

**MASS SCALE PRODUCTION OF SHIKIMIC ACID  
FROM DIFFERENT BIOLOGICAL SOURCES**

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FROM DIFFERENT BIOLOGICAL SOURCES**

*by*

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*Submitted*

*In fulfillment of the requirements of the degree of doctor of Philosophy*

*to the*



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*To my parents...  
&  
my Son, Ojswa....*

## **Certificate**

This is to certify that the thesis entitled “**Mass scale production of shikimic acid from different biological sources**”, being submitted by **Ms. Swati Shalini** 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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**Swati Shalini**

**Date**

**Place**

## **Mass scale production of shikimic acid from different biological sources**

### **ABSTRACT**

Shikimic acid (SA) is a raw material for chemical synthesis of drug Oseltamivir commercially known as Tamiflu, the only drug effective against influenza pandemics like “Swine flu” and “Bird flu”. It also prevents the spread of H5N1 and Influenza A virus. Besides its application in the production of antiviral drugs, shikimic acid is also used in chemotherapy and hormone replacement therapy. SA is potentially used as herbicide and antibacterial agent. The demand of shikimic acid is growing day by day in the pharmaceutical industries and its market value has risen to \$1000 /kg during pandemic situations, yet the supply of SA is inadequate to meet the growing demand of world market. Currently SA is recovered from the seeds of Chinese plant Star Anise using a complex extraction procedure. SA accumulation has been also reported in other Chinese plant species but their low content and restricted availability are the major limitations for their use as raw material for commercial SA production. Hence, there is an urgent need for the identification of alternate sources and/or development of production protocols for mass scale SA production which was the major motivation of the present study. Several alternate production routes have been explored in the recent past to supplement shikimic acid production e.g., microbial fermentation of genetically recombinant bacteria and enzymatic synthesis SA. But above production protocols are not suitable for large scale production of SA due to different reasons as indicated below. Fermentation by genetically modified organisms requires specific aromatic amino acids in the cultivation medium which are costly and their worldwide availability is less than their requirement for SA production. Besides, most of the microbes studied for production of SA are gram negative in nature having lipo-polysaccharides (endotoxins) in their cell membrane and so are not Generally Recognized As Safe (GRAS) for pharmaceutical use. Similarly, the enzymatic synthesis procedures require expensive

reagents, besides these production protocols are not easily scalable, with the result these production strategies are not considered as appropriate for commercial SA production.

In present study in-vitro plant cell cultivation technology was developed which can be regarded as a safe, scalable and efficient protocol for mass scale shikimic acid production. Two, literature reported Indian plants *Agathis borneensis* and *Melia azedarach* were selected for development of the sustainable production of shikimic acid. *A. borneensis* is already listed as endangered species as it belong to Gymnosperm group of plant and was reported to contain highest concentration of shikimic acid among all the Indian plant species studied so far. *A. borneensis* tree, generally, grows in association with fungal culture and due to this reason callus induction & the cell cultivation was difficult for this plant species. Also no reports were available for either callus induction or mass scale cell cultivation for this plant species.

The callus & suspension culture of *A. borneensis* were established and mass propagation was attempted in the bioreactor for the first time.

For this purpose the protocols for surface sterilization, callus induction and suspension culture initiation were standardized in the present study. The batch production kinetics studies of *A. borneensis* was examined in shake flask, 3L stirred tank bioreactor (STR) and even 3L air lift reactor (ALR). The cell suspension cultivation in shake flask with appropriate sucrose concentration and cultivation conditions resulted in reasonably high shikimic acid accumulation (8 % w/w on dry weight basis) 32% higher than the natural plant source. However the plant cells were observed to be very shear sensitive and therefore the biomass accumulation decreased by 12% and 22% while attempting the cell cultivation in STR and ALR respectively. The loss in cell viability of 60% and 57% resulted in reduction of SA production by 35 % and 40 % during cultivation in STR and ALR respectively. The cell

culture turned brown due to release of intracellular phenolic compounds which inhibited its growth in the bioreactors. The cell aggregates formed during shake flask cultivation significantly affected the development of homogenous suspension culture and eventually less shikimic acid accumulation. Due to above limitations *A. borneensis* cell suspension could not be used for in depth cultivation & optimization studies.

*Melia azedarach* leaves explants were collected from a local tree (IIT Delhi campus) and subjected for callus induction trials. The successful callus induction was followed by initiation of homogenous shake flask cell suspension culture. The medium components and shake flask cultivation conditions were, thereafter, statistically optimized for the enhanced production of biomass & shikimic acid. Batch cultivation of *M. azedarach* in the 3L STR with optimized cultivation conditions resulted in (465 mg/L) 4 folds increase in SA accumulation than the leaves of natural plants (120.6 mg/L).

