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Production of cell biomass and artemisinin via batch cell culture of *Artemisia annua*

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ABSTRACT

Production of cell biomass and artemisinin of *Artemisia annua* suspension cultures via batch cell culture system wereevaluated in this study to determine the best culture system for the production of artemisinin. Cell suspension cultures of *A. annua* TC2 variety of Vietnam origin in shake flasks have been found todemonstrate the ability to synthesize artemisinin, an anti-malarial drug. With the massive demand of artemisinin in the pharmaceutical industry due to its high potency, the commercial feasibility for mass culture of*A. annua*cells foresees lucrative potential.The selection of batchculture system as opposed to the intermittent medium replenishment batch culture system was determined in the cell suspension cultures of *A. annua* on a shake flask level. The effects of intermittent feeding batch culture system were evaluated based on the cell biomass productivity, artemisinin content and sucrose consumption. Results obtained indicatedthat a batch culture system without intermittent medium replenishment was superior for future scale-up production of *A. annua*.

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INTRODUCTION

*Artemisia annua*L. from the Asteraceae family has been found to be able to generate artemisinin, a potent anti-malarial drug, as its secondary metabolite, [1,16]. With its unique chemical structure containing a peroxide bridge, artemisininis able to act against chloroquine-resistantand sensitive strain of *Plasmodium falciparum* parasite that causes cerebral malaria [2]. However, the drawbacks of artemisinin production from *A. annua* due to factors such as the inconsistency with field-grown productions, low productivity from its natural resources as well as its inability to be chemically synthesized because of its complex chemical structure have driven the need for *in vitro*production of artemisinin to meet the global demand of this drug. The highest artemisinin content reported from the field grown plants of *A. annua* were 0.01-0.5% (dry weight) obtained from the leaf sections [11]. Cell suspension culture of *A. annua* possesses commercial potential for the mass production of artemisinin as its secondary metabolite. It was reported that artemisinin could be detected in *in vitro*cell suspension cultures of *A. annua* induced from friable leave calli with a suitable culture medium [7]. For the mass production of artemisinin via large volume culture vessels, the selection of the type of culture system; closed batch system or intermittent medium replenishment batch system is necessary for high productivity to be achieved. Closed-batch system does not involve the addition or the removal of any of the culture as opposed to fed-batch system, which involves intermittent medium replenishment.Intermittent medium replenishment in cell culture systems has been previously employed in many studies for the improvement of cell biomass as well as secondary metabolite production [3,9,15]. The intermittent medium replenishment strategy involves the removal of spent medium containing toxins and the replenishment with fresh medium at a selected interval within the culture period. This prevents the retardation of cell growth as well as unwanted feedback inhibition from other products in the culture medium [13]. In this study, we investigate the selection of closed batch system as opposed to intermittent medium replenishment batch system for the production of artemisinin in *A. annua* cell suspension cultures.

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MATERIALS AND METHODS

Preparation of cell suspension culture:

Friable callus line of *A. annua* (TC2) was used for the preparation of cell suspension culture. Calli of 2.50 ± 0.02 g was inoculated into 125 ml liquid Murashige and Skoog [10] (MS) medium supplemented with 0.5 mg l^{-1} BA and 0.5 mg l^{-1}NAA, 0.5 g l^{-1} casein hydrolysate and supplemented with 30 g l^{-1} sucrose in 500 ml Erlenmeyer flasks. The pH of the medium was adjusted to 5.75 ± 0.02 prior to autoclaving. The cultures were kept in a culture room at 25 ± 2 ^{o}C on a gyratory shaker at 100 rpm, under continuous lighting using cool white fluorescent lights of approximately 32.5 µmol m^{-2} s^{-1}light intensity.

Effect of intermittent medium replenishment volume on biomass and artemisinin content of Artemisia annua cells:

Medium replenishment was conducted on the *A. annua* cell cultures at various different replenishment volumes, 0, 25, 50, 75, and 100 % (v/v) of the total volume of the liquid medium. The replenishment of the liquid medium was carried out on the 12th day of the culture in which, spent medium were filtered and discarded, followed by the replenishment of fresh medium of the equal volumes discarded. The cells retained were returned to the culture flasks. The *A. annua*cells were harvested at every 4 days over a 40-day period. Cells were harvested via pump filtering through a Whatman No.1 filter paper. The harvested cells were then subjected to drying until constant weight was achieved for the determination of dried weight and the analysis of artemisinin content in the cells. The supernatant from the culture collected through filtering were used for residual sucrose profiling.

