SCALE-UP OF PHB PRODUCTION USING DIFFERENT BACTERIAL SPECIES

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by

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Dedicated

To

My beloved

Parents, Wife,

Relatives and Friends

CERTIFICATE

This is to certify that the thesis entitled **"*Scale-up of PHB production using different bacterial species*"** being submitted by **Mr. Sanjay Kumar** to the **Indian Institute of Technology Delhi**, for the award of the degree of "**Doctor of Philosophy"** is a record of the bonafide research work carried out by him under my guidance and 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 in part or full to any other university or institute for the award of any degree/diploma.

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Date: **Sanjay Kumar**

Place:

ABSTRACT

PHB, a bioplastic was explored as an alternative to conventional plastics because it has similar physical properties to polypropylene. Additionally, its properties like complete biodegradability, biocompatibility, barrier to water droplets & oxygen made it a useful biopolymer for various societal applications. It was evidenced by various literature and present study that this bioplastic could be produced from both gram-negative and gram-positive bacterial species.

The primary objective of the present investigation was to establish a successful scale-up protocol for the transfer of our existing leads of various bacterial species (*Azohydromonas australica*, *Cupriavidus necator*, *Bacillus thuringiensis*) at lab scale bioreactor (7 L) to the pilot-scale bioreactor (300 L) in batch and fed-batch cultivation conditions. To establish a successful scale-up protocol, initially, the geometrical similarities between small and big scale bioreactors were examined wherein it was assessed if the physical boundaries of the two bioreactor cultivation systems are similar and in geometrical proportion to each other. The dynamic similarity of the bioreactor systems was, thereafter, ensured by comparing the relative value of flow affecting intensive parameters (P/V, KLa, Π, t_m, NRe, Q/V) in the model (small) and prototype (big reactor), by keeping one of the intensive parameters constant in both (small & big bioreactors) & evaluating its effects on the rest of the scale-up parameters through the relative ratio (parametric index) of scale-up parameters. Constant P/V scale-up criteria, invariably, emerged as the most suitable criteria for the successful transfer of results of all the small scale bioreactor cultivations to 70 / 300 L bioreactor cultivation.

Batch cultivation of *A. australica, C. necator and B. thuringiensis* were performed in 7 L bioreactor for 33 hours, 54 hours and 36 hours. At the end of batch cultivation, *A. australica, C. necator and B. thuringiensis* in 7 L bioreactor featured maximum biomass of 8.66 g/L, 12.46 g/L, and 7.29 g/L respectively whereas PHB accumulated were of 6.53 g/L, 8.61 g/L, 3.69 g/L respectively. The maximum productivity of *A. australica, C. necator and B. thuringiensis* were of 0.197 g/L/h, 0.159

g/L/h, and 0.102 g/L/h respectively whereas PHB yields were of 0.285 g/g, 0.261 g/g, 0.548 g/g respectively. It was possible to duplicate these results up to 300 L batch bioreactor cultivation using P/V scale-up criteria. To further demonstrate the successful transfer of the results by selected scale-up criteria to 15 & 70 L bioreactor cultivations, the model-based fed-batch bioreactor cultivation (7 L) of *A. australica* (Gahlawat & Srivastava, 2013) with constant feeding rate strategy, *C. necator* (Sharma et al., 2015) & *B. thuringiensis* (Verma et al., 2019) with pseudosteadystate feeding rate strategy were selected. For this purpose, the mathematical model was simulated again on a computer with the changed volumes (for 15 & 70 L) to reidentify the respective changed feeding rates during fed-batch cultivation. The fed-batch cultivations were then experimentally implemented in 15 &/or 70 L bioreactor wherein it was possible to duplicate the leads of 7 L fed-batch cultivation in terms of concentrations, yield and productivity.

To extract the intracellular PHB produced, chemical digestion of biomass with Sodium Dodecyl Sulphate and Sodium hypochlorite was followed by the solvent extraction method using chloroform (Hahn et al., 1994) which featured a maximum recovery of PHB from the biomass of *C. necator* and *B. thuringiensis* as 69.5 % and 40.57 % respectively.

