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Optimization of Culture Conditions of *Talinum paniculatum* Gaertn. Adventitious Roots in Balloon Type Bubble Bioreactor Using Aeration Rate and Initial Inoculum Density

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ABSTRACT

Optimization of culture conditions of *Talinum paniculatum* Gaertn. adventitious roots in the balloon type bubble bioreactor have been done in order to increase its production of adventitious roots and saponin content. Culture conditions were used in this research were combination of aeration rate (0.25, 0.5 and 0.75 vvm) and initial inoculum density (0.5, 1, 2 g/400 mL). Bioreactor with a volume of 1000 mL was filled with 400 mL of liquid MS medium supplemented with IBA 2 mg L^{-1} were then given sterile air through a microfilter (0.2 μm) with different air flow rates. Into each bioreactor were added of different inoculum density of adventitious roots that had previously been induced from leaf explants of *T. paniculatum* on solid MS medium supplemented with IBA 2 mg L^{-1}. Cultures were maintained for 14 days and sampling was done for every two days to determine the sugar content, conductivity and pH of the medium. The results showed that the combination of aeration rate of 0.5 vvm and inoculum density of 1 g/400 mL was the best treatment that can increase biomass of adventitious roots, whereas the combination of aeration rate of 0.75 vvm and inoculum density of 2 g/400 mL was the best treatment that can be increased of saponin content.

Key words: *Talinum panicultum* Gaertn., adventitious root, balloon type bubble bioreactor, liquid culture, saponin

INTRODUCTION

Talinum paniculatum Gaertn which is in Indonesia also called java ginseng, has a bulging shape of the roots like *Panax ginseng* root and use as synchronized with *Panax ginseng*, especially its potential in increasing testosterone levels at the low testosterone condition, increasing number and motility of sperm (cell differentiation inductor (induce of sperm viability) and protection of human body from pathogen). In Indonesia, this plant usually was used as traditional medicine. One of the chemical content of the roots of this plant is saponins that are used as aphrodisiac. There are many kind of saponin, like saponin glycoside from *Gynostema penthaphyllum* known as gypnoside that are responsible for its pharmacological activities and ginsenoside from *Panax ginseng* (Yin *et al*., 2014). Since, saponin ginsenoside are the well-known biologically active constituents in Korean ginseng, *T. paniculatum* has also received considerable attention. But java ginseng roots grow very slowly in their natural habitat which is about 2-3 years to get more than

100 g of roots per plant. In addition, it is known that levels of saponins of java ginseng root age of 3 months was lower than the java ginseng roots grown in vitro for 28 days. Because of the necessary compounds in *T. paniculatum* was accumulated in root organ, the root culture technology is essential for the preservation of these plants developed in order to awake and efficacious compounds, especially saponin compounds that was accumulated in the root organs can be improved.

In many decades, adventitious root cultures have been used to produce many kind of secondary metabolites which have naturally in root. The advantages of adventitious root culture are the root not influenced by geotropism, growth of root branch fast and had a genetic stability. Many researches have been conducted to increase biomass and secondary metabolite by adventitious roots in bioreactor, for examples, producing whitanolide-A in adventitious root of *Whitania somnifera* (Praveen and Murthy, 2010; Sivanandhan *et al*., 2012), increasing biomass and secondary metabolite of *Glycyrrhiza uralensis* Fisch (Yin *et al*., 2014), increasing bioreactor capacity to produce biomass and cafeic acid derivatives in adventitious root culture of *Echinacea angustifolia* (Cui *et al*., 2013), production of adventitious root biomass of *Podophyllum hexandrum* Royle (Rajesh *et al*., 2014), *Astragalus membranaceus* (Wu *et al*., 2011), *Stevia reboudiana* Bertoni (Reis *et al*., 2011) and *Panax ginseng* (Wang *et al*., 2013).

