UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT AND BIOREACTOR CULTIVATION OF MORINDA ELLIPTICA CELL CULTURES FOR THE PRODUCTION OF ANTHRAQUINONES

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ESTABLISHMENT AND BIOREACTOR CULTIVATION OF *MORINDA ELLIPTICA* CELL CULTURES FOR THE PRODUCTION OF ANTHRAQUINONES

BY

MOHD AZMUDDIN ABDULLAH

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"DEDICATED TO ALL SINCERE SEEKERS OF TRUTH..."

Patriot tua, 'Rang muda dan Professor separuh abad
di suatu kelas kosong

Dewan kuliah P/C5, Isnin
Patriot tua terketar-ketar
dipimpin tongkat lendut pusaka
cari kerusi belakang, buat pelepas lelah tuanya
9.00 a.m
Professor separuh abad melangkah masuk
angkuh serba tahu, kuliah dimula
"...dari zat bernama protein, aku, engkau dan monyet melangkah,
dari kuat akal usaha, tongkat kucipta, buat mimpin Situa nyanyuk
Tuhan *Saddam Hussein* kosmik, Pemarah dan Penghukum
untuk apa Tuhan?"
Rang muda berambut 'blonde' di depan, hanyut
"...disko rancak muzik pesona
togok tonik bebuih lazat, kosong kapla lapang pikiran
rambut lembut,dada bosong gadismu
Firdausi, Kau pastinya di sini!"
Patriot tua bertasamuh
"...kukenal *Saccharomyces Cerevisiae*, punya kilang buat zat
kusuluh langit tujuh petala, berkelip *Kejora*, bintang seribu
di kegilaan dansa rewang, kudengar
James Bulger hancur dimamah, *Suzanne Capper* rentung dibakar.
Bingung aku
teori apa yang kau 'Eureka!'kan?
KEKOSONGAN!!?"

Bahu Patriot tua disapa 'rang muda berambut 'blonde'
tersentak
Professor sudah tiada
luar sana, nampak sekilas cahaya
Patriot tua bergegas keluar
mencari.....

Disember '93
UMIST

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LIST OF ABBREVIATIONS

mg	milligram
g	gram
ml	millilitre
l	litre
mM	milliMolar
s	Second
min	Minute
h	Hour
d	Day
v/v	Volume/volume
rpm	Revolution per minute
DW	Dry cell weight
AQ	Anthraquinones
2,4-D or D	2,4-Dichlorophenoxyacetic acid
NAA or N	α-Naphthaleneacetic acid
IAA	Indole-3-acetic acid,
K	6-Furfurylaminopurine (kinetin)
BAP	6-Benzylaminopurine
Z	γ-Hydroxymethyl adenine (zeatin)
ATP	Adenosine triphosphate
NAD	Nicotinamide adenine dinucleotide
DNA	Deoxyribonucleic acid
LM	Maintenance medium under illumination
LG, DG	Growth medium under illumination and in the dark, respectively
LP, DP	Production medium under illumination and in the dark, respectively
C_L	Oxygen concentration in the medium (mmol l^{-1})

C_E	Liquid phase oxygen concentration in equilibrium with the partial pressure of oxygen in the gas phase (mmol l^{-1})
K_L	Liquid film transfer coefficient (cm h^{-1})
a	Gas-liquid interfacial area per unit volume(cm^{-1})
Y_{x/O_2}	Yield coefficient (g biomass g^{-1} oxygen consumed)
m_{O_2}	Cell maintenance coefficient (g oxygen g^{-1} biomass d^{-1})
qO_2	Specific respiration rate (mmol g^{-1} h^{-1})
X, x	Cell concentration (g l^{-1})
S, S'	Substrate concentration in and out, respectively (g l^{-1})
P	Product content or yield (mg g^{-1} DW or g l^{-1}, respectively)
F, F'	Flowrates of medium in and out, respectively (l h^{-1})
μ	Specific growth rate (d^{-1} or h^{-1})
q_s	Specific substrate utilization rate (d^{-1} or h^{-1})
q_p	Specific product formation rate (d^{-1} or h^{-1})
α	Specific death rate (d^{-1} or h^{-1})
β	Specific product denaturation rate (d^{-1} or h^{-1})
$Y_{x/s}$	Biomass yield coefficient (g biomass g^{-1} substrate)
$Y_{p/s}$	Product yield coefficient (g product g^{-1} substrate)
M	Maintenance coefficient (g substrate g^{-1} biomass h^{-1})
t_d	Doubling time (d or h)
r_x	Rate of biomass formation (g l^{-1} d^{-1})
r_p	Rate of product formation (g l^{-1} d^{-1})
$-r_s$	Rate of sugar uptake (g l^{-1} d^{-1})
$-r_{(NO_3^-)}$	Rate of nitrate uptake (g l^{-1} d^{-1})
$-r_{(PO_4^{3-})}$	Rate of phosphate uptake (g l^{-1} d^{-1})
r^2	Linear regression correlation coefficient
n	Number of experimental points

