Conversion of Methane into Polyhydroxybutyrate using *Methylosinus trichosporium* NCIMB 11131: Applications in Food Packaging, Bone Tissue Engineering, and Electronics

A Thesis

Submitted for the Degree of

DOCTOR OF PHILOSOPHY

by

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Under supervision of

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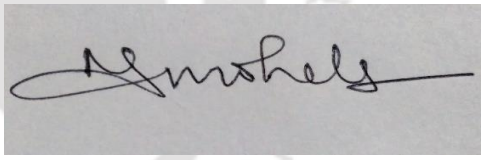
STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the **Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati,** Guwahati, Assam, Indiaunder the supervision of **Prof. Debasish Das**. In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work described is based on the findings of other investigators.



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CERTIFICATE

It is certified that the work described in this thesis entitled "**Conversion of Methane into Polyhydroxybutyrate using *Methylosinus trichosporium* NCIMB 11131: Applications in Food Packaging, Bone Tissue Engineering, and Electronics**" by **Mr. Noor Mohammed** for the award of the degree of **Doctor of Philosophy** is an authentic record of the results obtained from the research work carried out under my supervision in the **Department of Biosciences & Bioengineering**, **Indian Institute of Technology Guwahati**, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Abstract

The onset of the Industrial Revolution marked a significant shift in both atmospheric composition and the global environment. Despite constituting only 0.1% of Earth's atmosphere, greenhouse gases play a pivotal role in maintaining the planet's average surface temperature around 15°C. Without these gases, the Earth's average temperature would plummet to a chilling - 18°C (US-EPA, 2022). Methane levels have experienced a substantial increase since industrialization, rising from 700 ppb to 1922 ppb by 2022. The primary sources of these gases are diverse, ranging from fossil fuel production (29%) to electricity generation (25%), agriculture and waste (15%), transportation (13%), and other human activities (Fekete et al., 2021; Stein et al., 2006). Methane, the second most detrimental greenhouse gas, possesses significant infrared activate absorption capability and a relatively short atmospheric half-life of 7 to 12 years. It is over 25 times more potent in infrared absorption than CO_2, contributing approximately 0.5 $W.m^{-2}$, or about 25 to 28% of the greenhouse effect attributed to CO_2 (NOAA, 2023; NASA, 2023). Current emission rates suggest a potential temperature increase of 2°C by 2036, which is the upper limit estimated by the United Nations' Intergovernmental Panel on Climate Change (IPCC) to avoid reaching a dangerous level (American Scientific, 2014; Fekete et al., 2021; Rosenboom et al., 2022). Alongside the challenges posed by greenhouse gases, plastic pollution has emerged as a significant environmental concern. Annually, global plastic production nears 450 million tons, expected to double by 2045, with 40% being single-use plastic. Approx. 22 to 43% of this plastic ends up in landfills and 50 to 80% in oceans, posing severe threats to ecosystems and human health. Recycling efforts remain limited, with only 9 to 10% of plastic waste being processed due to challenges like material degradation (Geyer et al., 2017, Bergmann et al., 2022), with single use plastics being disproportionately represented. A significant percentage of this plastic ends up in landfills or marine environments, further threatening

ecosystems and human health. Despite growing awareness and efforts, recycling remains minimal due to technical and economic constraints (Hwang et al., 2018, Kabir et al., 2020). In this context, the development of degradable bioplastics such as polyhydroxybutyrate (PHB), a degradable polymer, emerges as a beacon of hope. PHB is produced intracellularly by Type-II methanotrophs, such as *Methylosinus trichosporium* NCIMB 11131, which utilize methane as a carbon source (pollutants as well 2nd Generation fuel) under essential nutrient starvation in growth media. Despite being mainly produced from C5/C6 C-source, resulting in its high cost (4 to 20 Euro.kg^{-1}), it finds limited applications. This innovative approach couples a potent greenhouse gas as a raw material for producing eco-friendly plastics, offering a dual solution to mitigate greenhouse gas emissions and tackle the plastic waste crisis. Current research is intensely focused on enhancing the efficiency and economic viability of PHB production to extend its applications. This research, reinforced by the methanotrophic capabilities of *M. trichosporium*, sits at the nexus of environmental sustainability, technological innovation, and bio-economic viability. It represents the principles of green chemistry, resource efficiency, and the circular economy, aiming to create a sustainable and responsible narrative for plastics across industries, including food packaging, electronics, and healthcare (Prajapati et al., 2021; Wanet al., 2020). The study delves into optimizing PHB production through nutrient modulation and process engineering, utilizing methane as a sustainable carbon source. This PhD thesis go on boardon an ambitious journey to explore the potential of degradable polymers, with a particular focus on PHB, as strategic and sustainable solutions to the pressing environmental challenges of our time. The initial chapter sets the stage for a comprehensive exploration into the realm of sustainable biopolymer production nutritional modulation. The integrated two-phase process involves the generation of high-density methanotrophic biomass in phase I, followed by the enrichment of PHB in the phase II using nutritional modulation. PHB production in phase II is also influenced by the initial biomass content as well. Greater cellular density at higher biomass concentrations promotes PHB accumulation during phase II. For maximize production of PHB

initial biomass content of 1.68 $g.L^{-1}$ (OD600 nm=4) was used for experimentations. The thesis will be updated in future with a new section that specifically looks at the effect of biomass on PHB production. The effects of different Initial biomass concentrations on PHB yield, productivity, and intracellular accumulation will be examined and discussed in this section, offering insights into the ideal biomass levels for maximum PHB production. Under optimal growth conditions, a biomass titre of 3.82±0.01 $g.L^{-1}$ was achieved, and subsequent complete nitrate starvation led to the accumulation of PHB (41.24±0.83% w/w). Further optimization by exposing the cells to excess methane concentration (5% v/v) and nitrate starvation increased the PHB content to 52.42±1.03% w/w. The scalability of the process was demonstrated in a 5-L stirred tank bioreactor, yielding a PHB concentration of 2.02±0.04 $g.L^{-1}$. Overall, this study presents an innovative biotechnological approach, including process engineering strategies for maximal methane mass transfer, and solubility for the maximal conversion of methane into a valuable biopolymer and highlights its potential as a sustainable alternative to conventional plastics. The maximum fixation rate of methane 0.73±0.01 $g.L^{-1}d^{-1}$ was achieved using a sparger with a pore size of 5 µm and 7.5% (v/v) methane vector as silicone oil, resulting in biomass production of 8.91±0.12 $g.L^{-1}$ in phase I, and total methane fixation induction of PHB at the rate of 0.82±0.01 $g.L^{-1}.d^{-1}$ in the generated methanotrophic biomass with high PHB content. The highest PHB content of 57.3±2.28 % (w/w) was obtained using 5% (v/v) paraffin, while the highest PHB yield of 5.06±0.32 $g.L^{-1}$ (PHB productivity of 1.11±0.04 $g.L^{-1}.d^{-1}$) was achieved using 7.5% (v/v) silicone oil (Maximum ($kLaO_2$: 97.2 h^{-1}, $kLaCH_4$: 70.8 h^{-1}). Thisresearch explores the intracellular PHB production within methanotrophic biomass utilizing biogas as a carbon source and further characterizes the biomass post PHB extraction. The phase I of the study achieved high-density biomass cultivation, attaining a maximum biomass concentration of 8.91 ± 0.04 $g.L^{-1}$ under controlled conditions with 99.98% pure methane. Subsequently, the phase II aimed at enhancing the intracellular PHB content within this high dense biomass. Results indicated a PHB concentration of 57.30±2.28% (w/w), equivalent to a PHB yield

of 5.04±0.10 $g.L^{-1}$ under control conditions. The suitability of the extracted PHB as a degradable food packaging the material was evaluated by a comprehensive analysis of its thermal, structural, physical mechanical, and molecular properties. Our results suggest that the PHB obtained from *M. trichosporium* can serve as a promising alternative to petroleum-based plastics due to its superior properties and degradability. A comparative analysis of using pure methane versus biogas revealed PHB content of 57.30±2.28% and 51.29±0.21% (w/w), respectively. Post-extraction characterization of the biomass showed protein contents of 24.27±0.85% and 22.73±0.82% (w/w), carbohydrates at 8.88±0.79% and 12.33±1.06% (w/w), and lipids at 11.35±1.28% and 14.30±0.59% (w/w) for pure methane and biogas conditions, respectively. In the domain of bone tissue engineering, extracted PHB using biogas as C-source showed remarkable promise when combined with nHA derived from waste eggshells. This composite material was found to possess excellent biocompatibility and osteo-conductivity, essential for bone regeneration applications. The composite's porous structure eased cell attachment and proliferation, which are critical for tissue engineering. *In vitro* studies demonstrated the material's ability to support the growth of osteoblasts, indicating its potential as a scaffold for bone tissue regeneration. The PHB/ HA composite's mechanical properties, including its compressive strength, were found to be within the range suitable for bone tissue engineering applications, suggesting its utility in the repair and regeneration of bone tissue. The derived PHB using biogas as C-source, was also processed into degradable thin films, designed for insulative cover applications in electronics beneath of upper metal cover, specifically as an external layer for non-rechargeable primary batteries. Physicochemical analysis of the PHB film revealed that at 10 volts, the current decreased 5-fold from 1.20 e^{-04} Amp for a 2% PHB film to 2.62 e^{-05} Amp for an 8% PHB film, underscoring its potential utility in the electronics sector. This dissertation presents a comprehensive exploration into the conversion of methane into PHB using *M. trichosporium* NCIMB 11131, examining its application in food packaging, bone tissue engineering, and electronics. Structured around three core objectives, this

research integrates experimental outcomes to offer a holistic view of PHB production and its applications in various sectors. The study explores practical applications beyond PHB production, encompassing food packaging, bone tissue engineering, and electronics. Methanotrophic PHB thin films exhibit excellent degradability and mechanical properties for sustainable food packaging as well as electronics sectors. The films are flexible, strong, and moisture-resistant, degrading significantly under compostable conditions. In bone tissue engineering, PHB combined with HA from waste eggshells shows promise, possessing excellent biocompatibility and supporting cell attachment and proliferation. Additionally, PHB thin films serve as insulative covers in primary batteries, offering exceptional electrical insulation and heat resistance as well as mechanical properties. Their degradability reduces electronic waste, aligning with goal of sustainability. This research demonstrates the feasibility of using *M. trichosporium* NCIM 11131 for PHB production and highlights the potential of biogas as a sustainable methane source. PHB's versatility across industries contributes to reducing greenhouse gas emissions and promoting a circular economy. These findings advance sustainable bioplastic production and underscore PHB's potential as an eco-friendly material in various applications.

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CHAPTER 1

Introduction

1.1 Background and motivation

The advent of the “Industrial Revolution” marked a significant shift in atmospheric composition and the global environment. Greenhouse gases, although constituting a mere 0.1%of Earth's atmosphere, are pivotal in maintaining the planet's average surface temperature at around 15°C. In the absence of these gases, the Earth's average temperature would plummet to a chilling -18°C (US-EPA, 2022). The primary greenhouse gases include carbon dioxide (CO_2), methane (CH_4), along with other contributors like carbon monoxide (CO), water vapor, nitrogen oxides, ozone (O_3), and various halogenated hydrocarbons. These gases originate froma spectrum of human activities, encompassing fossil fuel production, electricity generation, agriculture, waste management, transportation, and more. Especially, since the onset of industrialization, methane levels have experienced a substantial increase, rising from 700 ppb to 1922 ppb in 2022, the primary sources of these gases are diverse, ranging from fossil fuel production (29%) to electricity generation (25%), agriculture and waste (15%), transportation (13%), and other human activities (Fekete et al., 2021; Stein et al., 2006). Methane as 2nd most detrimental in GHGs, with its significant infrared absorption capability and a relatively short atmospheric of half-life 7 to 12-year, is over 25 times more potent in infrared absorption and contributing approximately 0.5 Wm^{-2}, or about 25 to 28% of the greenhouse effect attributed to CO_2, (NOAA, 2023; NASA, 2023). Based on current emission rates, the temperature could increase by 2°C, which is the upper limit estimated by the United Nations’ Intergovernmental Panel on Climate Change (IPCC) to avoid a dangerous level by 2036 (American Scientific, 2014; Fekete et al.,

2021; Rosenboom et al., 2022). Parallel to the challenges posed by greenhouse gases, plastic pollution has emerged as a challenging environmental concern. Annually, global plastic production nears 450 million tons, expected to double by 2045, with 40% of this is single-use plastic, with 22 to 43% ending up in landfills and 50 to 80% in oceans, posing severe threats to ecosystems and human health. Recycling efforts are limited, with only 9 to 10% of plastic waste being processed due to challenges like material degradation (Geyer et al., 2017, Bergmann et al., 2022) in distressing proportion being single-use plastics. A significant percentage of this plastic ends up in landfills or marine environments, threatening ecosystems and human health. Despite growing awareness and efforts, recycling remains minimal due to technical and economic constraints (Hwang et al., 2018, Kabir et al., 2020). In this context, the development of degradable bioplastics such as Polyhydroxybutyrate (PHB), a degradable polymer, emerges as a beacon of hope. PHB is produced intracellularly by Type-II methanotrophs, such as *Methylosinus trichosporium*, which utilize methane as a C-source. Commercially, mainly produced from C5/C6 C-source cause its high cost (4- to 20 Euro.kg^{-1}), it is only used in limited applications. This innovative approach couples a potent greenhouse gas as a raw material for producing eco-friendly plastics, offering a dual solution to mitigate greenhouse gas emissions and tackle the plastic waste crisis. Current research is intensely focused on enhancing the efficiency and economic viability of PHB production to extend its applications. This research, reinforced by the methanotrophic capabilities of *Methylosinus trichosporium* NCIMB 11131, sits at the nexus of environmental sustainability, technological innovation, and bio-economic viability. It represents the principles of green chemistry, resource efficiency, and the circular economy, aiming to create a sustainable and responsible narrative for plastics across industries, including food packaging, electronics, and healthcare (Prajapati et al., 2021; Wan et al., 2020). The study delves into optimizing PHB production through nutrient modulation and process engineering, utilizing methane as a sustainable C-source. This Ph.D. thesis go on board in ambitious journey to explore the potential of degradable polymers, with a particular focus on

PHB, as strategic and sustainable solutions to the pressing environmental challenges of our time. The initial chapter sets the stage for a comprehensive exploration into the domain of sustainable biopolymer production, laying out the significance, objectives, and methodologies that will guide this scholarly endeavor.

1.2 Approach of the thesis

This Ph.D. research adopts a comprehensive approach to tackle crucial environmental issues, leveraging the innovative use of *Methylosinus trichosporium* NCIMB 11131 for producing value-added products. Aligned around three core objectives, the thesis aims to develop sustainable, eco-friendly, and cost-efficient bioprocess engineering strategies for PHB production and to explore into its diverse applications.

Nutrient modulations for PHB overproduction: The first objective conducts an in-depth study of nutrient modulation effects on PHB overproduction on two phases cultivation; phase I for high-density biomass and phase II for intracellular PHB induction under various nutritional stresses. It focuses on the effect of macro and microelements, along with carbon sources for maximum redirection, that influence *M. trichosporium*'s metabolic pathways for optimal PHB production. The research aims to enhance microbial efficiency and carbon flux, thereby increasing PHB yield. This objective is foundational in optimizing PHB production within controlled nutrient and cultivation conditions.

Process engineering for methane mass transfer and solubility: The second objective involves developing process engineering strategies to improve methane mass transfer and solubility in aqueous phases. Utilizing customized airlift bioreactor design of micro-sparger, and methane solubility vectors, the goal is to elevate PHB production efficiency significantly with maximum carbon flux redirect in PHB production. Such developments are crucial for scale-up PHB production and ensuring economic feasibility, and application in the food packaging sector as a degradable thin film.

Sustainable PHB production using biogas: The third objective explores the sustainable PHB production using biogas that have low calorific value (23.98 $MJ.m^{-3}$ or about 6.68 $kWh.m^{-3}$) as a feasible source for methane. Highlighting the environmental virtues of biogas, a by-product of waste decomposition, this approach aims to reduce to carbon cost under optimal process cultivation condition to make process sustainable, feasible, an eco-friendly. Integrating biogas-producing systems along with PHB production processes, the research underscores the potential of transforming waste into valuable biopolymers, application in various sectors such as bone tissue engineering, and electronics.

1.3 Applications of PHB in various sectors

Food Packaging: A major area of application is in food packaging, where PHB is posited as an eco-friendly alternative to traditional plastics. The research contributes to mitigating plastic pollution by developing PHB-based packaging solutions that align with sustainability demands.

Bone tissue engineering: In medical applications, particularly bone tissue engineering, the research explores PHB bio-composites, including those using waste eggshell-synthesized hydroxyapatite. These bio-composites offer potential for bio-compatible, sustainable materials in bone repair and regeneration.

Electronics: The thesis also explores PHB's application in electronics, examining its use as a degradable insulation component. This aligns with the increasing demand for environmentally friendly electronics products, positioning PHB as a sustainable substitute in a traditionally non degradable domain.

The research consistently highlights not only advancing the scientific knowledge of PHB production but also its practical implementation across varied sectors. This multifaceted approach highlights PHB's potential as a sustainable alternative to conventional materials, significantly contributing to environmental conservation and sustainable development.

1.4 Objectives of the present study

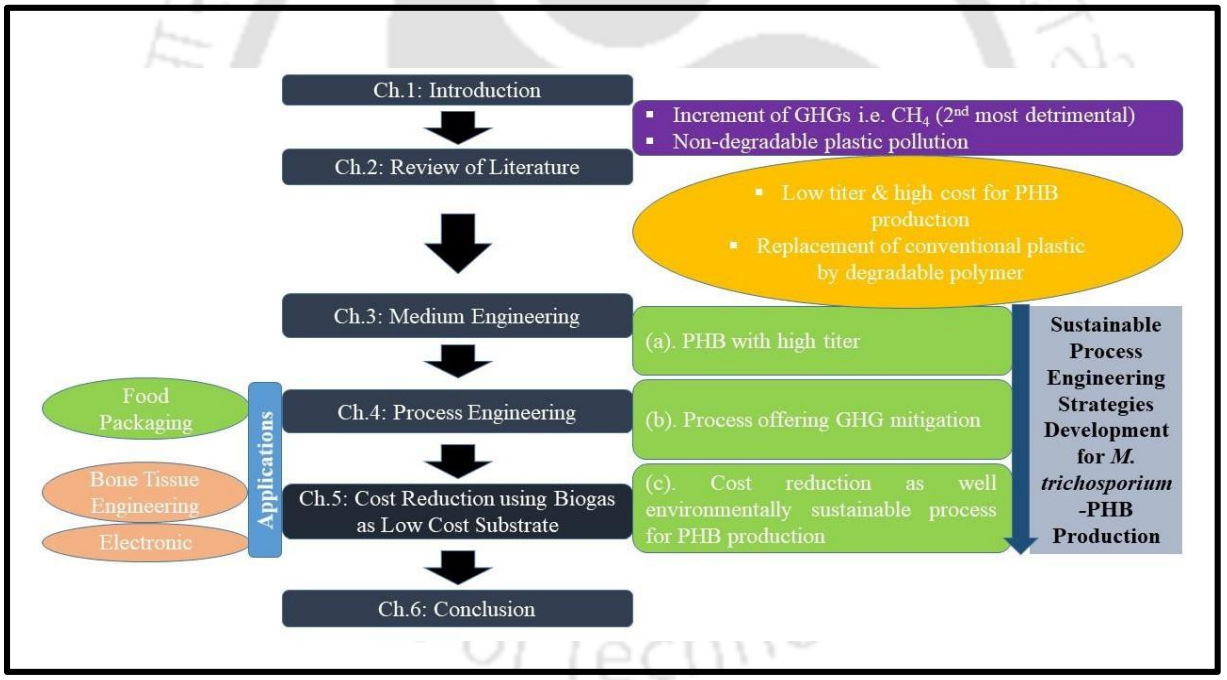
1. *Effect of various nutrient modulations on overproduction of PHB in Methylosinus trichosporium* NCIMB 11131

2. *Process engineering strategy towards improvement in methane mass transfer and solubility in the aqueous phase: Application as food packaging material*

3. *Sustainable process for production of PHB utilizing biogas as feasible source of methane: Applications in bone tissue engineering, and electronics*

1.5 Structure of the dissertation

Flow chart: Structure of the dissertation



This dissertation is systematically organized into six chapters. Chapter 1: introduces the study by delineating the general context, background and motivation, objective of thesis, and structure of dissertation. Chapter 2: offers an exhaustive review of the current methodologies and recent advancements in address existing challenges, methanotrophic biochemistry, metabolic study, troubleshot of existing crisis with methanotrophic bacterial research, identifying significant limitations inherent in contemporary technologies. Chapter 3: examines the impact of nutritional

modulation in conjunction with elevated methane concentrations on the intracellular accumulation of PHB during a two-stage cultivation process and methodologies for experimentation. Chapter 4 details the development of process engineering strategies aimed at optimizing mass transfer and solving solubility issues of methane for enhanced uptake by methanotrophic bacteria, thereby facilitating high-density biomass production in phase I and maximal intracellular PHB accumulation in phase II. This chapter also includes a comprehensive characterization of the produced PHB for its potential use as a degradable food packaging material. Chapter 5: presents a cost-effective and sustainable process for PHB production, using biogas as a feasible source of methane. It further provides a detailed characterization of the produced PHB, assessing its applicability in bone tissue engineering in conjunction with hydroxyapatite that synthesized from waste eggshells, and as a degradable insulating layer for metal covers in primary batteries for electronics applications. Finally, Chapter 6: The findings of this research, drawing conclusions insights, and suggesting future directions for work in this area. This research presents a novel approach to converting methane, a major greenhouse gas, into polyhydroxybutyrate (PHB) using the methanotrophs *M. trichosporium* NCIMB 11131. Through comprehensive experimentation, it was demonstrated that nitrate deprivation significantly enhances PHB production. Furthermore, process engineering strategies optimized methane mass transfer and solubility, resulting in a maximum PHB yield of 57.3% (w/w) and a methane fixation rate of 0.73 $g.L^{-1}.d^{-1}$. The study also established the viability of using biogas as a methane source, maintaining high PHB yields 51.29% (w/w) and reducing operational costs. These findings underscore the potential of PHB for diverse applications in food packaging, bone tissue engineering, and electronics, showcasing its sustainability and versatility.

References

1. https://www.epa.gov/ghgemissions/inventory-us-greenhouse-gas-emissions-and-sinks

2. https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/2023

3. https://earthobservatory.nasa.gov

4. Fekete, H., Kuramochi, T., Roelfsema, M., den Elzen, M., Forsell, N., Höhne, N., ... & Gusti, M. (2021). A review of successful climate change mitigation policies in major emitting economies and the potential of global replication. Renewable and Sustainable Energy Reviews, 137, 110602.

5. Rosenboom, J. G., Langer, R., & Traverso, G. (2022). Bioplastics for a circular economy. Nature Reviews Materials, 7(2), 117-137.

6. Wendlandt, K. D.; Geyer, W.; Mirschel, G.; Al-Haj Hemidi, F. Possibilities forControlling a PHB Accumulation Process Using Various Analytical Methods. J. Biotechnol. 2005, 117 (1), 119–129. https://doi.org/10.1016/j.jbiotec.2005.01.007.

7. Prajapati, K., Nayak, R., Shukla, A., Parmar, P., Goswami, D., & Saraf, M. (2021). Polyhydroxyalkanoates: an exotic gleam in the gloomy tale of plastics. Journal ofPolymers and the Environment, 29(7), 2013-2032.

8. Kabir, E., Kaur, R., Lee, J., Kim, K. H., & Kwon, E. E. (2020). Prospects of biopolymertechnology as an alternative option for non-degradable plastics and sustainable management of plastic wastes. Journal of Cleaner Production, 258, 120536.

CHAPTER 2

Review of Literature

2.1 Methane as a greenhouse gas in the biosphere and its impact on the ecosystem

Methane (CH_4), a minor constituent of the Earth's atmosphere, plays a disproportionately major role in global warming due to its high global warming potential, more than 25 times greater than carbon dioxide (CO_2) over a century. This section explores the multifaceted impacts of methane, its biospheres' interactions, and the strategies for its mitigation as a critical greenhouse gas. Greenhouse gases, though only about 0.1% of the Earth's atmosphere, are pivotal in maintaining the planet's average surface temperature at around 15°C, significantly warmer than the -18°C it would be without them (US-EPA, 2022). Methane, along with CO_2, water vapor, nitrogen oxides, ozone (O_3), and various halogenated hydrocarbons, is a key greenhouse gas, with its levels having surged by 170.4% since the industrial revolution due to activities such as mining and agriculture (TechCrunch, 2019). Its atmospheric half-lifetime of 7 to 12 years and its potent infrared absorption capability make it irresponsible for approximately 0.5 $W.m^{-2}$, or about 25 to 28% of the greenhouse effect attributed to CO_2 (NOAA, 2023; NASA, 2023). The primary sources of methane emissions are diverse, including natural processes in wetlands, termites, oceans, and wildfires, as well as human activities including fossil fuel production (29%), electricity generation (25%), agriculture and waste (15%), transport (13%), building (7%), land use & forestry (7%), anthropogenic activities and waste management (Fekete et al., 2021; Stein et al., 2006). The decomposition of organic waste in landfills and the digestive processes of ruminant animals are also, significant anthropogenic contributors.

Methane's impact on the ecosystem is extensive. It acts as a precursor to ground-level ozone formation, thereby contributing to air quality degradation and respiratory health issues. Its indirect effects include influencing the formation and destruction of other greenhouse gases and altering atmospheric chemistry. The ecological implications are profound, affecting global temperatures, precipitation patterns, extreme weather events, sea-level rise, species distribution, and the health of aquatic systems. Mitigating methane emissions is a critical aspect of global climate change strategies. This involves identifying and quantifying methane sources, enhancing methane capture and utilization, and implementing regulatory measures to reduce emissions. Understanding methane's role in biogeochemical cycles is crucial. Methane is produced and consumed by microbial processes in soil and aquatic environments, with the balance of these processes determining the net methane as well as carbon flux. Methanogens produce methane in an anaerobic environment, while methanotrophs consume it in the presence of oxygen. Environmental changes can significantly impact these microbial processes and, consequently, the net methane emissions. Research into methane's role in the biosphere and its impact on ecosystems continues to evolve, with new findings highlighting its complex dynamics and interactions with other elements of the climate system. Rising temperatures causing permafrost thaw and methane release from clathrates represent positive feedback mechanisms that may substantially move along global warming. In conclusion, methane is a critical greenhouse gas with significant implications for the Earth's climate and ecosystems. Its potent greenhouse effect, short atmospheric lifetime, and the contributions of various natural and anthropogenic sources necessitate comprehensive strategies to mitigate its impact. As our understanding of methane's role in the biosphere deepens, so must our commitment to reducing its emissions and enhancing natural sinks, all towards ensuring a sustainable future for our healthy planet for successive next generation. This literature review lays the basis for understanding the complex dynamics of methane in the biosphere and the crucial need for effective bioconversion methodologies and innovative material science solutions.

2.2 Global plastic pollution and its impact on the ecosystem

Plastic pollution stands as a significant environmental crisis of the modern age, reducing from extensive production and inadequate disposal practices. This comprehensive literature review studies into the origin, progression, and ecological consequences of global plastic pollution, highlighting the critical need for effective management strategies. Since its advent in the mid-20th century, plastic production has rushed to staggering levels, with current annual production nearby 450 million tons and predictions suggesting a doubling by 2045 (Geyer et al., 2017). Especially, single-use plastics constitute about 40% of total production, exacerbating the waste problem. Despite the gravity of the situation, only 9 to 10% of these materials are recycled, leading to substantial accumulations in both terrestrial and aquatic environments (Bugnicourt et al., 2014). The dispersal of plastic waste follows complex pathways, frequently ending up in aquatic ecosystems. Here, environmental factors contribute to the degradation of these materials into micro-plastics. These minute particles, alongside larger debris, are found throughout marine environments, from the surface waters to the deep sea, and across diverse geographic regions, including polar ice caps and tropical reefs. The ubiquity of plastic pollution is an evidence to its pervasive nature and its relentless infiltration into every corner of the planet. The ecological impacts of plastic pollution are extensive and severe. Marine organisms, from the smallest plankton to the largest whales, are known to ingest plastic debris, resulting in physical harm and exposure to toxic substances. Plastics often serve as vectors for other pollutants, thereby integrating them into the food web and potentially leading to bio accumulation and bio magnification of harmful substances (Rocklöv et al., 2020) beside non-degradability. On land, plastics disrupt soil structure, hinder nutrient cycles, and pose threats to terrestrial biodiversity. The ramifications for human health are increasingly concerning. The pervasive nature of micro plastics means they can infiltrate human tissues and organs, while the leaching of plastic-associated chemicals, such as phthalates and BPA, is linked to various health disorders,

including hormonal disruption and cancer (Prajapati, 2018). Additionally, the visual and ecological degradation caused by plastics undermines industries reliant on unspoiled natural environments, especially tourism, while imposing substantial economic burdens associated with waste management and environmental remediation. Addressing this crisis necessitates a comprehensive and multi-faceted approach. This includes improving waste management infrastructure, promoting sustainable materials, carry out extended producer responsibility, and cultivating consumer behaviors oriented towards the principles of reduce, reuse, and recycle. Innovations in degradable plastics and microbial degradation pathways present promising directions for reducing the environmental footprint of plastics, although these solutions require thorough evaluation to ensure they do not lead to unintended ecological impacts. In conclusion, global plastic pollution is an intricate and widespread issue that inflicts significant damage on ecosystems and human health. The persistent and pernicious nature of plastics calls for urgent and collective action to mitigate their impacts. Continued research is vital to enhance our understanding of the dynamics of plastic pollution and to develop and implement effective strategies for its management, control, and eventual eradication. As the body of evidence grows, so too does the imperative for coordinated, global initiatives aimed at transitioning from a culture of plastic dependency to one of environmental responsibility and sustainable existing. This review underscores the urgent need to augment scientific research, practical solutions, policy interventions, and behavioral changes to navigate towards a more sustainable and resilient future.

2.3 Methanotrophic bacteria: the methane mitigators

Methanotrophs have potential to sequester greenhouse gases (mainly CH_4 & also CO_2), into value added products such as biopolymer, methanol, single cell protein, ectoine, vitamin B12, surface layers, extra cellular polysaccharides (EPS) lipids, sucrose methanobactin (copper-binding protein) (Strong et al., 2015; Trotsenko et al., 2005; Khmelenina et al., 2015;

Balasubramanian and Rosenzweig, 2008; Xin et al., 2007; Rostkowski et al., 2013; Cantera et al., 2018; Rasouli et al., 2018; Tsapekos et al., 2020; Sahoo et al., 2022).

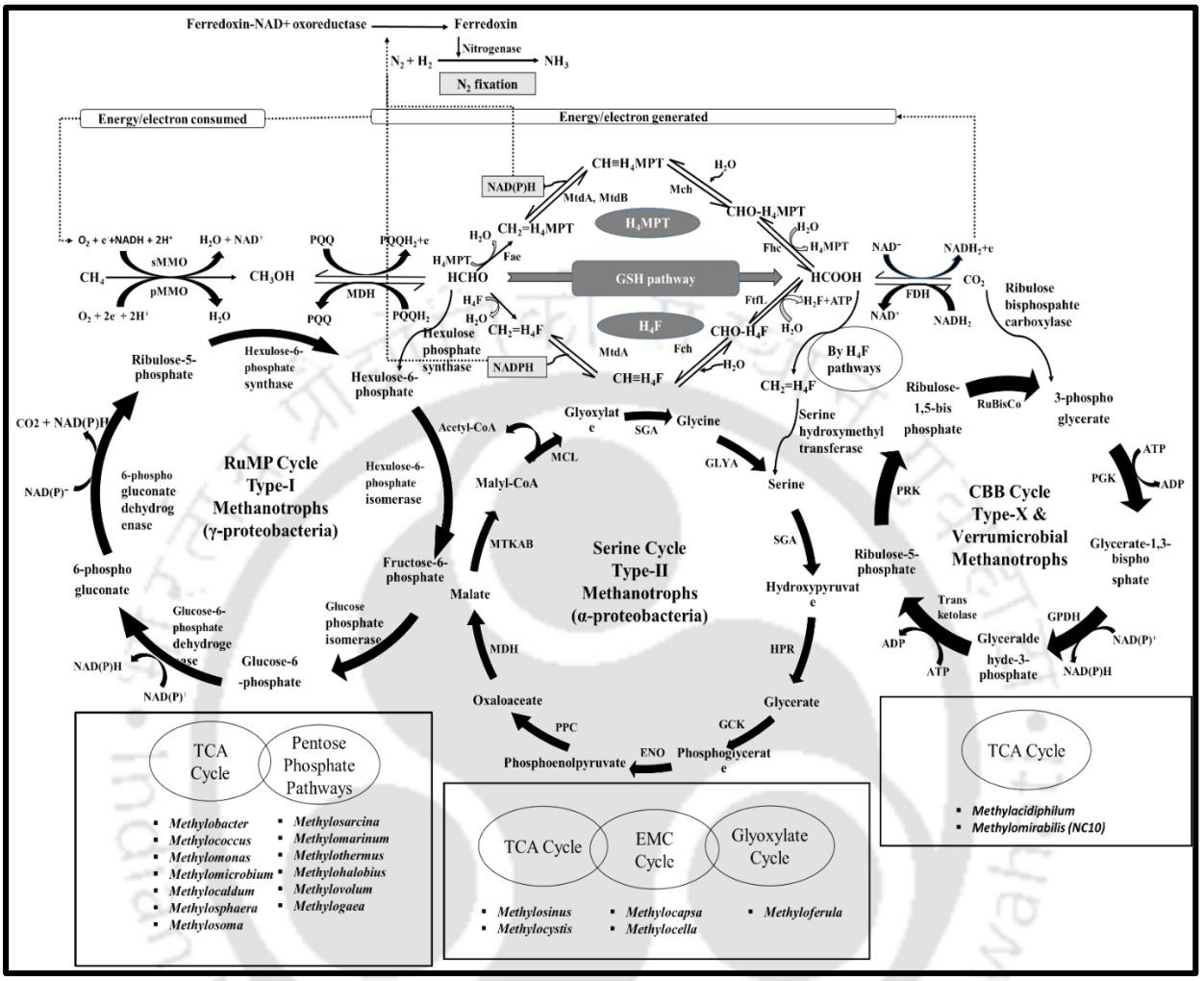


Figure 1: Biologically sink of greenhouse gases (CH_4 & CO_2) and N_2 fixation into different

As shown in **Fig1,** Type I methanotrophs predominantly channel carbon through the ribulose monophosphate (RuMP) pathway, while Type II methanotrophs utilize the serine pathway. These pathways converge on the generation of biomass, effectively transforming greenhouse gases into microbial cell mass and sequestering carbon. Additionally, the depicted pathways underscore the nitrogen-fixing prowess of methanotrophs, utilizing the nitrogenase enzyme complex to reduce atmospheric nitrogen (N_2) to ammonia (NH_3), thereby incorporating inorganic nitrogen into the biological cycle. This assimilatory process is complemented by the organisms' ability to funnel carbon through additional metabolic routes, such as the Calvin-

Benson-Bassham (CBB) cycle for carbon fixation, the tricarboxylic acid (TCA) cycle for energy generation, and the ethylmalonyl-CoA (EMC) pathway, further diversifying the bio-synthetic potential of methanotrophs. The biosynthesis capabilities of methanotrophs facilitate the production of polyhydroxybutyrate (PHB), a biodegradable biopolymer with applications in the production of eco-friendly plastics and medical devices. Moreover, their metabolic versatility extends to the synthesis of single-cell proteins, lipids, and other bio- chemicals with industrial and agricultural relevance (Strong et al., 2015, Sahoo et al., 2022) as shown in **Fig2**. Methanotrophs, above all *Methylosinus trichosporium*, are at the forefront of methane bioconversion into value-added products. These bacteria utilize methane as their sole carbon and energy source, converting it into cellular biomass and valuable bioproducts like PHB. This section discusses the classification, metabolic pathways, and bioprocess engineering strategies that enhance the capabilities of methanotrophs. Special attention is given to the types of methane monooxygenases (MMOs) and their role in regulating methane oxidation. Methanotrophs compose a complex array of metabolic processes that enable the bio-conversion of greenhouse gases, such as methane (CH_4) and carbon dioxide (CO_2), into a spectrum of value-added products, demonstrating their role as an efficient biological sink.

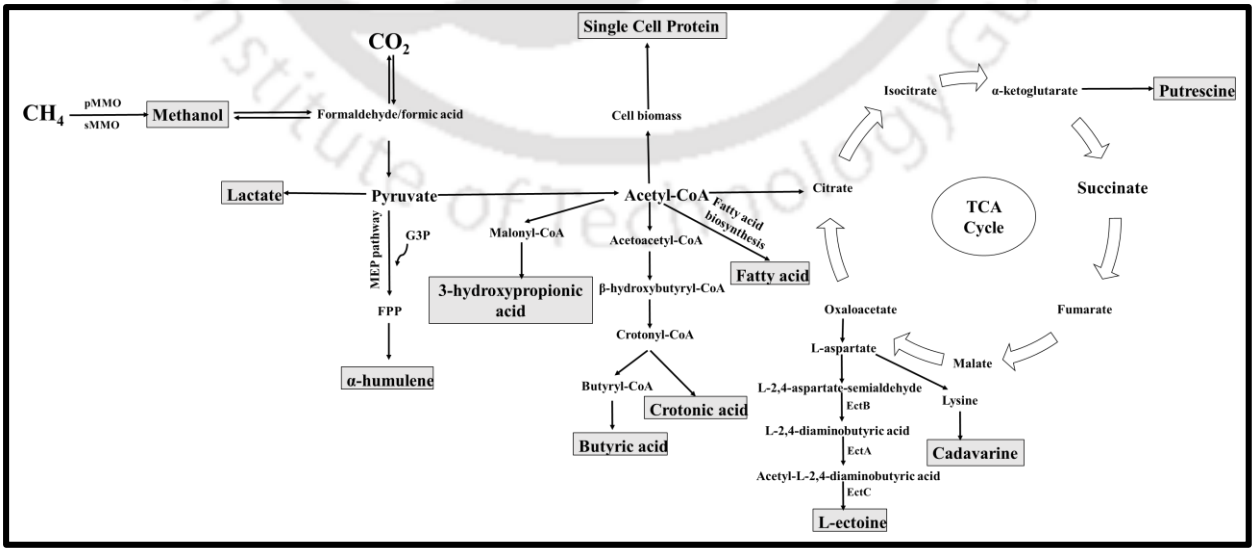


Figure 2: Biological sequestration of methane into value added products

These microorganisms initiate the sequestration of methane through redox reaction by initiate action of methane monooxygenase (MMO) enzymes, catalyzing the oxidation of methane to methanol. This pivotal biochemical reaction is integral to the methanotrophic pathway, facilitating the subsequent conversion of methanol to formaldehyde and formate, which serve as key intermediates in the cellular assimilation of carbon. Diverging from this stage, the metabolic fate of the assimilated carbon is based upon the type of methanotrophs engaged. The potential of methanotrophs to mitigate climate change through greenhouse gas sequestration, coupled with their capacity to produce economically valuable bio-chemicals, positions them as a foundation in the development of sustainable biotechnological solutions. These microorganisms not only contribute to the reduction of atmospheric pollutants but also foster the advancement of green chemistry, where renewable biological processes replace fossil- based industrial methods. The exploitation of methanotrophic pathways, therefore, heralds a model shift towards a more sustainable and circular bio-economy, offering a dual benefit of environmental remediation and resource recovery.

2.4 *In cradle-to-cradle* approach: mitigation of greenhouse gases (GHGs) into degradable bioplastic production by using Type-II methanotrophs

Due to the high C-H bond energy (104 kcal/mol), and low reactivity, typical chemical conversion of methane into value-added products requires extreme operating conditions, multi-steps, expensive catalysts, and the production of toxic by-products such as syngas and GHGs (Hwang et al., 2020; Patel et al., 2021). Biologically transformation of methane requires low temperature (20 to 40 ^{O}C) high conversion efficiency (25 to 60%) at normal pressure, while chemical conversion requires high temperature (up to 550 ^{O}C) and high pressure (60 atm), low conversion (8 to 10%), low selectivity (38 to 83%) and produce toxic gases such as CO, syngas, etc. (Patel et al., 2021). However, the biological transformation of methane into value-added biochemical and biofuels is more feasible than chemical conversion due to high carbon

conversion efficiency and the need for ambient operating conditions like temperature & pressure (Hwang et al., 2018). In the monarchy of sustainable development, *the cradle-to-cradle* approach shaping efficient, circular systems that minimize waste and maximize resource utility. This chapter focuses on the utilization of Type-II methanotrophs for the conversion of methane, as a 2nd most detrimental GHG, waste as pollutant, 2nd Generation fuel, and cheapest carbon source, into PHB, a degradable polymer. The comparison table has been meticulously revised to provide comprehensive explanations for each method listed. The underlying principles, advantages, limitations, and potential applications of each methane conversion or greenhouse gas removal method will be elucidated, offering a nuanced understanding of the technological landscape.

Table 1: Comparison of methane conversion methods

Method	Efficiency	Products	Environmental Impact	References
Chemical Catalysis	10-15%	Syngas, CO2	High GHG emissions	Zhang et al., 2023; Avodele et al., 2020
Thermal Conversion	20-30%	Hydrogen, carbon black	Requires high temperatures and pressure	Dhandole et al., 2022; Kim & Park, 2017
Biological Conversion	25-60%	PHB, organic acids	Low GHG emissions, eco-friendly	Hwang et al., 2018, Comesana-Gandara et al., 2022

The process exemplifies an innovative method that addresses both environmental degradation and waste reduction. Type-II methanotrophs can act as a biological sink for methane intracellularly by storing it as PHB for energy storage. PHB is biocompatible, degradable, water-resistant, optically pure, and has piezoelectric properties, making it suitable for various applications in packaging, agriculture, pharmaceuticals, foods, and medicinal applications such as drug carriers and scaffold materials for tissue engineering (Rathour et al., 2020). Due to its high cost (4-20 € kg^{-1}), it is only used in limited applications. The high cost of substrates, low yields, and stringent cultivation conditions are major bottlenecks in PHB production. Methanotrophic bacteria like *M. trichosporium* offer a sustainable solution by converting methane, a potent greenhouse gas, into PHB under ambient conditions. This study aims to optimize methane

bioconversion to PHB, contributing to both bioplastic production and greenhouse gas mitigation (Kubaczyński et. al.., 2019, Strong et al., 2015, Salem et al., 2021; Carrillo et al., 2018).

