PRODUCTION & CHARACTERISATION OF MEDICAL GRADE BIOPOLYMERS

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PRODUCTION & CHARACTERISATION OF MEDICAL GRADE BIOPOLYMERS

by

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Submitted

In fulfilment of the requirements of the degree of

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Dedicated

to

my dearly loved
parents, sisters,
relatives, and friends.

CERTIFICATE

This is to certify that the thesis entitled **"Production & characterisation of medical-grade biopolymers"** being submitted by **Mr. Navodit Kumar Singh** to the Indian Institute of Technology Delhi, for the 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 him, which has been prepared under our supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi. The research reports and results presented 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.

Date: 31/07/2024

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Navodit Kumar Singh

ABSTRACT

Polyhydroxybutyrate (P(3HB)) is a biodegradable polymer that is synthesised by several microorganisms to store carbon and energy within their cells. It is widely recognised as a biopolymer because of its notable characteristics, e.g., remarkable biocompatibility, biodegradability, and sustainability. It has undergone extensive research in recent decades, mostly due to its diverse applications, primarily, in the environment & medical areas.

Extensive cultivation studies & optimisation of P(3HB) accumulation in stirred tank reactor & different configurations of pneumatically agitated airlift bioreactors (using different air supply & mixing strategies) were done to economize the production of P(3HB) (polyhydroxy butyrate) by *Bacillus thuringiensis* IAM12077 with the by-product of biodiesel industry (glycerol) and glucose as substrate (at 7 pH & temperature of 30 °C) air (0.3-0.6 vvm). The P(3HB) accumulation in stirred tank bioreactor was observed to be 42.59 % (biopolymer accumulation in the total biomass) as compared to 37.07 % in outer aeration inner settling configuration of airlift bioreactor yet it emerged as a cost effective alternative for mass scale biopolymer production.

To further economize the polyhydroxybutyrate (P(3HB)) production cost, detailed studies were launched with yet another bacteria *Bacillus cereus* & crude glycerol as substrate. The medium composition for *B. cereus* was statistically optimised which resulted in a P(3HB) concentration of 4.13 g/L in 30-hour during shake flask cultivation, in comparison to model's predicted P(3HB) concentration of 3.75 g/L. Subsequently, 4.53 g/L biomass and a P(3HB) concentration of 1.95 g/L (content 43.05 %) were obtained in 39 hours during 3.5 liter bioreactor cultivation. The biopolymer production by *B. cereus* was then scaled up to 15 liter bioreactor using P/V as the scale

up criteria which resulted in a P(3HB) concentration of 1.81 g/L (as opposed to 1.95 g/L in 3.5 liter bioreactor) thereby confirming the feasibility of biopolymer production in higher scale bioreactor.

Following batch cultivations, biomass from *Bacillus thuringiensis* and *Bacillus cereus* underwent pretreatment using Sodium Dodecyl Sulphate (SDS) / Sodium Lauryl Sulfate (SLS) and Sodium hypochlorite (NaOCl), followed by chloroform extraction. The resulting biopolymer underwent comprehensive characterization, confirming its molecular structure through NMR and FTIR measurements. X-ray diffraction (XRD) analysis identified the orthorhombic crystal structure of the isolated polyhydroxybutyrate (P(3HB)) from *Bacillus* cultures. DSC analysis revealed melting temperatures of 156.47°C and 160.02°C for the extracted P(3HB) from *Bacillus thuringiensis* and *Bacillus cereus*, respectively. TGA studies established the maximum degradation temperatures as 603.6°C for *Bacillus thuringiensis* and 322.41°C for *Bacillus cereus* extracted P(3HB) respectively. GPC analysis indicated weight average molecular weights of 32.617 kDa for *Bacillus thuringiensis* and 45.808 kDa for *Bacillus cereus* isolated P(3HB) samples.

In addition, the extracted polyhydroxybutyrate (P(3HB)) obtained from *Bacillus cereus* was utilised to synthesise nanoparticles by a solvent evaporation method. The nanoparticles' hydrodynamic diameter (159.97 nm) and polydispersity index (13.5%) were identified using the dynamic light scattering (DLS) technique. The nanoparticles exhibited an average zeta potential of -3.5 millivolts (mV) while Field Emission Scanning Electron Microscopy (FESEM) analysis provided additional characterisation, showing that the particles exhibited a spherical shape and varied in size, ranging from 40 nm to 114 nm.

