

**PRODUCTION OF BIODEGRADABLE PLASTIC BY
POLYHYDROXYALKANOATES (PHAs) ACCUMULATING
BACTERIA AND ITS POTENTIAL ENVIRONMENTAL
APPLICATION**

**BY
SHIVA ALEY ACHARJEE**



Submitted to
NAGALAND UNIVERSITY

In Partial Fulfilment of the Requirement for The Award of The Degree

of

DOCTOR OF PHILOSOPHY

IN

ENVIRONMENTAL SCIENCE

DEPARTMENT OF ENVIRONMENTAL SCIENCE

NAGALAND UNIVERSITY

LUMAMI - 798627

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And

DR. PRANJAL BHARALI

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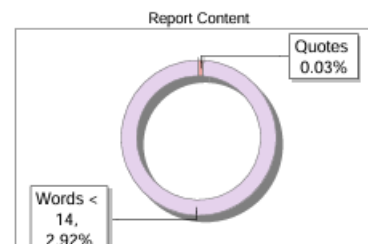
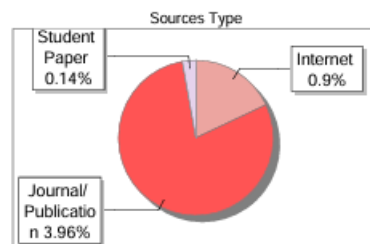
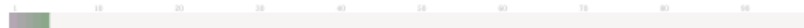
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LIST OF ABBREVIATIONS AND SYMBOLS IN THE TEXT

Å	Ångström
kV	Kilovolt
mA	Milliampere
a.u.	Arbitrary Units
BLAST	Basic Line Alignment Search Tool
cm	Centimeter
¹³ C	Carbon 13
¹ H	Hydrogen 1
CI	Crystallinity Index
Cu-Kα	Copper K-alpha
DMEM	Dulbecco's Modified Eagle's Medium
eV	Electron volt
GPa	Gigapascal
KBr	Potassium Bromide
mg	Milligram
m ²	Square meter
m ² /g	Square meter per gram
mm ²	Square millimeter
mm	Millimeter
min	Minute
MPa	Megapascal
GPa	Gigapascal
Mw	Weight Average Molar Mass
Mn	Number Average Molar Mass
MPs	Microplastics
N	Newton
PDI	Polydispersity Index
NCBI	National Centre for Biotechnology Information
Na ₂ HPO ₄	Disodium Hydrogen Phosphate
NO ₂	Nitrogen dioxide
SO ₂	Sulphur dioxide
VOCs	Volatile Organic Compounds
nm	Nanometer
O ₃	Ozone
O ₂ ⁻	Superoxide anions
3D	Three Dimensional

OH ⁻	Hydroxide ions
bp	Base pair
OH ⁺	Hydrogen ions
<i>p</i>	Probability
CV	Coefficient of Variation
HepG2	Human Hepatoblastoma Cell Line
NCCS	National Centre for Cell Science
PBS	Phosphate-buffered saline
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
RSM	Response Surface Methodology
PBD	Plackett-Burman Design
DCW	Dry Cell Weight
DoE	Design of Experiments
CCD	Central Composite Design
SD	Standard Deviation
PCR	Polymerase Chain Reaction
UV	Ultraviolet
v/v	Volume/volume
Hz	Hertz
MHz	Mega Hertz
g/g	Gram/Gram
h	Hour
S	Svedberg unit
F	Forward
H	Planck's constant
ν	Frequency
R	Reverse
Sec	Second
J/g	Joule/gram
&	And
w/v	Weight/volume
α	Alpha
mm	Millimetre
Wt%	Weight percent
N/mm ²	Newton per Square Millimetre
θ	Theta
ASTM	American Society for Testing and Materials
Mt	Million tonnes

±	Plus, minus
µg	Microgram
µm	Micrometre
<	Less than
>	Greater than
µL	Microliter
µg/mL	Microgram per Millilitre
µM	Micromolar
%	Percentage
X	Times
°	Degree
°C	Degree Celsius

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ABSTRACT

of

PRODUCTION OF BIODEGRADABLE PLASTIC BY POLYHYDROXYALKANOATES (PHAs) ACCUMULATING BACTERIA AND ITS POTENTIAL ENVIRONMENTAL APPLICATION

*Thesis submitted in partial fulfilment of the requirements for the award of
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ABSTRACT

Petroleum-derived plastics are linked to a variety of growing environmental issues throughout their lifecycle, including emission of greenhouse gases, accumulation in terrestrial and marine habitats, pollution, among others. There has been a lot of attention over the last decade in industrial and research communities in developing and producing eco-friendly polymers to deal with the current environmental issues. Bioplastics preferably are a fast-developing family of polymeric substances that are frequently promoted as substitutes to petroleum derived plastics. Polyhydroxy-alkanoates (PHAs), a type of bioplastic, are seen as potential substitutes for conventional petrochemical plastics because of their robust mechanical qualities that are comparable to those of thermoplastics derived from petroleum and their high level of biodegradability. PHAs are typically produced by microbes especially by bacteria intracellularly and stored as carbon and energy source in specialized sub-cellular organelles called carbonosomes, when they are exposed to nutritional limitations. Additionally, PHAs generation by bacteria has drawn notable interest in past few years due to its biocompatibility properties where it can be implanted in living tissues of human without triggering any immunological responses. PHAs have a number of appealing properties that make PHAs a feasible source material for bioplastics, either as a direct replacement of petroleum-derived plastics or as a blend with elements derived from natural origin, to fabricated biodegradable polymers, and/or non-biodegradable polymers. Among the most promising PHAs, polyhydroxybutyrate (PHB) is the most well-known and have a significant potential to replace traditional plastics. These biodegradable plastics decompose faster after disintegrate into carbon dioxide, water, and inorganic chemicals. Other properties like non-toxicity, insolubility in water, as well as its thermo-plasticity, make it an excellent alternative to conventional plastics.

Therefore, a potent PHB-producing strain was successfully isolated from kitchen waste effluent site in Lumami, Zunheboto, Nagaland, India, and identified through 16S rRNA gene sequencing as *Bacillus* sp. KE4. A range of organic wastes, such as Bael fruit pulp, sugarcane molasses, jackfruit wastes, papaya peels, pumpkin wastes, and glucose individually, were evaluated for their viability as

carbon sources for PHB production. Of the carbon sources examined, glucose emerged as the most effective substrate for PHB synthesis. Statistical methodologies such as Plackett-Burman Design (PBD) and Central Composite Design (CCD) within Response Surface Methodology (RSM) were employed to assess the significant variables affecting the yield of PHB. Physicochemical characterization revealed that the produced PHB possessed excellent structural, thermal, and mechanical properties, confirming its suitability for advanced technological applications. The weight average molar mass (M_w), number average molar mass (M_n), and polydispersity index (PDI) of the extracted PHB was 1,55,436 g/mol; 1,43,258 g/mol; and 1.08, respectively. Using open windrow composting method, the extracted PHB biopolymer film was assessed to determine its biodegradability. Within 28 days of soil burial, it was found that the biopolymer film had degraded by 61.34 %. Cytotoxicity assessment in HepG2 (human liver hepatocellular carcinoma cell line) cells indicated that PHB is non-cytotoxic in nature. The results have showed that the biomaterial is environmentally benign, biodegradable, and biocompatible; therefore, it may find its applications in diverse fields.

However, multifaceted industrial applications of PHB have been constrained by a lack of advanced features *viz.* poor physical and mechanical properties, low compatibility with standard thermal processing techniques, high susceptibility to thermal degradation, and high production costs. Thus, PHB need to be upgraded to enhance its inherent qualities. This upgradation can be primarily achieved by blending PHB either with natural or synthetic biodegradable polymers and adding reinforcements to develop novel PHB-based biocomposites and blends. Blending is effective and affordable, and when executed appropriate, it can yield new materials with enhanced properties. Therefore, in the present investigation an attempt is made to blend PHB with other polymers to enhance its properties for specific applications.

Generation of secondary contaminants, insufficient sorption and recovery, and degradability issues associated with the existing approaches render the management of hydrophobic pollutants very challenging. Hereby, a cost-effective, energy-efficient, non-cytotoxic, biocompatible, and biodegradable 3D (three-

dimensional) porous sorbent is fabricated employing a blend of PHB and PCL (Polycaprolactone) biopolymers using a facile salt leaching technique. The findings demonstrated an efficient biomaterial for addressing oil spill disasters while minimizing secondary contamination. The sorbent also fulfilled the zero-waste notion of the Sustainable Development Goals (SDGs) defined by the United Nations.

A combination of essential oils (EOs) of Cinnamon (*Cinnamomum verum*) and Clove (*Syzygium aromaticum*) as an antimicrobial agent was incorporated into PHB matrix by conventional solvent-casting method for food packaging applications. Migration values for active composite films for diverse food simulants were well within 10 mg/dm². EOs in PHB matrix have shown good antibacterial and antifungal effects against all tested food-spoiling pathogens. To test composite film's biocompatibility, primary rat hepatocytes were tested for cell viability, ROS (reactive oxygen species) production, and apoptosis. The composite film was found to be biocompatible, proving its non-toxicity. Additionally, the composite film degraded completely in 49 days in soil burial test.

Findings of the present investigation highlights the production of PHB from bacterial strain *Bacillus* sp. KE4, isolated from kitchen waste effluent and showed its versatility as an advanced biomaterial for environmental applications such as fabrication of 3D porous sorbent and active packaging by blending with other biopolymers and EOs. Furthermore, present investigation is in accordance with the principles of the circular bioeconomy and concept of SDGs and lays the path for the development of future eco-benign sustainable technologies.

Keywords: Polyhydroxybutyrate (PHB), Bioplastic, *Bacillus* sp. KE4, Environmental Applications, Circular bioeconomy, SDGs

CHAPTER-1

An Overview of Polyhydroxybutyrate (PHB): Properties, Production, and Applications

A part of this chapter has been published in:

Acharjee, S. A., Bharali, P., Gogoi, B., Sorhie, V., Walling, B., & Alemtoshi. (2023). PHA-based bioplastic: A potential alternative to address microplastic pollution. *Water, Air, & Soil Pollution*, 234(1), 21. <https://doi.org/10.1007/s11270->

General Introduction

Plastic products are incredibly versatile due to their low thermal and electric conductivity, lightweight, rigid, long-lasting and corrosion resistance, which enable them to serve as an oxygen and water barrier. Their low cost also contributes to their easy and widespread production, where they are utilized in numerous applications extending from food industry to medical and technological uses that benefit the society (Frias and Nash, 2019; Thompson et al., 2009). However, plastic waste has been noticed as a worldwide pervasive pollution over the last decade. Around the world, plastics, and therefore its wastes and residues, can be found in land, ocean, and fresh water habitats, including in the most remote locations. Although global plastic production and utility are increasing, but waste management and treatment in many places remains a major concern. Immoderate usage of plastics has wreaked havoc on the environment, with humans producing about 34 million tonnes of plastic recently. Only 7 % of production gets recycled, with remaining 93 % ending up in landfills, oceans, and seas (Shah et al., 2021). As a result, at a staggering pace of 31.9 million metric tonnes per year, plastic ends up in the natural surroundings (Bucci et al., 2020; Jambeck et al., 2015). Currently, only a small portion of this plastic is recycled when it is disposed of, and the remaining bulk is released into the environment, where it may stay for many years before totally degrading. Over the time, these plastics degrade in the environment, producing micro- and nano-plastics, which are tiny particles or fibers generally harmful to living organisms (Pellini et al., 2018). Arikan and Ozsoy (2015) mentioned several environmental and economic issues in their study caused by widespread use of plastics, including landfill problems, plastic accumulation in oceans, incineration of plastics, non-degradability or durability, and economic problems such as crude oil competition and energy security. These negative consequences have the potential to harm humans and animals, as well as to completely disrupt the biological cycles and ecosystems. Therefore, finding a substitute for petroleum derived polymers is becoming a pressing need to prevent the undesirable effects that traditional polymers have made in the environment due to their excessive utilization and improper disposal (Narayanan et al., 2020a). To

find alternatives, researchers developed entirely degradable polymers that can be discarded in the environment and easily degraded by microorganisms' enzymatic activity. These materials are known as "Biodegradable Plastics" (Marjadi and Dharaiya, 2010). Among the biodegradable polymer, polymeric material developed or synthesized from different biological systems *viz.* bacteria, fungi, algae, plants etc. are generally referred as "Biopolymer". Because of their similar physical and chemical qualities, as well as their ability to degrade faster in the environment than petroleum-derived plastics, bioplastics are regarded as a viable alternative for petroleum-derived synthetic polymers (Mascarenhas and Aruna, 2017). The growing demand for environment-friendly products, along with the need to cut carbon emissions and both micro- and macro-plastic pollution in earth's biosphere, has prompted the growth of new bioplastic supplies in recent years that meet a similar demand as existing plastics with lower environmental impact (Calero et al., 2021). Meereboer et al. (2020) examined recent developments and opportunities to use polyhydroxyalkanoates (PHA)-based bioplastics, a polymeric material of biological origin, as an alternative to petroleum-based plastics. In contrast to the negative effects of disposing of plastics in landfills, PHA based bioplastics exhibit biodegradable behaviour in all anaerobic and aerobic environments as described by American Society for Testing and Materials (ASTM) standards and can be used to make fully compostable, soil- and marine-biodegradable goods.

To produce bioplastics and associated biomaterials, PHAs may be extracted and refined from a variety of microbial sources especially from a number a bacterial species. This may help to reduce the dependency of the present world on petroleum-derived plastics, which will lead to reduce plastic pollution and safeguard the nature (Muneer et al., 2020). PHAs are typically produced intracellularly by microbes and stored as carbon and energy in specialized sub-cellular organelles (carbonosomes) when they are exposed to nutritional limitations (Tanamool et al., 2013). Usually, limiting nutritional factors like phosphorus (P) and nitrogen (N) restrictions or the availability of an excess carbon (C) supply is known to stimulate polyhydroxybutyrate (PHB), a type of PHA, production in bacteria. The requirements of C in living organisms are often greater than N. The proportion of these components (C:N) governs how bacteria utilize an organic substance.

According to Wei et al. (2011), when gluconic acid was utilized as the carbon source, the best C:N ratio for the efficient production of PHAs in *Cupriavidus taiwanensis* was 8:1, with a PHB concentration of 58.81 %. Likewise, at a C:N ratio of 8:1, *Bacillus thuringiensis* IAM 12077 produced the greatest PHB content of 72.3 % utilizing starch as the carbon source, according to the findings of Gowda and Shivakumar (2014). According to Lee et al. (2020), *Rhodobacter sphaeroides* produced the highest PHB content of 51.57 % at a C:N ratio of 15:1 when succinic acid was used as the carbon source. Thus, depending on the kind of bacterial species and the carbon sources, the C:N ratio for the optimal generation of PHB varies. However, the carbon need is almost always greater than the nitrogen requirement. Based on previous investigations, production of PHAs by various bacterial species using variety of carbon sources is presented in Table 1.1.

Table 1.1. Production of polyhydroxyalkanoates (PHAs) by various microorganisms using variety of carbon sources

Microorganisms	Carbon source	Dry cell weight (DCW) (g/L)	PHA yield (g/L)	PHAs accumulation (%)	References
<i>Burkholderia cepacia</i> ATCC 17759	Glycerol	5.8	4.8	82.76	Zhu et al. (2010)
<i>Bacillus aryabhatai</i>	sweet sorghum juice	3.02	1.74	57.62	Tanamool et al. (2013)
<i>Brevundimonas</i> sp. NAC1	Cardboard industry waste water	-	4.042	77.63	Bhuwal et al. (2013)
<i>Enterococcus</i> sp. NAP11	Cardboard industry waste water	-	5.236	79.27	

<i>Bacillus</i> NA10	sp.	Cardboard industry effluent	7.8	5.202	66.70	Bhuwal et al. (2014)
<i>Bacillus thuringiensis</i> IAM 12077		Starch	3.6	2.6	72.3	Gowda and Shivakumar (2014)
<i>Cupriavidus necator</i>		Fructose	11.6	7.48	64.48	Aramvash et al. (2015)
<i>Pseudomonas aeruginosa</i>		Mustard oil	-	9.01	20.1	Javed and Jamil (2015)
<i>Bacillus</i> sp.		Sugarcane bagasse	9.0	5.0	55.55	Getachew and Woldeesenbet (2016)
<i>Pseudomonas putida</i>		Waste frying oil	4.90	2.80	57	Gatea et al. (2018)
<i>Bacillus siamensis</i> PD-A10		Orange peel	2.66	2.16	81.2	Vijay and Tarika (2019)
<i>Bacillus subtilis</i> JCM 1465		Onion peel	3.33	2.93	88	
<i>Staphylococcus aureus</i> JH1		Onion peel	1.56	1.29	83	
<i>Bacillus megaterium</i>		Molasses	-	19.52	60.02	Joyline and Aruna (2019)
<i>Bacillus cereus</i> NDRMN001		Rice bran	36.26	33.19	91.54	Narayanan et al. (2020a)
<i>E. coli</i>		Sago molasses	33.26	27.1	81.47	Narayanan et al. (2020b)

<i>Paraburkholderia</i> sp. PFN29	Glucose	5.14	5	97.3	Sriyapai et al. (2022)

One of the most promising biopolymers is polyhydroxybutyrate (PHB), a biogenic short-chain polyhydroxyalkanoates synthesized by the process of glycolysis where preferably soluble mono- and di-saccharides are utilized to produce acetyl-CoA which later converted into PHB. PHB are additionally harmless to the environment and due to its biodegradability and biocompatibility nature as compared to petroleum-derived plastics. In contrast to traditional plastics, which have a life expectancy of between 100 and 1000 years, PHA-based bioplastic may degrade into water (H₂O) and carbon dioxide (CO₂) in 20 to 45 days if there is adequate humidity, oxygen, and a sufficient number of microbes available in the open environment (Moshood et al., 2022). Its characteristics *viz.* flexibility, crystallinity, elasticity, and thermoplasticity are comparable to those of thermoplastics that are petroleum-derived, and it can be biodegraded by depolymerase enzymes found in bacteria and fungi that break down PHB. Such bacteria and fungi exist extensively in various ecological systems (Awasthi et al., 2020). PHAs are utilized to develop bioplastics, which are of great interest to researchers in polymeric science due to their sustainability and effectiveness in achieving some of the related (e.g. environment; health; food security) sustainable development goals set forth by the United Nations. Moreover, they can help to reduce dependency on fossil fuels and uphold sustainability initiative (Muneer et al., 2020). However, the primary limiting factor that has restricted the marketable applicability of PHB is its cost of production. Therefore, the development of PHB as an ecologically benign plastic for commercial uses will require a cost-effective process and hyper-producing strains (Kavitha et al., 2018).

1.1. Brief History of Plastic

Bakelite, the first genuinely synthetic polymer, was manufactured in 1907 by Leo Baekeland, a Belgian chemist, and numerous more plastics emerged within the next few decades (Thompson et al., 2009). Plastic was produced in 60 million metric tonnes over the world in 1980. By the year 2000, production had risen to

187 million metric tonnes, followed by 265 million in 2010 (Chalmin, 2019). Global plastic production was 335 million tonnes (Mt) in 2016, 348 Mt in 2017, 359 Mt in 2018, 368 Mt in 2019, and 367 Mt in 2020, excluding the recycled plastics. There is no other commodity in the world that has seen such expansion (Plastics Europe, 2021). According to Indian plastic industry report (2019), the global market demand for major plastics from 2018 to 2019 was 280 Mt, whereas the Indian market demand for major plastics was 16 Mt. Furthermore, India's market share in the global market for Styrenics (high impact polystyrene) is 5.7 %. A study in Statista (2021) reported that a whopping 46 % of the plastic garbage produced worldwide in 2018 came from packaging. This was significantly more than any other industry, with textiles accounting for around 15 % of the entire bulk. Figure 1.1 (a) shows the global distribution of plastics in various sectors. In industrial facilities across the globe in 2019, 60 Mt of generated plastic trash, 6 Mt of residues from plastic recycling, and 1 Mt of collected litter were burned, whereas 162 Mt of generated garbage, 11 Mt of residues, and 1 Mt of collected litter were dumped in landfills (OECD, 2022). Figure 1.1 (b) shows that 15 % of global plastic wastes are collected for recycling, 17 % are incinerated, 22 % are mismanaged or littered, and remaining 46 % are dumped in landfills.

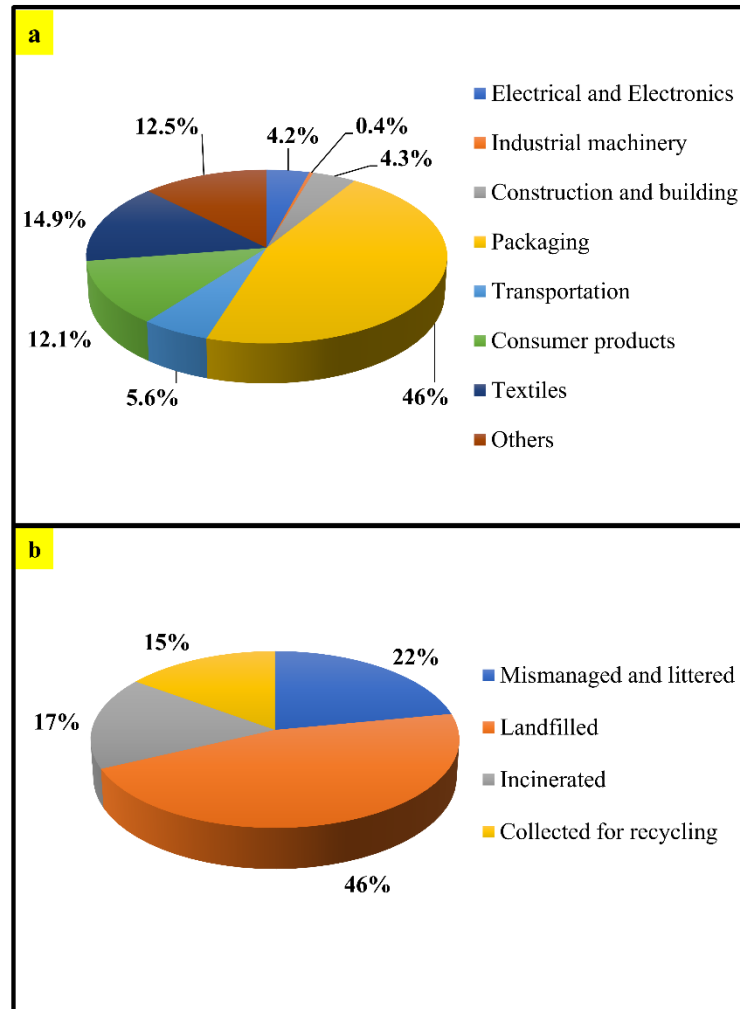


Fig. 1.1. (a) Pie chart of global distribution of plastics in various sectors (Statista, 2021) and (b) Pie chart of global plastic waste management before recycling, 2019 (OECD, 2022)

The European plastics value chain, which consists of plastics producers, plastics converters, plastics recyclers, and machinery producers, witnessed a decline in both its production and demand levels in 2020 as a consequence of the COVID crisis. Nevertheless, this industry managed to maintain a significant level of employment (Plastics Europe, 2021). The world's plastic manufacturing peaked in 2019 at 368 Mt, and then fell to 367 Mt in 2020, which was the first noticeable drop in the history of the plastics industry ever observed. However, the COVID-19 pandemic has induced a significant increase in plastic pollution due to overflow of medical waste, requirement for suitable personal protective equipment (PPE), rise

in online shopping and food delivery, prohibition on reusable bags, among others (Shams et al., 2021). Despite efforts to break the plastic cycle and its harm to the environment, it is projected that over 40 Mt of plastic will arrive into the environment annually, of which 11 Mt will be the macro- and micro- sized plastic debris that ends up in the ocean (Sorasan et al., 2022).

1.2. Overview of Plastic Degradation

Conventional plastics are generally not easily degraded and they usually remain in the environment for the next 1000 years depending on their properties and their surroundings. However, at a very slow pace the biotic and abiotic factors cause breakdown of plastics and bring changes in the inherent properties of the plastics (Zhang et al., 2021). The general process of plastic degradation is illustrated in figure 1.2. Over the time, degraded plastics give rise to fine plastic fragments that range between micrometre (μm) to nanometre (nm) and tend to accumulate in the nature. Fine fragments *i.e.* microplastic (MP) and nanoplastic easily released into to the aquatic environment and to the biotic system as a whole *via* ocean currents, tides, tsunamis or wind (Cai et al., 2018; Wang et al., 2016a, b).

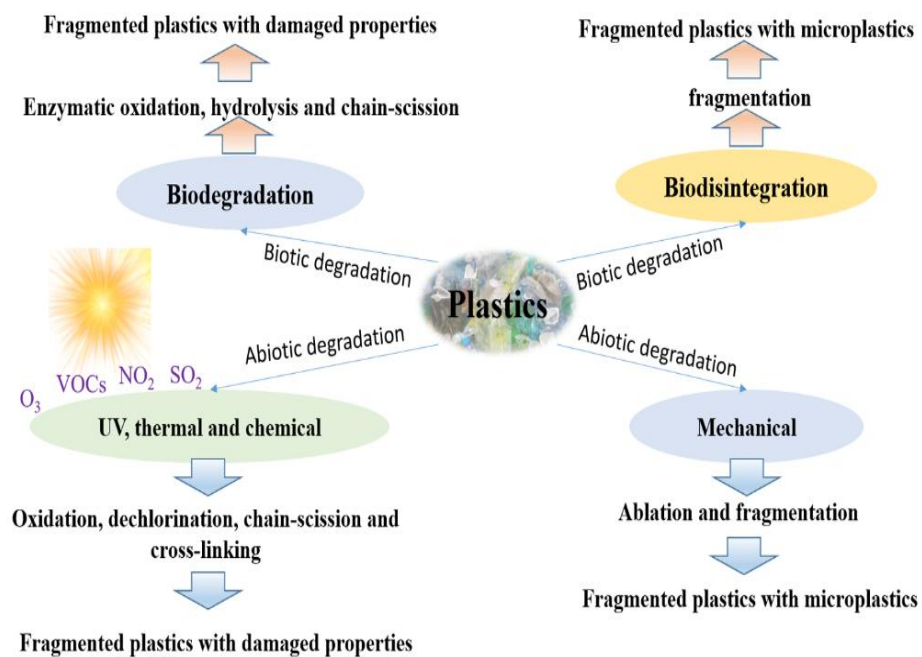


Fig. 1.2. An illustration of the mechanisms behind plastic deterioration (adapted from Zhang et al., 2021)

1.2.1 Abiotic Degradation of Plastics

In this type of plastic degradation, the abiotic factors like water, temperature, light, air and other forces (mechanical) bring physicochemical changes in the plastics (Zhang et al., 2021). Due to plastics' limited bioavailability, abiotic breakdown is typically anticipated to occur before biodegradation (Andrady, 2015). The abiotic degradation of plastics can occur generally *via* photodegradation, thermal, chemical or by mechanical means (Zhang et al., 2021).

1.2.1.1. Photodegradation of Plastics

One of the most significant environmental factors that triggers plastic breakdown is photodegradation. Plastics often undergo photodegradation through the use of free radical-mediated processes brought on by solar radiation, mainly due to ultraviolet (UV) radiation specifically by UV-A (315-400 nm) and UV-B (290-315 nm) irradiation (Liu et al., 2019; Zhang et al., 2021). In general, plastics such as polyethylene (PE) resists photodegradation due to absence of chromophores, but impurities or structural changes in the plastic act as chromophores like the carbonyl groups within the PE backbone which undergoes Norrish Type I and Type II reactions forming ketones, radicals and end-vinyls resulting in a main-chain scission (Zhang et al., 2021). The free radicals generally react with oxygen forming peroxy radicals that are further converted to peroxide moiety and finally dissociated into macro alkoxy and hydroxyl radicals that catalyze the subsequent reactions (Torikai et al., 1986). Similar to PE, impurities in polypropylene (PP) helps in forming radicals in presence of UV radiation and subsequent reactions lead to random cross-linking and chain scissions, with formation of low molecular weight degraded products (Su et al., 2019; Zhang et al., 2021). Likewise, in polyvinyl chloride (PVC), impurities act as chromophores which absorb UV radiations and forms free radicals which can further generate hydroperoxides that break the double bonds of the backbone chain resulting in small fragmented products (Law, 2017; Yang et al., 2018).

1.2.1.2. Thermal Degradation of Plastics

The term “thermal degradation” describes the degradation of polymers as a result of energy input at high temperatures. At high temperatures, plastics can experience thermo-oxidative reactions. Long polymer chains can be disrupted, creating radicals, when the polymer absorbs enough heat to break over the energy barrier (Peterson et al., 2001; Pirsheeb et al., 2020). Both molecule reduction and expansion may occur during heated degradation as an outcome of cross linking and chain scission. The temperature required for thermal deterioration is determined by the thermal characteristics of the plastic polymers and the availability of oxygen (Crawford and Quinn, 2017). Additionally, the deterioration of plastics may be accelerated by temperature and UV light, and the rate of oxidative processes also speeds up in response to temperature (Andrady et al., 2003; Zhang et al., 2021).

