

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

**RABAB ANJUM**



**DEPARTMENT OF BIOCHEMICAL ENGINEERING AND  
BIOTECHNOLOGY  
INDIAN INSTITUTE OF TECHNOLOGY DELHI  
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CULTURE**

by

**Rabab Anjum**

**Department of Biochemical Engineering and Biotechnology**

*Submitted*

*In fulfillment of the requirements of the degree of Doctor Of Philosophy*

*to the*



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**MARCH 2022**

*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.

**Date: -**



**Prof. Ashok K. Srivastava**  
**Department of Biochemical**  
**Engineering and Biotechnology,**  
**IIT Delhi**  
**New Delhi-110016**

**Prof. T. R. Sreekrishnan**  
**Department of Biochemical**  
**Engineering and Biotechnology,**  
**IIT Delhi**  
**New Delhi-110016**

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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 apetalum*, *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,  $(\text{NO}_3^-/\text{NH}_4^+)$  (40:20) 4.33g/L,  $\text{KH}_2\text{PO}_4$  0.09g/L,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  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,  $\text{GA}_3$ -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,  $(\text{NO}_3^-/\text{NH}_4^+)$  (40:20) 4.33g/L,  $\text{KH}_2\text{PO}_4$  0.09g/L,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.56 g/L, NAA 1.5mg/L, BAP 0.25mg/L, Charcoal 0.2 mg/L, CAH 0.8g/L,  $\text{GA}_3$  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 4<sup>th</sup> day

to 7<sup>th</sup> 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  $\Pi$ -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.

## प्लांट सेल कल्चर से शिकिमिक एसिड (टैमीफ्लू के लिए कच्चा माल) का बड़े पैमाने पर उत्पादन

### 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 इंप्लूएंजा 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 कॉलस 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<sub>3</sub>-/NH<sub>4</sub><sup>+</sup>) (40:20) 4.33g/L, KH<sub>2</sub>PO<sub>4</sub> 0.09g/L, CaCl<sub>2</sub>.2H<sub>2</sub>O 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<sub>3</sub>-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 बोर्नेसिस* 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<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup>) (40:20) 4.33g/L, KH<sub>2</sub>PO<sub>4</sub> 0.09g/L, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.56 g/L, NAA 1.5mg/L, BAP 0.25mg/L, चारकोल 0.2mg/L, CAH 0.8g/L, GA<sub>3</sub> 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<sub>1</sub>) और नाइट्रेट (एस<sub>2</sub>) के संभावित अवरोध की जांच की गई, जिसमें सुक्रोज और नाइट्रेट के संबंध में अधिकतम निरोधात्मक सांद्रता (एस<sub>एम</sub>) की पहचान 100g/L और 16.5g/L के रूप में की गई। *अगाथेस बोर्नेसिस* 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<sub>आरई</sub>) 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 ( $13.94 \pm 0.18 \text{g/L}$  15L,  $13.93 \pm 1.03 \text{g/L}$  100L में) और शिकिमिक एसिड सांद्रता ( $665.58 \pm 8.74 \text{mg/L}$  15L,  $658.89 \pm 4.8 \text{mg/L}$  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।

