Growth

E. longifolia callus dry weight was measured every 4 days, until the 20th day. Callus dry weight data were presented in growth curve and statistically analyzed by SPSS program, using one-way ANOVA (analysis of variance) and Duncan test, with 95% of confidence level to find out the significances of different treatment group (immobilization treatment and control)

D. Histochemical Analysis

Cells structure and secondary metabolites content of immobilized group and control were analyzed by using histochemical method. This analysis was performed using Dragendorff reagent for alkaloid test. Cells histochemical slides were observed with inverted microscope using 40x objective lens.

E. Secondary Metabolites Content Analysis

Cell-aggregates, medium, and alginate polymer from immobilization group and control were harvested on 4th, 12th, and 20th day to analyze its secondary metabolites content. Three replicate samples of cell-aggregates, medium, and alginate polymer from immobilized treatment as well as cell-aggregate and medium of control were extracted. Secondary metabolites extraction was performed by dissolving freeze-dried callus with 95% ethanol, then incubated overnight. The extract was then filtered by Whatman no. 1 filter paper to obtain fine secondary metabolites extract. Extract was analyzed by gas chromatography-mass spectrometry (GC-MS) method.

F. Protein Analysis

Cell-aggregates from immobilization group and control were harvested on 4th, 12th, and 20th day (out of 20 days of study) to analyze protein. A total of 1 gram samples of cell-aggregate were frozen and crushed with liquid nitrogen. Samples were then precipitated with 15 ml of solution trichloroacetic acid (10% w/v) with β -mercaptoethanol (2% v/v), followed by dissolving in acetone for 16 hours at a temperature of -20^oC. Samples were then centrifuged for 30 minutes at 5000 x g at 4^oC temperature. Supernatant was then removed and added to 10 mL of acetone, then centrifuged for 10 minutes. Samples were then added to 250 mL of buffer solution to obtained total protein extract. Samples protein value was determinate by Bradford method [11] in 595 nm wavelength. Determination of the amount of protein concentration was done by interpolating the absorbance of the sample with a standard curve using bovine serum albumin protein (BSA) at a concentration of 0.1 to 1 mg/ml at a wavelength of 595 nm.

Protein analysis was performed by 12% sodium dodecyl

sulfate method-polyacrylamide gel electrophoresis (SDS-PAGE) method. Protein samples [0.5 mg (w/v)] were injected into separating gel, then electrophoresis was performed with 100 W for two hours. Thermo Scientific Multicolor Broad Range Protein Ladder no. 26634 was used as protein marker to compare protein bands. Gel electrophoresis results were stained with silver staining method by Sambrook [12]. Analysis was performed by comparing protein bands from *E. longifolia* cell-aggregate with protein marker bands.

III. RESULTS AND DISCUSSION

The results (Fig.1) showed that the pattern of growth curve of immobilized cell resembled the control group, forming a sigmoid pattern. During 0-4th days, the biomass of cell-aggregate increased slowly. Cell-aggregate was then enter the logarithmic phase on 4th to 16th day as indicated by a rapid increase in biomass. In this phase the cells had been adapted to the environment and the absorption of nutrients was in an optimal point [13], then the cell biomass decreased from 16th to 20th day. This suggest that the cells begin to enter a senescence phase due to decreasing nutrients in medium as well as increasing the waste products of metabolism that are toxic to the cells [14] [15]. The maximum growth of immobilized cells could not be determined yet due to linearly and slowly but continually increasing of biomass until the end of study (20 days of study). Growth curve in Fig. 1 also showed that the growth of immobilized cell tended to be lower compared with control. Based on One Way ANOVA statistical test there was a significant difference between control and immobilization cell dry weight in the overall daily observations.

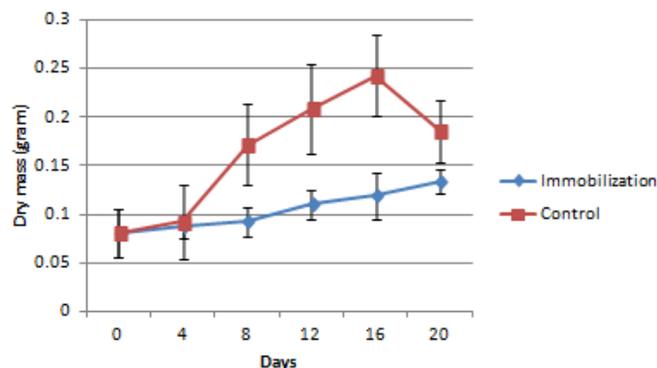


Fig.1 Growth of *E. longifolia* cell-aggregate in 20 days

Based on biomass analysis, immobilization treatment could inhibit cell growth of *E. longifolia*. This was also supported by previous research by Wulandari [9] which reported that immobilized cells of *Catharanthus roseus* showed lower biomass growth compared to control. According to Dornenburg [16], there will be time differences to achieve maximum growth between control and immobilized cells. This was also observed in this study that the control group reached its maximum growth at 16th day, whereas the immobilization group tend to increase slowly until the 20th day.

