MASS PRODUCTION OF SHIKIMIC ACID (RAW MATERIAL FOR TAMIFLU) FROM PLANT CELL CULTURE

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by

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Dedicated

To

My Beloved Brother

Dr. Sarfaraz Saeed

Certificate

This is to certify that the thesis entitled "**Mass Production of Shikimic Acid (Raw Material for Tamiflu) from Plant Cell Culture**", being submitted by **Ms. Rabab Anjum** to the Indian Institute of Technology, Delhi, for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research carried out by her, which has been prepared under my supervision in conformity with rules and regulations of the Indian Institute of Technology, Delhi. The research reports and results presented in the thesis have not been submitted for any degree or diploma in any other University or Institutes.

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Rabab Anjum

Mass Production of Shikimic Acid (Raw Material for Tamiflu) from Plant Cell Culture

Abstract

Shikimic acid (tri hydroxyl-1-cyclohexene-carboxylic acid) is the key intermediate for production of Oseltamivir phosphate (Tamiflu), which is potentially used to treat the diseases caused by entire influenza family, especially pandemic diseases like "Bird flu" and "Swine flu". The main source of Shikimic acid is the matured pods of Chinese plant Star Anise (*Illicium verum L.)*. However, its limited availability, low content, and high cost requires exploration of alternate protocols of production to meet the huge demand of Shikimic acid for Tamiflu production. The distinct ability of several indigenous plants e.g., *Arucaria excelsa, Pinus roxberghii, Pinanga dicksonii, Agathis borneensis, Calophyllum apetellum, Agele marmelos,* and *Melia azedarach,* etc. to produce Shikimic acid has been recently reported in the literature. However, it has been realized that the extraction of Shikimic acid from natural plant will not be able to cater to the huge demands of society. It was therefore, necessary to develop invitro mass cultivation protocols of specific cells containing high amount of Shikimic acid in the bioreactor & recover Shikimic acid from the growing cells to enhance the production & availability of Shikimic acid.

With above objectives in mind, induction of callus of *Agathis borneensis* was initiated in the medium (containing WPM 2.4g/L+ sucrose 30g/L+ NAA 1.5mg/L+BAP 1.25mg/L+ charcoal 0.2g/L+ agar 0.75%), and thereafter, the propagation of cell suspension culture in the shake flasks were established. The friability of callus of *A. borneensis* was increased by addition of 10%v/v coconut water while eliminating BAP in the callus induction media. This resulted in increase in the homogeneity of the suspension culture, which led to enhanced production of biomass (**11.2g/L)** and high Shikimic acid accumulation (of **240.38mg/L**). Shake flask cultivation studies on growth & Shikimic acid production from *Agathis* cells under statistically

optimized concentrations of medium components (sucrose 33.61g/L, (NO_3^-/NH_4^+) (40:20) 4.33g/L, KH_2PO_4 0.09g/L, $CaCl_2.2H_2O$ 0.56g/L, WPM 2.4g/L+ NAA 1.5mg/L+ BAP 0.25mg/L and charcoal 0.2g/L) & environmental conditions (pH 5.8; temperature 25°C; 16/8 h light/dark regime; 100 rpm) resulted in increased biomass growth **(13.8±1.1g/L)** and Shikimic acid content **(**of **339.505±43.60mg/L)**. The Shikimic acid yield was further increased by the combined addition of statistically optimized concentrations of selected elicitors (Casein acid hydrolysate, CAH-0.80g/L), vitamin (Pyridoxine HCl, PHCL-8.85mg/L), and growth factor (Gibberellic acid, GA_3-0.80mg/L) into nutrient medium which resulted in enhanced accumulation of biomass (**13.8g/L)** and Shikimic acid (**487.22mg/L).**

The batch cultivation of *A. borneensis* was performed in the (3L) stirred tank bioreactor using statistically optimized medium recipe (WPM 2.4g/L, sucrose 33.61g/L, (NO_3^-/NH_4^+) (40:20) 4.33g/L, KH_2PO_4 0.09g/L, $CaCl_2.2H_2O$ 0.56 g/L, NAA 1.5mg/L, BAP 0.25mg/L, Charcoal 0.2 mg/L, CAH 0.8g/L, GA_3 0.8mg/L, and PHCl 8.85mg/L) & optimal cultivation conditions (pH 5.8; temperature 25°C; 16/8 h light/dark regime; 100 rpm; aeration rate 0.2vvm), to establish the kinetics of batch growth and shikimic acid production, which resulted in highest biomass of **14.4g/L** and Shikimic acid accumulation of **706.54mg/L** after four days of cultivation.

