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Effect of Aeration and Inoculum Density on Biomass and Saponin Content of *Talinum Paniculatum* Gaertn. Hairy Roots in Balloon-Type Bubble Bioreactor

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ABSTRACT

Hairy roots have same or greater biosynthetic capacity for secondary metabolite production compare to their mother plants. *Agrobacterium rhizogenes* strain LB 510 has been known its ability to induce hairy roots of *Talinum paniculatum* from leaf explants. Cultivation of *T. paniculatum* hairy roots on MS medium in balloon-type bubble bioreactor(BTBB) under various aeration rates and inoculum densities were investigated in this research. Hairy root cultures on various aeration rates 0,25; 0,5; and 0,75 vvm had same inoculums density (2.5 g/L) and cultivated for 28 days. Biomass and saponin content at the end day of cultivation increased and higher than control (culture had no aeration). Saponin content in this research were represented by wide of saponin spot/0,1 g dry weight sample. Maximum biomass 0,93 g (dry weight) and saponin content 1,37 cm^2/0,1 g were obtained by culture at aeration rate 0,25 vvm. At hairy root cultures on various inoculums densities1.25; 2.5; 3.75 dan 5 g/L had same aeration rate (0,25 vvm) shown increasing of biomass and saponin content. The maximum biomass (1,91 g) and saponin content was achievedat inoculums density 5 g/L. Culture at inoculums density 1.25 g/L had maximum growth rate (0,057 g/day) and the other had relatively same (0,021-0,023 g/day). This result indicates that inoculums density at 5 g/L and aeration rate at 2 vvm were the best condition than others for biomass and saponin content.

KEY WORDS*:* aeration rate, inoculums density, *Talinum paniculatum*, hairy root, balloon-type bubble bioreactor

INTRODUCTION

Most of the pharmaceutical compound from plants are secondary metabolite that nonessential on the growth plant, produced in a small amount, and almost was accumulated in the special tissue, like tricome. Secondary metabolites usually have a complex structure, so organic synthesis was not effective, especially in cost. Extraction from a part of plant has become a main method for production of secondary metabolite until this era [1][2].

Hairy root culture haspotency as an alternative method to produce pharmaceutical compound in large scale. One of the advantages of hairy root culture is it has biosynthetic capacity same as or more than production of secondary metabolite from mother plant [3][4]. Cultivation of hairy root in large scale still need improved in various aspects [5].

Aeration in liquid culture has function as an oxygen supply. Oxygen transfer usually limited the work of biological system, because the limited dissolved oxygen in water. If the oxygen limited, cell growth and production of secondary metabolite will be influenced[6]. Inoculums density is a necessary parameter that influence on performance of cell culture. When the inoculums density is low, cell growth also low [5].It was known that when the inoculums density is high, culture has no lag phase period and cell growth became higher [7], and increasing of secondary metabolite also can get by increasing the inoculums density or using certain medium[8].

In Indonesia, especially in Java, java ginseng was used as a traditional medicine for diarrhea, antisepsis, aphrodisiac and improve vitality. Phytochemistry analysis of java ginseng showed that it contain saponin, triterpen or steroid, polifenol and essential oil [9]. Root extract of java ginseng can improve mice libido higher than root extract of Korean ginseng in the condition of low testosterone [10]. The aims of this research are to know the effect of aeration and inoculum density on the biomass and saponin content in hairy root culture of java ginseng (*Talinum paniculatum* Gaertn.) in balloon-type bubble bioreactor (BTBB).

MATERIALS AND METHODS

Hairy Root Culture

Agrobacterium rhizogenes LB510 was gotten from Research Center of Biotechnology Indonesia. Bacteri was cultivated in Luria Bertani (LB) medium in the rotary shaker incubator at 28^{o}C, 110 rpm for 2 days. Leaf explants of*T. paniculatum*were sterilized with 10% Clorox for 5 minutes, then the explants were submerged in

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the bacterial suspension and MS medium [11] free hormon (1:10) for 5 min. Acetosyringone 100mM was added to the medium. Leaf explants were dried on the sterile paper, then were planted in the Murashige and Skoog free hormone solid medium and were cultivated at 28°C without light for 2 weeks. Hairy root will be formed in the side of the leaf explants. Hairy roots were excised about 2-5cm and used as explants for liquid culture in balloon type bubble bioreactor.

