

OPTIMIZATION OF PHOSPHATE, NITRATE,
SUCROSE AND INCUBATION TIME ON THE
PRODUCTION OF ASCORBIC ACID IN
SUSPENDED CALLUS OF *Citrus grandis* (L.)
OSBECK.

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UNIVERSITI MALAYSIA PERLIS
2010



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By

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LIST OF ABBREVIATIONS

ABA	-	Absisic acid
ANOVA	-	Analysis of variance
ATP	-	Adenosine triphosphate
AQ	-	Anthraquinones
CCD	-	Central composite design
cm	-	centimeter
CV	-	Coefficient of variation
DNA	-	Deoxyribonucleic acid
DOE	-	Design of experiment
g	-	Gram
g/l	-	Gram per liter
HCl	-	Hydrochloric acid
kg	-	kilogram
L-AA	-	L-ascorbic acid
m	-	meter
MARDI	-	Malaysian Agriculture Research and Development Institute
mg	-	milligram
mg/ml	-	milligram per milliliter
mg/l	-	milligram per liter
min	-	minute
mM	-	millimolar
MS	-	Murashige & Skoog
N	-	normality
NaOH	-	Natrium hydroxide
NO ₃ ⁻	-	Nitric acid
ppm	-	part per million
PRESS	-	Prediction sum of squares
psi	-	pound per square inch
RM	-	Ringgit Malaysia
rpm	-	rotation per minute

RSM	-	Response surface methodology
SE	-	Standard error
SEM	-	Scanning electron microscope
UK	-	United Kingdom
USD	-	United State Dollar
UV	-	Ultra violet
wt/vol	-	Weight per volume
μm	-	micrometer
μM	-	micromolar
$^{\circ}\text{C}$	-	degree celcius
2,4-D	-	2,4- dichlorophenoxyacetic acid

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LIST OF SYMBOLS

σ^2	-	Variance
β	-	Beta
ϵ	-	random error term

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Pengoptimuman Fosfat, Nitrat, Sukros dan Masa Pengeraman untuk Penghasilan Asid Askorbik di dalam Kalus *C. grandis* yang Terampai

ABSTRAK

Tisu *Citrus grandis* yang berpotensi dalam menghasilkan asid askorbik yang tinggi telah dikaji dalam kajian ini. Di dalam teknik tisu kultur, nutrien dalam media merupakan faktor yang penting dalam memanipulasi hasil akhir yang diinginkan. Fosfat, nitrat dan sukros berserta dengan masa pengeraman telah dianalisis bagi mengetahui pengaruh bahan-bahan ini terhadap penghasilan asid askorbik di dalam kultur terendam *C. grandis*. Keputusan yang diperoleh di dalam rekabentuk faktorial telah mengesahkan yang diantara keempat-empat faktor tersebut, hanya sukros dan masa pengeraman memberikan kesan yang nyata apabila hadir secara sendiri. Walau bagaimanapun, kesemua faktor memberi kesan kepada pengeluaran apabila hadir bersama-sama. Hasil ini disahkan oleh interaksi yang nyata di antara keempat-empat faktor. Julat kepekatan yang sesuai bagi faktor-faktor ini kemudiannya digunakan di dalam kajian pengoptimuman. Dengan menggunakan metodologi permukaan sambutan (RSM) melalui rekabentuk komposit berpusat (CCD), keadaan yang optimum bagi faktor-faktor yang terpilih telah diperoleh. Keputusan kajian menunjukkan apabila kalus dibekalkan dengan 506.3 mg/l fosfat, 1916.7 mg/l nitrat dan 65.2 g/l sukros dan dieram di dalam bilik gelap selama 7 hari, hasil asid askorbik yang maksimum berjumlah 34.53 ± 2.20 mg/l diperoleh. Hasil yang diperoleh adalah dalam julat asid askorbik yang diramal dan oleh yang demikian, model kajian boleh diterima. Kemudian, pengkulturan kalus di dalam bioreaktor dijalankan. Kepekatan asid askorbik yang diperoleh adalah 9mg/l yang mana lebih rendah berbanding kepekatan yang diperoleh melalui pengkulturan di dalam kelalang. Hal ini disebabkan pengekangan yang dihasilkan oleh keadaan di dalam bioreaktor yang tidak sesuai untuk kalus.