The possible inhibition of major nutrients, Sucrose (S_1) and Nitrate (S_2) on the growth of *Agathis* cells were examined during shake flask cultivation, wherein the maximum inhibitory concentrations (S_m) with respect to Sucrose & Nitrate were identified as 100g/L & 16.5g/L. The batch growth kinetics of *A. borneensis* along with independently acquired substrate inhibition data was used to propose and identify the mathematical model. The batch model was, thereafter, extrapolated to fed-batch cultivation conditions by addition of dilution terms and was used to identify nutrient feeding strategy for enhanced biomass and Shikimic acid accumulation. It was observed that feeding of sucrose at concentration of 100g/L & rate 0.015mL/min during 4th day to 7^{th} day led to enhanced biomass (1.06 times) and Shikimic acid (1.14 times) concentrations respectively.

Scale-up of Shikimic acid production of 3L to 100L bioreactor cultivation was attempted in two steps (3L to 15L) and (15L to 100L) by maintaining identical geometrical and dynamic similarities of the two scales of bioreactor cultivation. The scale-up of batch cultivation of *Agathis* cells was attempted from 3L to 15L bioreactor using impeller tip velocity as scale-up criteria using statistically optimized media. The reactor operating conditions (agitation speed of 58 rpm and volumetric gas flow rate of 0.2vvm) corresponding to parametric indices similarity test of scale-up criteria (under Π-test) were chosen for operation of 15L bioreactor which upon experimental implementation resulted in relatively identical values of biomass and Shikimic acid concentrations in both 3L and 15L bioreactors (**14.4g/L in 3L vs 13.94g/L in 15L**), **(706.54mg/L in 3L vs 665.58mg/L in 15L).**

The scale-up of the batch cultivation of *Agathis* cells was, thereafter, attempted from 15L to 100L bioreactor using constant Reynolds number as the scale-up criteria using statistically optimized medium. The reactor operating conditions of 100-liter bioreactor (agitation speed of 58 rpm and volumetric gas flow rate of 0.2vvm) were identified by extensive scale-up calculations using same Reynold number (N_{Re}) criteria for the two bioreactor (15 & 100 liter) cultivations, which upon experimental implementation in 100L bioreactor, resulted in almost similar values of biomass **(13.94±0.18g/L in 15L vs 13.93±1.03g/L in 100L)** & Shikimic acid concentrations **(665.58±8.74mg/L in 15L vs 658.89±4.8mg/L in 100L).** It is believed that the developed optimized production protocol for mass production of Shikimic acid would supplement the Shikimic acid availability for production of Tamiflu in society.

<u>प्लांट सेल कल्चर से शिकिमिक एसिड (टैमीफ्लू के लिए कच्चा माल) का बड़े पैमाने पर उत्पादन</u>

