PRODUCTION OF BIOPOLYMER BY GRAM-POSITIVE BACTERIA (*BACILLUS THURINGIENSIS*)

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by

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CERTIFICATE

This is to certify that the thesis entitled "**Production of biopolymer by gram-positive bacteria (*Bacillus thuringiensis*)"** being submitted by **Mr. Jitendra Singh Verma** to the Indian Institute of Technology Delhi, for the award of **"Doctor of Philosophy"** in Biochemical Engineering and Biotechnology Department is a record of the original bonafide research work carried out by him, 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 the thesis has 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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Jitendra Singh Verma

Abstract

Poly-3-hydroxybutyrate (PHB) is a biodegradable polymer which has physical properties similar to polypropylene & is having wide range of societal applications and more importantly medicinal applications. Numerous gram-positive and gram-negative microorganisms have the capability to accumulate Polyhydroxyalkanoates (PHAs) under nutrient limiting conditions. However, the PHB produced by gram-negative bacteria contains lipopolysaccharides (LPS) endotoxins in their outer membrane, which makes it unfit for medical applications whereas PHB produced by gram-positive bacterial lacks LPS hence making it more suitable for medical applications. Therefore, in the present investigations research effortswere directed towards production of medical grade biopolymers using gram positive bacteria (*Bacillus thuringiensis* IAM12077).

The main objective of the present study was to establish PHB production process using *Bacillus thuringiensis* IAM12077 as a model organism. It was observed that *B. thuringiensis* was able to utilize glycerol as a major carbon source for PHB production, thereby indicating the possibility of utilization of inexpensive crude glycerol (as well) as key substrate, which is a by-product of the biofuel industry. To obtain maximum biomass PHB accumulation by *B. thuringiensis*, concentrations of different nutrients were statistically optimized. Batch cultivation in a 7L bioreactor using optimized medium recipe resulted in biomass concentration of 6.68 g/L with a PHB concentration of 2.71 g/L in 33 hours. Possibility of substrate inhibition by selected major limiting nutrient (Carbon source; glycerol) was also explored which demonstrated a decrease in specific growth rate of *B. thuringiensis* beyond 15g/L with respect to glycerol. To further enhance the PHB yield and productivity, cultivation of *B. thuringiensis* was attempted in 7 L bioreactor using optimized glycerol and glucose concentration in the ratio of 1:1, (at a total concentration of 15g/L) along with statistically optimized medium components which exhibited a biomass and PHB concentration of 8.45 g/L and 3.36 g/L, respectively

in 33 hours. This was significantly higher as compared to bioreactor batch cultivation conducted with glycerol as sole carbon source.

Successful attempts were then made to identify two different batch mathematical models, e.g., for glycerol as substrate and mixed substrate i.e. glycerol with glucose for production by *B. thuringiensis* IAM12077 using substrate inhibition studies & 7 L batch kinetics data. Both the models were then extrapolated to simulate different nutrient feeding strategies in fed-batch cultivation conditions which included constant feed rate of glycerol and maintenance of pseudo steady state of glycerol. It was observed that fed-batch cultivation using constant feed rate strategy of glycerol yielded PHB concentration of 10.4 g/L and 4.15 g/L, respectively in 33 hours the fed-batch cultivation conducted with mixed substrate & under pseudo steady state with respect to glycerol exhibited maximum biomass and PHB concentration of 8.42 g/L and 3.93 g/L, respectively in 36 hours. However, fed-batch cultivation conducted with mixed substrates & at constant feed strategy of mixed substrates resulted in biomass and PHB accumulation of 10.20 g/L and 4.57 g/L, in 27 hours respectively. On the other hand, the fed-batch cultivation strategy with feeding of mixed substrate (glucose and glycerol) to maintain pseudo steady state with respect to glycerol resulted in biomass and a reasonably high PHB accumulation of 12.74 g/L and 5.48 g/L, respectively in 33 hours.

The batch mathematical model for mixed substrate was used to develop an (integrated batch-fed-batch-continuous with cell retention) cultivation strategy to further enhance the biomass and PHB accumulation in the bioreactor. The experimental implementation of this model based unique cultivation strategy resulted in biomass and PHB accumulation of 24.1 g/L and 9.84 g/L, respectively, reported so far for *B. thuringiensis* IAM12077. The present studies also demonstrated the successful scale-up of batch PHB fermentation from 7L to 70L bioreactor using P_g/V criterion where in biomass and PHB concentration of 6.68 g/L and 2.71 g/L, respectively was obtained in both bioreactor cultivations.