Inducing the formation of adventitious roots of *T. paniculatum* using hypocotyls, epicotyls and leaves explants have been succeeded in solid and semisolid MS (Murashige and Skoog, 1962) medium supplemented with IBA 2 mg L^{-1}. But the growth in semi-solid media is limited by the availability of oxygen and culture space. This can be overcome by moving it into a liquid medium in a larger vessel such as flasks or bioreactors. Some of the advantages of liquid culture method in the bioreactor is oxygen demand that can be done by agitation or aeration, the culture space limitations can be overcome by using of the flask or bioreactor and nutrients can be accessed by all parts of the organ cultured. But growth in the bioreactor is influenced by many factors, such as shear stress, oxygen supply and gas composition (Kanokwaree and Doran, 1997).

Gas exchange between the gas and liquid phase is one of the factors that influence the cell and organ culture of plants in a liquid medium. In the bioreactor, strong aeration necessary for the supply of dissolved oxygen and increase the homogeneity of the liquid. Lee *et al*. (1998) reported that the concentration of dissolved oxygen through the aeration was beneficial for root growth and alkaloid production (tropan) in roots cultured of *Atropa belladonna*. In addition to aeration, inoculum density was also reported to affect the performance of cell suspension cultures and increased production of secondary metabolites. If the initial inoculum density was low, the cell growth was also low (Kanokwaree and Doran, 1997). But, if the initial inoculum density is high, the lag phase in cell culture does not occur because of the direct growth to be in the exponential phase. This suggests that increased secondary metabolites along with increased number of cells (Van Gulik *et al*., 1994; Sakurai *et al*., 1996). So, this experiment was conducted to optimization of the culture conditions of *T. paniculatum* Gaertn. adventitious roots in balloon type bubble bioreactor with the treatment of various aeration rate dan initial inoculum density.

MATERIALS AND METHODS

Materials: *Talinum paniculatum* Gaertn. was obtained from the Botanical Garden Purwodadi, Pasuruan, East Java Indonesia which further was nurtured in polybag with mix of soil and organic fertilizer (50:50%) and incubated at room temperature (28-31°C). Adventitious root was obtained from leaf explants of *T. paniculatum* Gaertn.

Induction and proliferation of adventitious roots: Leaves were washed with detergent and then rinsed thoroughly with tap water. Explants were sterilized with clorox 10% (v/v) and soaked for 10 min while gently agitated. After that, clorox solution discarded and rinse 3 times with sterile distilled water. Explants were placed in sterile filter paper in the petridishes and cut 1 cm^2 and then planted in MS medium supplemented with IBA 2 mg L^{-1}, 30 g L^{-1} sucrose and 7 g L^{-1} agar. Cultures were maintained in room culture at 25°C in the dark conditions. After 4 weeks, adventitious root were used for the next stage.

Adventitious root culture in balloon type bubble bioreactor: Adventitious roots obtained from the previous stage were used as inoculum. Balloon Type Bubble Bioreactor (BTBB) was designed by Gupta and Ibaraki (2008). Bioreactor with a capacity of 1 L was filled with 0.4 L of liquid MS medium supplemented with IBA 2 mg L^{-1}, 30 g L^{-1} sucrose and sterilized by autoclaving at 121°C for 20 min. The experiment consisted of 9 cultures that received the combinations of treatment aeration rate: 0.25, 0.5 and 0.75 vvm (volumes of gas per volume of liquid per minute) and the initial inoculum density: 0.5, 1.0 and 2 g/400 mL. The cultures were incubated at room temperature for 14 days and each treatment has 3 replications. Fresh weight and dry weight of adventitious roots and saponin content were analyzed at the end of the cultivation period. To determine the growth of adventitious roots, pH, total sugar content and conductivity of the medium were done. Measurements were done every two days by taking 1 mL of culture medium. Measurement of pH was done using a pH meter (Boeco, BT-600), the total sugar content was measured using a hand refractometer (Atago), while the conductivity was measured using conductometer (Ezdo, Cond5021).