## Table of contents

List of Figures.....	i
List of Tables .....	v
List of Abbreviations .....	viii
List of Symbols .....	x
<b>Chapter 1: Introduction and Objectives.....</b>	<b>1</b>
<b>1.1 Introduction.....</b>	<b>2</b>
<b>1.2 Aim and Objectives.....</b>	<b>4</b>
<b>Chapter 2: Literature Review.....</b>	<b>6</b>
<b>2.1 What is Shikimic acid? .....</b>	<b>7</b>
<b>2.2 Shikimic acid for the treatment of Swine Flu .....</b>	<b>7</b>
<b>2.3 Other Applications of Shikimic acid .....</b>	<b>9</b>
<b>2.3.1 Use of Shikimic acid for production of other useful drugs .....</b>	<b>9</b>
<b>2.3.2 Chemotherapy .....</b>	<b>9</b>
<b>2.3.3 Agriculture.....</b>	<b>9</b>
<b>2.3.4 Cosmetics .....</b>	<b>10</b>
<b>2.3.5 Use of Shikimic acid (Tamiflu) in the treatment of Coronavirus (Covid-19).....</b>	<b>10</b>
<b>2.4 Sources of Shikimic acid production.....</b>	<b>10</b>
<b>2.4.1 Extraction from Star Anise plant.....</b>	<b>11</b>
<b>2.4.2 Shikimic acid production from microbes .....</b>	<b>12</b>
2.4.2.1 Shikimate pathway .....	13
2.4.2.2 Shikimic acid production by metabolic engineering of microbes .....	14
2.4.2.3 Shikimic acid production from recombinant/mutated strains .....	15
<b>2.4.3 Chemical synthesis of Shikimic acid .....</b>	<b>19</b>
<b>2.4.4 Shikimic acid extraction from natural plants .....</b>	<b>19</b>
<b>2.5 Plant cell cultivation protocols.....</b>	<b>21</b>

2.5.1 Shikimic acid production from plant cell culture.....	23
2.5.2 Production of Shikimic acid from <i>Agathis borneensis</i> .....	24
2.6 Production of other valuable metabolites through plant cell culture methods .....	26
2.7 Plant metabolites production through cell cultivation methods .....	27
2.7.1. Strain improvement.....	27
2.7.2. Selection of medium components .....	28
2.7.3 Statistical Optimization of cultivation medium components concentration .....	30
2.7.4 Selection and Optimization of culture environment parameters.....	31
2.7.5 Influence of aeration and agitation on growth during plant cell cultivation .....	32
2.8 Production enhancement strategies .....	33
2.8.1 Elicitation.....	33
2.8.2 Growth promoting hormone: Gibberellic acid .....	35
2.8.3 Addition of Vitamins .....	35
2.9 Use of mathematical models.....	36
2.10 Plant cell Bioreactors.....	37
2.11 Scale-up studies on plant cell cultivation.....	43
2.12 Limitations in Scale-up during plant cell cultivation .....	48
Chapter 3: Scale-up Strategy and Calculations.....	51
3.1 Scale-up Principles.....	52
3.1.1 Geometrical similarity.....	53
3.1.2 Hydrodynamic similarity .....	56
3.2 Assessment of Dimensionless numbers .....	56
3.3 Scale-up based on keeping operating variables constant.....	57
3.3.1 Power to volume ratio (P/V).....	58

3.3.2 Volumetric gas flow rate ( $Q/V$ ).....	58
3.3.3 Impeller tip velocity ( $\Pi$ ) .....	58
3.3.4 Volumetric mass transfer coefficient ( $K_{La}$ ).....	59
3.3.5 Blend time ( $\Theta$ ) .....	60
3.3.6 Impeller Reynolds number ( $N_{Re}$ ).....	60
<b>3.4 Summary of steps of Scale-up strategy (3L to 15L then 15L to 100L) used for mass cultivation of <i>Agathis</i> cells .....</b>	<b>61</b>
<b>3.5 Scale-up calculations (3L to 15L Bioreactor).....</b>	<b>62</b>
<b>3.5.1 Maintenance of geometrical similarity .....</b>	<b>62</b>
3.5.1.1 Vessel geometry.....	62
3.5.1.2 Impeller geometry.....	63
<b>3.5.2 Maintenance of Dynamic similarity .....</b>	<b>64</b>
3.5.2.1 Scale-up parameter estimation for 3L Bioreactor.....	64
3.5.2.2 Scale-up parametric test calculations for 15L Stirred Tank Reactor.....	69
<b>3.6 Comparison of Scale-up parametric indices (3L to 15L bioreactor)....</b>	<b>96</b>
<b>3.7 Scale-up calculation from 15L to 100L Bioreactor.....</b>	<b>98</b>
<b>Chapter 4: Materials and Methods .....</b>	<b>99</b>
<b>4.1 Materials .....</b>	<b>100</b>
4.1.1 Plant material and Plant cell culture medium .....	100
4.1.2 Chemicals.....	101
4.1.3 Equipments and Instruments .....	102
<b>4.2 Methods.....</b>	<b>103</b>
4.2.1 Induction of callus from <i>Agathis borneensis</i> explants .....	103
4.2.2 Initiation and maintenance of uniform Suspension Culture in shake flask.....	104
4.2.3 Increasing friability of the <i>Agathis</i> callus .....	105
4.2.3.1 Manipulating the composition of media components.....	105