The batch kinetics of *M. azedarach* along with its substrate inhibition data with respect to specific growth rate was used to develop a mathematical model. The model was then extrapolated to simulate different nutrient feeding strategies for higher biomass and shikimic acid accumulation during fed-batch cultivation conditions. Selected model based nutrient feeding strategies for fed-batch cultivation were thereafter successfully implemented experimentally wherein 15 folds increase in SA was observed than the natural plant whereas in continuous cultivation with cell retention an 18 folds increase in SA than the natural plant was achieved by *M. azedarach* cells. To further improve the shikimic acid content “Integrated fed batch cultivation (with maintenance of pseudo steady state with respect to sucrose @ 23.5 g/L) and thereafter continuous cultivation @ Dilution rate 0.09 L/day) with in situ cell retention using spin filter (10 micron pore size)” was simulated by model and experimentally implemented. During this cultivation study, the biomass growth & shikimic acid accumulations were 80.6 g/L DCW & 8640 mg/L respectively (34 folds higher SA than

natural plant) on 26<sup>th</sup> day of cultivation. However, the cell suspension turned very viscous at such high cell density and it was difficult to maintain the dissolved oxygen (at 30% saturation value) which eventually led to a decrease in the shikimic acid (8640 mg/L) content after 26<sup>th</sup> day of cultivation as opposed to the model predicted SA accumulation of 9400 mg/L on 28<sup>th</sup> day of cultivation.

To address the oxygen limitation problem, the cell suspension of *M. azedarach* was successfully cultivated in different bioreactor configurations and it was observed that 3L Centrifugal Impeller Bioreactor (CIB) was most suitable configuration for the large scale cultivation of cells of *M. azedarach* which enhanced the biomass production and SA accumulation by 20% and 150% respectively than the shake flask cultivation. The Volumetric oxygen transfer coefficient ( $K_{La}$  value) of CIB ( $5.6 \text{ h}^{-1}$ ) was almost double than that of STB ( $2.6 \text{ h}^{-1}$ ). Which ensured the oxygen availability to growing cells at higher cell density? Mixing time of centrifugal impeller was also less (4.9 sec) than that of stirred tank bioreactor (5.3 sec). Shorter mixing time may have enhanced the growth of high density cell suspension by improving oxygen transfer capacity and reducing the dead zone formation thereby leading to biomass and SA concentrations of  $17.4 \pm 1.3 \text{ g/L DCW}$  and  $1150 \pm 21 \text{ mg/L}$  respectively.

## सार

शिमिमिक एसिड दवा के ओसिल्टामवीर के रासायनिक संश्लेषण के लिए एक कच्चा माल है जिसे वाणिज्यिक तौर पर टैमिफ्लू के नाम से जाना जाता है। टैमिफ्लू "सूअर फ्लू" और "बर्ड फ्लू" जैसे इन्फ्लुएंजा महामारी के खिलाफ एकमात्र दवा है। यह एच 5 एन 1 और इन्फ्लुएंजा ए वायरस के प्रसार को भी रोकता है। एंटीवायरल ड्रग्स के उत्पादन के अलावा शिमिमिक एसिड कामोथेरेपी और हार्मोन रिप्लेसमेंट थेरेपी में भी प्रयोग किया जाता है। शिमिमिक एसिड को संभावित रूप से जड़ी-बूटियों और जीवाणुरोधी एजेंट के रूप में प्रयोग किया जाता है। फार्मास्युटिकल उद्योगों में दिन-दर-दिन शिमिमिक एसिड की मांग बढ़ रही है और महामारी की स्थितियों में इसकी बाजार मूल्य बढ़कर 1000 डॉलर / किग्रा हो गई है। लेकिन शीलिक एसिड की आपूर्ति विश्व बाजार की बढ़ती मांग को पूरा करने के लिए अपर्याप्त है। वर्तमान में चाइकीमिक एसिड एक जटिल प्रक्रिया के साथ चीनी संयंत्र स्टार अनीस के बीज से निकाला जाता है। शैमिमिक एसिड संचय कुछ अन्य पौधों की प्रजातियों में भी दर्ज किया गया है लेकिन सामग्री कम होने की सूचना है और पौधों की उपलब्धता केवल कुछ भौगोलिक क्षेत्रों तक ही सीमित है। बड़े पैमाने पर शकीमिक एसिड उत्पादन के लिए वैकल्पिक मार्गों के विकास की एक जरूरी आवश्यकता है। हाल के दिनों में कई वैकल्पिक उत्पादन मार्गों का पता लगाया गया है ताकि उच्च शिमिमिक एसिड उत्पादन में वृद्धि हो सके। जैविक रूप से पुनः संयोजक बैक्टीरिया के माइक्रोबियल किण्वन और शिमिमिक एसिड के एंजाइमेटिक संश्लेषण लेकिन ऊपर बताए गए विभिन्न कारणों के कारण उत्पादन प्रोटोकॉल ऊपर शिमिमिक एसिड के बड़े पैमाने पर उत्पादन के लिए उपयुक्त नहीं हैं। सूक्ष्मजीव किण्वन की खेती के माध्यम से सुगंधित अमीनो एसिड की आवश्यकता होती है जो उत्पादन की लागत को बढ़ाती है। इसके अलावा शिमिमिक एसिड के उत्पादन के लिए अध्ययन किए जाने वाले अधिकांश रोगाणुओं की प्रकृति में ग्राम नकारात्मक है जो उनके सेल झिल्ली पर एंडोटोक्सिन है और इसलिए दवा के इस्तेमाल के लिए सुरक्षित नहीं माना जाता है। इसी तरह एंजाइमिक संश्लेषण प्रक्रिया में महंगा अभिकर्मकों की आवश्यकता होती है और शिकीमिक एसिड के आर्थिक वाणिज्यिक उत्पादन के लिए समस्याएं होती हैं।