Hairy Roots Culture in Balloon-Type Bubble Bioreactor

MS liqiud medium (0.4L) was added in Balloon-type bubble bioreactor (BTBB) with capacity 1 L [12] and sterilized on the autoclave at 121°C, for 15 min. Four BTBB were treated with different aeration rate, 0, 0.25 vvm, 0.5 vvm, and 0.75 vvm (volumes of gas per volume of liquid per minute) and the inoculums density was 2.5 g/L. Cultures were cultivated at 28°C, without light for 28 days. Best result of this experiment (highest biomass and saponin content) will be used in the next experiment. In the next step experiment, three BTBB were treated with different inoculums density, 1.25 g/L, 2.5 g/L, 3.75 g/L and 5 g/L. Cultures of hairy root were cultivated at 28°C, without light for 28 days and the pH, conductivity (Ezodo Cond521) and total sugar (hand refractometer Atago Master 10T) were measured every week. At the end of cultivation, biomass of the hairy roots was measured (fresh weight and dry weight).

Measurement of Fresh and Dry Weight of Hairy Roots

Measurement method of fresh weight was done by some step: the hairy roots were filteredand washed by aquades and then was drained at the moment. The hairy roots were ready to weighed [13]. For measurement of dry weight, the hairy roots were air dried for several days until the dry weight constant [14].

Extraction and Analysis of Saponin

For sample preparation, 100 mg of powdered dried of hairy roots were soaked in 10 mL ethanol, and then warmed in waterbath at 80°C for 30 minutes. Extract were saturated in water bath at 80°Cfor 3 hours until the volume was obtained 0.2 mL. Extract and saponin standard were spottedon silica gel GF_{254} and eluted in propanol: water (14:3). The spot was detected by anisaldehide-sulfic acid (Merck) and warmed in the oven at 110°C for 6-10 minutes. The saponin standard (Calbiochem) will give green to black color.

RESULTS AND DISCUSSION

In cultivation medium hairy roots started to form on the leaf explant after 5-7 days. Hairy roots had many root hairs. As an explant in the balloon-type bubble bioreactor, hairy roots were excised to separating from leaf explant and then subculture in semisolid medium. In this medium, the hairy roots still grow and form root hair.

In this research we tried to identify saponin content in hairy root in the different eluent of thin layer chromatography method. Result was shown in Figure 1. This figure showed that the hairy roots contained saponin content, which gave a green spot after spraying with anisaldehide-H_2SO_4. This result proved that source of explant did not influence the hairy root of java ginseng to produce saponin. It was also happened in the hairy root of *Lawsonia inermis* and *Artemisia annua* that produce of lawsone and artemisine in MS medium, whereasthese compounds only has produced in the aerial part of normal plants[15, 16]. Differences of the Rf value of saponin spot between hairy root and saponin standart showed that they were the different kind of saponin. The differences was clearly seen on the result of TLC with chloroform/methanol/water (5:4:1) and propanol/water (17:2) eluent.

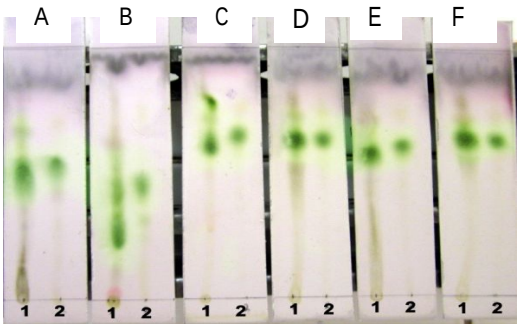

Figure 1. Result of thin layer chromatography of ethanol extract of java ginseng hairy root on silica gel GF_{254} used various eluen. (A-B) chloroform/metanol/water 4:4:1, 5:4:1, (C-F) propanol/water 17:2, 17:4, 14:3, 20:1. (1) saponin standart, (2) ethanol extract of hairy root.

Effect of Aeration Rate on Biomass and Saponin Content of Hairy Root

pH medium of four cultures on the different aeration rate experiment during cultivation were decreased (Figure 2A). In the first week of cultivation, the pH was decreased extremely. During cultivation, decreasing of pH was caused by MS medium that contained ammonium, so the decreasing of pH happened less than 2 weeks. Increasing of pH medium would be happened in culture without aeration (0 vvm) in 2 weeks cultivation, while

the pH medium with aeration rate of 0.75 vvm increased in 4 weeks of cultivation. Increasing of pH medium after decrease extremely was also happened in suspension culture of *Elaeis guinensis*which the pH medium increased from 4.0 to 4.4 in 25 days cultivation [17]. It was also happened in hairy root of *Catharanthus roseus*, which the pH medium increase vastly and the maximum was achieve in 25 days cultivation. Culture medium became alkaline was maybe caused by proton transport ofsymport mechanism to counterbalanceuptake of phosphat ion (PO_4^{3-}) and nitrat (NO_3^-) [18].