Residual sucrose measurement:

Residual sucrose content in the medium was determined via colorimetric phenol-sulphuric acid assay (Dubois et al., 1956). Supernatant of the medium was collected and diluted 5 times with distilled H_2O. Approximately 0.1 ml of the solution was transferred into a test tube and topped up with distilled H_2O to a total volume of 2 ml. Then, 1 ml of 5% phenol was added, followed by 5 ml of 95% sulphuric acid (H_2SO_4). The test tubes were left to stand at room temperature for 10 minutes before they were transferred into a water bath for 15 minutes at 30^{o}C. The absorbance of the solution was then analysed at 490 nm using UVMINI-1240 Spectrophotometer (Shimadzu Scientific Instrument). The readings were compared to a reference curve determined earlier through a series of dilution of the similar solution that was being tested.

Extraction and analysis of artemisinin content:

Dried cells of *A. annua* were grounded with a mortar and pestle. Approximately 0.5 g of the cells was ultrasonicated at 40^{o}C for 90 minutes in 3 ml of AR grade n-hexane. The supernatant of the hexane extract was then filtered through a filter paper of 0.45µm and collected in a test tube, followed by the addition of 3 ml of fresh n-hexane to the dried cells at every 30 minutes, and was repeated 3 times. The hexane extract obtained were then evaporated to dryness. Acetonitrile of 1 ml was then added to the extract, and the solution was filtered through a 0.45 µm Milipore membrane to be injected into the vials for UHPLC-UV (Agilent) analysis. Artemisinin content was detected in the UHPLC via C18 Reversed Phase column with a UV detector at 210 nm wavelengths. The mobile phase constituted of acetonitrile: double distilled H_2O at a ratio of 65:35, and the flow rate was set at 0.3ml/ min.

RESULTS AND DISCUSSIONS

Effect of intermittent medium replenishment on cell biomass of A. annua 2GdTC2

Intermittent feeding of selected volumes 0, 25, 50, 75 and 100 % (v/v) of medium were conducted on day 12 of the culture period, which corresponds with the late exponential phase of the *A. annua* cells, determined through a preliminary time-course growth profile. Intermittent medium replenishment has been previously employed in many studies for the improvement of cell culture performance in terms of cell biomass as well as the secondary metabolite of interest [3,15]. The removal of culture medium containing toxins and spent medium was replaced with fresh medium acts as a replenishment of substrates to prolong the growth profile from entering the stationary phase and eventually the death phase. Rao and Ravishankar [13] reported that it was possible that the by-products in the spent medium could lead to unwanted feedback inhibition towards the products of interest. Results obtained with intermittent medium replenishment and the cell biomass (dried weight) harvested at 4-day intervals for a period of 40 days demonstrated the increase in cell biomass from day 16 onwards for all intermittent feeding of 25, 50, 75, and 100 % (v/v) in comparison with the control (no intermittent feeding) (Fig. 1). Highest cell biomass was achieved with the intermittent feeding of 75 % (v/v) on day 32 of culture with a dried cell biomass of 26.3± 1.2g/L which was 2.4 folds higher compared to the cell biomass achieved by the control on day 32. Intermittent medium replenishment of 50, 75 and 100 % (v/v)