Extraction and analysis of saponins: Saponins contained in adventitious roots were analyzed qualitatively using thin layer chromatography and quantitatively using HPLC. Adventitious roots were dried at 50°C for 5 days and then were grinded used mortar. A 100 mg powder of adventitious roots were immersed in 10 mL of ethanol and then heated 80°C in water bath for 30 min. The extract was then concentrated in 80°C for 3 h until a volume of 0.2 mL was obtained. Extracts and saponins standard (Calbiochem) were spotted on silica gel GF254 and eluted using propanol: water (14:3). Spot were detected by spraying with a mixture of anisaldehyde 0.5 mL, acetic acid glacial 10 mL, ethanol 85 mL, sulfuric acid 5 mL and then heated at 110°C for 6-10 min. Saponin standard gave the dark green colour. Quantitatively measurement of saponin was done using HPLC system (Agilent Q-TOF 6530 L) equipped with c18 columns. The mobile phase was a mixture of reagent 0.1% formic acid in water grade and acetonitrile (40:60, v/v) and the volume inject were 0.2 μL. The detection wavelength was set at 299 nm.

RESULTS

Effect of combinations of treatment of initial inoculum density and aeration rate on adventitious root biomass: Combinations of treatment of initial inoculum density and aeration rate affect the growth of adventitious roots. Growth ratio of adventitious root indicated by root fresh weight at the end of the culture reduced the initial fresh weight (Fig. 1).

The growth of adventitious roots of ginseng java were highest in the combinations of treatment aeration rate 0.5 vvm and initial inoculum density 1 g/400 mL (0.635 g), where as the combinations of treatment aeration rate 0.25 vvm and initial inoculum density 0.5, 1 and 2 g/400 mL also showed high growth (Fig. 1). This is supported by the data of conductivity, sugar content and pH in culture medium for 14 days (Fig. 2).

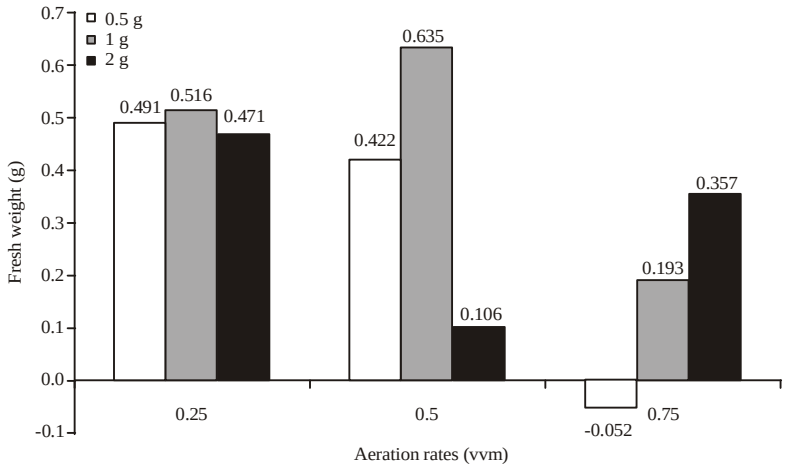

Fig. 1: Growth ratio of adventitious roots on combination of aeration rate and initial inoculum density on 14 days culture