Result of conductivity and total sugar measurement in culture medium on the different aeration showed that both of them had increased during cultivation because the evaporation of medium (Figure 2B,C). In the ideal condition, conductivity and total sugarwill decrease in line with time of culture, because the inorganic salt and sugar in the medium are used as nutrient by the explants. It's assumed that increasing of the conductivity and total sugar in medium is caused by highly evaporation medium, especially in the 3 culture with different aeration (0.25; 0.5; and 0.75 vvm). Highly evaporation will saturate inorganic salt and ion in the medium. In the end of cultivation, volume of medium was left 40-50% of first volume, but in the medium without aeration, the decreasing was only 1.5%.

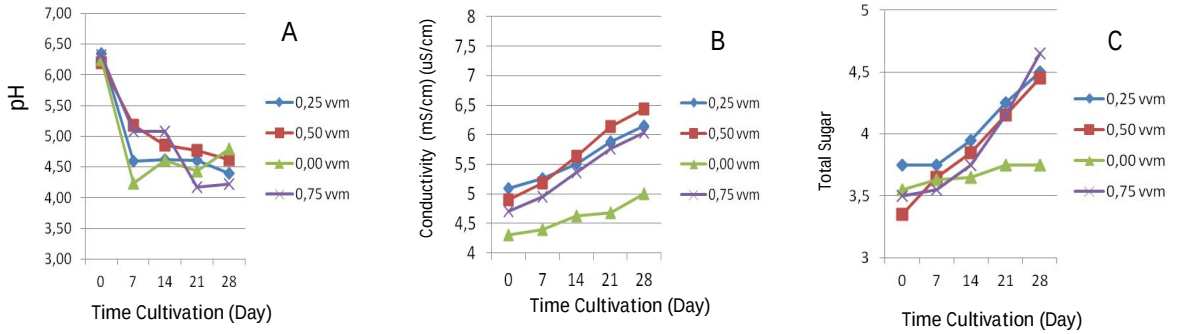

Figure 2. Effect of aeration on (A) pH level; (B) conductivity and (C) total sugar of MS liquid medium during java ginseng hairy root culture in BTBB with inoculums density of2.5 g/L.

Various aeration in balloon-type bubble bioreactor of java ginseng hairy root had a positive effect on the biomass and saponin production. Cultures with various aeration gave biomass and saponin production higher than culture without aeration (Table 1). Saponin content was describedas spot size of saponin/0.1 g of dry weight hairy root.

Table 1. Effect of aeration on biomass and saponin content of java ginseng hairy root for 28 days and inoculums density 2.5 g/L

Aeration (vvm)	Fresh Weight (g)	Dry Weight (g)	TLC spot size of saponin (cm^2/0,1 DW)
0,00	1,03	0,59	1,19
0,25	1,62	0,93	1,37
0,50	1,41	0,82	0,79
0,75	1,38	0,79	1,34

Scale up of cell or tissue culture in bioreactor need aeration to dissolve oxygen supply [19]. Highly aeration rate was known that will destroyon cell culture growth [20]. Impact ofhighly damage was also happended in cell culture growth of *Catharanthus roseus*[21]. Continuously aeration during cultivation in different rate of three treatment of java ginseng hairy roots show the decreasing of biomass and saponin content. Decreasing ofbiomass and saponin content was happened in aeration of 0.5 vvm and 0.75 vvm (Table 1). It was assumed it was caused by CO_2 concentration in the medium and the stress shear of hairy root. Growth explants and secondary metabolite accumulation in bioreactor was influenced by various factor, example stress shear, oxygen supply, and gas composition [5]. In this research, fragmentation of hairy root was happened during cultivation in aeration treatment of 0.5 and 0.75 vvm. Fragmentation was happenedalong with cultivation time, especially in old hairy root which have brown color. Fragmentations of hairy root werealso happened in air lift bioreactor or stirring bioreactor that gave decreasing hairy root production [22]. The best aeration rate in BTBB for biomass and saponin production of java ginseng hairy root for 28 days cultivation was 0.25 vvm.

Effect of Inoculum Density on Biomass and Saponin Production of Java Ginseng Hairy Root

In different inoculums density, the pH were decreased during cultivation, but conductivity and total sugar content wereincreased, so it could not be used as undirect indicator for biomass change (data was not shown). Evaporation of medium was assumed as a cause of the increasing of conductivity and total sugar content during

cultivation. Biomass and saponin content of java ginseng hairy root on the different inoculum density were shown in Table 2.