Abstract of the Dissertation presented to the Senate of Universiti Putra Malaysia in the fulfilment of the requirement for the Degree of Doctor of Philosophy

ESTABLISHMENT AND BIOREACTOR CULTIVATION OF *MORINDA ELLIPTICA* CELL CULTURES FOR THE PRODUCTION OF ANTHRAQUINONES

By

MOHD AZMUDDIN ABDULLAH

April 1999

Chairman : Assoc. Prof. Dr. Arbakariya Ariff

Faculty : Food Science and Biotechnology

Morinda elliptica (Rubiaceae) cell suspension cultures were established in shake flask and bioreactor systems for the production of anthraquinones (AQ). To improve AQ productivity at shake flask level, manipulations of media components such as carbon, nitrogen, phosphate and myo-inositol; and cultural conditions such as incubation temperature, light intensity, culture and inoculum age, were made. At bioreactor level, the study was aimed at finding the best bioreactor operation with minimum foaming and wall-growth problem. Several strategies such as mode of aeration, number of impellers, paddle orientation, antifoam addition and medium formulations, were applied.

Murashige and Skoog's basal medium was found to be the best medium in enhancing both cell growth and AQ production. By manipulation of sucrose concentration, hormone combination and concentration, culture age and inoculum

age, the type of medium formulation used to grow inoculum, incubation temperature and light intensity, three types of media were formulated – maintenance medium (M), growth medium (G) and production medium (P). The toxic effects of nitrogen were shown not a result of the individual effect of nitrogen toxicity per se but of both individual and collective effects of NH_4^+ and NO_3^- levels, in consonance with the level of sucrose and the medium formulation used. Reduction in pH for cultures grown in medium containing high concentration of NH_4^+ was another contributing factor for ammonium toxicity. Phosphate had little influence on cell growth and AQ production though its absence could suppress growth completely. The phosphate toxicity could also occur depending on sucrose level and medium formulation. Myo-inositol was not an absolute requirement in *M. elliptica* cell suspension culture.

The growth of cell suspension cultures of *M. elliptica* in G and P media were sigmoidal. The AQ yields in P medium, of 2.9 and 4.5 g l^{-1} with corresponding overall productivity of 0.14 and 0.21 g l^{-1} d^{-1}, under illumination and in the dark, respectively, were among the highest amount of secondary metabolites and productivities by plant cell suspension cultures. The formation of AQ displayed a non-growth associated characteristic. High sucrose, glucose and fructose concentration over the period of two weeks in P medium was suggested to cause osmotic pressure on the cells which hindered rapid growth, leading to higher accumulation of AQ. With increasing culture age to 36 month-old, the doubling time was increased by 30% to 1.5 days; and 100% to 1.6 days, in M and P medium, respectively. The maximum cell concentration in LP(36) was however 35% lower than LP(18) while the AQ yield dropped sharply from 2.92 g l^{-1} to a mere 0.55 g l^{-1}. The spent medium was observed more yellowish in LP(36) indicating that AQ was no longer retained in the cell vacuole but released into the medium. The faster rate of sucrose hydrolysis and uptake rate of glucose and fructose in LP(36) may have

reduced the osmotic pressure in the medium which allows rapid cell growth and diffusion of AQ.