सारांश

शिकिमिक एसिड (त्रि हाइड्रोक्सिल-1-साइक्लोहेक्सीन-कार्बोक्सिलिक एसिड) ओसेल्टामिविर फॉस्फेट (टैमीफ्लू) के उत्पादन के लिए महत्वपूर्ण मध्यवर्ती है, जिसका उपयोग संभावित रूप से पूरे इंफ्लूएंजा परिवार, विशेष रूप से महामारी रोगों जैसे "बर्ड फ्लू" और "स्वाइन फ्लू" के कारण होने वाली बीमारियों के इलाज के लिए किया जाता है। शिकिमिक एसिड का मुख्य स्रोत चीनी संयंत्र स्टार एनीज़ (इलिसियम वेरम एल) की परिपक्व फली है। हालांकि, इसकी सीमित उपलब्धता, कम सामग्री और उच्च लागत के कारण टैमीफ्लू उत्पादन के लिए शिकिमिक एसिड की भारी मांग को पूरा करने हेतु उत्पादन के वैकल्पिक प्रोटोकॉल की खोज की आवश्यकता होती है। कई स्वदेशी पौधों जैसे, *अरुकारिया एक्सेलसा, पिनस रॉक्सबर्गी, पिनांगा डिकसोनी, अगाथेस बोर्नेन्सिस, कैलोफिलम एपेटेलम, एजेल मार्मेलोस,और मेलिया अज़ेदराच,* आदि की विशिष्ट क्षमता हाल ही में साहित्य में सूचित किया गया है । हालांकि, यह महसूस किया गया है कि प्राकृतिक पौधे से शिकिमिक एसिड निकालने से समाज की भारी मांगों को पूरा नहीं किया जा सकेगा । इसलिए, बायोरिएक्टर में उच्च मात्रा में शिकिमिक एसिड युक्त विशिष्ट कोशिकाओं के इनविट्रो बड़े पैमाने पर खेती प्रोटोकॉल विकसित करना और शिकिमिक एसिड के उत्पादन और उपलब्धता को बढ़ाने के लिए बढ़ती कोशिकाओं से शिकिमिक एसिड को पुनर्प्राप्त करना आवश्यक था ।

मन में उपरोक्त उद्देश्यों के साथ, *अगाथेस बोर्नेन्सिस* के कॉलस को माध्यम (WPM 2.4 g/L+ सुक्रोज 30g/L+ NAA 1.5 mg/L + BAP 1.25 mg/L+ चारकोल 0.2 g/L+ अगार 0.75%) में शामिल करने की शुरुआत की गई थी। शेक फ्लास्क में सेल सस्पेंशन कल्चर का प्रचार-प्रसार स्थापित किया गया था। कॉलस इंडक्शन मीडिया में BAP को खत्म करते हुए 10% v/v नारियल पानी डालने स्वरूप *अगाथेस बोर्नेन्सिस* के कैलस की फ्रिबिलिटी बढ़ा दी गई थी । इसके परिणामस्वरूप सस्पेंशन कल्चर की

एकरूपता में वृद्धि हुई, जिसके कारण बायोमास (11.2g/L) और शिकिमिक एसिड संचय (240.38mg/L) का उत्पादन बढ़ा। मध्यम घटकों की सांख्यिकीय अनुकूलित सांद्रता के तहत *अगाथेस* कोशिकाओं से विकास और शिकिमिक एसिड उत्पादन पर शेक फ्लास्क खेती अध्ययन (सुक्रोज 33.61g/L, (NO_{3-}/NH_{4+}) (40:20) 4.33g/L, KH_2PO_4 0.09g/L, $CaCl_2.2H_2O$ 0.56g/L, WPM 2.4g/L+ NAA 1.5mg/L+ BAP 0.25mg/L और चारकोल 0.2g/L) और पर्यावरणीय स्थितियों (पीएच 5.8; तापमान 25 डिग्री सेल्सियस; 16/8 h प्रकाश / अंधेरा; 100 आरपीएम) के परिणामस्वरूप बायोमास वृद्धि (13.8±1.1g/L) और शिकिमिक एसिड संचय (339.505±43.60mg/L) में वृद्धि हुई। शिकिमिक एसिड की उपज को बढ़ाने के लिए चयनित एलिसिटर (केसिन एसिड हाइड्रोलिटेरेट, CAH-0.80g/L), विटामिन (पाइरिडोक्सिन HCl, PHCL-8.85mg/L), और विकास कारक (गिबरेलिक एसिड, GA_3-0.80mg/L) का उपयोग पोषक तत्व माध्यम में किया गया था जिसके परिणामस्वरूप बायोमास (13.8g/L) और शिकिमिक एसिड (487.22mg/L) का संचय बढ़ा।

