



Biomass Production of *Gynura procumbens* Adventitious Roots in Different Type of Liquid Culture

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Abstract

Gynura procumbens has a potency to produce raw material for pharmaceutical industry. Liquid culture systems have significant effects on multiplication rates of organ, so the propagation technology to increase biomass of this plant or organ using the liquid culture system was necessary. This research was conducted to compare biomass production of adventitious roots of *G. procumbens* in different liquid culture (shake flask, temporary immersion bioreactor and balloon-type bubble bioreactor). Adventitious roots culture was maintained in Murashige and Skoog liquid medium supplemented with IBA (indole butyric acid) 5 mg/L and different concentrations of sucrose (10, 30, 50 g/L). Result showed that the highest biomass production (fresh weight) was in shake flask that was 3.9-fold higher than initial explant, while in temporary immersion system was 5.12-fold higher than initial explant, and in balloon-type bubble bioreactor was 13.1-fold higher than initial explant. The highest enhancement of adventitious roots was occurred at sucrose supplementation of 50 g/L. Based on this research we found that the best type of liquid culture to increase biomass of *G. procumbens* adventitious roots was balloon-type bubble bioreactor. Scaling-up of adventitious root culture are necessary to fulfill the need of raw material for pharmaceutical industry and balloon-type bubble bioreactor was the most suitable method to scale up the performance.

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INTRODUCTION

Gynura procumbens is one species of Asteraceae known as a medicinal plant in Indonesia. It is a herb which have fast growth and widely distributed in South East Asia, including Indonesia, Malaysia, and Thailand. People use this plant as a traditional medicine for inflammation diseases, diabetes, cancer, and hypertension. This plant is potential to produce secondary metabolites containing alkaloid, flavonoid, and coumarin. Kaempferol, quercetin, rutin, and myricetin which belong to flavonoid have been isolated from *G. procumbens* leaves (Saiman *et al.*, 2012). Flavonoid is potential as an antioxidant, especially myricetin and kaempferol (Kaewseejan *et al.*, 2015). Ethyl acetate fraction of *G. procumbens* leaves have a potency as co-chemotherapy agent (Nurulita *et al.*, 2012) and flavonoids contained in this plant can affect the vasodilatation process (Hoe *et al.*, 2011; Ng *et al.*, 2013). Generally, secondary metabolite in plant was found in leaves, roots, and stems. Propagation technology of those organs have a potency to produce raw materials of the secondary metabolites for pharmaceutical industry.

In vitro propagation of organ culture has developed in agar-based system and liquid-based system. Liquid culture systems have significant effects on multiplication rates and morphology of shoots, somatic embryos, microtubers or bulblets produced *in vitro* (Hvoslef-Eide & Preil, 2005). Many researches have been developed to produced adventitious roots in liquid culture, such as production of biomass and bioactive compounds from adventitious roots of *Eurycoma longifolia* in ballon-type bubble bioreactor system (Lulu *et al.*, 2015), establishment of adventitious root culture of *Stevia reboudiana* Bertoni in a roller bottle system (Reis *et al.*, 2011), production of biomass and bioactive compounds of *Panax ginseng* adventitious root in ballon-type bubble bioreactor (Wang *et al.*, 2013), and production of biomass and metabolites accumulation in adventitious root culture of *Glycyrrhiza uralensis* Fisch (Yin *et al.*, 2014). In many cases, cultivation of roots in liquid culture have faster growth rate than in agar medium, such as growth rate of *Justicia gendarussa* hairy root (Wahyuni *et al.*, 2017).

Compared to agar-based system, liquid system are more adaptable to automation so it is more suitable to reduce the number of labor and production costs, beside that the media can be changed easily during scaling-up, and cleaning of culture vessels is simplified. Using different concentration of sucrose in liquid medium was also performed to determine the optimum sucro-

se concentration in enhancing the growth of adventitious roots.