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garnered their highest cell biomass concurrently on the 32nd day of culture while intermittent feeding of 25 v/v% achieved highest cell biomass 4 days earlier on the 28th day of culture with dried cell mass of 23.4 ± 0.8 g/L. It was observed that cell biomass increment was more prominent for higher replenishment volumes in the media, with the exception of a total replenishment volume of 100 % (v/v) There was also the absence of a stationary phase in cultures of medium replenishment volumes of 25, 50 and 75 % (v/v) as the decrease in cell biomass occurred right after the highest cell biomass was obtained. This indicated the lack of viability in the cells after achieving their highest cell biomass. However, this observation was not as evident in medium replenishment volumes of 100 % (v/v) where there was a short stationary phase occurring from day 32 to day 36 of culture before the reduction in cell biomass.The cells cultured without intermittent feeding (control group) experienced tissue browning within the culture media by the 28th day of culture period while the other cells cultured with intermittent feeding maintained as green cells (Fig 2.). The cell cultures with intermittent feeding, 25, 50, 75, and 100 %(v/v) remained green in the cultures until the end of the culture period. Browning of cells is commonly caused by the impending necrosis of the cells due to oxidation of the culture media [4]. Cells cultured with the treatment of intermittent feeding which did not experience browning on the 28th day of culture was largely due to presence of substrates that could be utilized by the cells for further mitosis and viability. Sucrose, which acts as a major carbon source in the cell culture media, has been found to be actively responsible for the activity of cell division (Riou-Khamlichi et al., 2000). Thus, there was no restriction or limitations in terms of growth for necrosis or oxidation to occur as substrates and the nutrients required were abundant.This observation was further studied by investigating the residual sucrose profile in the culture medium (Fig 3.). Initial sucrose supplied to the culture was detected at 29.0±0.5 g/L, where the sucrose level throughout the culture duration before intermittent replenishment of medium was conducted on day 12. After intermittent medium replenishment was conducted, there was a predictable spike in residual sucrose content in all the treatment groups except the control group on day 12 of culture period. However, by the 20th day of culture, sucrose in the culture medium was depleted for the treatment groups 0, 25, 50 and 75 % (v/v)medium replenished. In the culture media that was 100 % (v/v) intermittent medium replenished, there was residual sucrose of 7.3 g/L (28 %), which remained until it was completely depleted by the 28th day of culture.

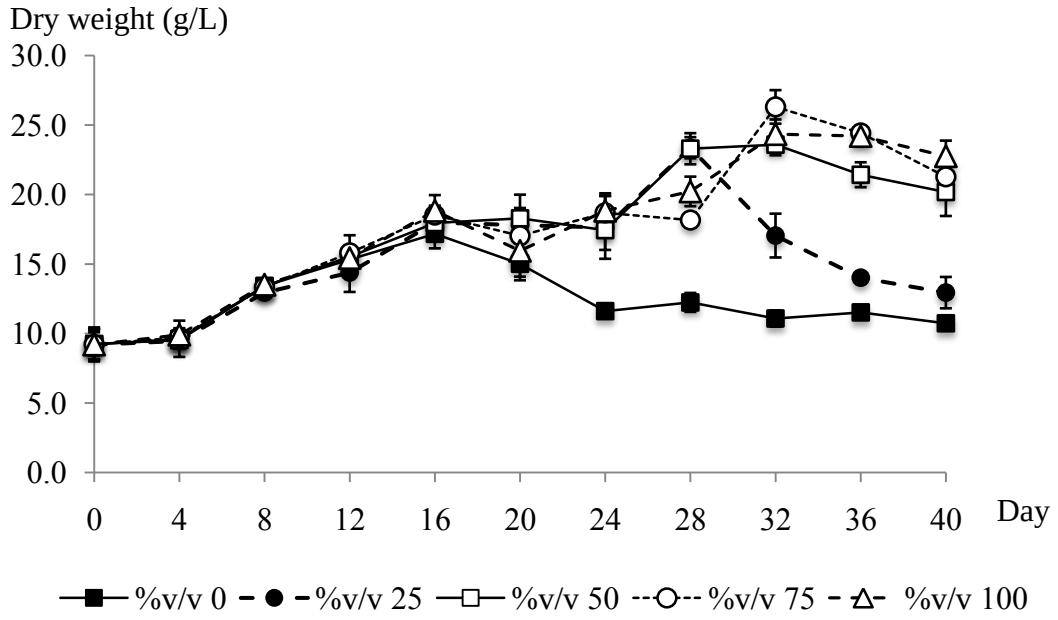

Fig. 1: Effects of intermittent medium replenishment on day 12 (0, 25, 50, 75, and 100 %(v/v) on the time-course profile of dry cell biomass of *A. annua* TC2. Bars represent the standard deviation of 6 replicates.

Fig. 2: Cell cultures of *A. annua* TC2 with intermittent medium replenishment (0, 25, 50, 75, and 100 % (v/v) observed on the 28th day of culture in shake flasks of 500 ml.