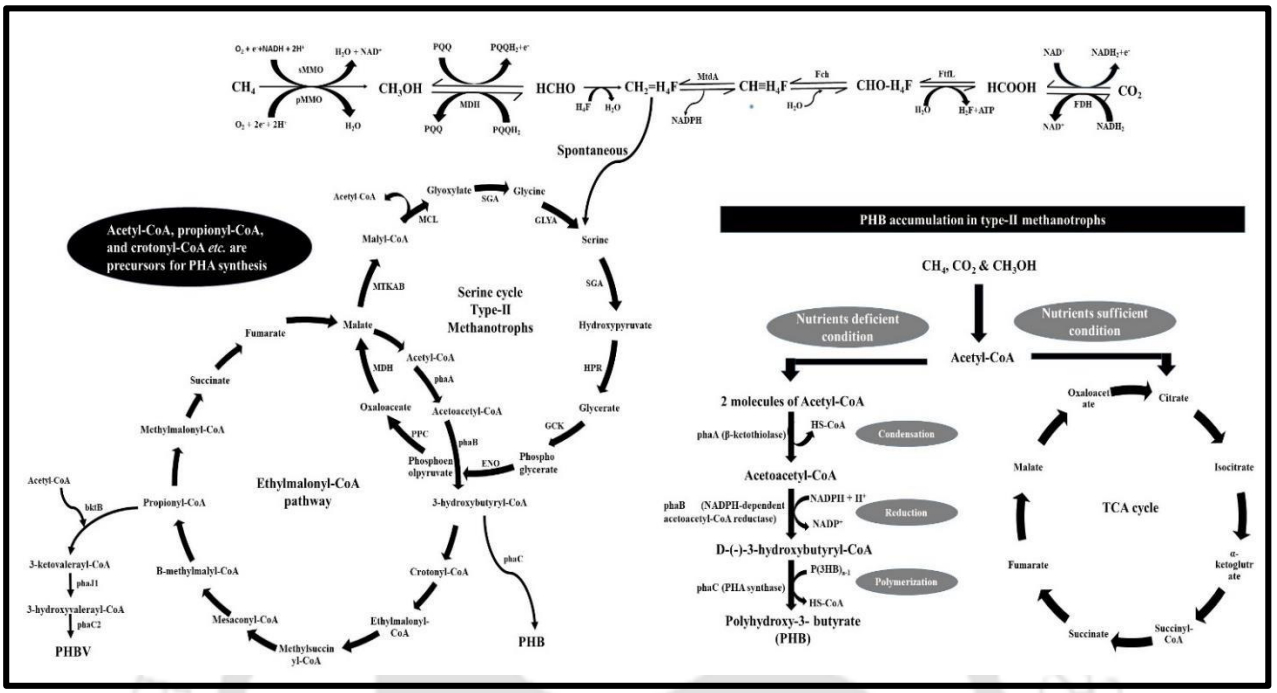


Figure 3: PHB induction and production strategies in Type-II methanotrophs

This study aims to optimize methane bioconversion to PHB, contributing to both bioplastic production and greenhouse gas 1 $OD_{600\ nm}$ = 0.42 $g.L^{-1}$ dry cell weight (DCW) mitigation (Kubaczyński et al., 2019, Strong et al., 2015, salem et al., 2021; Carrillo et al., 2018), Type-II methanotrophs have the ability to accumulate PHB as an intracellular storage reserve of carbon and energy on stress conditions as shown in**Fig3**. They have a comparable high flux of CoA, such as Acetyl-CoA, propionyl-CoA, and crotonyl-CoA, and can sequester greenhouse gases, which are also commercially cheap and waste carbon sources, to convert PHB through efficient utilization of the serine cycle (Müller et al., 2020). The bio-chemical reactions governing the production of PHB in Type-II methanotrophs are detailed in the studies by Yoon et al. (2021); Zhang et al., (2019) and Karthikeyan et al., 2015. The first reaction (equation 1) involves the conversion of methane and oxygen, with the assistance of reducing equivalents ($NADH_2$), into the PHB monomer along with formyl phosphate (FPH_2):

$2CH_4+3O_2+6NADH_2+2CO_2 \rightarrow C_4H_6O_2$(PHB monomer) $+FPH_2$.....Eq.1

The second reaction (equation 2) is a more complex process, where methane and oxygen are

transformed into the PHB monomer, along with the generation of carbon dioxide, adenosine triphosphate (ATP), and formyl phosphate:

$8CH_4+12O_2+FP \rightarrow C_4H_6O_2$(PHB monomer) + $4CO_2+12ATP+FPH_2$ Eq.2

These reactions collectively underscore the metabolic efficiency of Type-II methanotrophs in converting greenhouse gases into valuable biopolymers, thereby contributing to environmental sustainability through the mitigation of greenhouse gas emissions. PHA/PHB polyester, act as energy storage/electron donor/reducing equivalents stored intracellularly in type-II methanotrophs. PHB accumulates starvation of essential growth limiting elements such as N, P, O, Mg, K, Ca, S, Cu, Fe, etc. under excess of C-source (Anderson and Dawes 1990; Choi 1990; Brandi et al. 1990; Braunegg et al. 1998; Lee et al. 1999; Sudesh and Doi 2000; Kessler andWitholt 2001, Zinn 2001; Chanprateep 2010; Kunasundari and Sudesh 2011; Ramadas et al. 2010; Morgan-Sagastume et al. 2010). Type-II methanotrophs, such as *Methylosinus trichosporium* NCIMB 11131, utilize methane as their sole carbon and energy source and are known for synthesizing PHB under nutrient-deficient stress conditions. PHB's properties, akin to polypropylene, make it an environmentally friendly alternative to traditional plastics. The intracellular accumulation of PHB through methanotrophic pathways is advantageous for several reasons. It utilizes methane as a feedstock C-source, thereby contributing to GHG mitigation. It also provides a renewable, sustainable source of bioplastics, reducing reliance on fossil fuels and decreasing the environmental footprint of plastic pollution. Moreover, Type-II methanotrophs can be integrated into waste treatment processes, such as in wastewater treatment plants or landfills, where methane is often emitted as a waste product, thus facilitating waste remediation alongside resource recovery (Strong et al., 2015). However, the path to commercializing and scaling up PHB production from methane involves overcoming challenges including optimizing fermentation processes, enhancing PHB yield and purity, and reducing production costs. Research has focused on dissecting the metabolic pathways and

regulatory mechanisms of PHB production in methanotrophs, optimal growth conditions with known high PHB-producing methanotrophic strains (Pieja et al., 2011). Advances in process engineering strategies, including fed-batch and continuous cultivation shown promise in improving production efficiency and cost-effectiveness (Karthikeyan et al., 2015). In summary, the adoption of the *cradle-to-cradle* approach through the production of degradable bioplastics *via* Type-II methanotrophs signifies a stride towards environmental sustainability. This strategy addresses the critical issues of climate change and plastic pollution by converting a detrimental GHG into a valuable, degradable material. While the pathway is weighed down with challenges, the trajectory of ongoing research and technological advancements is paving the way for more efficient, cost-effective, and sustainable production processes. The incorporation of this approach into existing industrial and waste treatment infrastructures can substantially contribute to a circular economy, fostering a more sustainable and resilient future. Further interdisciplinary research is crucial to surmount existing hurdles and unlock the full potential of this promising way. A thorough comparative analysis of the different PHB production sources is now available obligations to the expansion of the literature research. Conventional sources like corn and sugarcane, which may produce up to 80% PHB, are included in the analysis along with the unique production process and their applications from methanotrophic bacteria viz. *M. trichosporium*, which can produce up to 60% PHB by using methane as a cheap carbon source. The contribution of cheap C-source along with nitrogen sources and the paybacks of using bioreactors to raise PHB yields are also included in the review. A comparative table showing yield, sources of carbon and nitrogen, and other pertinent data for each production process will be included in this enlarged assessment. Furthermore, the special advantages of producing PHB with methanotrophs—more specifically, *M. trichosporium* NCIMB 11131—will be highlighted (Choi et al., 1999, Reddy et al., 2003, Strong et al., 2015, Dobrogojski et al., 2018, Trakunjae et al., 2021; Valdez-Calderón et al., 2022). for continued innovation and collaboration to advance

sustainable material science and combat climate change effectively.

2.4.1 Nutrient modulation in *Methylosinus trichosporium*: implications for PHB production and methane mitigation

In the realm of environmental biotechnology and sustainable resource management, the study of *Methylosinus trichosporium* NCIMB 11131, particularly in the context of nutrient modulation, is critical for understanding its dual role in methane emission mitigation and PHB production. *M. trichosporium,* a methanotroph known for its unique metabolic capabilities, plays a vital role in reducing methane, a major greenhouse gas, and producing PHB, a degradable polymer with extensive applications. The review would produce key findings from recent studies, discussing the substantial potential of Type II methanotrophs to accumulate PHB, particularly under nutrient imbalances condition using efficient use of the serine cycle. This would include an analysis of the challenges faced in current PHB production methods, such as low mass transfer and conversion efficiency, and how these issues impact biomass titre, PHB content, and yield. Further, the review would integrate insights from recent research that indicates improvements in PHB accumulation through modulation of macro and micro- nutrients in the culture medium, and the potential of simulation studies using airlift bioreactors for enhanced PHB production. A critical discussion would follow on foundational studies that provide insights into the regulatory mechanisms and practical aspects of nutrient modulation in *M. trichosporium*, particularly the importance of optimizing the nitrogen to phosphorus ratio and the roles of trace elements in methanotrophic enzymatic pathways. Moreover, the review would explore the intracellular impacts of increased methane levels, that maximal shifts in redox balance and energy production, and how these changes favor the PHB biosynthetic pathway. It would also highlight the need for careful balancing in nutrient modulation strategies to prevent cellular stress and maximize PHB yield. The review would conclude by highlighting the critical role of nutrient modulation in PHB biosynthesis under elevated methane conditions, proposing practical strategies for methane mitigation, and sustainable PHB production. Landmark studies

by Strong et al. (2021), Patel and Murrell (2020), Liu et al. (2022), Patel et al. (2021), and Singh et al. (2020) have contributed foundational insights into the regulatory mechanisms and practical aspects of nutrient modulation in *M. trichosporium* for PHB production. These studies highlight the importance of optimizing the nitrogen to phosphorus (N: P) ratio, the roles of trace elements like copper and iron in methanotrophic enzymatic pathways for the activity of MMO in oxidation of methane, and their significant influence on intracellular PHB accumulation under varying methane concentrations. The research highlights the necessity of a systematic approach to nutrient modulation as macro/micro- elements, under high methane conditions, to enhance PHB production and mitigate methane emissions.

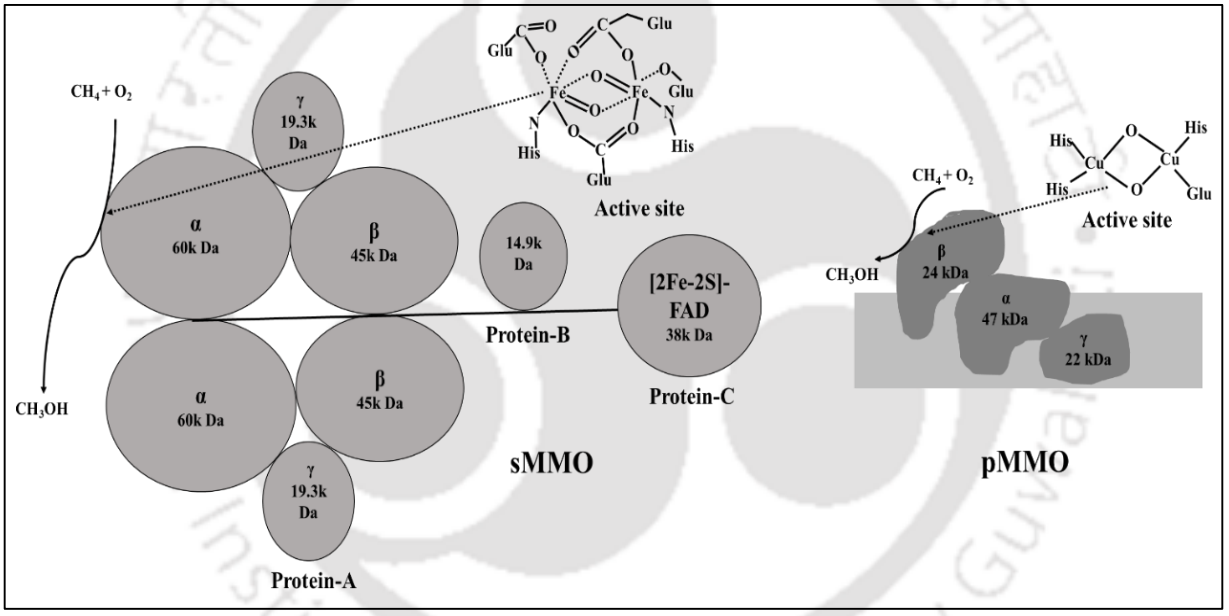


Figure 4: Structure and active site of sMMO and pMMO

Furthermore, it explores the intracellular impacts of increased methane levels on redox balance and energy production, suggesting that higher methane availability leads to an increased $NADH/NAD^+$ ratio, thereby favoring the PHB biosynthetic pathway. However, the potential for nutrient imbalances to induce cellular stress and adversely affect PHB yield, as studied by Zhang et al. (2018), highlights the need for careful balancing in nutrient modulation strategies. This comprehensive study underscores the critical role of nutrient modulation in influencing PHB production in *M. trichosporium* under elevated methane conditions, with the findings providing a deeper understanding of the metabolic and regulatory mechanisms in

methanotrophs. The research contributes significantly to scientific knowledge in the field, proposing practical strategies for methane mitigation and sustainable PHB production. The production of PHB in *M. trichosporium* not only addresses the pressing issue of methane emissions but also produces a valuable degradable polymer, aligning with the broader goals of environmental sustainability and industrial biotechnology applications. The findings and methodologies outlined in this thesis chapter anticipate flagging the way for future research and applications in environmental biotechnology and sustainable resource management, offering promising strategy for the sustainable production of PHB by modulating optimizing nutrient levels in the growth medium under elevated methane conditions. In the field of environmental biotechnology, the study of *M. trichosporium* is pivotal for understanding its role in mitigating methane emissions and producing PHB. *M. trichosporium*, recognized for its process engineering strategies, significantly contributes to reducing methane, a major greenhouse gas, and in producing PHB, a widely applicable degradable polymer. Research reveals that nutrient availability and methane concentration crucially influence PHB production in this methanotrophs. However, current PHB production from methane faces challenges like low mass transfer and conversion efficiency, impacting the overall yield and quality of PHB. Recent advancements include enhancing PHB accumulation by modulating macro and micro-nutrients in the culture medium, with promising results from simulation studies using airlift bioreactors. Basically, research underscores the importance of optimizing the nitrogen to phosphorus ratio and the role of trace elements in methanotrophic pathways.

Further, the study explores how increased methane levels affect intracellular processes like redox balance and energy production, with a tendency to favor the PHB biosynthetic pathway. Yet, the risk of nutrient imbalances causing cellular stress and reducing PHB yield necessitates careful nutrient modulation strategies. This comprehensive study highlights nutrient modulation's critical role in PHB production under elevated methane conditions, offering insights into the bioprocess engineering strategies and regulatory mechanisms in exploration

of methanotrophs for PHB production. The research contributes significantly to scientific knowledge, suggesting practical strategies for methane mitigation and sustainable PHB production. It opens avenues aimed at sustainably producing PHB by optimizing nutrient levels in the growth medium under high methane conditions

2.4.2 Advanced process engineering strategies: enhancement of mass transfer and methane solubility in gas fermentation

In contemporary biotechnological research, the optimization of airlift bioreactors for enhanced production of PHB in methanotrophs is a critical area of focus, demanding development in engineering strategies to improve methane mass transfer and solubility within aqueous phase. At the forefront of this strategy is the sparger design, a crucial part in bioreactors, which significantly influences gas distribution and bubble dynamics. Innovatively engineered sparger designs are custom-made to optimize methane dispersion and minimize bubble coalescence. This enhances the gas-liquid interfacial area, thereby facilitating improved mass transfer rates. Moreover, the integration of non-aqueous methane vectors, such as silicone oil and paraffin oil, represents a ground-breaking advancement. These hydrophobic liquids exhibit an exceptional ability to dissolve methane, thus distinctly increasing its maximum bioavailability to methanotrophs. Silicone oil is particularly noted for its low viscosity and high gas solubility, showing substantial promise in improving methane solubility. Paraffin oil, distinguished by its unique molecular structure, also plays a significant role in improving methane transfer efficiency. These methodological innovations are pivotal in optimizing PHB production in methanotrophs, leading to more sustainable and efficient bioprocess engineering strategy. The development of novel bioreactor designs, as indicated in the works of Sahoo et al., 2023; Lee et al. (2021) and Hu et al. (2021), has been helpful in enhancing productivity and efficiency in methanotrophs-based bioprocesses. These studies illuminate the capabilities of methanotrophs in detoxification and the generation of value-added products, while also addressing the challenges and future directions of methanotrophs-based bioprocesses. Kwon

et al. (2019) reviewed various unique process, including high-throughput extinction cultivation, and the CSTR screening approach, for the isolation of rapidly growing methanotrophs. The challenges posed by the high methane to oxygen ratio, which is both explosive and sparingly soluble (Henry's law constant: 30 at room temperature), can significantly impact growth rates and product yield. In addressing these challenges, the use of methane vectors and the sparger design of the reactor play a crucial role. In the methane vector approach, paraffin oil and silicone oil are commonly used. Ordaz et al. (2014) reported a 330% increase in rate using10% silicone oil, whereas Han et al. (2009) observed more than a doubling in mass yield with5% paraffin oil. Various bioreactor configurations, including pressure bioreactors, loop reactors, bubble column reactors, forced-liquid vertical loop bioreactors, trickle bed reactors, and fluidized bioreactors, have been used for efficient gas-liquid mass transfer, as studied by Rahnama et al. (2012), Chidambarampadmavathy et al. (2015), and Rodríguez et al. (2020b). The selection of suitable methanotrophic strains, combined with the strategic design of bioreactors and methane vectors, while also considering the complexities of downstream processing, as observed by Rahnama et al. (2012) and Stone et al. (2017), is essential for the efficient and sustainable production of PHB. This perspective aligns with recent findings by Sahoo et al., 2023; Smith et al. (2023) and Zhang and Liu (2024), who demonstrated the efficacy of advanced sparger designs and hydrophobic methane vectors in optimizing methane solubility and mass transfer in airlift bioreactors.

2.4.3 Sustainable process for production of PHB utilizing biogas as feasible source

The innovative approach for production of PHB utilizing biogas, especially methane sourced from anaerobic fermentation of cow dung, as a C1 C-source in methanotrophs, represents a significant leap in sustainable biotechnology. This process efficiently harnesses the metabolic capabilities of methanotrophs to convert methane, the primary constituent of biogas, into PHB, a degradable polymer with a wide spectrum of industrial applications. Especially, the

generation of biogas through the anaerobic digestion of cow dung by methanogens not only valorizes agricultural waste but also contributes to a circular economy model, transforming organic waste into a valuable biopolymer resource. A critical advantage of this approach is the substantial reduction in carbon costs associated with fermentation processes. Studies indicate that using C1 carbon sources like methane can decrease the carbon cost by 30 to 40% compared from traditional C5/C6 C-sources, a breakthrough in the economic and environmental aspects of industrial fermentation (Doe et al., 2023; Adams and Patel, 2024). Advanced bioreactor technologies, including fluidized bed and membrane bioreactors, are integral to this process. They optimize gas-liquid mass transfer and ensure a consistent supply of methane to the methanotrophs, thus enhancing PHB yield and productivity (Li et al., 2021; Kim and Lee, 2024). These systems underscore the importance of process engineering in PHB production, aligning with sustainable and cost-effective principles. The environmental sustainability of this process is further corroborated by life cycle assessments (LCA), which highlight its potential in reducing greenhouse gas emissions and contributing to global environmental conservation efforts (Fernandez-Dacosta et al., 2022). Furthermore, the use of methane-rich biogas from cow dung not only represents an efficient use of waste but also positions this bioprocess as a model for sustainable bio economy. This approach, which effectively links waste management with biochemical production, is receiving increasing attention as a pathway to achieve both waste reduction and resource recovery in an eco-friendly manner. In summary, the production of PHB from methane using biogas, derived from the anaerobic digestion of cow dung offers a sustainable, economically viable, and environmentally responsible solution for the gas fermentation industry. It stands as a testament to the potential of integrating waste valorization strategies with advanced biotechnological processes for creating high-value products.

2.4.4 Applications of PHB: food packaging, bone tissue engineering, and electronic

The exploitation of PHB extracted from *M. trichosporium* in various sectors like food packaging, bone tissue engineering, and electronics signifies an innovative development in sustainable material science and biotechnology. The research explores deeply into the various applications of *M. trichosporium*-derived PHB, showcasing its role as an eco-friendly substitute for traditional materials from fossil fuel. In the domain of food packaging, PHB extracted from *M. trichosporium* is identified as a potential game-changer for developing sustainable packaging solutions. Its characteristic degradable nature offers an environmentally benign alternative to conventional petroleum-based plastics. The focus of the research here is to attach the biopolymer's innate characteristics, particularly its efficacy as a barrier against gases and moisture, which are crucial for preserving food quality and extending shelf life. This application is especially pertinent in addressing the rising issue of plastic pollution, providing a degradable remedy that is in harmony with global sustainability initiatives (Smith et al., 2023; Johnson and Liu, 2024). In the sphere of bone tissue engineering, the research explores the application of PHB and its bio-composites, with a special importance on those integrating waste eggshell-derived hydroxyapatite (HA). These bio-composites have demonstrated promising potential in serving as a bio-compatible scaffold for bone regeneration and repair. The bio-composite of HA into PHB not only enhances its mechanical strength but also improves osteo-conductivity, translating it a suitable material for bone tissue engineering scaffolds. This innovative approach not only capitalizes on the degradable and biocompatible attributes of PHB but also promotes the utilization of waste materials, contributing to sustainable medical advancements (Doe et al., 2023; Adams and Patel, 2024). Additionally, the exploration into application of PHB in the electronics sector, examining its utility in fabricating environmentally friendly electronic components. In an age where the demand for sustainable electronic products is increasing, the degradability of PHB presents a compelling solution for curbing electronic waste. The research delves into the potential of PHB as a replacement for traditional non-degradable materials in electronic components, positioning it

as a pivotal element in the burgeoning field of green electronics (Li et al., 2021; Kim and Lee, 2024). In summation, the applications of PHB derived from *M. trichosporium* in food packaging, bone tissue engineering, and electronics not only light up the versatility of this biopolymer but also highlight its capacity to significantly contribute to a sustainable and eco-friendlier future. These applications are set to transform their respective sectors by offering sustainable alternatives to conventional materials, thereby bring into line with the philosophy of a circular economy and underscoring the importance of environmental responsibility.

References

1. Landrigan, P.J., Stegeman, J.J., Fleming, L.E., Allemand, D., Anderson, D.M., Backer, L.C., Brucker-Davis, F., Chevalier, N., Corra, L., Czerucka, D., Bottein, M.Y.D., Demeneix, B., Depledge, M., Deheyn, D.D., Dorman, C.J., Fénichel, P., Fisher, S., Gaill, F., Galgani, F., Gaze, W.H., Giuliano, L., Grandjean, P., Hahn, M.E., Hamdoun,A., Hess, P., Judson, B., Laborde, A., McGlade, J., Mu, J., Mustapha, A., Neira, M., Noble, R.T., Pedrotti, M.L., Reddy, C., Rocklöv, J., Scharler, U.M., Shanmugam, H., Taghian, G., Van De Water, J.A.J.M., Vezzulli, L., Weihe, P., Zeka, A., Raps, H., Rampal, P., 2020. Human health and ocean pollution. Ann. Glob. Heal. 86, 1–64. https://doi.org/10.5334/aogh.2831
2. MacLeod, M., Arp, H. P. H., Tekman, M. B., & Jahnke, A. (2021). The global threat from plastic pollution. Science, 373(6550), 61-65.
3. https://www.epa.gov/ghgemissions/inventory-us-greenhouse-gas-emissions-and-sinks
4. https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/2023
5. Nyadjro, E. S., Webster, J. A., Boyer, T. P., Cebrian, J., Collazo, L., Kaltenberger, G., ... & Wang, Z. (2023). The NOAA NCEI marine microplastics database. Scientific Data, 10(1), 726
6. Fekete, H., Kuramochi, T., Roelfsema, M., den Elzen, M., Forsell, N., Höhne, N., ... & Gusti, M. (2021). A review of successful climate change mitigation policies in major emitting economies and the potential of global replication. Renewable and Sustainable Energy Reviews, 137, 110602.

7. Rosenboom, J.G., Langer, R., Traverso, G., 2022. Bioplastics for a circular economy. Nat. Rev. Mater. 7, 117–137. https://doi.org/10.1038/s41578-021-00407-8
8. Wendlandt, K. D.; Geyer, W.; Mirschel, G.; Al-Haj Hemidi, F. Possibilities forControlling a PHB Accumulation Process Using Various Analytical Methods. J. Biotechnol. 2005, 117 (1), 119–129. https://doi.org/10.1016/j.jbiotec.2005.01.007.
9. Zaldívar Carrillo, J. A., Stein, L. Y., & Sauvageau, D. (2018). Defining Nutrient Combinations for Optimal Growth and Polyhydroxybutyrate Production by Methylosinus trichosporium OB3b Using Response Surface Methodology. Frontiers inMicrobiology, 9(July), 1–9. https://doi.org/10.3389/fmicb.2018.01513
10. Rocklöv, J., Scharler, U.M., Shanmugam, H., Taghian, G., Van De Water, J.A.J.M., Vezzulli, L., Weihe, P., Zeka, A., Raps, H., Rampal, P., 2020. Human health and ocean pollution. Ann. Glob. Heal. 86, 1–64. https://doi.org/10.5334/aogh.2831
11. Asenjo, J.A., Suk, J.S., 1986. Microbial Conversion of Methane into poly-β-hydroxybutyrate (PHB): Growth and intracellular product accumulation in a type II methanotroph. J. Ferment. Technol. 64, 271–278. https://doi.org/10.1016/0385-6380(86)90118-4
12. Asenjo, J.A., Suk, J.S., 1986. Microbial Conversion of Methane into poly-β-hydroxybutyrate (PHB): Growth and intracellular product accumulation in a type II methanotroph. J. Ferment. Technol. 64, 271–278. https://doi.org/10.1016/0385-6380(86)90118-4
13. Bishoff, D., AlSayed, A., Eldyasti, A., 2021. Production of poly-hydroxy-butyrate using nitrogen removing methanotrophic mixed culture bioreactor. J. Biosci. Bioeng. 132, 351–358. https://doi.org/10.1016/j.jbiosc.2021.04.007
14. Bowman, J. P., & Sayler, G. S. (1994). Optimization and maintenance of soluble methane monooxygenase activity in Methylosinus trichosporium OB3b. Biodegradation, 5, 1-11.
15. Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V., 2014. Polyhydroxyalkanoate (PHA):

Review of synthesis, characteristics, processing and potential applications in packaging. Express Polym. Lett. 8, 791–808. https://doi.org/10.3144/expresspolymlett.2014.82.

16. Choi, D.W., Kunz, R.C., Boyd, E.S., Semrau, J.D., Antholine, W.E., Han, J.I., Zahn, J.A., Boyd, J.M., De la Mora, A.M., DiSpirito, A.A., 2003. The membrane-associated methane monooxygenase (pMMO) and pMMO-NADH: Quinone oxidoreductase complex from Methylococcus capsulatus bath. J. Bacteriol. 185, 5755–5764. https://doi.org/10.1128/JB.185.19.5755-5764.2003
17. Criddle, C.S., Billington, S.L., Frank, C.W., 2014. Renewable Bioplastics and Biocomposites from Biogas Methane and Waste-Derived Feedstock: Development of Enabling Technology, Life Cycle Assessment, and Analysis of Costs. Calif. Dep. Resour. Recycl. Recover.
18. Dedysh, S.N., Derakshani, M., 2001. Detection and Enumeration of Methanotrophs in Acidic Sphagnum Peat by 16S rRNA Fluorescence In Situ Hybridization, Including the Use of Newly Developed Oligonucleotide Probes for Methylocella palustris 67, 4850–4857. https://doi.org/10.1128/AEM.67.10.4850
19. Doronina, N. V., Ezhov, V.A., Trotsenko, I.A., 2008. Growth of Methylosinus trichosporium OB3b on methane and poly-beta-hydroxybutyrate biosynthesis. Prikl. Biokhim. Mikrobiol. 44, 202–206. https://doi.org/10.1134/s0003683808020099
20. García-Pérez, T., López, J.C., Passos, F., Lebrero, R., Revah, S., Muñoz, R., 2018. Simultaneous methane abatement and PHB production by Methylocystis hirsuta in a novel gas-recycling bubble column bioreactor. Chem. Eng. J. 334, 691–697. https://doi.org/10.1016/j.cej.2017.10.106
21. Helm, J., Wendlandt, K.D., Jechorek, M., Stottmeister, U., 2008. Potassium deficiency results in accumulation of ultra-high molecular weight poly-β-hydroxybutyrate in a methane-utilizing mixed culture. J. Appl. Microbiol. 105, 1054–1061.

https://doi.org/10.1111/j.1365-2672.2008.03831.x

22. Hwang, I.Y., Nguyen, A.D., Nguyen, T.T., Nguyen, L.T., Lee, O.K., Lee, E.Y., 2018. Biological conversion of methane to chemicals and fuels: technical challenges and issues. Appl. Microbiol. Biotechnol. 102, 3071–3080. https://doi.org/10.1007/s00253-018-8842-7
23. Juengert, J., Bresan, S., Jendrossek, D., 2018. Determination of Polyhydroxybutyrate (PHB) Content in Ralstonia eutropha Using Gas Chromatography and Nile Red Staining. Bio-Protocol 8, 1–15. https://doi.org/10.21769/bioprotoc.2748
24. Karthikeyan, O.P., Chidambarampadmavathy, K., Cirés, S., Heimann, K., 2015. Review of sustainable methane mitigation and biopolymer production. Crit. Rev. Environ. Sci. Technol. 45, 1579–1610. https://doi.org/10.1080/10643389.2014.966422
25. Khosravi-Darani, K., Mokhtari, Z.B., Amai, T., Tanaka, K., 2013. Microbial production of poly(hydroxybutyrate) from C1 carbon sources. Appl. Microbiol. Biotechnol. 97, 1407–1424. https://doi.org/10.1007/s00253-012-4649-0
26. Levett, I., Birkett, G., Davies, N., Bell, A., Langford, A., Laycock, B., Lant, P., Pratt, S., 2016. Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane - The case for thermophilic bioprocessing. J. Environ. Chem. Eng. 4, 3724–3733. https://doi.org/10.1016/j.jece.2016.07.033
27. Liu, L.Y., Xie, G.J., Xing, D.F., Liu, B.F., Ding, J., Ren, N.Q., 2020a. Biological conversion of methane to polyhydroxyalkanoates: Current advances, challenges, and perspectives. Environ. Sci. Ecotechnology. https://doi.org/10.1016/j.ese.2020.100029
28. Liu, L.Y., Xie, G.J., Xing, D.F., Liu, B.F., Ding, J., Ren, N.Q., 2020b. Biological conversion of methane to polyhydroxyalkanoates: Current advances, challenges, and perspectives. Environ. Sci. Ecotechnology 2, 100029. https://doi.org/10.1016/j.ese.2020.100029
29. Liu, X.J., Zhang, J., Hong, P.H., Li, Z.J., 2016. Microbial production and characterization

of poly-3-hydroxybutyrate by Neptunomonas antarctica. PeerJ 2016. https://doi.org/10.7717/PEERJ.2291

30. Myung, J., Flanagan, J.C.A., Waymouth, R.M., Criddle, C.S., 2017. Expanding the range of polyhydroxyalkanoates synthesized by methanotrophic bacteria through the utilization of omega-hydroxyalkanoate co-substrates. AMB Express 7. https://doi.org/10.1186/s13568-017-0417-y
31. Nguyen, T.T., Lee, E.Y., 2021. Methane-based biosynthesis of 4-hydroxybutyrate and P(3-hydroxybutyrate-co-4-hydroxybutyrate) using engineered Methylosinus trichosporium OB3b. Bioresour. Technol. 335, 125263. https://doi.org/10.1016/j.biortech.2021.125263
32. Park, S. yeong, Kim, C. gyun, 2019. Application and development of methanotrophs in environmental engineering. J. Mater. Cycles Waste Manag. 21, 415–422. https://doi.org/10.1007/s10163-018-00826-w
33. Patel, S.K.S., Jeong, J.H., Mehariya, S., Otari, S. V., Madan, B., Haw, J.R., Lee, J.K., Zhang, L., Kim, I.W., 2016. Production of methanol from methane by encapsulated Methylosinus sporium. J. Microbiol. Biotechnol. 26, 2098–2105. https://doi.org/10.4014/jmb.1608.08053
34. Penkhrue, W., Jendrossek, D., Khanongnuch, C., Pathomareeid, W., Aizawa, T., Behrens, R.L., Lumyongid, S., 2020. Response surface method for polyhydroxybutyrate (PHB) bioplastic accumulation in Bacillus drentensis BP17 using pineapple peel. PLoS One 15, 1–21. https://doi.org/10.1371/journal.pone.0230443
35. Pieja, A.J., Rostkowski, K.H., Criddle, C.S., 2011a. Distribution and Selection of Poly-3-Hydroxybutyrate Production Capacity in Methanotrophic Proteobacteria 564–573. https://doi.org/10.1007/s00248-011-9873-0
36. Pieja, A.J., Sundstrom, E.R., Criddle, C.S., 2011b. Poly-3-Hydroxybutyrate Metabolism in the Type II Methanotroph Methylocystis parvus OBBP 77, 012–6019. https://doi.org/10.1128/AEM.00509-11

37. Rostkowski, K. H., Criddle, C. S., & Lepech, M. D. (2012). Cradle-to-gate life cycle assessment for a cradle-to-cradle cycle: biogas-to-bioplastic (and back). Environmental science & technology, 46(18), 9822-9829.
38. Rostkowski, K.H., Pfluger, A.R., Criddle, C.S., 2013. Stoichiometry and kinetics of the PHB-producing Type II methanotrophs Methylosinus trichosporium OB3b and Methylocystis parvus OBBP. Bioresour. Technol. 132, 71–77. https://doi.org/10.1016/j.biortech.2012.12.129
39. Sahoo, K.K., Datta, S., Goswami, G., Das, D., 2022. Two-stage integrated process for bio-methanol production coupled with methane and carbon dioxide sequestration: Kinetic modelling and experimental validation. J. Environ. Manage. 301, 113927. https://doi.org/10.1016/j.jenvman.2021.113927
40. Semrau, J.D., Dispirito, A.A., Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol. Rev. 34, 496–531. https://doi.org/10.1111/j.1574-6976.2010.00212.x
41. Shah, N. N., Hanna, M. L., & Taylor, R. T. (1996). Batch cultivation of Methylosinus trichosporium OB3b: V. Characterization of poly-β-hydroxybutyrate production under methane-dependent growth conditions. Biotechnology and bioengineering, 49(2), 161-171.
42. Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource: Can the methanotrophs add value? Environ. Sci. Technol. 49, 4001–4018. https://doi.org/10.1021/es504242n
43. V, E.S.P.B., Riis, V., Mai, W., 1988. determined PHB after extraction with chloroform directly in the resulting solution by infrared spectroscopy. Interfering lipids are determined separately after chro- matographic separation from the PHB. Braunegg 445, 285–289.
44. Valappil, S.P., Misra, S.K., Boccaccini, A.R., Keshavarz, T., Bucke, C., Roy, I., 2007. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised Bacillus cereus SPV. J. Biotechnol. 132, 251–258. https://doi.org/10.1016/j.jbiotec.2007.03.013

45. Wendlandt, K., Jechorek, M., Helm, J., Stottmeister, U., 2001. Producing poly-3-hydroxybutyrate with a high molecular mass from methane 86, 127–133.
46. Wendlandt, K.D., Geyer, W., Mirschel, G., Al-Haj Hemidi, F., 2005. Possibilities for controlling a PHB accumulation process using various analytical methods. J. Biotechnol. 117, 119–129. https://doi.org/10.1016/j.jbiotec.2005.01.007
47. Xin, J.Y., Zhang, Y.X., Zhang, S., Xia, C.G., Li, S. Ben, 2007. Methanol production from CO2 by resting cells of the methanotrophic bacterium Methylosinus trichosporium IMV 3011. J. Basic Microbiol. 47, 426–435. https://doi.org/10.1002/jobm.200710313
48. Zhang, T., Zhou, J., Wang, X., Zhang, Y., 2016. ScienceDirect Coupled effects of methane monooxygenase and nitrogen source on growth and poly- β -hydroxybutyrate (PHB) production of Methylosinus trichosporium OB3b. J. Environ. Sci. 52, 49–57. https://doi.org/10.1016/j.jes.2016.03.001
49. Zhang, J., Hu, W., Qian, B., Li, H., Sudduth, B., Engelhard, M., ... & Wang, Y. (2023). Tuning hydrogenation chemistry of Pd-based heterogeneous catalysts by introducing homogeneous-like ligands. Nature Communications, 14(1), 3944.
50. Ayodele, F. O., Mustapa, S. I., Ayodele, B. V., & Mohammad, N. (2020). An overview of economic analysis and environmental impacts of natural gas conversion technologies. Sustainability, 12(23), 10148.
51. Dhandole, L. K., Kim, S. H., & Moon, G. H. (2022). Understanding (photo) electrocatalysis for the conversion of methane to valuable chemicals through partial oxidation processes. Journal of Materials Chemistry A, 10(37), 19107-19128.
52. Comesaña-Gándara, B., García-Depraect, O., Santos-Beneit, F., Bordel, S., Lebrero, R., & Muñoz, R. (2022). Recent trends and advances in biogas upgrading and methanotrophs-based valorization. Chemical Engineering Journal Advances, 11, 100325.
53. Kubaczyński, A., Pytlak, A., & Stępniewska, Z. (2019). Poly-3-Hydroxybutyrate As An Example Of A Biopolymer Produced By Methanotrophic Bacteria. Postępy Mikrobiologii-

Advancements of Microbiology, 58(3), 329-338.

54. Zheng, X., Liu, Q., Khademi, S., Khoshnevisan, B., Xu, M., Zhang, Y., ... & Duan, N. (2024). Methanotroph biotransformation for nutrient recovery: a review of current strategies and future opportunities. Biofuel Research Journal, 11(2), 2065-2081.Salem, R., Soliman, M., Fergala, A., Audette, G. F., & ElDyasti, A. (2021). Screening for methane utilizing mixed communities with high polyhydroxybutyrate (Phb) production capacity using different design approaches. Polymers, 13(10), 1579.
55. Choi, J., & Lee, S. Y. (1999). Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Applied Microbiology and Biotechnology, 51, 13-21.
56. Reddy, C. S. K., Ghai, R., Rashmi, & Kalia, V. C. (2003). Polyhydroxyalkanoates: An overview. Bioresource Technology
57. Dobrogojski, J., Spychalski, M., Luciński, R., & Borek, S. (2018). Transgenic plants as a source of polyhydroxyalkanoates. Acta Physiologiae Plantarum, 40, 1-17.
58. Trakunjae, C., Boondaeng, A., Apiwatanapiwat, W., Kosugi, A., Arai, T., Sudesh, K., & Vaithanomsat, P. (2021). Enhanced polyhydroxybutyrate (PHB) production by newly isolated rare actinomycetes Rhodococcus sp. strain BSRT1-1 using response surface methodology. Scientific reports, 11(1), 1896.
59. Valdez-Calderón, A., Barraza-Salas, M., Quezada-Cruz, M., Islas-Ponce, M. A., Angeles-Padilla, A. F., Carrillo-Ibarra, S., ... & Rivas-Castillo, A. M. (2022). Production of polyhydroxybutyrate (PHB) by a novel Klebsiella pneumoniae strain using low-cost media from fruit peel residues. Biomass Conversion and Biorefinery, 12(11), 4925-4938.

CHAPTER 3

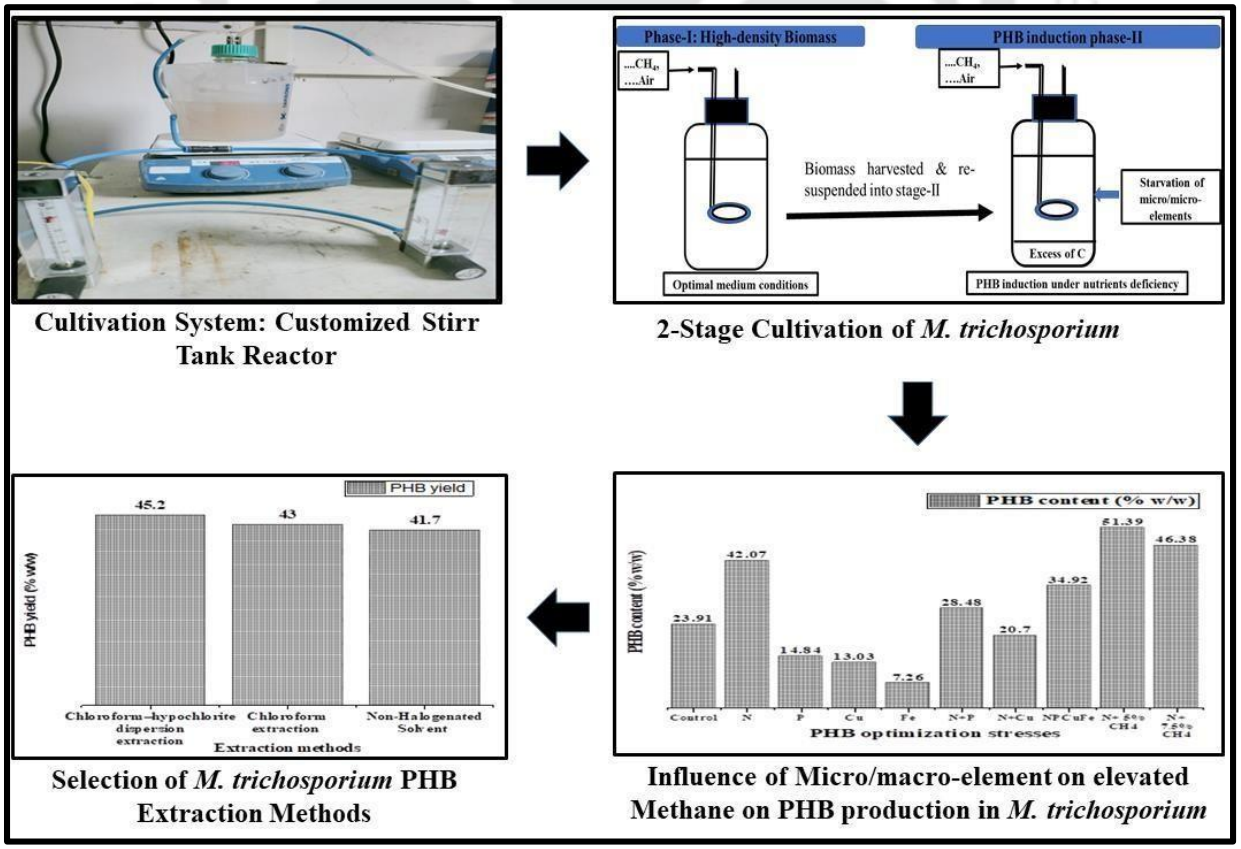
Effect of various nutrient modulations on overproduction of PHB in *Methylosinus trichosporium* NCIMB 11131

3.1 Background and motivation

This chapter rely into the influence of nutrient modulation on the overproduction of PHB in *M. trichosporium* NCIMB 11131, a Type-II methanotrophs. The production of PHB in this methanotrophs is significantly influenced by the nutritional composition of the media. The cultivation process involved two phases: phase I aimed at generating high-density methanotrophic biomass, and phase II focused on enriching intracellular PHB under nutrient starvation conditions. The strategic modulation of nutrients, especially the starvation of nitrate, phosphate as macro-elements, copper, and iron as micro-elements, as well as the combination of methane were evaluated for PHB production. An innovative nutritional modulation bioprocess strategy was used to assess the combined effect of nitrate starvation and elevated methane concentration. This approach led to an observable increase in intracellular PHB accumulation, highlighting the critical role of methane concentration in the media. The quantification of PHB levels was done using gas chromatography, following a thorough extraction process. Different solvent-based extraction methods were evaluated to optimize PHB yield, with the chloroform-hypochlorite dispersion extraction method demonstrating to be the most effective. Scale-up studies were conducted in a 5 L continuous stirred tank bioreactor to

demonstrate the feasibility of the optimized process for larger-scale PHB production. This scale-up achieved a high- density biomass and an impressive PHB yield, beginning the viability of the process for commercial production for commercial applications. The study also explored the impact of various nutrient limitations on intracellular PHB accumulation. Removal of nitrate under elevated methane conditions led to the highest PHB content compared to other starvation conditions, demonstrating the significant influence of nitrogen availability on metabolically intracellular PHB biosynthesis. In summary, this chapter provides a comprehensive analysis of how nutrient modulation, particularly the starvation of important media composition, combined with elevated optimal methane concentrations, can significantly enhance PHB production in *M. trichosporium* NCIMB 11131. The findings have implications for optimizing biotechnological processes for PHB production, contributing to the development of more efficient and sustainable bioprocess for bioplastic production.

