

# **STRATEGIES FOR PRODUCTION OF BIOPOLYMERS USING GASEOUS SUBSTRATES (METHANE & AIR)**

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BIOPOLYMERS USING GASEOUS SUBSTRATES  
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*by*

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*Submitted*

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*to the*



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**DEDICATED TO MY BELOVED PARENTS AND  
GRANDMOTHER**

# CERTIFICATE

This is to certify that the thesis entitled “**Strategies for the production of biopolymers using gaseous substrates (methane and air)**” being submitted by **Ms. Anika Goyal** to the Indian Institute of Technology Delhi, for award of degree of “**Doctor of Philosophy**” in Biochemical Engineering and Biotechnology Department is a record of the original bonafide research work carried out by her, which has been prepared under our supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi. The results contained in this thesis have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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# STRATEGIES FOR PRODUCTION OF BIOPOLYMERS USING GASEOUS SUBSTRATES (METHANE AND AIR)

## ABSTRACT

Economic production protocols of biopolymers are desperately required for replacement of petroleum-derived polypropylene polymers that have been extensively used in the society. PHA biopolymers can be naturally synthesized by certain microorganisms such as *Alcaligenes latus*, recombinant *Escherichia coli*, etc., under excess availability of carbon and limiting concentrations of minor nutrients such as nitrogen, potassium, phosphorous, and sulfur. It has been observed that the cost of biopolymers thus produced is relatively high, primarily due to the high cost of agricultural products (sugars, such as glucose, fructose, etc.), which are used as the major substrates during bioreactor cultivation. Recently, it has been reported that Type II *Methanotrophs* such as that *Methylosinus trichosporium*, *Methylobacterium organophilum*, and *Methylocystis hirsuta* can utilize methane gas and have a distinct ability to accumulate PHB based biopolymers.

Experimental protocols were successfully developed for cultivation of *M. organophilum* and *M. trichosporium* using Nitrate Mineral Salt (NMS) media and methanol as substrate. The inoculum propagation protocols for *M. organophilum*, *M. trichosporium*, and *M. hirsuta* were also established in crimp-sealed serum bottles/flasks containing NMS media with an overlay of methane gas, wherein *M. hirsuta* demonstrated the highest biomass production ability, and was therefore, identified as the key organism for in-depth studies in the present investigation. *M. hirsuta* featured 0.08 g/L biomass accumulation after 100 hours cultivation in the serum flasks. Inoculum development

protocols for *M. hirsuta* were successfully established in modified Borosil bottles, which facilitated continuous purging of methane with the result of which biomass growth of 2.32 g/l was obtained after 126 h in NMS media. Thereafter, study of batch growth kinetics of *M. hirsuta* was done in 2.5L bioreactor using NMS media under constant methane purging (at 0.05 vvm), which resulted in production of 2.5 g/l biomass with PHB accumulation of 0.55 g/l in 140 hours. To enhance the biomass growth further a fed-batch bioreactor cultivation of *M. hirsuta* was initiated in 3.5L bioreactor wherein sterilized media, (500 ml of concentrated (2.5X NMS) media having reduced (1X) concentration of Potassium nitrate) was supplemented after 200 hours and the overall biomass concentration of 10.57 g/L with PHB accumulation of 1.1 g/L was obtained after 800 hours.

The nutrient concentrations of NMS media were statistically optimized. The concentrations for  $\text{KNO}_3$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  emerged as 1 g/l, 1 g/l, 0.27 g/l and 0.71 g/l, respectively after statistical optimization. The dilutions of NMS media were done to figure out the possibility of improvement of growth rate of *M. hirsuta*, wherein it was observed that NMS/4 concentration was most favorable for the maintenance of exponential growth phase of *M. hirsuta* for a longer period of time. *M. hirsuta* was then cultivated in 2L stirred-tank reactor (STR) and 4L airlift reactor (ALR) using 0.25X NMS media with methane purging at 0.05 vvm. This led to biomass and PHB production of 0.59 g/l and 0.16 g/l, respectively, in 74 h in STR and biomass growth of 0.72 g/l and intracellular PHB accumulation of 0.21 g/l after 73 hours for ALR configuration.

Detailed nutrient limitation studies were performed in 2.5L STR and 4L ALR revealed

that the limiting concentration of nitrate ions along with constant (non-limiting) availability of methane featured high PHB production within the cells of *M. hirsuta*. The role of inoculum age on the PHB accumulation was identified for *M. hirsuta* cultivation by using the inoculum of different ages (48 & 100 h age) for bioreactor cultivation. It was observed that the culture age of 100h exhibited biomass and PHB productivity of 0.184 kg.m<sup>-3</sup>.d<sup>-1</sup> and 0.125 kg.m<sup>-3</sup>.d<sup>-1</sup>, respectively, which is the highest productivity reported for *M. hirsuta*.

