



**UNIVERSITI PUTRA MALAYSIA**

**OPTIMIZATION STRATEGIES, KINETICS AND MODELING OF  
CELL GROWTH IN *CENTELLA ASIATICA* CELL CULTURE**

**ROZITA OMAR**

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**OPTIMIZATION STRATEGIES, KINETICS AND MODELING OF  
CELL GROWTH IN *CENTELLA ASIATICA* CELL CULTURE**

**By**

**ROZITA OMAR**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in the Fulfilment of the Requirements for the Degree of Master of Science**

**June 2003**

To my mama and abah who believe in me,  
to my husband who loves me unconditionally and  
to my children, Hanzalah and Dayana who inspire me

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements of the degree of Master of Science

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**Faculty: Food Science and Biotechnology**

*C. asiatica* (Umbelliferae) or locally known as “pegaga” cell suspension culture is used as a model system to produce triterpenoids (TTPs). Cell growth and TTPs production were optimized at shake flask level. Different factors such as nutritional requirement and environmental conditions were screened and optimized using Response Surface Methodology (RSM) experimental design. Preliminary study of laboratory-scale bioreactor was done to study cell growth at regulated pH. Kinetics and modeling studies were carried out aimed at evaluating growth and production parameters for better understanding and control of the process. This study was conducted as foundation for production of triterpenoids at commercial scale.

Growth medium (G) was developed for optimum cell growth by manipulation of different inoculum age and size, sucrose concentration, hormone combination and concentration, incubation temperature, initial pH and light intensity.

Optimization strategies by RSM has established cell growth of *C. asiatica* above 16 g L<sup>-1</sup> at 25°C, pH 5.65 and light intensity of 734 lux; medium NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> ratio between 0.45 – 0.9, higher PO<sub>4</sub><sup>3-</sup> at 2.6 mM, sucrose concentration around 6.68% in combination with 0.84 mg L<sup>-1</sup> IAA and 1.17 mg L<sup>-1</sup> BAP and higher total number of nitrogen around 40 mM. Maximum cell dry weight around 27 g L<sup>-1</sup> was attained with G medium. However, TTPs production was not significantly affected by all factors because of very low production.

Cell growth rate at 0.09 day<sup>-1</sup> (t<sub>d</sub>=7.5 days) was 1.5 times higher when medium pH was controlled at pH 4 in stirred-tank bioreactor. However, maximum cell dry weight at 8.6 g L<sup>-1</sup> was 1.5 times higher when pH was not controlled, with in almost three times more efficient sucrose utilization at 0.28 g cell g<sup>-1</sup> sucrose. Higher growth rate at 0.18 day<sup>-1</sup> in bioreactor cultivation (B) was only 20% higher than shake flask cultivation. However, maximum cell dry weight at 10.5 g L<sup>-1</sup> in M was 14% higher than in B. A 97% confidence was achieved by fitting three unstructured growth models; Monod, Logistic and Gompertz equations to the cell growth data. Monod equation described cell growth in all cultures adequately. The specific growth rate however cannot be predicted using Logistic and Gompertz equation with deviation up to 73 and 393%, respectively. The deviation in Logistic and Gompertz models could be due the model was developed for substrate-independent growth and fungi growth, respectively.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan untuk ijazah Master Sains.

**STRATEGI PENGOPTIMAAAN, KINETIK DAN PERMODELAN UNTUK  
PERTUMBUHAN SEL DARI KULTUR SEL *CENTELLA ASIATICA***

Oleh

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Kultur cell ampaiian *C. asiatica* (Umbelliferae) atau dikenali sebagai pegaga digunakan sebagai sistem model untuk menghasilkan triterpenoids (TTPs). Pertumbuhan sel dan penghasilan TTPs dioptimakan di peringkat kelalang bergoncang. Faktor-faktor yang berbeza seperti keperluan nutrisi dan keadaan persekitaran disaring dan dioptimakan menggunakan rekabentuk eksperimen Kaedah Permukaan Respon (RSM). Kajian saringan menggunakan bioreactor berskala makmal telah dijalankan untuk mengkaji pertumbuhan sel dalam pH yang dikawal. Kajian kinetik dan permodelan telah dijalankan untuk menilai parameter pertumbuhan sel dan penghasilan produk bagi pemahaman dan kawalan proses yang lebih baik. Kajian ini telah dijalankan sebagai asas untuk penghasilan triterpenoids berskala komersial.