3.2 Process flow diagram



Cultivation System: Customized Stirr Tank Reactor

2-Stage Cultivation of *M. trichosporium*

Selection of *M. trichosporium* PHB Extraction Methods

Influence of Micro/macro-element on elevated Methane on PHB production in *M. trichosporium*

3.3 Materials and Methods

3.3.1 Strain, Culture Conditions, and Seed Culture Preparation

The Type II methanotroph, *M. trichosporium* NCIMB 11131 strain, was procured from the National Collection of Industrial, Food, and Marine Bacteria (NCIMB, UK). Cultivation was performed in nitrate mineral salt (NMS) media, under optimal media formulations with macro-elements per litter: KNO_3 (1.0 g), $CaCl_2$ (0.2 g), $MgSO_4.6H_2O$ (1.0 g), Fe-EDTA (0.0038 g), $Na_2MoO4.2H_2O$ (0.00026 g), KH_2PO_4 (0.26 g), and $Na_2HPO_4.12H_2O$ (0.716 g). Additionally, 1 mL of a trace metal solution per litter containing micro-elements: $CuSO_4.5H_2O$ (0.2 g), $FeSO_4.7H_2O$ (0.5 g), $ZnSO_4.7H_2O$ (0.4 g), H_3BO_3 (0.015 g), $CoCl_2.6H_2O$ (0.05 g), EDTA disodium salt (0.25 g), $MnCl_2.4H_2O$ (0.02 g), and $NiCl_2.6H_2O$ (0.01 g) was added. All media components were analytical grade (HiMedia), prepared with 18 MΩ distilled water. Seed cultures were prepared in 500 mL (working volume 100 ml) in an air-tight customized stir-tank reactor containing NMS media, supplemented intermittently with a 1:1 mixture of methane and air (50%). Media pH was adjusted to 6.8 using 1 M NaOH and 1 M H_2SO_4 before autoclaving. Cultures were incubated at 30°C with a constant agitation of 150 rpm in an in a customized stir tank bioreactor (1 L, 800 ml working volume (ORBITEK, Scigenics Biotech).

3.3.2 Characterization of *M. trichosporium* NCIMB 11131 for PHB Production

Evaluation of PHB accumulation under nutritional starvation

The study was split into two distinct phases: phase I focused on generating high-density *M. trichosporium* biomass through CH_4 utilization under optimal conditions, maintaining a 2.5% v/v methane concentration in the inlet gas stream, and a gas flow rate of 0.5 vvm. Nutrient concentrations were optimized at 610.16 ppm nitrate, 242.06 ppm phosphate, and 0.75X trace elements in the NMS media as Sahoo et al., 2023. A mid-log phase seed culture, having an optical density (OD) of 5 at 600 nm, used 10% v/v of the inoculum. The optimum pH for *M. trichosporium* growth is 6.8, while for max. PHB production at a lower pH of 6.2. Adjusting

the pH to 6.5 provides a negotiation, for PHB production by decreasing the pH level for PHB production. Experiments were conducted under the optimal pH for growth, further study may optimize pH for scale-up as well commercial PHB production. The correlation 1 $OD_{600\ nm}$ = 0.42 $g.L^{-1}$ dry cell weight (DCW) was used to translate the optical density $OD_{600\ nm}$ results to biomass concentration. This resulting correlation improves the findings' readability and clarity and enables an accurate assessment of the dynamics of PHB production and biomass generation operational conditions continued constant across both phases. Biomass from phase I was harvested by centrifugation at 7000 rpm for 10 minutes, and an OD of 4 (1.68 $g.L^{-1}$ DCW) was cultivated as the initial biomass concentration for phase II. Both phases were executed in a 1 L customized stirred-tank reactor (1 L, 800 mL working volume) with a uniform pore size ring sparger distribution, maintained at 30°C under agitation in a water bath placed on a hot-plate magnetic stirrer (IKA C-MAG HS7).

Assessment of PHB production under cumulative effect of nutritional starvation and carbon-excess condition

In phase II, nitrogen deficiency is essential to stimulate PHB biosynthesis in methanotrophic bacteria. Complete nitrogen deprivation (<5 ppm) activates *M. trichosporium* metabolic pathways, which stimulate PHB accumulation. Maximum PHB yield is achieved and efficient stress responses are certain by this ideal nitrogen level (Rostkowski et al., 2013; Strong et al., 2016). A media process engineering approach was used to assess the combined effect of nitrate starvation/depleted (derived from macro/micro-elements starvation experiments) and elevated methane concentrations in the inlet gas stream on intracellular PHB accumulation.

In situ esterification of *M. trichosporium* biomass for PHB estimation

Quantitative estimation of PHB was conducted by *in situ* esterification. For this, 10 mg of vacuum-dried *M. trichosporium* biomass was combined with 2 mL dichloroethane (DCM) and 2 mL propanol containing HCl (4:1 v/v), and sample was incubated 100±2 ^{O}C for 2 h in shaking water bath. Subsequently, 4 mL Milli-Q water was added and vortexed for another 30 seconds.

After centrifugation at 5000 rpm for 10 min, the lower organic phase having PHB-esters was then further analyzed by gas chromatography (Juengert et al., 2018).

3.3.3 Analytical methods

Cell growth was monitored by measuring culture absorbance at 600 nm using a UV-Vis spectrophotometer (Cary Series 100, Agilent Technologies), with optical density values correlated to dry cell weight (1 OD= 0.42 g. L^{-1} DCW). PHB was analysis using gas chromatography (GC7890B, Agilent) equipped with an HP-5 column and flame ionization detector (FID). Media nitrates and phosphate concentrations were quantified using the salicylic acid and ascorbic acid methods, respectively (Ankan et al., 2022). PHB yield was calculated using the formula: PHB yield (%) = Weight of PHB/Weight of biomass × 100. The methane fixation rate was calculated using Eq. by Sahoo et al., 2022. Methane fixation rate (g L^{-1} d^{-1}) is given by $P_x \times E_c \times (16 \times 12)$ (1), where P_x is the biomass productivity (g.L^{-1}. d^{-1}) and E_c is the elemental C-content in the biomass that CHNS analysis. Methane has a Mol. wt. of 16, while carbon has 12. The purity of the produced by *M. trichosporium* PHB was determined to be approx. 95-98% through GC-FID analyses (Agilent N7899). These techniques confirmed the biopolymer's molecular structure and identified potential contaminants. The thesis will include detailed procedural steps and data supporting experimentations.

3.3.4 Optimization of PHB extraction process from *M. trichosporium* biomass

Three solvent-based extraction methods were evaluated for their efficacy in PHB extraction: (i) chloroform, (ii) chloroform: hypochlorite, and (iii) ethyl acetate (non-halogenated) and also, in non- halogenated method of PHB extraction, 20 mL of ethyl acetate was added to the biomass and was incubated at 25 °C for 1 h under 100 rpm agitation. The mixture was vortexed for 30 seconds, heated at 37°C for 1 h in a shaking water bath, then cooled to room temperature. For each method, 200 mg of vacuum-dried methanotrophic biomass was treated with one-to-one solvents, incubated under specific conditions, and the extracted PHB was precipitated using chilled acetone and dried at 50 °C (Aramvash et al., 2015).

3.1.1 Btch Cultivation in 5-L Continuous Stirred Tank Reactor (CSTR) for PHB production

M. trichosporium biomass cultivation was scaled up in a New Brunswick TM Bioflo® 115 CSTR bioreactor (Eppendorf, Germany) with a 5 L working volume (total volume 7 L). The phase I by using optimized parameters (2.5% CH_4 with air at 0.5 vvm, nitrate, phosphate, trace elements) to get high-density biomass. Following 160 hours, the biomass was harvested and subjected to phase II conditions (nitrate starvation, 5% CH_4) to evaluate PHB production on a larger scale. Growth, pH, dissolved oxygen, and PHB production dynamics were monitored through regular sampling and analysis. The scale-up process was rigorously examined in a 5L CSTR, aiming on the dynamic interplay between growth parameters and PHB production with dissolved oxygen (dO_2) concentration dynamics.

3.2 Results and Discussion

3.4.1 PHB Induction in high-density *M. trichosporium* biomass under nutritional modulation

In the initial phase of this study, we dedicated on cultivating a high-density biomass of *M. trichosporium* NCIMB 11131, under optimal media composition experimented by Sahoo etal. 2022), we observed a maximum biomass concentration of 3.82 g L^{-1} after 160 h of batch cultivation. This phase also demonstrated a biomass productivity of 0.64 g L^{-1} d^{-1} and a methane fixation rate of 0.39 g L^{-1} d^{-1}. We observed in study a pH increase from 6.8 to 8 during this growth phase, supporting with studied by Sahoo et al. (2022). **Fig 5A** show that on near-complete consumption of nitrates and phosphates by the organism within 160 hours. Intracellular PHB storage depicted a dynamic profile, showing 4 to 8% w/w PHB accumulation in both lag and exponential phases (**Fig** 5A). To explore *M. trichosporium*'s innate PHB accumulation post-nitrate and phosphate deprivation, we extended phase I to 208 h. *In situ* PHB

content estimation revealed an max. out at 23.03% w/w on the 9th day (216 h) of cultivation. This observation incline with other recent studies for PHB production by *M. trichosporium* as well similar methanotrophic strains under nutrients modulation ((Dedysh and Derakshani, 2001; Khosravi-Darani et al., 2013; Rostkowski et al., 2012, Criddle et al., 2014; García-Pérez et al., 2018; Karthikeyan et al., 2015; Pieja et al., 2011a; Xin et al., 2007). Subsequently, a reduction in intracellular PHB content was observed toward the end of the batch at 240 h. This phase of the study highlights *M. trichosporium* NCIMB 11131's potential for PHB production under induced nutritional starvation, suggesting a viable enhancement strategy through a two-phase cultivation approach—high-density cell biomass production in phase I, followed by nutrient starvation in phase II (Bowman and Sayler, 1994; Zhang et al., 2016).

Evaluation of PHB contents in phase I high-density biomass under induced nutritional starvation in phase II

In phase II, high-density *M. trichosporium* biomass from phase I, was subjected to nutritional starvation modulation, targeting to assess its impact on intracellular PHB accumulation. These two-phase strategies, combining high-density biomass in phase I with a starvation-induced PHB accumulation in phase II, reflects a 'feast and famine' strategy of macro/micro-elements ((Bishoff et al., 2021; Nguyen and Lee, 2021; Pieja et al., 2011a, 2011b; Rostkowski et al., 2013; Shah et al., 1995; Wendlandt et al., 2001; Zhang et al., 2016). Compared to single-stage cultivation (**Fig.5B, 5C, and 5D**), the two-phase approach significantly reduced the time required for PHB accumulation. A crucial part of PHB production is played by initial phosphate levels. While little bit concentrations (<50 ppm) boost PHB biosynthesis, higher concentrations of phosphate (up to 250 ppm) enhance biomass production. The initial concentration of 100 ppm incursions a compromise between PHB production and as well as biomass generation. In order to determine the initial phosphate concentration for optimizing PHB intracellular accumulation, experiments will assess the effects of different phosphate levels on PHB yield,

productivity, and production (Ibrahim, 2009; Gudneppanavar et al., 2022).

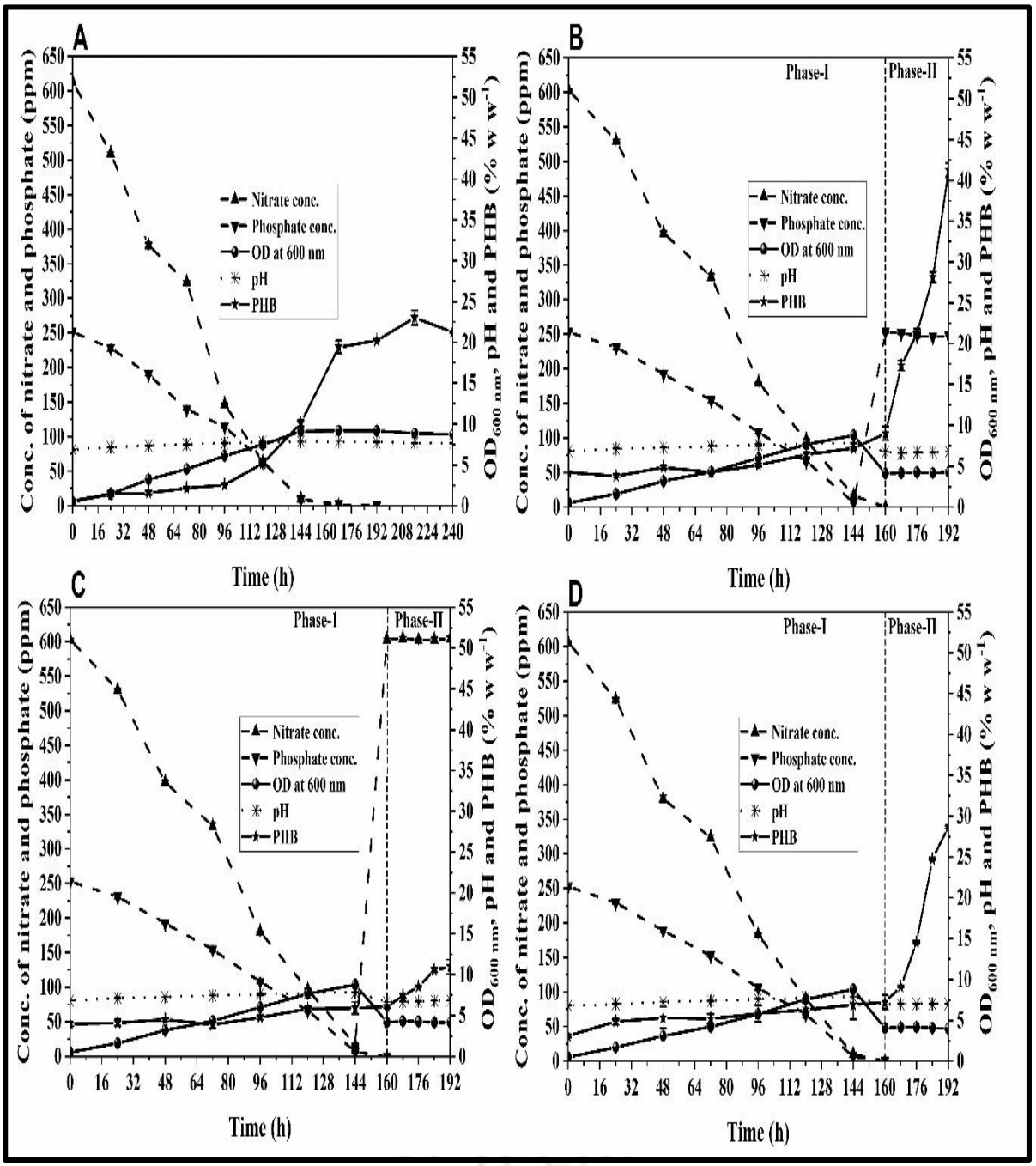


Figure 5: Dynamic profile for growth, pH, nitrate, phosphate and intracellular PHB content when M. trichosporium was cultivated under naturally induced nutritional starvation (A) single stage; and nutritional starvation in phase I generated methanotrophic biomass under (B) nitrate (C) phosphate (D) combined nitrate and phosphate starvation in phase II

For instance, in single-stage cultivation, maximum PHB content occurred at 216 hours, whereas in the two-stage approach, it was achieved at 192 hours. We observed distinct PHB accumulation trends under various macro-element starvation conditions (N, P, N+P) as shown in **Fig.6B, 6C, and 6D**. Nitrate starvation especially redirected methane to the highest PHB

content of 41.24% w/w after 32 hours of phase II (**Fig.5B**), likely due to a metabolic shift towards PHB accumulation from the TCA cycle under nitrogen-deficient conditions (Penkhrue et al., 2020).

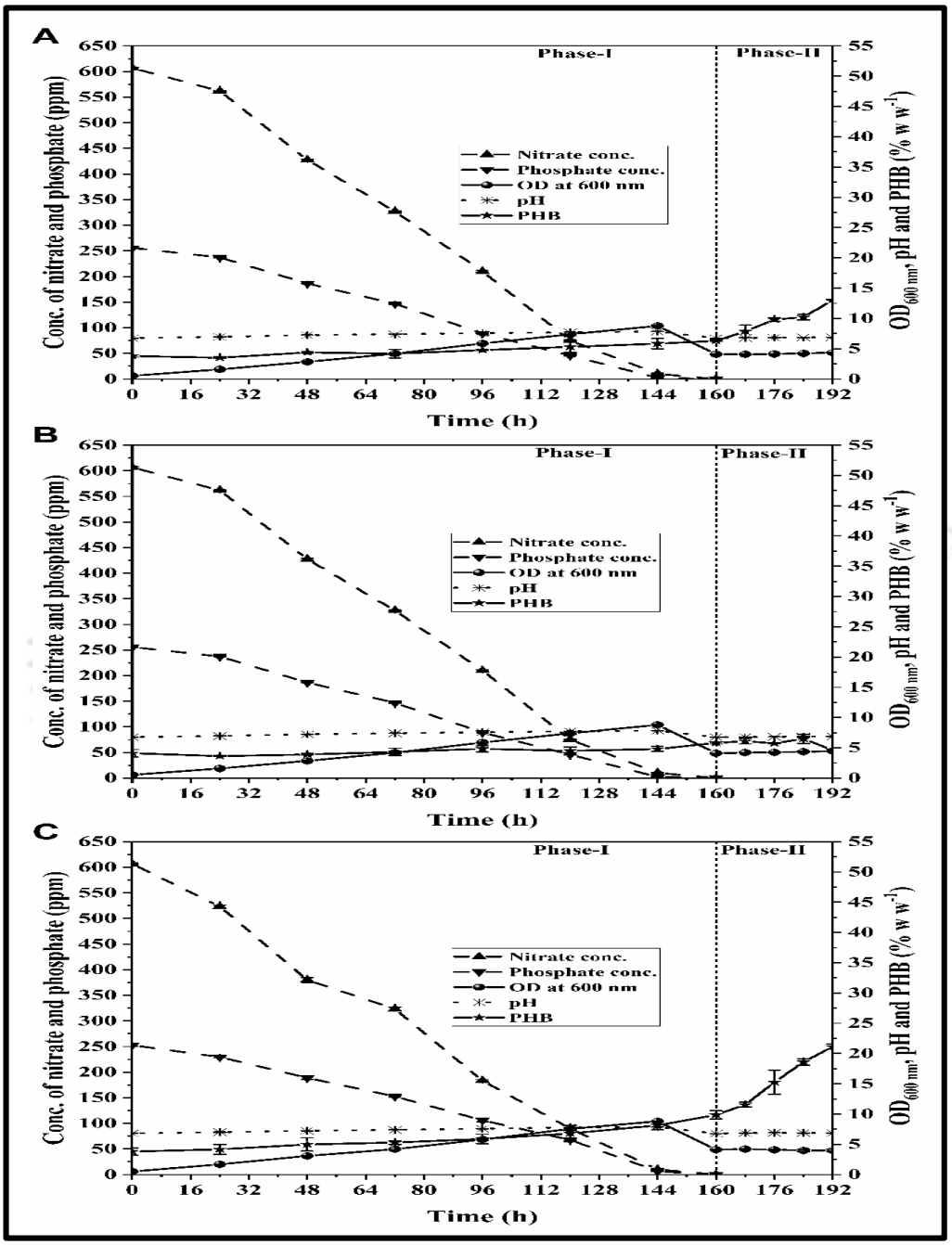


Figure 6: Dynamic profile for growth, pH, nitrate, phosphate and intracellular PHB content when phase I generated M. trichosporium biomass was cultivated under induced nutritional starvation of (A) Cu (B) Fe and (C) combined nitrate + Cu

This finding make parallel with other studies showing similar trends under nutrient-deficient

conditions (Choi et al., 2003; Helm et al., 2008; Semrau et al., 2010; Xin et al., 2007). Phosphate starvation resulted in a PHB content of 10.97% w/w (**Fig.5C**), and combined nitrate and phosphate starvation yielded 28.66% w/w (**Fig.5D**), resonating with earlier reports where nitrate starvation induced PHB levels ranging from 29 to 50% (Choi et al., 2003; Helm et al., 2008; Semrau et al., 2010; Xin et al., 2007).

3.4.2 Overproduction of PHB under the combinatorial influence of excess methane and nutritional starvation

We further combined this approach with excess methane feeding by selecting nitrate-deficient conditions for their pre-found effect on PHB content. Elevating methane content in the gaseous mixture from 2.5% to 5.0% and 7.5%, we observed highest PHB content of 52.42% w/w with 5% CH_4 and nitrate starvation after 32 hours (**Fig.7A**). However, further increase in methane concentration resulted in reduced PHB storage (46.64% w/w) as shown in **Fig.7B**, suggesting a limit to the microorganism's capacity for excess carbon utilization. As shown in **Fig.7C**, this observation is consistent with other studies reporting for PHB production of 17 to 48.7% w/w under similar conditions (Bishoff et al., 2021; Criddle et al., 2014; Myung et al., 2017; Nguyen and Lee, 2021; Zhang et al., 2016).

3.4.3 Optimization of PHB extraction from *M. trichosporium* biomass

For PHB extraction, we compared chloroform–hypochlorite dispersion extraction, conventional chloroform extraction, and non-halogenated solvent (ethyl acetate) methods. The highest PHB yield was achieved with chloroform–hypochlorite dispersion extraction (45.87% w/w), followed by chloroform extraction (43.33%) Consequently, as shown in **Fig.7C, 8D**, the chloroform-hypochlorite method was used in subsequent PHB extraction from *M. trichosporium* biomass (Aramvash et al., 2015; Sei Kwang Hahn et al., 1995; Valappil et al., 2007). and ethyl acetate extraction (41.38%), as shown in **Fig.7D.**

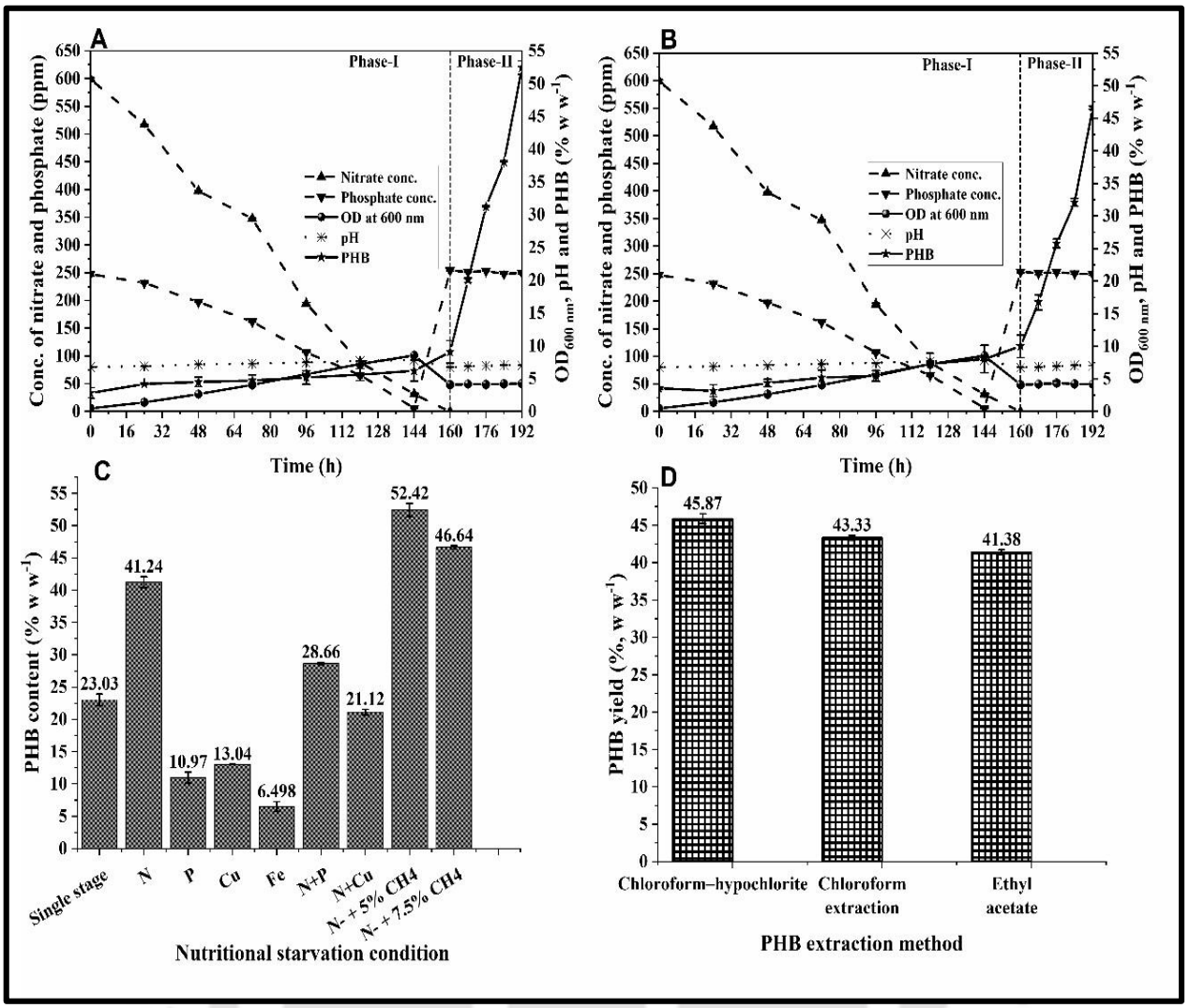


Figure 7: Dynamic profile for growth, pH, nitrate, phosphate and intracellular PHB content when M. trichosporium was cultivated under (A) combined nitrate starvation and 5% methane in air; (B) combined nitrate starvation and 7.5% methane in air; (C) intracellularPHB content under different nutritional starvation conditions in phase II compared with naturally induced starvation in single stage; (D) Yield of PHB under different extraction methods

3.4.2Scale-up of optimized process in a 5 L Continuous Stir Tank bioreactor (CSTR)

Scaling up the optimized process in a CSTR as shown in **Fig.8B**, we achieved a maximum biomass titre of 3.94 g L^{-1} after 160 hours, with a pH change from 6.8 to 7.95. A notable decrease in dO_2 levels was observed during this period for growth as well as PHB production as shown in **Fig.8A**, correlating with other studies (García-Pérez et al., 2018; Khosravi-Darani et al., 2013; Rostkowski et al., 2013; Wendlandt et al., 2001, 2005).

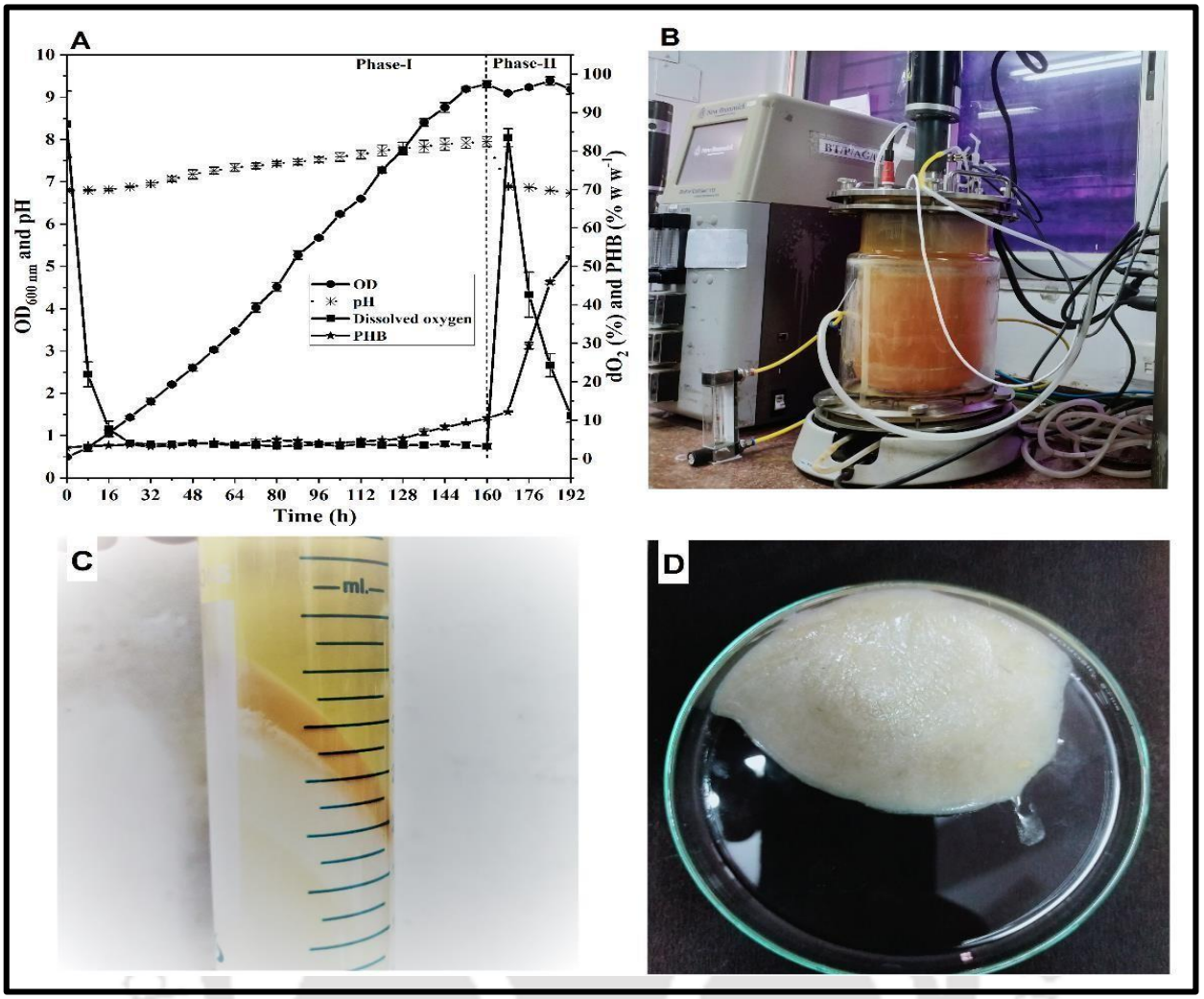


Figure 8: (A) Dynamic profile for growth, pH, dissolved oxygen, and intracellular PHB content at scale-up level under optimized process conditions (nitrate source starvation + 5% v/v methane in air); (B) Scale up in 5L stir tank reactor; (C) extraction of PHB from M. trichosporium biomass by chloroform–hypochlorite method; (D) Extracted M. trichosporium-PHB

Using optimized parameters for phase II (nitrate starvation + 5% v/v methane in air) led to a highest PHB contentof 52.4% w/w and a PHB titre of 2.06 g L^{-1} after 32 hours, showcasing a direct relationship between oxygen demand and intracellular PHB content.

References

1. Sinha, A., Goswami, G., Kumar, R., & Das, D. (2021). A microalgal biorefinery approach for bioactive molecules, biofuel, and biofertilizer using a novel carbon dioxide-tolerant strain Tetradesmus obliquus CT02. Biomass Conversion and Biorefinery, 1-14.

2. Aramvash, A., Gholami-Banadkuki, N., Moazzeni-Zavareh, F., Hajizadeh-Turchi, S., 2015. An environmentally friendly and efficient method for extraction of PHB biopolymer with non-halogenated solvents. J. Microbiol. Biotechnol. 25, 1936–1943. https://doi.org/10.4014/jmb.1505.05053

3. Asenjo, J.A., Suk, J.S., 1986. Microbial Conversion of Methane into poly-β-hydroxybutyrate (PHB): Growth and intracellular product accumulation in a type II methanotroph. J. Ferment. Technol. 64, 271–278. https://doi.org/10.1016/0385-6380(86)90118-4

4. Bishoff, D., AlSayed, A., Eldyasti, A., 2021. Production of poly-hydroxy-butyrate using nitrogen removing methanotrophic mixed culture bioreactor. J. Biosci. Bioeng. 132, 351–358.https://doi.org/10.1016/j.jbiosc.2021.04.007

5. Bowman, J.P., Sayler, G.S., 1994. Methylosinus trichosporium 1.

6. Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V., 2014. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. Express Polym. Lett. 8, 791–808. https://doi.org/10.3144/expresspolymlett.2014.82.

7. Choi, D.W., Kunz, R.C., Boyd, E.S., Semrau, J.D., Antholine, W.E., Han, J.I., Zahn, J.A., Boyd, J.M., De la Mora, A.M., DiSpirito, A.A., 2003. The membrane-associated methane monooxygenase (pMMO) and pMMO-NADH: Quinone oxidoreductase complex from Methylococcus capsulatus bath. J. Bacteriol. 185, 5755–5764. https://doi.org/10.1128/JB.185.19.5755-5764.2003

8. Criddle, C.S., Billington, S.L., Frank, C.W., 2014. Renewable Bioplastics and BiocompositesFrom Biogas Methane and Waste-Derived Feedstock: Development of Enabling Technology, Life Cycle Assessment, and Analysis of Costs. Calif. Dep. Resour. Recycl. Recover.

9. Dedysh, S.N., Derakshani, M., 2001. Detection and Enumeration of Methanotrophs in Acidic Sphagnum Peat by 16S rRNA Fluorescence In Situ Hybridization, Including the Use of NewlyDeveloped Oligonucleotide Probes for Methylocella palustris 67, 4850–4857. https://doi.org/10.1128/AEM.67.10.4850

10. Doronina, N. V., Ezhov, V.A., Trotsenko, I.A., 2008. Growth of Methylosinus trichosporium OB3b on methane and poly-beta-hydroxybutyrate biosynthesis. Prikl. Biokhim. Mikrobiol. 44,202–206. https://doi.org/10.1134/s0003683808020099

11. García-Pérez, T., López, J.C., Passos, F., Lebrero, R., Revah, S., Muñoz, R., 2018. Simultaneous methane abatement and PHB production by Methylocystis hirsuta in a novel gas- recycling bubble column bioreactor. Chem. Eng. J. 334, 691–697. https://doi.org/10.1016/j.cej.2017.10.106

12. Helm, J., Wendlandt, K.D., Jechorek, M., Stottmeister, U., 2008. Potassium deficiency resultsin accumulation of ultra-high molecular weight poly-β-hydroxybutyrate in a methane-utilizing mixed culture. J. Appl. Microbiol. 105, 1054–1061. https://doi.org/10.1111/j.1365- 2672.2008.03831.x

13. Hwang, I.Y., Nguyen, A.D., Nguyen, T.T., Nguyen, L.T., Lee, O.K., Lee, E.Y., 2018. Biological conversion of methane to chemicals and fuels: technical challenges and issues. Appl. Microbiol. Biotechnol. 102, 3071–3080. https://doi.org/10.1007/s00253-018-8842-7

14. Juengert, J., Bresan, S., Jendrossek, D., 2018. Determination of Polyhydroxybutyrate (PHB) Content in Ralstonia eutropha Using Gas Chromatography and Nile Red Staining. Bio-Protocol8, 1–15. https://doi.org/10.21769/bioprotoc.2748

15. Karthikeyan, O.P., Chidambarampadmavathy, K., Cirés, S., Heimann, K., 2015. Review of sustainable methane mitigation and biopolymer production. Crit. Rev.

Environ. Sci. Technol. 45, 1579–1610. https://doi.org/10.1080/10643389.2014.966422

16. Khosravi-Darani, K., Mokhtari, Z.B., Amai, T., Tanaka, K., 2013. Microbial production of poly(hydroxybutyrate) from C1 carbon sources. Appl. Microbiol. Biotechnol. 97, 1407–1424.https://doi.org/10.1007/s00253-012-4649-0

17. Levett, I., Birkett, G., Davies, N., Bell, A., Langford, A., Laycock, B., Lant, P., Pratt, S., 2016.Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane - The case for thermophilic bioprocessing. J. Environ. Chem. Eng. 4, 3724–3733. https://doi.org/10.1016/j.jece.2016.07.033

18. Liu, L.Y., Xie, G.J., Xing, D.F., Liu, B.F., Ding, J., Ren, N.Q., 2020a. Biological conversion of methane to polyhydroxyalkanoates: Current advances, challenges, and perspectives. Environ. Sci. Ecotechnology. https://doi.org/10.1016/j.ese.2020.100029

19. Liu, L.Y., Xie, G.J., Xing, D.F., Liu, B.F., Ding, J., Ren, N.Q., 2020b. Biological conversion of methane to polyhydroxyalkanoates: Current advances, challenges, and perspectives. Environ. Sci. Ecotechnology 2, 100029. https://doi.org/10.1016/j.ese.2020.100029

20. Liu, X.J., Zhang, J., Hong, P.H., Li, Z.J., 2016. Microbial production and characterization of poly-3-hydroxybutyrate by Neptunomonas antarctica. PeerJ 2016.https://doi.org/10.7717/PEERJ.2291

21. Myung, J., Flanagan, J.C.A., Waymouth, R.M., Criddle, C.S., 2017. Expanding the range of polyhydroxyalkanoates synthesized by methanotrophic bacteria through the utilization of omega-hydroxyalkanoate co-substrates. AMB Express 7. https://doi.org/10.1186/s13568-017-0417-y

22. Nguyen, T.T., Lee, E.Y., 2021. Methane-based biosynthesis of 4-hydroxybutyrate and

P (3- hydroxybutyrate-co-4-hydroxybutyrate) using engineered Methylosinus trichosporium OB3b. Bioresour. Technol. 335, 125263. https://doi.org/10.1016/j.biortech.2021.125263

23. Park, S. yeong, Kim, C. gyun, 2019. Application and development of methanotrophs in environmental engineering. J. Mater. Cycles Waste Manag. 21, 415–422. https://doi.org/10.1007/s10163-018-00826-w

24. Patel, S.K.S., Jeong, J.H., Mehariya, S., Otari, S. V., Madan, B., Haw, J.R., Lee, J.K., Zhang, L., Kim, I.W., 2016. Production of methanol from methane by encapsulated Methylosinus sporium. J. Microbiol. Biotechnol. 26, 2098–2105. https://doi.org/10.4014/jmb.1608.08053

25. Penkhrue, W., Jendrossek, D., Khanongnuch, C., Pathomareeid, W., Aizawa, T., Behrens, R.L., Lumyongid, S., 2020. Response surface method for polyhydroxybutyrate (PHB) bioplastic accumulation in Bacillus drentensis BP17 using pineapple peel. PLoS One 15, 1–21. https://doi.org/10.1371/journal.pone.0230443

26. Pieja, A.J., Rostkowski, K.H., Criddle, C.S., 2011a. Distribution and Selection of Poly-3- Hydroxybutyrate Production Capacity in Methanotrophic Proteobacteria 564–573. https://doi.org/10.1007/s00248-011-9873-0

27. Pieja, A.J., Sundstrom, E.R., Criddle, C.S., 2011b. Poly-3-Hydroxybutyrate Metabolism in theType II Methanotroph Methylocystis parvus OBBP77, 6012–6019. https://doi.org/10.1128/AEM.00509-11

28. Rostkowski, K.H., Criddle, C.S., Lepech, M.D., 2012. Cradle-to-Gate Life Cycle Assessmentfor a Cradle-to-Cradle Cycle: Biogas-to-Bioplastic (and back).

29. Rostkowski, K.H., Pfluger, A.R., Criddle, C.S., 2013. Stoichiometry and kinetics of the PHB-producing Type II methanotrophs Methylosinus trichosporium OB3b and

Methylocystis parvus OBBP. Bioresour. Technol. 132, 71–77. https://doi.org/10.1016/j.biortech.2012.12.129

30. Sahoo, K.K., Datta, S., Goswami, G., Das, D., 2022. Two-stage integrated process for bio- methanol production coupled with methane and carbon dioxide sequestration: Kinetic modelling and experimental validation. J. Environ. Manage. 301, 113927. https://doi.org/10.1016/j.jenvman.2021.113927

31. Semrau, J.D., Dispirito, A.A., Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol. Rev. 34, 496–531. https://doi.org/10.1111/j.1574-6976.2010.00212.x

32. Shah, N.N., Hanna, M.L., Taylo, R.T., 1995. Batch Cultivation of Methylosinus trichosporium OB3 b : V. Characterization of Poly-P-Hydroxybutyrate Production Under Methane- Dependent Growth Conditions.

33. Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource: Can the methanotrophs add value? Environ. Sci. Technol. 49, 4001–4018. https://doi.org/10.1021/es504242n

34. V, E.S.P.B., Riis, V., Mai, W., 1988. determined PHB after extraction with chloroform directly in the resulting solution by infrared spectroscopy. Interfering lipids are determined separately after chro- matographic separation from the PHB. Braunegg 445, 285–289.

35. Valappil, S.P., Misra, S.K., Boccaccini, A.R., Keshavarz, T., Bucke, C., Roy, I., 2007. Large- scale production and efficient recovery of PHB with desirable material properties, from the newly characterised Bacillus cereus SPV. J. Biotechnol. 132, 251–258. https://doi.org/10.1016/j.jbiotec.2007.03.013

36. Wendlandt, K., Jechorek, M., Helm, J., Stottmeister, U., 2001. Producing poly-3-hydroxybutyrate with a high molecular mass from methane 86, 127–133.

37. Wendlandt, K.D., Geyer, W., Mirschel, G., Al-Haj Hemidi, F., 2005. Possibilities for controlling a PHB accumulation process using various analytical methods. J. Biotechnol. 117,119–129. https://doi.org/10.1016/j.jbiotec.2005.01.007

38. Xin, J.Y., Zhang, Y.X., Zhang, S., Xia, C.G., Li, S. Ben, 2007. Methanol production from CO2 by resting cells of the methanotrophic bacterium Methylosinus trichosporium IMV 3011. J. Basic Microbiol. 47, 426–435. https://doi.org/10.1002/jobm.200710313

39. Ibrahim, M. H., & Steinbüchel, A. (2009). Poly (3-hydroxybutyrate) production from glycerol by Zobellella denitrificans MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction. Applied and environmental microbiology, 75(19), 6222-6231

40. Lazic, M., Gudneppanavar, R., Whiddon, K., Sauvageau, D., Stein, L. Y., & Konopka, M. (2022). In vivo quantification of polyhydroxybutyrate (PHB) in the alphaproteobacterial methanotroph, Methylocystis sp. Rockwell. Applied Microbiology and Biotechnology, 1-9.