The batch growth kinetics of *M. hirsuta* using 0.25XNMS media duly purged with methane (at 0.05 vvm) in 2.5L STR and 4L airlift bioreactor were used to develop respective mathematical models, which adequately described the observed batch growth kinetics of *M. hirsuta*. The models were extrapolated to simulate fed-batch cultivation to identify nitrate feeding for maintenance of pseudo steady state of nitrate for enhanced biopolymer accumulation. Upon experimental implementation of the nutrient feeding strategy during the fed-batch cultivations of *M. hirsuta* in STR & air lift bioreactor, it was observed that the developed mathematical model adequately described the experimental batch bioreactor cultivation of *M. hirsuta*. However, the model-predicted maintenance of constant nitrogen concentration during the fed-batch cultivation could not correlate well with the experimentally observed values of biomass and PHB.

Fed-batch cultivation with intermittent (need based) supplementation of NMS media was attempted for cultivation of *M. hirsuta* in 2.5L bioreactor containing 0.25X NMS media duly purged with methane at 0.05 vvm. The NMS media was supplemented in small pulses of 50 ml after 24, 48, 56 and 78 h. The feed concentration was then increased to

50 ml of 2.5X NMS at 150, 175 and 200 hours and 350 ml of 2.5X NMS media was finally added after 228 hours. The above fed-batch cultivation strategy led to biomass production and PHB accumulation of 3.77 g/l and 1.08 g/l respectively.

The co-cultivation of the two organisms (*M. hirsuta* to *M. organophillum*) in NMS-methane media resulted in slightly higher OD (0.65) than the respective pure culture of *M. hirsuta* (OD of 0.59) and *M. organophillum* (OD of 0.06), indicating that co-cultivation of these organisms can result in higher biomass and PHB accumulation.

गैसीय सबस्ट्रेट्स (मीथेन और वायु) का उपयोग करके बायोपॉलिमर्स के उत्पादन के लिए रणनीतियाँ अमूर्त

### ABSTRACT

समाज में बड़े पैमाने पर उपयोग किए जाने वाले पेट्रोलियम-व्युत्पन्न पॉलीप्रोपाइलीन पॉलिमर के प्रतिस्थापन के लिए बायोपॉलिमर्स के आर्थिक उत्पादन प्रोटोकॉल की सख्त आवश्यकता है। PHA बायोपॉलिमर्स को कार्बन की अधिक उपलब्धता और नाइट्रोजन, पोटेशियम, फॉस्फोरस, और सल्फर जैसे मामूली पोषक तत्वों की सांद्रता को सीमित करने के तहत कुछ सूक्ष्मजीवों जैसे अल्कलिजेनस लैटस, पुनः संयोजक एस्चेरिचिया कोलाई आदि द्वारा स्वाभाविक रूप से संश्लेषित किया जा सकता है। यह देखा गया है कि इस प्रकार उत्पादित बायोपॉलिमर्स की लागत अपेक्षाकृत अधिक है, मुख्य रूप से कृषि उत्पादों (चीनी, जैसे ग्लूकोज, फ्रुक्टोज, आदि) की उच्च लागत के कारण, जो कि बायोरिएक्टर की खेती के दौरान प्रमुख सबस्ट्रेट्स के रूप में उपयोग की जाती हैं। हाल ही में, यह बताया गया है कि टाइप II मेथनोट्रोफ्स जैसे कि मिथाइलोसिनस ट्राइकोस्पोरियम, मिथाइलोबैक्टीरियम ऑर्गोफिलम, और मिथाइलोसिस्टिस हिरसुता मीथेन गैस का उपयोग कर सकते हैं और पीएचबी आधारित बायोपॉलिमर जमा करने की एक अलग क्षमता रखते हैं।

सबस्ट्रेट के रूप में नाइट्रेट मिनरल साल्ट (एनएमएस) मीडिया और मेथनॉल का उपयोग करके एम. ऑर्गनोफिलम और एम. ट्राइकोस्पोरियम की खेती के लिए प्रायोगिक प्रोटोकॉल सफलतापूर्वक विकसित किए गए थे। एम. ऑर्गनोफिलम, एम. ट्राइकोस्पोरियम, और एम. हिर्सुटा के लिए इनोकुलम प्रसार प्रोटोकॉल भी मीथेन गैस के ओवरले के साथ एनएमएस मीडिया वाले क्रिम्प-सील्ड सीरम बोतलों/फ्लास्क में स्थापित किए गए थे, जिसमें एम. हिरसुता ने उच्चतम बायोमास उत्पादन क्षमता का प्रदर्शन किया था, और इसलिए, वर्तमान जांच में गहन अध्ययन के लिए प्रमुख जीव के रूप में पहचाना गया। एम. हिरसुता ने