Media pertumbuhan (G) telah dibangunkan untuk mengoptimakan pertumbuhan sel dengan memanipulasi umur dan saiz inokulum, kepekatan sukrosa,

kombinasi dan kepekatan hormon, suhu pengeraman, pH awal dan keamatan cahaya yang berlainan. Strategi pengoptimaan menggunakan eksperimen RSM membuktikan bahawa pertumbuhan sel *C. asiatica* telah ditingkatkan melebihi 16 g L<sup>-1</sup> pada 25°C, pH 5.65 dan keamatan cahaya 734 lux; nisbah NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> yang sederhana di antara 0.45 – 0.9, PO<sub>4</sub><sup>3-</sup> yang tinggi pada 2.6 mM, kepekatan sukrosa di sekitar 6% dengan kombinasi 0.84 mg L<sup>-1</sup> IAA dan 1.17 mg L<sup>-1</sup> BAP; dan jumlah nitrogen sekitar 40 mM. Kepekatan cell kering maksima sebanyak 27 g L<sup>-1</sup> telah dihasilkan dengan G medium. Walaubagaimanapun, penghasilan TTPs tidak dipengaruhi secara signifikan oleh semua factor kerana penghasilah yang sangat rendah.

Kadar pertumbuhan sel pada 0.09 hari<sup>-1</sup> ( $t_d=7.5$  hari) adalah 1.5 kali lebih tinggi apabila pH media dikawal pada pH 4 di dalam bioreaktor berpengaduk mekanikal. Manakala kepekatan maksimum sel pada 8.6 g L<sup>-1</sup> adalah lebih 1.5 kali lebih tinggi apabila pH tidak dikawal dengan pengambilan sukrosa hampir tiga kali lebih efisien pada 0.28 g sel g<sup>-1</sup> sukrosa. Kadar pertumbuhan yang lebih tinggi di paras 0.18 per hari di dalam kultur bioreaktor (B) adalah hanya 20% lebih tinggi daripada kultur kelalang bergoncang. Walaubagaimanapun, kepekatan maksimum sel pada 10.5 g L<sup>-1</sup> di dalam M hanya 14% lebih tinggi daripada B. 97% keyakinan telah dicapai dengan memadankan tiga model pertumbuhan tidak berstruktur; Monod, Logistic dan persamaan Gompertz kepada data pertumbuhan sel. Persamaan Monod dapat meramalkan pertumbuhan sel di dalam ketiga-tiga kultur. Kadar pertumbuhan spesifik maksimum tidak boleh diramalkan menggunakan persamaan Logistic dan Gompertz dengan sisihan sehingga 73 dan 393%, masing-masing. Persisihan di dalam model-model Logistic dan Gompertz mungkin disebabkan oleh

model tersebut dibangun untuk pertumbuhan bebas-substrat dan pertumbuhan fungi, masing-masing.



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## LIST OF ABBREVIATION

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	isopentyl- $\gamma,\gamma$ -dimethylallyl
<i>A</i>	maximum cell concentration (Gompertz equation)
AA	Asiatic Acid
AO	Asiaticoside
B	bioreactor culture
BAP	6-benzylaminopurine
CCD	central composite design
CPA	$\rho$ -chlorophenoxyacetic acid
DFT	Deep Flow Technique
DNA	deoxyribonucleic acid
DOT	dissolve oxygen tension
DW	dry cell weight ( $\text{g L}^{-1}$ )
<i>F'</i>	flow rate
<i>F<sub>i</sub></i>	bulk liquid flow in ( $\text{mass volume}^{-1} \text{time}^{-1}$ )
<i>F<sub>o</sub></i>	bulk liquid flow in ( $\text{mass volume}^{-1} \text{time}^{-1}$ )
FRIM	Forest Research Institute of Malaysia
G	growth medium
GZ	Gompertz
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IBA	indole-3-bytyric acid
<i>k'<sub>a</sub></i>	secretion constant in the non-viable cells ( $\text{g prod g}^{-1} \text{cell d}^{-1}$ )
<i>k<sub>t</sub></i>	specific product hydrolysis rate ( $\text{day}^{-1}$ )
<i>k<sub>a</sub></i>	secretion constant in the viable cells ( $\text{g prod g}^{-1} \text{cell d}^{-1}$ )
<i>k<sub>d</sub></i>	cell death coefficient
<i>K<sub>d</sub></i>	degradation constant of product ( $\text{day}^{-1}$ )
<i>k<sub>d</sub></i>	dry weight decay constant ( $\text{day}^{-1}$ )
<i>K<sub>i</sub></i>	inhibition constant ( $\text{mol L}^{-1}$ )
Kinetin	6-Furfurylaminopurine
<i>k<sub>L</sub>a</i>	volumetric oxygen transfer coefficient
<i>K<sub>m</sub></i>	saturation constant of maintenance ( $\text{g L}^{-1}$ )
<i>K<sub>MS</sub></i>	specific glucose consumption for maintenance ( $\text{mol mol}^{-1} \text{h}^{-1}$ )
<i>k<sub>nd</sub></i>	decay constant of non-viable cells ( $\text{d}^{-1}$ )
<i>K<sub>p</sub></i>	saturation constant of product ( $\text{g L}^{-1}$ )
<i>k<sub>p</sub></i>	product hydrolysis saturation constant ( $\text{g L}^{-1}$ )
<i>K<sub>s</sub></i>	saturation constant
<i>k<sub>vd</sub></i>	decay constant of viable cells ( $\text{d}^{-1}$ )
<i>K<sub>x</sub></i>	saturation constants of growth ( $\text{g L}^{-1}$ )
L	Logistic
<i>m</i>	maintenance coefficient
M	maintenance medium
MA	Madecassic Acid
MARDI	Malaysian Agricultural Research and Development Institute
MD	Monod
MIT	Massachusetts Institute of Technology