इसलिए वर्तमान अध्ययन में पौधे सेल खेती प्रौद्योगिकी विकसित की गई थी जिसे बड़े पैमाने पर शकीमिक एसिड उत्पादन के लिए एक सुरक्षित और कुशल प्रोटोकॉल माना जा सकता है। दो साहित्य भारतीय पौधों की रिपोर्ट करते हैं, अगाथासिस बोनेसेन्सिस और मेलिया अज़ेदारच का चयन बड़े पैमाने पर शकीमिक एसिड उत्पादन के लिए पौधे सेल की खेती प्रौद्योगिकी के विकास के लिए किया गया था। ए जन्मदारियों को पहले से ही लुप्तप्राय प्रजातियों के रूप में सूचीबद्ध किया गया है और यह पौधों के जिमनस्पर्म समूह से संबंधित है और सभी भारतीय पौधों की प्रजातियों में अब तक शैमिमिक एसिड के उच्च घनत्व शामिल

होने की खबर है। ए उत्पत्ति के पेड़ आम तौर पर कवक संस्कृति के साथ बढ़े और इस वजह से सेल की खेती इस पौधे की प्रजातियों के लिए मुश्किल थी। इस पौधे की प्रजातियों के लिए कॉलस प्रेरण और बढ़े पैमाने पर सेल की खेती के लिए कोई रिपोर्ट उपलब्ध नहीं हैं। निलंबन की संस्कृति शुरू की गई थी और पहली बार बायोरिएक्टर स्तर में बढ़े पैमाने पर प्रचार किया गया था। इस प्रयोजन के लिए वर्तमान अध्ययन के दौरान भूतल नसबंदी, माइक्रोप्रोपोगेशन और कॉलस प्रेरण के लिए प्रोटोकॉल स्थापित किए गए थे। ए। बेनेजेन्सिस के बैच प्रोडक्शन कैनेटीक्स का अध्ययन हिला फ्लास्क, उत्तेजित टैंक बायोरिएक्टर और एयर लिफ्ट रिएक्टर में किया गया था। खेती की स्थिति के अनुकूलन के परिणामस्वरूप शिमीमिक एसिड संचय में 8% w / w (सूखी वजन आधार पर) तक बढ़ गया।