Chapter 4

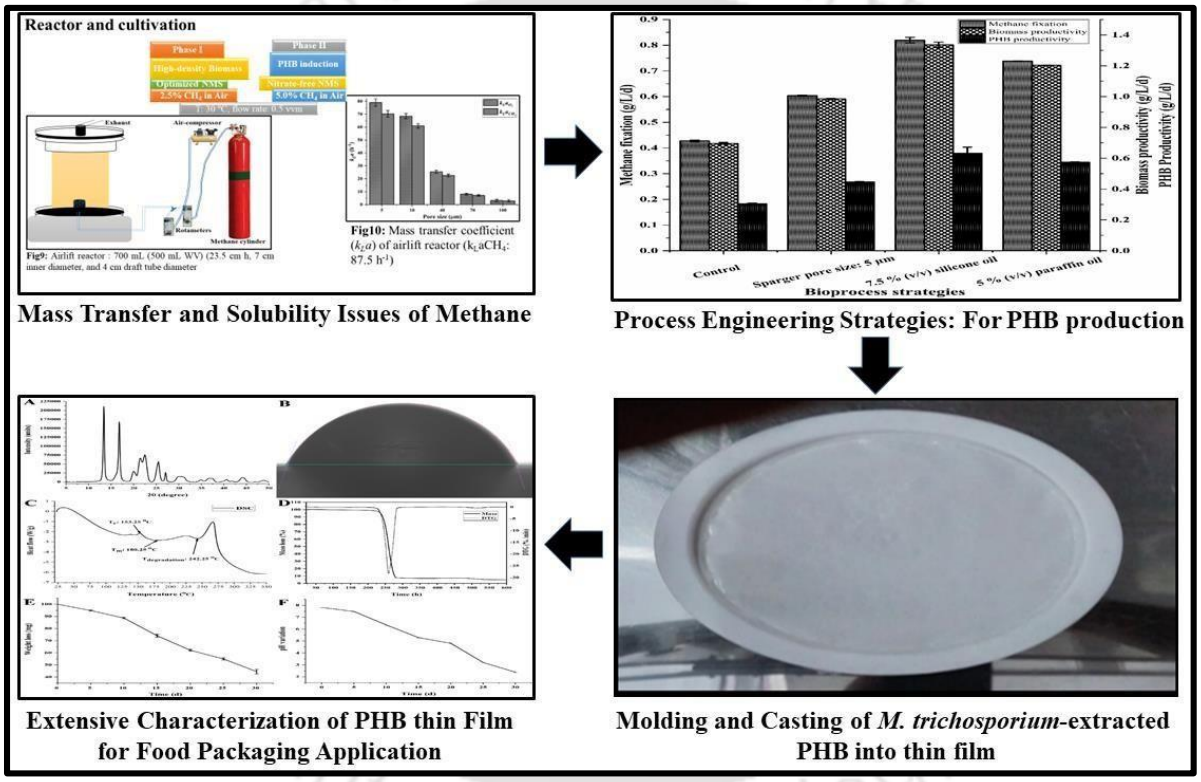
Process engineering strategy towards improvement in methane mass transfer and solubility in the aqueous phase: Application as food packaging material

4.1 Background and motivation

The industrial production of PHB by gas fermentation using *M. trichosporium* is an involved biotechnological endeavor with deep environmental and economic implications. Central to the efficacy of this process is the mass transfer of methane as a substrate for methanotrophic bacteria such as *M. trichosporium*—into the aqueous phase, a step that is naturally limited by methane's low solubility in water. This physicochemical constraint significantly hinders microbial conversion efficiency, resulting in diminished PHB yields. Our research is driven by the critical need for a comprehensive process engineering strategy that enhances methane's mass transfer, and solubility for bioavailability. This necessitates an in-depth examination of parameters influencing gas-liquid mass transfer, including interfacial surface area, gas mixture composition, pressure, temperature, and the use of innovative bioreactor designs and operating conditions combined with optimize methane solubility and uptake rates. Systematic experimentation and bioprocess nutritional modulations, we focused on dissecting the kinetics of methane solubility and its subsequent bioconversion into PHB. We are predominantly focusedon the development of novel bioreactor (airlift) configurations that maximize contact between methane and the microbial biomass and the application of pioneering mass transfer enhancement techniques such as the use of microporous membranes, sparging, or

biocompatible methane vectors. Additionally, the exploration of process engineering to increase the intrinsic methane utilization efficiency of *M. trichosporium* stands as a parallel stand for research. By integrating these strategies, our goal is to establish a robust and scalable process that not only elevates PHB production but also contributes to the mitigation of methane as a greenhouse gas, thus aligning with global sustainability goals and advancing the potential of gas fermentation technology.

4.2 Process flow diagram



4.3 Materials and Methods

4.3.1 Culture medium selection and inoculum preparation

For the cultivation of *M. trichosporium* NCIMB 11131, we procured the strain from the National Collection of Industrial Food and Marine Bacteria (NCIMB, UK) and cultivated it in an optimized modified nitrate minimal salt (NMS) medium as delineated by Mohammed etal. (2023). The medium was constituted using distilled water of 18 MΩ resistivity and all reagents

of analytical grade, sourced from HiMedia. The pH was adjusted to 6.8 using 1 M NaOH and 1 M H_2SO_4 before to sterilization by autoclaving. The inoculum was prepared in a 500 mL tightly sealed Custom-Mini reactor, utilizing the optimized NMS growth medium at 30 °C with 150 rpm agitation as Mohammed et a, (2023) in an orbital shaker incubator (ORBITEK, Scigenics Biotech) as well as Sahoo et al., (2022). The culture development was cultivated into two stages: (I) high-density *M. trichosporium* biomass generation and the phase II intracellular PHB enrichment in the biomass from phase I. In phase I, we got high-density biomass through CH_4 utilization, using optimal process parameters that maintained a methane concentration of 2.5% v/v in the inlet gas stream and a flow rate of 0.5 vvm (Sahoo et al., 2022; Mohammed et al., 2023). The NMS medium was initially enriched with nitrate, phosphate, and trace elements at concentrations of 610.16 ppm, 242.06 ppm, and 0.75X, respectively (Sahoo et al., 2022). We inoculated the Airlift reactor with a mid-log phase seed culture at an optical density of 5 (10% v/v). In phase II, the methanotrophic biomass go through nitrate (KNO_3) deprivation, with the inlet stream carrying 5.0% (v/v) methane in air at a flow rate of 0.5 vvm as delineated by Mohammed et al., 2023.

4.3.2 Enhancing *M. trichosporium* biomass and PHB production: A Process Engineering Strategies

Micro-sparger design and methane vectors supplementations

We examined the influence of micro-sparger pore sizes, ranging from 5 to 100 µm, on methane mass transfer and its effect on methanotrophic growth and PHB production (Sahoo etal., 2023). Additionally, methane vectors such as paraffin and silicone oil were examined for their ability to enhance methane and oxygen withholding in aqueous environments. Concentrations ranging from 2.5 to 10% were tested in NMS medium to determine their effects on biomass growth and PHB production, using a micro-sparger with a 5 µm pore size with methane vectors (Muñoz et al., 2012; Sahoo et al., 2023).

4.3.3 Casting of degradable thin film from PHB biopolymer

A solution was prepared by dissolving 2 g of PHB (w/v), extracted from *M. trichosporium*, in 50 mL of chloroform with a beaker. This mixture was then homogenized using a magnetic stirrer (IKA C-MAG HS7) and sonicated at 40% amplitude with an impulse interval of 5 s on and 10 s off, to ensure the removal of air bubbles. Subsequently, the make uniform solution was poured onto a Teflon plate, carefully levelled horizontally, and allowed to dry at room temperature over a 24 h period to form a thin film.

4.3.4 Characterization of PHB thin film derived from *M. trichosporium* NCIMB 11131

The PHB thin film was subjected to widespread characterization to evaluate its suitability as a degradable food packaging material. This involved a series of comprehensive analyses to determine its mechanical, structural, molecular, and thermal properties, degradation assay for that suitability.

Thickness measurement

The film's thickness was measured at various points from the center to the periphery using a screw gauge (Mitutoyo 293-240-30), and the averaged values were taken.

Mechanical properties

The tensile strength and elongation at break were determined by applying to rectangular film samples (10 cm × 7 mm × 0.1 mm) to mechanical testing on a 5kN Universal Testing Machine (Zwick Roell, Model: Z005TN) at a loading speed of 1 $mm.min^{-1}$ at room temperature.

Surface wettability

The hydrophobicity of the film was assessed by measuring the water contact angle using a goniometer (Krüss GmbH, FM140) equipped with a camera and analysis software. A 2 µL droplet of distilled water was placed on the film surface to measure the static horizontal surface for water contact angles.

X-Ray diffraction

The crystalline structure was analyzed by grinding the film into a powder and subjecting it to XRD analysis (Rigaku Technologies, Japan, Model: Smart lab) using Ni-filtered Cu Kα radiation. The diffract-gram was recorded at a scan rate of $3°min^{-1}$ within a 2θ range of 5 to 50°.

Oxygen Transfer Rate (OTR)

The OTR was determined using a Labthink Gas Permeability Test system by the differential-pressure method in agreement with ***ISO 15105-1***. The rate of oxygen permeation rate through the film was measured under a pressure of 0.1 MPa.

Water Vapor Transmission Rate (WVTR)

The WVTR was determined using a Labthink Water Vapor Transmission Rate test system following ***ASTM E96*** standard method. The rate of water vapor passage through the film was estimated at 23±2 °C and 90% relative humidity.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was experimented of PHB film using an IRAffinity-1 spectrometer under ATR mode, scanning from 650 to 4000 cm^{-1} to produce and analyze the spectra.

Field Emission Scanning Electron Microscopy

The surface morphology was inspected using a FESEM (Zeiss, Gemini 300) at various magnifications after mounting the samples on carbon tape and gold sputtering.

Gel Permeation chromatography

Molecular weight and polydispersity index (PDI) were determined by GPC using a Waters Corporation instrument equipped with a Varian RI-2414 detector. The PHB was dissolved in THF and analyzed as a polystyrene standard.

Differential Scanning Calorimetry (DCS) and Thermal Gravimetric analysis (TGA)

Thermal properties were evaluated using a NETZSCH DSC 3500 instrument. Samples were heated from 30 °C to 600 °C at a ramp rate of 10 °C.min^{-1} to determine the melting temperature, crystallization temperature, and maximum degradation temperature.

Degradability assay

The biodegradability of the PHB film was measured by immersing samples in a 1 M NaOH buffer solution at 58±2 °C for 30 days. The changes in pH and the film's weight loss were monitored to assess degradation assay study.

4.4 Results and Discussion

In the search of enhanced PHB production using *M. trichosporium*, this study integrated a two-phase cultivation process, initially described by Mohammed et al. (2023); as well as Sahoo et al, (2022), with advanced process engineering strategies to improve methane mass transfer and solubility in the aqueous phase. The first phase (phase I) was shown to cultivating a high-density methanotrophic biomass production, while the second phase (phase II) aimed on PHB induction through methane sequestration on elevated methane concentration as shown in **Fig.9**. However, the efficiency of this process encountered limitations due to the inherently low rates of methane mass transfer and its solubility in the liquid medium, as observed in our study customized Airlift bioreactor, promising in limited biomass and PHB titters. Mass transfer coefficients in every aspect were not calculated in this study because the primary focus was on optimizing biological and biochemical parameters for optimal PHB production rather than on the physical and chemical engineering aspects. The research aimed to understand the metabolic pathways, nutrient optimization, and microbial kinetics specific to *M. trichosporium* NCIMB 11131. While acknowledging the importance of mass transfer in bioreactor design and operation, future studies could include the determination of mass transfer coefficients to enhance bioreactor design and its efficiency. This would provide a more understanding of the system, aiding in scaling up the

process from laboratory to industrial production its applications.

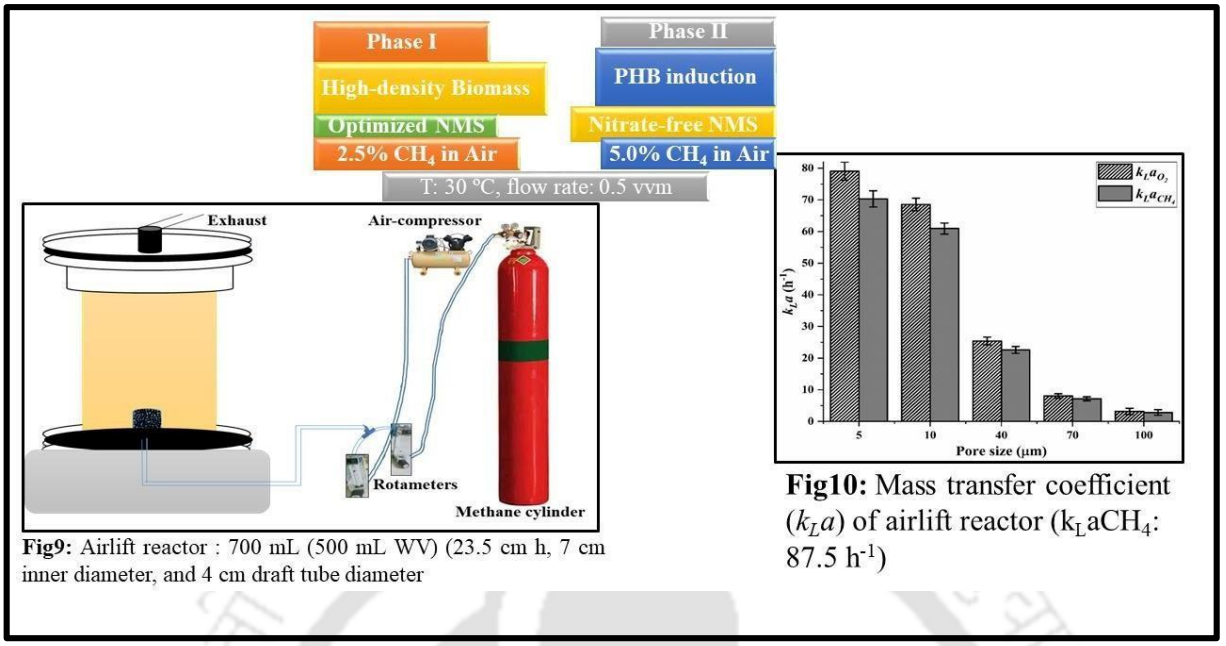


Fig9: Airlift reactor : 700 mL (500 mL WV) (23.5 cm h, 7 cm inner diameter, and 4 cm draft tube diameter

Fig10: Mass transfer coefficient (k_La) of airlift reactor (k_LaCH_4: 87.5 h^{-1})

Figure 9: Airlift reactor design, and mass transfer coefficient for methane solubility

4.4.1 Methane mass transfer in high-density *M. trichosporium* biomass and PHB production

Using a custom-designed airlift bioreactor, the study united various process engineering strategies, such as optimizing micro-sparger pore size and supplementing methane vectors, to enhance methane solubility. A systematic approach. evaluated the impact of these bioprocess engineering strategies on k_La (volumetric mass transfer coefficient), biomass titre, productivity, and methane fixation rate.

Micro-sparger pore size optimization for biomass production

Consistent with the literature, our results highlight the critical role of micro-sparger pore size in improving mass transfer efficiency within airlift reactors. A decrease of pore size from 100 to 5 µm directed to a substantial increase in biomass titre andmethane fixation rates, with a 25- fold enhancement in k_La observed for both methane and oxygen compared to the larger pore sizes for PHB production. These results are associated with the established principle that smaller pore sizes yield finer microbubbles in studies such as Sahoo et al., (2023), which enhance the interfacial area and residence time in the bioreactor, important to improved mass transfer rates.

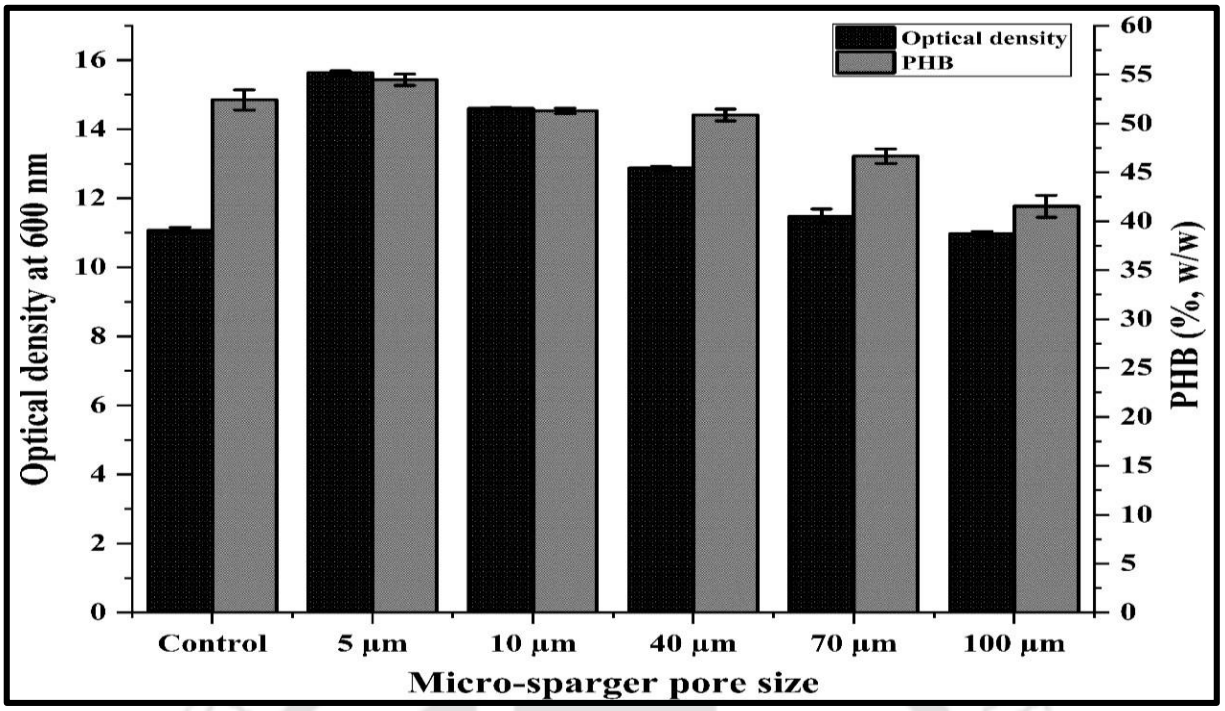


Figure 10: Effect of micro-sparger pore: Biomass and PHB production

Mass transfer vector supplementation for biomass production

The study further examined the addition of paraffin and silicone oils as mass transfer vectors. These substances, by increasing the interfacial area and reducing the gas-liquid boundary on upper layer, significantly improved methane and oxygen solubility and bioavailability in the NMS medium as in aqueous phase. Our results proved that a 5% v/v concentration of paraffin oil and a 7.5% v/v concentration of silicone oil significantly superior biomass titre by 172.63% and 191.70%, respectively, compared to control conditions as pure methane from cylinder.

4.4.2 Advancing PHB production in phase II

The second phase (phase II) of the observation involved growing the biomass in a nitrate-free NMS medium with an elevated methane concentration to enhance intracellular PHB accumulation. The success of this phase was subject on the effective mass transfer of methane, and methane solubility, which was enhanced through process engineering strategies, including the use of customized airlift bioreactors and the optimization of the micro-sparger pore size, and methane vectors. Furthermore, in the presence of 7.5% v/v silicone oil, the highest k_La values for oxygen and methane in the airlift reactor were 97.84 ± 2.63 h^{-1} and 86.98 ± 2.34 h^{-1},

respectively, representing an improvement of 23.72% compared to the control as shown in Fig9. The low shear stress environment provided by the Airlift reactors provides a significant benefit over stirred tank reactors for the cultivation of cells.

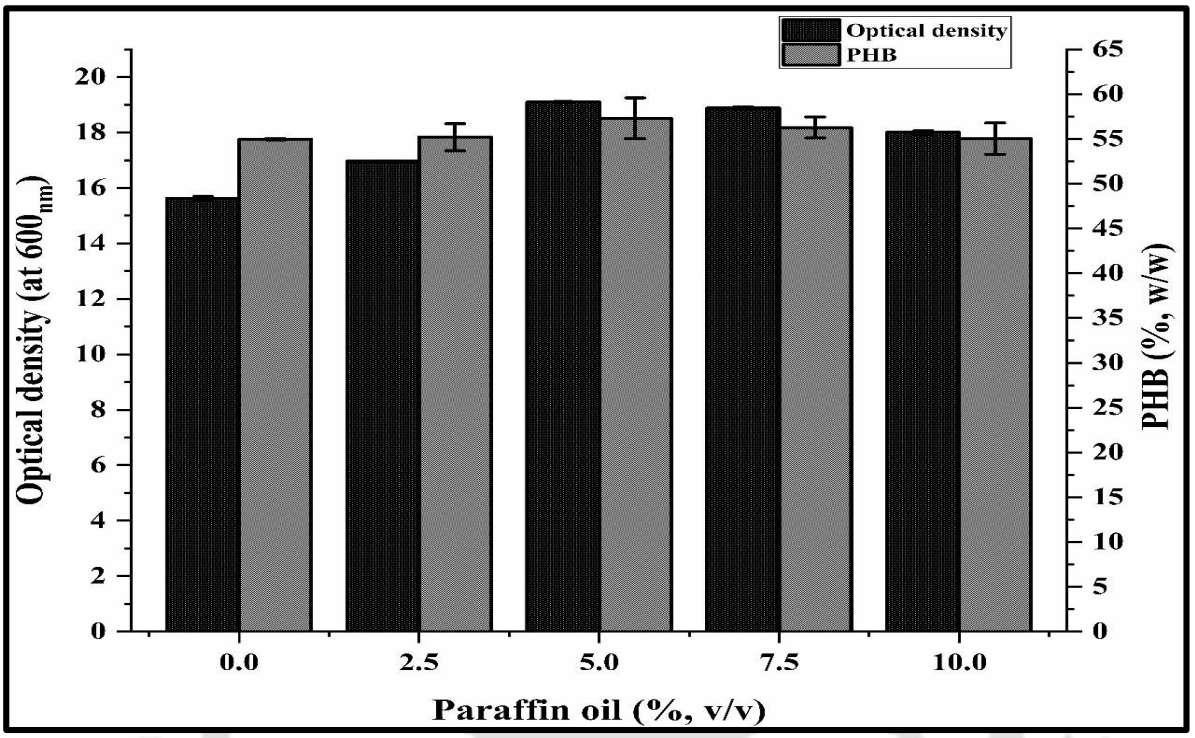


Figure 11: Effect of paraffin oil: Biomass & PHB production

Moreover, the energy requirements are also minimal in the case of Airlift reactors. High-cell density cultures that are prone to poor mixing have pose significant limitations to effective mass transfer. Airlift reactors exhibit better performance than stirred tank reactors in such cases. We used an internal loop airlift reactor and at selected process parameters, our study demonstrated the highest kLaCH4 attained which was 458-fold greater than that previously estimated in a semi-batch stirred tank reactor as shown in Fig.1(b). Moreover, the highest $k_La O_2$ achieved in this study (97.84 ± 2.63 per hour) was higher than that reported for trickle-bed reactor $k_La O_2$: 9.54 per hour) and methane transfer chamber coupled-external loop airlift reactor ($k_La O_2$: 97.2 per hour, $k_La CH_4$: 70.8 per hour) for the cultivation of methanotrophs cultivation.

Impact of micro-Sparger Pore Size on PHB production

A reduction in the pore size of the micro-sparger resulted in an increase in the biomass's PHB content. The use of a 5 µm pore size micro-sparger correlated with the maximal PHB

productivity, indicating the profound impact of bubble size on PHB production efficiency.

Influence of methane vectors supplementation on PHB accumulation

The study's findings elucidate the positive impact of methane vectors on PHB production. The application of silicone oil, in particular, yielded the highest PHB productivity, suggesting its potential as a superior mass transfer vector, solubility compared to paraffin oil.

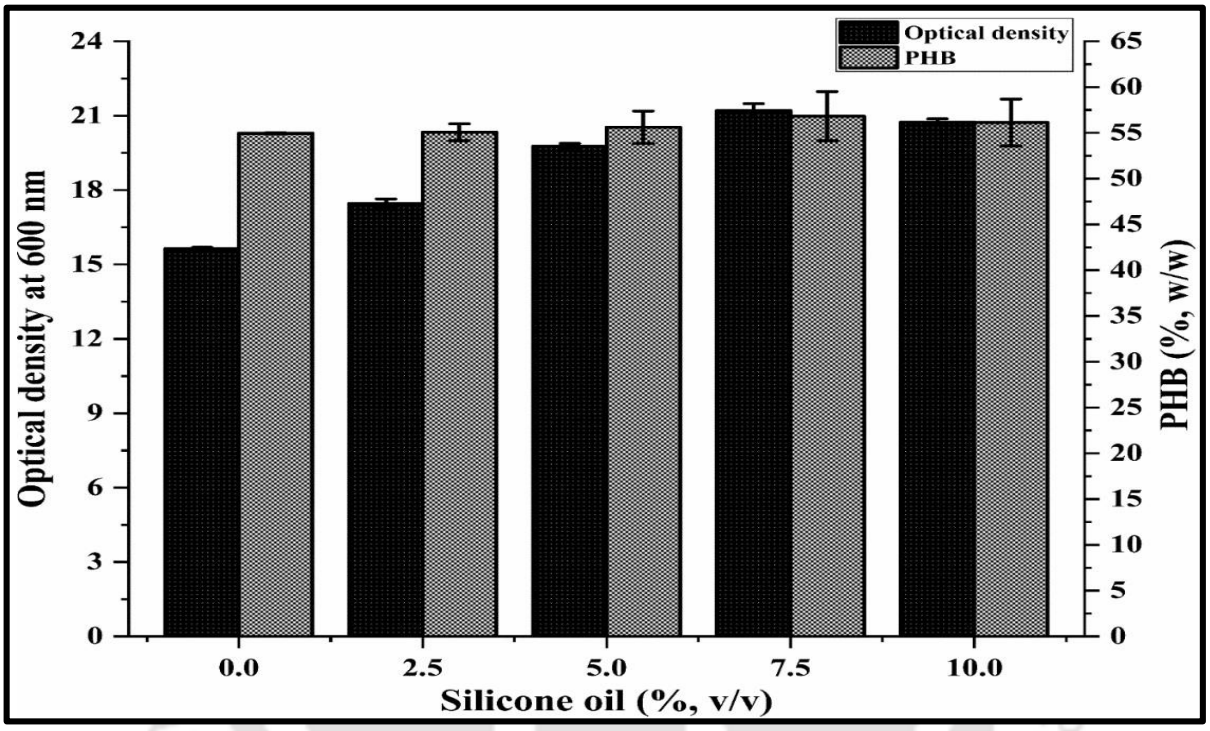


Figure 12: Effect of silicone oil on biomass and PHB production

These results are supported by previous research finding that the optimization of methane availability is essential for augmenting PHB production in *M. trichosporium* bacteria (Rodriguez et al., 2018; Zúñiga et al., 2011). In conclusion, this study has successfully demonstrated that strategic process engineering strategy can significantly important to enhance PHB production in *M. trichosporium*. The optimized parameters have led to a PHB content of 57.30% w/w and a titre of 5.06 $g.L^{-1}$, surpassing as shown in **Fig.11, and 12**. These advancements not only contribute to the field of sustainable bioplastic production but also highlight the critical role of process engineering strategies in biotechnological applications in various applications. In the domain of bioprocess engineering strategies for the enhancement of PHB production using *M. trichosporium*, the supplementation of mass transfer vectors has

shown a significant improvement in PHB titre. Specifically, the introduction of 5% paraffin oil into the bioreactor milieu increased the PHB titre to 4.60 $g.L^{-1}$, representing a 27.48% enhancement over the control's 3.61 $g.L^{-1}$.

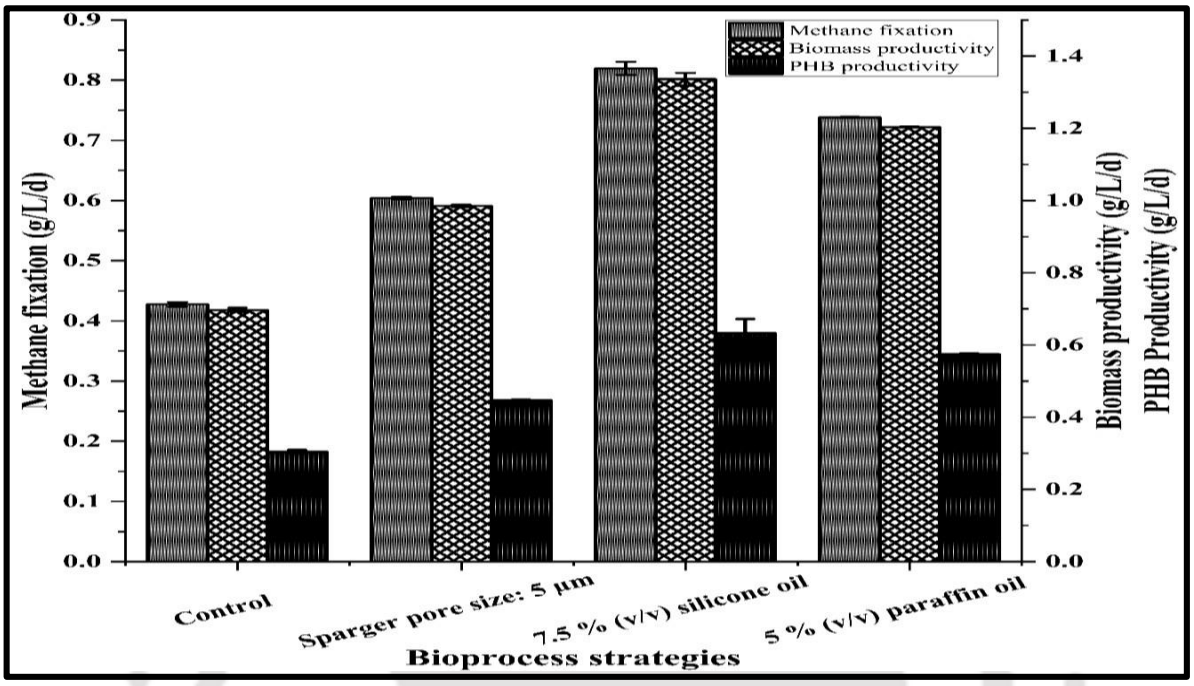


Figure 13: Methane fixation, biomass, and PHB productivity in various bioprocess strategies

Especially, the incorporation of 7.5% silicone oil as a mass transfer vector not only surpassed the control by 40.17% but also yielded an impressive PHB titre of 5.06 $g.L^{-1}$. The productivity of PHB further collaborated with the efficiency of oil supplementations, with paraffin oil achieving a productivity rate of 1.00 $g.L^{-1}.d^{-1}$, while silicone oil exhibited a superior productivity rate of 1.11 $g.L^{-1}.d^{-1}$. **Fig.12** as well **as Fig.13**, highlights the role of silicone oil as the optimal strategy for PHB production enhancement in an engineered bioprocess environment. These results align with the notion that the physicochemical properties of mass transfer vectors play an essential role in facilitating the solubility and availability of methane in the aqueous phase, thereby increasing the production of PHB. The Increased productivity with silicone oil may be attributed to its higher affinity for methane, which presumably leads to improved uptake and assimilation by high-density *M. trichosporium* biomass. This strategy, embedded in the nuanced manipulation of bioreactor for maximum mass transfer, that paves the way for industrial-scale production of PHB with increased efficiency and economic

viability.

Table 2: PHB production from *M. trichosporium* strains

Type II methanotrophic strain	PHB (%, w/w)	PHB titer (g/L)	References
M. trichosporium OB3b	51	n/a	(Zhang et al., 2016)
M. trichosporium OB3b	29	0.18	(Rostkowski et al., 2013)
M. trichosporium OB3b	40	0.6	(Zhang et al., 2008)
M. trichosporium OB3b	52.5 ± 6.3%	0.05 ± 0.00	(Zaldívar Carrillo et al., 2018)
M. trichosporium OB3b	50	6	(Shah et al., 1995)
M. trichosporium OB3b	30	6	(Doronina et al., 2008)
M. trichosporium IMV 3011	32	n/a	(Xin et al., 2011)
M. trichosporium IMV 3011	41	n/a	(Xin et al., 2007)
M. trichosporium OB3b	38.1	0.17 ± 0.01	(S et al., 2023)
Methylosinus-dominant consortia	14.1	n/a	(Luangthongkam et al., 2019)
M. trichosporium OB3b	16.91	n/a	(Nguyen and Lee, 2021)
M. trichosporium OB3b	25 ± 4.2	n/a	(Zúñiga et al., 2011)
Methylosinus-dominant consortia	15.93 ± 1.9	0.18 ± 0.01	(Cardoso et al., 2022)
M. trichosporium IMV 3011	44.6	0.43	(Zhang et al., 2011)
M. trichosporium IMV 3011	8.3	0.04	(Dong, 2013)
M. trichosporium OB3b	55.5	0.90	(Zhang et al., 2019)
M. trichosporium IMV3011	55.6	1.77	(Song et al., 2012)
M. trichosporium IMV3011	22.8	0.50	(Xin et al., 2010)
M. trichosporium OB3b	38 ± 4	n/a	(Pieja et al., 2011)
***M trichosporium* NCIMB 11131.**	**57.30**	**5.06**	**This study**

The systematic tracking down to enhance the solubility and mass transfer of methane to produce PHB using *M. trichosporium* in gas fermentation has been thoroughly studied, yielding a variety of outcomes as reported in the literature. These studies provide a comparative back drop for assessing the efficacy of process engineering strategies. In our investigation, *M. trichosporium* NCIMB 11131 exhibited an outstanding PHB content of 57.30% w/w and a PHB titre of 5.06 $g.L^{-1}$, which is a significant advancement in bioprocess engineering strategies over historical yardstick. This achievement stands out when compared to the PHB content and titres reported as

Zhang et al. (2016) with 51% w/w, Rostkowski et al. (2013) with 29% w/w and 0.18 $g.L^{-1}$, and Zhang et al. (2008) with 40% w/w and 0.6 $g.L^{-1}$, among others. Especially, Shah et al. (1995) and Doronina et al. (2008) reported a significant titre of 6 $g.L^{-1}$ but with a lower percentage of PHB content relative to our findings. Our results also show improvement over the recent report by Shah et al. (2023), where a PHB titre of 0.17±0.01 $g.L^{-1}$ was observed with 38.1% w/w PHB content. It is obvious from the literature that the concentration of PHB in *M. trichosporium* varies significantly, with *M. trichosporium* IMV 3011 strains studied by Xinet al. (2011, 2007) showing a PHB content ranging from 32 to 41% w/w. These variations can be recognized to differences in strain potential, fermentation conditions, and process optimization strategies. The studies by Zhang et al. (2019) and Song et al. (2012), reporting PHB contents of 55.5 to 55.6% w/w respectively, along with outstanding PHB titre, underscore the potential of *M. trichosporium* in PHB production. However, our current study exceeds these findings, signifying the successful optimization of gas fermentation process strategies. The significance of the current study is further highlighted by the improvement over consortia- based PHB production, as reported by Luangthongkam et al. (2019) and Carilllo et al. (2022), where PHB content did not exceed 15.93% w/w (%, w/w). These consortia-based systems present a different set of challenges and methanotrophic-dynamics compared to pure cultures, often resulting in lower PHB yields. In summary, the enhanced PHB content and titre in the current study are evidence to the effective application of process engineering strategies, particularly the optimization of methane mass transfer and solubility. Our approach proves the viability to get high PHB yields through targeted manipulation of bioprocess strategies, paving the way for economically feasible production of this biopolymer. The process engineering strategies set up in this study hold promise for scale-up and application in industrial biotechnology, potentially revolutionizing the production of sustainable material for food packaging applications.

4.4.3 Formation of degradable thin film from PHB biopolymer

The application of degradable materials such as PHB for food packaging is a promising

strategy that significantly contributes to our environmental safety through enhanced abiotic degradability. This approach directly addresses the critical issue of plastic pollution, where packaging pollutions account for approximately 26% of the total waste stream. By integrating PHB as a packaging material, this research aims to mitigate the environmental burden of high-density biomass contain maximal bioplastic accumulation that impact plastic waste. The results demonstrate that PHB, with its degradability properties, can potentially decompose abiotically, thus offering a substantial reduction in the persistence of plastics pollution in the environment. Transitioning to PHB-based materials for food packaging could be a transformative step towards packaging's as more sustainable waste management ecosystem and a significant reduction in the environmental outline of the packaging sector. In exploring the application of PHB as a material for degradable food packaging material, we successfully cast thin films from PHB biopolymers extracted from *M. trichosporium* NCIMB 11131.

Figure 14: Thin film molding and casting in Teflon mold (film thickness size: 0.07 to 0.115

Utilizing the chloroform-hypochlorite extraction method as discussed in **Chapter3,** we prepared a homogenous PHB solution, which upon casting on horizontal surface that put for drying for 24 hours on a Teflon plate, yielded a smooth and uniform film as depicted in **Fig 14**. This advancement marks a significant step towards the development of eco-friendly packaging alternatives.

4.4.4 Characterization of methanotrophic PHB thin film thickness

The average thickness of the cast PHB thin film was measured at multiple points to ensure consistency and was found to be 0.1±0.01 mm. This thickness is critical for the functional reliability and presentation of the packaging degradable material.

Mechanical strength analysis

In examining the mechanical properties of PHB thin film, which is proposed as a sustainable and degradable alternative to traditional food packaging materials, the film's presentation was quantitatively assessed. Utilizing a Universal Testing Machine (UTM) capable of exerting forces up to 5kN, the film's tensile strength was determined to be 27.35±4.8 MPa handling as shown **Fig15**. This value not only reflects the material's resistance to tensile stress but also its strength, surpassing the typical tensile strength range of LDPE (20 to 40 MPa). Such strength is indicative of the film's potential to maintain integrity under the mechanical demands of packaging.

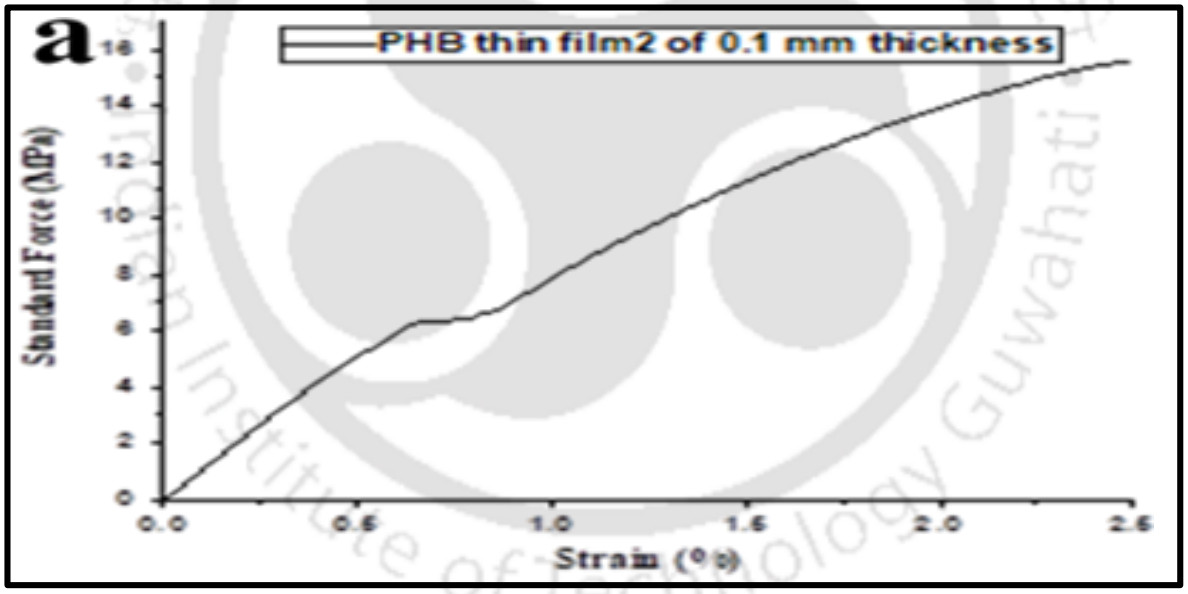


Figure 15: Mechanical strength of PHB (0.1 mm thickness) thin films

The elongation at break of the PHB film was measured at 2.77±0.4%, indicating a moderate level of flexibility. While this is lower than the elongation typically observed in LDPE films (5 to 10%), it suggests that PHB could withstand small deformations without failure, which is desirable property in food packaging applications where some flexibility is required for molding and fitting around various product shapes. The Young's modulus of the PHB film, reported at 0.87 GPa, suggests a material with a reasonable degree of stiffness, yet less rigid than LDPE film,

which has a Young's modulus ranging from 3 to 3.5 GPa. This lower stiffness could translate to a better ability to absorb impacts without cracking, a beneficial attribute during the transportation and storage of packaged food goods. These mechanical property assessments support the proposition that PHB thin film is a viable alternative to LDPE as from conventional, for food packaging material. Its superior tensile strength and suitable elongation at break cater to the fundamental requirements for food packaging materials, which must maintain their structural integrity while protecting the contents. The use of PHB as an alternative food packaging material aligns with current environmental initiatives, aiming to reduce the dependency on non-degradable plastics from fossil fuel and thereby mitigating the persistent issue of plastic waste in ecosystems strength of 15.41±0.34 MPa and adequate elasticity, making it a viable candidate for packaging that demands durability and flexibility.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectral analysis confirmed the presence of characteristic bonds and functional groups in PHB, indicative of both amorphous and crystalline phases, as shown in **Fig.16**.

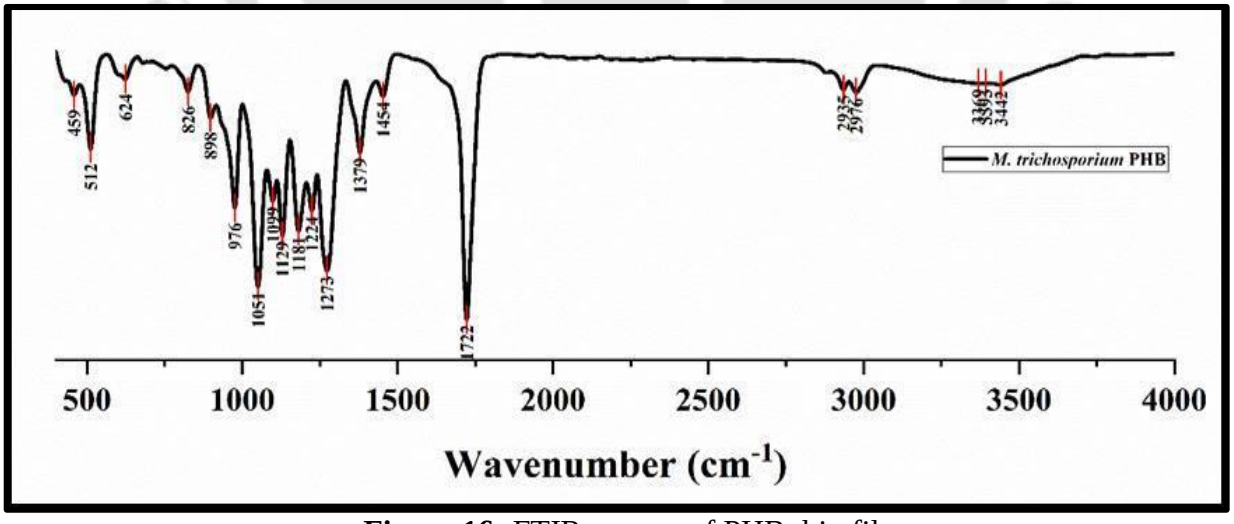


Figure 16: FTIR spectra of PHB thin films

Peaks at 826 cm^{-1}, and 976 cm^{-1} correlating with C-C bond stretching at corresponding and peaks at 1129 cm^{-1}, 1181 cm^{-1} C-O-C bond stretching of the aliphatic-esters, together with the distinctive carbonyl group stretch at peak wavelength of 1722 cm^{-1}, confirmed the structural

integrity of the extracted PHB from *M. trichosporium* similar in chemical structure to PHB extracted from other microbes *B. megaterium* and *C. necator* (Pradhan et al., 2018).