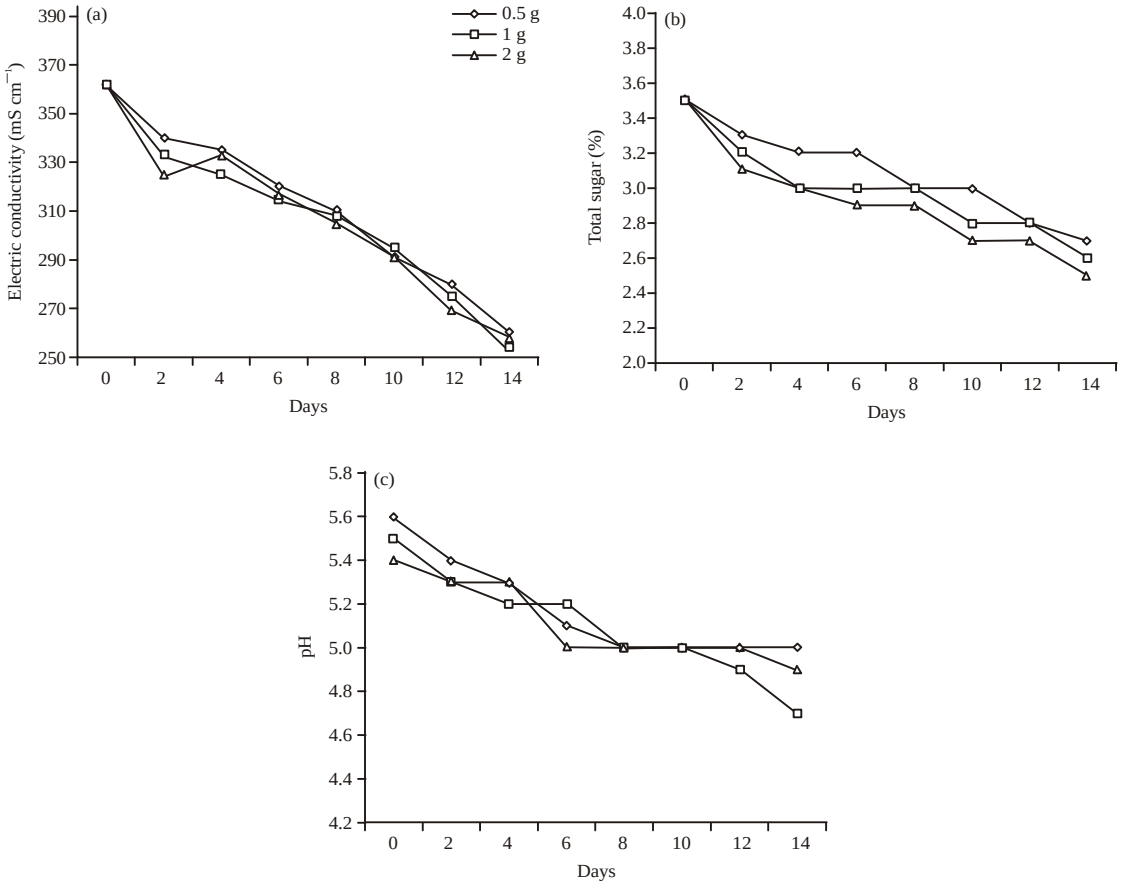

Fig. 2(a-c): (a) Conductivity, (b) Total sugar and (c) pH medium of adventitious root culture of *T. paniculatum* in balloon-type bubble bioreactor with combination of aeration rate (0.25 vvm) and initial inoculum density 0.5, 1 and 2 g/400 mL