4.2.3.2 Sub-culturing for increasing friability .....	106
<b>4.2.4 Optimization of nutrient medium for increased growth of <i>Agathis borneensis</i> .....</b>	<b>108</b>
4.2.4.1 Selection of sucrose as carbon source .....	108
4.2.4.2 Selection of suitable nitrate/ammonium concentrations as the nitrogen source .....	108
4.2.4.3 Selection of suitable Plant growth regulators (auxin-cytokinin) combination and concentration .....	109
<b>4.2.5 Optimization of cultivation conditions in shake flasks .....</b>	<b>111</b>
4.2.5.1 Selection of suitable photoperiod .....	111
4.2.5.2 Selection of suitable inoculum age/concentration .....	111
<b>4.2.6 Statistical Optimization of medium components .....</b>	<b>113</b>
4.2.6.1 Plackett Burman design .....	113
4.2.6.2 Response Surface Methodology .....	115
4.2.6.3 Experimental validation of the model predicted media in shake flask .....	118
<b>4.2.7 Study of Growth and Shikimic acid production kinetics of <i>Agathis borneensis</i> suspension culture in shake flask (with optimized medium composition).....</b>	<b>118</b>
<b>4.2.8 Yield enhancement strategies .....</b>	<b>119</b>
4.2.8.1 Addition of Elicitors for increasing Shikimic acid yield .....	119
4.2.8.2 Role of addition of Vitamins in increasing the production of Shikimic acid ...	120
4.2.8.3 Role of addition of Gibberellic acid (GA <sub>3</sub> ) in increasing the production of Shikimic acid .....	121
<b>4.2.9 Study of combined addition of Elicitors, Vitamins, and Gibberellic acid in increasing Shikimic acid production .....</b>	<b>122</b>
<b>4.2.10 Bioreactor Cultivation .....</b>	<b>124</b>
4.2.10.1 Preparation and Dressing of 3L Stirred Tank Bioreactor .....	124
4.2.10.2 Inoculum development for 3L Stirred Tank Bioreactor cultivation .....	126
4.2.10.3 Selection of suitable rotational speed for 3L Bioreactor .....	127