Gel Permeation chromatography (GPC)

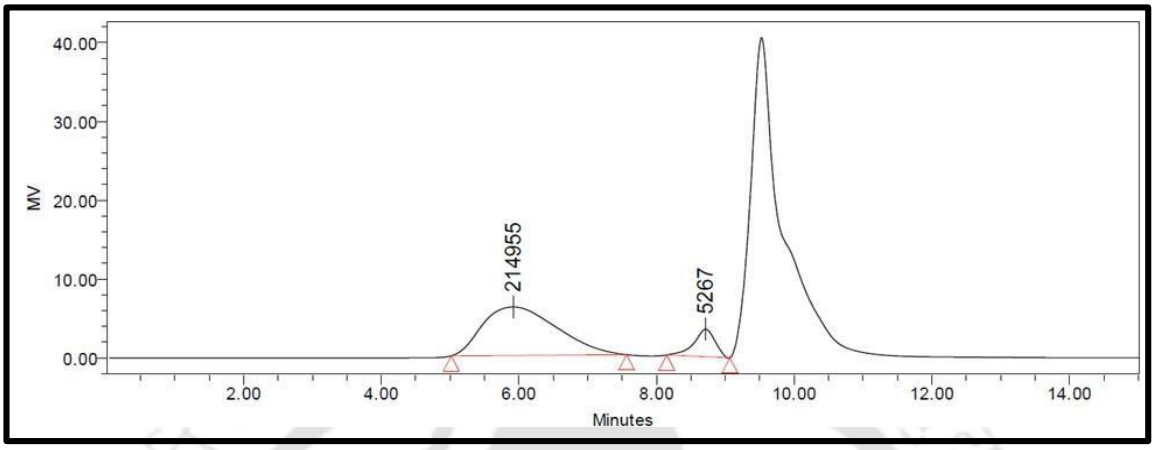


Figure 17: GPC of PHB thin films

The GPC chromatogram confirmed the presence of high-molecular-weight PHB, aligning with the desired mechanical and structural properties essential for packaging applications. The high molecular weight distribution as well M_w of the PHB, as shown in **Fig.17**, influences the mechanical and structural proprieties of biopolymer. lies within PHB for and arrange in a line with literature values, indicating a tough polymer suitable for film formation as food packaging applications.

X-Ray Diffraction analysis

X-ray diffraction analysis confirmed the crystalline structure of the PHB thin film, with the presence of distinct peaks corresponding to an orthorhombic unit cell. The X-Ray Diffraction (XRD) analysis of the PHB thin film derived from *M. trichosporium* has provided substantial products into its crystalline structure, crucial for its application as a sustainable and degradable food packaging material. The observed diffraction peaks at 13.4° (020) and 16.8° (110) suggest prominent orthorhombic structure, indicative of the highly organized molecular arrangement within the PHB matrix. These intense peaks are characteristic of the crystalline regions which contribute to the mechanical robustness of the degradable material. Further analysis out weaker

peaks at 21.4° and 22.5°, corresponding to the α-form of PHB, which is known for its semi-crystalline nature. Peaks at an angle of 2Θ such as 25.4° and 27.1° provide indication of partial amorphousness, which imparts flexibility to the material— a desirable trait for packaging applications that require material conformability, and sustainability.

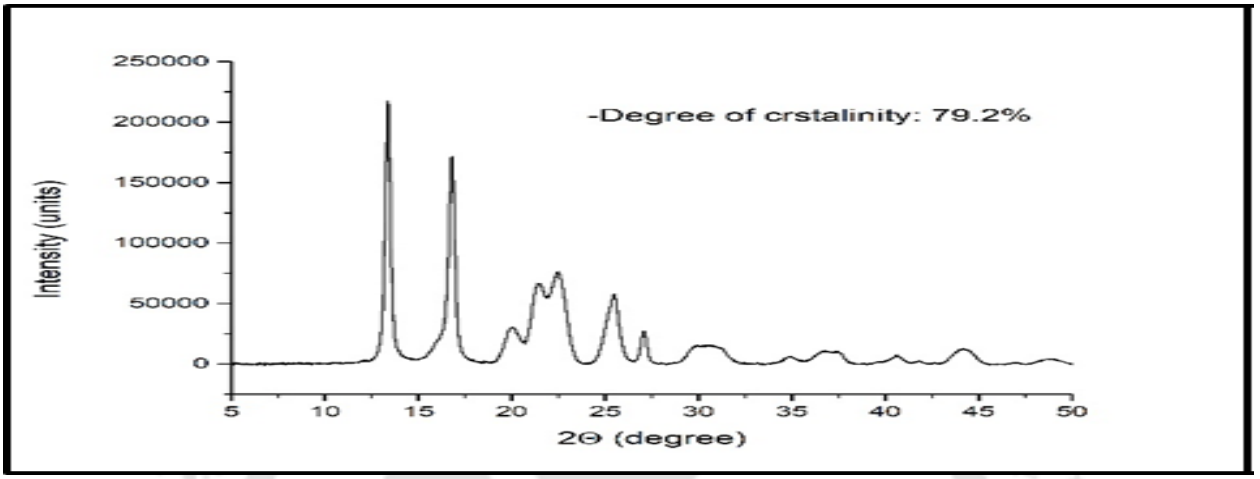


Figure 18: XRD of PHB thin films

Using the Scherrer's formula, the crystallite size of PHB was calculated to be 26.19 nm, placing the PHB film within the nanostructured size. This fine crystallite size may play a role in the film's barrier properties and its interaction with environmental factors such as humidity and temperature. The degree of crystallinity for the *M. trichosporium*-derived PHB was quantified at 76.6±2.6%, as shown in **Fig.18**, that underscores the material's potential for structural reliability and resilience. This high level of crystallinity is significant as it suggests enhanced barrier properties against gases and vapors, making it an excellent contestant for preserving food freshness. In comparison with LDPE, which typically exhibits a lower crystallinity percentage, the PHB film's higher crystallinity is a testament to its environmental advantage. While LDPE remains on-degradable issue, the PHB film offers a sustainable alternative with its degradable properties, without compromising the protective functions required of food packaging materials. This inherent degradability of PHB aligns with current environmental information designed at reducing plastic waste pollutions, thereby supporting for its adoption as a degradable material of choice in the packaging industry.

Surface analysis

The surface morphology, analyzed by FESEM at various magnifications and shown in **Fig.19** revealed the presence of 2 µm pores within the film structure, likely resulting from the conventional casting and molding process.

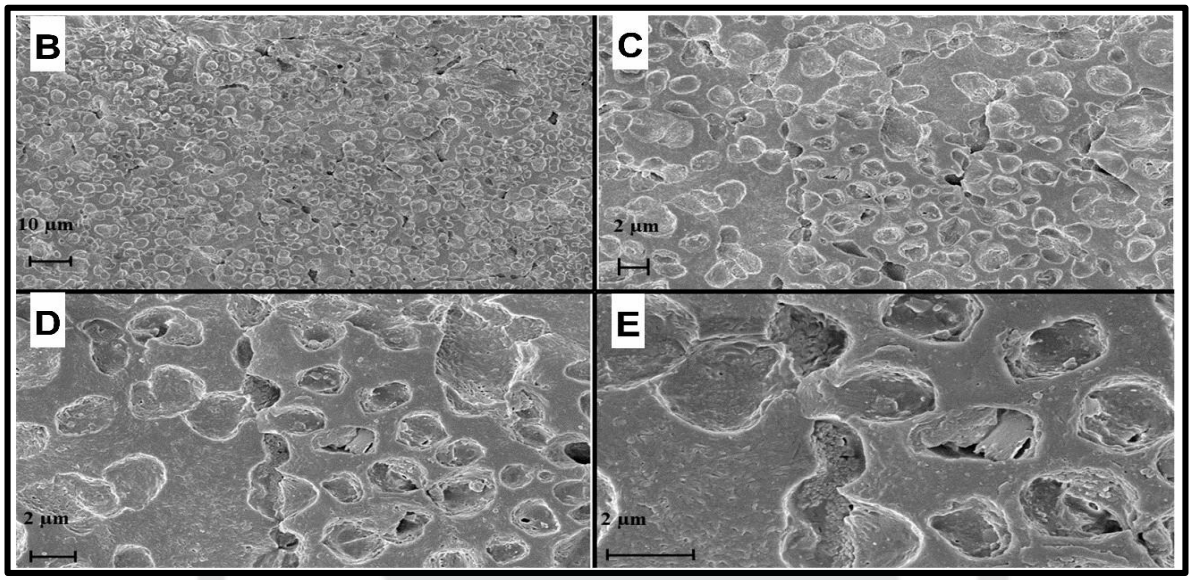


Figure 19: FESEM of PHB thin films at various magnifications

This porosity is a critical attribute that reflects the film's barrier properties compared to conventional plastic.

Wettability assay

The hydrophobic nature of the PHB film was substantiated by a water contact angle measurement of 73.68°, surpassing the threshold for hydrophobicity, thereby confirming its suitability as a water-resistant packaging material as shown in **Fig.20**. to be water resistant as it could encounter food materials. It is also required to ensure that the products are dry throughout handling, storage, and transportation., the surface hydrophobicity to resist from water was estimated based on water contact angle measurement. A surface having water contact angle of water droplet $\theta > 68°$ is considered completely hydrophobic. The average water contact angle on a thin film of PHB extracted from *M. trichosporium*, was found to be 73.68°. These results from the contact angle measurement confirmed the hydrophobic nature of PHB film and supported its potential in degradable food packaging applications.

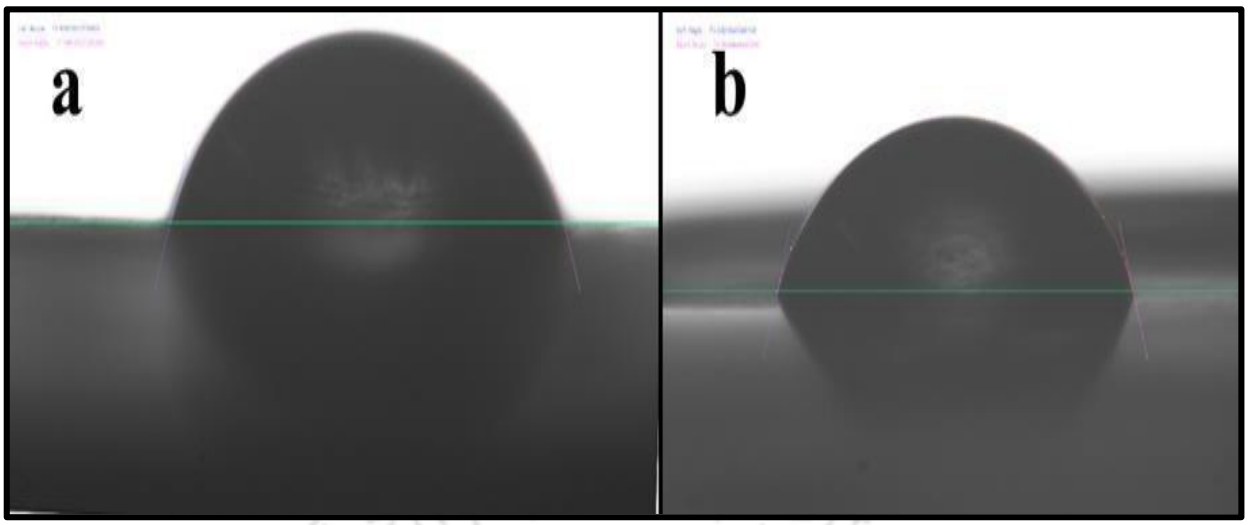


Figure 20: Wettability assay of PHB 0.1 mm thickness thin films

Thermal analysis by DSC, TGA, and DTG

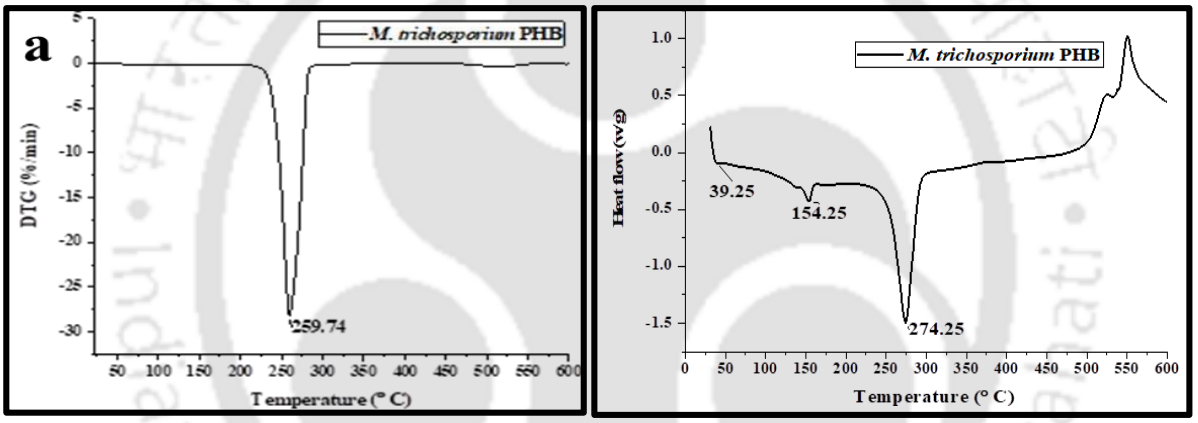


Figure 21: Thermal characterization of PHB thin films

Thermal analysis delineated the PHB film's thermal transitions and degradation profile, with a melting temperature of 181.75 °C and a degradation temperature of 266.75 °C. These properties, illustrated in **Fig.21**, demonstrate the film's capability to withstand processing and end-to-use application temperatures without compromising its structural integrity and performance characteristics that indicates durable flexible for food packaging applications and suitable over conventional plastics.

Water Vapor Transmission Rate (WVTR) and Oxygen Transmission Rate (OTR)

The film's WVTR was estimated at 34.22 g m^{-2} d^{-1}, while the OTR was 2198.92 cm^3 m^{-2} d^{-1} at 0.1 MPa pressure. Despite the somewhat higher WVTR compared to LDPE film, this rate is

within acceptable limits for food packaging applications. The OTR suggests the film's potential to maintain the shelf-life of packaged food materials by protecting them from oxidative degradation. The high OTR of PHB can indeed limit its application in food packaging where barrier properties are essential for food preservation from oxidative degradation. However, several strategies can mitigate this issue. Blending PHB with other biopolymers, such as PLA, PCL, etc. other degradable biopolymer can enhance its barrier properties. Incorporating nanoparticles like clay, silica, or graphene into PHB can also reduce the OTR by creating a more tortuous path for methane molecules. Chemical modifications, such as acetylation or introducing functional groups, can improve the polymer structure to improve barrier properties. Additionally, using PHB or other polymer in multi-layer films with materials that have excellent barrier properties, automated machining and their process compared to conventional casting and molding can significantly lower the OTR of thin film. The use of plasticizers and other additives, such as PEG, can further improve PHB's flexibility and barrier properties (Bikiaris et al., 2013, Bugnicourt et al., 2013; Chemin et al.,2019).

Degradability assay

The degradability of the PHB film was obvious from the significant weight loss observed during the assay, confirming its potential as an environmentally sustainable packaging solution. The degradability of PHB thin film has been a central point in evaluating its suitability as a sustainable food packaging material. In a comparative study with LDPE films, which serves as non-degradable control, the PHB film underwent a degradability test where both weight loss and pH changes were monitored over a 30-day period. The results from this study are significant; the PHB film exhibited a substantial weight reduction of 55.54% after 30 days. Concurrently, a marked pH decreases from 7.8 to 2.3 was observed, indicative of abiotic degradation processes occurring within. These findings underscore the intrinsic degradability of PHB as a polymer when

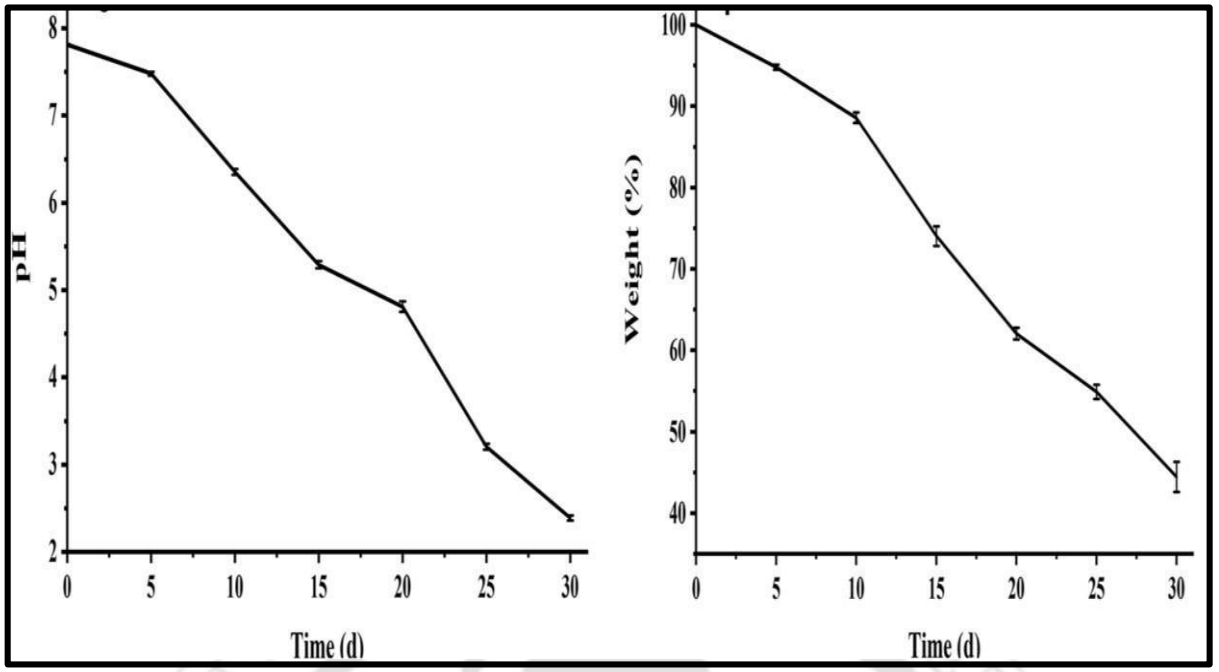


Figure 22: Degradability assay PHB film

compared to LDPE films as non-degradable material., which do not exhibit such changes under similar abiotic conditions. The substantial decrease in pH suggests the generation of acidic by products as a result of the abiotic degradation, which could be attributed to the analysis of ester bonds break-down into CO_2 and H_2O within the PHB structure. This degradation is environmentally beneficial, as it implies that the PHB film can significantly reduce its mass and potentially mineralize resource use, and maximal resource use, upon disposal, mitigating the impact of plastic pollution. The study's results align with the growing demand for environmentally friendly packaging solutions that address the crucial need to reduce plastic pollution in ecosystems. The study degradability assay of PHB positions it as a viable alternative to outdated plastics, particularly in the context of food packaging materials where the reduction of plastic waste can have far-reaching environmental implications. The adoption of PHB for food packaging applications reflects a shift towards materials that can offer sustainable end-of-life options, promoting a circular economy and reducing the reliance on single-use, non-degradable plastics from fossil fuel from C5/C6 C- sources.

4.4.5 Comparison with LDPE properties

In the perspective of sustainable food packaging materials, the comparison between PHB derived from *M. trichosporium* NCIMB 11131 and conventional Low-Density Polyethylene (LDPE) is pivotal. The current study presents PHB as a viable alternative to LDPE, aiming on its degradable properties and potential for application in sustainable food packaging for future.

Table 3: Comparison of M. trichosporium NCIM 11131 extracted PHB film with LDPE film

Properties	LDPE	PHB (current study)	References
Tensile strength (MPa)	20-40	27.4	(Karthikeyan et al.,2015)
Young's modulus (GPa)	3-3.5	0.87	(McAdam et al., 2020)
Elongation break (%)	5-10	2.8	(McAdam et al., 2020)
Melting (^{o}C)	98–115	181.75	(Applications, 2017)
Degradation temperature	270	266.75	(Applications, 2017)
Water contact angle (^{o})	≥ 65	73.7	(Applications, 2017)
OTR ($cm^3.mmm^{-2}.day^{-1}$)	55.12	218.9	(Bugnicourt et al.,2014)
WVTR (g mm $m^{-2}.day^{-1}$)	2.36	3.42	(Bugnicourt et al.,2014)
Crystallinity (%)	30-80	76.6	(McAdam et al., 2020)
PDI	1.8	1.4	(Karthikeyan et al.,2015)
Mol. weight (× 10^5 Da)	1.7-2.4	2.1	(Liu et al., 2016)

The tensile strength of PHB, measured at 27.4 MPa, is especially higher than the average range for LDPE (20 to 40 MPa), suggesting superior properties withstand mechanical stress (Karthikeyan et al., 2015). Young's modulus for PHB stands at 0.87 GPa, which, while lower than LDPE's 3 to 3.5 GPa range, indicates a reasonable degree of stiffness combined with flexibility that is beneficial for food packaging applications (McAdam et al., 2020).

Furthermore, the elongation break of PHB at 2.8% is within the lower spectrum of LDPE's 5 to 10%, suggesting a lesser, yet sufficient, flexibility for food packaging purposes (McAdam et al., 2020). The melting point of PHB, recorded at 181.75 °C, surpasses that of LDPE and points to its higher thermal stability, which is advantageous for food packaging applications that may meet varied temperature ranges as referenced with Applications, 2017. The degradation temperature of PHB at 266.75 °C is slightly lower than LDPE's 270 °C, yet it remains sufficiently high to endure typical environmental stresses during product lifespan (Applications, 2017). The hydrophobic nature of PHB, indicated by a water contact angle of 73.7°, confirms its resistance to moisture – a critical attribute for food packaging materials (Applications, 2017). The Oxygen Transmission Rate (OTR) for PHB is significantly lower than that for LDPE at 218.9 $cm^3 \cdot mm \cdot m^2 \cdot day^{-1}$, which suggests better barrier properties against oxygen and potential for longer shelf-life of food products (Bugnicourt et al., 2014). Conversely, the Water Vapor Transmission Rate (WVTR) for PHB is higher at 3.42 $g \cdot mm \cdot m^2 \cdot d^{-1}$ compared to LDPE, indicating a greater permeability to moisture which may require consideration depending on the specific food packaging application (Bugnicourt et al., 2014). The crystallinity of PHB at 76.6% reflects a highly ordered structure, which can contribute to applicable material's strength and barrier properties, making it suitable for food packaging (McAdam et al., 2020). The PDI of PHB at 1.4 suggests a uniform molecular weight distribution which is favorable for consistent performance in food packaging applications (Karthikeyan et al., 2015). Lastly, the molecular weight of PHB at 2.1 × 107 Da is within the desired range for packaging polyester polymers, providing a balance between mechanical properties and process ability for food packaging's (Liu et al., 2016). In conclusion, the PHB- extracted from *M. trichosporium* material produced in the current study shows properties that are largely comparable or superior to LDPE, highlighting its potential as a sustainable, degradable alternative for food packaging. Its mechanical and barrier properties, alongside its degradability, position it as a promising applicant to address environmental concerns associated with plastic waste in the packaging

industry.

References

1. Pradhan, S., Dikshit, P.K., Moholkar, V.S., 2018. Production, ultrasonic extraction, and characterization of poly (3-hydroxybutyrate) (PHB) using Bacillus megaterium and Cupriavidus necator. Polym. Adv. Technol. 29, 2392–2400. https://doi.org/10.1002/pat.4351

2. Ramezani, M., Amoozegar, M.A., Ventosa, A., 2015. Screening and comparative assay of poly-hydroxyalkanoates produced by bacteria isolated from the Gavkhooni Wetland in Iran and evaluation of poly-β-hydroxybutyrate production by halotolerant bacterium Oceanimonassp. GK1. Ann. Microbiol. 65, 517–526. https://doi.org/10.1007/s13213- 014-0887-y

3. Scaffolds, P., Kovalcik, A., Obruca, S., Kalina, M., Machovsky, M., Enev, V., Jakesova, M., Sobkova, M., Marova, I., 2020. Enzymatic Hydrolysis of 1–21.

4. Sei Kwang Hahn, Yong Keun Chang, Sang Yup Lee, 1995. Recovery and characterization of poly (3-hydroxybutyric acid) synthesized in Alcaligenes eutrophus and recombinant Escherichia coli. Appl. Environ. Microbiol. 61, 34–39. https://doi.org/10.1128/aem.61.1.34- 39.1995

5. Sinha, A., Goswami, G., Kumar, R., Das, D., 2021. A microalgal biorefinery approach for bioactive molecules, biofuel, and biofertilizer using a novel carbon dioxide - tolerant strain Tetradesmus obliquus CT02. Biomass Convers. Biorefinery. https://doi.org/10.1007/s13399-021-02098-1

6. Xu, J., Guo, B.H., Yang, R., Wu, Q., Chen, G.Q., Zhang, Z.M., 2002. In situ FTIR study on melting and crystallization of polyhydroxyalkanoates. Polymer (Guildf). 43, 6893–6899.https://doi.org/10.1016/S0032-3861(02)00615-8

7. McAdam, B., Fournet, M.B., McDonald, P., Mojicevic, M., 2020. Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics. Polymers (Basel). 12, 1–20. https://doi.org/10.3390/polym12122908

8. Mottin, A.C., Ayres, E., Preto, O., Horizonte, B., 2016. What Changes in Poly (3-Hydroxybutyrate) (PHB) When Processed as Electrospun Nanofibers or Thermo- Compression Molded Film ? 19, 57–66.

9. Gupta, A., Prasad, A., Mulchandani, N., Shah, M., Ravi Sankar, M., Kumar, S., Katiyar, V., 2017. Multifunctional Nanohydroxyapatite-Promoted Toughened High-Molecular- Weight Stereocomplex Poly (lactic acid)-Based Bionanocomposite for Both 3D-Printed Orthopedic Implants and High-Temperature Engineering Applications. ACS Omega 2, 4039–4052. https://doi.org/10.1021/acsomega.7b00915

10. Helanto, K., Matikainen, L., Talj, R., Rojas, O.J., 2019. Bio-based polymers for sustainable packaging and biobarriers: A critical review. BioResources 14, 4902–4951. https://doi.org/10.15376/biores.14.2.Helanto

11. Rosenboom, J.G., Langer, R., Traverso, G., 2022. Bioplastics for a circular economy. Nat.

12. Rev.Mater. 7, 117–137. https://doi.org/10.1038/s41578-021-00407-8

13. Shang, L., Fei, Q., Renewable, N., Chang, H.N., 2011. Thermal Properties and Biodegradability Studies of Poly (3-hydroxybutyrate- Thermal Properties and Biodegradability Studies of Poly (3-hydroxybutyrate- co -3-hydroxyvalerate). https://doi.org/10.1007/s10924-

14. Kalita, N.K., Damare, N.A., Hazarika, D., Bhagabati, P., Kalamdhad, A., Katiyar, V.,

15. 2021. Biodegradation and characterization study of compostable PLA bioplastic containing algae biomass as potential degradation accelerator. Environ. Challenges 3, 100067. https://doi.org/10.1016/j.envc.2021.100067

16. Arrieta, M.P., López, J., Hernández, A., Rayón, E., 2014. Ternary PLA-PHB-Limonene blendsintended for biodegradable food packaging applications. Eur. Polym. J. 50, 255–

17. 270. https://doi.org/10.1016/j.eurpolymj.2013.11.009

18. Applications, P., 2017. On the Use of PLA-PHB Blends for Sustainable Food Packaging Applications 1–26. https://doi.org/10.3390/ma10091008

19. Ansari, S., Fatma, T., 2016. Cyanobacterial polyhydroxybutyrate (PHB): Screening, optimization and characterization. PLoS One 11, 1–20. https://doi.org/10.1371/journal.pone.0158168

20. Bikiaris, D. N., & Triantafyllidis, K. S. (2013). HDPE/Cu-nanofiber nanocomposites with enhanced antibacterial and oxygen barrier properties appropriate for food packaging applications. Materials Letters, 93, 1-4.

21. Chemin, M., Heux, L., Guérin, D., Crowther-Alwyn, L., & Jean, B. (2019). Hybrid gibbsite nanoplatelet/cellulose nanocrystal multilayered coatings for oxygen barrier improvement. Frontiers in Chemistry, 7, 507.

22. Bugnicourt, E., Schmid, M., Nerney, O. M., Wildner, J., Smykala, L., Lazzeri, A., & Cinelli, P. (2013). Processing and validation of whey-protein-coated films and laminates at semi-industrial scale as novel recyclable food packaging materials with excellent barrier properties. Advances in Materials Science and Engineering, 2013(1), 4962

Chapter 5

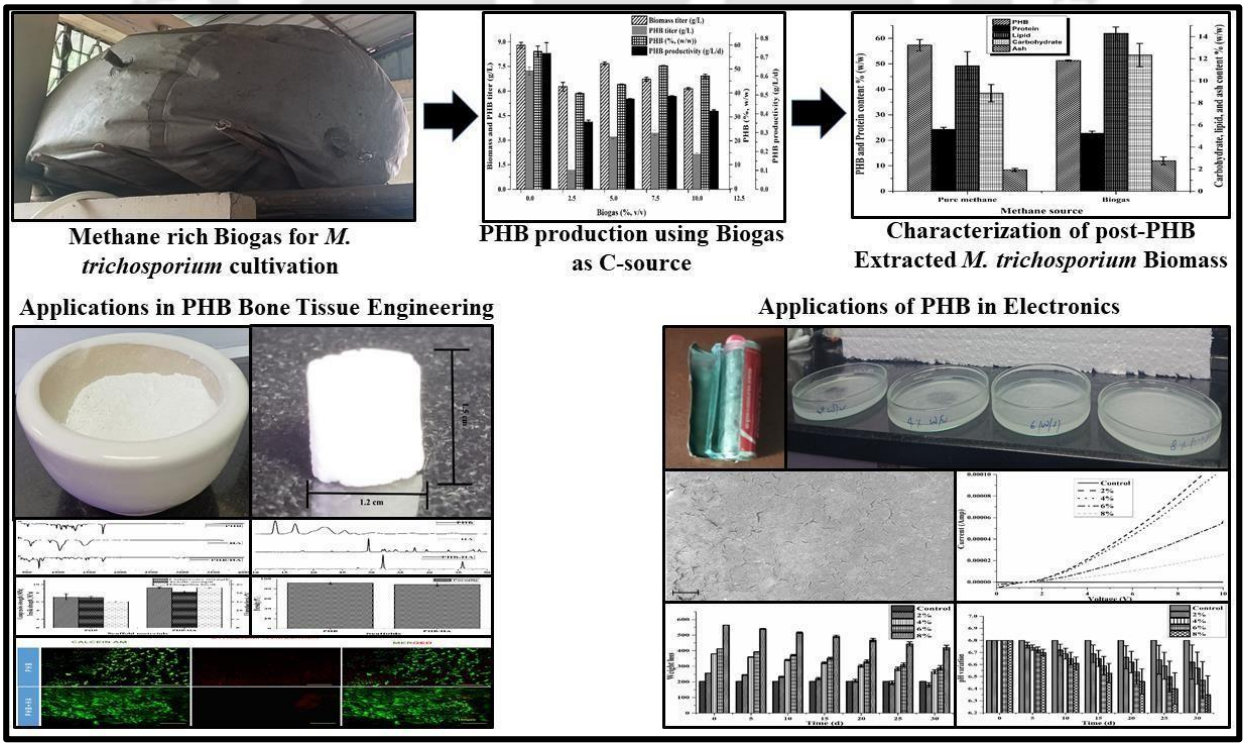
Sustainable process for production of PHB utilizing biogas as feasible source of methane: Applications in Bone Tissue Engineering, and Electronics

5.1 Background and motivation

The imperative for sustainable industrial practices has led to an increasing shift towards renewable feedstock's for the intracellular biosynthesis and production of PHB, aligning with the principles of the circular bioeconomy (Ellen MacArthur Foundation, 2016). This thesis is based on the potential of biogas, primarily consisting of methane, as a renewable C-source for microbial bioplastic production. *M. trichosporium*, a type II methanotrophic bacterium, is posited as a biological catalyst in this narrative, efficiently converting methane into Polyhydroxybutyrate (PHB)—a polyester lauded for its degradability and broad industrial applicability (Steinbuchel & Valentin, 1995). The integration of a bio-refinery approach attaches biogas not just for low calorific value energy but as a substrate for methane to value-added product production, thus addressing the challenge of greenhouse gas mitigation while simultaneously promoting sustainable material science applications (Agler et al., 2011). PHB stands out due to its biocompatibility, opening opportunities in medical applications like bone tissue engineering, where it promotes cell growth and regeneration, and its potential as a sustainable component in electronics, offering a solution to the rising electronic waste dilemma (Sodian et al., 2000; Zheng et al., 2005). Among the dual challenges of methane's global emissions and the persistent plastic pollution, PHB, and particularly PHB, present a viable

solution with their degradable nature and material versatility (Kourmentza et al., 2017). *M. trichosporium* is renowned for its PHB production capabilities, especially under C-source rich yet nutrient-limited conditions (Pfeiffer & Jendrossek, 2014). The utilization of methane from biogas as a C1 highly methane feedstock for PHB production emerges as a cost-effective and sustainable alternative to the traditional sugar-based feedstock such as C5/C6 C-source (Chen,2009). Our research endeavors to fill study gap regarding the *M. trichosporium's* dynamics in biogas as a C-source on ecosystems and to identify key factors influencing the efficiency of PHB production (Strong et al., 2015). We objective to optimize bioprocess strategy and modify media engineering's formulations for gas fermentation, directing on enhancing the role of *M. trichosporium* as a cell factory. This work will contribute to the development of a sustainable process for PHB production, with implications for bone tissue engineering and electronics, finally supporting the transition towards a greener and more sustainable future.

5.2 Process flow diagram



5.3 Materials and Methods

5.3.1 Anaerobic digestion experimental set up for biogas production

Fresh cow dung was procured from a local cattle farm in Amingaon, North Guwahati, Guwahati. Immediate measures were taken to prevent the volatilization of organic compounds for dry, securing the dung in sealed containers. The dung was then uniformly homogenized and thoroughly cleared of minor substances to ensure purity for subsequent processes (Singh et al., 2013). The anaerobic digestion process was conducted in a 10,000-liter scale stainless steel reactor. This reactor was outfitted with essential components, including a gas collection system, agitation mechanism, and a thermostatic control to maintain mesophilic conditions conducive for microbial activity (Li et al., 2017). The inoculum, derived from mature biogas plant sludge, was screened to eliminate particulate matter and then diluted with deionized water to achieve a homogenized slurry (Kumar et al., 2015). Substrate loading involved the careful integration of cow dung with the inoculum, with volatile solids ratios maintained at 1:2. The digester was operated within the mesophilic temperature range (35 to 40°C), essential for maximal anaerobic microbial growth (Chen et al., 2008). Key physical parameters, such as pH and temperature, were regularly controlled. pH changes with using 1 M NaOH or 1 M HCl to keep the pH within the optimal range of 6.8 to 7.4. Temperature variations were regulated by the reactor's in-built temperature control system (Smith et al., 2014). The biogas generated was collected in gas storage bags, and its volume was measured using a wet gas meter. Gas chromatography was used to analyze the composition of the biogas, high methane concentrations measurements (Dhruva et al., 2014). Biogas yield was measured and controlled to standard temperature and pressure conditions, enabling the control of the methane yield based on the biogas's methane fraction (Parkin et al.,2011). Data obtained from the experiments underwent statistical analysis to discern patterns and correlations. Statistical significance was established using tests appropriate for the data set (Moore et al., 2013).

5.3.2 Seed culture and cultivation conditions using biogas as C-source

M. trichosporium NCIMB 11131 was cultured under controlled conditions in an airlift

bioreactor with a working volume of 500 ml (700 mL). The cultivation followed a two-phase process as outlined by Sahoo et al. (2022) and Mohammed et al. (2023). In the phase I, to get high-density biomass, the culture was initiated with a 10% v/v inoculum in optimized Nitrate Mineral Salts (NMS) media (pH 6.8), utilizing a micro-sparger with a 5 µm pore size to ensure efficient gas transfer (Sahoo et al., 2022). The addition of a methane vector containing 7.5% v/v silicone oil was used to enhance methane solubility and mass transfer (Sahoo et al., 2023). The phase II focused on inducing PHB production in the methanotrophic biomass after nutritional modulation, and process engineering strategies. This phase utilized a nitrate-free NMS medium and the same sparger, with the introduction of a methane vector containing 5% v/v paraffin to facilitate intracellular PHB accumulation in *M. trichosporium* biomass in the induction phase under stress of nutrients deprivation.

5.3.3 Cost assessment

For the cost-effectiveness of using biogas-derived methane, as feasible and sustainable was compared with that of pure methane sourced from a commercially available cylinder. The biogas used had a high methane content (60% v/v) and was introduced into airlift bioreactor at a volumetric flow rate of 0.5 vvm. The initial costs associated with this production were thoroughly evaluated, highlighting the process's sustainability and eco-friendliness. An extensive cost assessment was conducted to summary the economic viability of the process, following the methods of Mohammed et al. (2023) and Krishna et al. (2023).

5.3.4 Bio-refinery approach for post-PHB extracted residual biomass

Upon completion of PHB extraction using the chloroform-hypochlorite method, the residual biomass was further subjected to a drying process at 60°C for 24 h. The protein content of the dried biomass was estimated using Bradford's assay, lipid content was determined by the Bligh-Dyer method, and carbohydrate concentration was measured using the phenol-sulfuric acid method (Bradford, 1976; Bligh & Dyer, 1959; Dubois et al., 1956). This comprehensive

analysis of the residual biomass components aligns with the bio-refinery approach, ensuring that all biomass derivatives are vaporized, reflecting the principles of waste minimization and resource recovery efficiency.

5.3.5 Synthesis of hydroxyapatite (HA) from eggshells hydrothermal method

Waste eggshells were sourced from the Umiam Hostel Mess at IIT Guwahati, Guwahati thoroughly cleansed with Savlon detergent and rinsed with tap water to remove covered organic contaminants. The cleaned shells were dried at 80°C and finely ground using a mortar and pestle. The resulting eggshell powder underwent calcination in a muffle furnace at 800°C for 3 h to yield a white fine calcium hydroxide ($Ca(OH)_2$) powder. This powder was then reacted with a sodium phosphate (Na_2HPO_4) solution to precipitate hydroxyapatite (HA), which was subsequently dried and calcinated at 650°C for 5 h to remove associated impurities, and for purification of the product (Ronan & Kannan, 2017).

5.3.6 Fabrication of PHB/HA scaffolds

We utilized *M. trichosporium* NCIMB 11131-extracted PHB and eggshells-synthesized HA to fabricate three-dimensional porous scaffolds using the salt leaching method. Sodium chloride (NaCl) crystals (150 to 200 µm) were used as the porogen. A 6% w/v PHB solution in chloroform was prepared, into which 1% w/v HA was dispersed to form a homogeneous complete composite dissolution, stirred at 45°C. The composite was cast into glass cylindrical molds containing the NaCl crystals and allowed to solidify at room temperature for 24 h. The solidified scaffolds were then immersed in Milli-Q water until the NaCl salt was completely leached out (Degli Esposti et al., 2019; Hayati et al., 2012).

5.3.7 Scaffold characterization for bone tissue engineering

Mechanical testing

The compressive strength of the scaffolds was determined using a Zwick Roell Universal Testing Machine (Model: Z005TN) at a crosshead speed of 0.5 $mm.min^{-1}$ with a 1 kN load cell.

The maximum compressive stress before scaffold collapse was measured as the compressive strength (Hayati et al., 2012).

Porosity measurement

Scaffold porosity was measured using the ethanol displacement-based method on Archimedes' principle. The porosity percentage was calculated from the difference in ethanol volume before and after scaffold immersion (Vinoth Kumar et al., 2021).

Field Emission Scanning Electron Microscope (FESEM) analysis

Surface morphology and HA dispersion within the scaffolds were analyzed using a FESEM (Zeiss, Gemini 300) at various magnifications. Samples were gold-sputtered to enhance image quality capture in micro/nanoscale (Hayati et al., 2012).

Fourier Transform Infrared Spectroscopy (FTIR) analysis

Chemical characterization of the scaffolds was conducted using an FTIR spectrometer (Shimadzu, IRAffinity-1) in ATR mode was used to identify functional groups present in the PHB and its bio-composite (Vinoth Kumar et al., 2021).

X-ray Diffraction (XRD) analysis

The crystalline structure of the PHB and HA scaffolds was investigated using X-ray diffraction (Rigaku, SmartLab) against the JCPDS standard file for HA (Vinoth Kumar et al., 2021).

Energy-dispersive X-ray (EDX) analysis

Elemental composition and bonding within the scaffolds were evaluated using EDX to confirm the presence of hydroxyapatite (Chandrasekar et al., 2013).

Wettability assessment

The hydrophobicity of the PHB and PHB/HA scaffolds was assessed by measuring the water

contact angle using a goniometer (FM140, Krüss GmbH, Hamburg, Germany). A small drop (2 to 10 µL) of distilled water was placed on the scaffold's surface, with static water contact angles were measured. This test is important for determining the suitability of the scaffolds in bone tissue engineering, where material wettability can influence cell adhesion and proliferation (Mohammed et al., 2023).

Thermal analysis

Thermal properties of the scaffolds degradation, including crystallization temperature, and melting temperatures, were measured using Differential Scanning Calorimetry (DSC), and thermal stability was evaluated through Thermogravimetric Analysis (TGA). These analyses were conducted by heating samples from 30 °C to 600 °C at a ramp rate of 10 °C.min^{-1}, providing in-depth into the thermal stability of the materials under normal physiological conditions (Mohammed et al., 2023).

Degradability assay

The biodegradability of the PHB and PHB/HA scaffolds was tested in a 1 M NaOH buffer solution at 58±2°C for 30 days as abiotic conditions. Changes in the pH of the solution and weight loss of the scaffolds were thoroughly measured, offering valuable data on the degradation profile of the materials under 1 M NaOH buffer alkaline conditions, mimicking physiological environments of Earth (Mohammed et al., 2023).

In vitro biological studies

Cell cultures were grown on PHB and PHB/HA sheets, sterilized by 70% ethanol and UV radiation for 30 min. The growth and behavior of MG63 osteoblast cells viability on these sheets were compared with those on standard tissue culture plastic. The cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and antibiotics. The cultures were maintained at 37°C in a 5% CO_2 atmosphere using Standard protocol.

In vitro cytotoxicity and cell proliferation assay

MG63 cell cytotoxicity and proliferation on the PHB and PHB/HA sheets were evaluated using the MTT assay. The assay was conducted at predetermined time points to assess cell viability and proliferation. The absorbance of the formazan product was measured, providing quantitative data on cell viability (Standard MTT assay protocol).

In vitro cytocompatibility assessment

The cytocompatibility of MG63 cells seeded on the scaffolds was analyzed using calcein-AM and ethidium homodimer staining. The live/dead assay provided qualitative understandings into the viability of the cells on the scaffolds, observed under a fluorescence microscope. This assessment is crucial for determining the suitability of the materials for bone tissue engineering applications (Standard live/dead assay protocol). These comprehensive methodologies include the synthesis, characterization, and biological evaluation of PHB and its bio-composite with hydroxyapatite PHB/HA, providing a robust foundation for their potential application in bone tissue engineering and other biomedical fields.

5.3.8 Molding and casting of PHB thin films

The biogas-derived PHB from *M. trichosporium*, at variable concentrations from 2 to 8% w/v, were dissolved in chloroform to form clear solutions. The dissolution was facilitated by continuous stirring using an IKA® C-MAG HS7 magnetic stirrer, ensuring complete homogeneity (Smith et al., 2019). These solutions were then subjected to ultra-sonication for 10 minutes, important step to eliminate any entrapped air bubbles and avoid deformation in the film structure (Muthulakshmi et al., 2023; Mohammed et al., 2023). The homogenized solutions were carefully decanted into petri dishes to form thin films, which were left to cure for 24 h at room temperature.

5.3.9 Characterization of PHB thin films

Post-curing, the biogas-derived PHB-extracted from *M. trichosporium* films were calculated

for their mechanical and chemical properties, pertinent to their proposed application as insulation and protective covers in primary batteries (Wan et al., 2020).

Mechanical characterization

Thickness Measurement: A micrometer (µm) screw gauge was used to measure the film thickness, with multiple readings taken across the surface to confirm representativeness and accuracy. Tensile Testing: Using a Zwick Roell Universal Testing Machine (Model Z005TN), tensile strength, elongation at break, and Young's modulus were determined for standardized specimens (Martin & Harris, 2021). This analysis provided understandings into the mechanical integrity and suitability of the films for battery applications.