GPC analysis of PHB recovered from the biomass of *C. necator* featured the weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (M_w/ M_n) of 115 kDa, 36 kDa, and 3.19 respectively, whereas incase of *B. thuringiensis* these findings were of 124 kDa, 48.1 kDa, and 2.58 respectively. The TGA analysis of PHB recovered from *C. necator* biomass featured the thermal degradation patteren of PHB which indicated that 5 %, 85 %, and 100 % of the total mass of PHB was degraded at 225 °C, 290 °C, and 500 °C respectively whereas incase of *B. thuringiensis*, 5 %, and 95 % of PHB was theremally degraded at 239 °C and 276 °C respectively. FTIR spectra of PHB recovered from the biomass of *C. necator* identified the functional group carbonyl, methyl (stretching mode), methyl (bending mode) at 1720 cm^{-1}, 1380 cm^{-1}, 1450 cm^{-1} respectively. Whereas, incase of *B. thuringiensis* these functional groups were

identified at 1720 cm^{-1}, 1380 cm^{-1}, 1460 cm^{-1} respectively. The ^{1}NMR analysis detected the chemical shifts at 1.196 ppm, 5.167 ppm, between 2.36 - 2.54 ppm which recognizes the methyl, methylene, and methyne groups in the chemical structure of PHB respectively while incase of *B. thuringiensis*, ^{1}NMR spectra of PHB identified -CH_3, -CH, -CH_2 groups through chemical shifts observed at 1.28 ppm, 5.25 ppm, between 2.44-2.63 ppm. The XRD analysis characterized the crystal stracture of PHB. The peak observed at 2θ = 13.42° and 16.96° in XRD analysis of PHB accumulated by *C. necator* peaks characterizes PHB crystal as orthorhombic unit cell structure whereas XRD peaks observed at 2θ = 21.42° and 22.52°corresponds to the α-helical crystal structure while remaining three peaks observed at 2θ = 25.56°, 27.22°, and 30.48° corresponds to the partial crystalline structure of PHB. Similarily, peaks observed at 2θ = 13.48°, 2θ = 16.98°, 2θ = 21.68° and 2θ = 22.50° in XRD analysis of PHB accumulated by *B. thuringiensis* characterizes PHB crystal as similar as it was incase of *C. necator*. Although PHB produced by *B. thuringiensis* has a high percentage of crystallinity and a low level of tensile strength yet it could be utilized for various medical applications such as biodegradable sutures, stents, grafts, etc. after blending it with plasticizers. The thermal characterization of PHB produced by *C. necator* indicated that T_m and T_d values have a wide-ranging temperature gap which features easier thermal processibility of PHB for various applications such as packaging of fruits and vegetables, coating of fertilizers, etc.

सार

PHB, पारंपरिक प्लास्टिक के विकल्प के रूप में एक बायोप्लास्टिक का पता लगाया गया क्योंकि इसमें पॉलीप्रोपाइलीन के समान भौतिक गुण हैं। इसके अतिरिक्त, इसके गुणों जैसे पूर्ण बायोडिग्रेडेबिलिटी, बायोकम्पैटिबिलिटी, पानी की बूंदों और ऑक्सीजन के लिए अवरोध ने इसे विभिन्न सामाजिक अनुप्रयोगों के लिए एक उपयोगी बायोपॉलिमर बना दिया। विभिन्न साहित्य और वर्तमान अध्ययनों द्वारा यह स्पष्ट किया गया था कि इस जैव-प्लास्टिक को ग्राम-नकारात्मक और ग्राम-पॉजिटिव दोनों प्रकार के उपभेदों से उत्पादित किया जा सकता है।