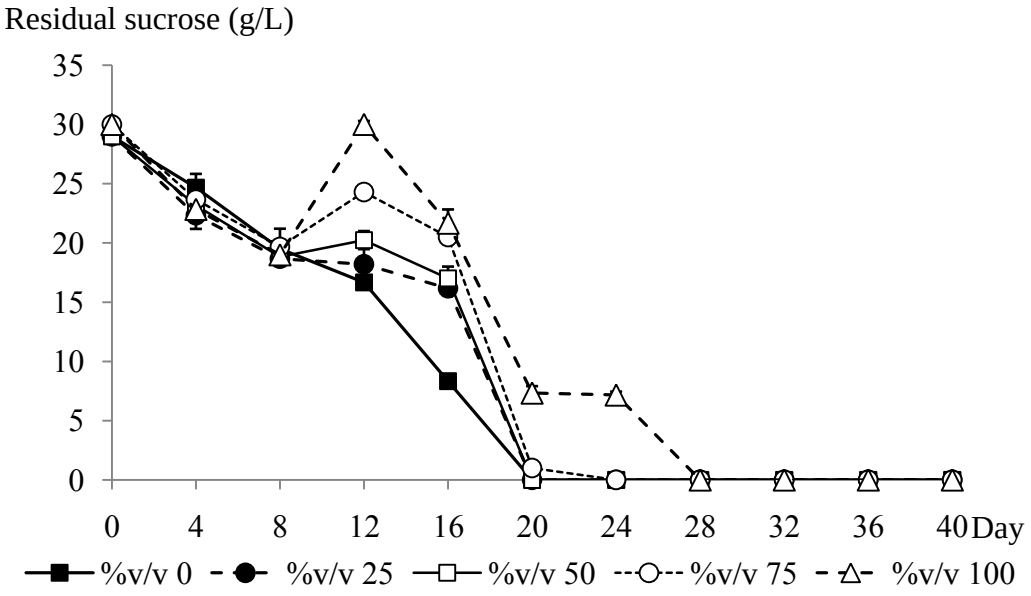

Fig. 3: Residual sucrose profiles of *A. annua* TC2 cell suspension cultures with intermittent medium replenishment 0, 25, 50, 75, and 100 % (v/v) on day 12 of culture.Bars represent the standard deviation of 6 replicates.

Effect of intermittent medium replenishment on the artemisinin production:

Medium replenishment strategies were previously employed in other studies for the improvement of cell biomass and the secondary metabolites that were being produced. Such examples include the manipulation of medium replenished in *Panax ginseng* Meyer cell cultures for the improvement of ginsenosides accumulation [6] as well as the improvement of *Cyperus aromaticus* cell biomass for the production of juvenile hormone III (JH III) [3]. In this study, the artemisinin content of interest in *A. annua* cell suspension cultures was observed throughout the culture period of 40 days. Results obtained (Fig. 4) indicated that the artemisinin content in the control group of *A. annua*, without intermittent medium replenishment, was the highest with 64.1±2.7 µg/g dry weight artemisinin produced on day 12, and began to demonstrate a declining trend with a slight decrease to 60.5 ±6.3 µg/g dry weight on day 16 followed by a significant 45% drop in artemisinin content from day 16 to day 20 of the culture period. This drop in artemisinin content corresponded with the exhaustion of sucrose content in the culture medium, which was completely exhausted by the 20th day of culture period (Fig 3.). Furthermore, earlier results in Fig 1. indicate that the cells were entering the stationary phase with the declining cell biomass from day 16 onwards. The *A. annua* cells maintained an average 26.2 µg/g dry weight artemisinin throughout the period from day 16 to day 32 while experiencing exhaustion of sucrose as carbon source in the medium and the impending death phase. For the treatment groups of cultures with intermittent medium replenishment, the significant drop in artemisinin content in the cellswas observed on day 16, where the lower medium replenishment volumes, 25 and 50 % (v/v) garnered 21.4 ±1.2 µg/g dry weight and 17.6 ±0.7 µg/g dry weight artemisinin respectively while the higher medium replenishment volumes 75 and 100 % (v/v) showed no artemisinin content.It was apparent that dried cell biomass increased with the abundance of carbon source from