सामान्यसारांश

Poly-3-hydroxybutyrate (PHB) एक जैवनिम्ननी पॉलीमर है जिसमें पॉलीप्रोपाइलीन के समान भौतिक गुण होते हैं और इसकी व्यापक श्रेणी के सामाजिक अनुप्रयोगों और अधिक महत्वपूर्ण रूप से औषधीय अनुप्रयोग होते हैं। कई ग्राम-पॉजिटिव और ग्राम-नेगेटिव सूक्ष्मजीवों में पोषक तत्वों की सीमित स्थिति के तहत पॉलीहाइड्रॉक्सीकल्कनेट्स (PHAs) को संचित करने की क्षमता होती है। हालांकि, ग्राम-नेगेटिव बैक्टीरिया द्वारा निर्मित PHB में उनके बाहरी झिल्ली में लिपोपॉलीसेकेराइड (LPS) एंडोटॉक्सिन होते हैं, जो इसे चिकित्सा अनुप्रयोगों के लिए अयोग्य बनाता है, जबकि ग्राम-पॉजिटिव बैक्टीरिया द्वारा उत्पादित PHB में LPS (एल-पि-एस) उपस्थित नहीं होता है जिसके कारण PHB मेडिकल अनुप्रयोगों के लिए अधिक उपयुक्त होता है।इसलिए, वर्तमान अध्ययन में ग्रामपॉजिटिव बैक्टीरिया (बैसिलस थुरिंगिनेसिस IAM12077) का उपयोग करके मेडिकल ग्रेड बायोपॉलिमर उत्पादन पर ध्यान केंद्रित किया गया था।

वर्तमान अध्ययन का मुख्य उद्देश्य बेसिलस थुरिंगिनेसिस IAM12077 का एक मॉडल जीव के रूप में उपयोग करके PHB उत्पादन प्रक्रिया स्थापित करना था। यह देखा गया कि बी. थ्युरिंगेंसिस ग्लिसरॉल को PHB उत्पादन के लिए एक प्रमुख कार्बन स्रोत के रूप में उपयोग करने में सक्षम था, जिससे महंगे कच्चे ग्लिसरॉल (साथ ही) प्रमुख सब्सट्रेट के उपयोग की संभावना का संकेत मिलता है, जो जैव ईंधन उद्योग का एक उप-उत्पाद है। । बी. थ्यूरिंगेंसिस द्वारा अधिकतम बायोमास (PHB) पीएचबी (PHB) संचय प्राप्त करने के लिए, विभिन्न पोषक तत्वों की सांद्रता को सांख्यिकीय रूप से अनुकूलित किया गया था।अनुकूलित माध्यम नुस्खा का उपयोग कर 7 लीटर बैच बायोरिएक्टर में उपज (cultivation) से बायोमास सांद्रण 6.68 ग्राम/लीटर हुआ एवं 2.71 ग्राम/लीटरकी PHB एकाग्रता का 33 घंटो में उतपादन हुआ। सब्सट्रेट इनहिबिटबी की प्रमुख सीमित पोषक तत्व (कार्बन स्रोत; ग्लिसरॉल) की संभावना भी पता लगाया गया था, जो कि 15ग्राम/लीटर ग्लिसरॉल से परे बी.

थ्यूरिंगेंसिस की विशिष्ट वृद्धि दर में कमी को प्रदर्शित करता है। PHB की उपज और उत्पादकता को और बढ़ाने के लिए, सांख्यिकीय रूप से अनुकूलित मध्यम घटकों के साथ, 1: 1 के अनुपात में ग्लिसरॉल और ग्लूकोज सांद्रता का उपयोग करते हुए बी. थुरिंगिएन्सिस की खेती का प्रयास 7 लीटरबायोरिएक्टर में किया गया। जिसमें 33 घंटे में क्रमशः 8.45 ग्राम/लीटर और 3.36 ग्राम/लीटर के बायोमास और पीएचबी एकाग्रता का प्रदर्शन किया गया। यह केवल कार्बन स्रोत के रूप में ग्लिसरॉल के साथ आयोजित बायोरिएक्टर बैच की खेती की तुलना में काफी अधिक था।