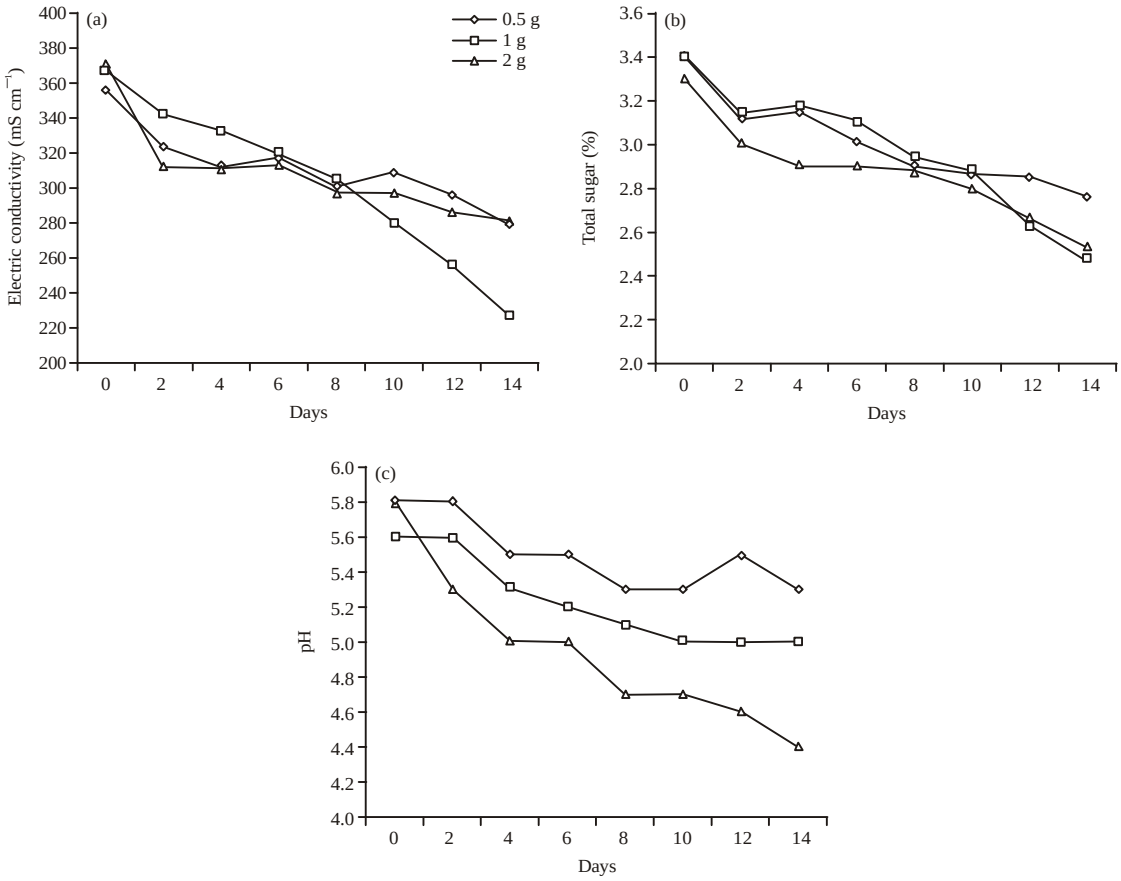

Fig. 3(a-c): (a) Conductivity, (b) Total sugar and (c) pH medium of adventitious root culture of *T. paniculatum* in balloon-type bubble bioreactor with combination of aeration rate (0.5 vvm) and initial inoculum density 0.5, 1 and 2 g/400 mL

Combination of treatment aeration rate 0.5 vvm and initial inoculum density 0,5 1 and 2 g have highest biomass at the treatment of 0.5 vvm and 1 g. Growth of the adventitious roots in that treatment were support of the medium condition include the conductivity, total sugar and pH medium during culture (Fig. 3). Final conductivity at the combinations of treatment 0.5 vvm and 1 g/400 mL was lower than two other combinations of treatment. It was indicate the maximum absorption of nutrient from medium. The same condition also happen in the absorption of sugar which were showed of concentration of total sugar at 14 days also lowest, whereas pH medium during culture were constant at 5-5.3. This condition induced the growth of adventitious root cells, so the biomass of adventitious root increased.

Combinations of treatment aeration rate 0.75 vvm and initial inoculum density 0.5, 1, 2 g/400 mL showed the negative growth at treatment 0.75 vvm and 0.5 g (Fig. 2). It was seen from the data of conductivity, total sugar and pH medium showed that condition not different with another treatment (Fig. 4). At the combination of aeration rate 0.75 vvm and initial inoculum density 1 g/400 mL and 2 g mL^{-1} have a positive growth, same as aeration rate 0.25 and 0.5 vvm, although the growth rate were lower. Negatively growth rate of the adventitious root at that treatment were caused by shear stress, so the adventitious root became cut to small pieces and

