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Enhancement Mulberroside A Production in *Morus alba* L. Cell Cultures by Calcium Alginate Immobilization and Elicitation

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Abstract

Introduction: Mulberroside A is a major stilbene glycoside in root bark extract of *Morus alba* L. (Moraceae). This compound has been used commercially as an active ingredient in whitening cosmetics due to its anti-tyrosinase and anti-oxidant activities. To enhance mulberroside A production, this study aimed to investigate effect of immobilization and elicitation on accumulation of mulberroside A in cell cultures of *M. alba*. **Methods:** *M. alba* cell cultures were immobilized in calcium alginate beads and cultivated in Murashige and Skoog (MS) liquid medium containing 0.1 mg/L thidiazuron and 1 mg/L napthalene acetic acid. Then, culture beads were elicited with methyl jasmonate and yeast extract and observed mulberroside A accumulation at 24, 48 and 72 hr after elicitation. **Results:** Cell immobiliz ation in calcium alginate enhanced the production of mulberroside A over control (un-immobilization) by 1.4-fold in 40 days and 9.4-fold in 120 days. Addition of 50 mg/mL yeast extract and 50 μM methyl jasmonate in 40 days, *M. alba* cell immobilized cultures resulted in greatest mulberroside A accumul ation by yielded 32.74 mg/g DW which is 2.4- and 1.9-fold higher than control and immobilized cells, respectively. In all conditions, more than 60% of total mulberroside A production was released into culture medium. **Conclusion:** The results may indicate that immobilization slowed the growth rate and stimulated mulberroside A release of *M. alba* cell cultures. Moreover, the combination between immobilization and elicitation showed a synergistic effect to enhance the production of mulberroside A.

Keywords: Mulberroside A, *Morus alba* L., Immobilization, Elicitation, Alginate

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1. Introduction

Genus *Morus* (Family Moraceae) con sists of 10-15 species and extensively planted throughout the warm climate over the world. In Thailand, *Morus alba* L. or White mulberry is the dominant cultivated species among all (Butt, *et al*., 2008). Mulberry leaves are an important source of food for silkworm due to high protein and carbohydrate contents. Moreover, their root barks which contain high levels of mulberroside A have also been used commercially as an active ingredient in whitening cosmetics. Previous stud ies were reported pharmacological activities of mulberroside A as an effective anti-tyrosinase and anti-oxidation activities (Kim, *et al*., 2010). Gener ally, the conventional propagation of mulberry tree required long period of cultivation. In addition, season of harvest also effect the production levels of secondary metabolite (Zhou, et al., 2013). Therefore, immobilization of plant cell cultures has considered as an alternative cultivation system for solving the limitation of conventional technique (Kamaraiah, *et al*., 2003). This culture technology also decreases maintaining cost of *in vitro* large scale culture and simplify collection process by harvest from culture medium. The use of high biomass for extended periods can reduce the cultivation cost and hence increase the productiv ity of target substances (Villergas, *et al*., 1999). Beside prolonged cultivation period by physical technique, elicitation is one of the effective chemical enhancement strategies for increasing product yield. In the previous study, yeast extract and methyl jasmonate are an effective exogenous elicitors which enhances variety of secondary metabolite (Sanchez-Sampedro, *et al*., 2005; Komaikul, *et al*., 2013). Therefore, this study is aim to investigate effect of immobilization and elicitation on cell immobilization cultures at various incubation times on accumulation of mulberroside A content in *M. alba* cell cultures.

2. Material and Methods

1) Chemicals: Mulberroside A was purchased from Chengdu biopurity, Ltd. Polyclo nal antibodies (PAb) against Mulberside A was produced by our research group as reported previously (Komaikul, et al., 2014). Peroxidase labeled anti-rabbit IgG was purchased from MP biomedical. 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), methyl jasmonate and yeast extract were obtained from Sigma chemical, Co. Alginic acid from brown algae and citric acid were purchased from Wako, Inc. Other chemical substances which used in the experiment are products of analyticallgrade.

2) Plant material: M. alba callus cul tures were cultivated in Murashige and Skoog (MS) solid medium contain 0.1 mg/L Thidiazuron and 1 mg/L Napthalene acetic acid. Cell suspen sion cultures were established in 250 ml-flask containing 30 ml MS (T0.1N1) liquid medium. All flasks were aerated in shaker machine at 80 rpm, 12 hr-light andl25±2l°C.