According to Cooksey [17], immobilized cell were in a pseudo-resting state phase, a state in which the cells did not grow logarithmically, which might be due to decreasing rate of nutrients and oxygen diffusion from the medium into the

entrapped cells. Alginate polymer restrain the movement of the cells and nutrient transport. With a high cell density and limited nutrient inside the immobilization matrix, it will suppress the cell growth so the cells do not grow logarithmically [8]. This is the main purpose of immobilizing cells, as the cells were in a slow-growth phase, the production of secondary metabolites can be improved [18].

Histochemical analysis was conducted by adding a Dragendorff reagents to detect the presence of alkaloid compounds in cell-aggregate of *E.longifolia* qualitatively, and it showed positive results, which revealed that both cell-aggregate group (control and immobilization treatment) produced alkaloids (Fig. 2). This analysis also showed that positive colour intensity was denser in immobilized cell than control cell (more brown) (Fig. 2). The use of NAA and kinetin as plant growth regulators as well as the implementation of immobilization method might increase the alkaloids content significantly.

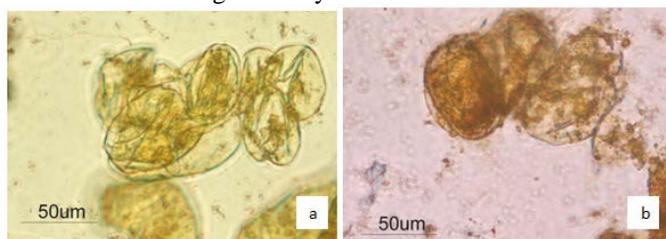


Fig. 2 Histochemical analysis results, (a) control group, (b) immobilization group with brown colour as a positive results of alkaloids.

According on previous study from Dornenburg [16], the stress reaction during immobilization procedure would improve the production of secondary metabolites. The flow of nutrients and energy that supposed was used for growth were shifted to biosynthesis to produced certain secondary metabolites. On immobilized cell of *E. longifolia*, it was focused on the alkaloid biosynthetic pathway. Confirmation of this results was carried out by further analysis using GC-MS.

GC-MS was performed on cell-aggregate, medium, and alginate polymer and the results showed more than 100 different compound. Some of them were alkaloid compounds that have medical properties, such as canthin-6-one and 4H-imidazol-4-one (Fig. 3). These alkaloids were the main compound that accumulated and evenly dispersed in all samples tested by GC-MS. Other alkaloid compounds were also detected in a small percentage, which were pyrrolidine, 3-amino-2-oxazolidinone, 2-4-diamino-6-piperidino pyrimidine, 2-acetyl-1-pyrroline, and 3-(2-piperidinoethyl)-indole.

Canthin-6-one was an alkaloid compound that was produced at most on immobilized cell-aggregate on 12th day which was 37.86%, while control group was 1.09%. Control group reached its maximum content of canthin-6-one on 20th day, which was 19.67%. Canthin-6-one belongs to monoterpene indole alkaloid, and generally has a function for cancer chemotherapy, anti-hypertensive, and anti-neoplast [19]. Earlier studies by Kanchanapoom *et al.* [20], Siregar [21], and Esyanti [22] also confirmed the existence of canthin-6-one in *E.longifolia* culture.

4H-imidazol-4-one also belongs to alkaloid group and was produced at most on immobilized cell-aggregate on 20th day

(58.82%). The control group also produced maximum content of 4H-imidazol-4-one on 20th day (33.72%). Immobilized group produced higher concentration on each day of sampling, in contrast to the control group that had 4H-imidazol-4-one decreased on 12th day. 4H-imidazol-4-one is an imidazol derivative compounds which are known to have a broad pharmacological activity, such as anti-inflammatory, anti-fungal, anti-neoplast, anti-helminthik, anti-viral, anti-ulcer, and anti-cancer [23]