1.2.1.3. Chemical Degradation of Plastics

Pollutants including ozone (O_3), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), and volatile organic compounds (VOCs) in the environment can either directly damage plastics or stimulate the creation of radicals through photochemical reactions, which may additionally cause plastics to degrade (Crawford and Quinn, 2017). Chain scission on the polymer results from the interaction of O_3 with double bonds. O_3 can likewise react with saturated polymers; however, it does so far more slowly (Cheremisinoff, 2001). Moreover, UV light has the ability to excite SO_2 , resulting in a reactive singlet or triplet state that directly interacts with the unsaturated carbon-to-carbon double bond ($C=C$) or produces O_3 through a photochemical reaction with O_2 . Furthermore, because of the odd electrons present in the molecule, NO_2 is highly reactive and may quickly react with the unsaturated $C=C$ in the polymer (McKeen, 2019). The pH and salinity of the water are the two chemical parameters that have the greatest impact on plastic decomposition in aquatic environments. Polyamides (PAs), a kind of plastic that is vulnerable to hydrolysis, may degrade when exposed to high concentrations of acidic or basic hydrogen ions (H^+) or hydroxide ions (OH^-) in an aqueous environment (Hocker et al., 2014).

1.2.1.4. Mechanical Degradation of Plastics

The term “mechanical deterioration” describes how polymers break down as a result of outside influences. Environmental external pressures might result

from plastics rubbing up against sand and rocks due to waves and wind. The mechanical breakdown of polymers can also be brought on by the thawing and freezing of plastics in aquatic conditions (Pal et al., 2018). Lower elongation at break values increases the likelihood that plastics may shatter in the presence of external tensile forces. Plastics that are subjected to constant stress eventually experience polymer chain scission (Sohma, 1989). The mechanical characteristics of plastics, notably their tensile modulus and tensile elongation at break, will be impacted by chain scissions of polymers during thermal, photo, and chemical degradation (Andrady, 2017; Zhang et al., 2021). Mechanical degradation is mostly relevant to synthetic fibers, which undergo degradation forming MPs fibers *via* stresses, shear or abrasion during laundering (Cesa et al., 2020).

1.2.2. Biotic Degradation of Plastics

The term “biotic degradation of plastics” describes how microorganisms erode polymers. Microorganisms can breakdown plastics either physically by digesting, biting, or chewing fragmentation or biologically by biochemical processes (Danso et al., 2019). The biological breakdown of plastics may be primarily caused by microorganisms, such as bacteria, fungi, and insects (Crawford and Quinn, 2017). Due to their solid form and the fact that only a very tiny portion of the polymer is exposed to potential degraders, conventional plastics often have a very poor bioavailability (Battin et al., 2016). Additionally, macromolecular polymers cannot be utilized directly by bacteria, extracellular enzymes must first break them down into smaller molecules before they can be absorbed by cells and used for further metabolization (Chen et al., 2019).

The bacterium *Azotobacter beijerinckii* was revealed to have “hydroquinone peroxidase” as the mechanism for polystyrene (PS) biodegradation (Nakamiya et al., 1997). Furthermore, it has been hypothesized that a number of fungi-excreted enzymes can shorten PE polymer strands (Sánchez, 2020). The oxidative breakdown of hydrolysable polymers may also be aided by enzymatic oxidation in addition to hydrolysis (Magnin et al., 2020). Plastics eventually have the potential to mineralize into CO₂ and H₂O in aerobic environments and into CO₂, methane (CH₄), organic acids, H₂O, and ammonium (NH₄) in anaerobic

environments, which promotes the formation of microbial biomass (Zhang et al., 2021).

1.3. Impact of Plastic on the Environment

Discarded plastic wastes is commonly found in a variety of ecosystems and frequently interacts with wildlife and humans, causing harmful repercussions. These plastic wastes have a significant impact on numerous ecosystems, including aquatic habitats, terrestrial ecosystems, aerial ecosystems, and the human biological system, which are briefly discussed below.

1.3.1. Impact on Aquatic Ecosystem

Most of the plastic waste discovered in coastal water ways is assumed to have originated from poor land management mainly due to illegal dumping of wastes, storm water runoff, and activities like fishing, shipping, and aquaculture. The rivers, sewage systems, surface runoffs, wind, and shore trash all carry plastic debris from the terrestrial to the marine (Ory et al., 2020). The persistent, bioaccumulative, and toxic (PBT) compounds, which include organic pollutants and heavy metals, are also carried by MPs and are a source of multiple stresses for life (Manzoor et al., 2021). MPs litter in the ocean is a potential contributor to biodiversity loss and a health risk to humans. Plastics and their degraded by-products are consumed by a wide range of aquatic creatures, from invertebrates to fish, with varying effects (Li et al., 2020). MPs also serve as a carrier for chemical contaminants in aquatic ecosystems by transporting chemicals to other aquatic organisms or locations (Ha and Yeo, 2018). Due to their tiny size, which is similar to plankton, MPs pose a risk to the health of the organism by being easily consumed by fish and other aquatic animal, along with any toxins they may be linked to. Even at higher trophic levels, birds consume fish that has already ingested MPs and hence accumulating it in their body (Manzoor et al., 2021). The effect of MPs in aquatic environment is shown in figure 1.3.

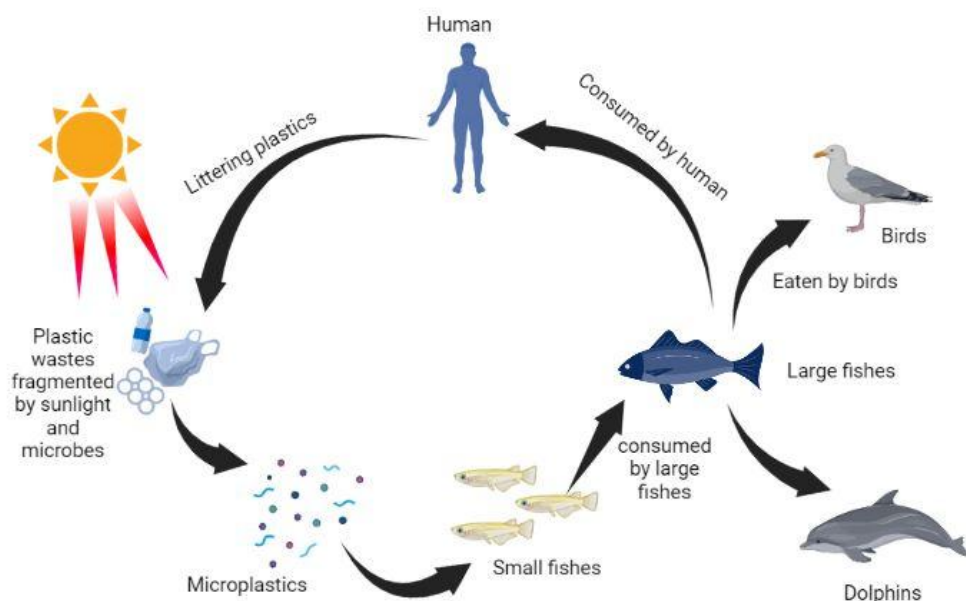


Fig. 1.3. Effect of microplastics in aquatic environment (modified from Issac and Kandasubramanian, 2021)

Since MPs has been found in numerous biotas at various trophic levels, it is clear that MPs has moved through the food chain over time. After entering aquatic habitats, MPs can be transported throughout the food chain, for instance, from small to large plankton or from mussel (*Mytilus edulis*) to green crab (*Carcinus maenas*) (Zhang et al., 2019). According to Kühn et al. (2015), MPs accumulation has increased in numerous marine species from 1997 to 2015. The number of afflicted species has dramatically expanded as a result of MPs accumulation. Nelms et al. (2019) reported that MPs were discovered to be tropically transported from wild mackerel (*Scomber scombrus*) to gray seals (*Halichoerus grypus*). Numerous fish species and other animals that are higher up the food chain have been found to exhibit biomagnification (Bhuyan, 2022). One of the most important components of the marine ecosystem, plankton, is also negatively impacted by MPs. Chlorophyll absorption is decreasing as a result of MPs entering phytoplankton cell walls. When exposed to MPs, heterotrophic plankton that goes through the phagocytosis process keeps this minute plastic pieces in their tissues (Chatterjee and Sharma, 2019). The occurrence of MPs in the digestive tracts of wild gudgeons (*Gobio gobio*) from French rivers showed that continental fish consumed MPs, with MPs contamination found in 12 % of collected fish (Sanchez et al., 2014). More,

the presence of meso-plastics and MPs in freshwater fish from China showed that all 21 sea species and 6 freshwater species of fish contained plastics (Jabeen et al., 2017). Traces or residues of consumption of foamed PS was also found in the stomach contents or feces of a variety of aquatic creatures, such as fish, crustaceans, turtles, birds, and mammals. As floating fragments are quite identical in size and color to conventional food items like fish eggs, larvae, and fish, foamed PS is frequently found in seabird's stomach too (Laist, 1987; Turner, 2020).

1.3.2. Impact on Terrestrial Ecosystems

In aquatic ecosystems, MPs are already seen as a serious issue. However, when compared to effects on aquatic species, understanding of MP's effects on the terrestrial ecosystem is rather limited. The threat of MPs transmission from agricultural land to the human food chain has recently been studied and established. Therefore, MPs should be viewed as a potential danger to sustainable agriculture and food supply (Sarker et al., 2020). MPs in aquatic habitats have been thoroughly studied, but research in terrestrial habitats has just begun to pick up the pace (He et al., 2020). Continuous inputs and fragmentation of massive plastic litters, the most of which are thought to come from terrestrial sources, have contributed to the abundance of MPs in oceans. Terrestrial domain like land is more prone to plastic contamination than oceans. The annual intake of MPs from sewage sludge/biosolids applied to agricultural areas is estimated to surpass the total quantity of MPs presently floating in the oceans (Wang et al., 2020). Since, soil microbial communities are the primary drivers of carbon processing, nitrogen cycling, and other biogeochemical processes vital to human life. Alteration in soil structures *viz.* water-holding capacity, water stable aggregates, bulk density, pore volume etc. are linked to the presence of MPs and may also have a direct impact on such processes. These modifications in physicochemical characteristics rely on the polymer, concentration, and shape of the MPs (Baho et al., 2021). The potential for MPs to disrupt important soil–water connections and the effects on soil structure and microbial activity were studied and showed that MPs had an impact on the water holding capacity, bulk density, and a link between water stable aggregates and microbial activity. Moreover, MPs were considered as significant anthropogenic stressors and key agents of climate change in terrestrial environment (de Souza

Machado et al., 2018). MPs are integrated into the soil by numerous physicochemical interactions, which impact growth of plants and soil bioactivity (Sarker et al., 2020). MPs pollution of soils may affect the biophysical and geochemical conditions as well as the characteristics of the soil. This may also have a significant impact on the structural and functional variety of soil microbial communities, perhaps leading to severe soil environmental issues (Khalid et al., 2020; Rillig and Bonkowski, 2018). When the Mung bean (*Vigna radiata*) was directly exposed to soil polluted with nano-plastics, Chae and An (2020) noticed 83.3 % decline in root development at a concentration of 100 mg/kg nano-plastics in the soil. Additionally, a build-up of MP particles in Mung bean leaves was noted. The African giant snail (*Achatina fulica*), which consumed the tainted Mung bean plant, was thereafter adversely affected in terms of growth rate. MPs have the ability to alter soil structure and characteristics, as well as plant performance. Several studies have confirmed that MPs delay seed germination, limit plant development, and cause genetic toxicity and ecotoxicity in plants, among other things, depending on the kind and quantity of MPs (Ge et al., 2021). Recently, a hydroponics experiment studied the aggregation and transfer of MPs in rice seedlings and showed that rice seedlings can aggregate MPs in their roots and relocate them to aerial parts, possibly transporting the amassed MPs to herbivores if feeds on such MPs loaded plant parts (Liu et al., 2022). Figure 1.4 shows the effect of microplastics to plants and organisms that live in soil.

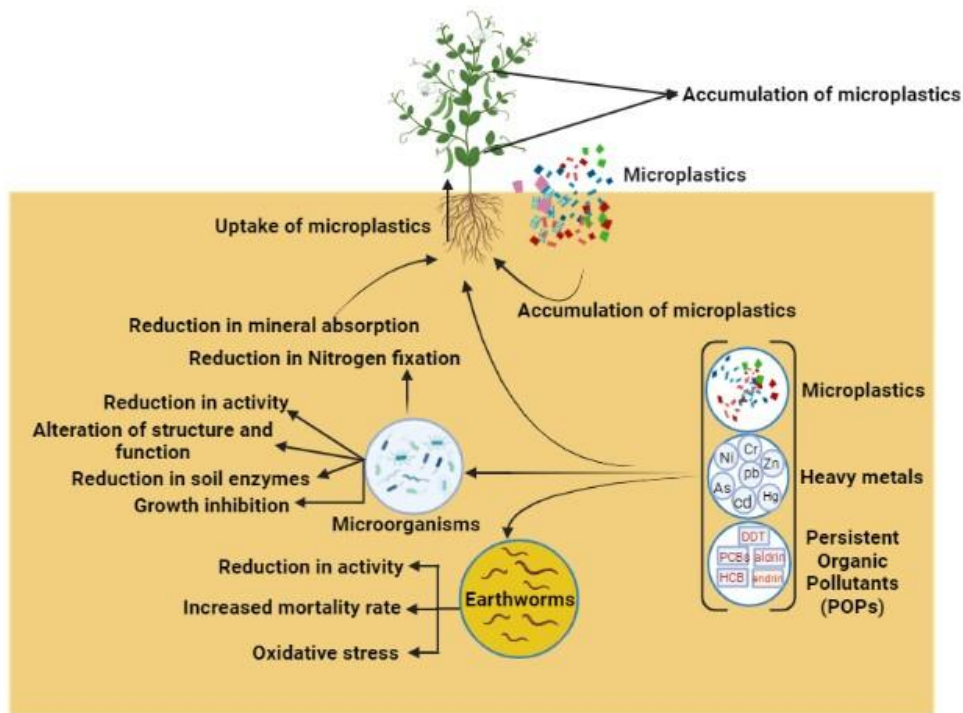


Fig. 1.4. Effect of microplastics to plants and organisms that live in soil (modified from Khalid et al., 2020)

1.3.3. Impact on Aerial Ecosystems

In addition to being found in water and soil, MPs have also been found in the air, which is often overlooked in many studies yet significant source of inhalation and oral exposure (Sridharan et al., 2021). MPs have been found in soils, densely inhabited areas, and many other locations all over the world, with the Antarctic and Arctic regions raising the most concern (Shao et al., 2022). Atmospheric transport may also play a crucial role in the spread of MPs to pristine habitats around the world (Sridharan et al., 2021). Furthermore, MPs were also discovered in Arctic snow, indicating considerable atmospheric contamination, showing that aerial transport plays a big part in the dispersal of MPs (Bergmann et al., 2019). Figure 1.5 schematizes the airborne MPs being deposited into global biosphere. In rainwater and air samples taken from 11 remote and protected sites in the Western US, the presence of MPs was also reported (Brahney et al., 2020). The data showed that more than 1000 tonnes of MPs enter these areas annually. However, compared to rainfall, snow MPs tend to be huge and more varied in their composition and shape. This may be because snowflakes are larger and have slower

terminal velocities than raindrops. It is therefore imperative to pay more attention to the substantial role that snowfall plays in the transference of MPs from the atmosphere to the soil and surface water (Abbasi et al., 2022).

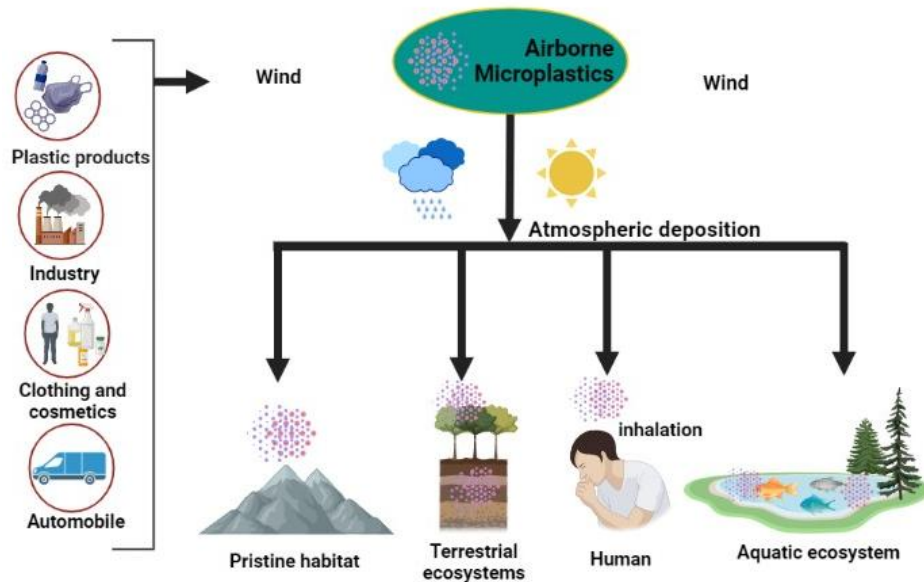


Fig. 1.5. Schematic representation of airborne micro plastics being deposited into global biosphere (modified from Shao et al., 2022)

MPs were reported in Arctic polar waters for the first time by Lusher et al. (2015). Most of the MPs discovered were fibers. The sources and routes by which MPs entered the Arctic are still unknown. Although it is probable that the northeast Atlantic and Norway's prevailing winds and surface water transport have an impact on the quantity of MPs that enter Arctic waters. More recently, the first evidence of MPs in Antarctic snow was documented by Aves et al. (2022). The most typical morphotype observed was a fiber, and PET was the most typical polymer discovered. The study also noted that MPs could also be transferred over extended distances by ocean currents, exchanged from the ocean to the atmosphere during evaporation, and transported over both short and long distances by the atmosphere. Airborne MPs are typically generated when bigger plastics are broken down by UV light, but they can also come from clothing and personal care items (Lett et al., 2021). Since they are microscopic and have a low density, these particles are easily swept into the atmosphere by air currents after degrading (Revell et al., 2021). The wind-driven dispersion of dried sludge by-products applied to agricultural soils

may also cause MPs to be released into the atmosphere. Synthetic fibers, microbeads, and particles have been identified in the wastewater treatment facilities' sludge and became airborne (Amato-Lourenco et al., 2020). Abrasions of car tyres are a main source of airborne MPs along with paint and abrasive cleaning agents (Verschoor, 2018). An important way that humans are susceptible to aerial MPs is by inhalation, and dust is a key inhalation medium. When inhaled, aerial MPs have the potential to cause a variety of ailments in the human cardiovascular and respiratory systems, as well as cancer (Sridharan et al., 2021). The immediate bronchial reactions (asthma-like) that occur when particles are inhaled are the first signs of toxicity, and the detrimental impacts of particle toxicity are primarily associated to inflammation caused by particle localization and immune cells that produce proteases, reactive oxygen species, and cytokines to fight the foreign substance. In addition, persistent inflammation may result in cancer (Enyoh et al., 2019). Prata (2018) has discussed occupational diseases in workers from a variety of industries that is caused by air borne MPs. Chronic bronchitis, extrinsic allergic alveolitis, chronic pneumonia, pneumothorax, dyspnea, and cancer are among these illnesses. However, a need for additional research is deemed necessary to completely comprehend the above-mentioned occupational diseases and establish safe exposure limits.

1.3.4. Impact on Human Biological System

There are significant environmental issues as an outcome of the massive quantities of waste plastic that have accumulated in the environment. Studies have revealed that these plastic wastes continue to harm wild animals' health and may be a concern to people's health as they are potential pathogen carriers (Sang et al., 2021). The size of plastic waste is a significant consideration not only for ingestion by organisms but also primarily for its ability to pass through biological barriers, which has an array of impacts at the cellular level (Binelli et al., 2020). MPs penetrate the human body by the intake of food containing them, inhalation of airborne MPs, and skin contact with these particles found in goods, fabrics, or dust. Microorganisms like *Vibrio* spp. may also colonize the surface of MPs. MPs might serve as vectors in this situation, bringing pathogens to the tissues, shielding them from the immune system, and inducing tissue injury that might encourage infection

(Prata et al., 2020). Figure 1.6 shows a schematic diagram of exposure route of MPs to human body and its associated effects.

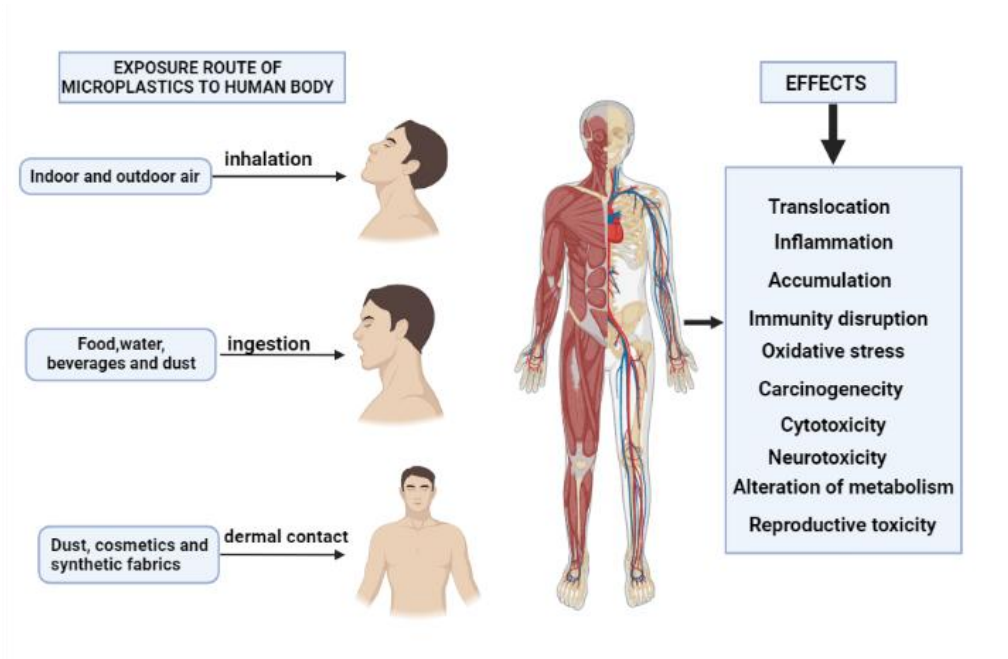


Fig. 1.6. Exposure route of microplastics to human body and its associated effects (modified from Ageel et al., 2022; Bhuyan, 2022)

MPs may be absorbed and consumed by species at the primary trophic level, such as zooplankton and phytoplankton, providing a possible route into the food chain (Lusher, 2015). The marine food web diversity in aquatic animals, such as turtles, seabirds, fish, crabs, and worms, have been documented for the ingestion of MPs. Several studies have shown that MPs accumulate in the guts of organisms, which are usually not directly ingested by humans. However, humans do eat shellfish, including clams, mussels, and some shrimps, along with their gut as a whole (Galloway, 2015). Thus, significant threat to human health is posed by MPs in seafood. There is a great possibility that intestinal MPs contamination will spread throughout the body. The different effects associated with MPs exposure to the body are oxidative stress, cytotoxicity, neurotoxicity, carcinogenicity, reproductive toxicity, immunity disruption, alteration of metabolism, translocation of cells, and inflammation (Bhuyan, 2022). The combined effects of plastics and their composites, often known as endocrine-disrupting compounds (EDCs), are harmful to both human and marine species health. All recent human study subjects had high

concentrations of these metabolites in their urine. Despite detoxifying, minute quantities of EDCs produced from plastic tend to bio-accumulate in bodily tissues (Biamis et al., 2021). MPs in human feces were first discovered by Harvey and Watts (2018). The most prevalent polymers were PP and PET and more than half of the global population is estimated to bear MPs in their feces. In addition, the occurrence of MPs in human placenta was first evidenced by Ragusa et al. (2021). Using Raman micro-spectroscopy, 12 MPs fragments (varying in size from 5 to 10 μm) with irregular shape were discovered in the placentas of four women with physiological pregnancies. More recently, Leslie et al. (2022) discovered and quantified MPs in human blood for the first-time. Baeza-Martínez et al. (2022) also reported the discovery of the first MPs evidence in human lower airways, in the form of microfibers. This discovery has enormous significance as it demonstrates that MPs can move throughout the body and could be lodged in organs.

1.4. Challenges Associated with the Strategies to Mitigate Plastic Pollution

1.4.1. Landfilling

Landfills are one of the simplest and cheapest ways to dispose polymers; however, there is a major issue due to the limited availability of landfill space. This works well with biodegradable polymers (Roohi et al., 2018). Due to the linear rather than cyclical nature of the material flow, landfills have a considerable negative effect from sustainability perspective, where none of the material resources used to make plastics is recovered (Hopewell et al., 2009).

1.4.2. Incineration

When disposed plastics are burned in an incinerator, it leads to the generation of secondary toxic air pollutants, which is known to have detrimental impacts on the environment and human health (Roohi et al., 2018). Hazardous compounds may be emitted into the atmosphere during the process of incineration. For instance, the presence of PVC and halogenated compounds in mixed plastic trash incineration raises the possibility of releasing dioxins, polychlorinated biphenyls and furans into the environment. Moreover, after these hazardous compounds are released, it binds with dust particles and can be transported over great distances by the wind. Therefore, incineration of plastic in context of solid waste-management is preferred lesser than landfilling and mechanical recycling,

primarily due to the detrimental effect on the various components of the environment (Hopewell et al., 2009). Moreover, strict pollution control guidelines imposed by the regulatory authorities have further restricted its wide scale operations, and only plastic incinerating plants for recovering energy are allowed to operate (Raubenheimer and McIlgorm, 2017).

1.4.3. Challenges in recycling processes

Recycling is a significant method found to be effective in reducing plastic waste, but it is projected that approximately 12 % (800 Mt) of plastics were incinerated and only 9 % (600 Mt) of the total (6300 Mt) amount of plastics produced have been recycled in the year between 1950 and 2015. The remaining 79 % (4900 Mt) of plastics has either been dumped in landfills or is still lying in the nature (Geyer et al., 2017). In addition, every reprocessing cycle includes degradation, due to exposure to hot processing temperatures that cause a breakdown of molecules into monomers and oligomers, which may migrate to packaged food products (Dey et al., 2021).

1.4.4. Clear-out activities

In addition to being tools for awareness-raising and citizen science, clean-up initiatives have been suggested as mitigation techniques. However, the pervasive pollution of the environment with plastic particles of all sizes makes it appear difficult to completely eradicate plastic pollution (Prata et al., 2019).

1.4.5. Reducing Plastics Consumption

Food safety has become a growing concern among the people throughout the globe. It has been estimated that approximately 450 people died in UK in the year 2006 because of foodborne diseases. The most convenient way to enhance food safety is through appropriate packaging, which minimizes food contamination. The majority of such food packaging industry depends entirely on single use plastics. Although the strategy of reducing the plastics consumption is beneficial, it is occasionally challenging because of food safety concerns and inconvenience (Beitzen-Heineke et al., 2017).

1.4.6. Using Paper Bags and Jute Bags

Although paper bags have been proposed as plastic bag substitutes, paper bag manufacture uses 40 % more energy than plastic bag production (Schnurr et al.,

2018). Paper bags are reusable and recyclable, but they use more fossil fuels to generate and manufacture than plastic bags because they are made from wood fiber pulp or recycled fibers. Jute bags are well recognized for their resilience and are created from renewable vegetable fibers. Jute bags, however, can pose a greater risk of microbial contamination due to cleaning difficulties, and when combined with chemicals to increase durability, they might raise the danger of environmental contamination when compost (Nwafor and Walker, 2020).

1.4.7. Ban on Single-Use Plastics

One of the typical measures recently implemented by governments of several nations all over the world to reduce the predicament of plastic pollution is to enforce a ban on shopping bags made of single-use plastic (SUP) (Dey et al., 2021). However, an outright ban on plastic bags won't do much to address the fact that a particular nation lacks an efficient environmental management strategy. Such measures, however judicious they may be, will not protect the environment from the negative consequences of a "throw away" mentality of the people that need to be more environmentally educated to become better aware of the extent of the problem and the collective efforts to be undertaken to solve this problem for their own salvation and benefit. Instead, better management rather than a constraint on the usage of thin plastic bags would probably be a better option and a possible solution to the issues they cause (Casanova, 2012). Planning and implementing sustainable low-cost solid waste management strategies is crucial for emerging countries like India. The lack of effective sanitation techniques is mostly caused by lack of awareness, inappropriate technical knowledge, a lack of funding, irresponsibility at all levels of the society, and the implementation and enforcement of law and policy (Rajmohan et al., 2019).

