THE INFLUENCE OF IMMOBILIZATION ON CELL GROWTH AND CATHARANTHINE PRODUCTION IN THE CELL AGREGATE CULTURE OF *Catharanthus roseus* (L.)G.Don

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Abstract. *Immobilization method is frequently used as one of the reliable techniques to increase in vitro secondary metabolite production. Research has been conducted to evaluate the influence of cell immobilization on cell growth and catharanthine production in C.roseus culture. Callus was first initiated in MS medium with 1,5 mg/L 2,4-D and 0,15 mg/L kinetin. The cell agregate derived from callus of Catharanthus roseus was cultured in MS medium with 2mg/L NAA and 0,2 mg/L kinetin, and subcultured every 10 days for 4 times before immobilization. Immobilization was conducted by using 2,5% Na-alginate. The cell growth and production pattern of catharanthine in the cells agregate were observed every 3 days for 27 days. Qualitative and quantitative analyses were conducted by using HPLC, eluted by using methanol : acetonitrile : diamoniumhydrogenphosphate (3:4:3), rate: 1ml/minute. The results showed that catharanthine was detected in the cells agregate and culture medium. The highest concentration of catharanthine in the control cell was detected on the 9th day of culture, i.e. 39,1843 µg/g, while in medium was obtained on the 12th day of culture, i.e. 5,6892 µg/L. Analysis on the immobilized cells showed that optimal content on the cells agregate was obtained on the 9th day (136,4230 µg/g), while in the medium was on the12th day of culture, i.e. 17,6306 µg/L. The average concentration of catharanthine in immobilized agregate cell culture increased 248 % compared to the control. The result also showed that catharanthine was still retained in the alginate matrix, and reached 3,6974 µg/L on the 18th day. Therefore further experiment is still needed to optimize the secretion of catharanthine into the medium.*

Keywords: *Catharanthine, Immobilization, Catharanthus roseus, aggregate cells, alginate, beads.*

1 Introduction

Plant secondary metabolite can be useful for human beings as raw material of medicine, flavours, fragrance, colours and insecticides (Fulzele, 2000). One of the plants that can be used as a raw material of medicine is *Catharanthus roseus*. Traditionally, these plants can be used to cure hypertention,malaria,diabetes and cancer (Scragg, 1994). *C.roseus* produces alkaloid, namely vincristine and vinblastine which are potencial as anticancer compounds. Naturally, in vivo production of secondary metabolite vincristine and vinblastine are not only quite low but also cannot be produced synthetically, so vincristine and vinblastine are produced semisynthetically in industrial scale (Zhao *et al*, 2001).

This procces need primary substances,catharanthine and vindolin. Vindolin is synthesized in plant in a high concentration,but catharanthine is only in a very small amount (Fowler & Warren, 1992). Therefore further experiment is needed to enhance catharanthine production.

Enhancing catharanthine production by using tissue culture method is among the alternatives, because this method is not significantly influenced by environmental factors, and the growth condition of the culture can be controlled (Gaines, 2004). There are various methods used to increase *in vitro* secondary metabolite production, among others is immobillization. Immobillization method is used to trap aggregate cell in matrix (e.g alginate). Trapping aggregate cell causes the decrease of cell growth, which will then could cause an increase production of secondary metabolite, (e.g catharanthine).

In the previous experiment, immobillization method with 2,5% alginate matrix could promote the production of ajmalicin to 122% in C.*roseus* culture using Zenk medium (Martin, 2004). Therefore these similar method was applied to enhance catharanthine production in the aggregate culture of *C. roseus* using Murashige & Skoog (MS) medium.

2 Materials and Method

Initiation of callus and aggregate cell of Catharanthus roseus

Callus culture *of Catharanthus roseus* was established from leaf explants on MS medium. The initiation medium contained 1.5 ppm 2,4-D and 0.15 ppm kinetin, whilst production medium contained 2 ppm NAA and 0.2 ppm kinetin. Callus was transferred into liquid medium for

secondary metabolite production. Cultures were placed on rotary shaker (120 rpm) and incubated at 25 ^{o}C, in 24 h light.

Immobillization method

Immobilization was conducted by using 2,5% Na-alginate. Aggregate cell was separated from the medium, before adding 2,5% Na-alginate solution. The cells were then dropped into 50 ml MS medium containing 50Mm CaCl2 to form beads. These beads were washed with 25 mM CaCl2 solution. After being washed, the beads were incubated in MS medium suplemented with the same growth regulator. The growth of the cells and production patterns of catharanthine in the cells aggregate as well as in medium and alginate matrix were observed every 3 days for 27 days.