In stirred-tank bioreactor, P medium not only promoted both growth and AQ production but also resulted in lower foaming and wall-growth without any necessity for antifoam addition. No significant different was observed between having vertically-inclined paddles or 45°-downwardly-inclined paddles. Lower number of impellers with consequent lower shear effects led to higher growth rate. Continuous aeration assisted mixing by providing greater turbulence than that could be achieved via intermittent air supply, and consequently promoting better cell growth. In G medium, the semi-continuous mode promoted AQ content, despite the addition of 0.012% (v/v) antifoam. In P medium, the contradiction between growth profile and AQ profile supports the common understanding that higher cell growth will normally be associated with lower secondary metabolite production and vice versa. The early entrance of cells into deceleration phase could be a result of high air flow rates and high $k_L a$ values with increased turbulence and shear; and the possible oxygen toxicity leading to inhibiton of metabolic activity. The suppression of growth rate at high air flow rates could be avoided through the application of semi-continuous mode and intermittent air supply. The oxygen uptake rate profile suggests that oxygen uptake could be more important in promoting biomass formation rather than AQ formation. The shorter duration of time spent under more acidic pH of below 4 could be one of the reason for the better performance of cell growth and AQ production in P as compared to G medium. Cell growth and AQ yield were better in shake flask (SF) than in the bioreactor (ST) systems while the sugar uptake rate in ST was lower than in SF.

Abstrak Disertasi yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi syarat-syarat bergraduasi Ijazah Doktor Falsafah

PENGKULTURAN DAN PENGGUNAAN BIOREAKTOR UNTUK AMPAIAN SEL *MORINDA ELLIPTICA* DALAM PENGHASILAN ANTRAKUINON

Oleh

MOHD AZMUDDIN ABDULLAH

April 1999

Pengerusi : Prof. Madya Dr. Arbakariya Ariff

Fakulti : Sains Makanan dan Bioteknologi

Ampaian sel *Morinda elliptica* (Rubiaceae) telah dikulturkan di dalam sistem kelalang bergoncang dan bioreaktor untuk penghasilan antrakuinon (AQ). Produktiviti AQ di dalam kelalang bergoncang ditingkatkan dengan memanipulasi komponen media seperti karbon, nitrogen, fosfat dan myo-inositol; dan keadaan pengkulturan seperti suhu pengeraman, keamatan cahaya, umur kultur dan inokulum. Di peringkat bioreaktor, penyelidikan dilakukan untuk menentukan operasi yang terbaik dengan masalah busa dan pertumbuhan di dinding bioreaktor pada tahap yang minima. Beberapa bentuk strategi dicuba, antaranya teknik pengudaraan, jumlah pengaduk, orientasi bilahan, penggunaan bahan kimia anti-busa dan formulasi media. Setakat ini, belum ada laporan berkenaan penggunaan ampaian sel *M. elliptica* untuk penghasilan AQ di dalam kedua-dua sistem.

Media Murashige and Skoog's adalah media terbaik dalam meningkatkan kedua-dua pertumbuhan sel dan penghasilan AQ. Dengan manipulasi kepekatan sukrosa, kombinasi dan kepekatan hormone, umur kultur dan inokulum, jenis media untuk pertumbuhan inokuluin, suhu pengeraman dan keamatan cahaya, tiga jenis formulasi media dicadangkan : – media penjagaan (M), media pertumbuhan (G) dan media penghasilan (P). Ketoksidan nitrogen bukan hanya fungsi paras ammonium dan nitrat secara individu atau serentak, tetapi adalah juga paras sukrosa dan formulasi media. Ketoksidan ammonium boleh disebabkan oleh paras ammonium yang tinggi yang menghasilkan pH yang lebih berasid dan membantut pertumbuhan sel dan kandungan AQ. Fosfat tidak menunjukkan kesan yang ketara ke atas pertumbuhan sel adan kandungan AQ walaupun ketiadaan fosfat mampu membantut pertumbuhan sel. Ketoksidan fosfat juga bergantung kepada paras sukrosa dan formulasi media. Myo-inositol bukanlah suatu keperluan utama untuk pertumbuhan ampaian sel *M. elliptica*.