1.5. A Possible Approach to Mitigate Plastic Pollution

A multimodal approach involving prevention, reuse, recycling, recovery, and disposal is necessary to address the world's plastic problem and biodegradable plastics are a crucial and integral part of this strategy (Flury and Narayan, 2021). Single-use plastic will be banned by 2030, according to the European Union's plastic strategy. Therefore, the packaging and distribution sectors were compelled to pioneer the usage of biodegradable polymers as an alternate to traditional plastics

(Loh and Chew, 2021). Polyhydroxyalkanoates (PHAs) are seen as potential substitutes for conventional petrochemical plastics because of their robust mechanical qualities that are comparable to those of thermoplastics derived from petroleum and their high level of biodegradability (Khatami et al., 2021). Additionally, PHAs generation by microbes has drawn notable interest in past few years in the field of medical science due to its biocompatibility properties where it can be implanted in living tissues of human without triggering any immunological responses. Other properties like non-toxicity, insolubility in water, as well as its thermo-plasticity, make it an excellent alternative to conventional plastics (Loh and Chew, 2021).

1.6. Polyhydroxyalkanoates (PHAs)

PHAs are a type of biopolyester and the most well-known are poly-hydroxybutyrate (PHB) and a copolymer of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). PHAs, including PHB, poly-hydroxyvalerate (PHV), poly-4-hydroxybutyrate (P4HB), as well as polyhydroxyhexanoate (PHH) and polyhydroxyoctanoate (PHO) (Ansari and Fatma, 2014) are biodegradable, environmentally benign, and non-toxic (Muneer et al., 2022). General structure of PHAs and some of its polymers is presented in figure 1.7. PHAs are made from lignocellulosic biomass and can be thermoformed into a variety of bio-based products (Reshmy et al., 2021). Both Gram-negative and Gram-positive bacteria (George et al., 2021) produce granules of PHAs, which are used as water-insoluble storage compounds when the environment is stressful. They are produced by biosynthesis, which occurs when there are a surplus of carbon and a limited supply of vital growth nutrients like nitrogen or phosphate (Możejko-Ciesielska and Kiewisz, 2016). According to their structural characteristics, these bacterial polyesters can be broadly separated into two groups: short-chain-length PHAs (scl-PHAs), which have 3-5 carbon atoms, and medium-chain-length PHAs (mcl-PHAs), which have 6-14 carbon atoms (Kim, 2002). However, compared to scl-PHAs production, the process development for mcl-PHAs production has been significantly less extensive (Sun et al., 2007). A few bacterial species can accumulate PHAs up to 90 % of the dry cell weight (George et al., 2021). Olive oil, fermented molasses, date syrup, pomace, effluents from paper mill and palm oil

mill are just a few of the wastes that have been used as carbon source and have accumulated between 40 and 70 % biodegradable PHAs (Abd El-malek et al., 2020). PHAs are a type of biopolymers that are not only ecologically friendly, but it also has a variety of material qualities that can be adjusted. The current market cost of PHAs is roughly €5 per kg, which is over 6 times more expensive than the cost of traditional plastics (€0.8-1.5 per kg). This is primarily due to the high cost of biodegradable polymers, as most techniques are still in the research and development stages (Khatami et al., 2021). In spite of its high manufacture cost, the production capacity of PHAs was 2.11 Mt globally in 2018 and is projected to surpass 2.6 Mt in 2023. With additional cost reductions and the increase in highly improved utilization techniques, it is expected to evolve into a valuable material that is marketable (Sun et al., 2018).

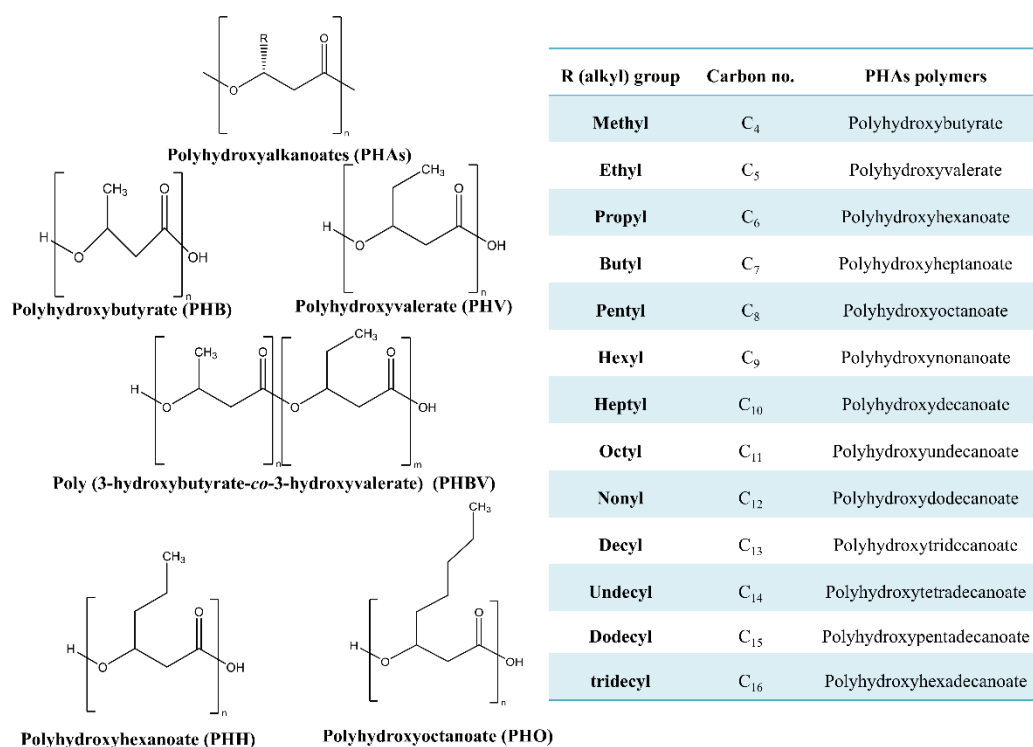


Fig. 1.7. General structure of PHAs and some of its polymers (modified from Akinmulewo and Nwinyi, 2019)

1.7. Polyhydroxybutyrate (PHB)

In 1925, Lemoigne made the first discovery of the linear polyester polyhydroxybutyrate (PHB) in bacteria. The biological synthesis of PHB from non-replenishing sources taking place in both Gram-negative and Gram-positive bacteria under the circumstance of nutritional imbalance is the most significant factor in its production (Rajan et al., 2019). PHB have a linear chain structure and both crystalline and amorphous phases, which results in its high degree of crystallinity. It is available either as a pure polymer or as a component of copolymers and blends. In a varied range of bacterial strains, it is produced as a carbon reserve and is then manufactured industrially through bacterial fermentation (McAdam et al., 2020). In their cells, bacterial species collect intracellular PHB granules as a source of carbon and energy. The role of carbon-rich culture media in PHB synthesis is crucial. Carbon is used by micro-organisms for both energy production and biosynthesis. For microbial growth and the production of PHB, an appropriate carbon supply is essential. Simple sugars like glucose, fructose, mannose, galactose, sucrose, and xylose, as well as polysaccharides like starch and lipids like oleates and glycerides, are examples of sources of defined carbon. Nitrogen is also a key component in fermentation media as a vital component of proteins, nucleic acids, and co-enzymes like vitamins (Allikian et al., 2019). Among the inexpensive carbon sources are molasses, maize steep liquor, starch, wheat bran, among others (Verlinden et al., 2007). PHB are a member of the PHAs family and are distinguished by an ester linkage group ($-\text{COOR}$) and methyl functional group ($-\text{CH}_3$); these functional groups are also responsible for the material's hydrophobic, high crystallinity, thermoplastic, and brittle properties. PHB and its by-products, for instance, have two major temperatures that define their thermal properties *i.e.* a melting temperature (T_m) for their crystalline phase and a glass transition temperature (T_g) for their amorphous phase (Grigore et al., 2019). PHB are an excellent alternative for synthetic polymers and have mechanical qualities similar to polypropylene (Verlinden et al., 2007).

The biochemical mechanism by which PHB are produced has three phases. Firstly, to form acetoacetyl-coenzyme A (CoA), two molecules of acetyl-CoA are linked utilizing 3-ketothiolase (PhaA). Secondly, utilizing the enzyme acetoacetyl-CoA reductase (PhaB), 3-hydroxybutyryl-CoA is generated by reducing

acetoacetyl-CoA by nicotinamide adenine dinucleotide (NAD). Finally, employing PHB synthase (PhaC), PHB is polymerized from 3-hydroxybutyryl-CoA, releasing CoA (Ross et al., 2017). The biochemical mechanism for PHB production is displayed in figure 1.8.

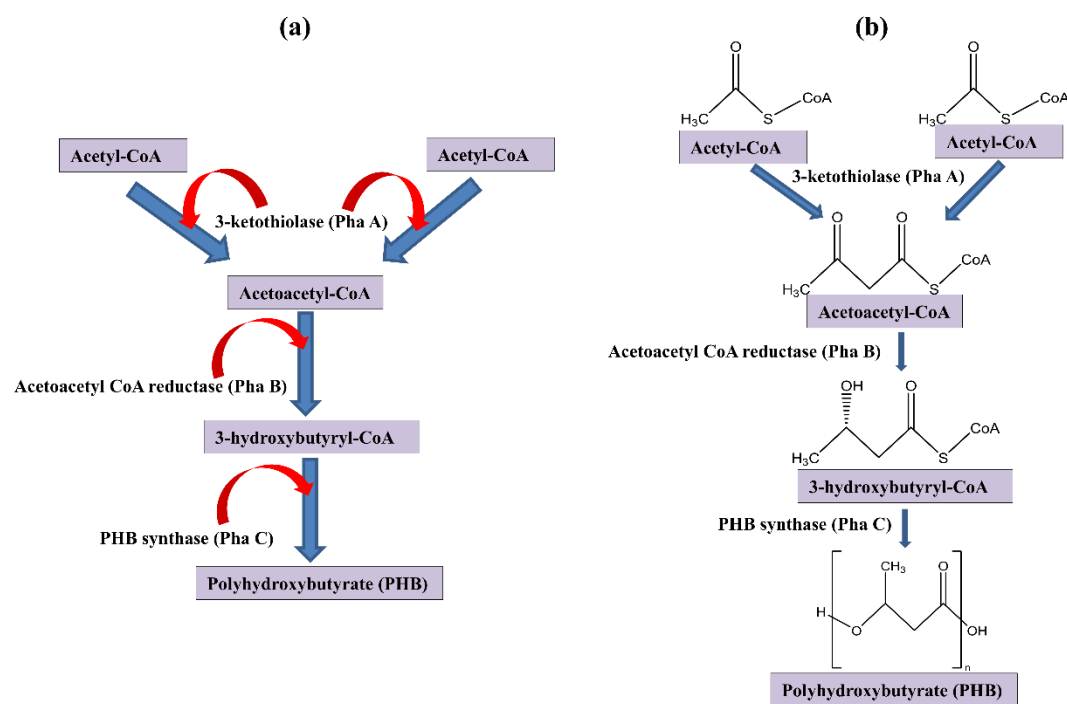


Fig. 1.8. Schematic diagram showing (a) metabolic pathway of PHB production and (b) chemical structure of intermediates and final products (modified from Ross et al., 2017)

A wide range of bacteria are the most researched, accepted, and renowned species in the bioplastics manufacturing process. PHAs granules are used by many bacterial species as a source of carbon stores and energy in their cells (Thiruchelvi et al., 2020). The microorganisms such as *Bacillus cereus* (Narayanan et al., 2020a), *Cupriavidus necator* (Aramvash et al., 2015), *Pseudomonas aeruginosa* (Javed and Jamil, 2015), *Azospirillum rubrum* (Jin and Nikolau, 2014), *Brevundimonas* spp., and *Enterococcus* spp. from cardboard industry waste water (Bhuwal et al., 2013), *Burkholderia cepacia* from biodiesel-glycerol (Zhu et al., 2010), *Pseudomonas putida* from vegetable oil wastes (Gatea et al., 2018), *Bacillus megaterium* (Joyline and Aruna, 2019), *Bacillus siamensis*, *Bacillus subtilis*, *Staphylococcus aureus* (Vijay and Tarika, 2019), *Paraburkholderia* spp. (Sriyapai

et al., 2022), *Methylocystis* spp. and *Rhizobium* spp. (Abd El-malek et al., 2020; Snell and Peoples, 2009), *Aeromonas hydrophila*, *Burkholderia sacchari*, *Acinetobacter* spp., *Halomonas boliviensis*, *Sphingobacterium* spp., *Caulobacter* spp., *Brochothrix* spp., *Ralstonia* spp., and *Yokenella* spp. (Mascarenhas and Aruna, 2017) are capable of producing PHB. These microorganisms utilize various substrates for the production of PHAs. For instance, different forms of PHAs can be created from a wide range of sustainable sources, comprising fatty acids, agricultural wastes, and by-products from industries. Furthermore, the market price of commonly utilized substrates, namely oils, pure sugars and fatty acids, accounts for half of the entire cost of PHAs production. Therefore, a number of research works has been done in order to incorporate low-cost carbon-rich substrates like agro industrial wastes (sugarcane bagasse, rice bran, wheat bran, wheat straw, and corn cob) for the production of PHAs (Abd El-malek et al., 2020). Tsang et al. (2019) reviewed on current technology for the synthesis of PHA-based bioplastics from food waste (FW). When FW is landfilled, it causes harmful effects such as groundwater contamination and emission of greenhouse gases. Therefore, production of PHA-based bioplastics seems to be an ideal method for FW disposal, and its production from FW is a renewable and sustainable method that uses resources that are carbon neutral. Nevertheless, only a few bacterial strains have so far been employed effectively on an industrial scale to produce PHAs due to high production cost (Możejko-Ciesielska and Kiewisz, 2016). To determine the efficacy of PHAs production, Mohapatra et al. (2017) have isolated and screened different *Bacillus* species. They found that under optimized condition (glucose concentration of 10 g/L, temperature 37 °C for 48 h, C:N ratio 6:1, pH 7) for microbial growth, the *Bacillus* species produced 3.09 g/L of PHAs, which was believed to be a greater yield than *Paenibacillus durus* (Hungund et al., 2013) that accumulated 0.9 g/L of PHAs using fructose as carbon source. To produce an eco-friendly biodegradable polymer of PHAs, Tanamool et al. (2013) used sweet Sorghum juice as carbon source. The study revealed that *Bacillus aryabhattai* had the highest PHAs accumulation of 1.74 g/L with 57.62 % of DCW. A study was conducted by Narayanan et al. (2020a) to identify the most common bacteria that produce PHB

in polluted lake soil samples and found that *Bacillus cereus* strain NDRMN001 has the ability to produce 91.54 % of PHB from 36.26 g/L culture biomass.

1.8. Properties of PHB

1.8.1. Physico-Mechanical Properties

PHB are a homopolymer with highly crystalline, rigid, but brittle material. It performs as a hard-elastic substance when spun into fibers. Copolymers with the majority of PHBs' other mechanical qualities, like PHBV or mcl-PHAs, are less brittle and stiff than PHB. The crystalline helical structure of homopolymer PHB appears to be shared by a number of different copolymers (Verlinden et al., 2007). Along with molecular weight, the length of the side chain, the kind of monomer, and the separation between the ester bond and the functional group all affect the mechanical properties of PHAs. The interaction of these variables can alter the glass transition (T_g) and melting (T_m) temperatures of polymers as well as their crystallinity (stiffness/flexibility) (Guimarães et al., 2022). PHAs with medium chain length (mcl) are elastic and flexible; they have high elongation at break ratios, moderate tensile strength, and low crystallinity. mcl-PHAs polymers exhibit higher elongation-to-break ratios, lower T_g temperatures, and lower T_m temperatures than scl-PHAs polymers (Sharma et al., 2017). PHB material's mechanical characteristics, such as its tensile strength (43 MPa) and Young's modulus (3.5 GPa), are comparable to those of isotactic poly propylene's tensile strength (38 MPa) and Young's modulus (1.7 GPa). Nevertheless, PHBs' extension to break (5 %) is significantly lesser than polypropylene's (400 %). In comparison to polypropylene, PHB looks to be a harder and more brittle material (Sudesh et al., 2000). PHB grow into incredibly thin lamellar crystals that are arranged as spherulites when it is produced from the melt or oblong lath-like single crystals in dilute solution. Lamellar thicknesses in PHB spherulites are normally in the range of 5 nm, though they can be noticeably smaller in crystals produced from solutions (Barham et al., 1984; Van der Walle et al., 2001).

1.8.2. Thermal Properties

At temperatures near its melting point, which is 160-180 °C, PHB exhibits severe instability. It is a thermoplastic polymer having a T_m in the range of 160-180 °C and a T_g of 0-5 °C. Around its T_m , it starts to degrade thermally. The melting

points of PHB vary based on the carbon source. Due to its semi-crystalline nature, PHB exhibits a melting behavior between crystalline and pure amorphous materials (Sreedevi et al., 2015). At temperatures exceeding 170 °C, there are reports of alterations in molecular weight and the generation of crotonic acid (Van der Walle et al., 2001). After being held at 190 °C for 1 h, a sample of PHB was found to lose nearly half of its initial molecular weight (Barham et al., 1984).

1.9. Applications of PHAs

Biopolymers such as PHAs are biodegradable and can be made without fossil fuels because they are mostly made from biomass, implying possible carbon neutrality. As a result, there has been a surge in the use of these materials in the form of blends or composites with degradable or non-degradable synthetic polymers (Nandakumar et al., 2021). PHAs are an essential source of packaging material because of qualities including thermoplasticity, hydrophobicity, insulation, and vapor barrier. PHA-derived jars, disposable cups, trays, containers, and foam-based packaging utensils are already available in the food industry (Rekhi et al., 2022). PHAs have various medical utilizations such as drug carrier, tissue engineering, heart valves, surgical sutures, medical implants, artificial skin, artificial organ reconstruction, chemotherapeutics, antibacterial, anti-cancer agents, memory boosters, and biocontrol agents in aquaculture (Ray and Kalia, 2017). PHAs have been presented as a possible medical device material since it is biocompatible with human bones and tissues, biodegradable, and has high mechanical properties. Articular cartilage restoration, cardiac patch fabrication, meniscal repair devices, adhesions barriers, repair patches, orthopaedic pins, tacks and rivets, screws and staples, stents, suture fastener, surgical mesh and other devices are among the most potential devices (Ray and Kalia, 2017; Kalia et al., 2021). Furthermore, when utilized *in-vivo*, PHAs have no acute or long-term negative impacts on health (Raza et al., 2018). PHAs are also employed in agriculture for a variety of purposes, including seed and fertilizer encapsulation, crop protection films, and biodegradable pesticide and insecticide carriers (George et al., 2021). Shade nets, clips, geotextiles, wires, and pheromone dispensers are examples of products used in agriculture to protect plants from severe environmental conditions (Kalia et al., 2021). Many farmers cover their

fields with plastic blankets to boost product production. Traditional polymers leave traces, whereas biodegradable plastic made from natural materials degrades completely. Biodegradable mulch film can be ploughed back into the field after its use, reducing labour and disposal cost (Rujnić-Sokele and Pilipović, 2017; Venkatachalam and Palaniswamy, 2020).

The automotive sector is pursuing sustainability efforts, such as the use of biopolymers. Toyota claims to be the first to utilize sugarcane-based PET in car liners as well as some interior surfaces. In one model, Mazda motors claim an interior fittings content of more than 80 % plant-derived content, as well as seat covers made entirely of plant-derived biofabric (Lackner, 2000). PHB, one of the PHAs, may prove to be a highly environment-friendly material for the automotive interior components (Mostafa et al., 2020). Furthermore, technological advancements have assured that the spectrum of biopolymers applications continues to expand, currently including keyboard parts, mobile phone coverings, microchips and ultracapacitors (Rekhi et al., 2022), and automobile components (Industry Experts, 2012). The applications of PHAs biopolymers are presented in figure 1.9.

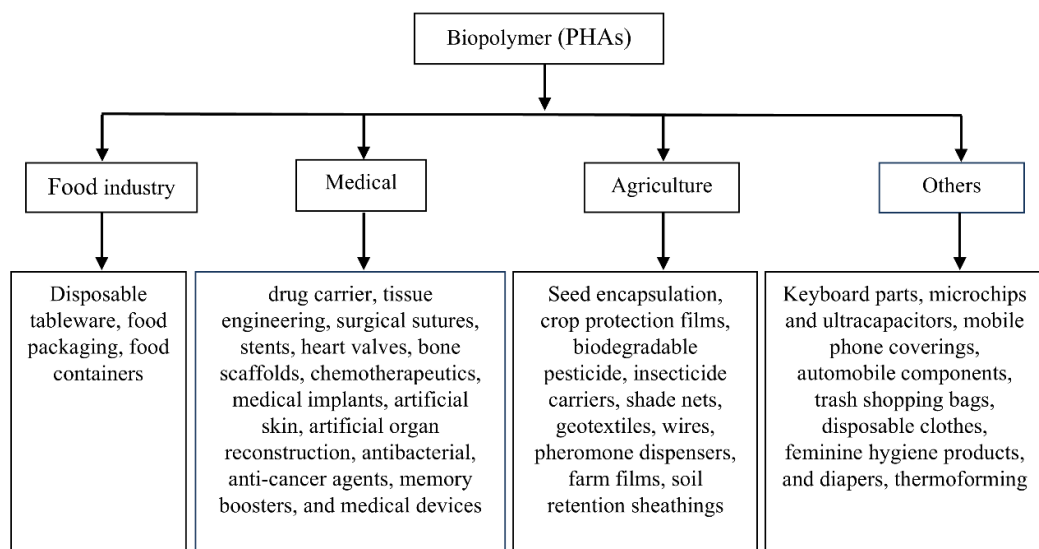


Fig. 1.9. Applications of PHAs biopolymers (modified from Abd El-malek et al., 2020)

1.10. Advantages of PHAs

In comparison to conventional plastics made from petrochemicals, PHAs particularly PHB have various benefits. PHB are completely biodegradable and are made from renewable resources. It is entirely non-toxic and has better physical qualities for food packaging applications than polypropylene (Ansari and Fatma, 2014). Green or biodegradable plastics provide a number of advantages over petroleum-based plastics, including non-toxic chemicals, ease of recycling, reduced use of fossil fuels, lower energy requirements, and are renewable and environmentally safe (Thiruchelvi et al., 2020). Another advantage of bioplastics' biodegradability is that the end products can be recycled as raw materials, removing the requirement for virgin raw materials and providing the product with several end-of-life alternatives (Nandakumar et al., 2021). The various advantages of PHAs are discussed below.

1.10.1. Biodegradability

PHAs are considered to be the most important biopolymers because of their high biodegradation efficiency, which is essential for their replacement of traditional plastics (Robledo-Ortíz et al., 2021). Biodegradation can take place in both anaerobic and aerobic environments when microorganisms like bacteria, fungi, or actinomycetes are present (Lammi et al., 2019). The final products under aerobic conditions are biomass, carbon dioxide (CO₂), and water, whereas the final products under anaerobic conditions are biomass, CO₂, methane (CH₄), and water (Gu, 2003). These products are not hazardous to the environment, and CO₂ and CH₄ in particular could be employed in power plants to produce electricity (Kuciel et al., 2019). During anabolic processes *viz.* photosynthesis, the by-products are further reabsorbed by microorganisms and plants, serving as a feedstock for the subsequent synthesis of PHAs. PHAs are a component of the biosphere's nutrient cycle as a result of this property (Bhola et al., 2021). The rate and time of biodegradation can be influenced by the polymer's crystallinity, surface morphology, side-chain length, and shape, as well as environmental factors like temperature, UV exposure, nutrition levels, mechanical force strength, types of bacteria present, pH, and oxygen levels. The average rate of PHAs biodegradation in the marine environment was found to be 0.04–0.09 mg/day/cm², indicating that it would take between 1.5 to 3.5 years for a PHAs water bottle to entirely decompose (Dilkes Hoffman et al.,

2019). In contrast, a conventional plastic takes about 500 to 1000 years to degrade in marine environment (Whiting, 2018). However, they do not decompose completely; instead, they photo-degrade and turn into tiny microplastics or nanoplastic, polluting various the environmental matrices (Zhang et al., 2021).

1.10.2. Biocompatibility

PHAs, particularly PHB are biocompatible with human bones and tissues; therefore, it can be inserted into the human body without triggering an inflammatory reaction. It can be employed as a carrier for the gradual release of drugs since it degrades gradually within the human body (Nair and Laurencin, 2007). The biocompatibility of PHB is also possible to assess in the context of tissue engineering, and this quality can be improved by sodium hydroxide and lipase treatment (Zhao et al., 2002). By amalgamation with other polymers, altering the surface, or integrating PHAs with different inorganic materials, can further improve PHAs mechanical and biocompatibility qualities, making them acceptable for a broader range of applications (Sreedevi et al., 2015).

1.10.3. Natural Feedstock

Biopolymers are made from substances that are naturally occurring and can be produced by a variety of metabolic processes in living cells, including microbes and plants, that involve chemical and thermo-chemical polymerization (Haddadi et al., 2019). Currently, using inexpensive raw materials are given some thought to reduce PHB cost. Biomasses from maintaining green spaces, wastes, and by-products of industry, including glycerol, sugarcane bagasse, and lignocellulosic biomass from agricultural or forest residues, and dairy wastes and food industry waste (Sirohi et al., 2020) are some of these potential substrates. The main benefit of such approaches is that, it turns wastes into products with added value (Angelini et al., 2015).

1.10.4. Sustainable Bioeconomy

If sustainability requirements are incorporated into the process and product design, PHAs can be desirable substance for a sustainable bioeconomy. The utilization of lignocellulosic biomass as a feedstock has potential for sustainable PHAs production because it naturally occurs as waste and can therefore reduce

feedstock greenhouse gas emissions, land use, and other feedstock-associated effects like water use and fertilizer (Dietrich et al., 2017).

1.11. Limitations of PHAs

PHAs, primarily PHB are a unique type of hydrophobic polymer with a high melting point and crystallinity that is genuinely biocompatible and biodegradable. However, for some applications, its strength and a few additional qualities, like thermal stability, solvent resistance, gas permeability, and flame retardance, are insufficient for end use (Maiti et al., 2007). The thermal instability of PHB, which causes its mass to fall at 170-185 °C by a random scission mechanism, is one of its most significant drawbacks. It has a relatively small processing window because its melting point, which is about 175 °C, is just a little below its degradation temperature, which starts at 185 °C (Robledo Ortiz et al., 2021). Moreover, PHB has very poor mechanical qualities with a low elongation at break as a result of its high crystallinity, which restricts the variety of its applications (Ansari and Fatma, 2014). Therefore, to enhance the thermal, physical, and mechanical properties of these materials, it is crucial to produce blends of components from natural sources, synthetic biodegradable polymers, and/or non-biodegradable polymers (Moreira et al., 2022). Blending, coating, adding nanoparticles, adding cellulose, chemical/physical alteration, are some of the options (Jabeen et al., 2015). PHAs are substantially more costly than conventional polymers and other bio-based biodegradable plastics, despite the fact that some PHAs improve ductility as their chain length rises (Meereboer et al., 2020). Furthermore, maintaining best bacterial growth conditions is one of the main hindrances to the industrial output of PHAs. At the completion of the cultivation of bacteria, the majority of fermentation techniques do not provide the maximum synthesis of PHAs granules (Sharma et al., 2021). Due to limitations in production techniques, the cost of bio-based polymers has historically been a significant barrier in commercial use. The emergence of microbial synthesis pathways and the accessibility of inexpensive carbon-rich precursors have made it easier to produce PHAs at a reasonable price for usage in medical and other commercial applications. However, the drawback of mixed cultures is that they are less expensive but produce poor yields and productivity in terms of volume. Moreover, consumer purchasing habits and attitudes toward

sustainable products also have an impact on the cost and competition issues (Maraveas, 2020). Due to the fact that consumers focus their purchasing decisions on a product's value in relation to its price, bio-based polymers are still currently unable to compete with traditional plastics on the commercial market (Kapferer and Laurent, 2016; Maraveas, 2020).

