THE EFFECT TRYPTOPHAN FEEDING ON GROWTH, PROTEIN CONTENT AND TDC ACTIVITY OF *CATHARANTHUS ROSEUS* (L) G. DON CELL AGGREGATE CULTURE IN THE AIRLIFT BIOREACTOR

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Abstract. There are various ways to increase secondary metabolite content in tissue culture, one of them is precursor feeding. Therefore research to study of the growth, protein content, and TDC activity of *Catharanthus roseus* (L) G. Don aggregate cell culture with the addition of tryptophan as a precursor has been conducted. The aim of the research were to evaluate the growth, protein content, and TDC activity that correlated to enhancement of catharanthine content. The research was carried out experimentally in the laboratory. The concentrations of tryptophan precursors used were 50-250 mg/L for the culture of cell aggregates. Growth was determined by measuring changes of weight and observing the morphological changes. Protein content was determined by the methods of spectrophotometry. TDC activity was determined by the methods of spectrofluorometry. The maximum growth of cell aggregate culture occurred in the treatment of tryptophan 150 mg/L in airlift bioreactor. The addition of tryptophan induced protein synthesis and TDC activity in aggregate cell culture of *C.roseus*. The highest of protein content and TDC activity occurred in the treatment of 250 mg/L after 10days of culture . The highest TDC activity of 3.459±0.001 units after 10 days of culture. The tryptophan treatment enhanced protein content and TDC activity. The results concluded that the treatment of tryptophan as a precursor increased protein content and TDC activity. The optimum growth on tryptophan concentration 150 mg/L after 10 days of culture.

Key words: Tryptophan Feeding, Cell Aggregates, *Catharanthus roseus,* Protein Content, TDC Activity

1 Introduction

Tapak dara (*Catharanthus roseus*) is an annual shrub that has been cultivated as a medicinal plant. Alexandrova *et al.* [1] states that this plant is useful for treating hypertension, diabetes, a bleeding of decrease the number of platelets, chorionic epithelioma, acute lymphocytic leukemia, acute monocytic leukemia, limphosarcoma and cell sarcoma reticulum [2]. Tapak dara ability in the treatment of various diseases indicates that these plants contain several active compounds

that act as medicines. Approximately 130 types of alkaloids have been identified at this plant [3, 4] such as anti-cancer alkaloid vinblastine, vincristine, catharanthine, leurosidine and leurosine. Anticancer compounds are commercialized mostly from this plant, especially the white flowering [5].

The enhancement of catharanthine production can be done with plant tissue culture techniques (*in vitro*). This is because plant tissue culture can be used to produce chemical compounds useful drug desired. Drug compounds that are classified as secondary metabolites in plants. One part of these secondary metabolites are alkaloids. Certain alkaloids produced in higher levels through tissue culture rather than the content of the parent plants [6,7]. Another advantage is the use of tissue cultures for alkaloid production are the production may be regulated, the quality and yield more consistent production, lower production costs and land use can be reduced [1].

Several methods and strategies for the enhancement of secondary metabolites, especially catharanthine in cell aggregate culture has been carried out by El-Sayed, *et al.*, [8]; Hong *et al.*, [9]; Whitmer *et al.*, [10]; Misawa, [11]; Esyanti, *et al.* , [12]; Pandiangan *et al.*, [13]. The methods that has been employed were the immobilization, elicitation, and addition of tryptophan precursor in *C.roseus* cell aggregate culture. The methods of tryptophan precursor feeding was a method that supports the increasing of biomass and a higher of catharanthine content controls [12,13]. The optimally growth and catharanthine content improvement are important for the production of secondary metabolites in a bioreactor. These two aspects, namely increasing of biomass and secondary metabolites, are occurred in the method of addition of tryptophan precursors.