4.2.10.4 Selection of suitable airflow rate for 3L Bioreactor .....	127
4.2.10.5 Study of Growth and Production kinetics of <i>Agathis borneensis</i> suspension culture in 3-liter Stirred Tank Reactor with low shear steric impeller .....	128
<b>4.2.11 Development of batch mathematical model .....</b>	<b>130</b>
4.2.11.1 Study of substrate inhibition kinetics.....	130
4.2.11.2 Analysis of batch kinetic data proposal of the mathematical model and determination of kinetic parameters.....	131
4.2.11.3 Extrapolation of batch model to simulate fed-batch cultivation.....	133
4.2.11.4 Model predicted fed-batch cultivation strategy using constant feed rate of sucrose .....	133
<b>4.2.12 Scale-up studies .....</b>	<b>133</b>
4.2.12.1 Development of Scale-up strategy for mass production of Shikimic acid by <i>Agathis borneensis</i> .....	133
<b>4.2.13 Scale-up production of Shikimic acid in 15-liter Stirred Tank Reactor .....</b>	<b>136</b>
4.2.13.1 Preparation of 15-liter Bioreactor .....	136
4.2.13.2 Inoculum preparation for 15-liter Bioreactor.....	137
4.2.13.3 Study of Growth and Shikimic acid production kinetics of <i>Agathis borneensis</i> suspension culture in 15-liter Stirred Tank Reactor with low shear steric impeller .....	138
<b>4.2.14 Scale-up production of Shikimic acid in 100-liter Stirred Tank Reactor .....</b>	<b>140</b>
4.2.14.1 Fabrication of Steric impeller for 100L Bioreactor .....	140
4.2.14.2 100L Reactor preparation .....	141
4.2.14.3 Inoculum preparation for 100-liter Bioreactor.....	142
4.2.14.4 Study of the Growth and Production kinetics of <i>Agathis borneensis</i> suspension culture in 100-liter Stirred Tank Reactor with low shear steric impeller .....	144
4.2.14.5 Summary of the steps involved in mass production of Shikimic acid from shake flasks to 100L Bioreactor .....	146
<b>4.2.15 Analytical protocols .....</b>	<b>147</b>

4.2.15.1 Estimation of Biomass .....	147
4.2.15.2 Estimation of residual sucrose concentration .....	147
4.2.15.3 Estimation of residual nitrate concentration .....	148
4.2.15.4 Estimation of Shikimic acid concentration .....	148
4.2.15.5 Estimation of mixing time .....	150
4.2.15.6 Estimation of Growth and production indicators during bioreactor cultivation .....	150
<b>Chapter 5: Results and Discussion .....</b>	<b>151</b>
<b>5.1 Induction of callus from <i>Agathis borneensis</i> explants .....</b>	<b>152</b>
<b>5.2 Initiation of uniform Suspension Culture in shake flask .....</b>	<b>153</b>
<b>5.3 Strategies to increase the friability of the <i>Agathis</i> callus .....</b>	<b>154</b>
<b>5.3.1 Use of modified media for <i>A. borneensis</i> callus induction .....</b>	<b>154</b>
<b>5.4 Optimization of nutrient medium for increased growth of <i>Agathis borneensis</i> .....</b>	<b>157</b>
<b>5.4.1 Sucrose as carbon source .....</b>	<b>157</b>
<b>5.4.2 Selection of suitable ratio of nitrate/ammonium concentrations as the nitrogen source .....</b>	<b>160</b>
<b>5.4.3 Selection of suitable Plant growth regulators (auxin-cytokinin) combination and their concentrations .....</b>	<b>162</b>
<b>5.5 Optimization of cultivation conditions in shake flasks .....</b>	<b>164</b>
<b>5.5.1 Selection of suitable photoperiod .....</b>	<b>164</b>
<b>5.5.2 Selection of suitable inoculum age/concentration .....</b>	<b>166</b>
<b>5.6 Statistical optimization of medium components .....</b>	<b>170</b>
<b>5.6.1 Plackett Burman's design.....</b>	<b>170</b>
<b>5.6.2 Central Composite Design (CCD) for Response Surface Methodology .....</b>	<b>173</b>
<b>5.6.3 Predicted values for optimized media.....</b>	<b>176</b>