1.12. Current Scenario of PHAs

The current predicament created by huge quantity of non-biodegradable wastes in the environment has led to spurring research into novel biodegradable materials build from natural resources like biomass, plants, and microorganisms. MPs being an emerging pollutant in both marine and terrestrial environment, it is necessary to reduce petrochemical based synthetic plastic application and opt for an eco-benign substitute such as bioplastics. The process of landfills needs a lot of space, an uninterrupted energy supply, and billions of dollars to continue operating in order to fix an issue that shouldn't have existed in the first place. The marine environment has already been irreparably damaged by ocean dumping (Bhola et al., 2021). A new plastic economy paradigm is required to reduce plastic waste from packaging. This idea of a circular economy includes bio-based and biodegradable polymers (Flury and Narayan, 2021). Bioplastics can help in solving environmental issues such as unregulated garbage dumping on land and disposal into the sea, as well as the hazardous compounds released as a result. However, to maximize the benefits of bioplastics, appropriate collection, sorting, and recycling processes, as well as public awareness, are required (Venkatachalam and Palaniswamy, 2020). This chapter gives a brief overview of PHAs, which have now gained interest for being prospective substitutes for traditional plastics because of their biological origin, biodegradability, and biocompatibility, and subsequently mitigating MPs pollution. Although bioplastic production would reduce fossil fuel consumption, CO₂ emissions, and plastic waste generation; the high cost of producing bioplastic from bacteria remains a key restriction when compared to the cost of producing petroleum-derived polymers (Ali et al., 2017). However, as bioengineering techniques are progressively improving, they have the ability to manufacture effective, high-performance high-yield PHB, even from waste stream resources, lowering costs and producing PHAs materials at competitive prices with the

necessary mechanical qualities to open up a wide range of advanced applications and market prospects (McAdam et al., 2020). Their range of applications will expand as production costs are brought reduced, culminating in a competitive pricing for these biodegradable polymers (Kavitha et al., 2018). Petroleum derived plastic is expected to be completely substituted once PHAs is more economically viable. This will give an opportunity for technological advancement without worrying too much about dumping and will benefit the economy in the sectors where petroleum-derived plastic is currently used (Bhola et al., 2021). In this regard, research on manufacturing of PHAs particularly PHB from low-cost raw materials, such as waste and by-products, is ongoing. To lower synthesis costs and create efficient recovery and downstream processing techniques for PHAs, as well as to increase their commercialization, significant research and development is still required (Keshavarz and Roy, 2010). The use of biodegradable plastics to reduce pollution is a discussed benefit; nevertheless, substituting conventional plastics with bioplastics is not the most efficient solution; rather, the problem still resides in throw away attitude. To solve the problem, eco-friendly mentality, strict rules, and an awareness programme must be carefully designed and implemented, and sustainable resources must be used. As the bioplastics area develops, much more emphasis should be paid on both production and disposal processes.

Research Objectives

The ever-growing demand for plastic is resulting in the exhaustion of natural resources like petroleum, besides the accumulation of plastic waste in the environment which leads to various types of pollutions. Non-degradable plastics or conventional plastics are extremely harmful to the environment, human and animal health, therefore biodegradable bioplastics are the need of the hour. PHB have gained popularity as an environmentally benign polymer for industrial applications due to its unique features such as biodegradability, thermoplasticity, and biocompatibility. Currently, the fabrication of PHB blends and composites is receiving a lot of interest. Blended PHB have numerous uses, and we can successfully use them together with their co-polymers and other biopolymers, to enhance its properties for specific applications.

With this in mind, the present study was undertaken with the following objectives:

- i. Isolation of PHB producing bacteria from natural environment.
- ii. Morphological characterization and molecular identification of the PHB producing isolate.
- iii. Physicochemical characterization of the PHB produced by the selected bacterial strain.
- iv. Optimization of PHB production media by Response surface methodology (RSM).
- v. Development and characterization of PHB and its composites and their possible application in removal of environmental pollutants, and active packaging.

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CHAPTER-2

**Polyhydroxybutyrate (PHB)-Based Sustainable Bioplastic derived
from *Bacillus* sp. KE4 isolated from kitchen waste effluent**

A part of this chapter has been published in:

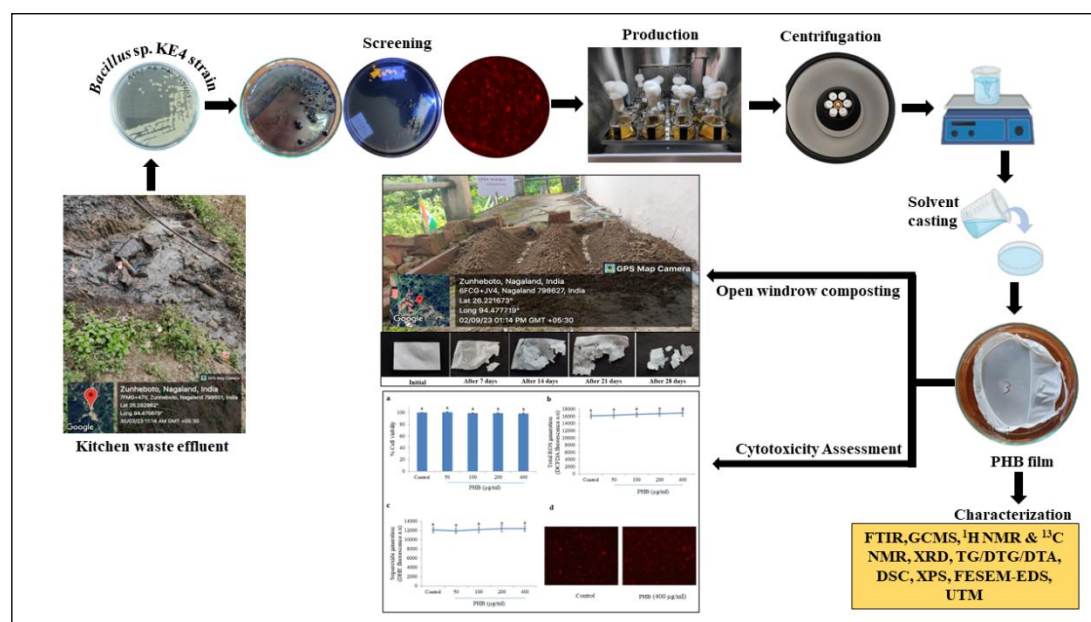
Acharjee, S. A., Bharali, P., Ramachandran, D., Kanagasabai, V., Hazarika, S., Vishawkarma, V. (2024). Polyhydroxybutyrate (PHB)-Based sustainable bioplastic derived from *Bacillus* sp. KE4 isolated from kitchen waste effluent. *Sustainable Chemistry and Pharmacy*, 39, 101507.

2.1. Abstract

Petrochemical-derived plastics pose a major threat to the biosphere and the environment due to their acute or long-term toxicity. Therefore, to ensure sustainability, it is essential to find an effective replacement for synthetic plastics with natural and ecofriendly biomaterial. In this context, production of biopolymeric material namely polyhydroxy-butyrates (PHB) from natural source could be an appropriate alternative. In the present study PHB was produced by the *Bacillus* sp. KE4 using glucose as the carbon source, and a maximum dry cell weight (DCW) of 4.4 g/L with a PHB concentration of 3.1 g/L, PHB yield of 0.15 g/g was obtained, and the PHB content was 70.4 % of the DCW. The extracted biopolymer was characterized using FTIR, GC-MS, ^1H NMR, and ^{13}C NMR which confirmed the structure of the biopolymer as PHB. The findings of TGA, DTG, DTA and DSC studies corresponded to PHB's excellent thermal stability. PHB biopolymer has a melting temperature of 168.7 °C and a maximum degradation temperature of 273 °C. The weight average molar mass (M_w), number average molar mass (M_n), and polydispersity index (PDI) of the extracted PHB was 1,55,436 g/mol; 1,43,258 g/mol; and 1.08, respectively. The XRD analysis validated the material's partly crystalline nature. FESEM-EDS and XPS were employed to determine the elemental composition of the purified PHB film. The Young's modulus, tensile strength and elongation at break showed rigid and brittle nature of PHB. Using open windrow composting method, the extracted PHB biopolymer film was assessed to determine its biodegradability. Within 28 days of soil burial, it was found that the biopolymer film had degraded by 61.34 %. Cytotoxicity assessment in HepG2 cells indicated that PHB is non-cytotoxic in nature. The results have showed that the biomaterial is environmentally benign,

biodegradable, and biocompatible; therefore, it may find its diverse application covering packaging, medicine, agriculture and allied fields.

Keywords: Polyhydroxybutyrate (PHB), *Bacillus* sp. KE4, Biopolymer, Biodegradable, Biocompatible



2.2. Introduction

The amount of non-degradable plastic products has surged in recent decades due to the rapid population growth. Evidently, due to the COVID-19 pandemic, the world has witnessed a massive increase in plastic pollution as a result of the overflow of medical waste, the need for appropriate personal protective equipment (PPE), masks, hand sanitizer bottles, the growth in online shopping and food delivery, the ban on reusable bags, and other factors (Shams et al., 2021). Globally, the amount of plastic produced has surged from 60 million metric tonnes in 1980 to 368 million tonnes (Mt) in 2019 and is projected to surpass 600 Mt in 2025. In the next 20 years, global production of plastics is anticipated to double if it keeps growing at the current pace (Tyagi et al., 2021). Petroleum-derived plastics linger in the environment for an extremely long time and eventually they degrade into micro- and nano-plastics, which are tiny fibres or particles that are often hazardous to living organisms and have a substantial impact on the quality of the soil, microbial activity, fauna, and flora. In addition, this poses a threat to human health since it gets into the food chain (Pellini et al., 2018). Microplastics (MPs) are

readily consumed by zooplanktons, fish, crustaceans and molluscs. Once ingested by zooplanktons, these pollutants pass to small fish and ultimately humans *via* the food chain. MPs are easily incorporated into the human food chain since they are consumed by both marine and terrestrial organisms. When MPs enter the human body, they can cause harmful health effects, including neurological and mental disorders, gastrointestinal disorders, respiratory disorders, autoimmune disorders, infertility, cancer, and chromosomal disorders (Al Mamun et al., 2023). Due to these potential health risks posed by MPs, federal governments are currently exploring alternatives to cut down on the use of petroleum-derived plastics. In this regard, biopolymers produced from biological sources are the best substitutes for polymers made from petrochemicals. Among various biological sources biopolymers, microbially-derived biopolymers such as polyhydroxyalkanoates (PHAs) have seized the attention among the scientific community due to its appealing qualities such as biodegradability, biocompatibility, thermoplasticity, sustainability and non-toxicity (Hamdy et al., 2022). Because of these eye-catching qualities, PHAs are used in numerous sectors, including medical, pharmacy, agriculture, food packaging, and automobiles (Ben Abdallah et al., 2020). PHAs-based bioplastics are anticipated to be a part of future circular economies due to their sustainability and efficiency in accomplishing some of the related Sustainable Development Goals (SDGs) set forth by the United Nations (Trakunjae et al., 2023). Additionally, they can support sustainability initiatives and lessen reliance on fossil fuels (Muneer et al., 2020).

PHAs are a family of microbial polyesters that are biosynthesized by a number of prokaryotic microorganisms under imbalanced growth conditions and stored as carbon and energy reserves in subcellular organelles (carbonosomes) (McAdam et al., 2020). Some recently reported microorganisms such as *Loktanella* sp. SM43 (Lee et al., 2022); *Brachybacterium paraconglomeratum* (Mandragutti and Sudhakar, 2023); *Priestia* sp. YH4 (Jung et al., 2023); *Halomonas* sp. YLGW01 (Kim et al., 2023) and *Paraburkholderia* sp. RG36^T (Chhetri et al., 2023) have been found to produce PHB. Other reported PHB-producing bacteria includes *Aeromonas hydrophila*, *Acinetobacter* spp., *Azospirillum rubrum*, *Bacillus megaterium*, *B. siamensis*, *B. subtilis*, *B. cereus*, *Brevundimonas* spp., *Burkholderia*

cepacia, *B. sacchari*, *Caulobacter* spp., *Cupriavidus necator*, *Enterococcus* spp., *Halomonas boliviensis*, *Methylocystis* spp., *Pseudomonas aeruginosa*, *P. putida*, *Ralstonia* spp., *Rhizobium* spp., *Sphingobacterium* spp., and *Yokenella* spp. (Bhagowati et al., 2015; Acharjee et al., 2023). PHB-producing bacteria thrive in environments high carbon sources compared to other nutrients like phosphorous and nitrogen (Susianingsih and Kadriah, 2020). Some potential sources for PHB-producing bacteria are wastewater treatment area; paper, pulp and cardboard industry sludge; oil manufacturing plants; sugar processing factory; cow and chicken dung slurry; Agricultural soil, kitchen waste soil; Garbage dumping sites; oil contaminated soil (Sangkharak and Prasertsan, 2012; Bhuwal et al., 2014; Susianingsih and Kadriah, 2020; Trakunjae et al., 2021).

Bacillus and *Pseudomonas* are the most researched genera of bacteria among all prokaryotic organisms. These bacteria are capable of producing PHAs by using a variety of inexpensive carbon sources. Various carbon sources such as cashew apple juice, Waste cooking oil, sugar cane molasses, macroalgal biomass, crude glycerol, wine industry waste; sugar-based syrup from cereal waste (Arumugam et al., 2020; Sangkharak et al., 2021; Jung et al., 2023; Bhatia et al., 2023; Kim et al., 2023; Drakonaki et al., 2023; Bellini et al., 2024) have been used to produce PHB. More research has been done on *Bacillus* spp. as a producer of PHAs than other microorganisms because of their abundance, even in extremely harsh environmental conditions; higher growth rates on complicated substrates, greater capacity to produce PHAs, and enzymatic machinery, which allows them to use a wide variety of complex compounds to produce PHAs (Morya et al., 2018).

The PHB-producing bacteria that are isolated from the environment can be screened using a variety of techniques. However, it is imperative to quickly screen a large number of bacterial samples in a brief period of time. Traditionally, Sudan Black B (SBB) staining has been used by researchers to identify PHB granules in bacterial cells. The use of SBB staining is regarded as a presumptive detection method for PHB (Godbole, 2016). While this approach offers a straightforward means of effectively screening PHB-producing microorganisms, it is prone to errors in detection; staining of other lipophilic compounds may result in false positives (Koller and Rodríguez-Contreras, 2015). Hence, Nile blue A (NBA), a more

specific stain for PHB, is further used to screen SBB positive isolates using a viable colony approach that is quicker, more sensitive, and more accurate (Bhuwal et al., 2014). When exposed to UV light, PHB granules stained with NBA dye gives it a red, pink, yellow, or orange color appearance. This makes it possible to identify and isolate the bacteria that produce PHB (Das et al., 2018).

PHAs can generally be divided into three groups based on the number of carbon atoms they contain: short-chain lengths (C_3 - C_5), medium-chain lengths (C_6 - C_{14}), and long-chain lengths ($>C_{14}$) (Ding et al. 2022). One of the most well-known PHAs, polyhydroxybutyrate (PHB) (a short-length chain PHAs), has gained tremendous recognition as a potential alternative to synthetic polymers because of its mechanical properties that are similar to those of conventional plastics (Mostafa et al., 2020). Additionally, PHB degrades in the presence of microorganisms such as bacteria, fungi, or actinomycetes in both aerobic and anaerobic environments. In one study, bacterial strain *Microbulbifer* sp. SOL66 was employed by Cho et al. (2022a) to study the microbial degradation of PHB/tributyl citrate film. After 3 days, the film was determined to have degraded over 90 %. However, *Microbulbifer* sp. SOL66 deteriorated 70 % of virgin PHB film in just 1 day. Similarly, *Bacillus* sp. JY35 was able to degrade a biodegradable plastic, poly (butylene adipate-co-terephthalate) (PBAT) by 50 % after 21 days (Cho et al., 2022b). Recently, Shin et al. (2023) have also reported on the use of *Bacillus* sp. NR4 in the degradation of polycaprolactone (PCL) and PCL/PBAT blends. After 10 days, the strain was able to degrade PCL by 85.6 %, and after 14 days, it was able to degrade PCL/PBAT blends by 88.3 %.

PHB material is broken down by these microbes into biomass, carbon dioxide (CO_2), and water as end products under aerobic conditions, whereas it breaks down into biomass, CO_2 , methane (CH_4), and water under anaerobic conditions (Lammi et al., 2019; Soni et al., 2023). Another prime attraction of PHB is that it can be introduced into the human body without causing an inflammatory response because they are biocompatible with human bones and tissues. Since it breaks down gradually in the human body, it can be used as a drug delivery system for a slow release of medication (Nair and Laurencin, 2007). In 2007, the Food and

drug administration (FDA) approved the first PHB film used for surgical purposes (Riaz et al., 2018).

Based on the backdrop information, the present study focuses on the isolation, biosynthesis and in-depth physico-chemical characterization of PHB biopolymer accumulated by *Bacillus* sp. KE4 strain, which has been isolated from kitchen waste effluent. Furthermore, biodegradability and cytotoxicity assay of PHB film were also performed to establish their environmentally benign nature.

2.3. Materials and methods

2.3.1. Chemicals and reagents

All the chemicals and reagents such as Na₂HPO₄, KH₂PO₄, NH₄Cl, NaCl, (NH₄)₂SO₄, MgSO₄, citric acid, FeSO₄.7H₂O, ZnSO₄.7H₂O, CuSO₄.5H₂O, MnSO₄.5H₂O, CaCl₂.2H₂O, Na₂B₄O₇.10H₂O, (NH₄)₆MO₇O₂₄, HCl, yeast extract, nutrient broth, agar, fructose, sucrose, glucose, maltose, xylose, mannitol, NaClO (4 % v/v), acetone, ethanol, chloroform, Sudan black-B dye and Nile blue A dye were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India.

2.3.2. Collection and processing of environmental sample

Soil sample was collected from a kitchen waste effluent site in Lumami, Zunheboto, Nagaland, India (Latitude 26.282962° Longitude 94.475679°). Using a sterile spatula and sterile glass container, the soil sample was collected. They were given labels with the collecting information and brought right away to the laboratory to be refrigerated at 4 °C before being processed further.

2.3.3. Isolation of PHB producing bacteria

For isolation of PHB-producing bacteria, 0.85 % (w/v) sterile saline solution was prepared for serial dilutions. It was prepared by dissolving 0.85 g of Sodium chloride in 100 mL distilled water (Hassan et al., 2016). During serial dilutions, osmotic pressure builds up on the bacterial cell and has the potential to damage the cells. For this reason, 0.85 % saline solution is used to prepare serial dilution. In 9 mL of sterilized saline solution, 1 g of soil sample was added. The soil sample was then serially diluted (10^{-5} to 10^{-7}) in sterile saline solution, with 100 µL of each dilution being spread out on a plate of carbon-enriched nutrient agar (Bhuwal et al., 2014). The carbon-enriched nutrient agar media contains the

following components per litre: 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 1 g of NH₄Cl, 0.5 g of NaCl, 0.05 g of yeast extract, 17 g of agar, and 10 g of glucose (Appendix II). The pH of the culture medium was adjusted to 7.0. After spreading the diluents, plates were incubated for 24 h at 37 °C (Shah and Kumar, 2021).

2.3.4. Screening of PHAs producing bacteria

To ensure the biosynthesis of PHB, the obtained bacterial isolates were grown in nutrient broth medium (Appendix II) for 24 h at 37 °C at 180 rpm in an orbital incubator shaker. An aliquot of 10 mL of the aforementioned pre-culture inoculums was inoculated in 250 mL Erlenmeyer flasks separately containing 100 mL of sterile PHB production media and maintained in an orbital incubator shaker for 72 h at 37 °C and 180 rpm. The pH of the culture media was adjusted to 7.0 prior to autoclaving. PHB production medium consists of (NH₄)₂SO₄ 2 g/L, KH₂PO₄ 13.3 g/L, MgSO₄ 1.3 g/L, citric acid 1.7 g/L, the trace element solution 10 mL/L (g/L, FeSO₄.7H₂O 10; ZnSO₄.7H₂O 2.25; CuSO₄.5H₂O 1; MnSO₄.5H₂O 0.5; CaCl₂.2H₂O 2.0; Na₂B₄O₇.10H₂O 0.23; (NH₄)₆MO₇O₂₄ 0.1; HCl 10 mL) and glucose 20 g/L (Appendix II) (Chaudhry et al., 2011). After 72 h, culture flasks were examined for the presence of increase in the turbidity of the culture medium in comparison to the control.

2.3.4.1. Sudan Black-B (SBB) Staining

To enable rapid detection and isolation of PHB-accumulating bacteria, the bacterial colonies were stained with a 0.02 % alcoholic solution of SBB (Appendix II) and the plates were left undisturbed for 30 minutes. Following this, the surplus dye was then drained, and was thoroughly rinsed by 100 % ethanol. After the completion of staining process, the colours of the bacterial colonies in the plates were examined (Bhuwal et al., 2014).

2.3.4.2. Nile blue A (NBA) staining

The SBB positive isolates were next screened using a viable colony method, which is quicker, more sensitive, and more precise, utilizing NBA, a more specific stain for PHB. The SBB positive isolates were first streaked over a plate of carbon-enriched nutrient agar that also included NBA dye at a concentration of 0.5 µg/mL. The plates were then kept at 34 °C for 24-48 h, during which cell proliferation occurs in the presence of the dye. After 48 h, plates were examined under an ultra

violet (UV) light source tuned at 312 nm to look for PHB accumulation in the bacterial colonies (Muneer et al., 2022).

2.3.4.3. Fluorescence microscopic study

Following the method of Ostle and Holt (1982), fluorescence microscopy (EVOS M5000 model) was used to determine the presence of PHB granules inside the growing bacterial cells after staining with NBA (Appendix II). A loopful of the bacterial cells were heat-fixed, stained with 1 % (w/v) NBA, and subjected to 8 % (v/v) aqueous acetic acid treatment in order to detect intracellular PHB granules. Next, fluorescence microscope (Euromex Microscopen BV Model: iScope, IS.3153-PLFi/6) was used to study the stained smear (Phukon et al., 2012).

2.3.5. Morphological, biochemical and molecular characterization

Morphological characterization of the selected PHB producing bacterium was done by observing the standard microbiological characters (colony shape, colony margin/edge, colony texture, elevation, opacity, colour, spore formation and Gram reaction) (Singh et al., 2013). In addition, the biochemical characterization of the isolate was done by a series of IMViC test (Appendix II) including indole test, methyl red test, voges-proskauer test and citrate test; motility, catalase and hydrogen sulfide (H₂S) tests were also performed. A test for the carbohydrate fermentation was conducted for sucrose, glucose, and lactose.

The isolate was further identified using 16S rRNA nucleotide sequencing. DNA extraction from the isolated bacterium strain was done according to the method described by Hesham (2014). PCR amplification of 16S rRNA was performed using universal primers 16sF-5' GGATGAGCCCGCGGCCTA 3' and 16sR-5' CGGTGTGTACAAGGCCCGG 3'. The PCR was performed in 25 cycles with initial denaturation at 96 °C for 5 minutes, followed by denaturation at 96 °C for 30 secs, hybridization at 50 °C for 30 secs, and elongation at 60 °C for 1 minute 30 secs. The ABI 3130 genetic analyzer and big dye terminator version 3.1 chemistry cycle sequencing kit were used for sequencing. The nucleotide sequence of the amplified 16S rRNA genes of the bacterium was submitted to the NCBI GenBank database and phylogenetic tree was constructed using MicrobiomeAnalyst 2.0 software.

2.3.6. Effect of carbon sources on PHB production

Different carbon sources were used and optimized (fructose, sucrose, glucose, maltose, xylose, and mannitol at 20 g/L) *via* shaking flask culture method in order to precisely validate which carbon sources is suitable for PHB production. The isolate was grown in 250 mL Erlenmeyer flasks with 100 mL of PHB production medium, but different carbon sources were taken (Appendix II). The production media was supplemented with 2 % (v/v) inoculum and were incubated for 72 h at 37 °C and 180 rpm. The PHB accumulated cells were extracted after 72 h and DCW was estimated. The experiment conducted was executed in triplicate for different carbon sources.

2.3.7. Effect of glucose concentration on PHB production

In the present study, different glucose concentrations were optimized during the PHB production process. Experiments were conducted on glucose concentrations varied from 10 to 30 g/L, with an increment of 5 g/L. The isolate was grown in multiple 250 mL Erlenmeyer flasks with 100 mL of production medium in each flask. The flasks were then incubated for 72 h at 37 °C and 180 rpm of agitation rate. Each experiment conducted in the flask was executed in triplicate.

2.3.8. Production Media and Culture Conditions

PHB was produced using PHB production medium as described by Chaudhry et al. (2011). To produce PHB, 250 mL Erlenmeyer flasks were used carrying 100 mL of PHB production medium (Appendix II) and were inoculated with 2 % (v/v) of 24 h old seed culture. After inoculation, the culture flasks were placed in an orbital incubator shaker and incubated for 72 h at 37 °C and 180 rpm.

2.3.9. Extraction, purification and quantification of PHB

PHB was extracted using the chloroform extraction technique. The bacterial cells underwent a 10-minutes centrifugation at 8000 rpm to form the pellet. The pellets were then dried in the oven overnight at 40 °C and the dry cell weight (DCW) (g/L) of the pellet was calculated. The pellet was subsequently suspended in 4 % sodium hypochlorite (NaClO) solution and incubated for 2 h at 37 °C in an orbital incubator shaker to make sure that all cell components, except for PHB, had completely digested. After the digestion step, the resulting mixture was centrifuged

at 8000 rpm for 10 minutes to separate the PHB granules, and the supernatant was discarded. In order to remove NaClO completely, the pellets were washed twice with distilled water, then finally washed with a solution of distilled water, ethanol and acetone (1:1:1). After that, the pellets were dissolved in boiling chloroform. To remove the undissolved components, the solution was filtered out using Whatman filter paper. Following that, the solution was poured on clean sterile glass petri dish and left overnight to evaporate chloroform. Finally, a thin biopolymer film was obtained after the chloroform evaporated (Girdhar et al., 2014; Mohapatra et al., 2017; Vijay and Tarika, 2019). For the quantitative estimation of PHB, the cell pellet's DCW was determined in triplicates. The amount of intracellular PHB accumulation was calculated as a percentage of the PHB content in the DCW (Shah and Kumar, 2021).

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of PHB (g/L)}}{\text{DCW of biomass (g/L)}} \times 100 \quad (2.1)$$

2.4. Characterization of the extracted biopolymer

2.4.1. Fourier transform infrared (FTIR) spectroscopy

To prepare the sample for FTIR analysis, 5 mg of the isolated PHB was initially mixed with dry potassium bromide (KBr) and the resultant mixture was compressed into an evacuable KBr pellet die. The sample was then examined using a spectrophotometer (Perkin Elmer, Spectrum two, 104140) for examination. To capture the spectrum at the 4000–400 cm^{-1} scan range, 64 scans were performed (Wendy et al., 2022).

2.4.2. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The monomeric composition of the isolated PHB was determined by using GCMS analysis as described by Rathika et al. (2019). A screw-capped tube containing 10 mg of the extracted biopolymer was suspended in a mixture of 1 mL of chloroform and 1 mL of methanol/sulphuric acid (85:15 v/v) before being heated at 100 °C for 2 h. After cooling, demineralized water (0.5 mL) was added and vortexed for 1 minute. Finally, GCMS (Thermo Scientific TRACE 1300 ISQ 7000) was used to examine the organic phase that contained the monomers of the resultant methyl esters. Helium was used as carrier gas, and a flow rate of 1 mL/minute was used in the VF-5ms column. Scan mode with m/z 40–600 range were recorded, and

the NIST 23 library was used to identify the compounds. The injector and detector were maintained at respective temperatures of 250 and 200 °C.

2.4.3. Nuclear Magnetic Resonance (¹H NMR and ¹³C NMR) spectroscopy

To determine the chemical structure of the extracted PHB biopolymer, the ¹H and ¹³C NMR spectra are used for analysis. Around 5 mg of PHB were dissolved in 1 mL of CDCl₃ (deuterated chloroform), and then they were examined at 400 MHz with a BRUKER AVANCE II 400 spectrophotometer. At a temperature of 25 °C, ¹H spectra were obtained using pulse widths of 5 ms (millisecond), a pulse angle of 45°, a spectral width of 1000 Hz, pulse repetition times of 3 secs, 32,000 data points, and 32 accumulations. ¹³C NMR analysis was carried out with 20 mg of PHB sample. At 25 °C, ¹³C spectra were acquired with pulse widths of 5 ms, pulse angles of 45°, pulse repetitions of 2 secs, spectral widths of 27,000 Hz, 16,384 data points, and 548 accumulations. Tetramethylsilane (TMS) was used as the internal standard (Stanley et al., 2020).

2.4.4. Determination of molecular weight by gel permeation chromatography (GPC)

GPC was used to calculate the extracted PHB's weight average molar mass (Mw), number average molar mass (Mn), and polydispersity index (PDI= Mw/Mn). Chloroform was used to dissolve the PHB, which was then run at room temperature through a GPC system (Agilent) for analysis. Polystyrene standards with a serially coupled Refractive Index detector were used to create a calibration curve. Tetrahydrofuran (THF) was used as the eluent, with a flow rate of 0.5 mL/minute and a temperature of 40 °C, to determine the molecular weight of the extracted PHB biopolymer (Phukon et al., 2012).