अगाथेस बोर्नेंसिस के बैच की खेती (3L) उभारा टैंक बायोरिएक्टर (STR) में सांख्यिकीय अनुकूलित मीडिया नुस्खा का उपयोग कर (WPM 2.4g/L, सुक्रोज 33.61g/L, (NO^{3-}/NH^{4+}) (40:20) 4.33g/L, KH_2PO_4 0.09g/L, $CaCl_2.2H_2O$0.56 g/L, NAA 1.5mg/L, BAP 0.25mg/L, चारकोल 0.2mg/L, CAH 0.8g/L, GA_3 0.8mg/L, और PHCl 8.85 mg/L) और इष्टतम खेती की स्थिति (पीएच 5.8; तापमान 25 डिग्री सेल्सियस; 16/8 h प्रकाश/ अंधेरा; 100 आरपीएम; वातारण दर 0.2vvm), में बैच विकास और शिकिमिक एसिड उत्पादन की गतिज स्थापित करने के लिए किया गया था, जिसके परिणामस्वरूप खेती के चार दिनों के बाद 14.4 g/L बायोमास और शिकिमिक एसिड 706.54mg/L का उच्चतम संचय हुआ। शेक फ्लास्क खेती के दौरान *अगाथेस* कोशिकाओं के विकास पर प्रमुख पोषक तत्वों, सुक्रोज (एस$_1$) और नाइट्रेट (एस$_2$) के संभावित अवरोध की जांच की गई, जिसमें सुक्रोज और नाइट्रेट के संबंध में अधिकतम निरोधात्मक सांद्रता (एस$_{एम}$) की पहचान 100g/L और 16.5g/L के रूप में की गई। *अगाथेस बोर्नेंसिस* के बैच विकास काइनेटिक्स के साथ-साथ स्वतंत्र रूप से अधिग्रहीत सब्सट्रेट अवरोध डेटा का उपयोग

गणितीय मॉडल का प्रस्ताव और पहचान करने के लिए किया गया था। इसके बाद बैच मॉडल को कमजोर पड़ने की शर्तों को जोड़कर फेड-बैच खेती की स्थिति में एक्सपेरिमेंट किया गया और इसका इस्तेमाल बायोमास और शिकिमिक एसिड संचय वृद्धि करने के लिए पोषक तत्वों को खिलाने की रणनीति की पहचान करने के लिए किया गया । यह देखा गया कि 4 दिन से 7 दिन के दौरान 100 g/L और दर 0.015mL/min की सांद्रता पर सुक्रोज को खिलाने से बायोमास (1.06 गुना) और शिकिमिक एसिड (1.14 गुना) सांद्रता में वृद्धि हुई।

बायोरिएक्टर खेती के दो पैमानों की समान ज्यामितीय और गतिशील समानताओं को बनाए रखते हुए 3L से 100L बायोरिएक्टर खेती के शिकिमिक एसिड उत्पादन को दो चरणों (3L से 15L) और (15L से 100L) में प्रयास किया गया था। *अगाथेस* कोशिकाओं के बैच की खेती के पैमाने को सांख्यिकीय रूप से अनुकूलित मीडिया का उपयोग करके स्केल-अप मापदंड के रूप में इम्पेलर टिप वेग का उपयोग करके 3L से 15L बायोरिएक्टर तक का प्रयास किया गया था। रिएक्टर ऑपरेटिंग स्थितियां (58 आरपीएम की आंदोलन गति और 0.2vvm की वॉल्यूमेट्रिक गैस प्रवाह दर) स्केल-अप मानदंडों के पैरामेट्रिक सूचकांक समानता परीक्षण के अनुरूप (इम्पेलर टिप वेग टेस्ट) को 15L बायोरिएक्टर के संचालन के लिए चुना गया था जो प्रायोगिक कार्यान्वयन के परिणामस्वरूप 3L और 15L बायोरिएक्टर में समान मूल्य बायोमास (3L में 14.4g/L, 15L में 13.94g/L) और शिकिमिक एसिड सांद्रता के अपेक्षाकृत समान मूल्य (15L में 665.58mg/L, 3L में 706.54mg/L) प्राप्त किया गया था।