The addition of tryptophan associated with the biosynthesis secondary metabolites such as catharanthine. The catharanthine production on a large scale requires the growth and the catharanthine content enhancement together. The addition of tryptophan can generated both. Tryptophan can be turned into tryptamine by tryptophan decarboxylase (TDC), generated by the presence of TDC gene on *C.roseus*. The TDC gene is expressed form enzyme to convert tryptophan into tryptamine on *C.roseus* [14]. Addition of tryptophan was associated with gene expression and activity of tryptophan decarboxylase [10; 15]. Therefore, the protein content and TDC activities will be interrelated and have been proven in this study.

2. Materials and Methods

Plants used as sources of explants in this study was *Catharanthus roseus* (L.) G. Don a white flowering. The leaves that are still conduct active growth at three to four of the shoot apex as explants. Callus were inducted by following aseptic technique or *in vitro* [16, 17]. Callus were subculture every 21 days. Callus were subcultured during the one year and two months old.

Aggregate cell culture were grown at 25 ^{o}C in 250 mL Erlenmeyer flask. Each Erlenmeyer containing 50 mL MS liquid medium with the same combination of growth regulators with callus production medium. Callus were grown approximately more one year old subcultured. The callus about 5 g were

transferred to 50 mL liquid MS medium, which added 2 mg/L NAA and 0.2 mg/L kinetin (medium NK or T0) [16]. Aggregate cell culture was incubated at room temperature and agitated at a speed of 120 rpm. Subcultures made after 14 days of liquid medium by replacing the old with the new liquid medium with a nutrient composition similar to the previous medium.

Culture results of cell aggregate were separated from the media, then media remaining on the cell aggregates are dried using sterile paper in pretridish, then weighed and then subcultured. Bioreactor that used were 1,5 L volume. Each Bioreactor containing 900 mL MS liquid medium with a combination of growth hormone similar to callus production medium but without agar. Subcultured in bioreactor with tryptophan treatment BT0 (Control) , BT1 (50), BT2 (100), BT2 (150), BT3 (200), BT4 (250) mg/L performed directly at the first subculture. Heavy inoculum used in the culture in bioreactor was 30 g of cell aggregates. The aeration was 0.15 L/min in airlift bioreactor with drough tube.

The growth of cell aggregate was determined by weighing the wet cell aggregates. Aggregate cells were weighed in the scales Digital with 4 decimals precision. Weight measurements performed on each day of harvest or sampling, on day 10 and 14.

Aggregates of cells were harvested 14 days old prioritized after subculture in tryptophan and each cell aggregates in bioreactor and wet weight measured. Cell aggregates were separated from the media and subsequently divided into two parts of the cell aggregates. One part for the analysis of proteins and cell aggregates TDC. One other part was dried in a freeze dryer to be used in the analysis of secondary metabolites such as HPLC catharanthine content. The sample was also taken with the support of the culture day 0 (approximately 6-8 hours after treatment), 10th and 14th, to assure further the role of the precursor tryptophan.

TDC activity determination performed by the following procedures:

a. **Extracts Preparation.** Extraction had been done according to the method of Li *et al.* [18] and Pandiangan, [19] was modified. From each sample 1 g of fresh cell aggregates. Samples were crushed in liquid nitrogen. Results scour added 3 mL buffer containing 0.05 M Tris HCl buffer (pH 8 at a temperature of 0^{o}C) and 0.15% (v/v) Triton X-100. Sentrifuge homogenate 14000 *g* speed for 10 minutes at a temperature of 0^{o}C. Supernatant was taken as an extract for the determination of protein content and enzyme activity of TDC.

b. Determination of Protein Concentration.
Determination of protein content was carried out by following the method of Lowry *et al.* [20]. Determination of protein content was conducted in two stages. The first step is the determination of BSA standard curve regression equation. The second step was to determine the concentration of protein samples. Dye reagent was used Folin-Ciocalteu from Sigma.