3) Cell immobilization cultures: M. alba cell suspension cultures were harvested in day 21 of growth. Desired amount of cells (20 g FW) were mixed with 100 ml of sterile alginic acid from brown algae (2%). Then, mixture was drop into calcium chloride solution (2%) by using 10 ml

cutting edge glass pipette. The beads were stirred in calcium chloride solution for 30 minutes and rinsed with distilled water 3 times. Cell immobiliza tion and free cell were cultured in the same medium as cell suspension. All flasks were aerated in shaker machine at 80 rpm with 12 hr-light and 25±2 °C. The effect of calcium alginate immobilization on mulberroside A accu mulation waslinvestigatedlwithinl120ldays.

4) Elicitation: Yeast extract (YS) and methyl jasmonate (MJ) in various concentrations (Table 1) were added to a 40 day M. alba L. cell immobilization cultures. The immobilized cells in each group were harvested at 24, 48 and 48 hoursLafterLelicitation.

Table 1 The concentration of yeast extract and methyl jasmonate for elicitation process.

Group (n=3)	Yeast extract (mg/mL)	Methyl Jasmonate (μM)
Control	-	-
YS	200	-
MJ	-	2
YS+MJ 1	50	0.5
YS+MJ 2	100	1
YS+MJ 3	200	2

*5) Sample extraction and mulberroside A analysis:*After harvested, the entrapped cells were recovered by immersed covering alginate for 30 minutes with 1.5% sodium citrate solution. Fresh cells were collected and dried by using hot air oven at 50 °C for 2 days. Then, 30 mg dried powdered were weight and extracted by sonicated four times using absolute methanol. The extracted solutions were combined, eva porated in water bath at temperature 50 °C and re-dissolved with 1 ml of absolute methanol. Mulberroside A was analyzed by indirect competitive ELISA using PAb against mulber roside A (Komaikul, *et al*., 2014). In detail, after coated 96 well plates with mulberroside A-OVA (1 μg/ml in 50 mM Carbonate buffer, pH 9.6), the plate was treated with 1% gelatin in phosphate buffer solution (PBS) to reduced non-specific binding. Then, 50 μl of extracted solution was dissolved in 20% MeOH and incubated with 50 μl PAb against mulberroside A for 1 hr in 37 °C. After washed plate with Tween 20 in phosphate buffer solution (TPBS) 3 times, 100 μl of 1,000 folds dilution peroxidase labeled anti-rabbit IgG solution was added and incubated again. After 1 hour, the plate was washed with TPBS and 100 μl of substrate solution (100 mM of Citrate buffer (pH 4.7) containing 0.003% H_2O_2) was added and incubated for 15 minutes. The absorbance was measured at 405 nm using micro plate reader (Model 550 Microplate reader, Biorad Labora tories). Culture medium and digested alginate medium were collect and analyzed the same method as fresh cell. All samples were analyzed in triplicate.

6) Statistic analysis: The data were presented as mean ±SD and tested the difference between samples by using one-way analysis of variance (ANOVA), turkey test at p-value < 0.05.

3. Results Effect of immobilization and elicitation on production of mulberroside A in *M. alba* L. cell cultures

Figure 1 displayed effect of calcium alginate immobilization on mulberoside A acc umulation in *M. alba* cell cultures within 120 days. The result exhibits that a major portion of mulbe rroside A was excreted into the culture medium (approximately 60% of total mulberroside A). Total production of mulberroside A in cell imm obilization was enhanced over control cell by 1.4-fold in 40 days (21.32±0.94 mg/g DW) and 9.4-fold in 120 days (19.21±2.06 mg/g DW). Then, day 40 of growth was chosen for addition of elicitor. After adding yeast extract and methyl jasmonate in various concentrations, the cells were collected, immersed and determined mulbe rroside A production as described previously. The result shows that addition of 50 mg/mL yeast extracts and 50 μM methyl jasmonate with 24 hours of incubation resulted in the greatest mulberroside A production by yielded 32.74 mg/g DW which is 2.4- and 1.9-fold higher than control and immobilized cells respectively (Figure. 2).