ABSTRACT

The potential of *C. grandis* tissue in producing high amount of ascorbic acid has been investigated in this study. In tissue culture technique, nutrients in the media have been a major factor in manipulating the final yield required. Phosphate, nitrate and sucrose with incubation time have been studied on their influence in production of ascorbic acid in *C. grandis* suspension cultures. The result obtained in factorial design indicates that among all four factors, only sucrose and incubation time give significant effect when present alone. However, all four factors have influence in the production when present together. This result was verified by the significant interaction among all the four factors. Then, suitable ranges of concentration for selected factors that obtained from factorial design were used in the optimization study. By using response surface methodology (RSM) through central composite design (CCD), the optimum conditions of selected factors were obtained. The result shows that when callus was supplied with 506.3mg/l phosphate, 1916.7 mg/l nitrate and 65.2 g/l sucrose and incubated in dark room for 7 days, maximum production of ascorbic acid at 34.53 ± 2.20 mg/l was obtained. The results obtained were in the range of predicted ascorbic acid and therefore the model of the study is acceptable. Then, the cultivation of callus in a bioreactor was carried out. The concentration of ascorbic acid obtained was 9 mg/l of which is very low compared to the production obtained in flask. This is due to the restriction provided by the unsuitable condition in the bioreactor for the growth of callus.

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CHAPTER 1

INTRODUCTION

1.1 Ascorbic Acid Production and Demand

Ascorbic acid has been firmly established as protective dietary antioxidants. Due to its beneficial effects on human health and ability to prevent various type of disease, this compound has become a highly demand product. Apart from that, ascorbic acid also contributes in product quality for food industry. It acts as a color improver and palatability of many food products such as in fruit juice. It is also used for maintaining meat color.

Through out the years, most of ascorbic acid that is consumed is mostly synthetic product. This synthetic ascorbic acid is obtained through chemical synthesis and fermentation. There is small production of natural ascorbic acid that is generated through extraction from plants. Through this technique, high cost of operation is required. Due to this matter, the product that generated cannot put up for sale on compatible price with synthetic ascorbic acid. High price of natural ascorbic acid has made manufacturer more interested in using synthetic product.

Nowadays, demand on natural products has increased as people are more health conscious. According to Mintel, a global market research company, the natural and organic personal care products market has grown 35 percent from USD 345 million to USD 465 million since 2005 (Granato, 2008). In Malaysia, the market size for natural

product had increased annually especially for herbal remedies that is estimated to be above RM 2 billion (MATRADE, 2008).

1.2 Ascorbic Acid in *Citrus grandis*

Natural ascorbic acid has been obtained widely from citrus family. One of the fruit in this family that is verified as a potential source of ascorbic acid is *Citrus grandis* (Guddadarangavvanahally, Jayaprakasha, Girennavar, & Patil, 2008; Jang, Chang, Chang, & Hsu, 2010). According to Department of Agriculture, there is about 44.8 mg ascorbic acid in 100g edible part of *C. grandis* fruits (Agriculture, 2009).

Several researches have been done in determining ascorbic acid in *C. grandis*. Tsai (2007) and his researchers had done a research on antioxidant content in *C. grandis* juice and freeze-dried product. They found that the content of ascorbic acid in the fruit is about 472 mg/ml respectively. Another research in Thailand also shows that there is high content of ascorbic acid in *C. grandis* juice samples they studied. They obtained about 37.03 mg to 57.59 mg ascorbic acid in 100 ml of *C. grandis* juice (Pichaiyongvongdee & Haruenkit, 2009). High content of ascorbic acid is also obtained in a research done by Xu et al. (2008) in their study on citrus varieties cultivated in China. The two varieties of *C. grandis* studied show ascorbic acid content around 390.57 mg/l and 314.19 mg/l. All the researches done verified that *C. grandis* is one of the fruits in citrus family that provides high amount of ascorbic acid.