Pertumbuhan kultur ampaian sel *M. elliptica* di dalam media G dan P menunjukkan kelok pertumbuhan berbentuk sigmoid. Penghasilan AQ di dalam media P sebanyak 2.9 dan 4.5 g l^{-1} dengan produktiviti keseluruhan sebanyak 0.14 and 0.21 g l^{-1} d^{-1}, di bawah suluhan cahaya atau di dalam gelap, mengikut urutan, adalah antara jumlah penghasilan metabolit sekunder dan produktiviti yang tertinggi yang dihasilkan oleh kultur ampaian sel tumbuhan. Penghasilan AQ menunjukkan kategori tidak-seiringan dengan pertumbuhan sel. Kepekatan sukrosa, glukosa dan fruktosa yang tinggi untuk tempoh dua minggu di dalam media P menyebabkan tekanan osmotik ke atas sel yang berkemungkinan membantut kadar pertumbuhan yang tinggi dan kesannya meningkatkan penghasilan AQ. Dengan pertambahan umur kultur kepada 36 bulan, masa gandaan dua bertambah 30% kepada 1.5 dan 100% kepada 1.6 hari, di dalam media M dan P, mengikut urutan. Kepekatan sel maksimum

di dalam LP(36) walaubagaimanapun 35% lebih rendah dari LP(18) sementara penghasilan AQ berkurangan dari 2.92 g l^{-1} kepada hanya 0.55 g l^{-1}. Sisa media selepas ditapis kelihatan lebih kuning menunjukkan kemungkinan AQ tidak lagi disimpan di dalam vakuol sel tetapi dilepaskan ke dalam media. Kadar hidrolisis sukrosa dan kadar pengambilan glukosa dan fructosa di dalam LP(36) yang lebih cepat dan berkemungkinan mengurangkan tekanan osmotik di dalam media yang menggalakkan pertumbuhan sel yang lebih cepat dan pengaliran keluar AQ dari sel.

Di dalam bioreaktor berpengaduk mekanikal, media P bukan sahaja menggalakkan pertumbuhan sel dan penghasilan AQ tetapi juga kesan busa dan pertumbuhan di dinding bioreaktor, yang lebih rendah tanpa memerlukan penggunaan bahan kimia anti-busa. Tiada perbezaan yang ketara antara penggunaan bilahan yang menegak atau 45°-condong ke bawah. Bilangan pengaduk yang lebih rendah dengan kesan daya ricihan yang lebih rendah menghasilkan pertumbuhan sel yang lebih cepat. Pengudaraan selanjar membantu pencampuran dengan menghasilkan darjah pengadukan yang lebih serata daripada yang diperolehi dengan pengudaraan secara terhenti-henti dan kesannya pertumbuhan sel yang lebih baik. Di dalam media G, teknik separa-selanjar yang digunakan berjaya meninggikan kandungan AQ, walaupun dengan penambahan anti-busa sebanyak 0.012% (v/v). Perbezaan antara profil pertumbuhan sel dan profil AQ di dalam P medium menyokong pendapat bahawa pertumbuhan sel yang tinggi selalunya disertai dengan penghasilan metabolit sekunder yang rendah, dan begitu juga sebaliknya. Kemasukan sel yang awal ke dalam fasa pertumbuhan yang perlahan boleh disebabkan oleh kadar pengudaraan yang tinggi dan nilai $k_L\sigma$ yang tinggi yang menyebabkan pertambahan daya ricih dan perolakan; dan kemungkinan kesan ketoksidan oksigen yang membantut aktiviti metabolisma. Pembantutan kadar pertumbuhan pada pengudaraan yang tinggi dapat dikurangkan dengan menggunakan teknik separa-selanjar dan pengudaraan pada

selang masa tertentu. Profil pengambilan oksigen mencadangkan yang pengambilan oksigen kemungkinan lebih penting di dalam menggalakkan pertumbuhan sel daripada penghasilan AQ. Tempoh masa pengkulturan yang lebih pendek pada pH di bawah 4 bagi kultur di dalam media P berbanding di dalam G, memungkinkan pertumbuhan sel dan kandungan AQ yang lebih baik. Pertumbuhan dan penghasilan AQ di dalam kelalang bergoncang (SF) adalah lebih baik daripada sistem bioreaktor (ST) sementara kadar pengambilan gula di dalam ST adalah lebih rendah berbanding di dalam SF.