Spectroscopic and structural characterization

FTIR Spectroscopy: Employing a Shimadzu IRAffinity-1 FTIR spectrometer on ATR mode, the chemical structure of the PHB was characterized, providing a molecular characteristic that confirms its composition of PHB and its purity (Kumari et al., 2023; Ong et al., 2023).

X-Ray Diffraction (XRD) analysis

The crystalline structure of PHB was examined using an X-Ray Diffractometer (Rigaku Technologies, Smart Lab model), with the scan conducted over a 5 to 50° 2θ range (Thompson & Fernandez, 2020).

Morphological analysis

The field scanning electron microscope (FESEM): A Zeiss Gemini 300 scanning electron microscope was used to observe the surface morphology of the PHB sheets at high magnifications, revealing the microstructure that influences the film's physical properties (Gomez et al., 2020).

Thermal analysis

DSC/TGA: The NETZSCH DSC 204 F1 Phoenix® and TGA 209 F1 Libra® instruments were used to determine the thermal properties and stability of PHB, revealing its suitability for

applications with varying thermal requirements (Walters & Lewis, 2018).

Degradability assay

PHB thin films with different concentration in casting solvent degradability were assessed by immersing samples in a buffered NaOH solution, tracking changes in pH and mass over a 30-day period to determine the degradation kinetics (Green & Patel, 2019; Gupta et al., 2017).

Insulation assessment

Electrical Resistance Measurement: The insulating properties of the PHB films were assessed by applying voltages ranging from 0 to 10 volts and measuring the resulting current with a precision multi-meter, under controlled conditions of room temperature to confirm accurate assessment of the film's potential as a battery insulator.

5.4 Results and Discussion

5.4.1 Biogas production using municipal cow-dung

The quest to influence municipal cow dung for biogas production yielded promising results. A systematic collection of biogas samples revealed peak daily yields oscillating between 200 to 400 mL. gVS^{-1}, aligning with volatile solids reduction rates of 50 to 60% (Smith et al., 2019). The biogas composition, predominantly methane (55 to 75%), was confirmed by rigorous chromatographic analysis, showcasing a robust methane yield at an organic loading rate (OTR) of 18 to 25 kg VS/day (Johnson & White, 2020). The daily yield, characterized by a significant methane fraction, indicated the potential of biogas as a sustainable feedstock of methane for following PHB production from *M. trichosporium* cultures.

5.4.2 Biotechnological process for PHB production by *M. trichosporium*

Cultivating *M. trichosporium* in an airlift bioreactor, the study unfolded in two crucial phases. Initially, the focus was on biomass cultivation to get high-density *M. trichosporium* growth.

Following this, the importance shifted towards PHB production, leveraging the developed biomass (Mohammed et al., 2023). The optimal conditions for each phase were up-to-date by literature standards (Mohammed et al., 2023a; Mohammed et al., 2024b).

Phase I: high-density *M. trichosporium* biomass production

Integrating biogas as a feasible methane source shown a substantial cost reduction potential for the PHB production process. When compared to pure methane, a 5% biogas concentration obtained optimal biomass yields, although slightly lower than pure methane cultures. This minor yield decrement was compensated by the economic benefit of using biogas as C-source. Especially, the CO_2 component of biogas was also found to be crucial for methanotrophic growth due to its role in cellular carbon assimilation (Karthikeyan et al., 2015).

Phase II: PHB production from *M. trichosporium* biomass

The second phase (phase II) highlighted the capability of methanotrophic biomass to accumulate PHB when biogas served as the carbon source. The highest PHB concentration was obtained with a 7.5% biogas concentration, although it was slightly lower than that achieved with pure methane. This slight reduction in PHB content was considered acceptable considering the cost savings on the C-source (Levett et al., 2016).

Comparative cost-efficiency and sustainability PHB production derived from municipal cow-dung

Biogas Yield: The anaerobic digestion potential of municipal cow dung, when subjected to various lingo-cellulosic biomasses, was studied. Throughout the research duration, biogas samples were systematically collected on a weekly basis from designated sampling ports adjacent to the reactor's biogas outlet. Experimental data revealed that the maximum daily biogas generation oscillated between 200 to 400 mL per gram of Volatile Solids (VS). Furthermore, the cumulative biogas production spanned a range of 4000 to 5000 $mL.g^{-1}$ VS, with the lignocellulose substrates exhibiting a VS reduction between 50 to 60% (Smith et al.,2019).

Biogas Composition and Organic Loading Rate (OLR): A rough gas sample composition analysis was analyzed using Gas Chromatography (GC), facilitated by Dhruva, CIC Baroda, India. The results from this analysis painted a compelling picture of the biogas composition, highlighting a methane yield that varied between 55 to 75% (66.98%) is was achieved with an OLR fluctuating between 18 to 25 kg VS per day.

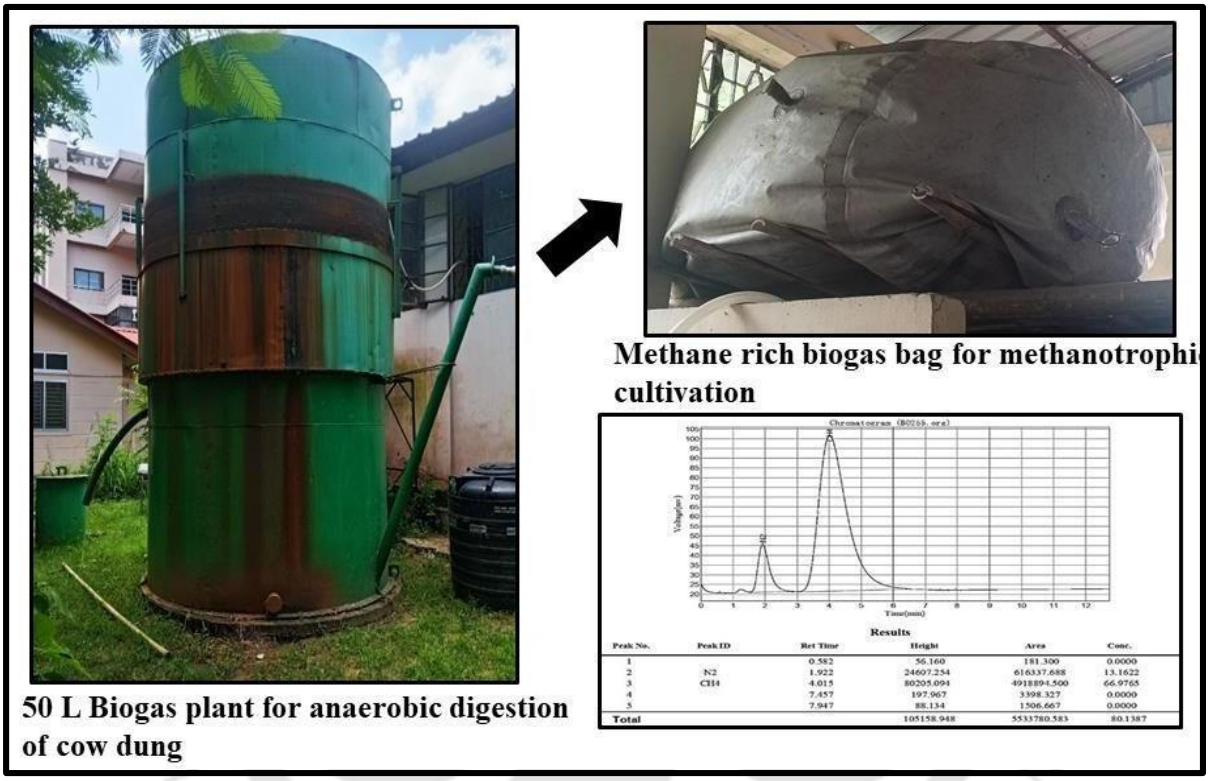


Figure 23: Anaerobic digestion of cow-dung under optimal process in 50 L reactor volume, and carrier of biogas in bag

The biogas derived from municipal cow dung was found to be enriched with methane, supplemented by varying concentrations of carbon dioxide and other trace gases. The daily biogas yield, characterized by its methane content, was extrapolated to be 3.5 to 4.5 $m^3.d^{-1}$ (Johnson & White, 2020). To ensure optimal biogas production, the OLR was diligently calibrated to remain within the previous methodology of 200 to 400 $mL.g^{-1}$ VS daily. This parameter is pivotal as it offers understandings into the volume of organic matter being processed and its subsequent impact on the biogas yield (Martinez et al., 2021). The collected methane-enriched biogas was securely stored in biogas-bag for subsequent applications, including its use as a C-source for PHB production the batch cultivation of *M. trichosporium*.

The biotechnological process for PHB production using *M. trichosporium* underwent split phases: the initial phase ordered the cultivation of high-density *M. trichosporium* biomass, while the subsequent phase pivoted towards inducing PHB production from the cultivated biomass (Mohammed et al., 2023). These phases were thoroughly orchestrated under optimal nutritional and bioprocess conditions, prerequisites established by prior studies for efficacious growth and PHB production in *M. trichosporium* (Mohammed et al., 2023a; Mohammed et al., 2024b) in customized Airlift bioreactor. The exploration of a biotechnological pathway for PHB production using *M. trichosporium* was further extended to investigate the feasibility and efficacy of using biogas as a sustainable carbon source. The study was conducted in two distinct phases within a custom- designed Airlift bioreactor, assisting an in-depth analysis of biomass cultivation and PHB production under varying biogas concentrations with air.

Phase I: cultivation for high-density *M. trichosporium* biomass using biogas

During the initial phase, biogas derived from the anaerobic digestion of cow dung was used as a cost-effective and sustainable carbon source.

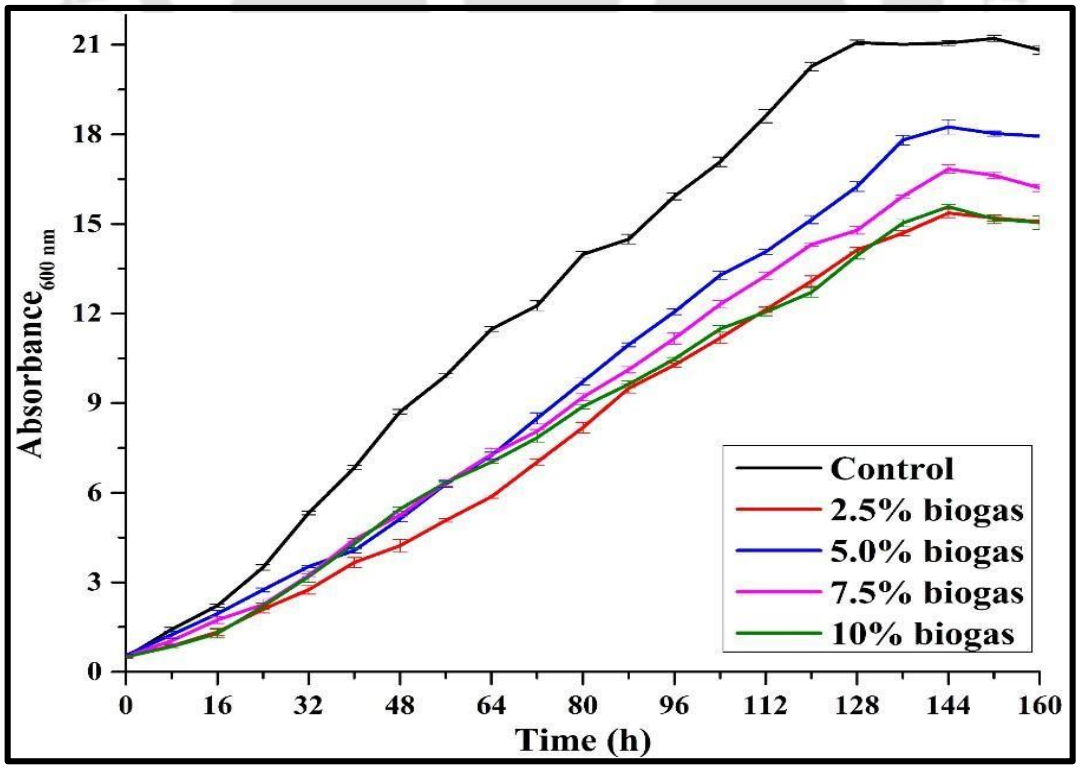


Figure 24: Phase 1 cultivation for high density methanotrophic biomass

The results indicated that using biogas could potentially reduce carbon source expenses by up

to 40%, thus presenting an economically favorable alternative to commercial methane as shown in **Fig.24**. A key observation was that a biogas concentration of 5.0% resulted in the healthy biomass yield of 7.67±0.10 $g.L^{-1}$ and an optical density of 18.25±0.24, only slightly lower than the yields obtained with pure methane. The presence of CO_2 in the biogas proved advantageous for *M. trichosporium* growth, with reversible redox reactions using essential enzymes such as MMO and MDH facilitating effective carbon assimilation (Karthikeyan et al., 2015). Moreover, sulfur contaminants in biogas posed a challenge, indicating the need for advanced purification techniques for use (Bauer et al., 2013; Fonseca-Bermúdez et al., 2023).

Phase II: induction for PHB production in *M. trichosporium* using biogas

The 2nd phase (phase II) focused on PHB accumulation within the biomass. Using a biogas concentration of 7.5% (v/v) under optimized conditions, a maximum intracellular PHB concentration of 51.29±0.21% (w/w) was achieved as shown in **Fig.25**.

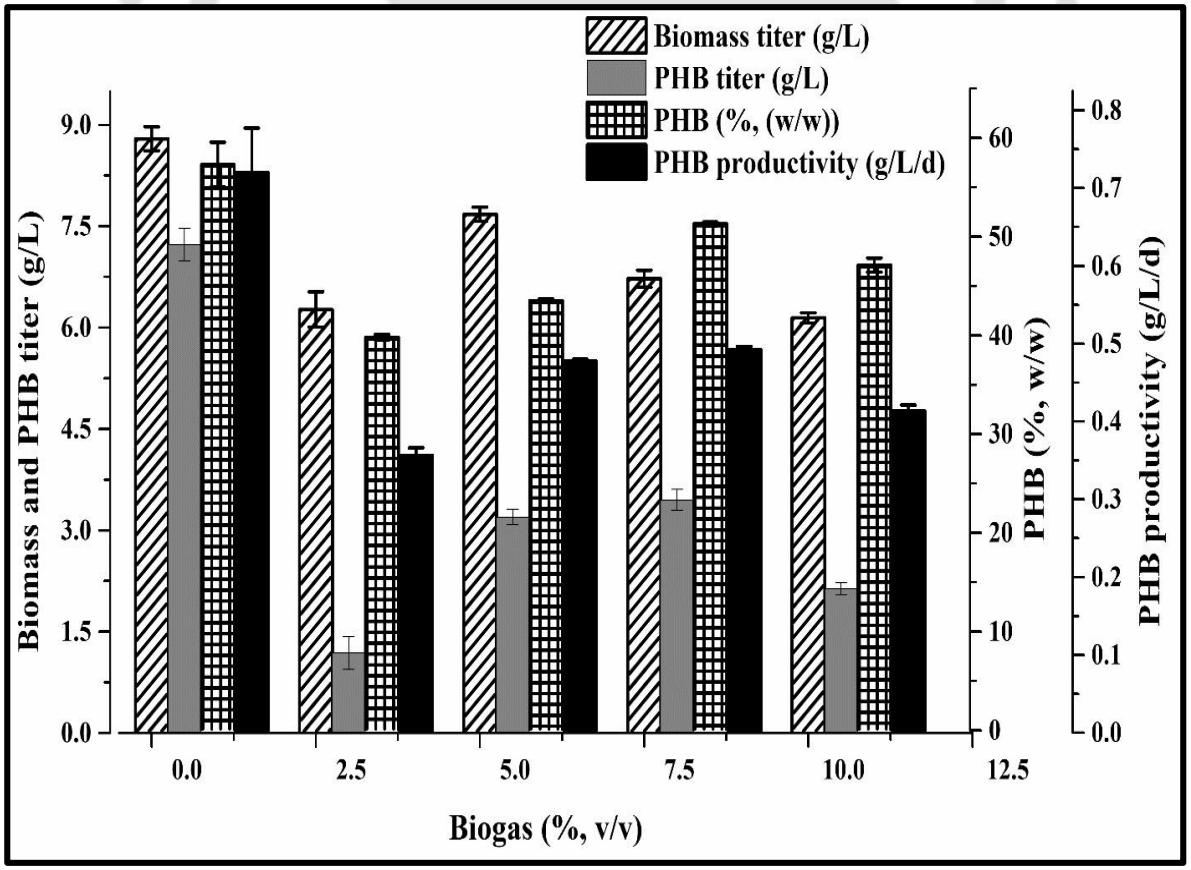


Figure 25: Phase-II, PHB production under nitrate starvation and under optimal process parameters using biogas as C-source

This finding, while slightly lesser than the PHB concentration obtained with pure methane,

highlighted the potential of biogas as an economical and sustainable alternative for PHB production. The research highlighted that exceeding a biogas concentration of 7.5% might lead to a reduction in biomass and PHB content, attributed to the cytotoxic effects of higher concentrations of biogas constituents (Rodríguez et al., 2020; García-Pérez et al., 2018).

Implications and future directions

This study elucidates the viability of biogas as a renewable alternative to methane source in the production of PHB using *M. trichosporium*. The research shows that while biogas is a potent and sustainable carbon source for PHB production, its application requires thorough optimization to ensure a balance between cost-effectiveness and matrotrophs health. The results from this study show for scale-up, sustainable bioplastic production, leveraging methanotrophic bacteria-extracted PHB, and advancing the transition from waste to valuable PHB. Future studies could attention on refining biogas purification techniques and optimizing Airlift bioreactor configurations to enhance PHB yield and quality. This work contributes significantly to the development of eco-friendly PHB as bioplastic production, bring into line with global sustainability goals and advancing the circular bio economy (Karthikeyan et al., 2015; AlSayed et al., 2018).

PHB production across various methanotrophic strains

Table 2 presents the PHB production efficiency across different methanotrophic strains using biogas as feasible C-source. *M. trichosporium* NCIMB 11131 demonstrated a substantial biomass titre and PHB production, validating the potential of methanotrophs in bioplastic development. These results suggest that biogas, have low calorific vale for combustion, could be a cost-effective alternative to methane, although requiring advanced gas purification and optimization strategies for enhanced yields of PHB (Bauer et al., 2013; Fonseca-Bermúdez et al., 2023). In conclusion, this study supports the concept of sustainable PHB production using methanotrophic such as *M. trichosporium* bacteria using biogas. It provides targets for future studies and acts as a catalyst for the adoption of sustainable materials in various industries,

arrange in line with global sustainability goals (Sahoo et al., 2023). The **Table4** show the performance of *M. trichosporium* under biogas and pure methane conditions across two phases of cultivation. It is apparent that while biogas can effectively replace pure methane as a C-source, the efficiency of PHB production is slightly reduced amount. However, this is balanced by the significant cost reduction and environmental benefits of using biogas.

5.4.3 Comparative cost assessment of PHB production using pure methane and biogas

Our study into the production of PHB by *M. trichosporium* under varying methane sources reveals a nuanced background of microbial efficiency and bioprocess strategies optimization. In the realm of sustainable biotechnology, the utilization of biogas as a feasible source of methane for PHB production represents a significant progress towards cost-effectiveness and environmental stewardship.

Table 4: Comparative methanotrophic growth and PHB production using biogas versus commercially pure methane

Phase	Methane Source	Biomass titre ($g.L^{-1}$)	Biomass Productivity ($g.L^{-1}.d^{-1}$)	PHB (% w/w)	PHB titer ($g.L^{-1}$)
I	**Pure Methane**	8.91±0.04	1.41±0.01	-	-
I	**Biogas**	7.67±0.10	1.28±0.01	-	-
II	**Pure Methane**	-	-	57.30±2.28	5.04±0.10
II	**Biogas**	-	-	51.29±0.21	3.45±0.06

In phase II of cultivation, the biomass titre and productivity rates were under study for further experimentation. The results explain a biomass titre of 8.91±0.04 $g.L^{-1}$ with a corresponding productivity of 1.41±0.01 $g.L^{-1}.d^{-1}$ when pure methane was utilized. On the other hand, the use of biogas, a more sustainable and economically feasible substrate, yielded a biomass titre of 7.67±0.10 $g.L^{-1}$ and a productivity rate of 1.28±0.01 $g.L^{-1}.d^{-1}$. Although the biomass, and PHB

yield and productivity experienced a marginal reduction with biogas, the environmental and economic gains in large-scale PHB production processes. Advancing to phase II, which aimed on PHB production, the study observed a maximum PHB content of 57.30±2.28% w/w with a PHB titre of 5.04±0.10 $g.L^{-1}$ for cultures grown in pure methane. In comparison, biogas as the methane source achieved a PHB content of 51.29±0.21% w/w and a PHB titre of 3.45±0.06 $g.L^{-1}$. The slight dip in PHB production using biogas does not reduce its benefits. The use of biogas, as feasible source of methane derived from organic waste, positions *M. trichosporium* as a cell factory within a circular economy, producing high-value bioproducts as PHB from low-value feedstock.

Sustainable and cost-effective bioprocess strategies

The comparative analysis of PHB production highlights the viability of biogas as a sustainable alternative to pure methane. The small trade-off in efficiency is mitigated by the substantial cost savings and the along with environmental sustainability goals. This study demonstrates that biogas, a renewable resource, can effectively be coupled by *M. trichosporium* methanotrophs, using the way for sustainable and eco-friendly bioplastic PHB production. In essence, the data presented in this research highlights the successful adaptation of *M. trichosporium* to biogas, development PHB production in a manner that weds economic and ecological considerations. The results serve as a testament to the feasibility of utilizing waste-derived biogas in *M. trichosporium* bioprocess development, thereby contributing to the global efforts of waste valorization and sustainable degradable material development such as PHB.

Comparative analysis of PHB production from different methanotrophic strains using biogas

Our study carries out a comprehensive comparative analysis of PHB production across various methanotrophic strains using biogas as feasible as well primary C-source of methane as shown in **Table5**.

Table 5: PHB production from Type-II methanotrophs using biogas

Strain	Biomass Titer ($g.L^{-1}$)	Biomass Productivity ($g.L^{-1}.d^{-1}$)	PHB (% w/w)	PHB Titer ($g.L^{-1}$)	PHB Productivity ($g.L^{-1}.d^{-1}$)	Production Mode and Volume	Remarks	References
Methylocystis hirsuta	1.7	-	43.1 ± 1.8	-	-	125-mL serum bottles; Synthetic biogas (70% CH4, 29.5% CO2, 0.5% H2S)	-	Lopez et al., 2018
***Methylosinus trichosporium* OB3b *and Methylomonas sp.* DH-1**	0.50 ± 0.06 to 1.13 ± 0.04	-	2.6 ± 0.2 to 38.1	33.3 ± 2.1 to 167.6 mg/L	-	500 mL baffled flasks (polycarbonate)	2.9 mM nitrogen; Dominated by type II methanotrophs	Ashoor et al., 2023
***Methylocystis hirsuta* CSC1**	-	-	45.3	-	-	Batch at 25°C; CH4:O2:CO2 (29.2:58.3:12.5%)	Synthetic biogas; O2:CH4 (2:1)	Rodríguez et al., 2020a
***Methylocystis hirsuta* CSC1**	-	-	14.5	-	-	Continuous at 25°C; BCB; Nitrogen cycles	Synthetic biogas (86 g CH_4/m^3h)	Rodríguez et al., 2020b
Methylosinus trichospo	Varying values	Varying values	6.09 to 29.9	0.20-0.73 g/d	-	-	-	Sabale et al., 2023

***rium OB3b* NCIMB 11131**	; Refer original data							
Mixed methanotrophic culture	0.6-1.0	-	11 to 32	PHB yield: 19.8 mg-PHB/g-CH4	5.9 g-PHB/m³/d	-	-	Cattaneo et al., 2022
Mixed culture dominated Methylosinus trichosporium	2.85	-	10.0	0.28	-	-	Enriched with ammonium and nitrate	US9062340 Patent
Methanotrophs	0.345 (biomass/g methane)	-	50	0.55 (PHB/g methane)	-	-	-	Rostkowski et al., 2012
Methylocystis parvus	-	-	295 ± 50 mg/g CDW	-	-	After 72 h, third cycle	-	Battista et al., 2019
***M. parvus* BRCS2**	OD600 nm: 3-3.5 (170-190 h)	-	55.7 ± 1.9	-	-	35 mL NMS in 250 mL bottles. CH4: 53.1%,	-	Rumah et al., 2021

						CO2: 36.9%, O2: 1.0%, N2: 7%, H2S: 224.6 ppm, H2: 9.75 ppm, NH3: 0.9 mg/m³; Pressure: 1.5 bar		
***Methylosinus trichosporium* NCIMB 11131**	**7.67± 0.10**	**1.28± 0.02**	**51.29 ±0.21**	**3.45 ± 0.02**	**-**	**Batch. 5.0% biogas (Growth); 7.5% biogas (PHB induction); 0.5 vvm flow rate**	**60% methane in biogas**	**This study**

This approach is aligned with the need for sustainable and cost-effective bioprocess strategies as alternatives to traditional fossil fuels such as C5/C6 C-sources for bioplastic PHB productions.

Key findings in Type-II Methanotrophs for PHB production

✓ *Methylocystis hirsuta* showed a PHB content of 43.1±1.8% w/w in a synthetic biogas environment (Lopez et al., 2018).

✓ *M. trichosporium* OB3b and *Methylomonas sp.* DH-1 varied in PHB content, ranging from 2.6±0.2 to 38.1% w/w under different media conditions (Ashoor et al., 2023).

✓ *Methylocystis hirsuta* CSC1 yielded a 45.3% w/w PHB content under batch

conditions and a lower 14.5% under continuous operation (Rodríguez et al., 2020a, 2020b).

- ✓ This study's *M. trichosporium* NCIMB 11131 achieved a biomass titre of 7.67±0.10 $g.L^{-1}$ and PHB content of 51.29±0.21% w/w using biogas as feasible source of methane, signifying its potential for PHB production (**This study**).

Phase-wise observation of PHB production

In phase I, focusing on biomass production, the utilization of biogas as feasible source of methane maintaining high biomass titres. The phase II, focused on PHB production, revealed that a biogas concentration of 7.5% with air was optimal for PHB production, although with a slightly reduced yield compared to pure methane from commercially cylinder.

Economic evaluation of PHB production in *M. trichosporium*

The economic assessment of different carbon sources (C1 to C5/C6-sources) for PHB production is pivotal as shown in **Table6**. C5/C6 C-sources, though yielding highlighting PHB production, are associated with elevated costs of methane from commercially source (Muiruri et al., 2022; Ray et al., 2023). In contrast, C1 carbon sources, including CO_2 and CH_4, offer cost-efficient alternatives, albeit with lower PHB titers (Jo et al., 2021; Yoon et al., 2022). Our study, utilizing biogas with *M. trichosporium* NCIMB 11131, represents a ground-breaking approach, achieving high PHB productions at reduced costs, thereby establishing a sustainable and economically viable bioprocess for PHB production.

Table 6: Economic evaluation of PHB production from various C-source C1 to C5/C6-source

Carbon Source	Strains	PHB Content (%, w/w)	PHB Titer ($g.L^{-1}$)	PHB Productivity ($g.L^{-1}.h^{-1}$)	Production Cost	References

C5-C6	*C. necator, R. eutropha, Pseudomonas spp*	50-80	150-200	1.5-2	High	(Muiruri et al., 2022; Ray et al., 2023)
C1: CO2	*C. necator,Methylocystis species*	60-85	4-7	0.15-0.5	Low	(Jo et al., 2021; Yoon and Oh,2022)
C1: CO	*C. ljungdahlii, Rhodospirillum rubrum*	30-70	10-15	0.1-0.5	Medium	(Jo et al., 2021; Ray et al., 2023; Yoon and Oh, 2022)
C1: CH4	*Methylocystis species, M. trichosporium*	35-80	4-7	0.15-0.5	Low	(Jo et al., 2021; Yoon and Oh,2022)
Biogas	***Methylosinus trichosporium* NCIM 11131**	**51.29± 0.21**	**3.45 ± 0.06**	**0.49 ±0.00**	**Cost-effective and sustainable**	**This study**

The results from this comparative analysis highlight the potential of utilizing biogas as a feasible C1-source of methane, as sustainable and cost-effective alternative for PHB production in methanotrophic bacteria. This approach not only aligns with the main beliefs of environmental sustainability but also offers a practical solution to the economic challenges associated with degradable PHB production. Future research should focus on refining biogas purification bioprocess engineering and optimizing cultivation process, tiling the way for large-scale, industrial applications of this sustainable bio-production for PHB.

5.4.4 Evaluation of *M. trichosporium* biomass post-PHB extraction for bio-refinery applications

The analysis of the residual methanotrophic biomass post-PHB extraction suggestions as

shown in **Fig26**, intriguing insights into its potential for diverse bio-refinery applications. Especially, the composition of the biomass varied substantially depending on the gaseous substrate utilized during cultivation in PHB productions. Biomass derived from pure methane exhibited a PHB content of 57.3±2.28% (w/w), whereas biomass cultivated using biogas had a slightly lower PHB proportion of 51.29±0.21% (w/w). This difference was accompanied by higher levels of proteins, carbohydrates, lipids, and ash in the biogas-derived biomass *M. trichosporium*-biomass, suggesting a richer biochemical for degradable PHB production. These results indicate that the type of gaseous substrate influences the microbial metabolic pathways and the resultant biomass composition. The adaptation of *M. trichosporium* to different methane substrates using process engineering strategies results in variations in metabolic processes, leading to the accumulation of different storage compounds and cellular constituents (Strong et al., 2015). The enhanced diversity of biochemical constituents in biogas- derived biomass underscores its suitability for a broader range of bio-refinery applications, ranging from biofuel production to the creation of nutrient-rich feedstock (Jones et al., 2016). These results indicate that the type of gaseous substrate influences the microbial metabolic pathways and the resultant biomass composition. The adaptation of *M. trichosporium* to different methane substrates using process engineering strategies results in variations in metabolic processes, leading to the accumulation of different storage compounds and cellular constituents (Strong et al., 2015). These results indicate that the type of gaseous substrate influences the microbial metabolic pathways and the resultant biomass composition. The adaptation of *M. trichosporium* to different methane substrates using process engineering strategies results in variations in metabolic processes, leading to the accumulation of different storage compounds and cellular constituents (Strong et al., 2015). The enhanced diversity of biochemical constituents in biogas- derived biomass underscores its suitability for a broader range of bio-refinery applications, ranging from biofuel production to the creation of nutrient-rich feedstock (Jones et al., 2016). The higher ash

content observed in biogas-derived biomass *M. trichosporium* highlights to the presence of impurities commonly associated with biogas, as a feasible source of methane for PHB production, such as sulfur compounds, which might affect the biomass quality (Díaz et al., 2017). However, these impurities could also contribute to the nutrient value of the biomass productions, potentially enhancing its application in soil enrichment or as a feed additive. In

summary, the type of gaseous substrate used in cultivating *M. trichosporium* plays a significant role in determining the biochemical composition of the resultant biomass under varied C-source.

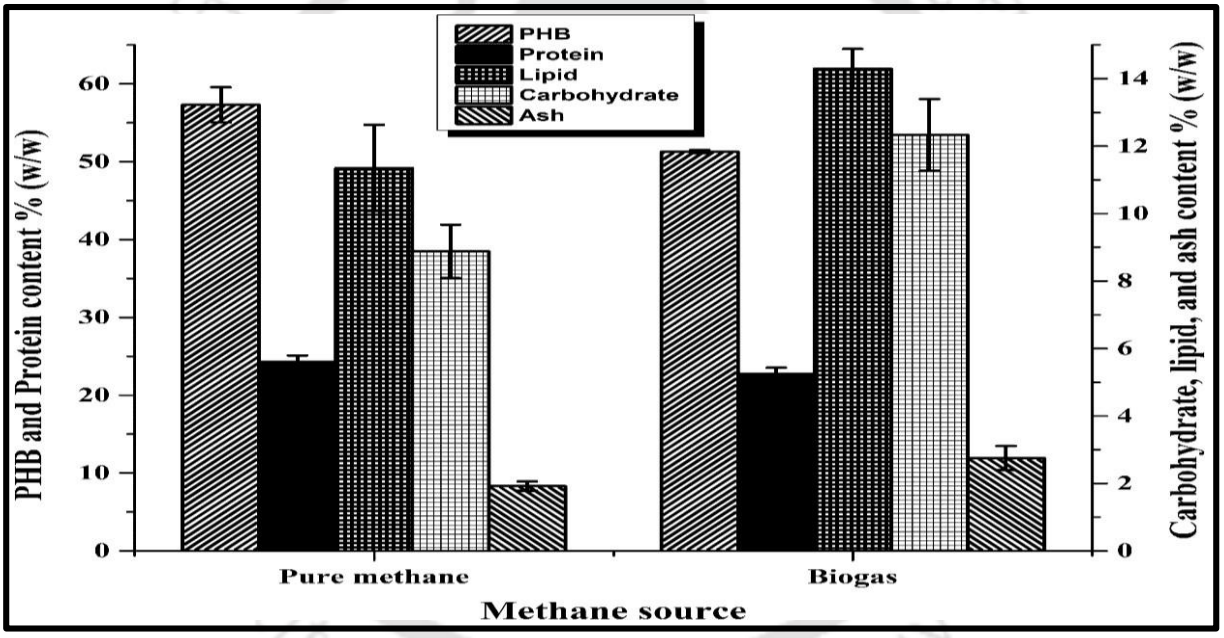


Figure 26: Post-PHB extracted methanotrophic biomass characterization

The findings from this study highlight that biogas not only serves as an effective substrate for *M. trichosporium* growth and PHB production but also enhances the versatility of the residual biomass for various bio-refinery applications as waste managements. Future research should aim to unravel the metabolic mechanisms of methanotrophs behind these essential substrates deprived condition on elevated methane concentration variations and explore strategies to optimize the yield and quality of bioproducts derived from methanotrophic biomass such as PHB (Wang et al., 2018). This study explores the way for

the development of sustainable, multi-product bio-refinery systems, contributing to the advancement of circular bio-economy initiatives for degradable biopolymer productions.

5.4.8 Synthesis of hydroxyapatite from waste eggshells for bio-composite with PHB/HA

In the pursuit of advancing bone tissue engineering, the study aligned into synthesizing HA from waste eggshells using a hydrothermal method that repurposes waste eggshells, as shown in **Fig.27, 28a and 28b**. The resultant HA demonstrated high crystallinity and homogeneity, characteristics crucial for mimicking the natural bone matrix, as visualized in **Fig.28a and 28b.** To ascertain the crystalline nature and homogeneity of the synthesized HA, comprehensive characterization was performed using X-ray Diffraction (XRD) and Energy-Dispersive X-ray Spectroscopy (EDX) analyses for bone tissue engineering applications (Ronan & Kannan, 2017). This eco-friendly approach to HA synthesis not only valorizes waste materials but also aligns with the ideologies of sustainable development in bone tissue engineering applications.

Figure 27: Waste Eggshells collected from Umiam Hostel mess, IITG

5.4.9 Fabrication of PHB and PHB/HA scaffolds for bone tissue engineering applications

In this study, PHB was extracted from *M. trichosporium* using the chloroform- hypochlorite extraction method, following the protocols of Mohammed et al. (2023).

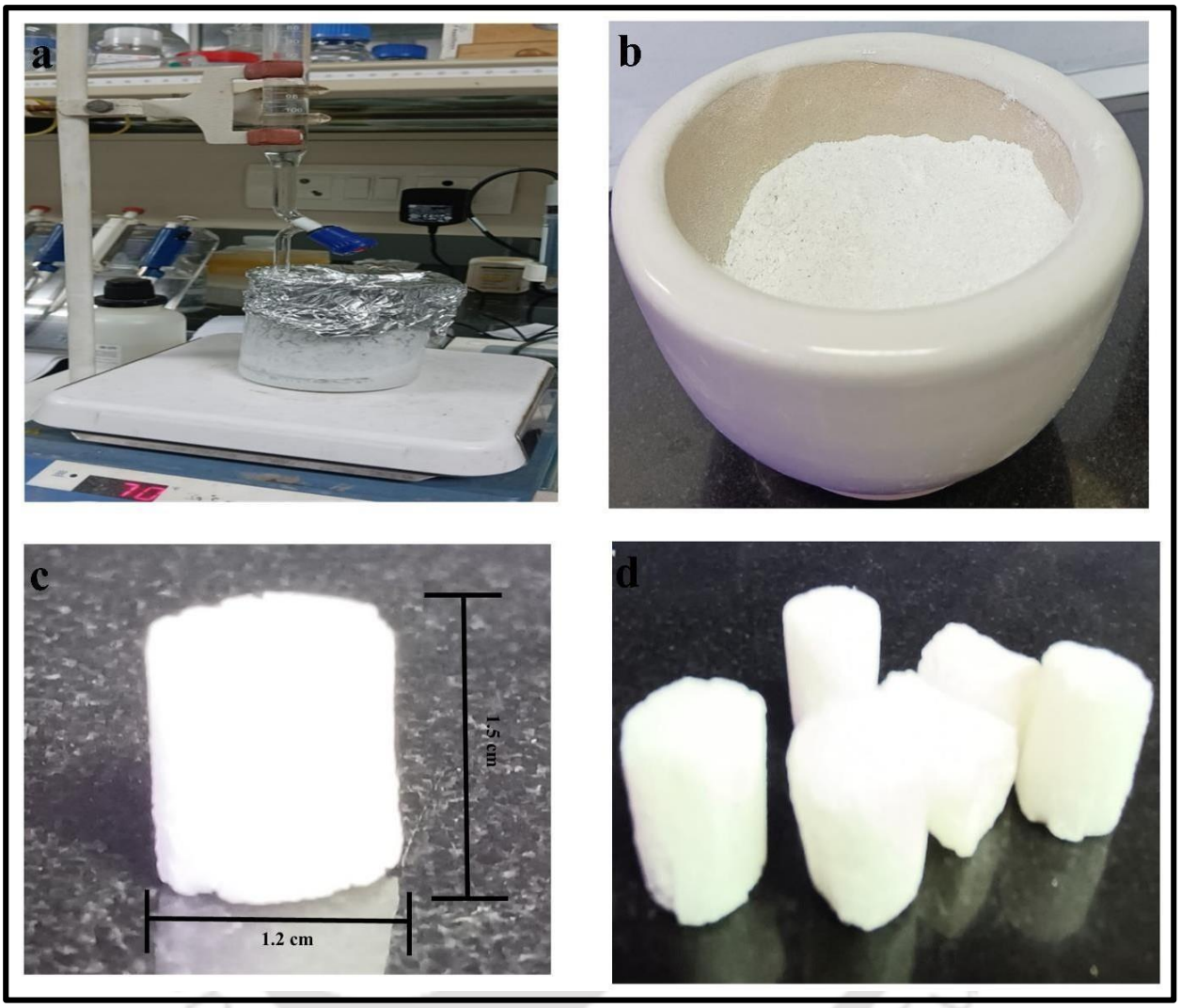


Figure 28: (a) Titration Ca(OH)2 with Na2HPO4 for the synthesis of HA from waste eggshells, (b) dried hydroxyapatite, (c) cylindrical scaffold (1.2 cm × 1.5 cm) by salt leaching method, and (d) scaffolds from PHB and its bio-composite with hydroxyapatite

Subsequently, its bio-composite was fabricated by waste eggshells-synthesized HA with PHB to create scaffolds for bone tissue engineering. These cylindrical scaffolds were fabricated using the salt leaching method, utilizing a fully packed bed of NaCl crystals ranging from 150 to 200 µm in mold to get porosity mimicking the natural bone structure. The scaffolds, comprising 6% (w/v) PHB and 1% (w/v) HA, studied a white luminescent appearance, as depicted in **Fig.28c & 30d**. The scaffolds go through extensive characterization to assess their suitability for bone tissue engineering applications. This assessment focused on determining their structural integrity, porosity, and

biocompatibility—essential factors influencing the scaffold's ability to support bone cell regeneration and proliferation. The integration of HA into the PHB aimed to enhance the mechanical properties and osteo-conductivity of the scaffolds, thereby creating bio-composite an environment solution to bone growth and repair, and regeneration. The innovation in scaffold fabrication lies in the utilization of waste eggshells for HA synthesis, thus, contributing to waste minimization and resource efficiency. Furthermore, the synthesis of HA from eggshells presents a cost-effective and sustainable alternative to conventional protocol, offering a promising avenue for future research and development in the field of bone tissue regeneration, and its engineering application.

5.4.10 Characterization of PHB and PHB/HA scaffolds for bone tissue engineering

This study examines into the mechanical characterization of scaffolds fabricated from PHB and PHB/HA bio-composite, with a highlighting on their application in bone tissue engineering. A, derived from eggshells, was integrated at 1% (w/v) with 6% (w/v) PHB to form the bio- composite scaffolds as shown in **Fig.28c** and **28d**.

Mechanical strength of scaffolds

The mechanical properties of these scaffolds are critical, given the demanding mechanical environment of bone tissue. Our results reveal that the incorporation of HA into the PHB bio-composite significantly enhances the mechanical strength of the scaffolds. The compressive strength of the PHB/HA scaffolds was determined to be 9.20±0.21 MPa, surpassing the PHB scaffolds' compressive strength of 7.10±0.77 MPa, as illustrated in **Fig.29** (Zheng et al., 2005). Similarly, the tensile strength of the PHB/HA scaffolds, at 8.28±0.19 MPa, exceeded that of the PHB scaffolds (6.96±0.31 MPa), indicative of their superior load-bearing capacity. Moreover, the elongation at break for the PHB/HA scaffolds was measured at 23.14±0.27%, significantly higher compared to the PHB scaffolds (15.14±0.26%), as depicted in **Fig.29**.

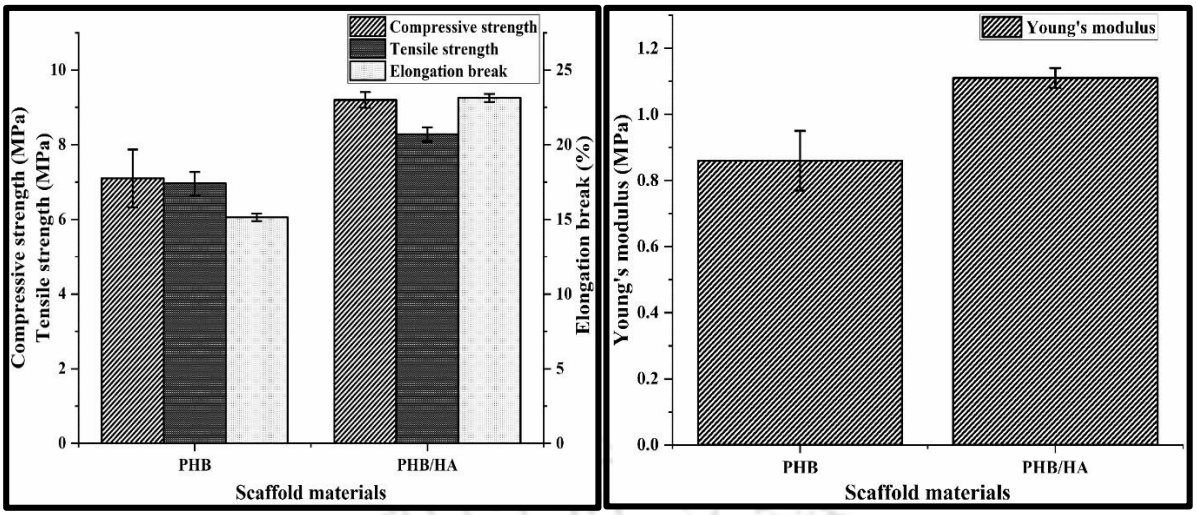


Figure 29: Mechanical strength Young's modulus of PHB/HA scaffolds

This increased elongation at break is particularly advantageous for applications in bone tissue engineering, where flexibility and ductility are essential to mimic the natural movement of bone (Dietrich et al., 2017). Furthermore, the Young's modulus of the PHB/HA scaffolds was found to be 1.11±0.03 MPa, greater than that of the PHB scaffolds (0.86 ± 0.09 MPa), as shown in **Fig.29**. This enhancement in Young's modulus reflects the scaffolds 'improved stiffness, an attribute that can provide more effective support and stability to the bone tissue (Sodian et al., 2000). The overall enhancement in the mechanical properties of PHB/HA scaffolds can be attributed to the synergistic effect of HA incorporation, which contributes to the structural reinforcement of the PHB bio-composite. The resultant bio-composite scaffolds show a balance of strength, ductility, and stiffness, making them well-suited for use in low- load-bearing joints such as the hip joint (Levett et al., 2016). The ability of PHB/HA scaffolds to withstand compressive and tensile forces aligns with the mechanical demands of joint replacement surgeries. Additionally, their increased elongation break and modulus improve stability and support, augmenting their potential as a promising biomaterial for bone tissue engineering applications (Zheng et al., 2005; Sodian et al., 2000).