इसके बाद *अगाथेस* कोशिकाओं के बैच की खेती का पैमाना 15L से 100L बायोरिएक्टर तक का प्रयास किया गया, जो सांख्यिकीय रूप से अनुकूलित माध्यम का उपयोग करके स्केल-अप मानदंड के रूप में लगातार रेनॉल्ड्स संख्या का उपयोग करके किया गया था। 100 लीटर बायोरिएक्टर (58 आरपीएम की आंदोलन गति और 0.2vvm की वॉल्यूमेट्रिक गैस प्रवाह दर) की रिएक्टर परिचालन स्थितियों की पहचान दो बायोरिएक्टर (15 और 100 लीटर) खेती के लिए एक ही रेनॉल्ड नंबर ($एन_{आरई}$) मानदंडों का उपयोग करके व्यापक पैमाने पर गणना द्वारा की गई थी, जो 100L बायोरिएक्टर में प्रायोगिक कार्यान्वयन पर

बायोमास के लगभग समान मूल्यों के परिणामस्वरूप (13.94±0.18g/L 15L, 13.93±1.03g/L 100L में) और शिकिमिक एसिड सांद्रता (665.58±8.74mg/L 15L, 658.89±4.8mg/L 100L में) । यह माना जाता है कि शिकिमिक एसिड के बड़े पैमाने पर उत्पादन के लिए विकसित अनुकूलित उत्पादन प्रोटोकॉल समाज में टैमीफ्लू के उत्पादन के लिए शिकिमिक एसिड उपलब्धता का पूरक होगा।

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List of Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
ALR	Air lift reactor
ANOVA	Analysis of Variance
BAP	6-Benzylamino purine
CAH	Casein acid hydrolysate
CCD	Central Composite Design
CCRD	Central Composite Rotatable Design
CDC	Centre of Disease Control
CH	Chitosan
DCW	Dry Cell Weight
DO	Dissolved oxygen
FW	Fresh weight
GA	Gibberellic acid
h	Hour
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
JA	Jasmonic Acid
Kn	Kinetin
Lpm	Liters per minute
MJ	Methyl Jasmonate
MS	Murashige-Skoog
NA	Nicotinic acid
NAA	Naphthalene Acetic Acid
OVAT	One Variable at a Time
PI	Parametric index
PHCl	Pyridoxine HCL
RSM	Response Surface Methodology
SA	Shikimic acid
SSWR	Sum of Squares of Weighed Residues

STR	Stirred tank reactor
THCl	Thymine HCl
TTC	Triphenyl Tetrazolium Chloride
vvm	Volume per Volume per Minute
WPM	Woody plant medium
YE	Yeast Extract
YECF	Yeast Extract Carbohydrate Fraction

List of Symbols

dX/dt	Rate of Biomass formation (g/L.d)
dS/dt	Rate of Substrate consumption (g/L.d)
dP/dt	Rate of Product formation (g/L.d)
K_{S_1}	Saturation constant for Sucrose (g/L)
K_{S_2}	Saturation constant for Nitrate (g/L)
K_1	Growth associated product formation constant (g/g)
K_2	Non-growth associated product formation constant (g/g.d)
S_1	Initial Sucrose concentration (g/L)
S_2	Initial Nitrate concentration (g/L)
S_{max1}	Maximum value of Sucrose concentration at which $\mu = 0$ (g/L)
S_{max2}	Maximum value of Nitrate concentration at which $\mu = 0$ (g/L)
qs_1	Substrate consumption rate for Sucrose
qs_2	Substrate consumption rate for Nitrate
m_{S_1}	Maintenance coefficient for Sucrose (g/g.d)
m_{S_2}	Maintenance coefficient for Nitrate (g/g.d)
n_1, n_2	Degree of interaction of substrats (carbon and nitrogen)
V	Volume (L)
X	Biomass (g/L)
N	Rotation Speed
P/V	Power to volume ratio
Q/V	Volumetric gas flow rate
K_La	Volumetric mass transfer coefficient
N_{Re}	Reynold number
Π	Impeller tip velocity
Θ	Blend time
Y_{X/S_1}	Yield of Biomass with respect to Sucrose (g/g)
Y_{X/S_2}	Yield of Biomass with respect to Nitrate (g/g)
$Y_{P/S}$	Product yield with respect to substrates (Sucrose or Nitrate)

Greek Letters:

μ	Specific growth rate (d^{-1})
μ_m	Maximum specific growth rate (d^{-1})
Wj	Weight of each process variable
Δij	Difference between the model and experimental values for i^{th} data point and j^{th} process variable