5.7 Study of the Growth and Shikimic acid production kinetics under shake flask cultivation studies using <i>Agathis borneensis</i> suspension (using optimized medium & cultivation conditions) .....	178
5.8 Shikimic acid productivity enhancement strategies .....	179
5.8.1 Addition of Elicitors for increasing Shikimic acid yield .....	179
5.8.2 Addition of Vitamins in increasing the production of Shikimic acid .....	182
5.8.3 Effect of Gibberellic acid (GA <sub>3</sub> ) on increasing the production of Shikimic acid .....	184
5.8.4 Combined effect of Elicitors, Vitamins, and Gibberellic acid .....	186
5.8.5 Predicted values of elicitor/precursor combination .....	189
5.9 Selection of suitable cultivation conditions for cultivation of <i>Agathis borneensis</i> in (3L) Bioreactor .....	190
5.9.1 Selection of suitable agitation speed.....	190
5.9.2 Selection of suitable aeration rate .....	192
5.10 Batch cultivation studies of <i>Agathis borneensis</i> cells in Stirred Tank Bioreactor for mass production of Shikimic acid .....	194
5.10.1 Study of Growth and Production kinetics of <i>Agathis borneensis</i> in 3-liter Stirred Tank Bioreactor with low shearing steric impeller.....	194
5.11 Development of batch mathematical model for growth and Shikimic acid production.....	197
5.11.1 Study of Growth and Shikimic acid production kinetics of <i>Agathis borneensis</i> in 3L Bioreactor for model development .....	197
5.11.2 Substrate inhibition studies for <i>Agathis borneensis</i> cell suspension .....	198
5.11.3 Development of batch mathematical model for growth and Shikimic acid production and optimization of model parameters .....	201
5.11.4 Evaluation of model parameters .....	203

5.11.5 Extrapolation of batch model to simulate fed-batch cultivation..	206
5.12 Scale-up of optimized Shikimic acid production from 3-liter to 100-liter Bioreactor.....	209
5.12.1 Scale-up of batch cultivation of <i>Agathis borneensis</i> from 3L to 15L Bioreactor.....	209
5.12.2 Study of Growth and Production kinetics of <i>Agathis borneensis</i> in 15-liter Stirred Tank Reactor using Constant Impeller tip velocity as Scale-up Criterion .....	214
5.13 Scale-up studies on batch cultivation of <i>Agathis borneensis</i> for mass production of Shikimic acid in 100L Bioreactor.....	217
5.13.1 Study of Growth and Production kinetics of <i>Agathis</i> suspension culture in 100L Bioreactor Using Constant Reynold number as the Scale-up Criterion.....	222
5.14 Summary of Growth and Production kinetics of <i>Agathis</i> cultivation in all three scales (3L, 15L and 100L) of bioreactor cultivation .....	224
5.15 Cost effectiveness of the current study .....	228
Chapter 6: Summary and Conclusions.....	230
6.1 Summary.....	231
6.2 Conclusions.....	238
Future studies .....	241
Chapter 7: References .....	242
Appendix.....	262
Resume	

## List of Figures

Figure No.	Title	Page No.
2.1	Chemical Structure of Shikimic acid	7
2.2	Tamiflu <sup>R</sup> tablets	8
2.3	Star Anise flowers	11
2.4	Shikimic acid pathway	14
2.5	<i>Agathis borneensis</i> plant (whole) and its leaves	25
2.6 (A-I)	Diagrammatic representation of different types of bioreactors	41
3.1 (a)	Schematic representation depicting the height to diameter ratio of model and prototype reactor(s)	53
3.1 (b)	Diagrammatic representation depicting the height to diameter ratio of steric impeller of both model and prototype reactor(s)	53
3.2	Relationship between Power number and Reynolds number	66
3.3	Graphical representation of Scale-up Parametric Indices under P/V test	73
3.4	Graphical representation of Scale-up Parametric Indices under K <sub>LA</sub> test	81
3.5	Graphical representation of Scale-up Parametric Indices under impeller tip velocity test	86
3.6	Graphical representation of Scale-up Parametric Indices under blend time test	90
3.7	Graphical representation of Scale-up Parametric Indices under Reynolds No. test	95
3.8	Graphical representation of Scale-up Parametric Indices under impeller tip velocity test	96
4.1	Callus initiation from leaves of <i>Agathis</i>	103
4.2	Callus subculture of <i>A. borneensis</i> on solid agar medium	103
4.3a	Non-friable, compact, dark brown color callus	106
4.3b	Friable, loosely aggregated, light brown/beige color callus	106
4.3c-d	Highly friable callus (left) inoculated into liquid medium to form uniform suspension culture (right)	106
4.4a	Three Liter Stirred Tank Reactor	125
4.4b	Low shear steric impeller	125
4.4c	Chilling unit	125
4.5	Inoculation Flask of volume 1L (for 3-liter bioreactor)	126