c. Determination of TDC activity. TDC activity (EC.4.1.1.28) determination was performed according to Li *et al.*, [18] with some modifications. Determination carried out in two stages. The first step was determining the standard curve

regression equation tryptamine. The second phase determined the concentration of TDC activity samples. Extract about 10-100 µL (30 µg protein) mixed with tryptamine test buffer (100nM Tris HCl, 5 mM b-merkaptoetanol, 10% glycerol pH 8.0) until the volume of 1 mL. TDC activity determined by incubation time 0 minutes and 30 minutes with Tris HCl buffer as a blank. Organic phase or the phluorometric top used for analysis using Shimadzu RF-540 spectrofluorometer. Tryptamine detected at an excitation wavelength of 280 nm and emission 345 nm, was measured in duplicate.

d. Data Analysis. Statistical test using STATISTICA program releases 6 and Excel releases 7. The statistical test used to determine the effect of treatment was done by analysis of variance (ANOVA) in completely randomized design (CRD) that the factorial of 95% confidence level or α = 0.05. If looks very real differences, then followed by a Post Hoc Test DMRT (Duncan's multiple range test) at 95% confidence level.

3. Results and Discussion

3.1 The Observation of Growth

Visual observation of form cell aggregates in the treatment of tryptophan in the airlift bioreactor was generally similar (Figure 1). Effect of tryptophan treatment on the growth of cells on culture in the bioreactor also showed qualitatively that tryptophan is always improving cell growth up to an optimum concentration of tryptophan. The visual observation showed that the days of observation also determine the difference effect on aggregate cell culture. Cell aggregates in BT2, BT3 and BT4 seems more and more resistant to contaminants. BT2-BT4 culture rarely experienced contamination, while BT0 very difficult to get the condition that no contamination. It's probably something to do with the treatment of tryptophan which can lead to optimal condition for growth of cells aggregates cultured in BT2, BT4. Culture also has a pH of about 5.38 -5.68 after 10 days of culture and 5.48 to 5.78 after 14 days of culture and represents the optimum pH. Conditions of culture medium can cause both for the aggregate growth of cells that can enhance cell resistance against contaminant factor.

Optimum cell growth occurs also in BT3-BT4 (tryptophan concentration 150-200 mg/L) either on days 10 and 14 after culture. Days of observation compared with the results of research before was the culture on day 14 maximum. In addition to other technical reasons related to culture in the airlift bioreactor using a draft tube.

Effect of tryptophan treatment on cell growth or the quantitative measurement of wet weight of cell aggregates in the bioreactor shown that tryptophan treatment did not always increase the growth of cells with increasing concentration of tryptophan. Statistic analysis results showed that the days of observation also determine the difference effect on aggregate cell culture. At tryptophan concentrations 100-200 mg/L or cell aggregates BT2, BT3 and BT4 showed optimum growth of cells on day 14. Previous research found in the culture in Erlenmeyer that at 10-14 days is a real effect of tryptophan treatment [13]. This

phase of the cell aggregate culture using the resources that have been subcultured callus during the first year two months, showing optimum growth at day 14 only (Figure 2). On day 10 that BT3 and BT4 were not significantly different had been testing DMRT. Therefore, we recommend adequate harvest and subculture on day 10 when treated with 200 mg/L tryptophan. But for BT3 should harvest on day 14. Besides tryptophan treatment significantly affect the cell aggregate biomass changes in the treatment BT3 with 135.25% enhancement at day 14 after culture in tryptophan treatment.

The observations of growth Tapak dara aggregate cells in the bioreactor days 10 and 14 have a generally similar pattern. However, on day 14 of treatment seems BT3 has a larger growth. The optimum treatment on growth of tryptophan contained in BT3 or treatment of tryptophan 150 mg/L on day 14 while at day 10 in the treatment of optimum growth BT3 and BT4. BT5 treatment day 14 showed decreased cell biomass. It can also caused by inhibition mechanism behind on protein synthesis and expression of genes that promote growth [21, 22].