MO	Madecassoside
MS	Murashige and Skoog
$m_s$	maintenance coefficient of glucose ( $\text{mol mol}^{-1} \text{h}^{-1}$ )
N	nitrogen
NA	not applicable
NAA	$\alpha$ -naphthaleneacetic acid
NaOH	Sodium Hydroxide
$n_C$	number of center runs
ND	not detected
$n_F$	number of full runs
OUR	oxygen uptake rate
$P$	product concentration ( $\text{g L}^{-1}$ )
$P_1$	intracellular polysaccharide concentration per volume of culture ( $\text{g L}^{-1}$ )
$P_2$	extracellular polysaccharide concentration per volume of culture ( $\text{g L}^{-1}$ )
PGRs	plant growth regulators
$\text{pH}_f$	final pH
$\text{pH}_i$	initial pH
$\text{P}_i$	intracellular phosphate
PID	Proportional, Integral, Derivative control
$q_B$	specific conversion rate of structural biomass ( $\text{mol mol}^{-1} \text{h}^{-1}$ )
$q_C$	specific conversion rate of storage carbohydrates ( $\text{mol mol}^{-1} \text{h}^{-1}$ )
$q_E$	specific conversion rate of precursors ( $\text{mol mol}^{-1} \text{h}^{-1}$ )
$q_L$	specific conversion rate of lysis products ( $\text{mol mol}^{-1} \text{h}^{-1}$ )
$q_p$	specific conversion rate of product ( $\text{g prod g}^{-1} \text{ cell DW d}^{-1}$ )
$q_{s,max}$	maximum specific rate of substrate utilization ( $\text{g subs g}^{-1} \text{ cell DW d}^{-1}$ )
$R^2$	coefficient of determination
$r_B$	rate of production of structural biomass ( $\text{mol L}^{-1} \text{h}^{-1}$ )
$r_C$	rate of production of storage carbohydrates ( $\text{mol L}^{-1} \text{h}^{-1}$ )
$r_{C,ms}$	rate of storage carbohydrate consumption for maintenance ( $\text{mol L}^{-1} \text{h}^{-1}$ )
$r_E$	rate of production of precursors ( $\text{mol L}^{-1} \text{h}^{-1}$ )
$r_L$	rate of production of lysis products ( $\text{mol L}^{-1} \text{h}^{-1}$ )
RM	Ringgit Malaysia
RNA	ribonucleic acid
$r_d$	rate of cell death ( $\text{mass volume}^{-1} \text{time}^{-1}$ )
$r_p$	rate of product formation ( $\text{g L}^{-1} \text{d}^{-1}$ )
$r_s$	rate of substrate consumed ( $\text{g L}^{-1} \text{d}^{-1}$ )
RSM	Response Surface Methodology
$r_x$	rate of cell growth ( $\text{g L}^{-1} \text{d}^{-1}$ )
$S$	substrate concentration ( $\text{g L}^{-1}$ )
SELDI-TOF	Surface Enhanced Laser Desorption / Ionization - Time of Flight
SNP	Single Nucleotide Polymorphism
T	temperature
$t$	cultivation time
$t_d$	doubling time (day)
TTPs	triterpenoids
$V$	volume ( $\text{m}^3$ )