<b>4.6</b>	<i>Agathis</i> cell cultivation in 3-liter stirred tank bioreactor equipped with low shear steric impeller	128
<b>4.7</b>	Sterilization of 15-liter bioreactor in horizontal autoclave	136
<b>4.8</b>	Transfer of inoculum from 3L reactor to 15L stirred tank bioreactor (low shear steric impeller)	137
<b>4.9</b>	<i>Agathis borneensis</i> suspension culture cultivation in 15L STR (with low shear steric impeller)	138
<b>4.10a</b>	Low shear Steric impeller (3L reactor)	139
<b>4.10b</b>	Low shear Steric impeller (100L reactor)	139
<b>4.11</b>	In-situ sterilization of 100L Bioreactor with the help of steam generator	141
<b>4.12</b>	Inoculum bottle (10L volume) for 100L bioreactor	142
<b>4.13</b>	Customized 10L inoculation bottle for transfer of inoculum from 15L reactor to 100L bioreactor	142
<b>4.14</b>	<i>Agathis borneensis</i> suspension culture cultivation in 100L STR (with low shear steric impeller)	144
<b>4.15</b>	Process conditions of 100L bioreactor cultivation displayed on the bio controller	144
<b>4.16</b>	Steps for scale-up production of Shikimic acid	145
<b>4.17</b>	Sugar estimation by DNS method	147
<b>4.18</b>	Extraction of Shikimic acid from dried biomass	148
<b>5.1a-c</b>	Steps of callus induction from <i>Agathis</i> explants	151
<b>5.2</b>	Study of Growth kinetics of <i>Agathis borneensis</i> in shake flask suspension culture	153
<b>5.3a-b</b>	Morphological changes in callus texture; (5.3a) shows hard, compact callus; (5.3b) shows loosely aggregated friable callus	155
<b>5.4a</b>	Effect of different sucrose concentration on biomass production by <i>A. borneensis</i> cell suspension	158
<b>5.4b</b>	Effect of different sucrose concentration on Shikimic acid accumulation by <i>A. borneensis</i> cell suspension	158
<b>5.5a</b>	Effect of different nitrate to ammonium ion ratio on biomass production by <i>A. borneensis</i> cell suspension	160
<b>5.5b</b>	Effect of different nitrate to ammonium ion ratio on Shikimic acid accumulation by <i>A. borneensis</i> cell suspension	161
<b>5.6a</b>	Effect of different plant growth regulators combination on biomass production by <i>A. borneensis</i> cell suspension	163
<b>5.6b</b>	Effect of different plant growth regulators combination on Shikimic acid accumulation by <i>A. borneensis</i> cell suspension	163