4. Discussions

Observation of this study is in agreement with the previous report in which immobilization technique reduced rate of growth in variety of plant cell cultures by decreasing the effect of shear stress and protease enzyme (Brodelious, *et al*., 1985)(Abdemajeed, *et al*., 2012). Moreover, entrapment with alginate beads also stimulated the release of secondary metabolite into culture medium (Kamaraiah, *et al*., 2003; Villegas, *et al*., 1999). In addition, elicitations of combination exogenous yeast extracts and methyl jasmonate were effective signal transductions to induce mulberroside A production in *M. alba* cell immo bilization cultures. Yeast extracts stimulated plant defense response of microbe while methyl jasmonate regulated chemical defense responses (Sanchez-Sampedro, *et al*., 2005). This may indicate that immobilization is an effective phy sical stimulation by prolongation period of growth and easy harvesting method. Moreover, combin ation technique between immobilization and elicitation showed a synergistic effect to enhanced mulberroside A production in cell cultures of *M. alba*. Furthermore, the application of immobiliza tion and combination immobilization with elicitation may apply for the industrial scale for improvement the production of mulberroside A in *M. alba* cell cultures.

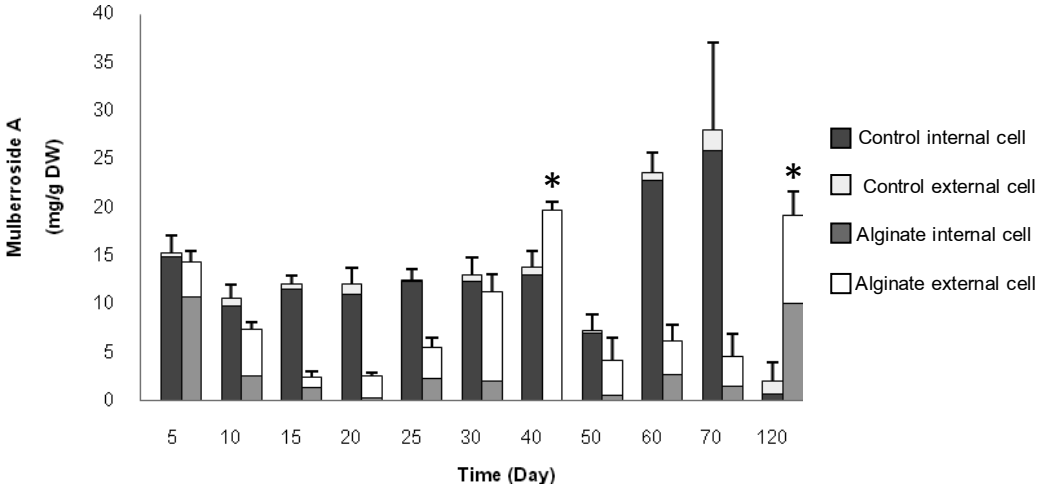

Figure 1 Effect of calcium alginate immobilization on mulberoside A accumulation in *M. alba* cell cultures

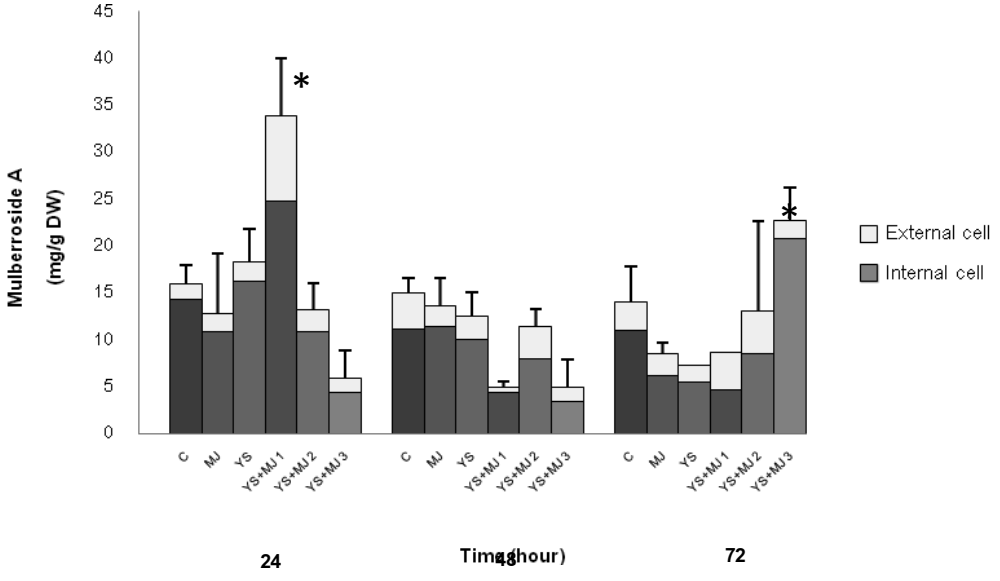

Figure 2 Effect of yeast extract and methyl jasmonate on mulberroside A production in *M. alba* cell immobilization cultures after 24, 48 and 72 hours of elicitation

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