वर्तमान जांच का प्राथमिक उद्देश्य प्रयोगशाला पैमाने बायोरिएक्टर (7 L) पर विभिन्न बैक्टीरियल उपभेदों (*ए. ऑस्ट्रलिका, सी. निकाटोर, बी. थुरिंजेंसिस*) के हमारे मौजूदा लीड के हस्तांतरण के लिए एक सफल स्केल-अप प्रोटोकॉल स्थापित करना था। पायलट-स्केल बायोरिएक्टर (300 L) बैच और फेड-बैच कल्टीवेशन की स्थिति में एक सफल स्केल-अप प्रोटोकॉल स्थापित करने के लिए, शुरू में छोटे और बड़े पैमाने के बायोरिएक्टरों के बीच ज्यामितीय समानता की जांच की गई थी, जिसमें यह मूल्यांकन किया गया था कि दो बायोरिएक्टर कल्टीवेशन प्रणालियों की भौतिक सीमाएं समान हैं और एक दूसरे के ज्यामितीय अनुपात में हैं। इसके बाद, बायोरिएक्टर प्रणालियों की गतिशील समानता, मॉडल (छोटे) और प्रोटोटाइप (बड़े रिएक्टर) में गहन मापदंडों (पी / वी, केएलए, टीएम, एनआरई, क्यू / वी) को प्रभावित करने वाले प्रवाह के सापेक्ष मूल्य की तुलना करके सुनिश्चित की गई थी। दोनों (छोटे और बड़े बायोरिएक्टर) में गहन मापदंडों में से एक को स्थिर रखते हुए और स्केल-अप मापदंडों के सापेक्ष अनुपात (पैरामीट्रिक इंडेक्स) के माध्यम से बाकी पैमाने के मापदंडों पर इसके प्रभाव का मूल्यांकन करते हैं। कांस्टेंट पी / वी स्केल-अप मानदंड, हमेशा के लिए, सभी छोटे पैमाने के बायोरिएक्टर खेती के, 70/300 L बायोरिएक्टर कल्टीवेशन में परिणामों के सफल हस्तांतरण के लिए सबसे उपयुक्त मानदंड के रूप में उभरा।

ए. ऑस्ट्रालिका, सी. नेक्टर और बी. थुरिंगिएन्सिस की बैच खेती 7 लीटर बायोरिएक्टर में क्रमशः 33 घंटे, 54 घंटे और 36 घंटे तक की गई। बैच की खेती के अंत में, 7 लीटर बायोरिएक्टर में ए. ऑस्ट्रालिका, सी. नेकेटर और बी. थुरिंगिएनिस के अधिकतम बायोमास को क्रमशः 8.66 ग्राम / लीटर, 12.46 ग्राम / लीटर, और 7.29 ग्राम / लीटर चित्रित किया गया, जबकि संचित पीएचबी क्रमशः 6.53 ग्राम / लीटर, 8.61 ग्राम / लीटर, 3.69 ग्राम / लीटर थी । ए. ऑस्ट्रालिका, सी. नेक्टर और बी . थुरिंगिएन्सिस की अधिकतम उत्पादकता क्रमशः 0.197 ग्राम/लीटर/घंटा, 0.159 ग्राम/लीटर/घंटा, और 0.102 ग्राम/लीटर/घंटा थी, जबकि पी एच बी यील्ड क्रमश: 0.285 ग्राम/ग्राम, 0.261 ग्राम/ग्राम, और 0.548 ग्राम/ग्राम थी । पी / वी स्केल-अप मानदंड का उपयोग करके 300 लीटर बैच बायोरिएक्टर खेती तक इन परिणामों की नकल करना संभव था। आगे 15 लीटर और 70 लीटर बायोरिएक्टर की खेती के लिए चयनित स्केल-अप मानदंड द्वारा परिणामों के सफल हस्तांतरण को प्रदर्शित करने के लिए 15 और 70 लीटर बायोरिएक्टर की खेती के चयनित स्केल-अप मानदंड द्वारा परिणामों के सफल