5.7	Effect of light conditions on biomass and Shikimic acid production by <i>A. borneensis</i> cell suspension	165
5.8a-b	Effect of different inoculum concentration on biomass and growth rate	167
5.9	Effect of inoculum age on biomass concentration	168
5.10	<i>A. borneensis</i> growth kinetics when shake flasks were inoculated with (optimized inoculum) 6-day old, 5g/L (DCW) callus	169
5.11	3D Contour plots for biomass accumulation by <i>A. borneensis</i> suspension culture	176
5.12	Biomass growth, substrate consumption and Shikimic acid accumulation by <i>A. borneensis</i> cells during shake flask cultivation (using statistically optimized media)	178
5.13	Effect of different elicitors on the biomass and Shikimic acid production by <i>A. borneensis</i> cell suspension	181
5.14a	Effect of different Vitamins on the biomass by <i>A. borneensis</i> cell suspension	183
5.14b	Effect of different Vitamins on Shikimic acid production by <i>A. borneensis</i> cell suspension	183
5.15a-b	Effect of different concentrations of Gibberellic acid on the biomass and Shikimic acid production by <i>A. borneensis</i> cell suspension	185
5.16	3D Contour plots for Shikimic acid accumulation by <i>A. borneensis</i> suspension culture	188
5.17	Effect of different agitation speed on the biomass and Shikimic acid production by <i>A. borneensis</i> cell suspension	191
5.18	Effect of different aeration rate on the biomass and Shikimic acid production by <i>A. borneensis</i> cell suspension	192
5.19	Biomass growth, substrate consumption and Shikimic acid accumulation by <i>A. borneensis</i> cells in 3L STR fitted with low shear steric impeller	195
5.20	Growth and Shikimic acid production kinetics of <i>Agathis</i> in 3L STR fitted with low shear steric impeller for model generation	197
5.21	Effect of increasing initial sucrose concentration on <i>Agathis</i> growth	199
5.22	Effect of increasing initial nitrate concentration on <i>Agathis</i> growth	199
5.23	Comparison of model-simulation (smooth lines) of batch kinetics with the experimental data (points) of <i>Agathis borneensis</i> cell suspension during stirred tank bioreactor cultivation	205
5.24	Model predicted fed-batch cultivation strategy using constant feed rate of sucrose during 4 <sup>th</sup> day to 7 <sup>th</sup> day	206
5.25	Summary of effect of maintenance of different equal scale-up criteria in 3L & 15L	212

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<b>5.26</b>	Biomass growth, substrate consumption and Shikimic acid accumulation by <i>A. borneensis</i> cells in 15L STR fitted with low shearing steric impeller	214
<b>5.27</b>	Comparative study of growth and production kinetics of <i>Agathis borneensis</i> in 3L and 15L bioreactors	216
<b>5.28</b>	Summary of effect of maintenance of different equal scale-up criteria in 15L & 100L	220
<b>5.29</b>	Comparative study of growth and production kinetics of <i>Agathis</i> cells in 15L and 100L bioreactors	223

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## List of Tables

Table No.	Title	Page No.
2.1	Shikimic acid yield from different microorganism	16
2.2	Shikimic acid yield from metabolically engineered strains of <i>E. coli</i>	17
2.3	Shikimic acid content of plants available in Russia	20
2.4	Studies of Shikimic acid production from plant cell cultivation methods	25
2.5	List of Vitamins and their functions in plant cell culture medium	36
2.6	Mass cultivation of plant cell in stirred tank bioreactors	45
2.7	List of different reactors for plant cell culture scale-up	46-47
2.8	List of scale-up strategies/parameters used during large scale bioreactor cultivation	49
3.1	Values of intensive scale-up parameters for 3L bioreactor under constant P/V Test	68
3.2	Values of intensive scale-up parameters for 15L bioreactor under constant P/V Test	72
3.3	Summary of the values of scale-up parametric indices under P/V Test	72
3.4	Values of intensive scale-up parameters for 15L bioreactor under constant $K_{La}$ Test	80
3.5	Summary of the values of scale-up parametric indices under $K_{La}$ Test	81
3.6	Values of intensive scale-up parameters for 15L bioreactor under constant Impeller tip velocity Test	85
3.7	Summary of the values of scale-up parametric indices under impeller tip velocity Test	85
3.8	Values of intensive scale-up parameters for 15L bioreactor under constant blend time Test	89
3.9	Summary of the values of scale-up parametric indices under blend time Test	90
3.10	Values of intensive scale-up parameters for 15L bioreactor under constant Reynolds No. Test	94
3.11	Summary of the values of scale-up parametric indices under Reynolds No. Test	94
3.12	Matrix of scale-up parametric indices for overall comparison of scale-up intensive parameters (3L to 15L)	96
4.1	Composition of WPM (PT026)	99
4.2	The list of chemicals used and their sources	100
4.3	List of all the Equipment/Instruments used and their sources	101-102
4.4	Different combinations of auxin and cytokinin concentrations	109
4.5	High and low concentration ranges of different effectors (media components) selected for Plackett-Burman design	113
4.6	Experimental recipe in Plackett-Burman design for medium optimization	113