1.3 Advantages of Tissue Culture Technique for Secondary Metabolites

Production

Plant tissue culture is an *in vitro* technique to produce and multiply plant cells under sterile condition (Debergh & Zimmerman, 1991). In today's world, plant tissue culture had been a successful technology in producing variety of products. This is due to various benefits provided by the technique. Through tissue culture, several advantages are achieved such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free plants, non-seasonal production, germplasm conservation and facilitating their easy international exchange (Govil & Gupta, 1997).

Plant cell and tissue culture has been developed as an attractive alternative for the production of commercially important biochemical products. The major valuable chemicals from plant cell culture are the secondary metabolites. As being stated by Mulabagal and Tsay in 2004, the evolving commercial importance of secondary metabolites has in recent years resulted in a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted.

1.4 Factors Influence Secondary Metabolites Production in Culture

Tissue culture cells typically accumulate large amounts of secondary compounds only under specific conditions. Several products were found to be accumulating in cultured cells at a higher level than those in native plants through optimization of cultural conditions. The accumulation of secondary products in plant cultures depends on the composition of culture medium, including the types and amounts of plant growth regulators, mineral salts and carbon sources used and environment conditions, including temperature, light and gas composition during culture (Matkowski, 2008; Rao & Ravishankar, 2002; Rout, Samantaray, & Das, 2000).

Culture environmental conditions such as light, temperature, medium pH and oxygen have been examined for their effect upon secondary metabolite accumulation in many types of cultures (Ganga, Zhen-Kuan, Yong-Xiang, Li-Ye, & Hong-Bo, 2007; Hou et al., 2010; Montanaro, Dichio, Xiloyannis, & Celano, 2006; Wahid, Gelani, Ashraf, & Foolad, 2007). For example is the effect of temperatures to secondary metabolites production. Each plant species may favor a different temperature. A study done by Zobayed and his fellow researchers (2005) found that temperature is an important environmental factor to optimized the secondary metabolite. They established that treatment at high temperature about 35°C increased secondary metabolites in shoot tissues of St. John's wort. The same result was obtained in a study done in 2006. Their results showed that change in environment can lead to high variation in the concentration of bioactive compound. Their study confirmed that different temperature enhanced different types of secondary metabolites (Couceiro, Afreen, Zobayed, & Kozai, 2006).

However, manipulation of nutritional elements in culture is perhaps the most fundamental approach for optimization of culture productivity (Mulabagal & Tsay, 2004). Medium optimization has been shown to be an effective means to improve productivity (Verpoorte, Heijden, Hoopen, & Memelink, 1999). Sugar, nitrate and phosphate were known as major nutrient in medium. These nutrients had been investigated widely for their effects on secondary metabolite production (Bondarev, Reshetnyak, & Nosov, 2003; Rao & Ravishankar, 2002).

1.5 Large scale production of Selected Compounds

Due to high demand on natural products, large scale production of the natural compounds is required. The first step that was done in generating large scale production was through tissue culture technique (Akin-Idowu, Ibitoye, & Ademoyegun, 2009; Stockigt, Obitz, Flakenhagen, Lutterbach, & Endress, 1995). This technique allows large amount of production from small pieces of the stock plant (explants) in a short time of period. Then, the method for developing large scale production improves to production in a bioreactor.

Bioreactor is a large culture vessel with a microprocessor control unit and designed depending upon requirement of process applied. Through bioreactor, large scale production can be established. Furthermore, it also provides better control on the system. Cultivation of various plant species in bioreactor had been done for the past years. Mostly, it concerned on propagation of cells and production of secondary metabolites. Producing secondary metabolite using bioreactor had been considered necessary especially for commercial production. In recent years, plant cell have been