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Effect of sucrose and potassium nitrate on biomass and saponin content of *Talinum paniculatum* Gaertn. hairy root in balloon-type bubble bioreactor

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ABSTRACT

Objective: To increase biomass and saponin production in hairy root culture of *Talinum paniculatum* Gaertn. (*T. paniculatum*) in balloon-type bubble bioreactor (BTBB).
Methods: Hairy roots which were collected from leaf explants of *T. paniculatum* were infected by *Agrobacterium rhizogenes* strain LB510. The hairy roots were cultivated at 400 mL Murashige and Skoog liquid medium without growth regulator (MS0) in 1 000 mL BTBB. Each BTBB had 2 g hairy roots as initial inoculum and these cultures were treated with various concentrations of sucrose (3%, 4%, 5%, 6% w/v) and potassium nitrate (0.5, 1.0, 1.5 and 2.0 strength of MS medium). Cultures were maintained for 14 days. Fresh and dry weights of hairy roots at the end of culture were investigated.
Results: Various concentrations of sucrose influenced the biomass accumulation of hairy roots. Maximum biomass was reached by MS medium supplemented with 6% sucrose and it was approximately threefold higher than control. Culture supplemented with potassium nitrate at 2.0 strength of MS0 could increase biomass accumulation of hairy roots until 0.14 g dry weight and it was almost threefold higher than control. However, the maximum saponin content was obtained by MS medium supplemented with 5% sucrose and 2.0 strength potassium nitrate of MS.
Conclusions: Based on this research, those conditions can be used to produce biomass and saponin of hairy root of *T. paniculatum* in the large scale.

1. Introduction

Java ginseng [*Talinum paniculatum* Gaertn. (*T. paniculatum*)] has been used in pharmaceutical industries for source of saponins, flavonoids, tannins, triterpenes or sterols, and polyphenols. Saponins of *T. paniculatum* are accumulated in roots. Ability and effectiveness of saponins on many medicinal treatments have been scientifically proven. Saponins were reported to be able to enhance viability, motility and number of spermatozoa. Saponins also act as a anti-inflammatory agent, have androgenic potency, are able to induce cell differentiation through receptor cells [1], and could increase body resistance to disease [2]. *T. paniculatum* needs 3–4 years to produce the maximum saponins in the root. Root culture technology could be a solution to fill saponins demand in the market. This technology is important to be developed for plant preservation and increasing saponin content in roots.

Transformation by using *Agrobacterium rhizogenes* (*A. rhizogenes*) as a mediator to transfer transfer-DNA (T-DNA) into plant DNA is shortly alternative to produce roots. The T-DNA contains genes encoding enzymes for the synthesis of the phytohormones cytokinin and auxin, and of specific opine. The expression of oncogenes in Ri plasmid is indicated by adventive roots formation in infected area of explants. These adventive

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roots are called hairy roots. In recent decades, hairy root culture has been widely used to produce various types of secondary metabolites that are naturally present in the roots. It was previously reported that *rolC* from *A. rhizogenes* T-DNA was shown to stimulate the production of secondary metabolites in the transformed plant cells of different plants. It was revealed that hairy roots enhanced the amount of glycyrhizin in *Glycyrrhiza glabra* [3], plumbagine in *Plumbago rosea* [4], saponin in *Bacopa monnieri* [5], anthraquinones in *Polygonum multiflorum* [6], and polyphenols in *Momordica charantia* [7]. Organic nutrients in plants play a role in growth, development and accumulation of secondary metabolites. Growth and synthesis of secondary metabolite in hairy roots are also influenced by the nutritional quality. The effect of sucrose and nitrate concentrations in culture medium had been investigated. Biomass growth and withanolide A, production of *Withania somnifera* hairy roots were affected by different carbon sources in the Murashige and Skoog liquid medium (MS medium) [8]. Biomass and metabolite accumulation were also affected by NH_4: NO_3 ratio in balloon-type bubble bioreactor (BTBB) culture of *Eurycoma longifolia* adventitious roots [9].

In previous studies, hairy roots of *T. paniculatum* that were cultivated in solid MS medium without growth regulator (MS0 medium) grew slowly. Hairy root growth was accelerated after subculture in semi-solid MS0 medium, but limited by oxygen supply and space growth. Therefore, hairy roots must be subcultured to liquid medium. Liquid culture has some advantages, for example, oxygen demand is filled by agitation or aeration, culture space limitation is solved by widening bioreactor chamber, pH medium is under control, nutrients are more homogenized and available for all parts of explants. Culture of *T. paniculatum* hairy roots in BTBB has mainly focused on aeration rates and inoculum densities. The best aeration rate and inoculum density for biomass and saponin content were reached at 2 g/400 mL and 0.25 vvm respectively [10]. In this research, various concentrations of sucrose and nitrogen are determined to increase biomass and saponin content.