सार

पॉलीहाइड्रॉक्सीब्यूटाइरेट (पीएचबी) एक बायोडिग्रेडेबल पॉलिमर है जिसे कई सूक्ष्मजीवों द्वारा अपनी कोशिकाओं के भीतर कार्बन और ऊर्जा संग्रहीत करने के लिए संश्लेषित किया जाता है। इसकी उल्लेखनीय विशेषताओं, जैसे, उल्लेखनीय बायोकम्पैटिबिलिटी, बायोडिग्रेडेबिलिटी और स्थिरता के कारण इसे बायोपॉलिमर के रूप में व्यापक रूप से मान्यता प्राप्त है। हाल के दशकों में इस पर व्यापक शोध हुआ है, जिसका मुख्य कारण मुख्य रूप से पर्यावरण और चिकित्सा क्षेत्रों में इसके विविध अनुप्रयोग हैं।

उप-उत्पाद के साथ बेसिलस थुरिंगिएन्सिस IAM12077 द्वारा P(3HB) (पॉलीहाइड्रॉक्सी ब्यूटायरेट) के उत्पादन को कम करने के लिए व्यापक खेती अध्ययन और उत्तेजित टैंक रिएक्टर में P(3HB) संचय का अनुकूलन और वायवीय रूप से उत्तेजित एयरलिफ्ट बायोरिएक्टर (विभिन्न वायु आपूर्ति और मिश्रण रणनीतियों का उपयोग करके) के विभिन्न विन्यास किए गए थे। बायोडीजल उद्योग (ग्लिसरॉल) और ग्लूकोज सब्सट्रेट के रूप में (7 पीएच और 30 डिग्री सेल्सियस के तापमान पर) हवा (0.3-0.6 वीवीएम)। एयरलिफ्ट बायोरिएक्टर के बाहरी वातन आंतरिक सेटलिंग कॉन्फ़िगरेशन में 37.07% की तुलना में स्टिरर्ड टैंक बायोरिएक्टर में P(3HB) संचय 42.59% देखा गया, फिर भी यह बड़े पैमाने पर बायोपॉलिमर उत्पादन के लिए एक लागत प्रभावी विकल्प के रूप में उभरा।

पॉलीहाइड्रॉक्सीब्यूटाइरेट (पीएचबी) उत्पादन लागत को और कम करने के लिए, सब्सट्रेट के रूप में एक और बैक्टीरिया बैसिलस सेरेस और क्रूड ग्लिसरॉल के साथ विस्तृत अध्ययन शुरू किया गया था। बी. सेरेस के लिए मध्यम संरचना को सांख्यिकीय रूप से अनुकूलित किया गया था, जिसके परिणामस्वरूप मॉडल की अनुमानित P(3HB) सांद्रता 3.75 g/L की तुलना में शेक फ्लास्क

खेती के दौरान 30 घंटे में 4.13 g/L की P(3HB) सांद्रता प्राप्त हुई। इसके बाद, 3.5 लीटर बायोरिएक्टर खेती के दौरान 39 घंटे में 4.53 ग्राम/लीटर बायोमास और 1.95 ग्राम/लीटर की पी(3HB) सांद्रता (सामग्री 43.05%) प्राप्त की गई। बी. सेरेस द्वारा बायोपॉलिमर उत्पादन को स्केल अप मानदंड के रूप में पी/वी का उपयोग करके 15 लीटर बायोरिएक्टर तक बढ़ाया गया, जिसके परिणामस्वरूप पीएचबी एकाग्रता 1.81 ग्राम/लीटर (3.5 लीटर बायोरिएक्टर में 1.95 ग्राम/लीटर के विपरीत) हुई, जिससे इसकी पुष्टि हुई। उच्च स्तरीय बायोरिएक्टर में बायोपॉलिमर उत्पादन की व्यवहार्यता।

बैच खेती के बाद, बैसिलस थुरिंगिएन्सिस और बैसिलस सेरेस के बायोमास को सोडियम डोडेसिल सल्फेट (एसडीएस) और सोडियम हाइपोक्लोराइट (NaOCl) का उपयोग करके पूर्व-उपचार किया गया, इसके बाद क्लोरोफॉर्म निष्कर्षण किया गया। परिणामी बायोपॉलिमर का व्यापक लक्षण वर्णन किया गया, एनएमआर और एफटीआईआर माप के माध्यम से इसकी आणविक संरचना की पुष्टि की गई। एक्स-रे विवर्तन (एक्सआरडी) विश्लेषण ने बैसिलस संस्कृतियों से पृथक पॉलीहाइड्रॉक्सीब्यूटाइरेट (पीएचबी) की ऑर्थोरोम्बिक क्रिस्टल संरचना की पहचान की। डीएससी विश्लेषण से बैसिलस थुरिंजिएन्सिस और बैसिलस सेरेस से निकाले गए पीएचबी के लिए क्रमशः 156.47 डिग्री सेल्सियस और 160.02 डिग्री सेल्सियस के पिघलने का तापमान पता चला। टीजीए अध्ययनों ने बैसिलस थुरिंजिएन्सिस के लिए अधिकतम गिरावट तापमान क्रमशः 603.6 डिग्री सेल्सियस और बैसिलस सेरेस निकाले गए P(3HB) के लिए 322.41 डिग्री सेल्सियस स्थापित किया। जीपीसी विश्लेषण ने बैसिलस थुरिंजिएन्सिस के लिए 32.617 केडीए और बैसिलस सेरेस पृथक पीएचबी नमूनों के लिए 45.808 केडीए के औसत आणविक भार का संकेत दिया।