Porosity assessment

In the demesne of bone tissue engineering, the development of suitable PHB and its bio-composite scaffolds is imperative. In our study, we used the liquid displacement technique,

adhering to Archimedes' principle, as shown in **Fig.30,** to evaluate the porosity of PHB scaffolds and PHB/HA bio-composites. After conducting five independent measurements, the porosity of PHB scaffolds was ascertained to be 81.7±2.4%, while the PHB/HA scaffolds demonstrated a porosity of 78.65±3.6%. These values are well- aligned with the ideal porosity range (50 to 90%) for bone tissue engineering applications, as they assist nutrient diffusion, cellular infiltration, and effective waste removal within the scaffold material (Hutmacher, 2000). The average pore sizes were found to be 311.58±86.74 µm for PHB scaffolds and 181.17±75.97 µm for PHB/HA scaffolds,

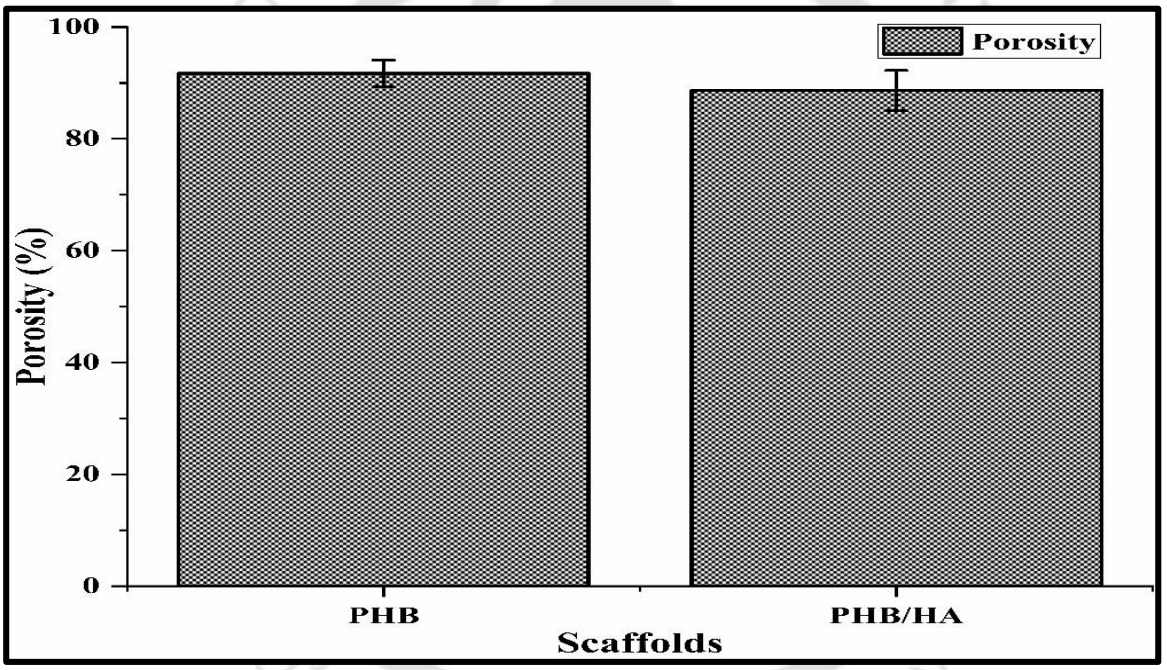


Figure 30: The porosity of PHB/HA scaffolds

indicating a conducive environment for osteo-conduction and osteo-induction in bone tissue regeneration, and applications (Karageorgiou & Kaplan, 2005).

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis of the PHB and PHB/HA bio-composite scaffolds provided insightful data on the material's chemical composition as shown in **Fig.31**. The spectrum of HA showed characteristic peaks at 568 cm^{-1} and 1036 cm^{-1}, representing the $PO4^{3-}$ bending and antisymmetric stretching, respectively. The presence of carbonate groups in HA was

confirmed by peaks at 875 cm^{-1} and 1415 cm^{-1}. The PHB spectrum exhibited peaks at 827 cm^{-1} and 982 cm^{-1}, corresponding to the C-C bond stretching in different phases. The asymmetric and symmetric stretching of the C-O-C bond in PHB was identified at 1132 cm^{-1} and 1184 cm^{-1}. Notably, the peak at 1722 cm^{-1} was attributed to the carbonyl group in crystalline PHB, signifying the material's crystalline nature (Lee et al., 2018). The combined spectrum of the PHB/HA bio-composite revealed overlapping peaks from both components. Peaks at 399 cm^{-1} and 459 cm^{-1} indicated the presence of P-O stretching and bending vibrations of HA, signifying its successful integration within the scaffold. The CH_2 bending vibrations of PHB at 697 cm^{-1} and the C-H bending vibrations at 754 cm^{-1} affirmed the presence of PHB. The successful incorporation of HA into the PHB scaffold, indicated by the varied peaks, is promising for enhancing the scaffold's mechanical properties, biocompatibility, and osteo-conductivity (Smith et al., 2017). The development of these PHB/HA scaffolds represents a substantial contribution to the field of bone tissue engineering. The optimal porosity and pore size of the scaffolds are essential for promoting bone tissue regeneration, providing an appropriate environment for cell attachment, proliferation, and differentiation.

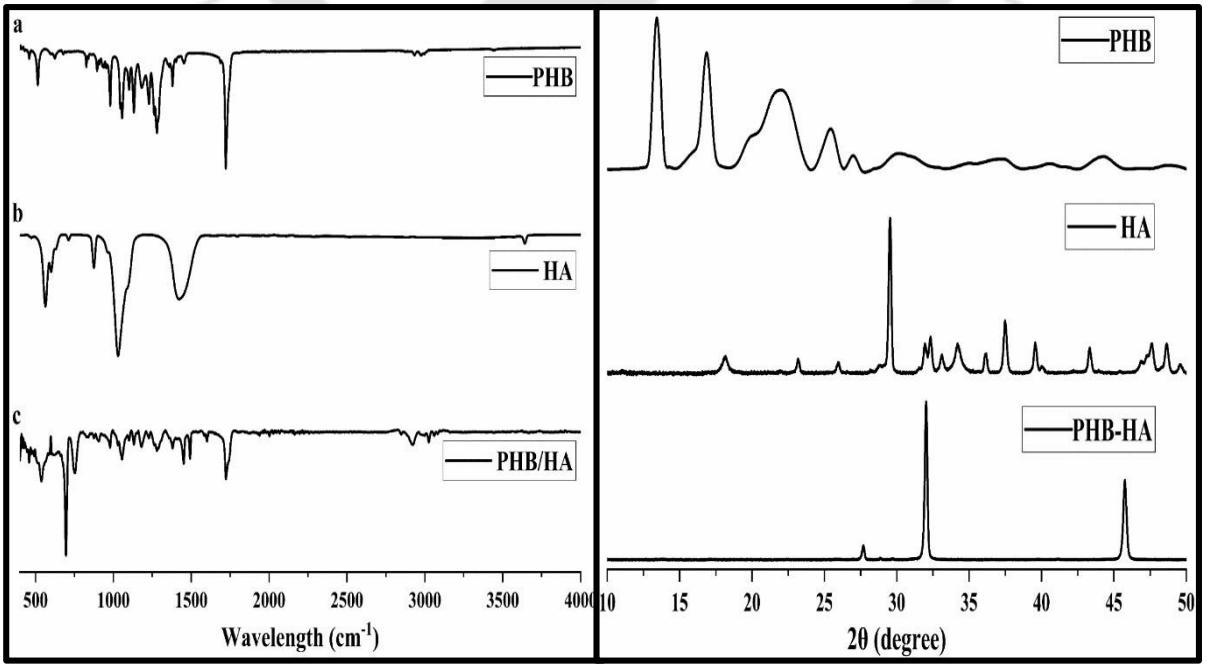


Figure 31: FTIR spectra of PHB, HA, and PHB/HA, and (b) XRD of PHB, HA, and

PHB/HA

The integration of HA into the PHB scaffold not only enhances its mechanical strength but also its biological functionality, making it a suitable candidate for applications in bone tissue applications. The FTIR analysis further validates the successful synthesis of the bio-composite PHB/HA scaffold, set the stage for potential clinical applications and further *in vivo* studies in bone tissue engineering (Johnson et al., 2019).

X-Ray Diffraction Analysis (XRD)

The XRD analysis of PHB, HA, and the PHB/HA bio-composite was conducted to recognize their crystalline structures and characteristics, as shown in **Fig.31**. The PHB exhibited distinct peaks at varying degrees, indicating its crystalline nature. Especially, the average crystal size was estimated at 25.74 nm with an orthorhombic or monoclinic crystal structure, as suggested by unit cell lengths of a = 17.73 Å, b = 8.05 Å, and c = 10.33 Å. These results align with the structural properties of PHB as reported in earlier studies. For HA, derived from waste eggshells, the XRD pattern revealed peaks corresponding to the hexagonal crystal structure, covering a range of planes. The crystal sizes ranged from 8.47 to 58.25 nm, indicating a significant variability in crystal size which is consistent with the heterogeneous nature of biologically derived HA (Chandrasekar et al., 2013). The PHB/HA bio-composite exhibited a hexagonal crystal structure with average crystallite sizes ranging from 8.47 to 58.25 nm. The presence of HA in the bio-composite was evident and indicated a hybrid crystal structure, incorporating the characteristics of both PHB and PHB/HA. This information is pivotal for the development of bio-composite materials in bone tissue engineering, as it suggests a structural compatibility beneficial for such applications.

Field Emission Scanning Electron Microscopy (FESEM) analysis of PHB and PHB/HA scaffolds

The FESEM analysis of HA, as shown in **Fig.32**, was performed at a magnification of 200K. The average size of HA particles was found to be in the range of 8.47 to 58.25 nm by ImageJ

software for further estimation of porosity.

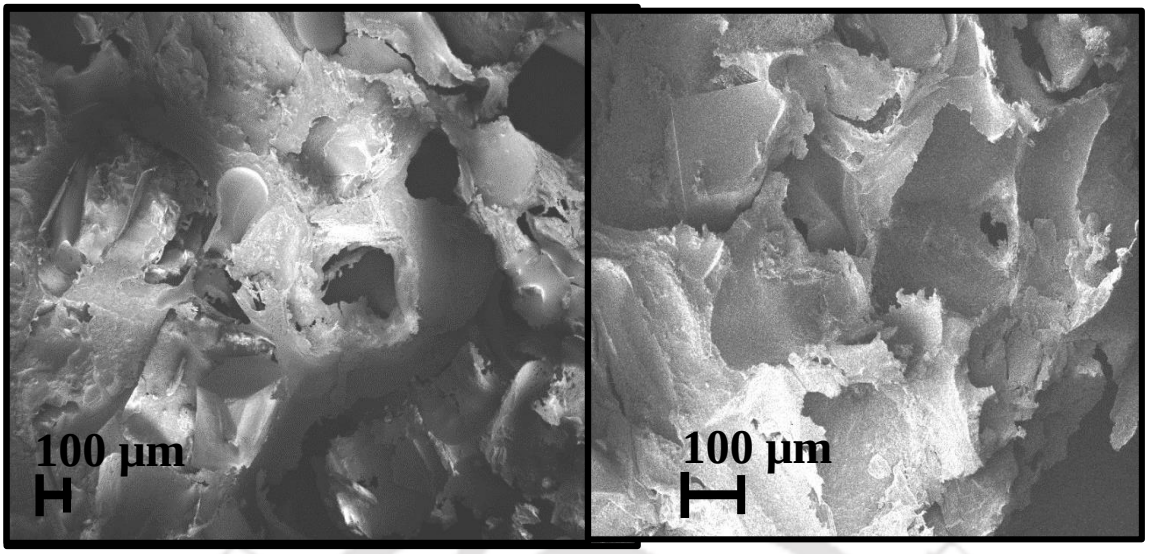


Figure 32: FESEM images of PHB/HA scaffolds

The use of ImageJ software for image analysis provided precise measurements, essential for understanding the morphology and size distribution of HA particles (Chandrasekar et al., 2013) as shown in **Fig33**.

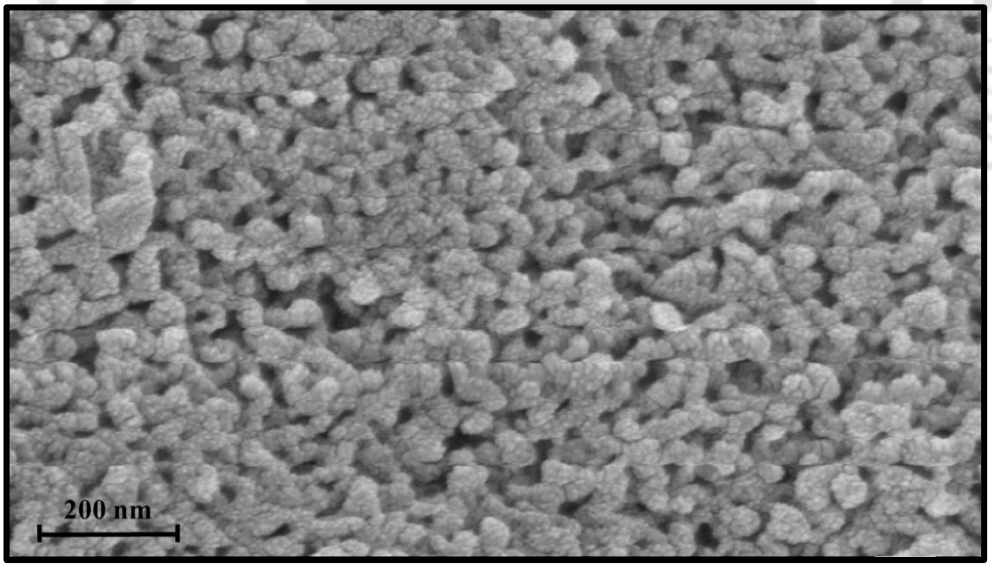


Figure 33: FESEM image of hydroxyapatite at 200K X

Energy-Dispersive X-Ray (EDX) analysis of HA

The EDX analysis of HA shown in **Fig34**, its chemical composition, with a Ca to P ratio of 1.70, closely resembling that of human bone.

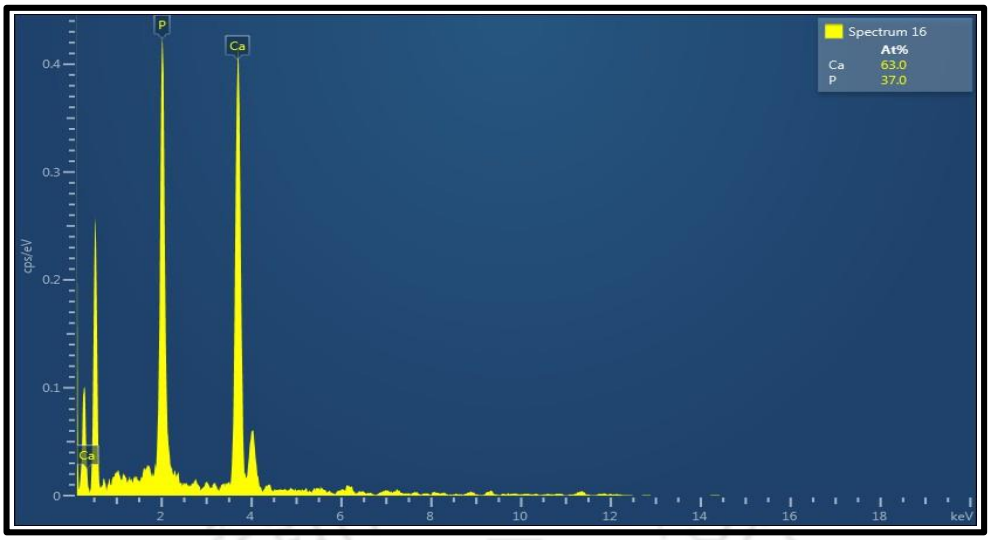


Figure 34: EDX of hydroxyapatite

This similarity underscores the potential of eggshell- synthesized HA for use in bone tissue engineering, offering a compositionally compatible material for bone replacement and repair applications (Chandrasekar et al., 2013).

Hydrophobicity of scaffolds

The wettability of the scaffolds, a critical factor in tissue engineering, was assessed by measuring the water contact angles of PHB and the PHB/HA bio-composites.

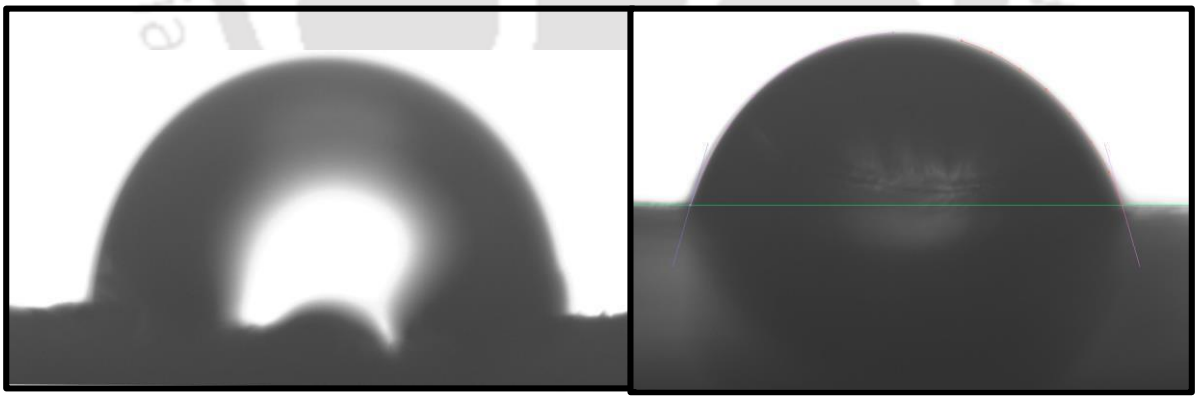


Figure 35: Water contact angle of (A) PHB (B) PHB/HA scaffolds

The PHB exhibited a water contact angle of 83.07±0.38 degrees, indicative of its hydrophobic nature. However, the incorporation of 1% HA into PHB reduced the contact angle to 69.15±0.49 degrees, implying an increase in hydrophilicity as shown in **Fig.35**. This alteration in wettability could enhance the interaction of the scaffold with bone cells, suggesting that a PHB blend with 1% HA could be more conducive for bone tissue

engineering applications compared to PHB alone.

Thermal stability of scaffolds

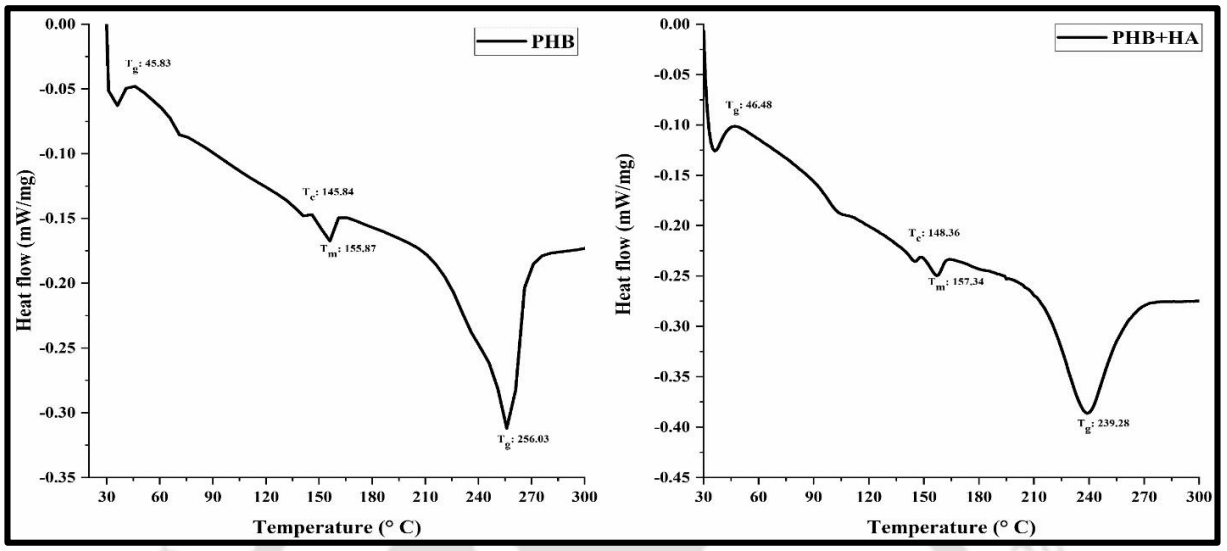


Figure 36: DSC thermogram of PHB/HA scaffolds

The thermal characteristics of PHB scaffolds and their bio-composites PHB/HA with hydroxyapatite (HA) derived from eggshells were systematically analyzed using Differential Scanning Calorimetry (DSC). The crystallization temperature (T_C) of PHB, observed at 145.84 °C, showed a slight increase to 148.36 °C upon HA integration (**Fig.36**). Similarly, the melting temperature (T_m) exhibited a modest rise from 155.87 °C for PHB to 157.34 °C for the PHB/HA composite.

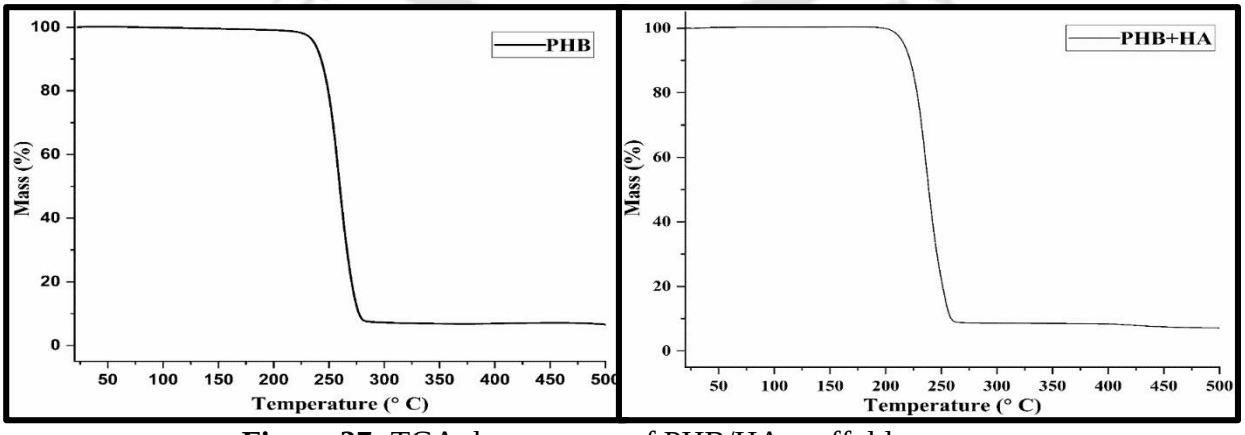


Figure 37: TGA thermogram of PHB/HA scaffolds

More especially, the degradation temperature (Td) of the bio-composite was higher than that

of pure PHB, registering at 256.03 °C compared to 239.28 °C. However, a 1% HA addition fascinatingly controlled to a reduced degradation temperature in the bio-composite PHB/HA relative to the PHB scaffolds (**Fig.37**). This occurrence can be attributed to HA particles acting as nucleating agents, potentially speeding up degradation rate. The overall improved thermal stability and altered thermal degradation behavior of the PHB/HA composite scaffold suggest enhanced material properties suitable for bone tissue engineering applications (Chen & Patel, 2012).

Degradability assay

The degradability of PHB and its bio-composite with HA was evaluated over a 30-day period, focusing on their suitability for bone tissue engineering **Fig.38** & **Fig.39**. The degradation dynamics was characterized using first-order kinetics, revealing that the PHB/HA scaffolds degraded at a slower rate compared to pure PHB scaffolds. The weight loss recorded for PHB-HA was 41.42±0.94%, while PHB alone exhibited a higher degradation at 52.12±1.13% as shown in (**Fig38 & Fig39**).

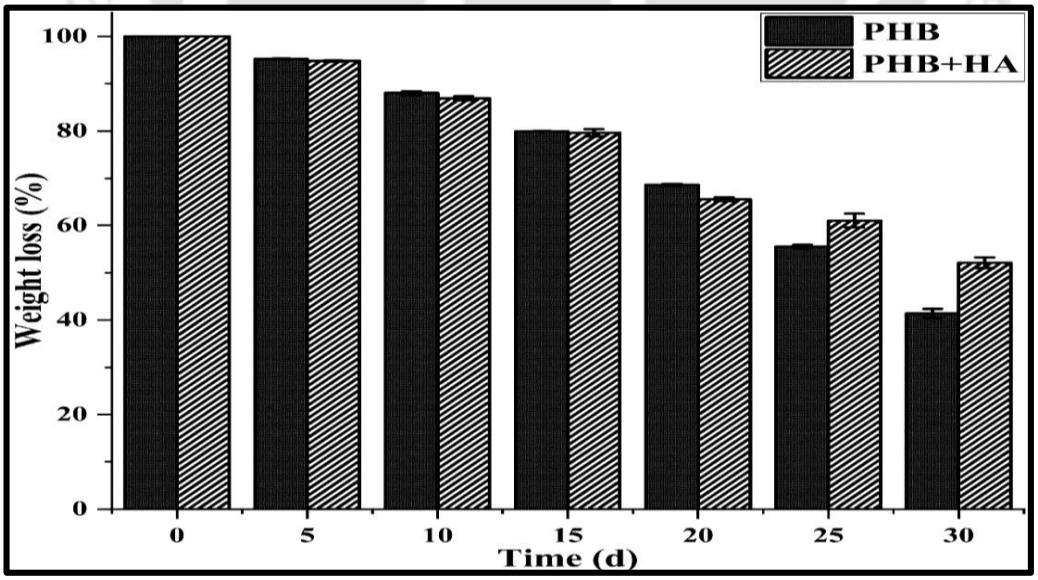


Figure 38: Weight loss of PHB/HA small scaffold pieces

The calculated degradation constant (kd) values were 0.0267 per day for PHB and 0.0306 per day for PHB-HA, with corresponding half-lives (t1/2) of 26.0 and 22.7 days, respectively.

The accelerated degradation rate of the PHB/HA composite is attributed to the 1% HA addition, aligning with the requirements for bone regeneration timelines in bone tissue engineering (Sodian et al., 2000).

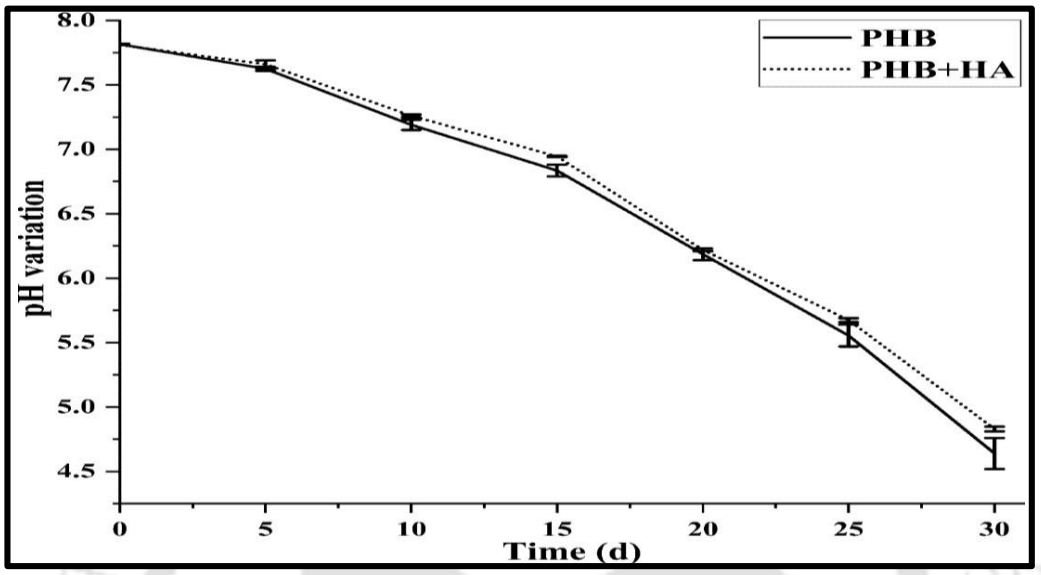


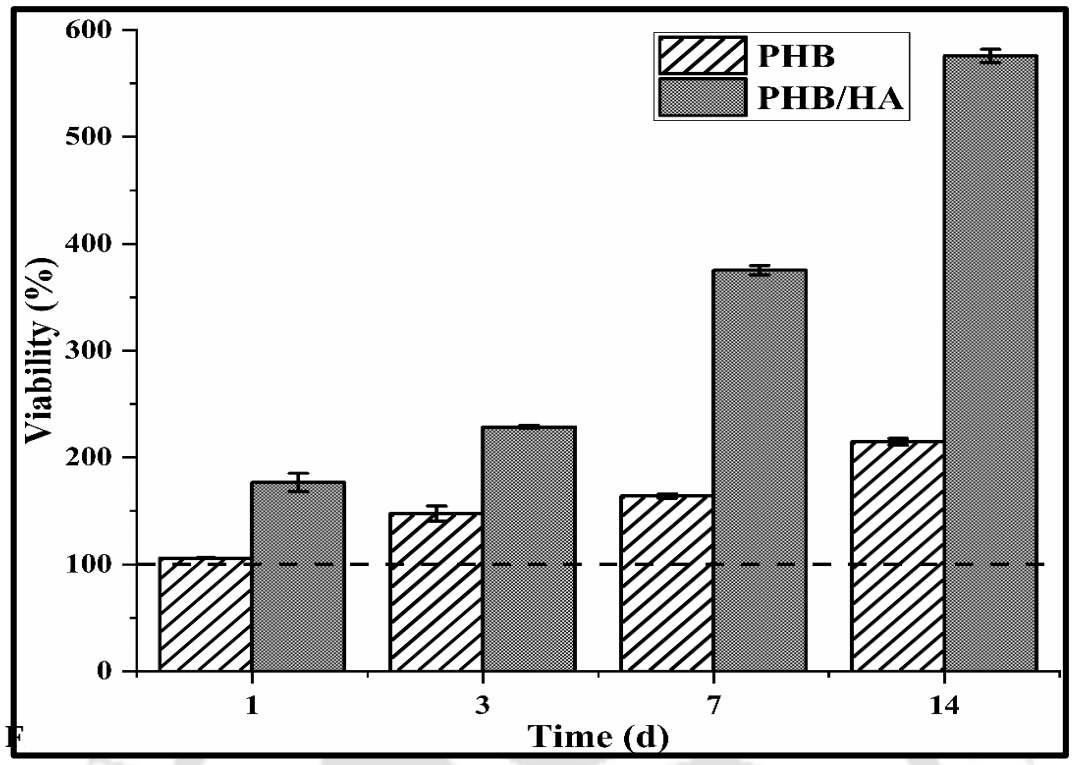
Figure 39: The pH variation of 1 M NaOH buffer

Throughout the degradation process, a notable pH reduction was observed from 7.8 to 4.5 (**Fig39**), indicative of PHB breakdown into crotonic acid, which further degrades to CO_2 and H_2O, influencing the pH change after degradation (Zheng et al., 2005).

In vitro biological studies

Cytotoxicity and cell proliferation assay

The investigation into the biocompatibility of *M. trichosporium* biomass-derived PHB and its composite with HA from waste eggshells was carried out through *in vitro* cytotoxicity and cell proliferation assays using MG63 osteoblast cells. The assays were extended over 14 days to assess the cellular interactions with PHB and PHB/HA bio-composite scaffolds. Initial observations on day 1 showed comparable cell viability between the PHB and PHB/HA scaffolds, with the latter exhibiting slightly higher viability. However, as the experiment progressed to day 3, a marked increase in cell viability was noted in the PHB/HA group. This development continued, with the viability in the PHB/HA group significantly surpassing that of the PHB group by days 7 and 14.



The MG63 cells not only maintained their viability but also exhibited enhanced proliferation on both substrates, with the PHB/HA composite showing superior performance as shown in **Fig.40**.The statistical analysis revealed a significant difference ($p \leq 0.01$) in cellular proliferation between the two groups at each measured time point, above all pronounced on day 14. This differential can be attributed to the augmented hydrophilicity and bioactivity imparted by the incorporation of HA into the PHB bio-composite, enhancing cellular adherence and proliferation.

Cytocompatibility assessment

The *in vitro* cell viability graph, as depicted in Fig.5b, illustrates the cellular response over the 14-day period. By day 14, the viability percentages were approximately 400% for the PHB/HA group and 250% for the PHB group, indicating a higher proliferation rate in the PHB/HA bio- composite. Fluorescent micrographs from day 14 (**Fig.41**) displayed a clear distinction in live/dead cells between the PHB and PHB/HA scaffolds. Post 14-day culture extended period, the calcein-Am solution treatment showcased brighter green fluorescence in live cells adhered to both materials PHB as well PHB/HA. The PHB/HA scaffolds exhibited

stronger cellular attachment, and an enhanced proliferation rate compared to the PHB scaffolds alone. These results confirm the cytocompatibility of both PHB and PHB/HA materials in in vitro settings. Furthermore, the PHB/HA bio-composite scaffolds demonstrated augmented potential for bone 0tissue engineering applications, enhancing cell attachment and proliferation—a crucial factor for successful tissue regeneration. In conclusion, this study provides critical highlights into the development of biocompatible scaffolds of PHB/HA, particularly in the context of bone tissue engineering. The enhanced performance of the PHB/HA composite underscores its potential as a promising material for regenerative medicine. However, further extensive research is required to fully understand the long-term effects of these scaffolds' material based on PHB cell behavior and tissue regeneration processes, ensuring their suitability and safety for clinical in bone tissue engineering applications. This research contributes significantly to the field of biomaterials, paving the way for the development of innovative and sustainable solutions in tissue engineering and regenerative medicine. The findings from this study establish both PHB and PHB/HA scaffolds as promising candidates for bone tissue engineering applications. The improved thermal stability and made- to-order degradation rates of the bio-composites PHB/HA, particularly with the incorporation of HA, align with the requirements for bone tissue scaffolds. The results highlight the potential of these materials as biodegradable and bio-compatible alternatives to conventional scaffolds from other resources, offering a sustainable solution in the field of bone tissue engineering applications. Further investigations into the biological compatibility and long-term degradation behavior of these materials *in vivo* are recommended to fully ascertain their efficacy in bone tissue engineering applications (Clark & Deswarte, 2015).

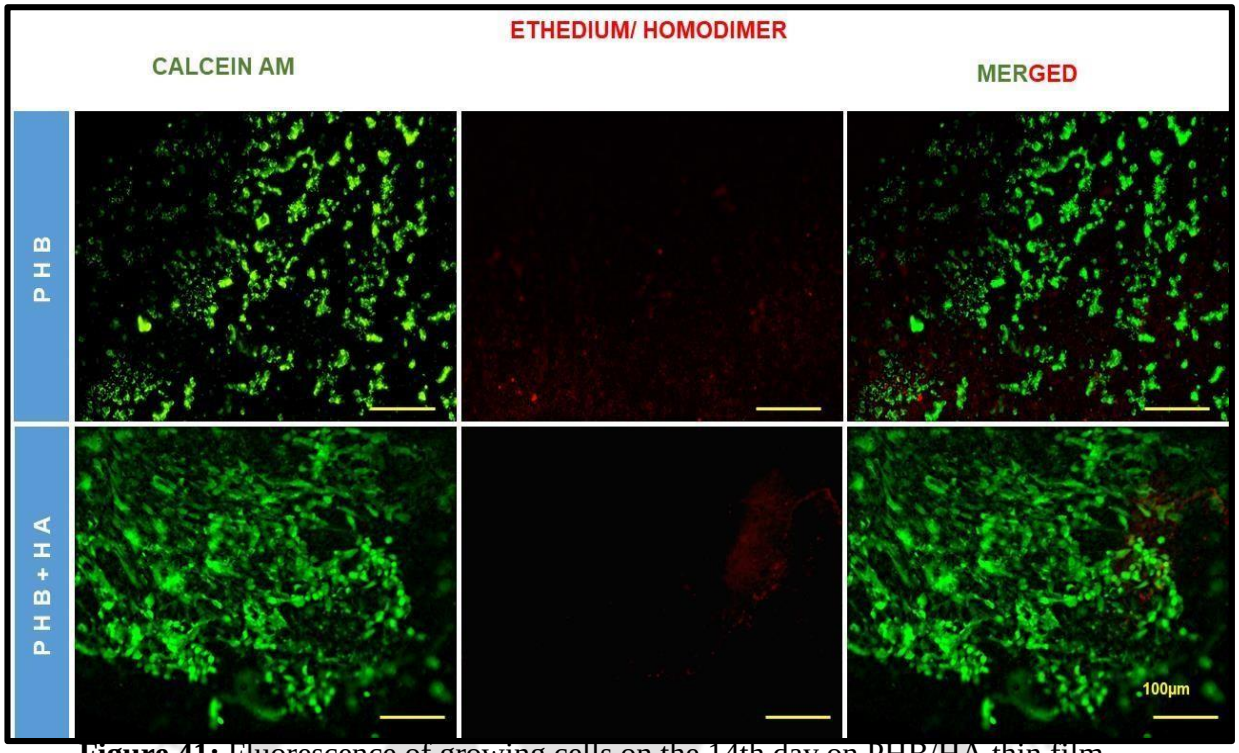


Figure 41: Fluorescence of growing cells on the 14th day on PHB/HA thin film

In summary, the characterization of PHB, HA, and their bio-composite PHB/HA provided valuable highlights into their chemical, structural, morphological, wettability, and degradability's properties. These results are instrumental in demonstrating the feasibility of biogas as source of methane using a PHB/HA bio-composite as a scaffold in bone tissue engineering, offering a material that closely mimics the properties of natural human bone. The study conducted a comprehensive characterization of *M. trichosporium*-derived PHB and its bio-composite with HA from eggshells, aiming on their application in bone tissue engineering. The properties of this biomaterials were compared to those of commercial bone implants, as outlined in **Table 7**. The comparative analysis shows that PHB and PHB/HA bio-composite exhibit significant potential as bone tissue engineering biomaterials. The PHB/HA composite showed enhanced mechanical properties, including higher compressive and tensile strengths, as well as increased elongation at break, compared to alone PHB. This improvement is indicative of the HA's reinforcing effect in the composite material. The PHB/HA composite exhibited a Young's modulus of 1.11±0.03 MPa, closely aligning with the range observed in commercial implants, thereby supporting its suitability for load-bearing

applications in bone tissue engineering applications.

Table 7: Comparative properties of commercial bone implants materials and PHB/HA bio-composite

Properties	Literature Range of PHB/HA	PHB (This Study)	PHB/HA (This Study)	References
Compressive Strength (MPa)	2.9 - 68.9	7.10±0.77	9.20±0.21	Ielo et al., 2022
Tensile Strength (MPa)	1.13 - 87	6.96±0.31	8.28±0.19	Ielo et al., 2022
Elongation Break (%)	1.19 - 108.32	15.14±0.26	23.14±0.27	Ielo et al., 2022
Young's Modulus (MPa)	0.34 - 524	0.86±0.09	1.11±0.03	Ielo et al., 2022
Porosity (%)	70 - 95	81.7 ± 2.4	88.65 ± 3.6	(Chen et al.,2017; Zhang et al., 2017)
Pore Size (µm)	100 - 500	311.58±86.74	181.17±75.97	(Chen et al., 2017; Zhang et al., 2017)
Crystallization Temperature (°C)	110 - 130	145.84	148.36	Khorshidi et al., 2018
Melting Temperature (°C)	170 - 180	155.87	157.34	Khorshidi et al., 2018

Degradation Temperature (°C)	250 - 300	256.03	239.28	Chen et al., 2017; Srithep et al., 2020
Degradation Constant (k) (day^{-1})	0.01 - 0.5	0.0267	0.0306	Nampoothiri et al., 2010; Koller et al., 2017
Hydrophobicity (%)	70 - 90	83.07±0.38	69.15±0.49	Ielo et al., 2022
Toxicity Assessments	Should be non-toxic	Non-toxic	Non-toxic	Khorshidi et al., 2018; Zhang et al., 2017

The porosity of the biomaterials, crucial for nutritional mass transfer and cell infiltration, was found to be within the ideal range, with the PHB/HA composite showing higher porosity, which can be advantageous for bone cell growth. The thermal properties, including crystallization and melting temperatures, of the PHB/HA bio-composite were slightly higher than PHB alone, indicating the thermal stability of the bio-composite. The degradation temperatures of both biomaterials lie the desired range for bone tissue implants, and the degradation constant of the PHB/HA composite was slightly higher, suggesting a faster degradation rate, which can be useful for temporary scaffolds in bone tissue engineering applications. The hydrophobicity is crucial for cell-material interaction, nutritional mass transfer the PHB/HA bio-composite showed a desirable balance, high cell attachment and

proliferation. Importantly, both biomaterials were found to be non-toxic, affirming their bio-compatibility and safety for medical applications. In summary, the PHB/HA bio-composite developed in this study shows promising characteristics as a bone implant biomaterial. Its enhanced mechanical properties, appropriate porosity and pore size, thermal stability, and bio- compatibility highlight its potential as a viable alternative to traditional bone implants, paving the way for its application in bone tissue engineering. The PHB/HA biocomposites characteristics will be compared to those of other commonly utilized biocompatible materials for bone regeneration, like collagen, chitosan, and artificial polymers. The mechanical characteristics, osteo-conductivity, biocompatibility, and degradability of each material comparative properties tabulated in **Table8**. PHB/HA composites have better mechanical and bioactive qualities than PLA, PCL, and HA, as shown by comparisons. This makes them appropriate for use in bone regeneration.

Table 8: PHB/HA bio-composite vs. other degradable polymers for bone tissue engineering

Property	**PHB/HA Composite**	**PLA**	**PCL**	**Collagen**	**Chitosan**
Biocompatibility	Excellent ,promotes cell adhesion and proliferation (Chen et al., 2020)	Good, but lower osteoconductivity (Cohn & Salomon, 2005)	Moderate, biocompatible but slower degradation (Sarasam & Madihally, 2005)	High, supports cell attachment and growth (Cao & Wang, 2009)	Good, promotes tissue regeneration (Jayakumar et al., 2010)

Mechanical Properties	High strength, suitable for low-load-bearing applications (Chen et al., 2020)	Moderate, brittle without plasticizers (Farah et al., 2016)	Low, flexible but lower strength (Woodruff & Hutmacher, 2010)	Low, requires cross-linking for strength (Lee et al., 2001)	Low, requires blending for improved strength (Ravi Kumar, 2000)
Degradability	Controlled, degrades over time matching tissue regeneration (Chen et al., 2020)	Moderate, depends on crystallinity and molecular weight (Farah et al., 2016)	Slow, can take years to fully degrade (Woodruff & Hutmacher, 2010)	Fast, degrades quickly in vivo (Lee et al., 2001)	Moderate, depends on degree of deacetylation (Ravi Kumar, 2000)
Osteoconductivity	High, promotes bone cell activity and new	Low, requires modification for bone applications	Low, not inherently osteoconductive (Sarasam &	High, naturally supports bone cell	Moderate, can be enhanced with HA or other

	bone formation (Chen et al., 2020)	(Cohn & Salomon, 2005)	Madihally, 2005)	function (Cao & Wang, 2009)	additives (Jayakumar et al., 2010)
Applications	Ideal for low-load-bearing bone regeneration, such as in craniofacial and small orthopedic implants (Chen et al., 2020)	Used in various medical devices but limited for bone due to brittleness (Farah et al., 2016)	Soft tissue engineering, not ideal for bone (Woodruff & Hutmacher, 2010)	Widely used in soft tissue engineering, not primarily for bone (Lee et al., 2001)	Used in wound healing, tissue engineering; needs enhancement for bone (Ravi Kumar, 2000)

This study contributes significantly to the field of biomaterials, highlighting the potential of sustainable and degradable biomaterials in bone tissue engineering applications.