Figure 1. Aggregate cell culture of *C.roseus* in the bioreactor after 1 day culture in media treatment of the precursor tryptophan. BT3 (150 mg/L), BT4 (200 mg/L) and BT5 (250 mg/L).

3.2 Total Protein Content

Protein content of Tapak dara cell aggregates were cultured in the volume of 1.5 L airlift bioreactor can be seen in Table 1. In tables can be seen the average content of protein with tryptophan treatment (mg/L) control (BT0), 50 (BT1), 100 (BT2), 150 (BT3), 200 (BT4), 250 (BT5). The day was the observations made on days 0, 10 and 14 after culture in treatment medium. Each measurement is always carried out together with the culture in Erlenmeyer.

Table 1. Average total protein content (mg/mL) of cell aggregate *C.roseus* in 1.5 L airlift bioreactor with tryptophan treatment (mg/L) control (BT0), 50 (BT1), 100 (BT2), 150 (BT3), 200 (BT4), and 250 (BT5).

Tryptophan Treatment	Day 0		Day 10		Day 14	
	Protein Average	± SD	Protein Average	±SD	Protein Average	±SD
BT0	0.72 **a**	±0.01	1.30 **c**	±0.01	1.34 **de**	±0.03
BT1	0.72 **a**	±0.01	1.33 **d**	±0.03	1.36 **e**	±0.01
BT2	0.73 **ab**	±0.01	1.40 **f**	±0.01	1.40 **f**	±0.02
BT3	0.73 **ab**	±0.01	1.75 **i**	±0.02	1.78 **j**	±0.01
BT4	0.75 **b**	±0.01	1.80 **h**	±0.01	1.66 **h**	±0.02
BT5	0.75 **b**	±0.01	2.07 **k**	±0.02	1.60 **g**	±0.02

Notes: Average obtained from three times of measurement. Each duplo measurements were carried out.
Letters on the same figures showed no significant difference at 95% confidence level

Tryptophan treatment increased the protein content by increasing tryptophan concentration occurred on day 10 after treatment. The highest protein content peak occurs on day 10 of 2.07 ± 0.006 Total (mg/mL) as shown in Table 1. But on the observation day-to-14 precisely reduced from BT3 on the treatment of tryptophan 200 to 250 mg/L (BT4 and BT5), and higher than BT0 (control). Reduced levels of these proteins may be subject to degradation of proteins and denaturation of proteins [21, 23].

Protein content in the bioreactor by tryptophan treatment showed significant differences in effect, it shows that the process of protein synthesis and degradation in either the process or system is a different culture [25]. Synthesis of different proteins can caused by different environmental conditions, which can ultimately lead to different protein levels [26]. Different protein levels in cells would also lead to differences in cell volume that can alter its growth. Besides, presumably related to the enzyme that catalyzes the synthesis of protein secondary metabolic reactions that produce secondary metabolites. It will be presented at further research, like catharanthine content.

Results of statistical analysis (Table 1) showed that the treatment tryptophan from BT1 to BT5 days 10 and 14 showed a significant effect on serum proteins and generally increased with increasing concentration of tryptophan treatment. However, on day 0 or early did not differ significantly between BT0 up until BT5, BT3 and BT2. The highest protein content was 2.07 ± 0.02 mg/mL.

In general, the effect of tryptophan treatment on total protein aggregates Tapak dara cells on day 10 after culture was varies. But on day 14 in treatment BT4 and BT5 lower than BT3. This indicates that the day 14th in treatment is alleged to

have been degraded or proteins undergo modification such as transamination, deamination glikolisasi and into other compounds by specific enzymes as the adjustment of cell aggregates of the condition or physiological and biochemical processes aggregates cells [21, 22]. Another possibility is the competitive reaction between the precursor tryptophan protein synthesis and synthesis of secondary metabolites [24]. Due to the possibility of the BT4 and BT5 on day 14 had a lower protein content than BT3.