Sample extraction and analysis of catharanthine

Catharanthine was extracted from cell aggregates and medium according to the modification of method described in Lee *et al.*(1981 in Asada & Shuler, 1989). HPLC analysis was performed by using a shimpak VP-ODS C18 (0.15 x 0.6). The solvent system was methanol : acetonitrile : diamonium hydrogen phosphate (3:4:3), eluted isochraticaly. The samples were injected at 1.0 mL min−1. Peak identification was based on a comparison of retention time with authentic catharanthine standard and wave length(τ) is 220 nm.

3 Result and Disscusion

Aggregate Culture establishment

Callus was first observed after the leaf explant was cultured for one week in MS medium supplemented with 1.5 ppm 2,4 D and 0.15 ppm kinetin (see fig.1). Callus was in the form of compact structure. Compact structure grew slowly but it could produce high secondary metabolite, which might be due to high integration of cells to each other, so that the differentiation procces which induced the synthethis of secondary metabolite would increase accordingly. Several studies showed that dark and compact callus usually contained higher alkaloid, such as catharanthine, vindoline, ajmalicine, and serpentine (Morris, 1986 in Fowler and Warren, 1992). In order to produce high yielding catharanthine line, callus was transferred into MS medium supplemented with 2 ppm NAA and 0.2 ppm kinetin so that it produced agreggate cells (see fig.2). The source of auxin, (2,4-D) in initiaton medium was replaced by NAA in production medium. NAA did not induce synthesis of celulase and pectinase enzyme, therefore, it was suitable for inducing cell aggregate formation (Krishnamoorty, 1981).

Figure 1. Callus of *Catharanthus roseus*.

Figure 2. Agreggate cells.

Cell immobillization

The beads which were formed by dropping cells in alginate into medium containing CaCl2 experienced ion change between Ca2+ and Na+. Both ions switched their places, Na-alginat gel which was not soluble in medium formed a layer in a ball shape in the outer cell (see fig.3&4).

Figure 3. Immobillized cell.

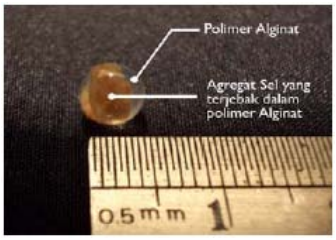

Figure 4. The Catharanthus roseus cell in alginate matrix.

Alginate matrix which in liquid MS medium covered agregat cells could influence growh procces of cells. Figure 5 showed a growth pattern of cells, which indicated that cells trapped in alginate matrix experienced a growth slow. Result of some experiments showed that alginate matrix could cause obstruction of nutrition and oxygen diffusions. Consequently, the cells trapped in the alginate grew more slowly than the free cells (Mattiason, 1982).

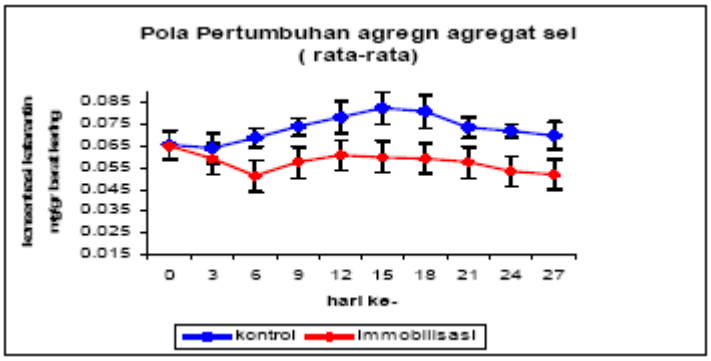

Figure 5. Growth pattern of *C.roseus* control and agregate cells immobilized.

Catharanthine accumulation in cell and medium

Catharanthine in agregate cell was observed being synthized starting on the first day (see fig.6). and reached the highest concertation on the 9thday (39,1843 µg/g) It was estimated that the cells during that periode produced catharanthine optimally,because nutrition was available in a great amount. Nutrition was not only used to grow the cell but also used to produce secondary metabolite (Jolicoeur, 2002). Catharanthine which was synthized in the cells could be secreted into the medium. The secretion of catharanthine occured on the 3rdday and increased to reach a maximal amount on the 12thday (5,6892 µg/L) (see fig.6) Most of alkaloids (catharanthine) was secreted using ion transport mechanism and passive diffusions (Knorr & Dornenburg, 1995).

