UNIVERSITI PUTRA MALAYSIA

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

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By

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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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Chairman: **Mohd. Azmuddin Abdullah, Ph.D.**

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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^{-1} at 25°C, pH 5.65 and light intensity of 734 lux; medium NH_4^+:NO_3^- ratio between 0.45 – 0.9, higher PO_4^{3-} at 2.6 mM, sucrose concentration around 6.68% in combination with 0.84 mg L^{-1} IAA and 1.17 mg L^{-1} BAP and higher total number of nitrogen around 40 mM. Maximum cell dry weight around 27 g L^{-1} 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^{-1} (t_d=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^{-1} 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^{-1} sucrose. Higher growth rate at 0.18 day^{-1} in bioreactor cultivation (B) was only 20% higher than shake flask cultivation However, maximum cell dry weight at 10.5 g L^{-1} 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 PENGOPTIMAAN, KINETIK DAN PERMODELAN UNTUK PERTUMBUHAN SEL DARI KULTUR SEL *CENTELLA ASIATICA*

Oleh

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Jun 2003

Pengerusi: **Mohd. Azmuddin Abdullah, Ph.D.**

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Kultur cell ampaian *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^{-1} pada 25°C, pH 5.65 dan keamatan cahaya 734 lux; nisbah NH_4^+:NO_3^- yang sederhana di antara 0.45 – 0.9, PO_4^{3-} yang tinggi pada 2.6 mM, kepekatan sukrosa di sekitar 6% dengan kombinasi 0.84 mg L^{-1} IAA dan 1.17 mg L^{-1} BAP; dan jumlah nitrogen sekitar 40 mM. Kepekatan cell kering maksima sebanyak 27 g L^{-1} 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 $hari^{-1}$ (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^{-1} 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^{-1} 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^{-1} 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 dibangunkan 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-γ,γ-dimethylallyl
A	maximum cell concentration (Gompertz equation)
AA	Asiatic Acid
AO	Asiaticoside
B	bioreactor culture
BAP	6-benzylaminopurine
CCD	central composite design
CPA	ρ-chlorophenoxyacetic acid
DFT	Deep Flow Technique
DNA	deoxyribonucleic acid
DOT	dissolve oxygen tension
DW	dry cell weight (g L^{-1})
F'	flow rate
F_i	bulk liquid flow in (mass $volume^{-1}$ $time^{-1}$)
F_o	bulk liquid flow in (mass $volume^{-1}$ $time^{-1}$)
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
k'_a	secretion constant in the non-viable cells (g prod g^{-1} cell d^{-1})
k_4	specific product hydrolysis rate (day^{-1})
k_a	secretion constant in the viable cells (g prod g^{-1} cell d^{-1})
k_d	cell death coefficient
K_d	degradation constant of product (day^{-1})
k_d	dry weight decay constant (day^{-1})
K_I	inhibition constant (mol L^{-1})
Kinetin	6-Furfurylaminopurine
k_La	volumetric oxygen transfer coefficient
K_m	saturation constant of maintenance (g L^{-1})
K_{MS}	specific glucose consumption for maintenance (mol mol^{-1} h^{-1})
k_{nd}	decay constant of non-viable cells (d^{-1})
K_p	saturation constant of product (g L^{-1})
k_P	product hydrolysis saturation constant (g L^{-1})
K_s	saturation constant
k_{vd}	decay constant of viable cells (d^{-1})
K_x	saturation constants of growth (g L^{-1})
L	Logistic
m	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 (mol mol^{-1} h^{-1})
N	nitrogen
NA	not applicable
NAA	α–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 (g L^{-1})
P_1	intracellular polysaccharide concentration per volume of culture (g L^{-1})
P_2	extracellular polysaccharide concentration per volume of culture (g L^{-1})
PGRs	plant growth regulators
pH_f	final pH
pH_i	initial pH
Pi	intracellular phosphate
PID	Proportional, Integral, Derivative control
q_B	specific conversion rate of structural biomass (mol mol^{-1} h^{-1})
q_C	specific conversion rate of storage carbohydrates (mol mol^{-1} h^{-1})
q_E	specific conversion rate of precursors (mol mol^{-1} h^{-1})
q_L	specific conversion rate of lysis products (mol mol^{-1} h^{-1})
q_p	specific conversion rate of product (g prod g^{-1} cell DW d^{-1})
$q_{s,max}$	maximum specific rate of substrate utilization (g subs g^{-1} cell DW d^{-1})
R^2	coefficient of determination
r_B	rate of production of structural biomass (mol L^{-1} h^{-1})
r_C	rate of production of storage carbohydrates (mol L^{-1} h^{-1})
$r_{C,ms}$	rate of storage carbohydrate consumption for maintenance (mol L^{-1} h^{-1})
r_E	rate of production of precursors (mol L^{-1} h^{-1})
r_L	rate of production of lysis products (mol L^{-1} h^{-1})
RM	Ringgit Malaysia
RNA	ribonucleic acid
r_d	rate of cell death (mass $volume^{-1}$ $time^{-1}$)
r_p	rate of product formation (g L^{-1} d^{-1})
r_s	rate of substrate consumed (g L^{-1} d^{-1})
RSM	Response Surface Methodology
r_x	rate of cell growth (g L^{-1} d^{-1})
S	substrate concentration (g L^{-1})
SELDI-TOF	Surfaced 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 (m^3)

v/v	volume per volume ($m^3 m^{-3}$)
w/v	weight per volume ($kg\ m^{-3}$)
X	cell concentration ($g\ L^{-1}$)
X_d	dry cell weight ($g\ L^{-1}$)
X_f	fresh cell weight ($g\ L^{-1}$)
X_i	initial cell concentration ($mass\ volume^{-1}$)
X_{max}	maximum cell concentration ($g\ L^{-1}$)
X_{nd}	non-viable dry weight ($g\ L^{-1}$)
X_o	initial cell concentration ($g\ L^{-1}$)
X_{vd}	viable dry weight ($g\ L^{-1}$)
y	response
$Y_{ao/s}$	asiaticoside yield coefficient
$Y_{ao/s}$	asiatic acid yield coefficient
$Y_{ma/s}$	madecassic acid yield coefficient
$Y_{mo/s}$	madecassocide yield coefficient
$Y_{x/p}$	product yield coefficient
$Y_{x/s}$	growth yield coefficient
Zeatin	γ-hydroxy-methyl-adenine
α	growth associated product coefficient ($g\ prod\ g^{-1}$ cell)
α	star points
β	non-growth associated product coefficient ($g\ prod\ g^{-1}$ cell)
κ_i	mortality coefficient (day^{-1})
λ	lag time (day)
μ	specific growth rate (d^{-1})
μ_{max}	maximum specific growth rate (d^{-1})
ω_B	concentration of intracellular compound structural biomass ($mol\ mol^{-1}$)
ω_C	concentration of intracellular compound storage carbohydrate ($mol\ mol^{-1}$)
ω_E	concentration of intracellular compound precursors ($mol\ mol^{-1}$)
ω_P	concentration of intracellular compound phosphate ($mol\ mol^{-1}$)

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^{th} 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 then 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