2.4.5. X-ray diffraction (XRD) analysis

In a Miniflex Benchtop X-ray diffractometer system (RIGAKU), the crystallinity of the extracted PHB biopolymer was evaluated using Cu K α radiation at a wavelength of 1.54 Å (40 kV, 30 mA). Each scan was performed in the 10 to 45° (2 θ degree) range at a speed of 3°/minute, where θ is the angle at which the X-ray beam incident on the sample.

According to Frone et al. (2020), the crystallinity index (CI) of the extracted PHB biopolymer was calculated by the following formula:

$$CI (\%) = \frac{\sum Ac}{\sum (Ac + Aa)} \quad (2.2)$$

Where, Ac is the sum area under the respective crystalline peaks, and Aa is the sum area of the amorphous halo.

2.4.6. Thermal analysis (Thermogravimetry/ Derivative Thermogravimetry/ Differential Thermal Analysis) (TG/DTG/DTA)

The thermostability of the extracted PHB was investigated using a Thermogravimetric analyzer (Netzsch STA 2500 Regulus). For the analysis, a sample weighing approximately 4.25 mg was used. The sample was heated from 30-600 °C at a rate of 10 °C/minute in the presence of nitrogen at a flow rate of 40 mL/minute in order to prevent sample oxidation (Stanley et al., 2020).

2.4.7. Differential scanning calorimetry (DSC)

DSC was used to determine the melting temperature (T_m) and crystallinity degree (X_c) of the extracted PHB biopolymer produced by the *Bacillus* sp. KE4 strain. On a Thermal Analyzer Instruments system (Shimadzu, DSC-60, Serial no. C30724300032SA) equipped with cooling accessory, DSC measurements were performed. Heat-cool method, as described by Wada et al. (2023) was used to acquire the data. Cast film sample that weighed 5–10 mg was placed in an aluminum pan and heated under nitrogen environment at a scanning rate of 10 °C/minute from 20 to 300 °C. From DSC endothermal peaks, the melting temperature (T_m) and melting enthalpy (ΔH_m) were calculated. The sample was annealed for one minute before being cooled to 20 °C (Biradar et al., 2018).

By using the equation as described by Hernández-Núñez et al. (2019), the crystallinity degree (X_c) of PHB is determined:

$$Xc = \left[\frac{\Delta H_m}{\Delta H^\circ} \right] \times 100 \quad (2.3)$$

where ΔH_m is the sample's melting enthalpy (J/g). ΔH° is the estimated melting enthalpy of 100 % crystalline PHB, which is 146 J/g.

2.4.8. Elemental analysis and morphological characterization of PHB film

Field emission scanning electron microscopy (FE-SEM) with energy dispersive X-ray spectroscopy (EDS) and X-ray photoelectron spectroscopy (XPS) were employed to ascertain the elemental composition of the purified PHB film.

PHB films were placed on aluminium stumps, cleaned in a buffer, then coated with gold in a sputter coater before being examined under a microscope. Then, imaging was carried out using a FE-SEM (JEOL, JSM7610F) with a 15 kV accelerating voltage (Maity et al., 2020). The Mg-K α line ($h\nu = 1253.6$ eV) was used at 10 eV binding energy at low pressure for XPS (Shimadzu-Axis SUPRA) study. Before being dried in a vacuum chamber, samples were cleaned with ultrasonic and deionized water to prevent contamination (Kumari et al., 2022).

2.4.9. Mechanical properties

The mechanical properties of 0.075 mm-thick PHB films were investigated at crosshead speed of 10 mm/minute in a universal testing machine (Lloyd LRX, Lloyd Instruments Ltd., UK) at 30 °C. The test was achieved in triplicates. Subsequently, elongation at break (%), tensile strength (MPa), and Young's modulus (GPa) were evaluated (Napathorn, 2014).

2.4.10. Biodegradability of PHB film

The film was cut into small rectangles that measured 2 cm by 1.3 cm and had a thickness of 0.075 mm. Next, PHB film was placed in the open windrow at a depth of 10 cm. Open windrow composting was made with soil collected from Nagaland University campus area, Lumami, India (Latitude 26.221674° Longitude 94.477508°). It was found that the experimental windrow had a pH of 6.5 and an environmental temperature of 30 °C. The weight loss and morphological deterioration of PHB film was recorded at an interval of 7 days up to 28 days. The film was taken out of the soil in order to assess any morphological changes, and any leftover soil particles were carefully rinsed away with distilled water (Maity et al., 2020). The method described by Chathalingath et al. (2023) was used to compute the weight and the percentage of degradation.

$$\text{Degradation \%} = \frac{W_1 - W_2}{W_1} \times 100 \quad (2.4)$$

Here, W1 denotes the film's initial weight and W2 its final weight after degradation.

2.4.11. Cytotoxicity assay of PHB film on human cell line

2.4.11.1. Cell culture and PHB exposure

The HepG2 cells (human liver hepatocellular carcinoma cell line) were obtained from NCCS, Pune India. The cells were cultivated in DMEM (Dulbecco's

Modified Eagle Medium) media (Appendix II) supplemented with 10 % FBS (Fetal Bovine Serum) penicillin (100 units/mL) and streptomycin (100 µg/mL). The cell cultures flasks were kept in 5 % CO₂ atmosphere at 37 °C in an incubator. After confluence, the viable cells (90 % viability using trypan blue exclusion assay) were transferred to tissue culture grade clear bottom 96 well block plates (Invitrogen, USA) for exposure and microplate-based fluorescence assays.

2.4.11.2. PHB exposure to cells

The PHB samples were exposed to HepG2 cells with different concentration range (50, 100, 200 and 400 µg/mL) as described previously (Abdo et al., 2023). Briefly, cells were exposed to PHB in 96 well plates and incubated for 24 h and kept in 5 % CO₂ atmosphere at 37 °C. After treatment schedule, the cells were subjected to various assays.

2.4.11.3. Cell viability – MTT assay

Cell viability was examined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay as previously described by Li et al. (2015), based on the conversion of MTT into formazan crystals by mitochondrial dehydrogenases. In general, cells were plated in 96-well plates at a density of 2×10^3 cells/well. Subsequently, MTT medium (Appendix II) was added to the culture medium at a final concentration of 0.45 mg/mL. This solution was then withdrawn after 4 h of incubation at 37 °C, and the formazan was dissolved in 100 µL of dimethyl sulfoxide. Using a multimode microplate reader, the absorbance was measured at 570 nm. Viability of the cells was determined by comparing the results to control cells that were considered to be 100 % viable.

2.4.11.4. Cellular oxidative stress – DCFDA assay

Intracellular ROS (reactive oxygen species) generation level was measured in cells using the general ROS indicator CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) (Invitrogen, USA). After deacetylation by intracellular esterases, free radicals oxidize H₂DCF (2',7'-dichlorodihydrofluorescein) to produce green fluorescent DCF (Dichlorofluorescein) (Ng et al., 2021). In a nutshell, treated cells were incubated at 37 °C for 45 minutes in a CO₂ incubator with 10 µM CM-H₂DCFDA. Later, DCF was washed twice with PBS (Phosphate buffered saline) (Appendix II) and relative

intensity of DCF was quantified (Excitation/Emission: 485/530 nm) using multimode microplate reader and expressed in arbitrary unit (a.u.) as described previously (Maadurshni et al., 2022).

2.4.11.5. Cellular superoxide generation – DHE assay

In current study, cellular superoxide generation in live cells was measured using Dihydroethidium (DHE, Sigma Aldrich) fluorescence method as reported earlier by Nagarajan et al. (2023). Briefly, after the treatment schedule, cells were washed with PBS and incubated at 37 °C in the dark with 5 µM DHE for 30 minutes in cell culture incubator. After incubation, cells were washed twice in PBS to eliminate excess staining solution. The fluorescence intensity of DHE was quantified (Excitation/Emission: 510/590 nm) using multimode microplate reader and expressed in arbitrary unit (a.u.).

2.4.11.6. Cellular superoxide generation - DHE imaging

For validation fluorescence imaging experiments, the cells were seeded on glass coverslips placed in 24 well plates. After treatment schedule, the cells were exposed to DHE with the concentration of 5 µM and incubated for 30 minutes under 37 °C. The stained cells were then observed under a fluorescence microscope equipped with high-resolution (12 MP) camera (Amscope, USA) using appropriate Excitation/Emission filters and images were captured with similar exposure time. The fluorescence images were analyzed through ImageJ software.

2.4.11.7. Statistical analysis

Statistical analysis was performed by GNU PSPP software. For illustrative purposes, the experimental values were presented as means ± SD (standard deviation). The present study used a one-way analysis of variance (ANOVA) to evaluate the statistical significance between means of values obtained from the cellular parameters. In this study, a *p* value of < 0.05 was deemed statistically significant.

2.5. Results and discussion

2.5.1. Isolation, screening and selection of PHB producing bacteria

In the current study, a total of 40 distinct bacterial isolates were isolated from various environmental samples. Initially, the colony-staining method using SBB was used to screen the bacterial isolates. In viable colony method, the colonies

of bacterial isolates were flooded with SBB and it appeared bluish black, which confirms the production of PHB (figure 2.1a). For further confirmation, the bacterial isolates were grown in the carbon-enriched nutrient agar media containing NBA dye, a more specialized stain for PHB. Under UV light (312 nm), the bacterial isolates fluoresced bright yellow-orange (figure 2.1b) confirming the accumulation of PHB inside the bacterial cell. Soni et al. (2023) also used NBA staining method to confirm the accumulation of PHB and the result was similar to the present study.

To further validate the PHB accumulation, a fluorometric study was performed on the bacterial isolates. The isolates were observed under a fluorescence microscope after NBA staining. When observed at 40X magnification, the NBA-stained cells fluoresced brightly in an orange-red colour (figure 2.1c), thus confirming the accumulation of PHB. The result was found to be consistent with the previous reports of Bhagowati et al. (2015) and Das et al. (2018). As a result, out of total 40 isolates, only 3 isolates showed positive result. These 3 positive isolates were isolated from kitchen waste effluent, garbage dumping site and chicken waste disposal site and were assigned as KE4, GS2, and CW1, respectively. However, out of these 3 isolates only strain KE4 was selected for detailed study as it was found to accumulate highest PHB content.

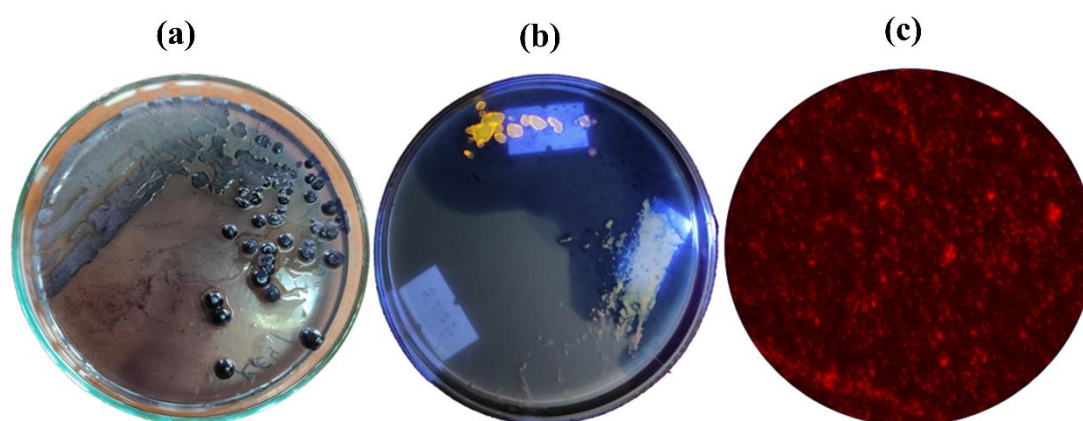


Fig. 2.1. Screening of potential isolate KE4 for PHB accumulation. (a) Bluish black colour observed by SBB staining, (b) Yellow-orange fluorescence emitted by colony under UV light (312 nm), and (c) Orange-red fluorescence of PHB granules using NBA staining under fluorescence microscopy

2.5.2. Morphological, biochemical and molecular characterization

The PHB producing bacterium was morphologically and biochemically characterized. The isolate was identified phenotypically as a round, entire, butyrous, flat, opaque, white, rod-shaped, endospore positive and gram-positive bacterium (*Bacillus* sp.). The IMViC test results for the indole and Voges-Proskauer tests were negative, whereas the results for the methyl red and citrate tests were positive. Motility and hydrogen sulfide (H₂S) tests were negative whereas catalase test was positive. Carbohydrate fermentation tests for glucose and sucrose were positive, whereas the result for lactose was negative.

Using a ribotyping technique based on the polymerase chain reaction (PCR), the competent isolate was further chosen for taxonomic identification. PCR yielded amplicons of 963 bp in length, which were then sequenced by comparing the sequence of PCR product with the available sequences of NCBI Genbank database, the selected bacterial strain was identified as *Bacillus* sp. Later, the sequence was added to the NCBI database with accession number OR775565. Figure 2.2 shows the phylogenetic tree that was created for the selected bacterial strain. The sequences used in Phylogenetic Tree Builder are aligned using the System Software aligner. Using the Jukes-Cantor corrected distance model, a distance matrix is created. Alignment inserts are not taken into consideration while creating the distance matrix; the minimum comparable position is 200. Only alignment model positions were utilized. Weighbor was used to construct the tree, with an alphabet size of 4 and a length of 1000. Weighbor is a weighted variant of Neighbor Joining that assigns slightly less weight to longer distances in the distance matrix. The weights are derived from the anticipated variances and covariances in a basic Jukes-Cantor model. Result clearly indicates the presence of significant variations and could be considered as different species of *Bacillus*. The alignment results showed that the 16S rRNA sequences of our strain KE4 was found to be vastly homologous with 97.72 % similarities to *Bacillus* sp. BCK-7 (accession no. KP992120.1).

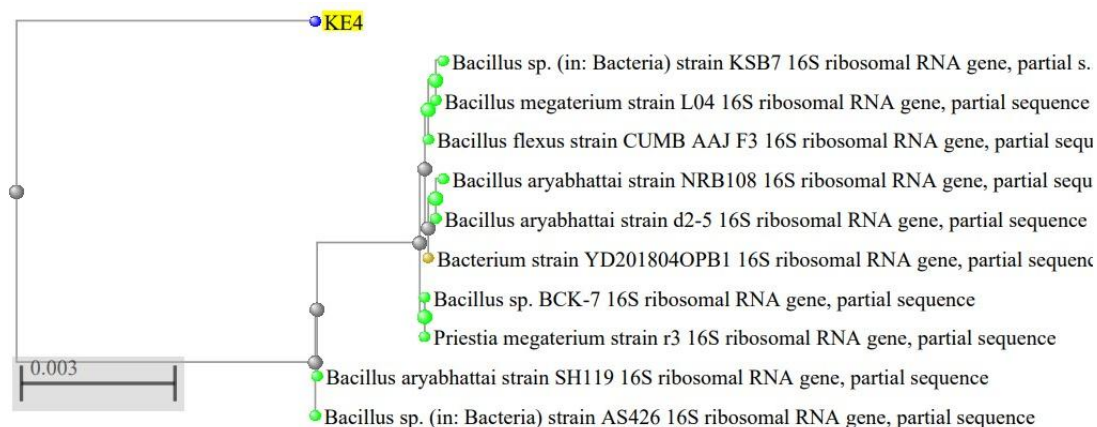


Fig. 2.2. Phylogenetic tree of the isolate KE4 based on 16S rRNA

2.5.3. Effect of carbon sources on PHB production

In the present study, *Bacillus* sp. KE4 strain was grown in several 250 mL Erlenmeyer flasks with 100 mL of PHB production medium containing different carbon sources, and were incubated for 72 h at 37 °C and 180 rpm. The findings indicated that the DCW (g/L) was highest in glucose with 4.4 g/L, followed by fructose (4.1 g/L), sucrose (3.9 g/L), maltose (2.9 g/L), mannitol (2.5 g/L), and xylose (1.8 g/L), respectively (figure 2.3a). Therefore, glucose was used as the carbon source for PHB production, based on the isolate's highest DCW production.

2.5.4. Determination of glucose concentration

PHB production and growth of cells are dependent on the concentration of glucose present in the culture media. Figure 2.3b and 2.3c shows the profiles of DCW (g/L), PHB concentration (g/L), PHB content (%), and PHB yield (g/g) produced in different glucose concentration. Five different glucose concentrations (10, 15, 20, 25, and 30 g/L) were used in the experiments. It is clear that as the concentration of glucose was raised from 10 to 20 g/L, DCW, PHB concentration, PHB content and PHB yield improved. However, a notable drop in PHB concentration, PHB content and PHB yield was noted from 25 g/L to 30 g/L. Up to 20 g/L, there was optimum cell growth and glucose utilization. Therefore, 20 g/L of glucose concentration was used in the present study to produce PHB.

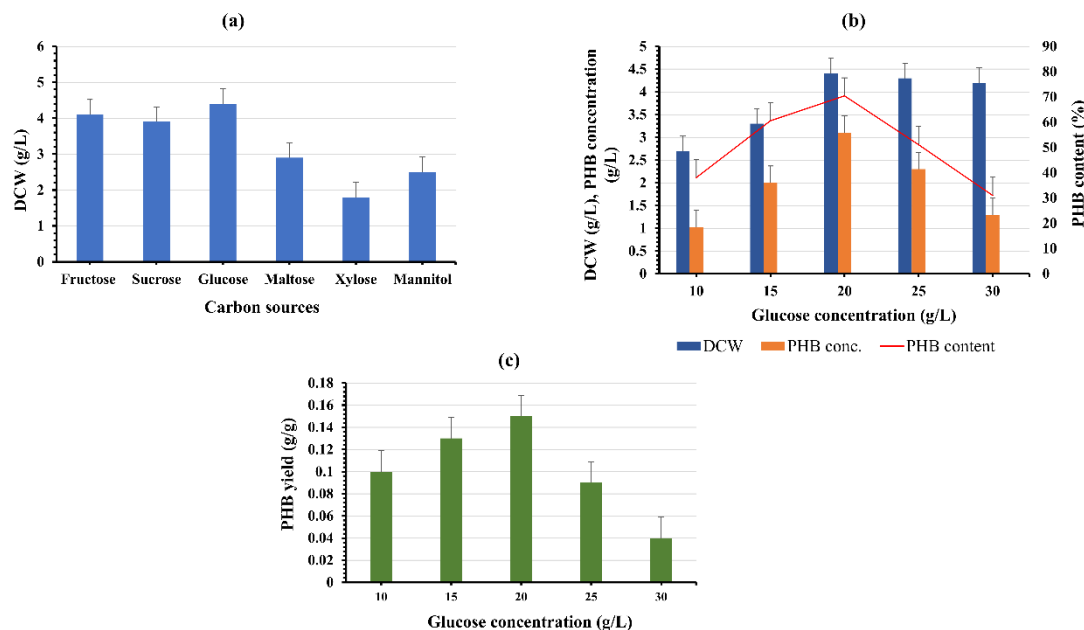


Fig. 2.3. Effect of different carbon sources on (a) DCW, (b) PHB content, and (c) PHB yield

2.5.5. Determination of DCW and PHB accumulation (%)

After allowing the chloroform to evaporate overnight, a thin biopolymer film was produced. As described by Shah and Kumar (2021), the DCW of the cell pellet and the percentage of PHB present in the DCW's composition were determined for the quantitative evaluation of PHB. Small-scale fermentation was used to measure PHB accumulation. *Bacillus* sp. KE4 strain was able to accumulate 70.4 % of PHB as DCW of 4.4 g/L with a PHB concentration of 3.1 g/L and PHB yield 0.15 g/g under culture conditions of 2 % (v/v) inoculum, 20 g/L of glucose (carbon source), pH 7, temperature 37 °C, and 72 h of incubation at 180 rpm. Several studies have reported the production of PHB by various microorganisms using glucose as carbon source. In one study, Sangkharak and Prasertsan (2012) reported PHB production by strain *Bacillus licheniformis* PHA 007 and was able to accumulate 68.80 % of PHB as DCW of 2.10 g/L with a PHB concentration of 1.83 g/L. Mohammed et al. (2019) reported PHB production by strain *Bacillus* sp. BPPI-14 and *Bacillus* sp. BPPI-19. *Bacillus* sp. BPPI-14 was able to accumulate 50 % of PHB as DCW of 0.94 g/L with a PHB concentration of 0.47 g/L and *Bacillus* sp. BPPI-19 was able to accumulate 48 % of PHB as DCW of 1.05 g/L with a PHB

concentration of 0.5 g/L. Similarly, Mohanrasu et al. (2020) also reported PHB production by strain *Bacillus megaterium* and was able to accumulate 54 % of PHB as DCW of 10.52 g/L with a PHB concentration of 5.65 g/L. As compared to the other reports, our *Bacillus* sp. KE4 strain have potentially accumulated the highest PHB content with 70.4 % of DCW.

2.6. Characterization of the extracted biopolymer

2.6.1. FTIR spectroscopy

To identify the functional groups of the extracted biopolymer, FTIR analysis was performed (figure 2.4). The broad absorption band at 3446 cm^{-1} attributes to hydroxyl stretch (-OH). The bands observed around 2900 cm^{-1} spectral region is attributed to the CH stretching bond, which is a characteristic of PHB polymers. The absorption bands at 1720 cm^{-1} and 1283 cm^{-1} are attributed to carbonyl (C=O) stretching of an ester group and C–O group, respectively. These two bands are identified as PHB indicators. The absorption band at 1641 cm^{-1} correspond to C=O bond extended for conjugated carbonyl or amide group. The absorption bands at 1453 and 1380 cm^{-1} are assigned to asymmetric and symmetric deformation of the -CH_3 groups, respectively. The absorption band at 1045 cm^{-1} correspond to C–O–C group stretching vibration. The obtained absorption bands are consistent with the previous studies on FTIR analysis of PHB (Getachew and Woldeesenbet, 2016; Kovalcik et al., 2017; Herrera-Kao et al., 2018; Mostafa et al., 2020; Sirohi, 2021; Siripurapu et al., 2022).

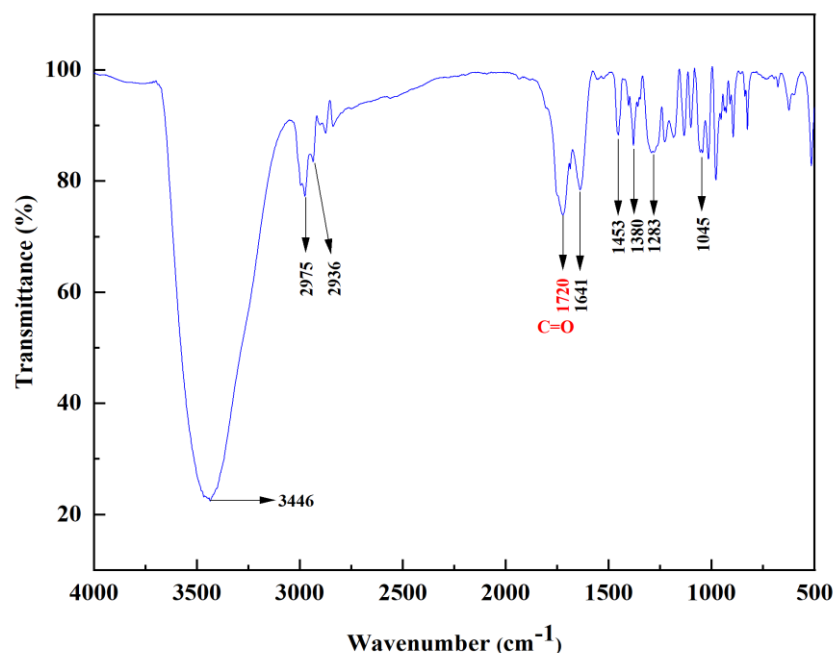


Fig. 2.4. FTIR spectra of PHB biopolymer extracted from *Bacillus* sp. KE4 strain

2.6.2. GC-MS Analysis

To evaluate the monomeric composition of the extracted biopolymer, GCMS analysis was performed. The total ion chromatogram (TIC) of the biopolymer showed significant peaks at retention times of 9.01, 11.33, 14.46, and 17.77 minute, which corresponding to Cyclopentanebutanoic acid, 2-oxo-, methyl ester; Tridecanoic acid, 12-methyl-, methyl ester; Hexadecanoic acid, methyl ester; and Octadecanoic acid, methyl ester, respectively (figure 2.5). The fragmentation peaks of PHB sample were obtained with m/z values of 43, 63, 74, and 87, which are identical with m/z values of MS library for R-3-hydroxybutyrate (Stanley et al., 2020). This result is in accordance with the previous reports of Gholamveisi et al. (2017), Evangeline and Sridharan (2019) and Rathika et al. (2019).

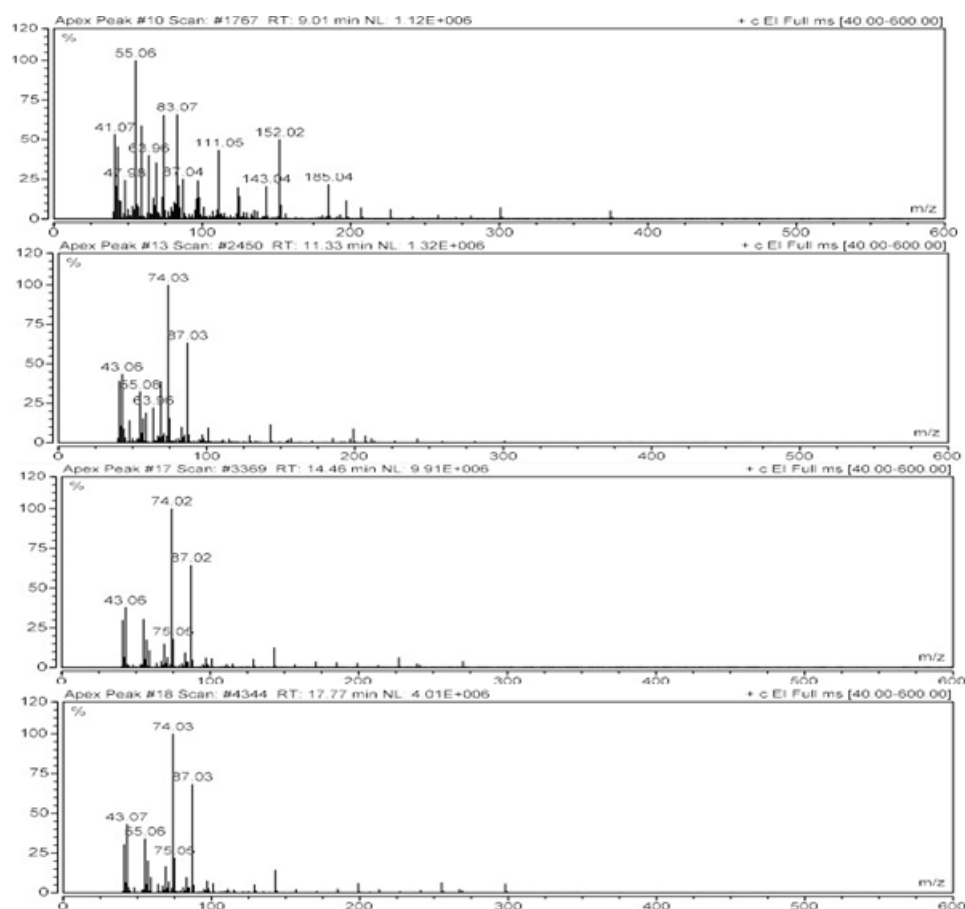


Fig. 2.5. GC-MS analysis of PHB biopolymer extracted from *Bacillus* sp. KE4 strain

2.6.3. ^1H and ^{13}C NMR spectroscopy

NMR spectroscopy is a crucial technique for confirming if the polymer structure accurately represents the structure of PHB. Using ^1H and ^{13}C NMR, the chemical structure of the biopolymer was examined. The ^1H NMR spectral analysis showing the presence of 1, 2, and 3 peaks at chemical shifts of 1.27, 2.45-2.60, and 5.27, respectively, revealed the characteristic signals for three distinct groups, namely methyl ($-\text{CH}_3$), methylene ($-\text{CH}_2$), and methine ($-\text{CH}$) groups (figure 2.6). The polymer's ^{13}C NMR spectra revealed chemical shift signals at 19.77, 40.77, 67.62, and 169.19 ppm, which corresponded to the methyl ($-\text{CH}_3$), methylene ($-\text{CH}_2$), methine ($-\text{CH}$), and carbonyl ($-\text{C}=\text{O}$) groups, respectively (figure 7). The ^1H and ^{13}C NMR results of present study is consistent with reports of Mostafa et al. (2020), Stanley et al. (2020) and Siripurapu et al. (2022).