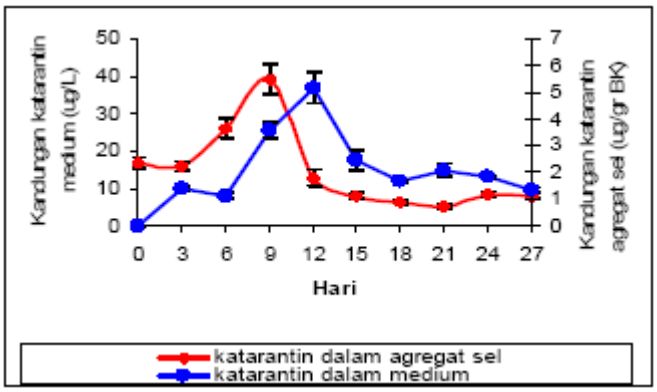

Figure 6. Catharanthine content in control.

Our research (fig 6) immobilization treatment showed that catharanthine was detected in medium,cells and alginate matrix. Catharanthine biosynthesis in aggregate cell was optimal on 9thday (136,4230 µg/g), while in the medium was on the 12thday of culture, i.e. 17,6306 µg/L (fig.7).

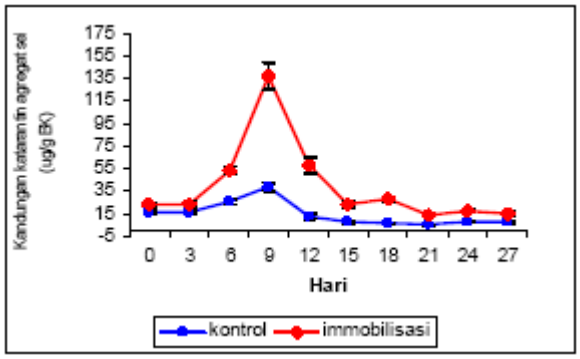

Figure 7. Catharanthine content culture which was immobilized.

However immobillized cell, catharanthine was also retained in alginate matrix, so that catharanthine could not be secreted into medium optimally. Catharanthine was still retained in the alginate matrix at the concentration of 3,6974 µg/L on the 18th day (fig.7)

Comparison of Catharanthine content in control and immobillized cell

The average concentration of catharanthine in immobilized agregate cell culture was 248 %, higher compared to that in the control (see fig 8 & 9).

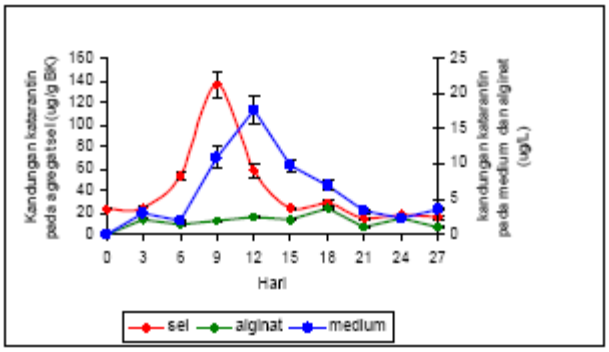

Figure 8. The comparison of catharanthine content between control and the cells was immobilized in the cell.

Several experiments showed that alginate matrix also induced stress condition in the immobillized cell. Therefore, beside creating more contact among cells, it could also cause the obstruction of cell growth, so cells growth was reduced in immobilized cells. Since cell growth was reduced in immobilized cultures, substrate consumption and flux of energy was directed to

the desired secondary metabolite pathway and so improved product yields of the process. Growth reduction and stress, in combination resulted in higher metabolite production. The same result was observed in *Datura innoxia* culture which was immobilized, immobilization could promote scopolamine production (Robin, 1986). Immobilization using pectin capsulation in *Vitis vinifera* also increased anthraquinone production (Dornenburg, 2004).

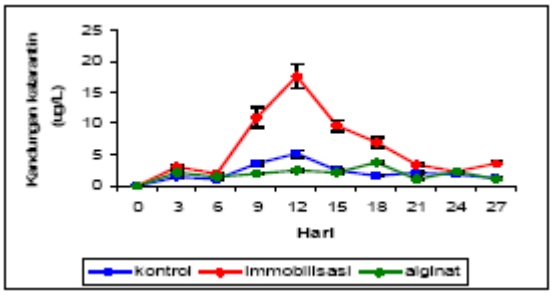

Figure 9. The comparison of catharanthine content between control and the cells was immobilized in the medium.

Results in figure 9 also indicated that imobillization increased catharanthine released into the medium by 82.66 %. This result was also supported by Jordin *et al.* (1991). Their experiment showed that immobilization caused a released of alkaloids to 50% particulary ajmalicin, into medium compare to control cell which release only 10-25 % of alkaloids into the medium.

However the secretion process was not optimal, it was detected that catharanthine was still retained in the alginate matrix. The secretion of catharanthine into medium was greatly influenced by the size of alginate pores and alginate concentration which were used to make beads (Facchini & Dicosmo, 1991). Therefore, further experiment is still needed to optimize the secreation of catharanthine into the medium.

Acknowledgements

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