3.3 TDC Activity

Figure 2 shows the average TDC activity of the cell aggregates treated with tryptophan (mg/L) control (BT0), 50 (BT1), 100 (BT2), 150 (BT3), 200 (BT4), 250 (BT5), on days 0, 10 and 14 after culture in treatment medium. Similarly, total protein content, that the treatment of tryptophan increased TDC activity with increasing concentrations of tryptophan on day 10 after culture (Figure 2). However, total protein content BT4 and BT5 was lower than BT3 on day 14, though still remains at the top day 0. Despite the lower protein content but the activity of TDC in BT4, BT5 remained higher on day 14. This indicates that the protein is not subject to degradation or modification of protein TDC, even the synthesis or activation of TDC increased with increasing precursor tryptophan. Highest TDC activity occurred in BT5 after 10 days of culture that is 3.459 ± 0.002 (Unit).

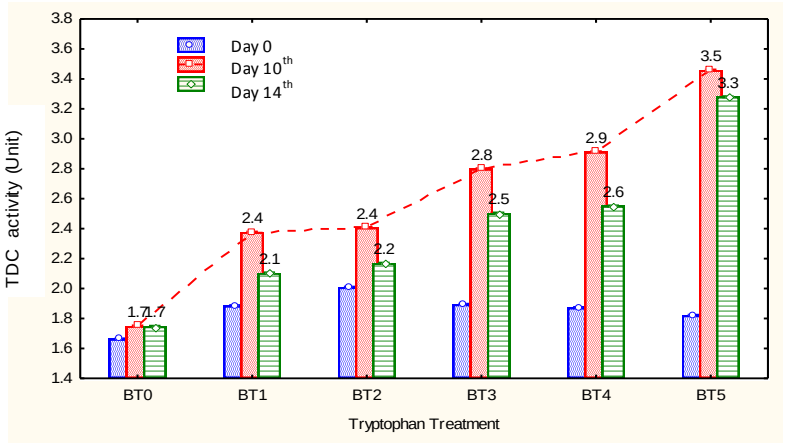

Figure 2. TDC activity in cell aggregates culture were cultured in 1.5 L airlift bioreactor treated with tryptophan (mg/L) Control (BT0), 50 (BT1), 100 (BT2), 150 (BT3), 200 (BT4), and 250 (BT5).

The effect of tryptophan on cell aggregate culture in the airlift bioreactor was significantly different. ANOVA results showed that tryptophan BT1 until BT5 had a significant effect on TDC activity in the bioreactor. These activities also increased with increasing concentration of tryptophan after treatment of cultured 10 and 14 days (Figure 2). These results show that the days of culture also influences the protein content of cells generated due to the treatment. The hypothesis is that treatment can affect the activity of the precursor tryptophan TDC proven and

acceptable. Generated is a positive influence that is increasing activity with increasing concentrations of TDC tryptophan. TDC activity on day 10 was higher than the 14th day after culture.

4. Conclusions

1. The growth of cell aggregate of Tapak dara (*C.roseus*) were increased at all concentrations of tryptophan from 50-250 mg/L of control and optimum at 150 mg/L after 14 days of culture.
2. Addition of tryptophan to the cell aggregate culture of *C.roseus* could increase the protein content in line with increasing concentrations of tryptophan and the highest protein content occurred in the treatment of 250 mg/L is 2.07 ± 0.06 mg/mL after 10 days of culture.
3. Addition of tryptophan to the cell aggregate culture *C.roseus* can enhance TDC activity in line with the addition of increasing concentrations of tryptophan, and the highest activity occurred in the treatment of 250 mg/L tryptophan with the highest activity of 3.459±0.001 Units after 10 days of culture.

Suggestions

It needs a follow-up experiment to see how the links between proteins, TDC activity increased with higher levels of catharanthine content of cell aggregates. This relationship can be determined at the stage of catharanthine analysis and its relation to other metabolites.

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