सीरम फ्लास्क में 100 घंटे की खेती के बाद 0.08 ग्राम/एल बायोमास संचय दिखाया। एम. हिरसुता के लिए इनोकुलम विकास प्रोटोकॉल को संशोधित बोरोसिल बोटलों में सफलतापूर्वक स्थापित किया गया था, जिससे मीथेन की निरंतर शुद्धिकरण की सुविधा मिली, जिसके परिणामस्वरूप एनएमएस मीडिया में 126 घंटे के बाद 2.32 जी/एल की बायोमास वृद्धि प्राप्त हुई। इसके बाद, एम. हिरसुता के बैच ग्रोथ कैनेटीक्स का अध्ययन 2.5L बायोरिएक्टर में लगातार मीथेन पर्जिंग (0.05 vvm पर) के तहत NMS मीडिया का उपयोग करके किया गया, जिसके परिणामस्वरूप 140 में 0.55 g/l के PHB संचय के साथ 2.5 g/l बायोमास का उत्पादन हुआ घंटे। बायोमास वृद्धि को और बढ़ाने के लिए एम. हिरसुता की एक फेड-बैच बायोरिएक्टर खेती 3.5 एल बायोरिएक्टर में शुरू की गई थी जिसमें निर्जमीकृत मीडिया, (500 मिलीलीटर केंद्रित (2.5X एनएमएस) मीडिया कम (1X) पोटेशियम नाइट्रेट की एकाग्रता) के बाद पूरक किया गया था। 200 घंटे और 10.57 g/L की समग्र बायोमास सांद्रता 1.1 g/L के PHB संचय के साथ 800 घंटे के बाद प्राप्त की गई।

एनएमएस मीडिया के पोषक तत्वों की सांद्रता को सांख्यिकीय रूप से अनुकूलित किया गया था।  $\text{KNO}_3$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{KH}_2\text{PO}_4$  और  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  के लिए सांद्रता सांख्यिकीय अनुकूलन के बाद क्रमशः 1 g/l, 1 g/l, 0.27 g/l और 0.71 g/l के रूप में उभरा। एम हिरसुता की विकास दर में सुधार की संभावना का पता लगाने के लिए एनएमएस मीडिया को कमजोर किया गया था, जिसमें यह देखा गया था कि एनएमएस / 4 एकाग्रता लंबे समय तक एम हिरसुता के घातीय वृद्धि चरण के रखरखाव के लिए सबसे अनुकूल थी। . एम. हिरसुता को तब 0.05 वीवीएम पर मीथेन पर्जिंग के साथ 0.25X एनएमएस मीडिया का उपयोग करके 2एल स्टिरर्ड-टैंक रिएक्टर (एसटीआर) और 4एल एयरफ्लिफ्ट रिएक्टर (एएलआर) में खेती की गई थी। इससे एसटीआर में 74 घंटे में क्रमशः 0.59 g/l और 0.16 g/l का बायोमास और PHB उत्पादन हुआ और ALR कॉन्फिगरेशन के लिए 73 घंटे के बाद 0.72 g/l की बायोमास वृद्धि और 0.21

g/l का इंटरसेल्युलर PHB संचय हुआ।

2.5एल एसटीआर और 4एल एएलआर में विस्तृत पोषक तत्व सीमा अध्ययन किए गए थे, जिससे पता चला कि मीथेन की निरंतर (गैर-सीमित) उपलब्धता के साथ नाइट्रेट आयनों की सीमित सांद्रता एम. हिरसुता की कोशिकाओं के भीतर उच्च पीएचबी उत्पादन को दर्शाती है। बायोरिएक्टर की खेती के लिए विभिन्न आयु (48 और 100 घंटे की उम्र) के इनोकुलम का उपयोग करके एम. हिरसुता की खेती के लिए पीएचबी संचय पर इनोकुलम उम्र की भूमिका की पहचान की गई थी। यह देखा गया कि 100 घंटे की कल्चर आयु में क्रमशः 0.184 kg.m<sup>-3</sup>.d<sup>-1</sup> और 0.125 kg.m<sup>-3</sup>.d<sup>-1</sup> की बायोमास और PHB उत्पादकता प्रदर्शित हुई, जो एम. हिरसुता के लिए रिपोर्ट की गई उच्चतम उत्पादकता है।