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the intermittent medium replenishment. This phenomenon did not create sufficient stress to the cell cultures, in which a certain level of environmental stress was found tocontribute tothe production of artemisinin as a secondary metabolite in *A. annua* [8]. All the treatment groups with intermittent medium replenishment did not produceartemisinin withcontent that was superior than the control group throughout the culture duration with the exception on day 32 where the artemisinin content achieved from 25 % (v/v) medium replenishment was slightly higher (29.3±4.2 µg/g dry weight) than the control group of 0 % (v/v) medium replenishment (22.9±3.3 µg/g dry weight). Various studies conducted on medium replenishment treatments, have indicated the slight enhancement of secondary metabolite production due to the manipulation of sucrose [3,17]. In this study, at 25 % (v/v) intermittent medium replenishment, artemisinin production in *A. annua* was only enhanced slightly (28%) compared to the control group of the similar culture duration of 32 days. However, the artemisinin content attained on day 32 with 25 % (v/v) intermittent medium replenishment (29.3±4.2 µg/g dry weight) was still considerably lower than achieved on day 12 (60.6 ±2.4 µg/g dry weight) even though there was a 16% increase in cell biomass from day 12 to day 32. Therefore, the artemisinin production was most efficient on day 12, without the additional 20 days of culture duration for the 27.9% increase in artemisinin content (compared to the control group) without requiring intermittent medium replenishment. From the results obtained, we gather that the artemisinin production in *A. annua* cell cultures without medium replenishment, were growth-associated. However, with the medium replenishment strategy conducted on the *A. annua* cell cultures, the artemisinin production was conversely demonstrated non-growth associated production.This indicates the possibility of artemisinin production in the replenished culture medium to be associated with the by-products that were present in the spent medium. It is also postulated that this interesting phenomenon observed in the medium replenished cultures could be due to the secretion of artemisinin at later stages of culture into the spent culture medium that was removed during medium replenishment [12]. The production of artemisinin from *A. annua* cells was also identified as more cost efficient with the shorter cultivation duration with higher yields. Furthermore, fed-batch system with intermittent medium replenishment also presents its own set of drawbacks such as the increased risk of contamination and the increased cost for a prolonged production period.

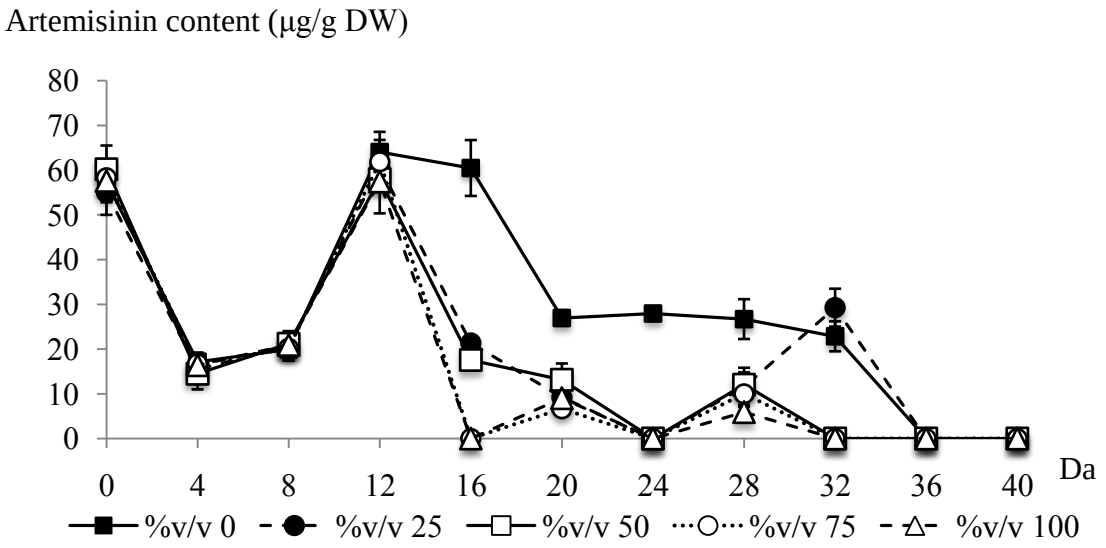

Fig. 4: Artemisinin content in *A. annua* TC2 cell suspension cultures, with day 12 intermittent medium replenishment of 0, 25, 50, 75, and 100%(v/v).Bars represent the standard deviation of 6 replicates.

Conclusion:

High cell biomass production of *A. annua*could be achieved with intermittent medium replenishment strategy of 75% (v/v) harvested on day 32.To achieve the high artemisinin content from the cells, cells should be harvested on day 12 without intermittent medium replenishment.The batch system cultures of *A. annua* without medium replenishment achieved the highest biomass yield at day 16, with 60.5 µg/gdry weightof artemisinin content. We conclude that the selection of a closed batch culture system without medium replenishment was identified as most favorable for the production of cell biomass of *A. annua* and artemisinin production.

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Abbreviations:

BA-6-benzylaminopurine; NAA-Naphthaleneacetic acid.

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