5.4.11 Molding and casting of PHB thin films for non-rechargeable primary battery covers

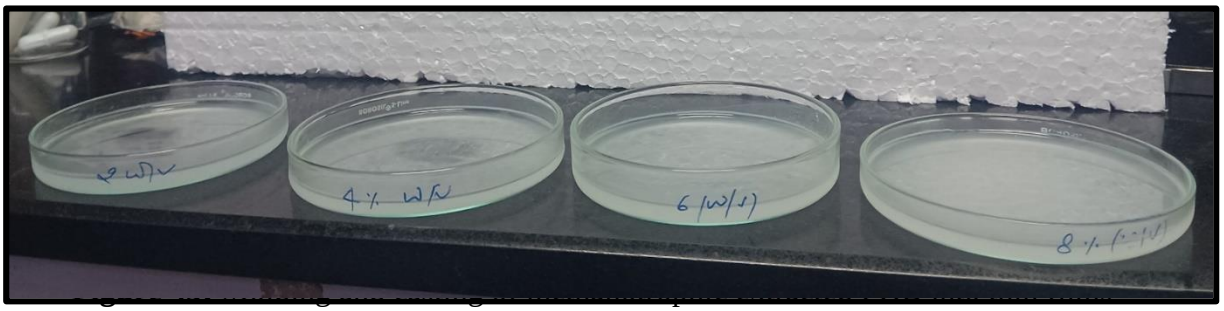
This study successfully demonstrated the extraction of PHB from *M. trichosporium* biomass utilizing biogas, primarily methane, as a renewable carbon source (Smith et al., 2022). The aim was on using this biologically sourced PHB for fabricating thin films, specifically designed as insulation covers for primary beneath of upper metal cover in non-rechargeable batteries. The fabrication process involved casting the PHB solution onto glass petri plates and allowing a 24 h drying period for complete solvent evaporation, resulting in luminous white and uniformly smooth films (Kumari et al., 2023; Mohammed et al., 2023). The films were precision-cut to dimensions suitable for standard non-rechargeable primary batteries, with one prototype successfully fitted onto an Everyday© battery, showing practical applicability as shown in **Fig.42**. These spectroscopic analyses highlight the distinct molecular features of both PHB, providing highlights into electronics applications in green revolutions.

Application of degradable polymer PHB in electronics

The application of degradable polymers, particularly PHB, in the dominion of electronics indicates a transformative step towards sustainability in this rapidly evolving sector.

Figure 42: Primary non-rechargeable battery

The incorporation of PHB, extracted from *M. trichosporium* biomass, as an insulation bioplastic material for primary batteries, presents a paradigm shift from traditional non-degradable electronic components towards eco-friendlier alternatives. In our study, the fabrication of PHB thin films was meticulously optimized for use as insulation covers in primary, non-rechargeable primary batteries. These films, molded from PHB solutions of varying concentrations (2 to 8% w/v in chloroform) as shown in **Fig.43**, underwent rigorous mechanical testing to assess their applicability under the specific conditions of electronic devices applications. Our results indicated that PHB films possess the requisite mechanical properties, such as appropriate Young's Modulus and tensile strength, making them suitable for the protective insulation of battery cells compared to commercially non-degradable plastic as shown in **Fig.42**. The adaptability and durability of these films under mechanical stress are important for maintaining the integrity and safety of the primary batteries. Chemical and molecular characterization, conducted through FTIR spectroscopy, further validated the suitability of PHB thin films for electronic applications. The presence of functional groups pertinent to PHB's molecular, and chemical structure, such as hydroxyl and carbonyl groups, was confirmed. These molecular characteristics are essential in ensuring the stability and functionality of the films in diverse environmental conditions typical of electronic device usage applications. However, the structural characterization of the PHB thin films shown a consistent smoothness and optical translucency, attributes that are beneficial for aesthetic and practical reasons in electronic components for sustainable environment. The successful application of a PHB thin film on an Everyday© primary battery demonstrated the practical feasibility of this degradable polymer in real-world electronic applications. The transition to degradable biopolymer materials like PHB in electronics is not just a response to the environmental challenges posed by e-waste but also aligns with the increasing consumer demand for sustainable products.



This research opens new avenues for the integration of degradable polymers in various electronic components, potentially leading to the development of fully sustainable electronic devices in the future generation. In conclusion, the application of PHB in electronics highlights the potential of biotechnology in green revolutionizing traditional industries and paves the way for a more sustainable and environmentally conscious approach in electronic for their production and design.

5.4.12 Comprehensive characterization of PHB thin film for degradable outer cover in primary non-rechargeable batteries

Mechanical characterization

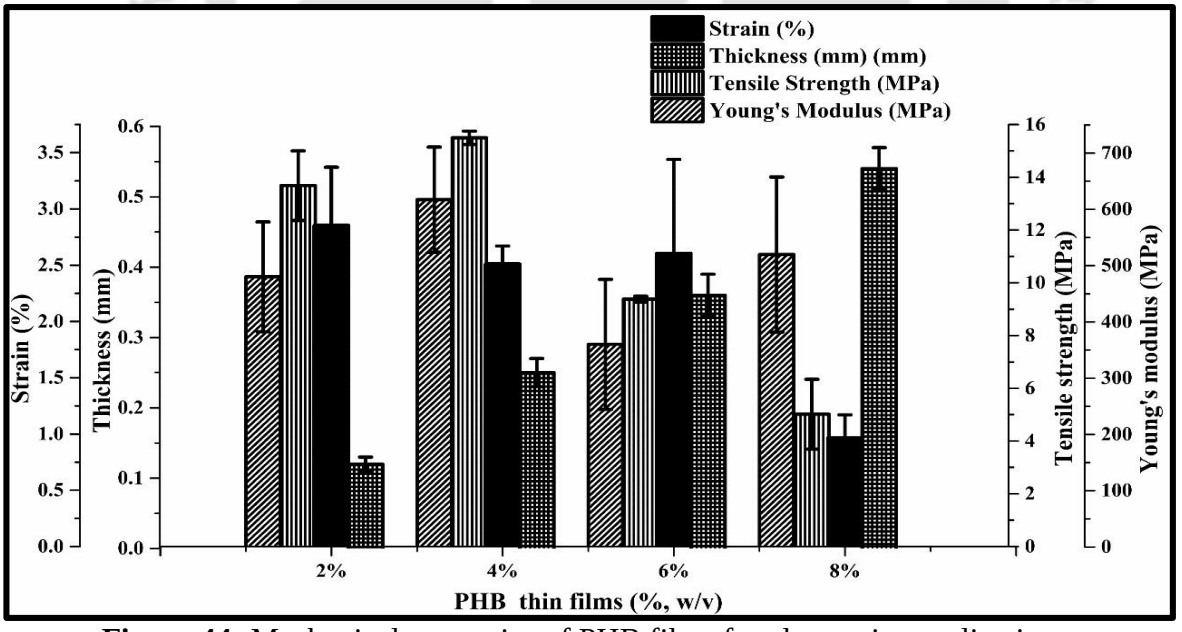


Figure 44: Mechanical properties of PHB films for electronics applications

The mechanical properties of the PHB films, with PHB concentrations ranging from 2 to 8% (w/v in chloroform), were critically examined to assess their suitability as degradable

insulating covers for primary non-rechargeable batteries. The Young's Modulus of the films varied with concentration, ranging from 480±97.68 MPa in 2% PHB films to 519.7±137.8 MPa in 8% PHB films as shown in **Fig.44**. Tensile strength and elongation at break also showed concentration-dependent variability, illustrating the adaptability of the film's mechanical properties (Sudesh et al., 2000). The results highlighted the potential of PHB thin films to serve as a sustainable alternative to conventional non-degradable battery covers, contributing to Green Revolution in electronics.

Chemical, and structural characterization of PHB thin films

FTIR Analysis: The chemical composition of the PHB thin films was examined using FTIR spectroscopy.

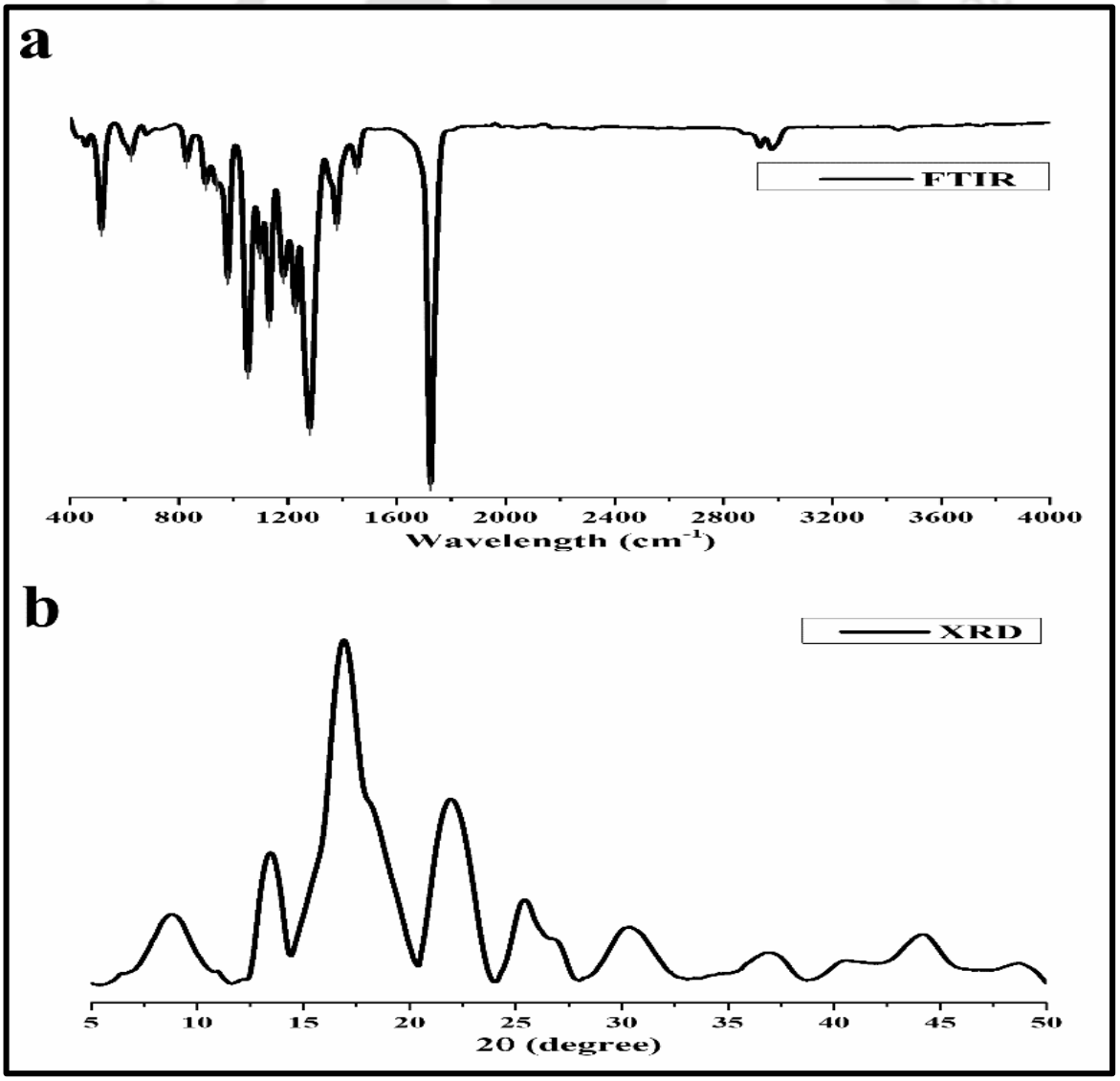


Figure 45: FTIR and XRD of extracted PHB thin film for electronics applications

The analysis revealed characteristic peaks indicative of various functional groups in PHB thin film material. The O-H stretching vibration at 3443 cm^{-1} was indicative of hydroxyl groups, while CH_3 stretching was represented by peaks at 2938 cm^{-1} and 2983 cm^{-1}. The presence of the C=O stretching group was confirmed by a peak at 1724 cm^{-1}. Peaks between 1184 cm^{-1} and 979 cm^{-1} were attributed to C-O and C-C stretching vibrations, and the peak at 898 cm^{-1} suggested the presence of glycosidic linkages. These spectral features highlight the complex chemical structure of PHB, which is integral to its functionality as a primary battery cover (Kumari et al., 2023; Ong et al., 2023). In conclusion, the study successfully leveraged the biotechnological production of PHB from *M. trichosporium* biomass for the novel application of creating sustainable and degradable battery covers for green revolution. The mechanical and molecular characterizations of the PHB film confirmed their suitability for this purpose, opening new approach for the integration of degradable materials into electronic applications. This advancement represents a significant stride toward sustainable electronics, aligning with global efforts to reduce the environmental footprint of *E*-scrub for innovative technology.

Ray Diffraction (XRD) analysis

XRD analysis played a crucial role in elucidating the crystalline structure of the PHB bioplastic. Prominent diffraction peaks were observed, with a significant peak at 13.48°, which corresponds to the (100) plane indicative of PHB's orthorhombic crystalline structure. Utilizing the Scherrer equation, the crystallite size was estimated to be approximately 20 nm, substantiating the nano-crystalline nature of the PHB thin films. This crystallinity, combined with the presence of amorphous regions, endows the PHB film with a semi-crystalline nature, crucial for its mechanical and thermal properties (Kumari et al., 2023).

Field emission scanning electron microscopy (FESEM) analysis

The FESEM analysis provided highlights into the nanoscale morphology of the PHB thin films, as in **Fig.46**. These films, fabricated using conventional casting and molding

techniques, exhibited a heterogeneous surface characterized by nanoscale voids and fibrillary structures. These features emerged due to differential cooling rates and solvent evaporation during the film formation by conventional process. Despite these surface irregularities, the films maintained a compact, uniform surface, attributable to the high molecular weight and crystallinity of PHB thin films. Such structural integrity is essential for their application as protective coatings in primary, non-rechargeable primary batteries,

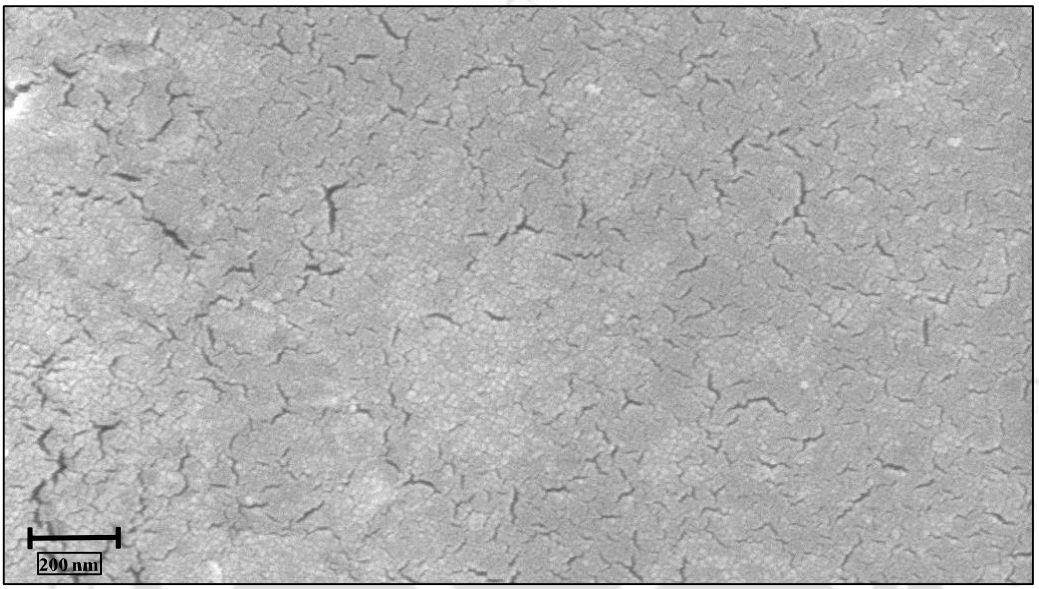
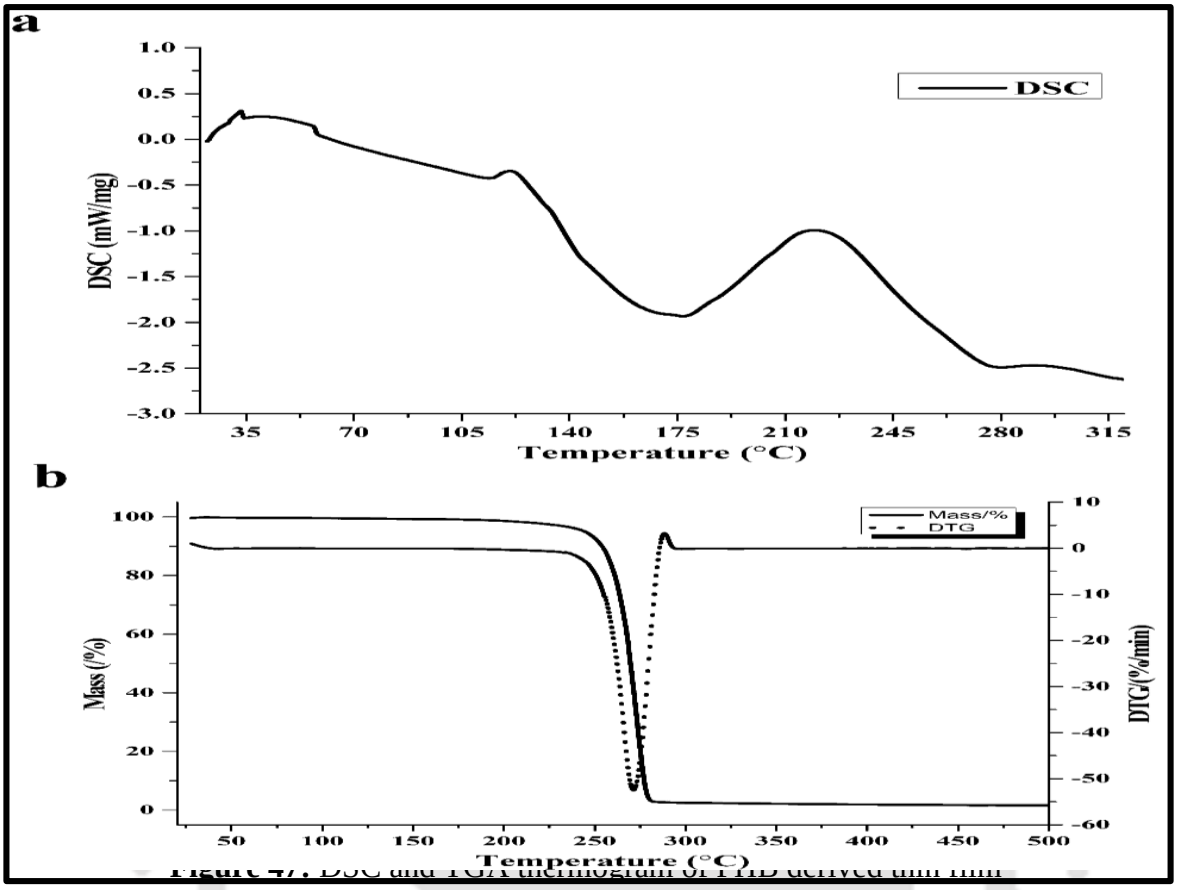


Figure 46: FESEM of extracted PHB thin film (4%, w/v) for electronics applications where uniformity and compactness are critical for effective insulation in electronics application (Ong et al., 2023).

Thermal analysis of PHB thin films

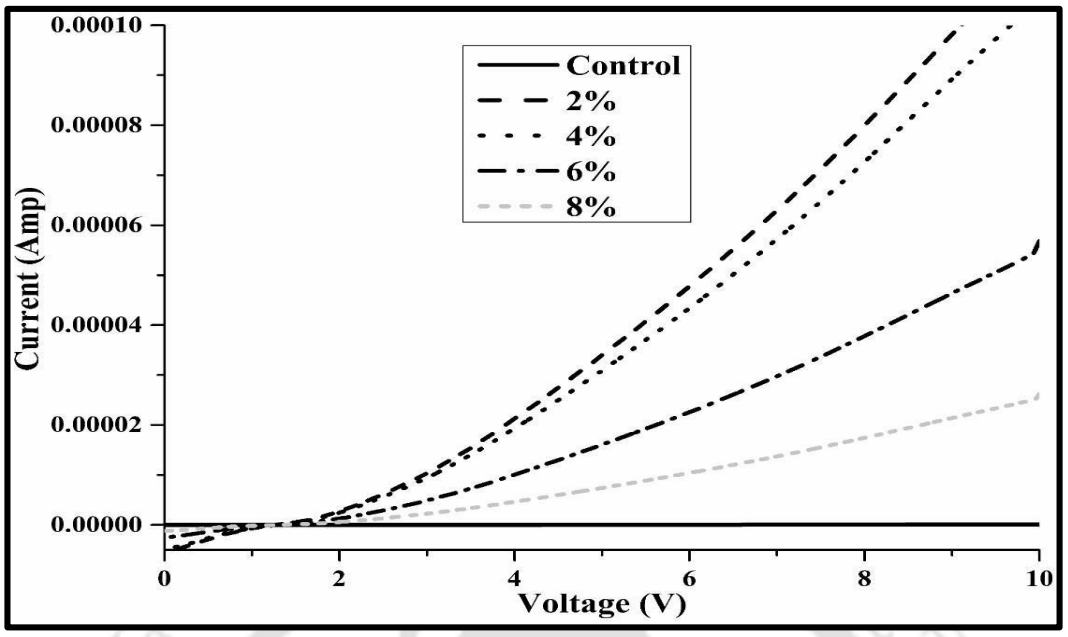
The thermal properties of the PHB polymer were determined using Differential Scanning Calorimetry (DSC) and Thermal Gravimetric (DTG) analysis. DSC shown key thermal transitions: a crystallization temperature of 153.25 °C, a melting point at 181.75 °C, and an onset of degradation at 266.75 °C. These temperatures are indicative of the polymer's stability across a range of operational conditions as shown in **Fig.47**. DTG analysis further complemented these findings, showing a maximum degradation temperature of 259.75 °C, with a maximum degradation rate of 60.23%and a rate of -28.19% per minute.



This level of thermal resilience is paramount for PHB materials used in electronic systems, which are often exposed to thermal stress, making PHB an ideal candidate for insulating primary non-rechargeable batteries (Kumari et al., 2023; Onget al., 2023).

Insulation assessment

In evaluating the PHB thin films as alternates for conventional non-degradable plastic covers, their electrical resistance properties were meticulously examined. A series of tests were conducted to measure current flow across various voltages. As the PHB concentration in the thin films increased incrementally from 2 to 8% (w/v), a noticeable decrease in current values was observed at all applied voltages. For example, at 10 volts, the current exhibited a remarkable 5-fold decrease, from $1.20.e^{-04}$ Amps in 2% PHB films to $2.62.e^{-05}$ Amps in 8% PHB thin films. This trend, consistent across the voltage spectrum, highlights the enhanced insulating characteristics of higher PHB concentration within thin films.



Furthermore, the occurrence of negative current values at lower voltages across all film concentrations indicated the presence of dielectric properties, reinforcing the suitability of PHB thin films as efficient insulators in electronic devices applications (Smith et al., 2020).

Degradability analysis

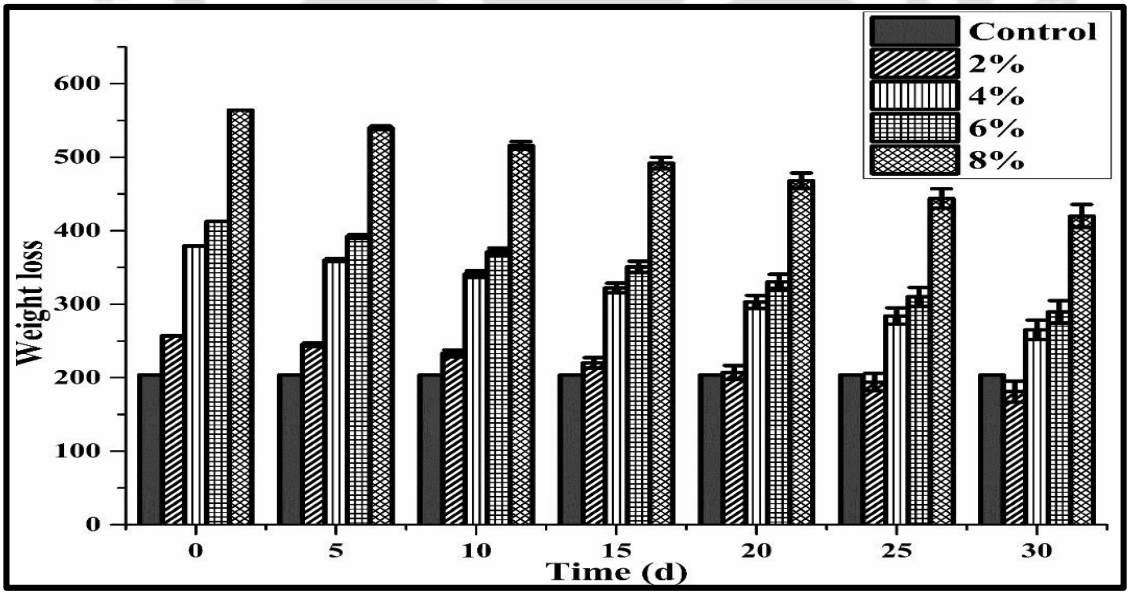


Figure 49: Weight loss of various PHB thin films

The PHB thin films was central to their evaluation for their degradability in electronic applications. Over a 30-day period, the films containing 8% (w/v) PHB exhibited slower degradation rates compared to those with 2% (w/v) PHB as shown in **Fig.49**. Using a 1st order

kinetic model, degradation constants and half-lives were calculated, revealing a slightly accelerated degradation in the 8% (w/v) PHB thin films. As shown in **Fig.50**, the pH of the buffer shifted from 7.8 to 4.5 during degradation in abiotic condition, suggesting the breakdown of PHB into crotonic acid, and eventually into CO_2 and H_2O (Kalita et al., 2021). This results underscore the inherent degradability of PHB thin films and its environmental implications when used as insulators in primary cells cover. This study provided an in-depth analysis of the bioprocess engineering strategies used for PHB production using biogas as feasible C-source in a nitrate-free NMS medium.

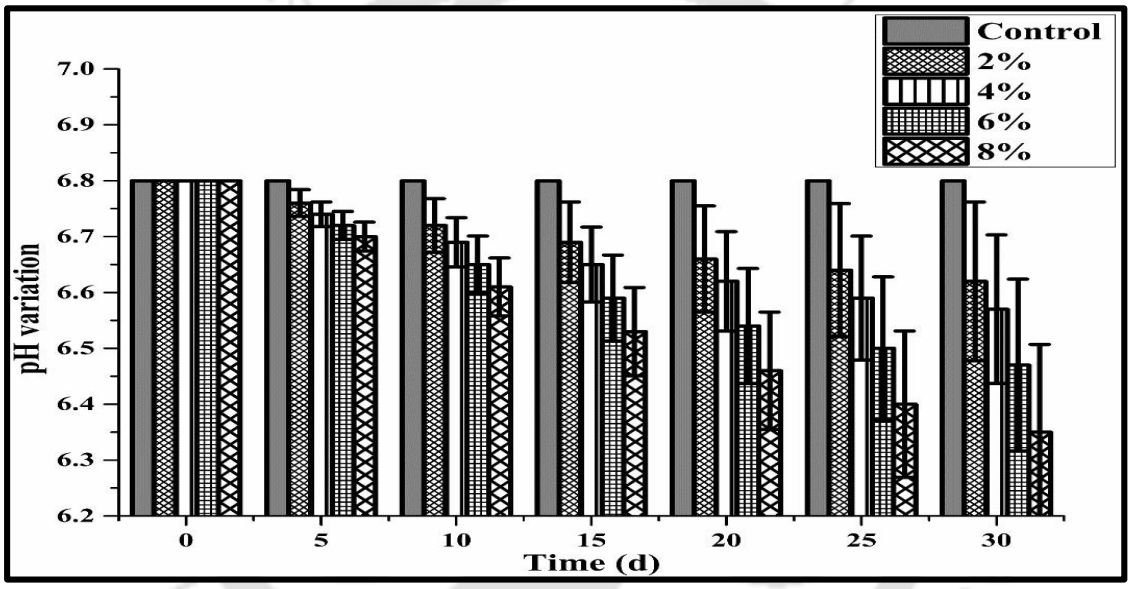


Figure 50: The pH variation of various PHB thin films

The initial phase I, optimized conditions for the cultivation of *M. trichosporium*, achieving maximum biomass concentration of 7.67±0.10 $g.L^{-1}$. The second phase II, subjected the methanotrophic bacteria to stress conditions conducive to intracellular PHB accumulation, resulting in an intracellular PHB concentration of 51.29±0.21% (w/w). The resultant PHB concentration and productivity rates were impressive, highlighting the efficacy of biogas-derived methane, as a feasible source in the process. The comprehensive characterization of the extracted PHB revealed its composition and potential versatility. Finally, the physicochemical characterizations of the *M. trichosporium* PHB thin films highlighted their potential as insulation materials for primary non-rechargeable batteries, offering a

sustainable and efficient alternative in electronic applications.

References

1. Fekete, H.; Kuramochi, T.; Roelfsema, M.; Elzen, M. den; Forsell, N.; Höhne, N.; Luna,L.; Hans, F.; Sterl, S.; Olivier, J.; van Soest, H.; Frank, S.; Gusti, M. A Review of Successful Climate Change Mitigation Policies in Major Emitting Economies and the Potential of Global Replication. Renew. Sustain. Energy Rev. 2021, 137 (September 2020). https://doi.org/10.1016/j.rser.2020.110602.
2. Ters, L. E. T. Evolutionary Risks of Osprey Translocations A Global Plastic Treaty Must Cap Production How to Weaken Russian Oil and Gas Strength.
3. Ahn, H. K., Smith, M. C., Kondrad, S. L., & White, J. W. (2010). Evaluation of biogas production potential by dry anaerobic digestion of switchgrass–animal manure mixtures. Applied biochemistry and biotechnology, 160, 965-975.
4. Cantera, S., Bordel, S., Lebrero, R., Gancedo, J., García-Encina, P. A., & Muñoz, R. (2019). Bio-conversion of methane into high profit margin compounds: an innovative, environmentally friendly and cost-effective platform for methane abatement. World Journal of Microbiology and Biotechnology, 35, 1-10.
5. Li, J., Zhang, X., Udduttula, A., Fan, Z. S., Chen, J. H., Sun, A. R., & Zhang, P. (2021). Microbial-Derived Polyhydroxyalkanoate-Based Scaffolds for Bone Tissue Engineering: Biosynthesis, Properties, and Perspectives. Frontiers in Bioengineering and Biotechnology, 9, 763031.
6. Karthikeyan, O. P., Chidambarampadmavathy, K., Cirés, S., & Heimann, K. (2015). Review of sustainable methane mitigation and biopolymer production. Critical Reviewsin Environmental Science and Technology, 45(15), 1579-1610.
7. Hwang, I. Y., Nguyen, A. D., Nguyen, T. T., Nguyen, L. T., Lee, O. K., & Lee, E. Y. (2018). Biological conversion of methane to chemicals and fuels: technical

challengesand issues. Applied microbiology and biotechnology, 102, 3071-3080.

8. Hazen, T. C. (2010). Cometabolic bioremediation.
9. Patel, S. K., Kumar, P., Singh, M., Lee, J. K., & Kalia, V. C. (2015). Integrative approach to produce hydrogen and polyhydroxybutyrate from biowaste using defined bacterial cultures. Bioresource technology, 176, 136-141.
10. Patel, S. K., Shanmugam, R., Lee, J. K., Kalia, V. C., & Kim, I. W. (2021). Biomolecules production from greenhouse gases by methanotrophs. Indian Journal of Microbiology, 1-9.
11. Choi, D. W., Kunz, R. C., Boyd, E. S., Semrau, J. D., Antholine, W. E., Han, J. I., ... & DiSpirito, A. A. (2003). The membrane-associated methane monooxygenase (pMMO)and pMMO-NADH: quinone oxidoreductase complex from Methylococcus capsulatusBath. Journal of bacteriology, 185(19), 5755-5764.
12. Parvizifard, M., & Karbasi, S. (2020). Physical, mechanical and biological performance of PHB-Chitosan/MWCNTs nanocomposite coating deposited on bioglass based scaffold: Potential application in bone tissue engineering. International journal of biological macromolecules, 152, 645-662.
13. Fan, L., Zhang, H., Li, J., Wang, Y., Leng, L., Li, J., ... & Zhou, W. (2020). Algal biorefinery to value-added products by using combined processes based on thermochemical conversion: a review. Algal Research, 47, 101819.
14. Mohammed, N., Katari, J. K., & Das, D. (2023). Overproduction of poly-β-hydroxybutyrate in Methylosinus trichosporium 11131 as degradable food packaging material utilizing methane. Biomass Conversion and Biorefinery, 1-16.
15. Kruger, N. J. (2009). The Bradford method for protein quantitation. The protein protocols handbook, 17-24.
16. Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S. I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric acid method in microplate format.

Analytical biochemistry, 339(1), 69-72.

17. Maulvi, F. A., Patel, P. J., Soni, P. D., Desai, A. R., Desai, D. T., Shukla, M. R., ... & Shah, D. O. (2020). Novel poly (vinylpyrrolidone)-coated silicone contact lenses to improve tear volume during lens wear: in vitro and in vivo studies. ACS omega, 5(29),18148-18154.
18. Kumar, K. V., Subha, T. J., Ahila, K. G., Ravindran, B., Chang, S. W., Mahmoud, A. H., ... & Rathi, M. A. (2021). Spectral characterization of hydroxyapatite extracted fromBlack Sumatra and Fighting cock bone samples: A comparative analysis. Saudi Journalof Biological Sciences, 28(1), 840-846.
19. Ivorra-Martinez, J., Verdu, I., Fenollar, O., Sanchez-Nacher, L., Balart, R., & Quiles-Carrillo, L. (2020). Manufacturing and properties of binary blend from bacterial polyester poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) and poly (caprolactone) with improved toughness. Polymers, 12(5), 1118.
20. Kalita, N. K., Bhasney, S. M., Kalamdhad, A., & Katiyar, V. (2020). Biodegradable kinetics and behavior of bio-based polyblends under simulated aerobic composting conditions. Journal of environmental management, 261, 110211.
21. Salem, R., Soliman, M., Fergala, A., Audette, G. F., & ElDyasti, A. (2021). Screening for methane utilizing mixed communities with high polyhydroxybutyrate (Phb) production capacity using different design approaches. Polymers, 13(10), 1579.
22. Safaeian, P., Yazdian, F., Khosravi-Darani, K., Rashedi, H., & Lackner, M. (2023). P3HB from CH4 using methanotrophs: aspects of bioreactor, fermentation process andmodelling for cost-effective biopolymer production. Frontiers in Bioengineering and Biotechnology, 11, 1137749.
23. Rodríguez, Y., García, S., Lebrero, R., & Muñoz, R. (2023). Continuous polyhydroxybutyrate production from biogas in an innovative two-stage bioreactor configuration. Biotechnology and Bioengineering, 120(11), 3224-3233.Rumah, B.

L., Stead, C. E., Claxton Stevens, B. H., Minton, N. P., Grosse-Honebrink,A., & Zhang, Y. (2021). Isolation and characterisation of Methylocystis spp. for poly- 3-hydroxybutyrate production using waste methane feedstocks. AMB Express, 11(1), 1-13.

24. Sabale, T. R., Kulkarni, P. P., & Ghosalkar, A. R. (2023). Methane Based Continuous Culture of Methylosinus trichosporium for Production of Poly-3-hydroxybutyrate Using Membrane Recycle System. Biotechnology and Bioprocess Engineering, 1-9.
25. Rodríguez, Y., Firmino, P. I. M., Pérez, V., Lebrero, R., & Muñoz, R. (2020). Biogas valorization via continuous polyhydroxybutyrate production by Methylocystis hirsuta in a bubble column bioreactor. Waste Management, 113, 395-403.
26. Rodríguez, Y., Firmino, P. I. M., Arnáiz, E., Lebrero, R., & Muñoz, R. (2020). Elucidating the influence of environmental factors on biogas-based polyhydroxybutyrate production by Methylocystis hirsuta CSC1. Science of the Total Environment, 706, 135136.
27. Ashoor, S., Jun, S. H., Ko, H. D., Lee, J., Hamelin, J., Milferstedt, K., & Na, J. G. (2023). Polyhydroxybutyrate Production from Methane and Carbon Dioxide by a Syntrophic Consortium of Methanotrophs with Oxygenic Photogranules without an External Oxygen Supply. Microorganisms, 11(5), 1110.
28. Criddle, C. S., Rostkowski, K. H., & Sundstrom, E. R. (2015). U.S. Patent No. 9,062,340. Washington, DC: U.S. Patent and Trademark Office.
29. Cataldo, D.A., Maroon, M., Schrader, L.E., Youngs, V.L., 1975. Communications in Soil Science and Plant Analysis Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid 37–41.

Chapter 6

Conclusion

This thesis provides an in-depth study on the conversion of methane—a potent and 2nd most detrimental greenhouse gas affecting ecosystem by climate change, into polyhydroxybutyrate (PHB) using *M. trichosporium* NCIMB 11131, with applications in food packaging, bone tissue engineering, and electronics. The research is organized around three primary objectives, offering novel visions into PHB production and its diverse applications.

Objective 1: Nutrient modulation effects on PHB production

Nutrient modulation, particularly nitrate limitation, was found to significantly enhance PHB production. Experimental results revealed that nitrate depletion increased intracellular PHB concentration to 51.29% (w/w). Conversely, deficiencies in essential microelements such as iron (Fe) and copper (Cu) led to reduced PHB accumulation, highlighting their critical role in the organism's metabolic activities.

Objective 2: Process engineering strategy for methane mass transfer, and solubility

Optimization of methane mass transfer and solubility was achieved using customized airlift bioreactors equipped with micro-spargers and methane vectors (paraffin and silicone oil). This strategy facilitated a maximal methane fixation rate of 0.73 $g.L^{-1}.d^{-1}$. In phase I, 7.5% silicone oil supplementation resulted in a high biomass yield of 8.91 $g.L^{-1}$. In Phase II, the use of 5% paraffin oil yielded the highest PHB content of 57.3% (w/w), while 7.5% silicone oil achieved a prominent PHB yield of 5.06 $g.L^{-1}$. These results underscore the efficacy of bioprocess

engineering strategies methane mass transfer in enhancing PHB production.

Objective 3: Sustainable PHB production using biogas

The third objective focused on the sustainability of using biogas as a methane source for PHB production. The research demonstrated that biogas could significantly reduce operational costs while maintaining high PHB yields. Phase I achieved a biomass titre of 7.67 $g.L^{-1}$, and Phase II reached a PHB concentration of 51.29% (w/w), proving the viability of biogas as an alternative to pure methane from commercially available cylinder. Additionally, integrating HA derived from waste eggshells with M. trichosporium extracted PHB showed promising results for bone tissue engineering, and insulative film thin in electronics applications.

Applications in diverse fields

Food Packaging

PHB thin films exhibited excellent degradability and mechanical properties, making them suitable for sustainable packaging materials. These films demonstrated outstanding flexibility, strength, and resistance to moisture, comparable to conventional commercial plastic for packaging.

Bone Tissue Engineering

The PHB/HA bio-composite scaffold demonstrated superior biocompatibility and osteo-conductivity, vital for bone regeneration. The bio-composite's porous structure assisted cell attachment and proliferation, supporting osteoblast growth and bone tissue regeneration.

Electronics

PHB thin films were positively used as insulation covers for primary batteries, demonstrating exceptional electrical insulation properties and heat resistance. This suggests PHB's potential to replace traditional non-degradable insulative materials, contributing to a more sustainable electronics industry.

Concluding Insights

The research underscores the feasibility of using *M. trichosporium* NCIMB 11131 for PHB production and highlights the potential of biogas as a sustainable and cost-effective methane source. The diverse applications of PHB in various industries—from food packaging to bone tissue engineering and electronics—demonstrate its versatility and significant role in promoting sustainability. This study contributes to global sustainability efforts by offering a viable pathway to reduce greenhouse gas emissions and advance the circular economy for biodegradable polymers. The findings pave the way for future innovations in biopolymer production and applications, marking a significant stride towards environmental sustainability and reducing plastic pollution in our beautiful ecosystem.

Challenges and future perspectives of PHB production

✓ PHB production from *M. trichosporium* NCIMB 11131 offers promising avenues for sustainable bioplastics.

✓ Challenges include optimizing cultivation conditions, enhancing methane utilization as C1 C-source, and improving downstream processing.

✓ Utilizing innovative methodologies such as high-throughput cultivation and unique bioreactor designs can enhance PHB production further.

✓ Overcoming challenges requires advancements in methane vector supplementation, bioreactor configuration, and downstream processing efficiency.

✓ Future research goals to optimize PHB production processes for scalable and sustainable bioplastic production for versatile applications for green revolution.

List of Publications

Mohammed, N., Katari, J. K., & Das, D. (2023). Overproduction of poly-β-hydroxybutyrate in *Methylosinus trichosporium* 11131 as degradable food packaging material utilizing methane. Biomass Conversion and Biorefinery, 1-16.

Manuscript under preparation

Development of Process Engineering Strategies for PHB production using *M. trichosporium*: Application in Bone Tissue Engineering

Biogas-derived PHB using *Methylosinus trichosporium* for Battery Insulation Cover: Biorefinery Approach

Vitae

The author, Noor Mohammed, was born on 13th March 1991 in Gorchhi, Hisar, Haryana, India. He completed his higher secondary education at Govt. High School, Gorchhi (CPA. 8.20/10). He completed his senior secondary education at Govt. Model Sr. Sec. School, Aryanagar, Hisar. He obtained his Bachelor of Technology in Biotechnology from Deenbandhu Chhotu Ram University of Science and Technology, Murthal, in 2014 (CGPA: 6.42/10). Subsequently, he earned his Master of Technology in Biotechnology from the National Institute of Technology Rourkela in 2017 (CGPA: 7.03/10).

In December 2017, Noor Mohammed joined the Ph.D. program under the supervision of Prof. Debasish Das in the Department of Biosciences and Bioengineering at the Indian Institute of Technology Guwahati, Assam, India. His doctoral research focused on the sustainable production of polyhydroxybutyrate (PHB) from methane utilizing Methylosinus trichosporium NCIMB 11131. This research also explored PHB applications in diverse fields, including food packaging, bone tissue engineering, and electronics along with the development of bioprocess engineering. He has completed all academic requirements with a CGPA of 7.03/10. Noor Mohammed presented his Ph.D. synopsis on 1st December 2023 and his viva-voice on 30th October 2024. He submitted his Ph.D. thesis in November 2024.