2.5L STR और 4L एयरलिफ्ट बायोरिएक्टर में मीथेन (0.05 vvm पर) के साथ विधिवत शुद्ध 0.25XNMS मीडिया का उपयोग करके एम. हिरसुता के बैच ग्रोथ कैनेटीक्स का उपयोग संबंधित गणितीय मॉडल विकसित करने के लिए किया गया था, जो एम. हिरसुता के बैच ग्रोथ कैनेटीक्स का पर्याप्त रूप से वर्णन करता है। बढ़ाया बायोपॉलिमर संचय के लिए नाइट्रेट की छद्म स्थिर स्थिति के रखरखाव के लिए नाइट्रेट फीडिंग की पहचान करने के लिए फेड-बैच की खेती का अनुकरण करने के लिए मॉडल को एक्सट्रपलेशन किया गया था। एसटीआर और एयर लिफ्ट बायोरिएक्टर में एम. हिरसुता की फेड-बैच खेती के दौरान पोषक आहार रणनीति के प्रायोगिक कार्यान्वयन पर, यह देखा गया कि विकसित गणितीय मॉडल ने एम. हिरसुता के प्रायोगिक बैच बायोरिएक्टर की खेती का पर्याप्त वर्णन किया। हालांकि, फेड-बैच की खेती के दौरान निरंतर नाइट्रोजन एकाग्रता के मॉडल-अनुमानित रखरखाव बायोमास और पीएचबी के प्रयोगात्मक रूप से देखे गए मूल्यों के साथ अच्छी तरह से संबंध नहीं बना सका।

एनएमएस मीडिया के आंतरायिक (आवश्यकता आधारित) पूरकता के साथ फेड-बैच की खेती को 0.05 वीवीएम पर मीथेन के साथ विधिवत शुद्ध 0.25X एनएमएस मीडिया वाले 2.5एल बायोरिएक्टर में एम.

हिरसुता की खेती के लिए प्रयास किया गया था। एनएमएस मीडिया को 24, 48, 56 और 78 घंटे के बाद 50 मिलीलीटर की छोटी दालों में पूरक किया गया। फ़ीड एकाग्रता को 150, 175 और 200 घंटों में 2.5X NMS के 50 मिलीलीटर तक बढ़ाया गया और 2.5X NMS मीडिया के 350 मिलीलीटर को 228 घंटों के बाद जोड़ा गया। उपरोक्त फेड-बैच खेती की रणनीति से बायोमास उत्पादन और क्रमशः 3.77 g/l और 1.08 g/l का PHB संचय हुआ।

एनएमएस-मीथेन मीडिया में दो जीवों (एम. हिरसुता से एम. ऑर्गनोफिलम) की सह-खेती के परिणामस्वरूप एम. हिरसुता (0.59 का ओडी) और एम. ऑर्गनोफिलम (ओडी) की संबंधित शुद्ध संस्कृति की तुलना में थोड़ा अधिक ओडी (0.65) हुआ। 0.06 की), यह दर्शाता है कि इन जीवों की सह-खेती के परिणामस्वरूप उच्च बायोमास और PHB संचय हो सकता है।

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## List of abbreviations

PHA	Poly hydroxyalkanoic acid
PHB	Poly (3-hydroxybutyric acid)
PHBV	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
NMS	Nitrate Mineral Salt
ALR	Airlift reactor
CCD	Central Composite Design
CoA	Co-enzyme A
DCW	Dry cell weight
DO	Dissolved oxygen
DoE	Design-of-Experiment
LCL	Long chain length
MCL	Medium chain length
RSM	Response Surface Methodology
SCL	Short chain length
SDS	Sodium dodecyl sulphate
SSWR	Sum of Squares of Weighed Residues
STR	Stirred tank reactor
TCA	Tricarboxylic acid
v/v	Volume by volume
vvm	Volume/Volume/min

## 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)
$F$	Total flow rate (L/d)
$K_s$	Saturation constant (g/L)
$K_1$	Growth associated product formation constant (g/g)
$K_2$	Non-growth associated product formation constant (g/g.d)
$S_0$	Initial Nitrate concentration (g/L)
$m_s$	Maintenance coefficient for Nitrate (g/g.d)
$V$	Volume (L)
$X$	Biomass (g/L)
$Y_{X/S}$	Yield of Biomass with respect to Nitrate (g/g)
<i>Greek Letters:</i>	
$\mu$	Specific growth rate ( $d^{-1}$ )
$\mu_m$	Maximum specific growth rate ( $d^{-1}$ )