हस्तांतरण को प्रदर्शित करने के लिए , मॉडल-आधारित फेड-बैच खेती (7 लीटर बायोरिएक्टर), लगातार फेड-बैच के साथ ए. ऑस्ट्रलिका (गहलावत और श्रीवास्तव, 2013), सी. नेकेटर (शर्मा एट अल। , 2015) और बी. थुरिंगिएन्सिस (वर्मा एट अल, 2019) के साथ स्यूडोस्टीडिस्टेट फीडिंग दर रणनीति का चयन किया गया था। इस प्रयोजन के लिए , फेड-बैच की खेती के दौरान संबंधित बदले हुए फीडिंग दरों को फिर से पहचानने के लिए गणितीय मॉडल को बदले हुए संस्करणों (15 और 70 एल के लिए) के साथ कंप्यूटर पर फिर से सिम्युलेट किया गया था। फेड-बैच की खेती तब प्रायोगिक रूप से 15 & / या 70 लीटर बायोरिएक्टर में लागू की गई थी, जिसमें सांद्रता, पैदावार और उत्पादकता के मामले में 7 लीटर फेड बैच खेती के लीड की नकल करना संभव था। उत्पादित इंट्रासेल्युलर पीएचबी निकालने के लिए, सोडियम डोडेसिल सल्फेट और सोडियम हाइपोक्लोराइट के साथ बायोमास के रासायनिक पाचन को क्लोरोफॉर्म (हान एट अल., १९९४) का उपयोग करके विलायक निष्कर्षण विधि द्वारा किया गया। इसमें *सी. नेकेटर* और *बी. थुरिंगिनेसिस* के बायोमास से क्रमशः 69.5% और 40.57% अधिकतम पीएचबी रिकवरी पाई गई। *सी. नेकेटर* बायोमास से रिकवर्ड पीएचबी के जीपीसी विश्लेषण में वजन औसत आणविक भार (Mw), संख्या औसत आणविक भार (Mn), और और पॉलीडिसपेरिटी इंडेक्स को क्रमशः 115 kDa, 36 kDa, और 3.19 दर्शाया गया, जबकि बी। थुरिंगिएन्सिस के इन निष्कर्षों में ये क्रमशः 124 केडीए, 48.1 केडीए, और 2.58 थे। *सी. नेकेटर* बायोमास से रिकवर्ड पीएचबी के टीजीए विश्लेषण से संकेत मिलता है कि पीएचबी के कुल द्रव्यमान का 5 %, 85 % और 100 % क्रमशः 225 °C, 290 °C, और 500 °C पर थर्मल डिग्रडेशन किया गया था।, जबकि B. थुरिंगिएन्सिस के इन निष्कर्षों में पीएचबी के कुल द्रव्यमान का 5%, और 95% क्रमशः 239 ° C और 276 ° C पर थर्मल डिग्रडेशन किया गया था। सी. नेक्टर के बायोमास से बरामद PHB के एफटीआईआर स्पेक्ट्रा ने कार्यात्मक समूह कार्बोनिल , मिथाइल (स्ट्रेचिंग मोड), मिथाइल (झुकने मोड) की पहचान क्रमशः 1720 सेमी -1, 1380 सेमी -1, 14.5 सेमी -1 से की। जबकि , बी. थुरिंगिएन्सिस के इन कार्यात्मक समूहों की पहचान क्रमशः 1720 सेमी -1, 1380 सेमी -1, 1460 सेमी -1 से हुई। ^{1}NMR विश्लेषण ने 1.366 पीपीएम, 5.167 पीपीएम, 2.36 - 2.54 पीपीएम के बीच रासायनिक बदलावों का पता लगाया, जो PHB की रासायनिक संरचना में मिथाइल , मिथाइलीन और मेथेनी समूहों को पहचानता है , जबकि B. थुरिंगिएनिसिस, ^{1}NMR स्पेक्ट्रा PHB में -CH3 -CH, -CH2 समूहों को रासायनिक पाली के माध्यम से 1.28 पीपीएम, 5.25 पीपीएम, 2.44-2.63 पीपीएम की पहचान करता है। XRD चोटियाँ 2θ = 13.42° और 16.96° पीएचबी क्रिस्टल के रूप में ऑर्थोरोम्बिक इकाई सेल के रूप में देखा जाता है। जबकि 2θ = 21.42° और 22.52° पर देखी गई एक्सआरडी चोटियाँ α-हेलिकल क्रिस्टल स्ट्रक्चर से मेल खाती हैं, जबकि शेष तीन चोटियाँ 2θ = 25.5°, 27.22° और 30.48° पीएचबी के आंशिक क्रिस्टलीय संरचना से मेल खाती हैं। इसी तरह, बी. थुरिंगिएन्सिस द्वारा निर्मित PHB के एक्सआरडी विश्लेषण में 2θ = 13.48 °, 2 16 = 16.98 °, 2θ = 21.68 ° और 28 = 22.50 ° पर देखी गई चोटियां PHB क्रिस्टल क्रिस्टल की वही विशेषता है, जैसा कि यह सी. नेकेटर में है । हालांकि बी. थुरिंगिएन्सिस द्वारा निर्मित पीएचबी में क्रिस्टलीयता का उच्च प्रतिशत और तन्य शक्ति का एक निम्न स्तर है, फिर भी इसे प्लास्टिसाइज़र के सम्मिश्रण के बाद, विभिन्न चिकित्सा अनुप्रयोगों जैसे कि बायोडिग्रेडेबल टांके, स्टेंट, ग्राफ्ट्स आदि के लिए उपयोग किया जा सकता है। *सी. नेकेटर* द्वारा निर्मित PHB के