Plant cell cultures are usually grown heterotrophically using simple form of sugar such as glucose, fructose, maltose, sucrose and their combination as an energy and carbon source. Among the various tested sugar, sucrose was found to be an ideal carbohydrate source for the biomass accumulation. For example, the various level of sucrose (1-8%) tested in *Gymnema sylvestre* cell culture, 3% sucrose in the medium showed the maximum accumulation of biomass (Murthy *et al.*, 2014); the same result was found in *Ginkgo biloba* cell cultures (Park *et al.*, 2004); but in *Bacopa monnieri* shoot cultures, 2% sucrose was found optimal for biomass accumulation (Naik *et al.*, 2010). This research was conducted to know the effect of sucrose concentration on biomass production of adventitious roots of *G. procumbens* in three liquid culture medium: shake flask culture, temporary immersion system, and ballon-type bubble bioreactor. Results of this research can be used as the basic method to scale up the production of biomass from *G. procumbens* adventitious roots.

METHODS

Gynura procumbens (Lours.) Merr was obtained from the Botanical Garden Purwodadi, Pasuruan, East Java, Indonesia. Adventitious roots were obtained from leaves, which were grown in MS (Murashige and Skoog) medium supplemented with IBA (indol butyric acid) 5 mg/L, sucrose 30 g/L and agar 8 g/L.

Leaves were washed with detergent and then rinse thoroughly with tap water. Explant were sterilized with clorox 10% (v/v) for 10 min and rinsed three times with sterile distilled water. Leaves were placed in sterile filter paper in petri dishes, cut 1 cm² and then planted in MS medium supplemented with IBA 5 mg/L, sucrose 30 g/L and agar 8 g/L. Cultured were maintained in incubator room with temperature 25±3°C in dark condition. After 15 days of culture, adventitious roots were separated from leaves and used as explants for liquid culture method.

Adventitious root explants obtained from leaves explants, were pondered 2±0.5 g and then planted in 250 mL liquid MS supplemented with IBA 5 mg/L and various sucrose concentration (10, 30, 50 g/L). Culture were maintained in shaker incubator and agitated at 100 rpm, temperature 25±3°C for 28 days. Treatments were replicated 3 times. At the end of culture process, adventitious roots were harvested and their fresh

and dry weight were measured.

Liquid MS medium (200 mL) containing IBA 5 mg/L were used to cultivate the adventitious roots (2 ± 0.1 g) in temporary immersion bioreactor. There were three bioreactors which had immersion frequency of 15 min each 12 h and various concentration of sucrose (10; 30; 50 g/L). Treatments were replicated 3 times. Culture were incubated at $25\pm 3^\circ\text{C}$ using 3 hours in light (General electric cool white fluorescent tubes) and 21 hours in dark for 28 days. Temporary immersion bioreactor were designed by modification of BIT[®] (Escalona *et al.*, 1999). At the end of culture process adventitious roots were harvested and their fresh and dry weight were measured.

Volumes of BTBB vessel were 1 L with working volumes between 200-500 mL. Liquid MS medium (400 mL) supplemented with IBA 5 mg/L and various sucrose (10, 30, 50 g/L) were used to cultivate the adventitious roots. The adventitious roots were weighed 2 ± 0.1 g then planted in BTBB; and aeration volume was established at 0.25 vvm (volume of gas per volume of liquid per minute). Cultures were incubated at $25\pm 3^\circ\text{C}$ for 28 days. Treatment were replicated three times. Ballon-type bubble bioreactor were designed by modification of Paek *et al.*, 2005.

RESULTS AND DISCUSSION

Based on fresh weight and dry weight, at the end of culture (28 days), it was known that in low concentration of sucrose (10 g/L), biomass of adventitious roots did not significantly different between the three type of liquid culture, but in higher sucrose concentration (30, 50 g/L) there was a significant different. Supplementation of sucrose 50 g/L significantly increase the growth of adventitious roots. It could be seen that there was an enhancement of fresh weight compared

to initial inoculum (2 g). In shake flask culture, it was obtained the final fresh weight of 7.8 g (3.9 fold higher than initial explant), in TIS was 10.24 g (5.12 fold higher than initial explant), and in BTBB was 26.20 g (13.1 fold higher than initial explant) (Table 1).