4.7	RSM experimental design for media optimization	115
4.8	Different Elicitors and their concentrations	118
4.9	Different Vitamins and their concentrations used in the investigation	119
4.10	RSM experimental design to study the combined effect of Elicitor, Vitamin, and Gibberellic acid on Shikimic acid production	122
5.1	Trial experiments to increase the friability of callus	156
5.2	Effect of different combinations and concentrations of auxins and cytokinin on biomass and Shikimic acid concentrations	162
5.3	Effect of different photoperiods on biomass and Shikimic acid concentrations	165
5.4	Effect of inoculum concentration on biomass and growth rate	166
5.5	Effect of inoculum age on biomass and growth rate	168
5.6	Plackett-Burman experimental design and responses with respect to biomass and Shikimic acid production	171
5.7	Values of 't' coefficient of different factors selected for Plackett-Burman study	171
5.8	RSM experimental design for media optimization	173
5.9	The experimental and model-predicted values of biomass and Shikimic acid obtained in the optimized medium	177
5.10	Addition of Elicitors in increasing the production of Shikimic acid	180
5.11	Role of addition of Vitamins in increasing the production of Shikimic acid	182
5.12	Addition of Gibberellic acid to enhance the production of Shikimic acid	185
5.13	RSM experimental design for the study of combined effect of Elicitor, Vitamin, and Gibberellic acid	186
5.14	Optimized values of model parameters for batch cultivation of <i>Agathis</i> cells	204
5.15	Comparison of the growth and Shikimic acid production kinetics for <i>A. borneensis</i> batch cultivation and model simulated fed-batch cultivation in 3L STR	207
5.16	Comparison of various scale-up criteria for scale-up from 3L to 15L bioreactor cultivation	211
5.17	Comparison of various scale-up criteria for scale-up from 15L to 100L bioreactor cultivation	219
5.18	Comparison of growth and production characteristics of the <i>A. borneensis</i> cultivation in 3L, 15L and 100L Bioreactor	225
5.19	Comparison of the overall Biomass Growth parameters for <i>A. borneensis</i> cultivation in 3L, 15L and 100L Bioreactor	225

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<b>5.20</b>	Comparison of the overall Substrate (Sucrose) consumption for <i>A. borneensis</i> cultivation in 3L, 15L and 100L Bioreactor	226
<b>5.21</b>	Comparison of the Product (Shikimic acid) formation for <i>A. borneensis</i> cultivation in 3L, 15L and 100L Bioreactor	226
<b>5.22</b>	Comparison of Biomass and Product Yield for <i>A. borneensis</i> cultivation in 3L, 15L and 100L Bioreactor	226
<b>5.23</b>	Comparison of Shikimic acid yield and productivity from <i>Agathis</i> cultivation (present study) with literature studies	227
<b>5.24</b>	Comparison of cost effectiveness of Shikimic acid production	228

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## List of Abbreviations

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

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

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## List of Symbols

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$dX/dt$	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
$\Pi$	Impeller tip velocity
$\Theta$	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)

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***Greek Letters:***

$\mu$	Specific growth rate ( $d^{-1}$ )
$\mu_m$	Maximum specific growth rate ( $d^{-1}$ )
$W_j$	Weight of each process variable
$\Delta_{ij}$	Difference between the model and experimental values for $i^{\text{th}}$ data point and $j^{\text{th}}$ process variable

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