Table 2. Effect of inoculum density on biomass and saponin production of java ginseng hairy root

Inoculum density (g/L)	Fresh weight (g)	Dry weight (g)	Growth rate (g/day)	TLC spot size of saponin (cm^2/0,1 DW)
1.25	1,29	0,93	0,057	1,33
2.5	1,62	0,93	0,022	1,37
3.75	2,39	1,73	0,021	1,67
5.0	3,31	1,91	0,023	4,92

The highest biomass was obtained from the treatment of inoculums density of 2 g/400 mL. The lowest biomass was 0.93 gobtain from inoculums density 1.25 g/L,but the growth rate was same in three treatments, that was 0.02 g/days. Measurement of growth rate was (final fresh weight – inoculums fresh weight) / inoculums fresh weight / days of culture [23]. Growth profil of java ginseng hairy root culture in inoculums density of 1.25/L for 28 days cultivation is shown in Figure 3.

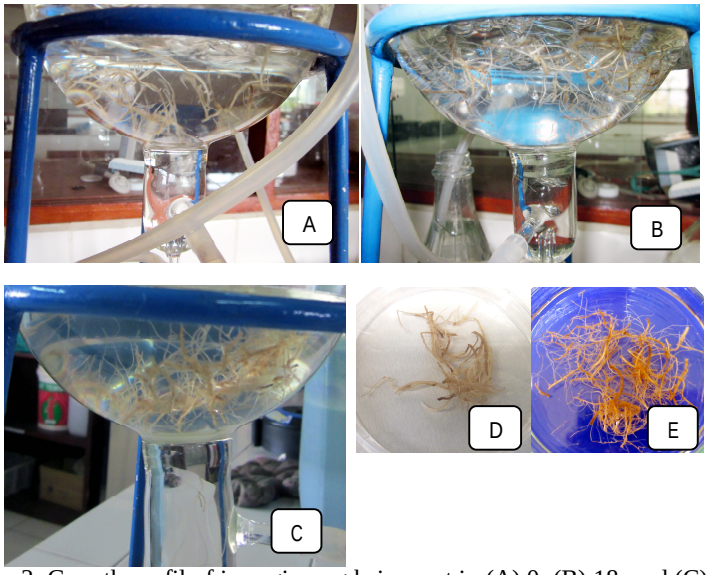

Figure 3. Growth profil of java ginseng hairy root in (A) 0, (B) 18, and (C) 28 days cultivation in BTBB at 0.25 vvm aeration, (D) Hairy root in early inoculums, (E) hairy root after 28 days cultivation

Based on result of growth rate (Table 2) it was indicated that concentration of sucrose in culture medium could not support the need ofculture at inoculums density 2.5, 3.75 and 5 g/L to improve biomass. Concentration of sucrose 30 g/L could support the need of culture at inoculums density of1.25 g/L with high growth rate and biomass multiplication of 2.6 times from initial biomass (fresh weight). Inoculums density more than 1.25 g/L has biomass multiplication 1.6 times from initial biomass. Its means sucrose in medium (30 g/L) not enough to support growth of hairy root culture. This phenomenon showed that content of sucrose have correlation with inoculums density. Carbohydrate, especially sucrose was necessary as a source of carbon and energy for cell. Concentration of initial sucrose influenced any parameter of culture, such as growth rate and content of secondary metabolite in cell culture [24].

Result of thin layer chromatography analysis showed that inoculums density influenced the spot size of saponin (Table 2). The highest spot size was givenby inoculums density of5 g/L. Inoculums density was reported that would significant influence on cell growth, saponin and polysacharide production in cell culture of *Panax notoginseng*[25]. The differences between cell suspension culture and hairy root culture was production bulk of root mass, so it was inhibited the water flow and oxygen supply [26-28]. Differences of inoculums density cause differences of cell density and culture parameter, such as DO and finally influence of cell metabolism that detail of this mechanism did not know [23].

The highest biomass and saponin content was achieved in java ginseng hairy root culture at inoculums density of 5 g/L and aeration rate of 0.25 vvm. This result might become a suggestion in future research to improve biomass and saponin content of ginseng java hairy root in scale up of bioreactor.

Conclusions

The highest biomass and saponin content was achievedby java ginseng hairy root culture at inoculums density of 5 g/L and aeration rate of 0.25 vvm. This result may become a suggestion in future research to improve biomass and saponin content of ginseng java hairy root in scale up of bioreactor.

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