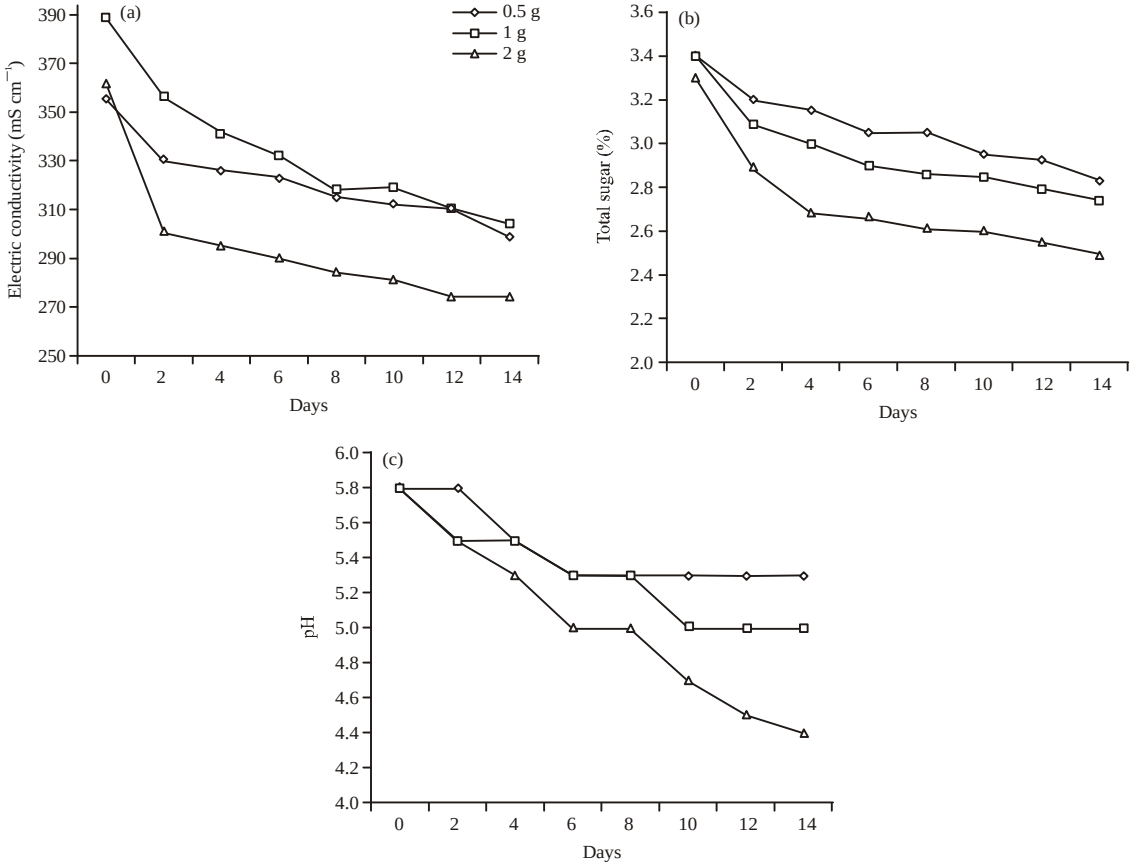

Fig. 4(a-c): (a) Conductivity, (b) Total sugar and (c) pH medium of adventitious root culture of *T. paniculatum* in balloon-type bauble bioreactor with combination of aeration rate (0.75 vvm) and initial inoculum density 0.5, 1 and 2 (g mL^{-1})

growth of root branch were rare, it can be seen in Fig. 5c and e. But at the combinations of treatment aeration rate 0.5 vvm and initial inoculum density 1 g/400 mL were the best condition of culture. It was indicated by highest of biomass and growth of root branches much more than other combinations of treatment (Fig. 5b and d). At the aeration rate 0.75 vvm adventitious root culture will get the shear stress caused by produce of size and number of bubble. When aeration rate increase, produce of bubble will be increased, so it can inhibit the growth of adventitious root. It also happened in hairy root culture of *T. panicultum* which got the shear stress in treatment of aeration rate 0.75 vvm (Manuhara *et al*., 2012).

Effect of combinations of treatment of initial inoculum density and aeration rate on saponin content of adventitious root: Qualitative analyzed of saponin at adventitious root showed that all of the adventitious roots of java ginseng in all combination of treatment were posit if in thin layer chromatography which indicate of dark green color and Rf value same as standard saponin (data not shown). Result of saponin content used high performance liquid chromatography were showed at Table 1. The highest saponin was gotten at the combinations of treatment of aeration rate 0.75 vvm and initial inoculum density 2 g/400 mL whereas, at the aeration rate