इसके अलावा, बैसिलस सेरेस से प्राप्त पॉलीहाइड्रॉक्सीब्यूटाइरेट (पीएचबी) का उपयोग विलायक वाष्पीकरण विधि द्वारा नैनोकणों को संश्लेषित करने के लिए किया गया था। डायनेमिक लाइट स्कैटरिंग (डीएलएस) तकनीक का उपयोग करके नैनोकणों के हाइड्रोडायनामिक व्यास (159.97

नैनोमीटर) और पॉलीडिस्पर्सिटी इंडेक्स (13.5%) की पहचान की गई। नैनोकणों ने -3.5 मिलीवोल्ट (एमवी) की औसत ज़ेटा क्षमता प्रदर्शित की, जबकि फील्ड एमिशन स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी (एफईएसईएम) विश्लेषण ने अतिरिक्त लक्षण वर्णन प्रदान किया, जिससे पता चला कि कण एक गोलाकार आकार प्रदर्शित करते हैं और आकार में भिन्न होते हैं, 40 नैनोमीटर से 114 नैनोमीटर तक।

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LIST OF ABBREVIATIONS

PHA	Poly-3-hydroxyalkanoates
P3HB	Poly-3-hydroxybutyrate
SCL	Short Chain Length
MCL	Medium Chain Length
LCL	Long Chain Length
DCW	Dry Cell Weight
DO	Dissolved Oxygen
NB	Nutrient broth
RSM	Response Surface Methodology
OD	Optical density
GC	Gas chromatography
HPLC	High Performance Liquid Chromatography
CHNS	Carbon hydrogen nitrogen sulphur analyzer
SDS	Sodium Dodecyl Sulfate
NaOCl	Sodium hypochlorite
STB	Stirred Tank Bioreactor
OAIS	Outer Aeration Inner Settling
IAOS	Inner Aeration Outer Settling
BC	Bubble Column
DSC	Differential scanning calorimetry
TEM	Transmission Electron Microscope
FTIR	Fourier Transform Infrared Spectroscopy

GPC	Gel permeation chromatography
TGA	Thermogravimetric analysis
XRD	X-ray diffraction
NMR	Nuclear Magnetic Resonance
PHV	Poly-3-hydroxyvalerate
PP	Polypropylene
FID	Flame ionization detector
NPCM	Non-polymer cellular material
DOE	Design of experiments
CCD	Central composite Design

LIST OF SYMBOLS

$\boldsymbol{\mu_m}$	Maximum specific growth rate (h^{-1})
$\boldsymbol{\mu}$	Specific growth rate (h-1)
L	Liter
K_La	Volumetric Mass Transfer Coefficient (h^{-1})
Fc	Correction factor
H_T	Height of Tank (cm)
H_L	Height of the liquid within the bioreactor
D_T	Diameter of Tank (cm)
N	Impeller rotation speed (rpm)
D_I	Diameter of Impeller (cm)
v/v	volume/volume
μ	Medium viscosity (Kg/cm/s)
N_{Re}	Reynold‟s Number
Q/V	Volumetric airflow rate (lpm)
Π	Impeller Tip Velocity (m/s)
P_{ug}	Un-gassed power (W)
Q	Airflow rate (lpm)
Vs	Superficial Gas Velocity (m/s)
ρ	Medium density (kg/m3)
Ө	Blend Time
P_g	Gassed power (W)
T_c	Crystalline Temperature (°C)

T_m	Melting Temperature (°C)
T_d	Thermal degradation Temperature (°C)
M_w/M_n	Polydispersity index (kDa)
T_g	Glass transition Temperature (°C)
M_n	Number average molecular weight (kDa)
P/V	Power per unit Volume (W/m3)
M_w	Weight average molecular weight (kDa)
t_m	Mixing time
M_Z	Z average molecular weight (kDa)
X_c	Degree of crystallinity