2. Materials and methods

2.1. Materials

T. paniculatum was obtained from Botanical Garden of Purwodadi, Indonesia. *T. paniculatum* leaves were used as explants to initiate hairy roots. *A. rhizogenes* LB510 was infected to *T. paniculatum* leaves to induce hairy roots. The bacteria were obtained from Research Center of Biotechnology, Indonesian Institute of Sciences, Indonesia.

2.2. Explant sterilization and induction of hairy roots

The leaf explants of *T. paniculatum* were washed briefly with detergent and rinsed with running tap water. After rinsing, the explants were sterilized with 10% (v/v) clorox for 5 min, and then rinsed 3 times with sterile water. After sterilized and rinsed, the explants were shaken gently. The sterile leaf explants were cut ± 1 cm^2 and ready to be infected by *A. rhizogenes* LB510. The bacteria were grown in liquid Luria-Bertani medium at 110 r/min, and (28 ± 2) °C for 2 days. The suspension of *A. rhizogenes* LB510 was diluted with liquid MS0 medium, then 100 μmol/L acetosyringone was added. The explants were submerged in the solution for 5 min and were shaken gently. After that, the explants were drained on sterile filter paper and then transferred to MS0 agar. The explants were incubated at (28 ± 2) °C under dark condition for 2 days. In the next step, the explants were transferred to MS0 solid medium supplemented with 500 mg/L cefotaxime. The explants were incubated at (28 ± 2) °C under dark condition for a week, and then were moved into MS0 semi-solid medium (5 g/L agar) for a week. Successful transformation was known with hairy root formation from the edge of the leaf explants. In the end of incubation time, hairy roots were excised from explants and then transferred to 250 mL Erlenmeyer flask. The flask contained 50 mL liquid MS0 supplemented with 500 mg/L cefotaxime. Hairy roots cultures were agitated at 90 r/min, (28 ± 2) °C, under dark condition for a week. At the end of culture, hairy roots were ready to use as inoculum on liquid culture in BTBB.

2.3. Liquid culture condition in BBTB

Volumes of BBTB vessel were 1 000 mL with working volumes between 200 and 500 mL. About 400 mL of MS0 medium was placed in 1 000 mL Erlenmeyer flask and sterilized on autoclave at 121 °C for 20 min. Liquid MS0 medium was transferred into BTBB aseptically. Initial inoculum was 2 g hairy roots and all BTBB cultures were aerated at flow rate of 0.25 vvm.

2.4. Treatment of sucrose and potassium nitrate at various concentrations

These experiments had eight BTBB cultures. Each culture was in the same conditions, such as 2 g hairy roots as inoculum, 0.25 vvm of air flow rate and 400 mL volume of MS0 medium. In this test, eight BTBB were treated with various concentrations of sucrose [3%, 4%, 5%, 6% (w/v)] and various concentrations of potassium nitrate (0.5, 1.0, 1.5 and 2.0 strength of MS medium). Cultures were incubated under dark condition at 25 °C for 14 days. The changes of pH, conductivity and total sugar in culture medium were checked every two days. Conductivity and total sugar content in medium culture were checked with hand conductometer (Ezdo) and hand refractometer (Atago). At the end of cultivation, biomass and saponin content were measured.

2.5. Saponin analyses

Saponin content of hairy roots was analyzed qualitatively by using thin layer chromatography and quantitatively by using high performance liquid chromatography (HPLC). Hairy roots were dried at 50 °C for 5 days and then were ground with mortar. About 100 mg powders of hairy roots were immersed in 10 mL ethanol, and then heated at 80 °C in water bath for 30 min. The hairy root extract was concentrated at 80 °C for 3 h until a volume of 0.2 mL. Extract and standard saponin (Calbiochem) were spotted on silica gel GF254 and were eluted by using eluent propanol: water (14:3). Spot was detected by spraying with anisaldehyde 0.5 mL, acetic acid glacial 10.0 mL, ethanol 85.0 mL, and sulfuric acid 5.0 mL and then heated at 110 °C for 6–10 min. Standard saponin will be dark green color.