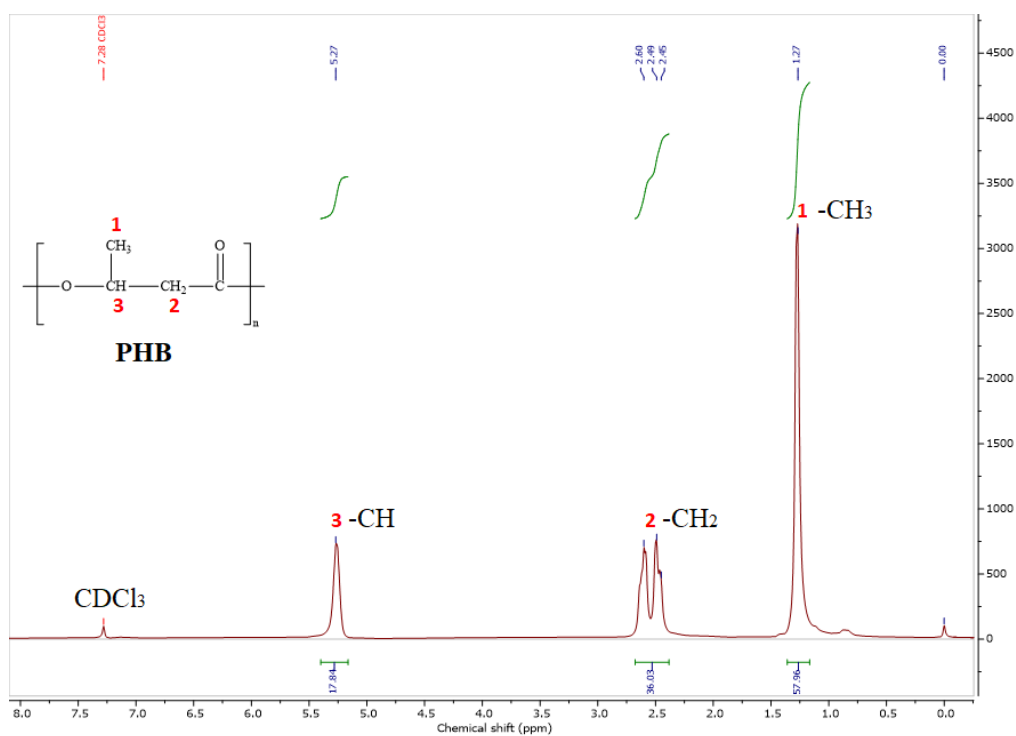


Fig. 2.6. ¹H NMR spectrum of PHB biopolymer extracted from *Bacillus* sp. KE4 strain

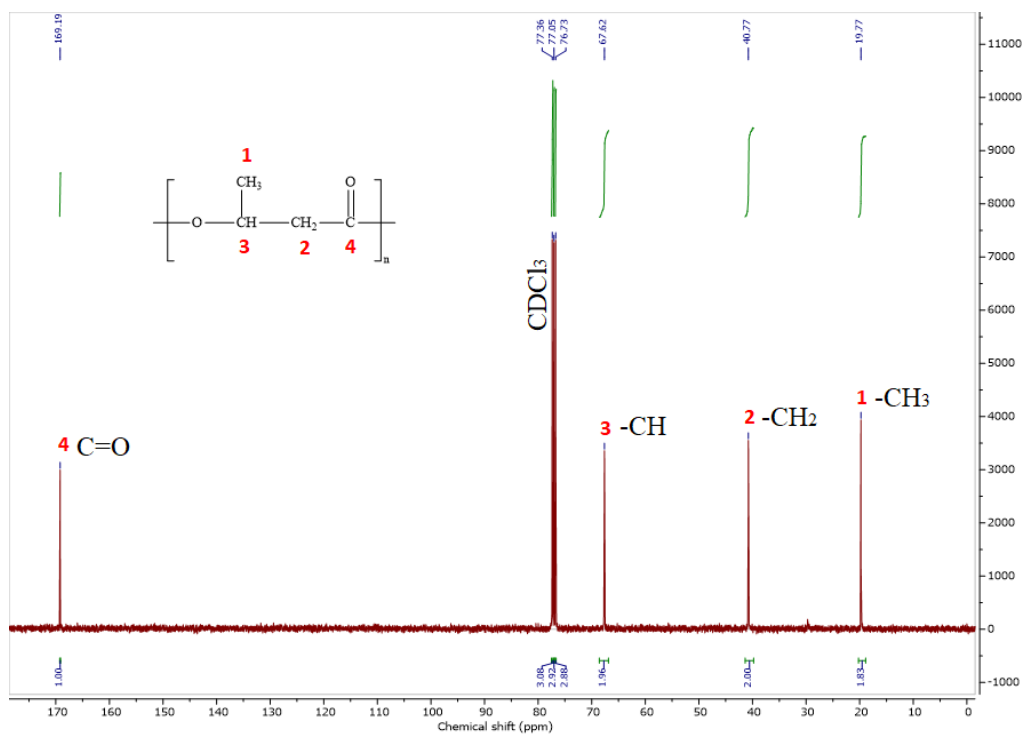


Fig. 2.7. ¹³C NMR spectrum of PHB biopolymer extracted from *Bacillus* sp. KE4 strain

2.6.4. GPC Analysis

The GPC analysis showed that the Mw, Mn, and PDI of the extracted PHB was 1,55,436 g/mol, 1,43,258 g/mol, and 1.08, respectively. Galego et al. (2000) reported that the Mw of the extracted PHB was 1,77,000 g/mol, and its Mn was about 91,000 g/mol with PDI of 1.95, which is relatively higher than the PDI of the present PHB biopolymer. The PDI of the present PHB biopolymer is also comparably lower than the PHB biopolymer reported from *Halomonas salina*, which had a PDI of 1.22 (Hernández-Núñez et al., 2019). The extracted PHB biopolymer have lower PDI, demonstrating that they can be used commercially without undergoing significant processing to uniformize the chain length.

2.6.5. X-ray diffraction (XRD) analysis

The X-ray diffractogram of the extracted PHB has been presented in figure 2.8. Significant peaks in the XRD patterns at $2\theta = 13.59^\circ$, 16.89° , 20.2° , 22.42° , 25.81° and 27.19° , which correspond to the reflections (020), (110), (101), (111), (121) and (040), respectively. The peak at 13.59° and 16.89° characterized the crystalline nature of the biopolymer; the peaks at 20.2° and 22.42° showed the existence of orthorhombic crystals, that is attributed to α -PHB crystals; the minor peaks at 25.81° and 27.19° designates the partial crystallinity nature of PHB. Similar peaks were visible in the XRD pattern of the standard PHB reported by Maity et al. (2020) at $2\theta = 13.35^\circ$, 16.86° , 22.41° , 25.45° and 27.09° . The XRD result of the current study is also consistent with other reports that have already been published (Macedo et al., 2010; Pradhan et al., 2018; Kumar et al., 2021; Etxabide et al., 2022). CI (%) was determined using the formula mentioned in equation 2.2 and it was found to be 25.7 %. It has been reported that polymers with lower crystallinity are less brittle and have a broader spectrum of applications in the field of food packaging, biomedical and agriculture (Pradhan et al., 2018).

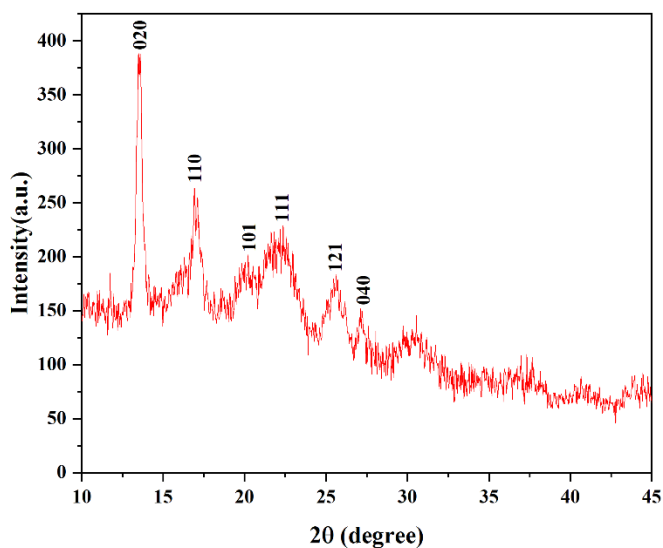


Fig. 2.8. X-ray diffractogram of PHB from *Bacillus* sp. KE4 strain indicating crystalline nature

2.6.6. TG/DTG/DT Analysis

TGA was used to ascertain the thermostability of the extracted PHB and the same is shown in figure 2.9. The first stage of mass loss took place at temperatures between 100 °C and 170 °C. This mass loss, which made up about 1.22 % of the overall mass, is due to evaporation of solvents (water, methanol, and chloroform) that is physically adhered to the biopolymer. The second or primary step of the polymer's decomposition begins at 200 °C, which is after PHB's melting point (Abdelmalek et al., 2022). The highest degrading temperature of biopolymer has been found to be between 220 °C and 303 °C, with around 43.59 % of the mass lost. It is clear that the obtained biopolymer has improved thermal stability and can therefore be employed in a number of applications including food industry, biomedical and agriculture industries. Pradhan et al. (2018) reported similar result, where a gradual weight loss of PHB obtained from *Cupriavidus necator* was between 245 °C and 289 °C. Similarly, Bhuwal et al. (2014) also reported a thermal degradation between 250 °C to 290 °C of PHB obtained from *Bacillus* sp. NA10.

DTG analysis is carried out to determine the rate at which biopolymer mass is lost in proportion to temperature and the same is shown in figure 2.9. The extracted PHB has a maximum degradation temperature (T_d) of about 273 °C and

a maximum mass-loss rate of 17.89 %/minute (figure 2.9). Similar results were observed from the PHB biopolymer obtained from *Bacillus megaterium* (248 °C, 26 %/minute) and *Cupriavidus necator* (277 °C, 70 %/minute) (Pradhan et al., 2018). Moreover, the maximal mass-loss rate of PHB in our investigation was considerably slower than that of the *Bacillus megaterium* and *Cupriavidus necator* strains. Therefore, it can be concluded that the PHB biopolymer of the present study has an outstanding thermal stability.

DTA is carried out to determine the disintegration heat. A DTA thermogram would show an exothermic peak if cross-linking processes were involved in the disintegration of PHB (Saranya and Shenbagarathai, 2011). The DTA curve exhibited a small exothermic peak at 163 °C, followed by an intense exothermic peak at 278 °C, which specified a phase shift and the burning of hydrocarbons in PHB structure. The curing temperature (point at which cross-linking occur) of the obtained PHB biopolymer was observed at 278 °C.

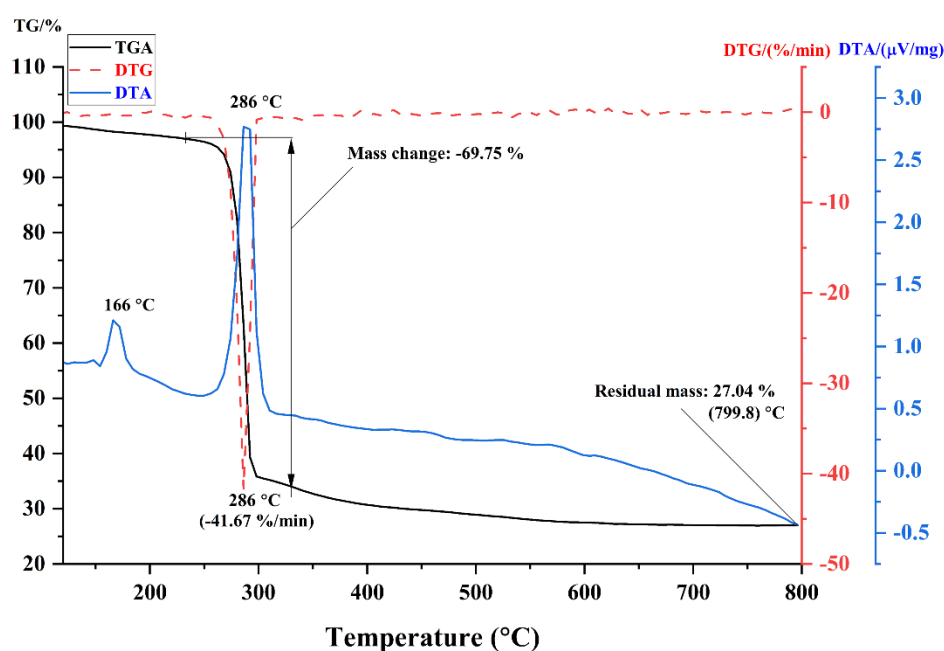


Fig. 2.9. TG/DTG/DTA curves of PHB biopolymer

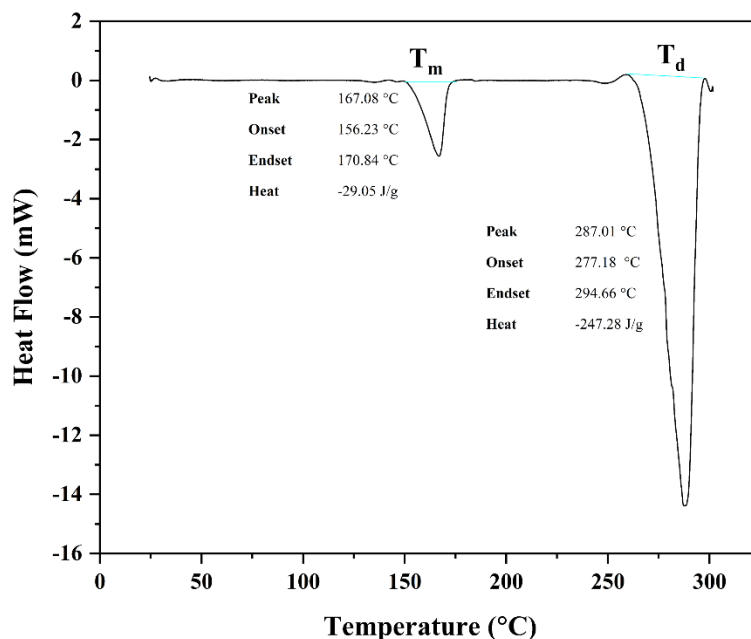


Fig. 2.10. DSC thermogram of PHB biopolymer

2.6.7. DSC Analysis

Using DSC, the melting temperature (T_m) and crystallinity degree (X_c) of the extracted PHB film were measured. The X_c of the polymer is an important aspect to describe the mechanical properties of the material. Polymers that have high crystallinity tend to be more brittle and have a narrow spectrum of applications. The extracted PHB revealed an endothermic peak on the DSC thermogram (figure 2.10), which corresponds to T_m (168.7 °C) and has a melting enthalpy (ΔH_m) of 63.21 J/g. At a high temperature (273 °C), the other endothermic peak is observed due to the degradation of biopolymer. The X_c of the extracted PHB biopolymer was calculated from equation 2.3 and found to be 43.2 %, which has lower crystallinity degree than PHB produced from *Cupriavidus necator* with X_c of 44 % (ΔH_m = 64 J/g) (Pradhan et al., 2018) and *Bacillus megaterium* ATCC 6748 with X_c of 60 % (ΔH_m = 87.7 J/g) (Chaijamrus and Udpuay, 2008). Due to the use of different methods, the crystallinity values of PHB biopolymer derived from XRD (25.7 %) and DSC (43.2 %) differ; XRD emphasizes surface crystallinity whereas DSC depicts bulk behaviour (Frone et al., 2020; de Sousa Junior et al., 2022). Unlike DSC, which bases its determination on the melting or crystallization

enthalpy, XRD bases its analysis on a comparison of the integrated intensities of properties related to the crystalline and amorphous phases (Tarani et al., 2023).

2.6.8. FESEM-EDS Analysis

FESEM was used to examine the topographical features of the extracted PHB film. The images obtained from the FESEM revealed irregular surface with the presence of pores with varied shapes (figure 2.11a). In one study, it has been reported that porosity in the biomaterial can be beneficial in various biomedical and microbiological applications which need exceptional ability to load, retain and release fluids (Udenni Gunathilake et al., 2017). Babruwad et al. (2016) also reported the presence of pores with varied sizes in the PHB film obtained from *Bacillus cereus* PW3A. An EDS study was carried out to analyse the chemical composition of the PHB film (figure 2.11b, 2.11c, 2.11d and 2.11e). This analysis demonstrates the presence of carbon and oxygen as main elements with 71.80 wt % and 28.20 wt %, respectively. EDS and XPS are both employed to determine the elemental composition. However, XPS offers information on the elemental composition as well as oxidation state and distinct binding energies, whereas EDS can only identify the elements that are present in the product (Walzak et al., 1998).

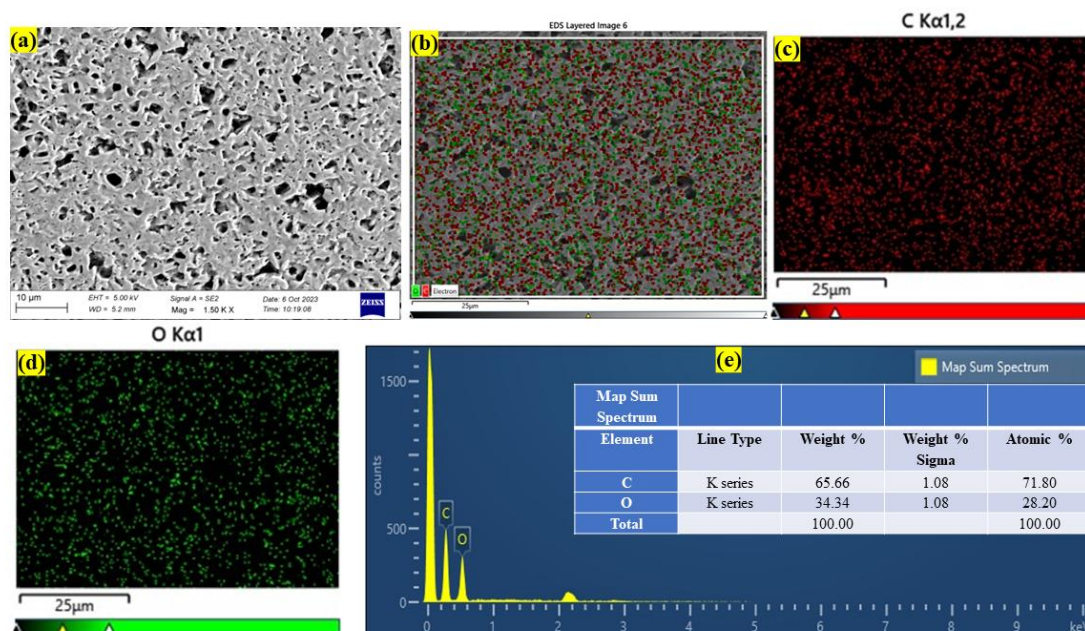


Fig. 2.11. FESEM micrograph of PHB film from *Bacillus* sp. KE4 strain. (a) 1.50 K X magnification, (b) EDS spectrum of PHB film indicates the homogenous distribution of carbon and oxygen in the area, (c) Elemental mapping of PHB film

corresponding to carbon, (d) Elemental mapping of PHB film corresponding to oxygen, and (e) SEM-EDS map sum spectrum of PHB film

2.6.9. XPS Analysis

Using XPS, the elemental composition and atomic percent (%) of the elements present in the extracted PHB surface layers was determined. Figure 2.12a shows the wide-scan survey XPS spectrum and figure 2.12b shows C1s core-level spectrum of extracted PHB. The wide-scan survey XPS spectrum indicated the presence of carbon 1s (C1s) between 280 and 290 eV and oxygen 1s (O1s) between 530 and 535 eV. The atomic percent (%) of C1s is found to be 73.13 %, while atomic percent of O1s is found to be 26.87 %. Frone et al. (2020) also reported the presence of C1s and O1s in the surface layer of PHB biopolymer with atomic percent of 69.99 % and 30.01 %, respectively. The deconvoluted C1s spectrum of extracted PHB exhibited three photoemission peaks at 284.8 eV, 286.4 eV and 288.9 eV corresponding to C–C accounting for 65.96 % area, C–O accounting for 20.62 % area, and C=O accounting for 13.41 % area, respectively. Similar results were reported in the literature with binding energy peaks corresponding to polyester (Mohapatra et al., 2016; Frone et al., 2020; de Sousa Junior et al., 2022; Kumari et al., 2022).

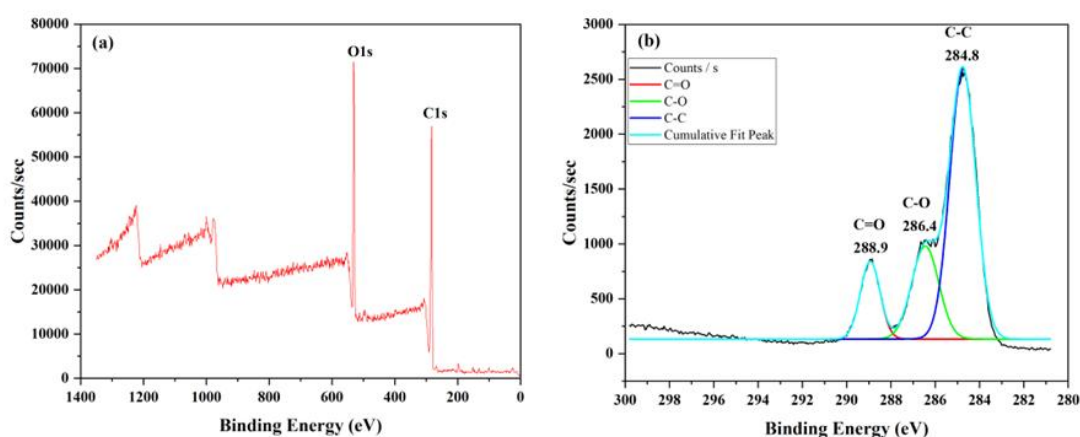


Fig. 2.12. (a) Wide-scan survey XPS spectrum of PHB from *Bacillus* sp. KE4 strain
(b) XPS C1s core-level spectrum of PHB

2.6.10. Mechanical properties

To assess the mechanical properties of the biopolymer, tensile strength, elongation at break, and Young's modulus were measured. The amount of force needed to pull a material until it breaks is measured by its tensile strength. Elongation at break (%) measures how far a material can stretch from its initial length before breaking. Young's modulus gives an indication of the stiffness of the biopolymer, which can vary from the highly ductile to rigid biopolymer (Stanley et al., 2020). The tensile strength, elongation at break, and Young's modulus of our PHB biopolymer were 26.6 MPa, 1.44 % and 1.84 GPa, respectively. The findings indicated the rigid and brittle nature of the PHB biopolymer, which is in accordance to other reported articles. The tensile strength, elongation at break, and Young's modulus of PHB biopolymer produced from *Paracoccus denitrificans* ATCC 17741 were 20.96 MPa, 1.57 % and 1.54 GPa, respectively (Napathorn, 2014). Stanley et al. (2020) reported the tensile strength (26 MPa), elongation at break (1.15 %), and Young's modulus (3.1 GPa) of PHB biopolymer produced from *Halomonas venusta* KT832796. Maity et al. (2020) reported the tensile strength (10.3 MPa), elongation at break (1.4 %), and Young's modulus (0.7 GPa) of PHB biopolymer produced from *Zobellella tiwanensis* DD5.

2.6.11. Biodegradation of PHB film

As the film degraded, its weight dramatically decreased. The biodegradation percentage rises in tandem with the degradation time (Fernandes et al., 2020). The initial weight of the PHB film before burying into the soil was 7.06 ± 0.06 mg. After 7 days, the weight of the film decreased to 6.83 ± 0.07 mg with only 3.25 % biodegradation percentage, followed by 5.79 ± 0.3 mg after 14 days with 17.9 % biodegradation percentage, and after 21 days, the weight of the film was 4.77 ± 0.38 mg with 32.4 % biodegradation percentage. Finally, after 28 days, the weight of the PHB film was found to be 2.73 ± 0.19 mg with biodegradation percentage of 61.34 %. During the experiment the physical morphological alterations of the film's surface, which included holes, ruptures, breaches, and a gradual deterioration of its parts, were visible to the unaided eye (figure 2.13a). Figure 2.13b presents the mass loss graphically and the weight loss and degradation percentage of PHB film is presented in supplementary table 2.1. Findings firmly confirm that the isolated PHB is biodegradable. The crystalline nature and irregular surface of the PHB film,

which facilitate microbial attachment and subsequent development of micropores on the surface, may be the cause of the film's faster degradation under natural conditions (Boyandin et al., 2012).

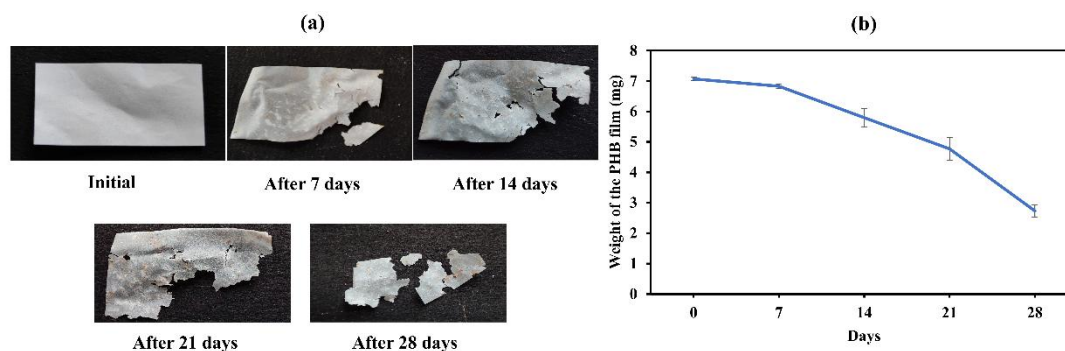


Fig. 2.13. (a) The morphological changes of PHB film buried in soil were studied for four weeks, and (b) Reduction in mass of PHB film

2.6.12. Cytotoxicity assessment

PHB has attracted much attention because of its biodegradation and excellent biocompatibility (Hamdy et al., 2022). Therefore, to determine the quantitative level of biocompatibility of the extracted PHB film, an *in-vitro* cytotoxicity test was performed. A significant aspect in determining the biocompatibility of PHB films is cell viability; a higher viability value often denotes better cell proliferation (Zhuikov et al., 2020). The cell viability (MTT assay) study in HepG2 cells indicates that PHB exposure up to 400 $\mu\text{g/mL}$ does not induce any significant ($p < 0.05$) changes in viability (figure 2.14a) which indicates its non-cytotoxic nature. When the cell viability value is $< 50\%$ in comparison to the control cells, PHB material can show considerable toxicity (Abdo et al., 2023). However, the cell viability for our biomaterial was over 90 % in all the tested concentrations, suggesting that these PHB films are safe and may be a good option for benign packaging and biomedical applications.

Reactive oxygen species (ROS) are naturally occurring byproducts from normal cell activity that take part in cellular signaling. The superoxide anion ($\text{O}_2^{\bullet-}$) is the main ROS. Oxidative stress is caused by a rise in ROS levels, which negatively impacts cell homeostasis, structures, and functions (Snezhkina et al., 2019). To determine whether the PHB film induces cellular oxidative stress in

human cell, oxidative stress assay was performed. The result revealed that PHB does not induce concentration dependent cellular total ROS generation (figure 2.14b) and superoxide generation (figure 2.14c) in a significant manner ($p < 0.05$). Also, the validation study on HepG2 cells indicates that, there was no induction of superoxide generation (DHE fluorescence) during PHB exposure (figure 2.14d). The above results cumulatively indicate the biocompatible nature of the PHB. In one study, Maity et al. (2020) used the human embryonic kidney cell line (HEK-293) to examine the cytocompatibility of PHB. The result showed that the cell viability increased up to 300 $\mu\text{g/mL}$ and then decreased, which confirmed non-cytotoxic nature of the PHB.