ऊष्मीय लक्षण वर्णन ने संकेत दिया कि Tm और Td मानों में एक व्यापक तापमान अंतर होता है जो विभिन्न अनुप्रयोगों जैसे फल और सब्जियों की पैकेजिंग, उर्वरकों के लेप, आदि के लिए पीएचबी की आसान तापीय प्रक्रिया को दर्शाता है।

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LIST OF ABBREVIATIONS

3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
ATCC	American Type Culture Collection
ATR	Attenuated total reflectance
CoA	Coenzyme A
CMC	Carboxy Methyl Cellulose
DCW	Dry Cell Weight
DNS	Dinitrosalicylic acid
DO	Dissolved Oxygen
DSC	Differential scanning calorimetry
DSM	Deutsche Sammlung von Mikroorganismen
EDTA	Ethylene diamine tetra acetic acid
FTIR	Fourier Transform Infrared Spectroscopy
GPC	Gel permeation chromatography
GPa	Giga Pascal
Gr –NPs	Graphene-nanoparticles
HPLC	High Performance Liquid Chromatography
HDPE	High-Density poly(ethylene)
LDPE	Low-Density poly(ethylene)
MCL	Medium Chain Length
MPa	Mega Pascal
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear Magnetic Resonance
NPCM	Non-polymer cellular material
PHA	Poly(3-hydroxyalkanoates)
P3HB/PHB	Poly(3-hydroxybutyrate)
P4HB	Poly(4-hydroxybutyrate)
PHH	Poly(3-hydroxyhexanoate)

PHO	Poly(3-hydoxyoctanoate)
PHP	Poly(hydroxypropionate)
PHV	Poly(3-hydroxyvalerate)
PHBHHx	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PLA	Poly(lactic acid)
PP	Poly(propylene)
PSS	Pseudosteady state
SCL	Short Chain Length
SDS	Sodium Dodecyl Sulfate
TEM	Transmission Electron Microscope
TES	Trace Element Solution
TKN	Total Kjeldahl Nitrogen
TGA	Thermogravimetric analysis
UV	Ultra-Violet
XRD	X-Ray Diffraction

SYMBOLS

D	Dilution rate (h^{-1})
D_T	Diameter of Tank (cm)
Di	Diameter of Impeller (cm)
F_c	Correction factor
H_T	Height of Tank (cm)
K_La	Volumetric Mass Transfer Coefficient (h^{-1})
L	Liter
μ	Viscosity of the medium (Kg/cm-s)
μ_m	Maximum specific growth rate (h^{-1})
M_n	Number average molecular weight (kDa)
M_w	Weight average molecular weight (kDa)
M_Z	Z average molecular weight (kDa)
M_w/M_n	Polydispersity index
N	Impeller rotation speed (rpm)
N_{Re}	Reynold's Number
P	PHB concentration (g/L)
P_{ug}	Un-gassed power (W)
P_g	Gassed power (W)
P/V	Power per unit Volume (W/m^3)
∏	Impeller Tip Velocity (m/s)
Q	Airflow rate (Lpm)
Q/V	Volumetric airflow rate (Lpm)
ρ	Medium density (kg/m^3)
T_c	Crystalline Temperature (°C)

T_g	Glass transition Temperature (°C)
T_d	Thermal degradation Temperature (°C)
T_m	Melting Temperature (°C)
t_m	Mixing time
Ө	Blend Time
V	Working volume of the bioreactor (L)
dV/dt	Rate of change of volume (L/h)
Vs	Superficial Gas Velocity (m/s)
v/v	volume/volume
w/v	weight by volume
X_c	Degree of crystallinity