मेलिया अज़ेदार व्याख्यान एक स्थानीय पेड़ (आईआईटी दिल्ली परिसर) से एकत्र किए गए और कॉलस या बालों के रूट प्रेरण परीक्षणों के अधीन रहे। एम। अज़ेदारच के पत्तों और नोडल व्याख्याओं से कंधे को प्रेरित करने के लिए सफल प्रयास किए गए। श्लेष्म कोशिका द्रव्यमान का उपयोग समरूप सेल निलंबन शुरू करने के लिए किया गया था। शमीमिक एसिड के बढ़ाए उत्पादन के लिए मध्यम घटकों और खेती की स्थिति सांख्यिकीय रूप से अनुकूलित थी। इसके बाद, सेल निलंबन को उपन्यास बायोरेक्टर्स कॉन्फ़िगरेशन में सफलतापूर्वक लगाया गया और यह पाया गया कि केन्द्रापसारक प्रेरित करनेवाला बायोरेक्टर कारक एम। अज़ेदार के बढ़े पैमाने पर खेती के लिए सबसे उपयुक्त विन्यास था। बायोमास की वृद्धि और एम। अज़ेदारच के शिमीमिक एसिड प्रोडक्शन कैनेटीक्स को एक गणितीय मॉडल विकसित करने के लिए सबस्ट्रेट अवरोधन डेटा के साथ उभरा हुआ टैंक बायोरिएक्टर में उपयोग किया गया था। उच्च बायोमास और शिमीमिक एसिड संचय के लिए विभिन्न पोषक आहार खिलाड़ियों की ऑफ़लाइन अनुकार के लिए एक मॉडल के रूप में इस मॉडल का उपयोग किया गया था। मॉडल आधारित चयनित पोषक तत्व खिला रणनीतियों को तत्काल तंग बैच और निरंतर खेती के लिए सफलतापूर्वक लागू किया गया (सेल प्रतिधारण के साथ) एम। अजार्ड कोशिकाओं के बढ़े पैमाने पर उत्पादन के लिए। सबसे अच्छी खेती की रणनीति एकीकृत फेड बैच की खेती के रूप में पहचान की गई थी, साथ ही स्पिरफिल्टर फिल्टर का उपयोग करके सेल प्रतिधारण के साथ सुक्रोज़ और निरंतर खेती के संबंध में छद्म स्थिर राज्य को बनाए रखने के साथ। इस खेती के दौरान बायोमास की वृद्धि 80.6 ग्राम / एल और शिमीमिक एसिड संचय की खेती के 26 वें दिन पर 8.6 ग्राम / एल का अध्ययन किया गया।

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## LIST OF ABBREVIATIONS

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SA	Shikimic Acid
ALR	Airlift reactor
CIB	Centrifugal Impeller bioreactor
IAOS	Inner Aeration Outer Settling
OAIS	Outer Aeration Inner settling
CCD	Central Composite Design
CoA	Co-Enzyme A
DCW	Dry cell weight
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen
DoE	Design of experiment
EPSP	Enoyl pyruvil Shikimate-3phospate
SIA	Salycilic acid
JA	Jasmonic acid
MJ	Methyl Jasmonate
Y.E.	Yeast extract
PVP	Poly Vinyl Pyrrolidone
RSM	Response Surface Methodology
SAP	Super Absorbent Polymer
SSWR	Sum of Squares of Weighted Residues
STR	Stirred Tank Reactor
TTC	2,3,5-Triphenyl-tetrazolium chloride
v/v	Volume by volume

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## LIST OF SYMBOLS

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$a_1$	Exponent indicating type of relation between $S_1$ (Sucrose) and $\mu$
$a_2$	Exponent indicating type of relation between $S_2$ (Nitrate) and $\mu$
$K_{S_1}$	Saturation constant for sucrose consumption (g/L)
$K_{S_2}$	Saturation constant for nitrogen consumption (g/L)
$1/Y_{X/S_1}$	Yield of biomass on sucrose consumption (g/g)
$1/Y_{X/S_2}$	Yield of biomass on nitrogen consumption (g/g)
$ms_1$	Maintenance coefficient for sucrose (g/g.h)
$ms_2$	Maintenance coefficient for nitrogen (g/g.h)
$qs_1$	Specific sucrose consumption rate ( $h^{-1}$ )
$qs_2$	Specific nitrogen consumption rate ( $h^{-1}$ )
$Qp$	Specific product formation rate ( $h^{-1}$ )
$D$	Dilution rate ( $h^{-1}$ )
$F$	Total flow rate (L/h)
$F_1$	Flow rate for sucrose (L/h)
$F_2$	Flow rate for nitrogen (L/h)
$K_1$	Growth associated product formation constant (g/g)
$n_1$	Constant in equation
$n_2$	Constant in equation
$So_1$	Inlet concentration of sucrose in the feed bottle (g/L)
$So_2$	Inlet concentration of nitrogen in feed bottle (g/L)

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$S_1$	Sucrose concentration (g/L)
$S_2$	Nitrogen concentration (g/L)
$S_j$	Variance of the error of a residual
$S_{m_1}$	Sucrose concentration at which complete inhibition occurs (g/L)
$S_{m_2}$	Nitrogen concentration at which complete inhibition occurs (g/L)
$V$	Working volume of the bioreactor (L)
$W_j$	Weight of each variable
$X$	Biomass concentration (g/L)
$dV/dt$	Rate of change of volume (L/h)

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### **Greek symbols**

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

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