तब सब्सट्रेट (ग्लिसरॉल) और मिश्रित कार्बन सब्सट्रेट (ग्लिसरॉल और ग्लूकोज) के रूप में ग्लिसरॉल के लिए दो अलग-अलग बैच गणितीय मॉडल, जैसे, सब्सट्रेट और मिश्रित सब्सट्रेट के रूप में ग्लिसरॉल के लिए यानी ग्लिसरॉल के साथ ग्लूकोज के साथ उत्पादन के लिए बी. थुरिंगिएन्सिस IAM12077 सब्सट्रेट निषेध अध्ययन और 7 लीटर बैच कैनेटीक्स डेटा का उपयोग करते हुएउत्पादन के लिए सफल प्रयासों की पहचान की गई। दोनों मॉडलों को तब अलग-अलग पोषक तत्वों की खिला रणनीतियों का अनुकरण करने के लिए अतिरिक्त रूप से तैयार किया गया था, फीड-बैच की खेती की स्थितियों में जिसमें ग्लिसरॉल की निरंतर फ़ीड दर और ग्लिसरॉल के छद्म स्थिर अवस्था का रखरखाव शामिल था। यह देखा गया कि ग्लिसरॉल की निरंतर फ़ीड दर रणनीति का उपयोग करके फीड-बैच (fed-batch)की खेती ने 10.4 ग्राम/लीटर और 4.15 ग्राम/लीटर की PHB सांद्रता प्राप्त की, 33 घंटे में मिश्रित सब्सट्रेट और छद्म राज्य के संबंध में आयोजित फीड-बैच की खेती के संबंध में ग्लिसरॉल ने अधिकतम बायोमास और PHBसांद्रता क्रमश: 8.42 ग्राम/लीटर और 3.93 ग्राम/लीटर, को 36 घंटे में प्रदर्शित किया। हालांकि, मिश्रित सब्सट्रेट्स और मिश्रित सब्सट्रेट्स की निरंतर फ़ीड रणनीति के साथ आयोजित बैच बैच की खेती के परिणामस्वरूप क्रमशः 27 घंटे में 10.20ग्राम/लीटर और 4.57ग्राम/लीटर का बायोमास संचय होता है। दूसरी ओर, मिश्रित-बैच सब्सट्रेट (ग्लूकोज और ग्लिसरॉल) की फीड-बैच की खेती की रणनीति के साथ-साथ ग्लिसरॉल के संबंध में छद्म स्थिर स्थिति बनाए रखने के लिए बायोमास और परिणामस्वरूपउच्च PHB, क्रमशः 12.74 ग्राम/लीटर और 5.48 ग्राम/लीटर 33 घंटे में हुआ।

किण्वन के शोरबे (fermentation broth) में बायोमास और PHB संचय को और बढ़ाने के लिए, एक एकीकृत बैच-फेड-बैच-निरंतर उतपादन की रणनीति विकसित मॉडल की मदद से विकसित की गई। ग्लूकोज और अन्य सांख्यिकीय रूप से अनुकूलित मीडिया घटकों के साथ ग्लिसरॉल के संबंध में छद्म स्थिर अवस्था में फेड-बैच की खेती के बाद बायोरिएक्टर में निरंतर मोड में ताजा पोषक तत्वों को खिलाने से जुड़ी इस अभिनव रणनीति को लागु किया गया। इस उतपादन की रणनीति से 24.1

ग्राम/लीटर बायोमास एवं 9.84 ग्राम/लीटर पीएचबी (PHB) सांद्रित प्राप्त हुआ, जिसमें PHB उत्पादकता स्थिर अवस्था में 0.298 ग्राम/लीटर.घंटे थी, जो कि बी. थुरिंगिएन्सिस उतपादन की सबसे उत्तम रणनीति का प्रमाण है।

वर्तमान अध्ययनों ने Pg/V मानदंड का उपयोग करते हुए 7लीटरसे 70लीटरबायोरिएक्टर तक बैच PHB किण्वन के सफल पैमाने-अप का प्रदर्शन किया, जहां बायोपास और PHB एकाग्रता क्रमशः6.68 ग्राम/लीटर और 2.71 ग्राम/लीटर,दोनों बायोरिएक्टर की खेती में प्राप्त किया गया था।