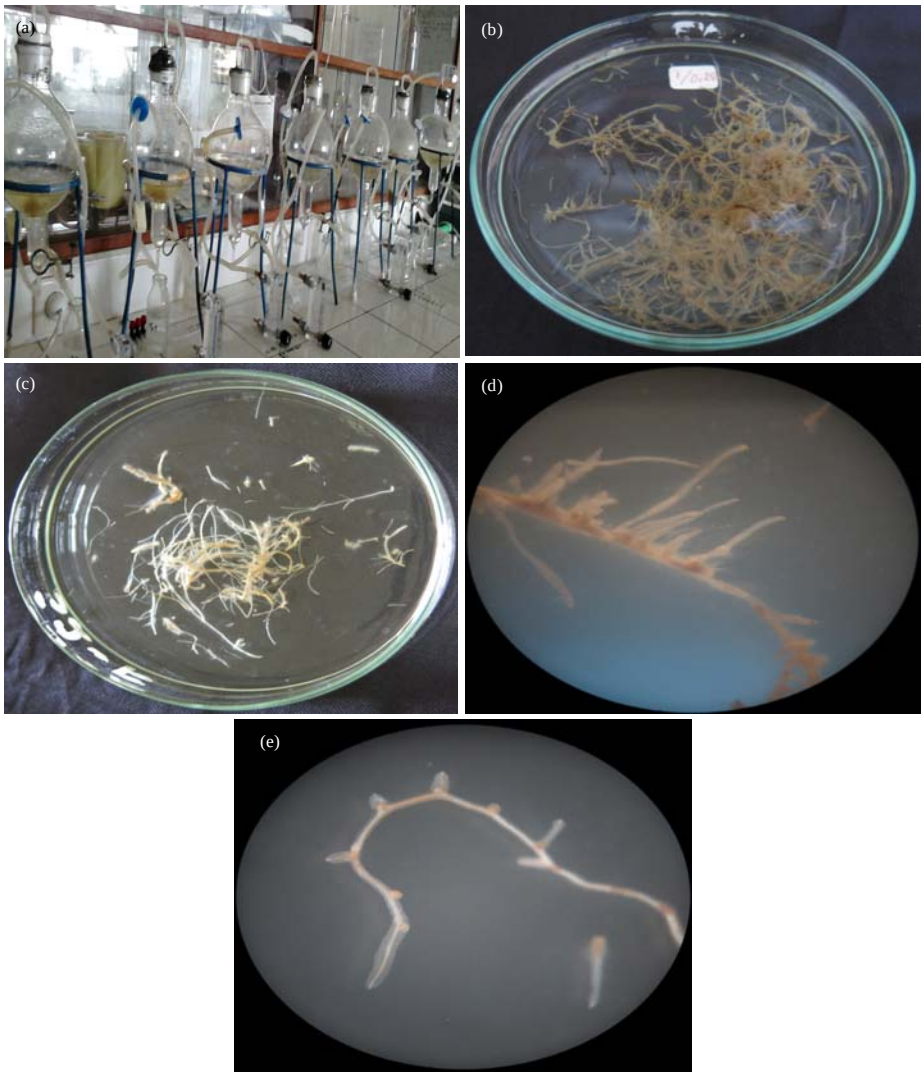

Fig. 5(a-e): (a) Adventitious root cultures of *T. paniculatum* in balloon type bubble bioreactor, (b, d) Macroscopic and microcopies (40x) pictures of adventitious root at the combinations of treatment of aeration rate and initial inoculum density: 0.5 vvm, 1 g/400 mL and (c, e) 0.75 vvm, 0.5 g/400 mL

Table 1: Saponin content (ppm/g dry weight) of adventitious roots of *T. paniculatum* at various aeration rate and initial inoculum density

	Aeration rate (vvm)		
Initial inoculum density (g/400 mL)	0.25	0.5	0.75
0.5	n.d	3.790	1.1500
1.0	1.070	6.390	2.2100
2.0	2.400	16450	25.500

n.d: Not detected

0.25 vvm and initial inoculum density 0.5 g/400 mL saponin content was not detected, although in the qualitat if test showed the posit if result. Its maybe caused the saponin content was very low. At combinations of treatment of aeration rate 0.5 vvm and initial inoculum density (0.5, 1 and

2 g/400 mL) showed saponin content increased, although its content lower than combinations of treatment of aeration rate 0.75 vvm and initial inoculum density 2 g/400 mL.