The plants propagated in liquid culture are usually submerged in medium. Since most plants propagated are terrestrial plants (not aquatic), and under their natural habitat, submerged condition is usually harmful to the plants. The shake culture method is considered to be an intermediate in establishing bioreactor technique. The growth characteristic in liquid medium are quite different, so the optimization of cultured condition is needed. There are many problems in liquid culture, such as asphyxia (less oxygen) and hyperhydricity which was caused by low oxygen and submerged condition in medium. In order to overcome these problems, temporary immersion system and ballon-type bubble bioreactor can be adopted.

Temporary immersion system (TIS) is a system which was used to eliminate hyperhydricity and asphyxia by adjusting the immersion times (Etienne & Berthouly, 2002). This research was obtained the biomass of *G. procumbens* adventitious roots in TIS higher than in shake flask culture. Fresh weight of adventitious roots in shake flask culture had average biomass of 3.2 fold higher than initial inoculum at different concentration of sucrose, whereas in TIS, the average of adventitious roots biomass was 4 fold higher than initial inoculum (Table 1). Immersion frequency of 15 min each 12 h provided opportunities for adventitious roots to absorb nutrient and oxygen as well as reducing the occurrence of hyperhydricity and asphyxia. Meanwhile, when the explant did not immerse in liquid medium, respiration of the cell still could take place because the oxygen

Table 1. Fresh weight and dry weight of *Gynura procumbens* adventitious roots in three different liquid culture and different sucrose concentrations

Type of liquid culture	Sucrose concentration (g/L)	Initial inoculum (g)	Fresh weight (g)	Dry weight (g)
Shake flask	10	2.02	5.00 \pm 1.2	0.13 \pm 0.03
TIS		2.04	5.30 \pm 0.6	0.11 \pm 0.02
BTBB		2.04	5.20 \pm 2.8	0.15 \pm 0.03
Shake flask	30	2.01	6.50 \pm 1.3	0.14 \pm 0.08
TIS		2.00	8.70 \pm 0.7	0.15 \pm 0.06
BTBB		2.06	14.95 \pm 0.8	0.49 \pm 0.33
Shake flask	50	2.00	7.80 \pm 1.9	0.28 \pm 0.01
TIS		2.02	10.24 \pm 0.6	0.19 \pm 0.06
BTBB		2.10	26.20 \pm 1.9	1.52 \pm 1.11

in the room culture were available. In shake flask culture, oxygen dissolved in liquid medium was limited resulted in slower growth of explants and more occurrence of hyperhydricity.

Different type of liquid culture influenced adventitious roots growth rate. Beside that, increasing of sucrose concentration also increased the adventitious roots biomass. Different type of liquid culture gave influence in growth of adventitious roots if in culture media had enough sucrose to support carbon source which was needed by plant cell for metabolism. Between these three different types of liquid culture, the best technique to increase the adventitious roots of *G. procumbens* was ballon-type bubble bioreactor (Figure 1c-e), because this technique could increase the growth of adventitious roots highest than the others. Morphology of root produced in three culture method had no significant different (Figure 1f-h).

Culture in balloon-type bubble bioreactor use liquid medium with forced aeration which stimulates the growth rate. The bubble creates less shear forces, therefore BTBB was suitable for plant organ cultures (Paek *et al.*, 2005). In balloon-type bubble bioreactor system, the average of increasing biomass was 13.1 fold higher than initial inoculum and was almost 2 fold higher than temporary immersion system. Root biomass was also found to be higher in the bioreactor cultures than in the shake flask culture of *Astragalus membranaceus* (Wu *et al.*, 2011) and *Eurycoma longifolia* (Lulu *et al.*, 2015). In medium supplemented with sucrose 50 g/L adventitious roots growth rate was highest. In BTBB system adventitious root growth rate was highest, especially in medium supplemented with sucrose 50 g/L.