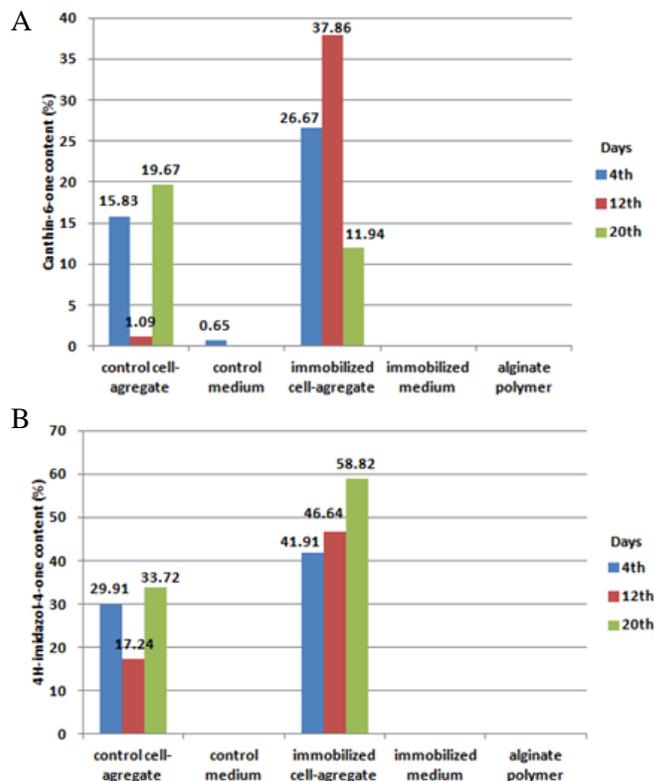


Fig. 3 Alkaloid production from cell-aggregate culture; A (canthin-6-one); B (4H-imidazol-4-one)

Both alkaloid compounds that found in this study was produced in cell-aggregate and very little or not at all found in the medium or alginate polymer. Alkaloid compounds are generally alkaline, and is usually stored in the protonated form inside vacuola which has an acidic properties in physiological pH. The alkaloids that has been protonated will be difficult to penetrate out from tonoplast, so the alkaloids will stay to accumulate inside the vacuole [24]. This phenomenon was confirmed by Wink [25], that the alkaloids were hydrophilic and stored in the vacuole. Based on this theory, alkaloid compounds will accumulate in cell-aggregate and may not penetrated out into the medium or alginate polymer, unless in the acidic medium [26]. Quantitative test results by GC-MS showed that immobilization treatment could increase the production of alkaloids. Thus, it clarified the previous histochemical analysis stating that the positive colour intensity was denser on immobilized cell than in control cell.

Protein analysis using SDS PAGE was performed to determined the protein profiles of control and immobilized cell-aggregate on each day of sampling (4th, 12th, 20th day). The results showed that there were ± 25 kDa and ± 40 kDa

molecular weight protein band (Fig. 4). Protein with molecular weight of ± 25 kDa was predicted as a typical Simaroubaceae protein, which was also found in previous studies from Rahmalia [27] and Parikrama *et al.* [28]. Baycu *et al.* [29] also found a typical protein weight 22.9-24.1 kDa in each sample of *Alianthus altissima* and *Alianthus excels* that also belongs to Simarobaceae. Protein at a molecular weight of ± 40 kDa was suspected as strictosydine synthase enzyme that had a role in alkaloids biosynthesis, especially in indole alkaloid group [30], in this study it was canthin-6-one and 4H-imidazol-4-one.

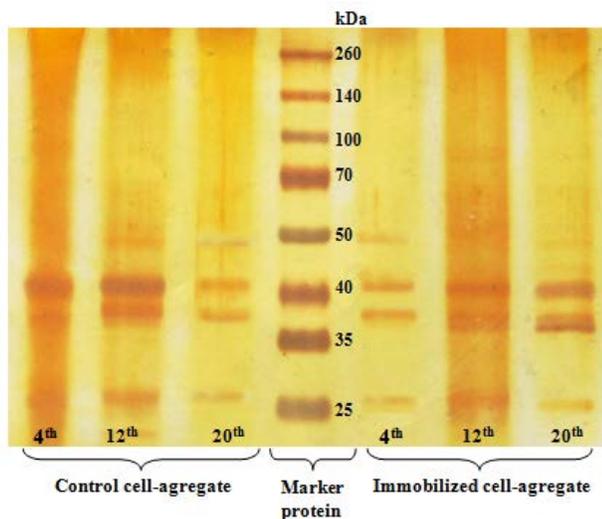


Fig. 4 SDS PAGE analysis results

IV. CONCLUSION

Based on the results of this study, immobilization that was conducted on cell-aggregate of *Eurycoma longifolia* could inhibit cell growth. Production of alkaloids from *E. longifolia* cell-aggregate culture, particularly canthin-6-one and 4H-imidazol-4-one can be enhanced by using immobilization method of trapping cell-aggregate in alginate polymer and reached a maximum percentage (37.86% and 58.82% respectively) on days 12th and 20th during 20 days of study.

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