DISCUSSION

The conductivity and sugar levels during the culture medium decreased. Its showed that absorption of inorganic compound and sucrose by adventitious root are effectively, so the growth of cell increase and it cause the biomass of adventitious root also increase. Beside that, decreasing of pH medium from 5.8-4.5-5.2 at the 14th day did not inhibit absorption of inorganic compound in the medium. Its also happened in hairy root culture of *Arachis hypogaea* in agitated liquid medium. Until 9 days culture, pH medium was decreased from 5.5-4.8 (Bolivar *et al*., 2007). In the cell suspension culture of *Elaeis guineensis* Jacq. decreased of pH medium from 5.6-4 was take place very fast during 9 days culture (Gorret *et al*., 2004), whereas hairy root culture of *Talinum paniculatum* Gaertn in balloon-type bubble bioreactor at the first week pH of culture was decreased from 6-4 (Manuhara *et al*., 2012). Vani (1996) also reported that decreasing of pH during culture of *Catharanthus roseus* hairy root in the circulated bioreactor was take place at 10-15 days culture. Decreasing pH of MS medium during hairy root culture was caused by MS medium consist of ammonium. Source of ammonium in MS medium was ammonium nitrate that necessary as a buffer and source of nitrogen. When cell need nitrogen, cell uptake the ammonium and release H^+. Releasing of H^+ into the medium caused acid condition.

Conductivity was used as indirect method to biomass estimated in the cell culture at bioprocess technology with efficient and accurate result. Conductivity was reflected of uptake inorganic compound by the cell during cultivation which was showed by the decrease of it's concentration and on the contrary the cell population or biomass increased (Thanh *et al*., 2006). Carbon source of MS medium is sucrose, which in the beginning of culture have been hydrolyzed to glucose and fructose. Cell consumed its sugar during cultivation, so measurement of total sugar also reflected growth of the cell (Gorret *et al*., 2004).

The lower growth of adventitious root in combinations of treatment of aeration rate 0.5 vvm and initial inoculum density 2 g were supported by the value of conductivity which was still high and the low of pH medium at the end of cultivation (Fig. 3). The high conductivity of medium showed that the adventitious root unavailable to absorb inorganic compound from medium. These conditions were also supported by the decreased pH of medium at the end of cultivation. Decreasing pH of medium can cause the low ability of cell to absorb inorganic compound from the medium. At the normal condition, organic compound will be absorbed at pH 5-6.

The highest saponin content in this research was achieved at aeration 0.75 vvm and initial inoculum density 2 g/400 mL. The same result was obtained in hairy root culture of *T. paniculatum* that showed the increasing of initial inoculum density can cause increasing of saponin content (Manuhara *et al*., 2012). According to Van Gulik *et al*. (1994), the high of initial inoculum density can eliminated lag phase of growth curve, so specific growth rate became maximum. The high of growth rate indicated that the culture have long of exponential phase. At the end of culture was assumed that content of sucrose in the culture medium still support to growth acceleration, so culture still in exponential phase. Culture with initial inoculum density 2 g/400 mL was assumed have lag and exponential phase shorter because sucrose in medium not enough to support growth acceleration. At the end of cultivation, culture was on the early stationer phase, so saponin accumulation higher than other culture. Bhojwani and Razdan (1996) stated that production of secondary metabolite generally happened at early to the end of stationer phase of growth curve,

when the source of nutrient limited. Sakurai *et al*. (1996) also state that increasing production of secondary metabolite and change of profile product was carried out by increasing inoculum density or modification of medium. Inoculums density was also reported that would significant influence on cell growth, saponin and polysaccharide production in cell culture of *Panax notoginseng* (Zhang and Zhong, 1997). In this study, the highest saponin content in adventitious root of *T. paniculatum* was achieved at aeration rate 0.75 vvm but in hairy root culture of *T. paniculatum*, the highest of saponin content was achieved at aeration rate 0.25 vvm (Manuhara *et al.*, 2012). It's maybe caused by supply of oxygen in culture medium can support cell to produce secondary metabolite, so saponin content in adventitious root of *T. paniculatum* was increased.

CONCLUSION

Based on nine combinations of treatments of aeration rate and initial inoculum density, the best combination to produce biomass of adventitious root of *T. paniculatum* in balloon-type bubble bioreactor was aeration rate 0.5 vvm and initial inoculum density 1 g/400 mL, whereas, the best treatment to produce saponin was aeration rate 0.75 vvm and initial inoculum density 2 g/400 mL. This result could be used as base to improve large scale of adventitious root of *T. paniculatum* in balloon-type bubble bioreactor.

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