Many factors could influence the bioreactor system. Proper oxygen supply and gaseous exchange are two critical factors. The existence

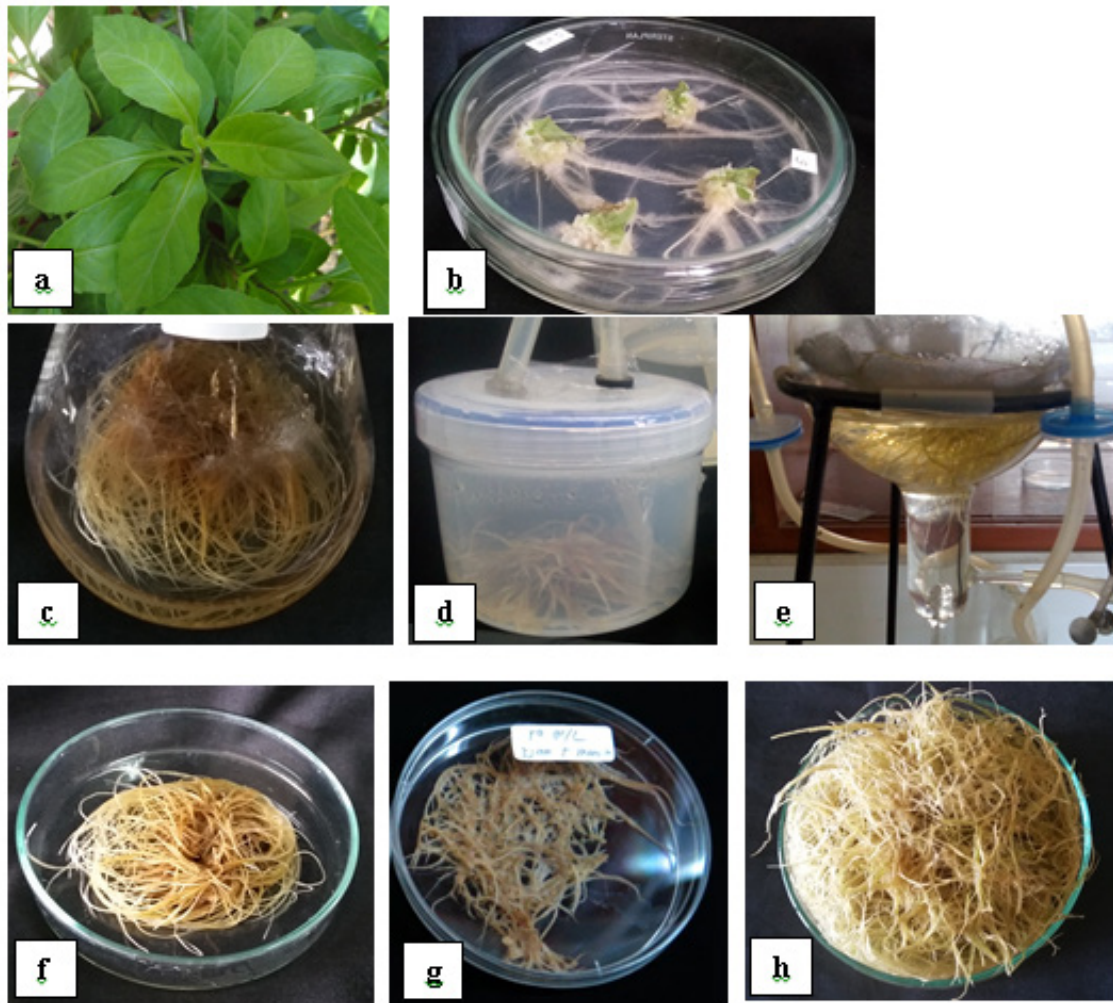


Figure 1. Adventitious roots of *G. procumbens* after 28 days culture in different type of liquid culture and 50 g/L sucrose concentration, (a) mother plant, (b) adventitious root induction in MS solid medium, (c) shake flask culture, (d) temporary immersion bioreactor, (e) ballon-type bubble bioreactor, (f-h) morphology of adventitious root.