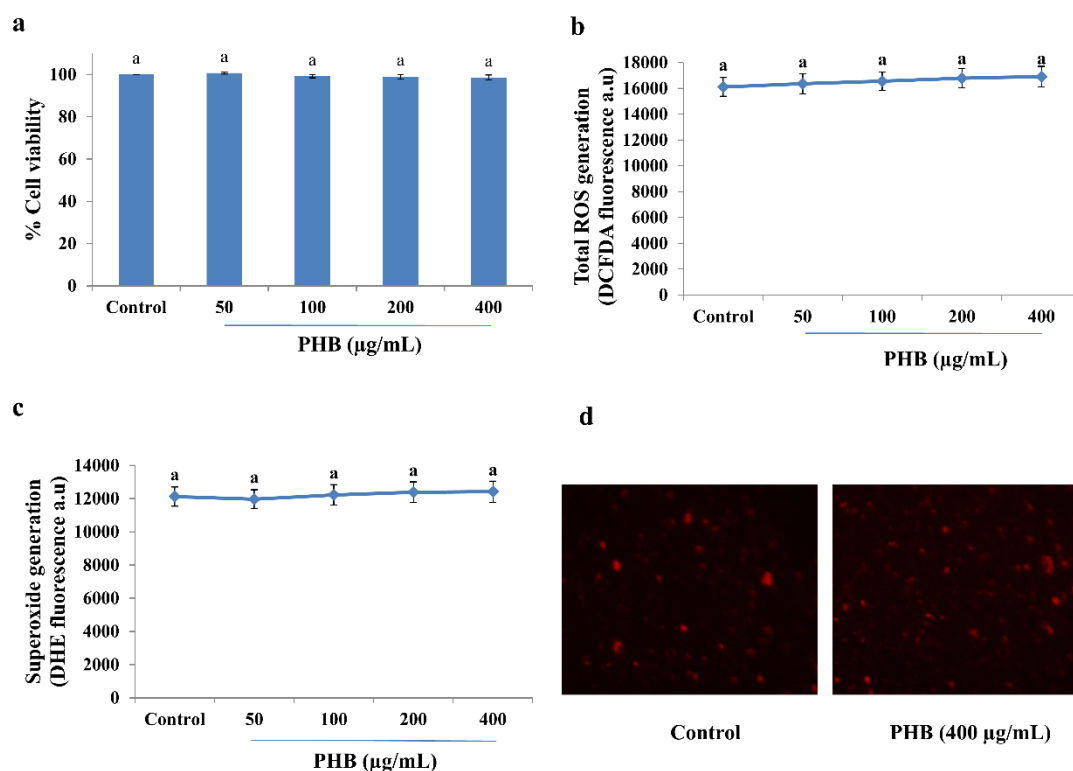


Fig. 2.14. Biocompatibility studies - Effect of PHB on cytotoxicity and oxidative stress. (a) Concentration dependent effect of PHB on cell viability, (b) cellular oxidative stress by means of total ROS generation, (c) cellular superoxide generation, and (d) its representative cellular images of dihydroethidium (DHE) fluorescence for control and PHB exposure. Data represented are mean $\pm$ SD of

three identical experiments. Values labeled with distinct letter indicate statistically significant difference with each other ($p < 0.05$)

2.7. Conclusion

In the present study, PHB producing bacterium was isolated from kitchen effluent waste and identified as *Bacillus* sp. KE4 using 16S rRNA analysis. The biopolymer produced by *Bacillus* sp. KE4 was confirmed to be polyhydroxybutyrate from FTIR, GC-MS, ^1H NMR, and ^{13}C NMR results. The isolate accumulated PHB up to 70.4 % of the DCW 4.4 g/L with a PHB concentration of 3.1 g/L and PHB yield of 0.15 g/g. The tensile strength (26.6 MPa), elongation at break (1.44 %) and Young's modulus (1.84 GPa) showed rigid and brittle nature of PHB. When PHB film was buried under the soil to determine its biodegradability, it was found to be degraded by 61.34 %. In addition, PHB film was also exposed to HepG2 cells for an *in-vitro* cytotoxicity assay to determine its biocompatibility. The result revealed that PHB exposure up to 400 $\mu\text{g/mL}$ does not induce any significant ($p < 0.05$) changes in viability which indicates its non-cytotoxic nature. Moreover, PHB biomaterial did not induce concentration dependent cellular total ROS generation and superoxide generation, exhibiting its biocompatible nature. Its biodegradable and biocompatible qualities make it an excellent option for the food packaging, biomedical, and agricultural industries. However, future research and development are imperative towards optimizing the culture conditions in order to achieve more improved quality and yield of PHB, along with its uses and efficiency.

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CHAPTER-3

Evaluation of Various Carbon Feedstock and Modelling of Fermentation Process for Scaling-up of Polyhydroxybutyrate (PHB) Production from *Bacillus* sp. KE4

3.1. Abstract

The synthesis of biopolymers from microorganisms plays a significant role in biotechnological applications for sustainable ecological management. This work aims to enhance the fermentation process for PHB synthesis by strain *Bacillus* sp. KE4, isolated from kitchen waste effluent. A range of organic wastes, such as Bael fruit pulp, sugarcane molasses, jackfruit wastes, papaya peels, pumpkin wastes, and glucose individually, were evaluated for their viability as carbon sources for PHB production. Of the carbon sources examined, glucose emerged as the most effective substrate for PHB synthesis. Statistical methodologies such as Plackett-Burman Design (PBD) and Central Composite Design (CCD) within Response Surface Methodology (RSM) were employed to assess the significant variables affecting the yield of PHB. Initially, three parameters (glucose, ammonium sulfate, and temperature) were selected from PBD for further optimization using CCD, wherein the interactions among these factors were examined in depth, resulting in a final optimized medium for enhanced biomass and PHB yield. The optimized medium produced a maximum biomass dry cell weight (DCW) yield of 4.81 g/L and a PHB yield of 3.48 g/L. The maximum yield was attained under ideal conditions of 25.28 g glucose concentration, 1.97 g ammonium sulfate concentration, and a constant temperature of 37 °C. The primary focus of the study was to evaluate different organic wastes and glucose as carbon sources for PHB production, and optimizing the production processes to attain maximum yield using the RSM model.

Keywords: Polyhydroxybutyrate (PHB), Sustainable, Plackett-Burman Design (PBD), Central Composite Design (CCD), Response Surface Methodology (RSM), Optimization

3.2. Introduction

The extensive use of petroleum-based plastics has caused significant environmental problems due to their non-biodegradable characteristics, leading to accumulation of used plastic wastes and accountable for the generation of noxious micro- and nano-plastic contaminants along with various ecological disturbance (Hassan et al., 2019). The global production of plastic has escalated from 60 million metric tonnes in 1980 to 368 million tonnes (Mt) in 2019 and is anticipated to exceed 600 Mt by 2025. Global plastic production is projected to double in the next 20 years if it continues to expand at the current rate (Tyagi et al., 2021). Despite several recycling initiatives, plastics discarded in municipal landfills persist causing significant issues. Consequently, there has been a growing number of initiatives to identify renewable, biodegradable, and recyclable materials, also referred to as green materials, that contribute to sustainability (Adnan et al., 2022). Therefore, global interest in bio-based polymers has surged as an environmentally sustainable alternative that mimics plastic in its physicochemical features, addressing the heightened demands in quickly advancing sectors such as biomedical and industrial domains (Geethu et al., 2021). Accordingly, it is anticipated that the capacity of the world to produce bioplastics would rise dramatically, from about 2.47 Mt in 2024 to around 5.73 Mt in 2029 (European Bioplastics, 2025). Thus, the circular economy and environmental consciousness have driven research into sustainable and renewable bio-based polymers as substitutes for synthetic plastics. In this context, alternative biodegradable and environmentally sustainable biopolymers, such as polyhydroxybutyrate (PHB), possess the potential to supplant linear usage and disposal patterns of plastics with a completely circular lifecycle (Guimaraes et al., 2025).

Several microorganisms produce polyhydroxybutyrate (PHB), a monomer of polyhydroxyalkanoates (PHAs), which is biosynthesised and accumulates in intracellular lipid granules. PHB is a low molecular weight ($5\text{-}10\times 10^5$); brittle, low-elasticity, thermoplastic, renewable, biodegradable, non-toxic, biocompatible, eco-

benign, resilient and hydrophobic material, exhibiting physical, thermal, and mechanical properties comparable to those of synthetic polymers. PHB biopolymer possesses several applications in medicine, agriculture, automotive, packaging, and cosmetic sectors (Nagajothi and Murugesan, 2023). Research on *Bacillus* spp., a well-known producer of PHAs, have been studied extensively compared to other microorganisms due to their prevalence in extreme environmental conditions, superior growth rates on complex substrates, enhanced capacity for PHA production, and enzymatic systems that enable the utilization of diverse complex compounds for PHA synthesis (Morya et al., 2018). Microorganisms utilize carbon for energy generation and biosynthetic processes. Certain bacterial species accumulate intracellular PHB granules within their cells as a carbon and energy source and can be produced in a larger amount industrially *via* bacterial fermentation (McAdam et al., 2020). The significance of carbon-rich culture media in the synthesis of PHBs is vital. An adequate carbon supply is crucial for microbial development and the synthesis of PHBs. Examples of specified carbon sources include simple sugars such as glucose, sucrose, fructose, mannose, xylose, galactose, and as well as polysaccharides like starch and lipids such as glycerides and oleates. Nitrogen is also an essential element in fermentation media, serving as a crucial constituent of proteins, nucleic acids, and coenzymes such as vitamins (Allikian et al., 2019).

When it comes to the point of large-scale production of PHB for industrial application, optimization of fermentation process is very crucial. Manual or classical optimization is labour-intensive, susceptible to errors, and inadequate for analysing interactions among several variables; statistical designs offer a range of important tools for developing a well-structured bioprocess methodology (Weuster-Botz, 2000). Optimizing the fermentation process increases the production rate of the final product significantly. Statistical optimization employing Plackett-Burman design (PBD) and Central Composite Design (CCD) of Response Surface Methodology (RSM) are some of common statistical tool that are extensively utilized to formulate effective bioprocess strategies (Sabapathy et al., 2019). Numerous studies have been conducted to enhance the production of PHB utilizing these tools. For instance, Evangeline and Sridharan (2019) examined various media

components and investigated the production of PHB by a *Bacillus cereus* strain VIT-SSR1 through the application of RSM. Recently, Kumar et al. (2025) also investigated the production of PHB by a strain *Klebsiella* spp. MK3 utilizing RSM. The optimization of physical variables that significantly influence the fermentation process was also extensively conducted by Hassan et al. (2019) and Adnan et al. (2022).

In the present investigation, we screened various organic wastes, including Bael fruit pulp (*Aegle marmelos* L. Corrêa), sugarcane (*Saccharum officinarum* L.) molasses, jackfruit (*Artocarpus heterophyllus* Lam.) wastes, papaya (*Carica papaya* L.) peel, and pumpkin (*Cucurbita pepo* L.) wastes, and glucose individually, for their potential as carbon sources for PHB production. The optimal carbon source was identified, and optimization was performed through RSM, focusing on experimental factors that significantly influence PHB production.

3.3. Materials and methods

3.3.1. Chemicals and reagents

All the chemicals and reagents such as ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate (MgSO_4), citric acid, ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), manganese (II) sulfate pentahydrate ($\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), sodium tetraborate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), ammonium heptamolybdate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}]$, and glucose were purchased from Merck Specialities Private Limited, Mumbai and Qualigens, Thermo Fisher Scientific, India Private Limited. Sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), sodium hypochlorite (NaClO , 4 %), acetone, ethanol, chloroform, and hydrochloric acid (HCl) were purchased from Merck Specialities Private Limited, Mumbai, India. Nutrient broth and bacteriological agar purchased from Himedia Private Limited, Mumbai.

3.3.2. Microorganism and culture maintenance

The bacterial strain of interest, *Bacillus* sp. KE4 (accession number OR775565), has previously been isolated from a kitchen waste effluent site located

in Lumami, Zunheboto, Nagaland, India (Acharjee et al., 2024). The strain was routinely sub-cultured on nutrient agar medium (Himedia) and preserved at 4 °C.

3.3.3. Substrate collection and hydrolysate preparation

Locally collected organic wastes were shredded into pieces and dried in a hot air oven at 50 °C for 2-3 days before being pulverized into fine particles. The resulting powder was subjected to simple acid pretreatment followed by thermal hydrolysis under optimum conditions as described by Raju et al. (2024). 20 % (w/v) of the organic waste was acid pretreated using 5 % (v/v) H₂SO₄. It was then thermally hydrolysed at 121 °C for 20 minutes and filtered using Whatman filter paper. The supernatant was neutralized by 1 N NaOH, and again centrifuged at 13,000 rpm for 15 minutes. Finally, a clear hydrolysate was obtained which can be substituted as a carbon source for PHB production. The hydrolysates extract at 4 % concentration, supplemented with the media component except for carbon source, were used as production media for PHB biosynthesis by *Bacillus* sp. KE4 (Danial et al., 2021). Sugarcane molasses was autoclaved for 20 minutes at 121 °C and subsequently centrifuged for 10 minutes at 6000 rpm to eliminate debris, after which a 4 % (v/v) concentration was added to the medium component (Shin et al., 2024).

3.3.4. PHB production under different carbon sources

PHB production medium comprises; (NH₄)₂SO₄ 2 g/L, KH₂PO₄ 13.3 g/L, MgSO₄ 1.3 g/L, citric acid 1.7 g/L, the trace element solution 10 mL/L (g/L, FeSO₄·7H₂O 10, ZnSO₄·7H₂O 2.25, CuSO₄·5H₂O 1.0, MnSO₄·5H₂O 0.5, CaCl₂·2H₂O 2.0, Na₂B₄O₇·10H₂O 0.23, (NH₄)₆MO₇O₂₄ 0.1, and HCl 10 mL) and glucose 20 g/L (Appendix II). PHB was produced in 250 mL Erlenmeyer flasks containing 100 mL of production medium, which were inoculated with 2 % (v/v) of a 24 h old seed culture. Subsequent to inoculation, the cultures were grown using shaking culture method for 72 h at 37 °C and 180 rpm (Acharjee et al., 2024).

3.3.5. Extraction, purification and quantification of PHB

Initially, the bacterial cells were centrifuged at 10,000 rpm for 10 minutes to extract the pellets, which were subsequently dried overnight at 40 °C to ascertain the DCW in g/L. Subsequently, the pellets were suspended in a 4 % (v/v) NaClO solution and incubated in an orbital shaking incubator for 2 h at 37 °C to ensure

complete digestion of all non-PHB cells. Following to the digestion phase, the resultant mixture underwent centrifugation at 10,000 rpm for 10 minutes for separating the PHB granules, after which the supernatant was discarded. To eliminate NaClO entirely, the pellets were rinsed twice with distilled water and subsequently washed with a solution of ethanol, distilled water, and acetone in a 1:1:1 (v/v/v) ratio. The pellets were then introduced into the boiling chloroform, while undissolved substances were eliminated from the solution using filtration, and the filtrate was transferred to sterilized glass Petri dish. Ultimately, the chloroform evaporated, resulting in the formation of a thin biopolymer film. The DCW of the cell pellet was determined for the quantitative evaluation of PHB. The amount of PHB in the DCW's composition was utilized to ascertain the extent of intracellular PHB accumulation (Shah and Kumar, 2021).

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of obtained PHB (gm/L)}}{\text{DCW of bacterial biomass (gm/L)}} \times 100 \quad (3.1)$$

3.3.6. Transmission electron microscopy (TEM) analysis

PHB granules within the cells of *Bacillus* sp. KE4 were identified using High-Resolution Scanning Transmission Electron Microscopy (HR-STEM). Samples were centrifuged at 8,000 rpm for 10 minutes at ambient temperature to obtain a pellet. Pellets were subsequently rinsed with phosphate-buffered saline (PBS, pH 7) (Appendix II), fixed with 2 % glutaraldehyde, and stained with 1 % (w/v) osmium tetroxide (Munir and Jamil, 2017) to enhance stability during the microscopy processes such as dehydration (gradient steps of ethanol series 30 %, 50 %, 70 %, 90 %, and 100 %), embedding, and electron bombardment. Moreover, it also prevents degradation and deformation of fine cellular ultrastructure of the bacterial cells. TEM analysis was conducted by making grids of the bacterial samples and thereafter viewing these grids using a Thermo Fisher Scientific Talos F200X G2 200 kV HR-STEM microscope. For grid preparation, the sample was positioned on 3 mm carbon-coated copper grids, which were subsequently stained with phosphate tungsten acetate stain. The prepared grids were positioned in a single tilt holder and introduced into the TEM assembly. The acquired TEM images were examined and analysed using Thermo Scientific Velox software.

3.3.7. Fourier transform infrared (FTIR) spectroscopy

Initially, 5 mg of the extracted PHB film was thoroughly mixed with dry potassium bromide (KBr), and the resulting mixture was crushed into a KBr pellet die. The material was subsequently analysed with a spectrophotometer (PerkinElmer, Spectrum Two). 64 scans were conducted to capture the spectra within the 4000-400 cm^{-1} range (Wendy et al., 2022).

3.3.8. Screening and optimization of PHB production media components using statistical experimental design

3.3.8.1. Plackett–Burman design (PBD)

The implementation of Design of Experiments (DoE), namely *via* the Plackett-Burman design (PBD), provides an effective approach for identifying significant variables in PHB production. This method yields precise estimations of direct impacts by carefully structuring experiments and reducing the number of variables tested. Utilizing PBD, the core values of independent variables were encoded as High (+1) and Low (−1) levels, and the resultant response was analysed comparatively at a 95 % significance level. This technique assists in identifying important factors, establishing suitable ranges, and averting experimental failures, so promoting the advancement of sustainable PHB biosynthesis processes (Kumar et al., 2025). The choices of fermentation variables for screening, together with their respective lower and upper values, was determined based on previously published studies (Sharma and Bajaj, 2015; Adnan et al., 2022; Grigary and Umesh, 2023).

PHB production was conducted in a 250 mL Erlenmeyer flask, fitted with a cotton plug, containing a working volume of 100 mL of production medium. Eleven variables, including glucose, ammonium sulfate, potassium dihydrogen phosphate, magnesium sulfate heptahydrate, citric acid, trace elements, pH, temperature, incubation period, inoculum size, and agitation speed, were considered. A total of 12 experiments was generated using Design Expert software version 13. The submerged fermentation process was conducted, and the produced PHB was quantified. To ascertain reproducibility, every phase of the experiment conducted in the flask was performed in triplicate, with results presented as means $\pm$ standard deviations (SDs). PBD is limited to screening the most efficient parameters and is

not suitable for optimization. Consequently, a central composite design (CCD) was implemented for effective optimization (Sehgal et al., 2023).

3.3.8.2. Central composite design (CCD)

CCD was conducted to assess the impact of key variables identified through the Plackett-Burman design. Critical factors were identified using a half-normal plot and Pareto chart, leading to the selection of three variables: glucose, ammonium sulfate, and temperature. An experimental design comprising 20 trials was established. The study aimed to elucidate the impact of these parameters on responses such as biomass DCW and PHB yield (Geethu et al., 2021).

3.3.9. Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA) to validate the experimental model. The significance ($p < 0.05$), quality, and relevance of the model equation were assessed using the coefficient of determination (R^2), and Fisher's test (F-test) to evaluate the overall significance of the regression model.

3.4. Results and discussion

3.4.1. Screening of best carbon source for PHB production

PHB production from different carbon sources was calculated (Table 3.1) and the same is represented in figure 3.1. From different carbon sources, medium supplemented with 20 g/L glucose showed a maximum biomass DCW of 4.4 g/L, PHB yield of 3.1 g/L, and PHB content of 70.4 %. Therefore, production medium supplemented with glucose as a carbon source was used for further optimization using Response Surface Methodology (RSM). It is well known that the bacterium *Bacillus* species prefer glucose as its primary carbon and energy source. In the phosphoenolpyruvate:sugar phosphotransferase system, the glucose permease (PtsG) transports and simultaneously phosphorylates glucose (Bachem et al., 1997). The biosynthesis of PHB commences with the metabolism of glucose, producing acetyl-CoA and NADPH (Nicotinamide adenine dinucleotide phosphate) *via* the glycolytic and pentose phosphate pathways (PPP). Subsequently, the two acetyl-CoA molecules undergo condensation by β -ketothiolase (PhaA) to form acetoacetyl-CoA, which was then reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA dehydrogenase (PhaB) utilizing NADPH as a cofactor, and

ultimately polymerized into PHB *via* PHB polymerase (PhaC) (Mohapatra et al., 2017).

Table 3.1. Comparison of Biomass DCW, PHB yield, and PHB content using different carbon sources

Carbon sources	Biomass DCW (g/L)	PHB yield (g/L)	PHB content (%)
Glucose	4.4 ± 0.05	3.1 ± 0.03	70.4
Molasses	4.09 ± 0.21	1.86 ± 0.07	45.47
Jackfruit waste	3.13 ± 0.35	1.22 ± 0.05	38.9
Papaya peel	3.63 ± 0.2	1.05 ± 0.07	28.92
Pumpkin waste	2.66 ± 0.25	0.23 ± 0.04	8.64
Bael pulp	2.13 ± 0.2	0.11 ± 0.03	5.16

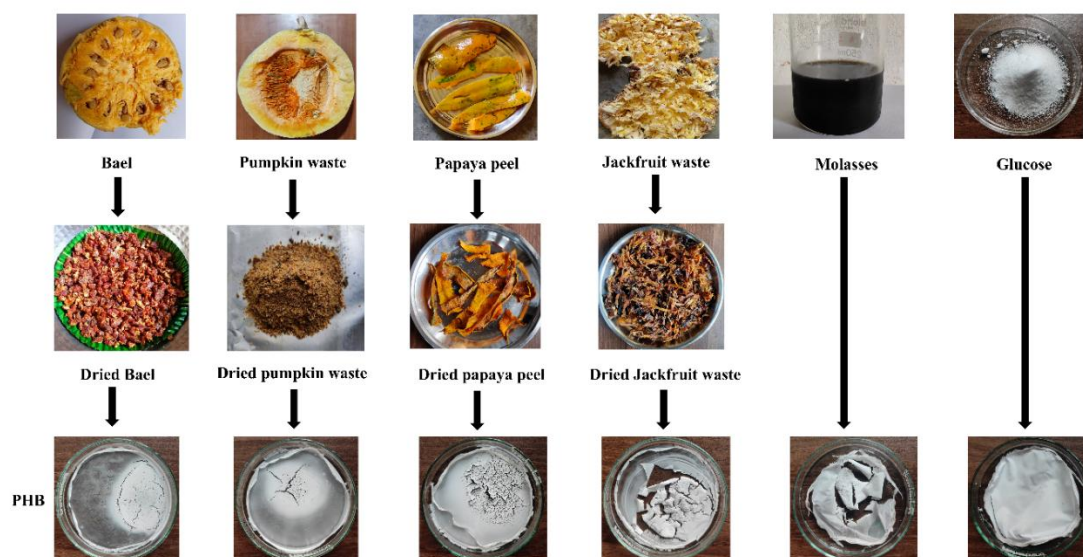


Fig. 3.1. PHB film produced from different carbon sources

3.4.2. TEM analysis

The TEM images of intracellular PHB granules stored within *Bacillus* sp. KE4, using glucose as the sole carbon source is presented in figure 3.2. TEM examination demonstrated the aggregation of dense PHB granules within the bacterium's cytosol. The cytosol included two large granules with average diameters of 1.525 μm and 0.425 μm . Numerous researches have documented the presence of PHB granules of varying sizes within the cytosol (Poomipuk et al., 2014; Munir and Jamil, 2017; Sriyapai et al., 2022). Consequently, TEM images validated the findings of quantitative analyses, demonstrating that the bacterial strain is a proficient producer of PHB.

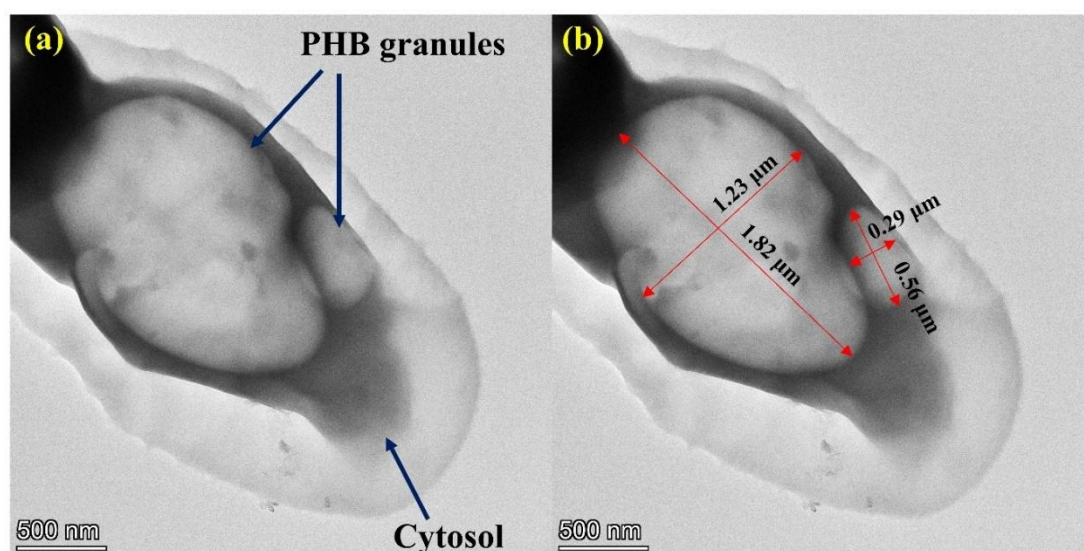


Fig 3.2. (a) TEM images of intracellular PHB granules stored inside *Bacillus* sp. KE4 (b) image showing the dimensions of the PHB granules

3.4.3. FTIR spectroscopy

Figure 3.3 demonstrates the identification of functional groups in the extracted biopolymers from various carbon feedstocks by FTIR analysis. The absorption bands detected in the 2800-2900 cm^{-1} spectral area are ascribed to the CH stretching bond, a distinctive feature of PHB biopolymers. The absorption bands between 1720-1740 cm^{-1} are ascribed to the carbonyl (C=O) stretching of an ester group, recognized as markers of PHB. The observed absorption bands align with prior research on FTIR analysis of PHB (Kovalcik et al., 2017; Mostafa et al., 2020; Siripurapu et al., 2022).

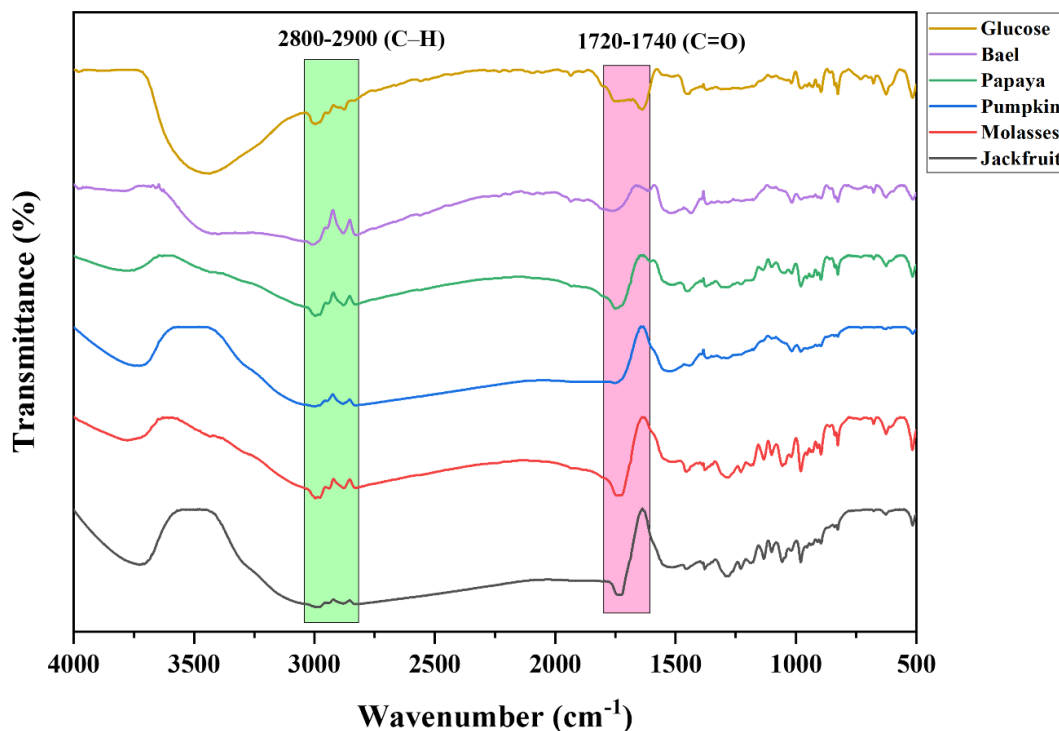


Fig 3.3. FTIR spectra of PHB produced from various carbon feedstocks

3.4.4. Optimization of PHB Production from *Bacillus* sp. KE4 Using Response Surface Methodology (RSM) approach

3.4.4.1. Plackett Burman Design (PBD)

The PBD was utilized to identify the factors that significantly influence PHB accumulation. Each variable was examined at two levels: lower and higher, as presented in Table 3.2. A half-normal plot and a Pareto chart were generated to illustrate the factors significantly affecting PHB production (Sehgal et al. 2023). Table 3.3 shows the impact of these factors, while Table 3.4 shows the analysis of variance (ANOVA) and fit statistics for the experimental design for the PBD analysis. Probability values (p-values) below 0.05 indicate that the variables significantly affect the response variables, namely biomass and PHB yield. The model F-value of 259 validated the model's significance. The likelihood that this model F-value was caused by noise is a mere 0.01%. P-values below 0.05 signify that the model terms are statistically significant. The Predicted R^2 of 0.9771 aligns closely with the Adjusted R^2 of 0.9860, with a difference of less than 0.2. Adequate precision quantifies the signal-to-noise ratio, with a ratio exceeding 4 being

considered optimal. The ratio of 41.832 signifies an adequate signal, demonstrating the model's adequacy. The coefficient of variation (CV) was measured at 5.38, which is within the acceptable range, as a CV value of less than 10 is considered desirable. The model was utilized for navigating the design space. In this scenario, glucose, ammonium sulfate, and temperature are identified as significant terms within the model. When the values exceed 0.1000, the model terms are considered to be non-significant.