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List of Abbreviations

3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
3HHD	3-hydroxy-hexa-decanoate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HOD	3-hydroxy-octa-decanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATPS	Aqueous Two-Phase System
CCD	Central Composite Design
CoA	Coenzyme A
CSTR	Continuous Stirred Tank Reactor
DCW	Dry Cell Weight
DO	Dissolved Oxygen
FTIR	Fourier transform infrared spectroscopy
HA	Hydroxyapatite
HBA	Hydroxybutyric acid
HPLC	High Performance Liquid Chromatography
LCL	Long Chain Length
LPS	Lipo-poly-saccharide
MCL	Medium Chain Length

NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
PBD	Plackett Burman Design
PHA	Poly -3-hydroxyalkanoates
PHB	Poly-3-hydroxybutyrate
PHO	Poly-3-hydroxyoctanoate
PHV	Poly-3-hydroxyvalerate
PHBV	Poly-(3hydroxybutyrate-co-3hydroxyvalerate)
PHBHHx	Poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate)
RSM	Response Surface Methodology
SCF	Supercritical Fluid Extraction
SCL	Short Chain Length
SDS	Sodium Dodecyl Sulfate
SSWR	Sum of the Squares of the Weighed Residues
TCA	Tricarboxylic Acid
TKN	Total Kjeldahl Nitrogen

List of Symbols

$1/Y_{[X+P/S_1]}$	**Yield of biomass and product on glycerol consumption (g/g)**
$1/Y_{[X+P/S_2]}$	**Yield of biomass and product on TKN consumption (g/g)**
$1/Y_{[X/S_3]}$	**Yield of biomass and product on glucose consumption (g/g)**
D	**Dilution rate (h^{-1})**
D_I	**Impeller diameter (cm)**
D_T	**Diameter of Tank (cm)**
dV/dt	**Rate of change of volume (L/h)**
f_C	**Correction factor**
H_T	**Height of Tank (cm)**
K_1	**Growth associated product formation constant (g/g)**
K_2	**Non-growth associated product formation constant (g/g)**
K_{I_1}	**Substrate inhibition constant for glycerol consumption (g/L)**
K_{I_2}	**Substrate inhibition constant for glucose consumption (g/L)**
K_La	**Volumetric Mass Transfer Coefficient (h^{-1})**
K_{S_1}	**Saturation constant for glycerol consumption (g/L)**
K_{S_2}	**Saturation constant for TKN consumption (g/L)**
K_{S_3}	**Saturation constant for glucose consumption (g/L)**
m_{S_1}	**Maintenance coefficient for glycerol (g/g.h)**
m_{S_2}	**Maintenance coefficient for TKN (g/g.h)**
m_{S_3}	**Maintenance coefficient for glucose (g/g.h)**
N	**Impeller rotation speed (s^{-1})**
N_{Re}	**Reynold's Number**
P	**PHB concentration (g/L)**

P_{ung}	**Un-gassed power (W)**
P_g	**Gassed power (W)**
Q	**Volumetric air flow rate (Lpm)**
rpm	**revolution per minute**
rps	**revolution per second**
q_p	**Volumetric productivity of PHB (g/L.h)**
q_{S_1}	**Specific glycerol consumption rate (h^{-1})**
q_{S_2}	**Specific TKN consumption rate (h^{-1})**
q_{S_3}	**Specific glucose consumption rate (h^{-1})**
S_{01}	**Inlet concentration of glycerol in the feed bottle (g/L)**
S_{02}	**Inlet concentration of nitrogen in the feed bottle (g/L)**
S_{03}	**Inlet concentration of glucose in the feed bottle (g/L)**
S_1	**Glycerol concentration (g/L)**
S_2	**Total Kjeldahl Nitrogen concentration (g/L)**
S_3	**Glucose concentration (g/L)**
V	**Working volume of the bioreactor (L)**
Vs	**Superficial Gas Velocity (m/s)**
W_j	**Weight of each variable**
Δ_j	**Mean residual of each variable (j)**
Δ_{ij}	**Difference between the model and experimental values for i^{th} data point and j^{th} process variable**
Ө	**Blend Time**
ϑ	**Kinematic Viscosity**
∏	**Impeller Tip Velocity (m/s)**
μ	**Specific growth rate (h^{-1})**
μ_m	**Maximum specific growth rate (h^{-1})**
ν	**Viscosity of the medium (Kg/cm-s)**
ρ	**Medium density (kg/m^3)**