of gaseous flow more than required by the culture can caused evaporation of some essential components, especially water. Improper aeration gives rise to foaming problem. Temperature should be in appropriate range so it will not affect the bioprocess activities in the culture with the maintenance of the metabolite production. The optimum temperature should be at 20-30°C but can be vary from species to species as well as with the type of products. Consideration of shear stress is also necessary for the hairy roots as it can causing reduction in productivity. Immersion time is a critical factor for root cultures and over lodging can cause hyperhydricity. Inoculum size can also affect the productivity, in which too many tissue mass formulated can cause problems during scaling up (Sharma *et al.*, 2014).

In some research, temporary immersion system have been used for many purposes, for example to increase the somatic embryogenesis of peach palm (Steinmacher *et al.*, 2011), enhance the propagation of Chinese water chestnut (Gao *et al.*, 2015), and micropropagation of embryogenic callus of oil palm (*Elaeis guineensis* Jacq.) (Marbun *et al.*, 2015; Gomes *et al.*, 2016). In some cases, temporary immersion system could reduce the hyperhydricity, because hyperhydricity is a major problem during *in vitro* culture of many crops in liquid culture system. Hyperhydricity is a disorder of tissue-cultured plants where leaves become translucent and stem become swollen, distorted and brittle. Although numerous hypotheses have been put forward to explain hyperhydricity but there is still a lack of knowledge about the nature of signals which was responsible for hyperhydricity (Dewir *et al.*, 2014). This study showed that the percentage of dry weight adventitious roots in the balloon-type bubble bioreactor was higher than flask culture and temporary immersion system.

In all type of liquid cultures, supplementation of sucrose that was higher than 10 g/L could increase the biomass (FW and DW) of adventitious roots. Sucrose is hydrolyzed into two monosaccharides, glucose and fructose by invertase which bounds extracellularly and/or cell wall during the initial culture period (Cui *et al.*, 2010). In this study, the higher sucrose supplementation can increase the biomass production. The highest enhancement was occurred at 50 g/L of sucrose supplementation. This result also occurred in adventitious root culture of *Echinacea angustifolia* (Baque *et al.*, 2012) High concentration of sucrose had significant effect on the production of ginseng saponin of *Panax notoginseng*. A high sugar level is able to the synthesis the ginseng saponin,

it may be due to the high osmotic pressure which was caused by high sugar concentration. The cell growth was reduced at sucrose concentration of 60 g/L, the maximum production of crude ginseng saponin was achieved at sucrose concentration of 40 g/L. A dual role of sucrose as a carbon source and an osmotic agent was observed in *Solanum melongena* (Mukherjee *et al.*, 1991). Recently, sugars have been recognized as a signaling molecules that affect the growth, development and metabolism of cultured cells (Wang & Weathers, 2007). Therefore, the selection of suitable carbohydrate source at an appropriate concentration is key criterion for biomass and secondary metabolite production in cell and organ cultures.

Based on this research we found that high concentration of sucrose could increase the biomass of *G. procumbens* adventitious roots in different type of liquid culture, so at the next study to scale up of adventitious roots production, sucrose concentration was necessary things to be noted.

CONCLUSION

Adventitious roots of *G. procumbens* was successfully developed in three types of liquid cultures, but the highest biomass was obtained in balloon-type bubble bioreactor system. Scaling-up of adventitious root culture are necessary to develop in the next study because this plant was potential as a source of raw materials for pharmaceutical industry.

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