v/v	volume per volume ( $\text{m}^3 \text{m}^{-3}$ )
w/v	weight per volume ( $\text{kg m}^{-3}$ )
$X$	cell concentration ( $\text{g L}^{-1}$ )
$X_d$	dry cell weight ( $\text{g L}^{-1}$ )
$X_f$	fresh cell weight ( $\text{g L}^{-1}$ )
$X_i$	initial cell concentration ( $\text{mass volume}^{-1}$ )
$X_{max}$	maximum cell concentration ( $\text{g L}^{-1}$ )
$X_{nd}$	non-viable dry weight ( $\text{g L}^{-1}$ )
$X_o$	initial cell concentration ( $\text{g L}^{-1}$ )
$X_{vd}$	viable dry weight ( $\text{g L}^{-1}$ )
$y$	response
$Y_{ao/s}$	asiaticoside yield coefficient
$Y_{ao/s}$	asiatic acid yield coefficient
$Y_{ma/s}$	madecassic acid yield coefficient
$Y_{mo/s}$	madecassoside yield coefficient
$Y_{x/p}$	product yield coefficient
$Y_{x/s}$	growth yield coefficient
Zeatin	$\gamma$ -hydroxy-methyl-adenine
$\alpha$	growth associated product coefficient ( $\text{g prod g}^{-1} \text{cell}$ )
$\alpha$	star points
$\beta$	non-growth associated product coefficient ( $\text{g prod g}^{-1} \text{cell}$ )
$\kappa_i$	mortality coefficient ( $\text{day}^{-1}$ )
$\lambda$	lag time (day)
$\mu$	specific growth rate ( $\text{d}^{-1}$ )
$\mu_{max}$	maximum specific growth rate ( $\text{d}^{-1}$ )
$\omega_B$	concentration of intracellular compound structural biomass ( $\text{mol mol}^{-1}$ )
$\omega_C$	concentration of intracellular compound storage carbohydrate ( $\text{mol mol}^{-1}$ )
$\omega_E$	concentration of intracellular compound precursors ( $\text{mol mol}^{-1}$ )
$\omega_P$	concentration of intracellular compound phosphate ( $\text{mol mol}^{-1}$ )

## **CHAPTER 1**

### **INTRODUCTION**

“A world without plant is a world without life”. Being the lowest in the food chain, plants provide not only food through the supply of carbohydrates, vitamins, fats, fiber and minerals, but also oxygen via photosynthesis. Since early civilizations, plants have been used for medicinal purposes. For example, a book on medicinal plants “Pen Tsao”, was compiled during the era of Chinese Emperor Chi'en Nung who rules in 3737 to 2697 B.C. (James & Hussian, 1998). The Sumerians are reported to record their medical prescriptions on clay tablets from the use of crude plant extracts around 3500 B.C. (National Institute of General Medical Sciences, 1997). The trend then changes to the use of pure active substances. In 18<sup>th</sup> century, Karl Scheele is the first to isolate organic acids from plants (Sengbusch, 2002).

There is still widespread belief that natural medicine is safer with no side effect than synthetically-derived drugs. More than 20,000 different chemical compounds, accounting 25% of total drugs in the market have been extracted from plants. These compounds cannot be chemically synthesized due to complex structures, elaborate processes, or requirements for specific stereoisomers (Petersen & Alfermann, 1993).

Quinine for example an anti-malarial drug from plant, has regained its popularity over synthetic drug for its potency on resistant malarial strain (Merillon & Ramawat, 1999).

The plant bioactive compounds for medicinal usage are usually secondary products, which are extractable from different parts such as leaves, roots, barks or fruits. Secondary metabolites are the result of synthesis, metabolism and catabolism of endogenous compounds by specialized proteins (Endress, 1994). Some secondary metabolites are not essential for the growth of host organisms, but could give some ecological advantages in terms of symbiotic relationships between plants, animals and human. The majority of natural products used medicinally are terpenoids, quinines, lignans, flavanoids and alkaloids (Phillipson, 1990). In fact, certain valuable medicinal products such as alkaloids, steroids and hormones can only be obtained from plants.

For the past three decades, scientists have worked on plant organ, tissue and cell culture technology as alternative sources to the whole plant for the production of specialty drugs. Taxol, an anti-cancer drug, provides excellent example on the use of plant cell culture technology as a viable alternative. As only 1 gram of taxol can be isolated from 7 kg of the bark of Oregon yew tree (*Taxus brevifolia*) and other *Taxus* species, almost one million *Taxus* trees need to be cut to meet the demand of 200 kg taxol per year (Stockigt et al., 1995). *In vitro* cultivation offers several advantages over conventional plantation due to the possibility of controlling the environmental, physiological and nutritional factors; and independence from pests, diseases and political uncertainties. It provides opportunities for genetic manipulation of the cells or tissues to