Table 3.2. Different levels of experimental factors selected for PHB production by *Bacillus* sp. KE4 using PBD

No.	Components (factors)	Low level (-1)	High level (+1)
1	Glucose	10	30
2	Ammonium sulfate	1	3
3	Potassium dihydrogen phosphate	10	15
4	Magnesium sulfate	1	2
5	Citric acid	1	2
6	Trace elements	8	12
7	pH	6	8
8	Temperature	30	40
9	Incubation period	48	96
10	Inoculum size	1	3
11	Agitation speed	80	160

		Fa ct or 1	Fa ct or 2	Fa ct or 3	Fa ct or 4	Fa ct or 5	Fa ct or 6	Fa ct or 7	Fa ct or 8	Fa ct or 9	Fact or 10	Fact or 11	Response 1	Response 2			
S t r u c t u r e	Ru n	A: Gl uc os e	B: A m m on iu m su lfa te	C: Po tas si u m di hy dr og en ph os ph at e	D: M ag ne si u m su lfa te he pt ah yd rat e	E: Ci tri c aci d	F: Tr ac e ele m en ts	G: p H	H: Te m pe rat ur e	J:I nc ub ati on pe rio d	K:In ocul um size	L:A gitat ion spee d	Biomass DCW	PHB yield			
		g/ L	g/ L	g/ L	g/ L	g/ L	m L		°C	h	%	rpm	g/L	g/L			
														Actual value	Predic ted	Actual value	Predic ted

													value (± 0.12 8 SD)	value (± 0.10 4 SD)			
3	1	30	1	15	2	1	12	8	40	48	1	80	3.86 $\pm$ 0.0 36	3.93	0.92 $\pm$ 0.0 78	0.9233	
6	2	10	1	10	2	1	12	8	30	96	3	160	1.73 $\pm$ 0.0 50	1.61	0.18 $\pm$ 0.0 20	0.1633	
1 2	3	10	1	10	1	1	8	6	30	48	1	80	1.54 $\pm$ 0.0 45	1.61	0.14 $\pm$ 0.0 35	0.1633	
5	4	10	1	15	1	2	12	6	40	96	3	80	1.95 $\pm$ 0.0 55	2.08	0.22 $\pm$ 0.0 41	0.3533	
8	5	30	3	10	1	1	12	6	40	96	1	160	3.12 $\pm$ 0.0 15	3.16	0.60 $\pm$ 0.0 50	0.6633	
7	6	30	1	10	1	2	8	8	40	48	3	160	4.18 $\pm$ 0.0 62	3.93	1.12 $\pm$ 0.0 58	0.9233	
9	7	30	3	15	1	1	8	8	30	96	3	80	2.72 $\pm$ 0.2 91	2.69	0.46 $\pm$ 0.0 91	0.4733	
1	8	30	3	10	2	2	12	6	30	48	3	80	2.63 $\pm$ 0.2 90	2.69	0.41 $\pm$ 0.0 95	0.4733	
4	9	10	3	10	2	2	8	8	40	96	1	80	1.28 $\pm$ 0.0 15	1.31	0.08 $\pm$ 0.0 15	0.0933	
2	10	10	3	15	1	2	12	8	30	48	1	160	0.92 $\pm$ 0.1 18	0.8350	0.04 $\pm$ 0.0 2	- 0.0967	
1 1	11	30	1	15	2	2	8	6	30	96	1	160	3.34 $\pm$ 0.2 85	3.46	0.68 $\pm$ 0.0 55	0.7333	

1	12	10	3	15	2	1	8	6	40	48	3	160	1.31±0.0	1.31	0.11±0.0	0.0933
0													45		26	

Table 3.3. PBD showing impact of eleven different variables on PHB production from *Bacillus* sp. KE4

Table 3.4. ANOVA and fit statistics for the experimental design for the PBD analysis

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	12.75	3	4.25	259.00	< 0.0001	significant
A-Glucose	10.30	1	10.30	628.17	< 0.0001	
B-Ammonium sulfate	1.78	1	1.78	108.43	< 0.0001	
H-Temperature	0.6627	1	0.6627	40.40	0.0002	
Residual	0.1312	8	0.0164			
Cor Total	12.88	11				
Std. Dev.	0.1281		R ²	0.9898		
Mean	2.38		Adjusted R ²	0.9860		
C.V. %	5.38		Predicted R ²	0.9771		
			Adeq Precision	41.8322		

A half-normal plot and Pareto chart, illustrated in figure 3.4, demonstrate the relative significance of factors in a PBD concerning biomass and PHB yield. In half-normal plots, variables exhibiting significant effects are likely to be positioned far to the right of the plot, deviating from the straight line that represents the majority of the points. A substantial disparity between significant and insignificant effects is frequently noted. Variables exhibiting insignificant effects are likely to cluster near the origin, adjacent to the straight line (Daniel, 1959). Three factors *i.e.*, glucose, ammonium sulfate, and temperature were observed to deviate significantly from the straight line on the plot, indicating a substantial impact on the response variables.

In the Pareto chart, the three factors surpass the t-value threshold of 2.306, demonstrating their significant influence on the response variables. The height of the bars on the chart indicates the magnitude of each factor's impact, facilitating straightforward comparisons. This chart serves as an effective instrument for identifying the primary factors influencing the response variable and emphasizing those that require additional examination or enhancement. Furthermore, it offers a succinct and precise method for conveying the results of the PBD (Kumar et al., 2025). The Pareto chart indicates that glucose and temperature positively influence PHB production, suggesting that increasing these factors results in favourable changes in the response variables. Ammonium sulfate exhibited a negative effect on PHB production, suggesting that a reduction in the factor's level results in an improvement in the response variables. Evangeline and Sridharan (2019) identified three critical factors: molasses, ammonium sulphate, and pH, that are essential for the production of PHB. In another investigation, findings were similarly derived, indicating that rice bran, peptone, and temperature emerged as the most significant factors influencing production (Adnan et al., 2022). The literature indicates that the sources and concentrations of carbon and nitrogen are essential for the synthesis of PHB. Nevertheless, certain research has indicated that various mineral salts can also enhance the production of PHB (Grigary and Umesh, 2023; Nagajothi and Murugesan, 2023).

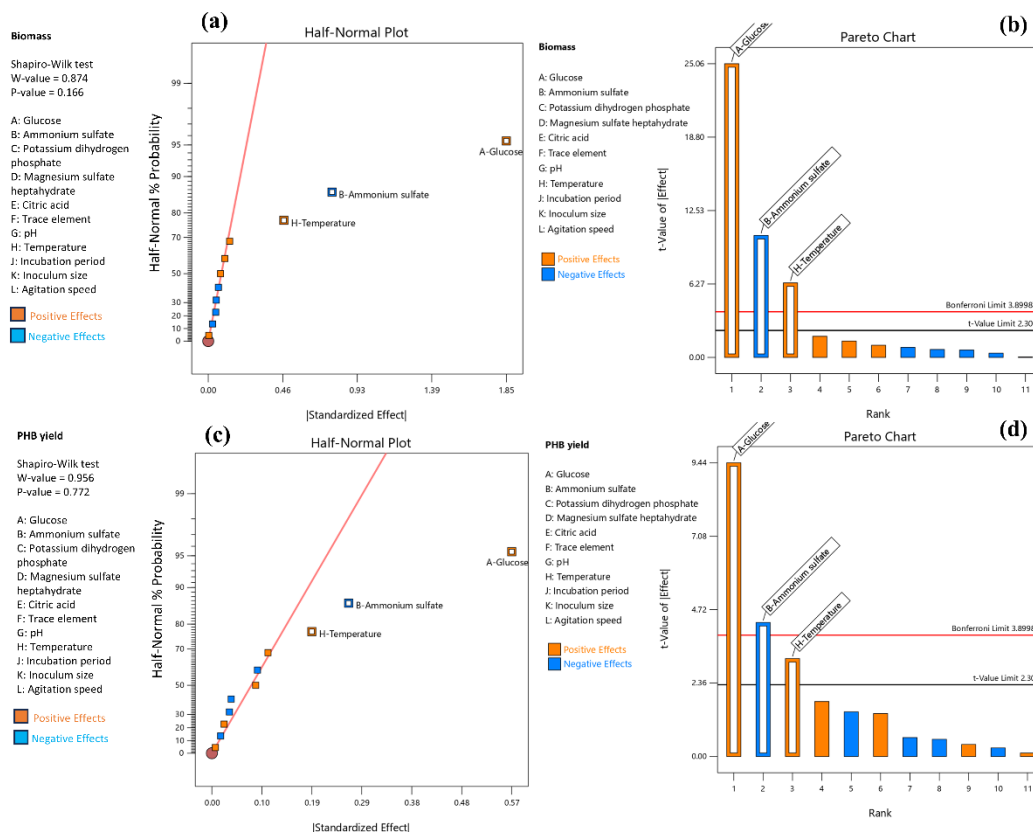


Fig 3.4. Half-Normal plot and Pareto chart showing most significant factors affecting biomass DCW and PHB yield

3.4.4.2. Predicted values vs actual values

The significance of the graph that compares predicted and actual values is rooted in its ability to evaluate the model's goodness of fit. The model is considered accurate and reliable when the predicted values align closely with the actual values. On the other hand, significant discrepancies between the predicted and actual values indicate that the model may require additional refinement or study. The data from 12 runs involving eleven factors, as shown in figure 3.5, align along a straight line, demonstrating the accuracy of the data and confirming the absence of outliers.

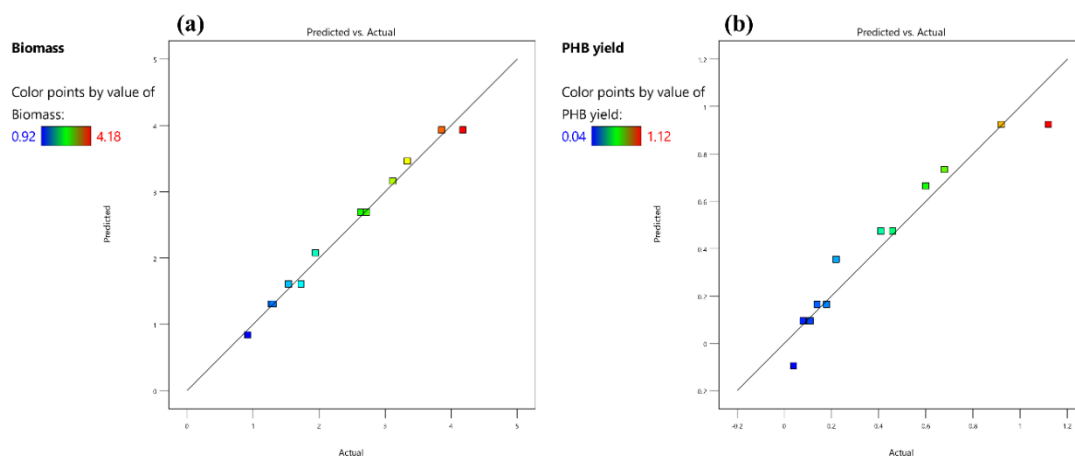


Fig. 3.5. Predicted and actual value of biomass and PHB yield in graphical form derived from PBD analysis

3.4.4.3. Central Composite Design

Three variables found from PBD tests were optimized using central composite design (CCD) of response surface methodology (RSM), while maintaining the remaining variables at a fixed level. Twenty experimental runs generated by Design Expert 13 were conducted, and the experimental values were inputted into the response column. Table 3.5 presents the variables and levels for CCD. The responses from CCD were analysed using standard ANOVA, which demonstrated that the model was highly reliable (Table 3.6). The quadratic model was determined to be the most effective and utilized for subsequent investigation (Table 3.7 and 3.8). A p-value < 0.05 signifies that the model terms are statistically significant. In this scenario, A, B, C, AB, AC, A², and B² are significant model terms. A larger t-test magnitude and a smaller p-value indicate a more significant coefficient. The ANOVA results for the quadratic model are displayed in Table 3.7.

Table 3.5. Low and high levels for 3 factors screened for PHB production by *Bacillus* sp. KE4 using CCD

No.	Components (factors)	Units	Low level (-1)	High level (+1)
1	Glucose	g/L	10	30
2	Ammonium sulfate	g/L	1	3
3	Temperature	°C	30	40

Table 3.6. RSM-CCD actual and predicted values of dependent factors for PHB production by *Bacillus* sp KE4

		Factor 1	Factor 2	Factor 3	Response 1		Response 2	
S	R	A:Glucose	B:Ammonium sulfate	C:Temperature	Biomass DCW		PHB yield	
		g/L	g/L	°C	g/L		g/L	
					Actual value	Predicted value	Actual value	Predicted value
						(±0.028 SD)		(±0.010 SD)
6	1	30	1	40	3.95±0.026	3.94	2.46±0.026	2.45
7	2	10	3	40	2.11±0.026	2.09	0.91±0.055	0.9107
8	3	30	3	40	4.37±0.02	4.39	2.77±0.015	2.76
14	4	20	2	43.409	3.45±0.032	3.45	1.80±0.055	1.83

1	5	36.817	2	35	4.12±0.0	4.11	2.61±0.0	2.63
0		9			11		32	
1	6	20	3.68179	35	3.79±0.0	3.77	2.30±0.0	2.31
2					85		35	
1	7	20	0.318207	35	3.51±0.0	3.49	2.13±0.0	2.11
1					25		83	
1	8	20	2	35	4.52±0.0	4.53	3.25±0.0	3.27
5					25		55	
4	9	30	3	30	4.07±0.0	4.06	2.26±0.0	2.25
					2		41	
1	1	20	2	35	4.56±0.0	4.53	3.27±0.0	3.27
8	0				25		45	
2	1	30	1	30	3.63±0.0	3.67	1.93±0.0	1.92
	1				55		25	
2	1	20	2	35	4.50±0.0	4.53	3.25±0.0	3.27
0	2				49		15	
5	1	10	1	40	2.12±0.0	2.15	1.03±0.1	1.01
	3				3			

1	1	20	2	35	4.51±0.0	4.53	3.29±0.0	3.27
9	4				52		45	
1	1	10	1	30	2.05±0.0	2.04	0.66±0.0	0.6856
	5				36		17	
1	1	20	2	35	4.56±0.0	4.53	3.28±0.0	3.27
7	6				05		5	
9	1	3.1820	2	35	0.81±0.0	0.8037	0.04±0.0	0.0324
	7	7			3		2	
1	1	20	2	26.591	3.09±0.0	3.07	1.15±0.0	1.13
3	8				11		55	
1	1	20	2	35	4.52±0.0	4.53	3.26±0.0	3.27
6	9				32		52	
3	2	10	3	30	1.89±0.0	1.92	0.63±0.0	0.6112
	0				2		2	

Table 3.7. Lack of fit for CCD showing model summary of Biomass yield

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
Linear	0.0037	< 0.0001	0.4764	0.3797	
2FI	0.9792	< 0.0001	0.3646	-0.0766	
Quadratic	< 0.0001	0.3368	0.9994	0.9980	Suggested
Cubic	0.3180	0.3119	0.9995	0.9922	Aliased

Table 3.8. Lack of fit for CCD showing model summary of PHB yield

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
Linear	0.0334	< 0.0001	0.3002	0.1920	
2FI	0.9904	< 0.0001	0.1458	-0.4502	
Quadratic	< 0.0001	0.2063	0.9999	0.9997	Suggested

Cubic	0.3015	0.153	0.9999	0.9979	Aliased
3					

Table 3.9. ANOVA and fit statistics of quadratic model showing its significance for Biomass (g/L)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	24.04	9	2.67	3269.77	< 0.0001	significant
A-Glucose	13.18	1	13.18	16137.82	< 0.0001	
B-Ammonium sulfate	0.0930	1	0.0930	113.92	< 0.0001	
C-Temperature	0.1682	1	0.1682	205.89	< 0.0001	
AB	0.1326	1	0.1326	162.36	< 0.0001	
AC	0.0136	1	0.0136	16.67	0.0022	
BC	0.0021	1	0.0021	2.59	0.1389	
A ²	7.74	1	7.74	9477.54	< 0.0001	
B ²	1.45	1	1.45	1778.39	< 0.0001	
C ²	2.90	1	2.90	3545.88	< 0.0001	
Residual	0.0082	10	0.0008			
Lack of Fit	0.0049	5	0.0010	1.49	0.3368	not significant
Pure Error	0.0033	5	0.0007			

Cor Total	24.04	19		
Std. Dev.	0.0286		R ²	0.9997
Mean	3.51		Adjusted	0.9994
			R ²	
C.V. %	0.8153		Predicted	0.9980
			R ²	
			Adeq	184.3373
			Precision	

Table 3.10. ANOVA and fit statistics of quadratic model showing its significance for PHB yield (g/L)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	21.32	9	2.37	21282.75	< 0.0001	significant
A-Glucose	8.12	1	8.12	72989.63	< 0.0001	
B-Ammonium sulfate	0.0456	1	0.0456	410.18	< 0.0001	
C-Temperature	0.5866	1	0.5866	5271.44	< 0.0001	
AB	0.0820	1	0.0820	736.98	< 0.0001	
AC	0.0210	1	0.0210	188.82	< 0.0001	
BC	0.0003	1	0.0003	2.81	0.1247	

A ²	6.82	1	6.82	61257.36	<	
					0.0001	
B ²	2.04	1	2.04	18370.81	<	
					0.0001	
C ²	5.81	1	5.81	52174.60	<	
					0.0001	
Residual	0.0011	10	0.0001			
Lack of Fit	0.0008	5	0.0002	2.18	0.2063	not significant
Pure Error	0.0003	5	0.0001			
Cor Total	21.32	19				
Std. Dev.	0.0105		R ²	0.9999		
Mean	2.11		Adjusted	0.9999		
			R ²			
C.V. %	0.4989		Predicted	0.9997		
			R ²			
			Adeq	434.6653		
			Precision			

The F-values for biomass of 3269.77 (Table 3.9), and PHB yield of 21282.75 (Table 3.10), indicate that the model is significant. The Lack of Fit F-values for biomass (1.49) and PHB yield (2.18) indicate that the Lack of Fit is not significant in comparison to the pure error. For biomass, there exists a 33.68% probability that a Lack of Fit F-value of this magnitude may arise from noise. In the instance of PHB yield, there exists a 20.63% probability that a Lack of Fit F-value of this magnitude may arise from noise. This indicates that the model is well-suited for the data. The maximum coefficient of determination was observed, with R² values for biomass at 0.9997 and PHB yield at 0.9999. The predicted R² values for biomass were 0.9980 and 0.9997 for PHB Yield, whereas the adjusted R² values for biomass were 0.9994 and 0.9999 for PHB Yield. A value of R² close to 1 indicates a stronger correlation between the experimental and predicted values. The

signal-to-noise ratio was assessed by Adequate Precision, with a preferred ratio exceeding 4. The biomass yield ratio is 184.3373, while the PHB yield is 434.6653, indicating an adequate signal. The biomass yield ratio is 184.3373, while the PHB yield is 434.6653, indicating an adequate signal. The coefficient of variation (CV) serves as an indicator of experimental reliability and provides insight into the precision with which the experiments were conducted (Sharma and Bajaj, 2015). In the current study, the CV for biomass were 0.8153 and 0.4989 for PHB yield, with a desired CV value of less than 10. All statistical measures, including standard deviation, CV, predicted R^2 , adjusted R^2 , and adequate precision, were determined to be suitable and statistically significant. The results obtained clearly indicate that a quadratic model may be employed to navigate the design space.

The regression equation for the proposed quadratic model, expressed in terms of coded factors is outlined below as equation 3.2 and 3.3

$$\text{Biomass (R1)} = 4.5288539425023 + 0.98241762150155 *A + 0.082542151753127 *B + 0.11096592167548 *C + 0.12875 *AB + 0.04125 *AC + 0.01625 *BC + 0.7329009547998 *A^2 - 0.3174757208527 *B^2 - 0.44829047537222 *C^2 \quad (3.2)$$

$$\text{PHB yield (R2)} = 3.274745650645 + 0.77120304441966 *A + 0.057812774697054 *B + 0.20725395477918 *C + 0.10125 *AB + 0.05125 *AC - 0.0062500000000001 *BC - 0.68776680282322 *A^2 - 0.37663981910114 *B^2 - 0.63473379423423 *C^2$$

(3.3)

where, A: Glucose; B: Ammonium sulfate; C: Temperature

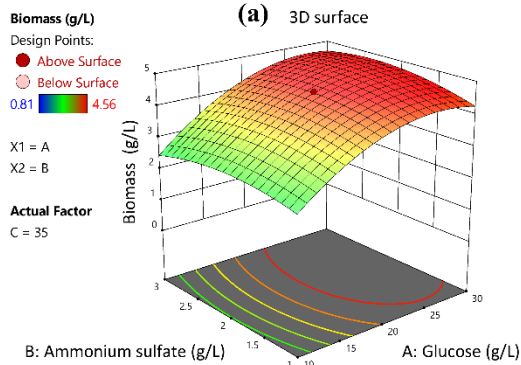
The regression equation was illustrated through 3D surface plots and 2D contour plots. The 3D response surfaces facilitate the examination of the relationship between independent variables and their impact on biomass DCW and PHB yield. The ideal conditions and cumulative impact of several parameters were analysed by creating 3D plots with two factors varied while maintaining other factors at optimal values (Sehgal., 2023). The 2D contour plot is typically employed to visually depict regression equations for analysing the interaction effects between independent and dependent variables. These plots illustrate two variables while maintaining the other variable constant, and the degree of interaction is typically assessed by the plot's shape (Geethu et al., 2021).

Figure 3.6 (a-c) illustrate 3D surface plots that employ Response Surface Methodology (RSM) to examine the interactions among critical factors (glucose, ammonium sulfate, and temperature) affecting biomass yield. These visuals clearly illustrate how different combinations of these factors affect biomass yield, offering insights into ideal operational circumstances. Plots A and B examine the relationship between glucose (X1) and ammonium sulfate (X2) concentrations, with temperature maintained at 35°C. The data indicate that optimal biomass yield is achieved at a glucose concentration of roughly 20 g/L and an ammonium sulfate concentration of 2 g/L. The red areas in 3D plots denote regions of optimal biomass yield, indicating that these represent the most advantageous conditions for biomass yield within the examined range. Plots A and C investigate the impacts of glucose concentration (X1) and temperature (X2), with ammonium sulfate maintained at a constant 2 g/L. The results indicate that optimal biomass yield occurs at a glucose concentration of approximately 20 g/L and a temperature of roughly 35 °C. Finally, plots B and C examine the interaction between ammonium sulfate concentration (X1) and temperature (X2), with glucose maintained at 20 g/L. The graphs demonstrate that optimizing ammonium sulfate content to around 2 g/L for nitrogen and temperature to 35 °C increases biomass yield.

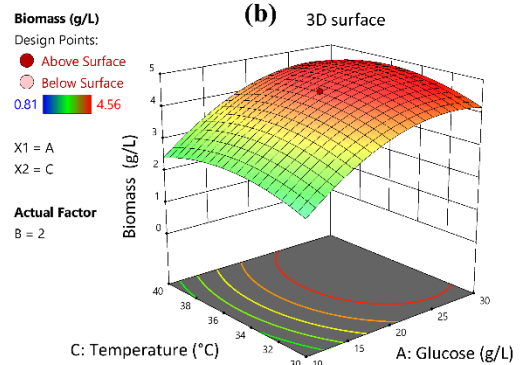
The perturbation plot (figure 3.6d) illustrates the individual influence of all factors on biomass yield. The graph illustrates the optimal values of all factors evaluated in the CCD. The independent influence of glucose concentration on biomass yield was predominant relative to other factors, as evidenced by the perturbation plot. The deviation from the reference point is more pronounced in A (*i.e.*, glucose concentration) compared to the other components affecting biomass yield, which do not deviate to that greater degree.

The 2D contour plots (figure 3.7a-c) demonstrate that moderate increases in both glucose concentration and temperature result in elevated biomass DCW, as evidenced by the growing red zone, which delineates the area of optimal production. Similar outcome was observed in case of PHB yield as depicted in 3D surface plots (figure 3.8a-c), perturbation plot (figure 3.8d), and 2D contour plots (figure 3.9a-c).

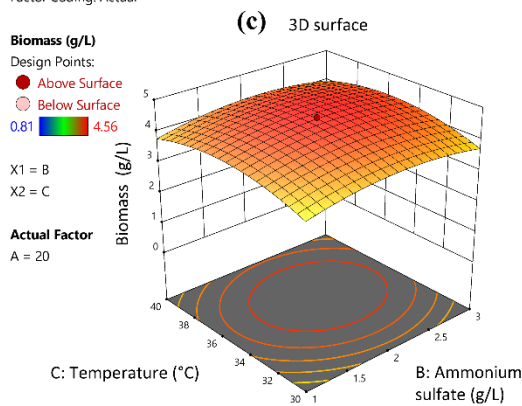
Factor Coding: Actual



Factor Coding: Actual



Factor Coding: Actual



Factor Coding: Actual

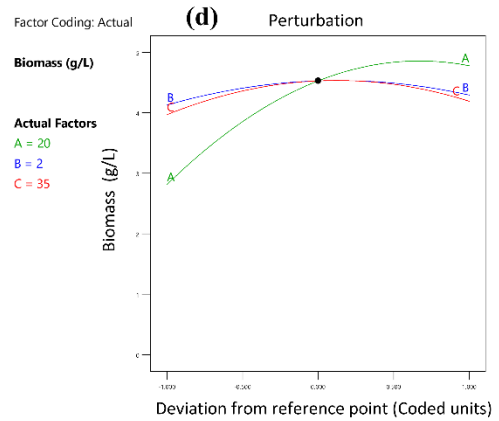


Fig. 3.6. 3D Surface plots showing the interaction between (a) AB, (b) AC, and (c) BC while the other factor is constant, (d) Perturbation plot illustrating the independent effect of each factor on biomass yield. A, B, and C represent the variables namely glucose, ammonium sulfate, and temperature, respectively

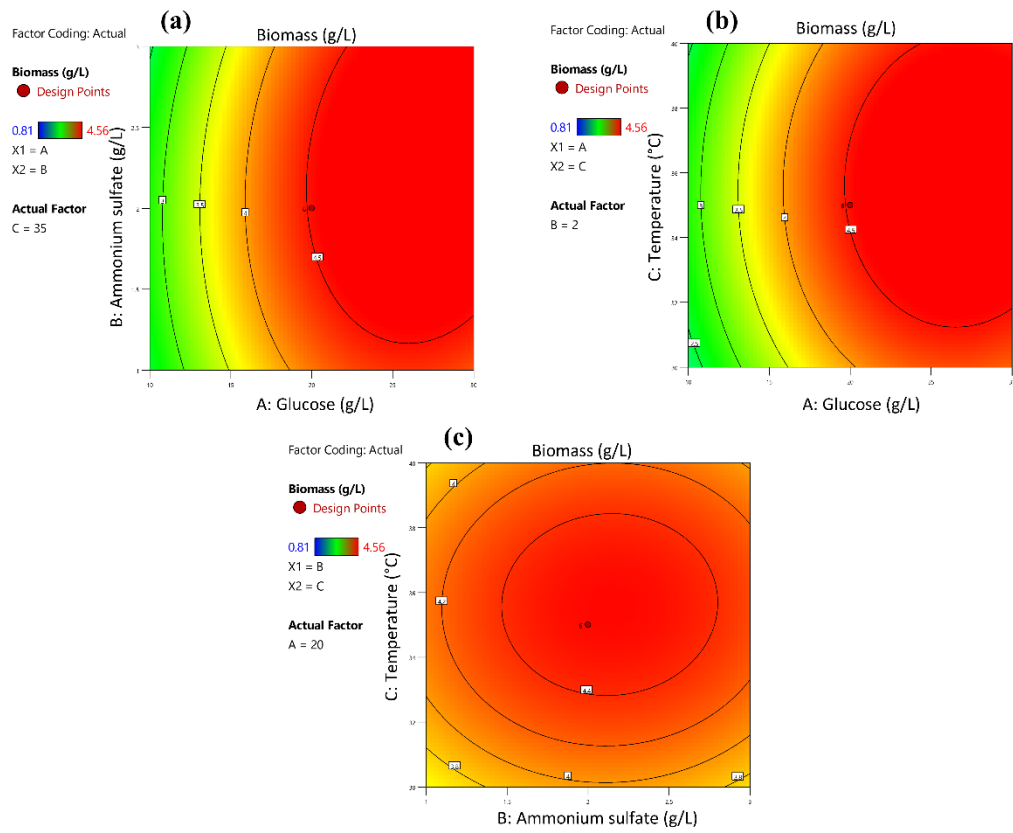


Fig. 3.7. 2D contour plots illustrating 2-way interaction among (a) AB, (b) AC, and (c) BC of biomass yield

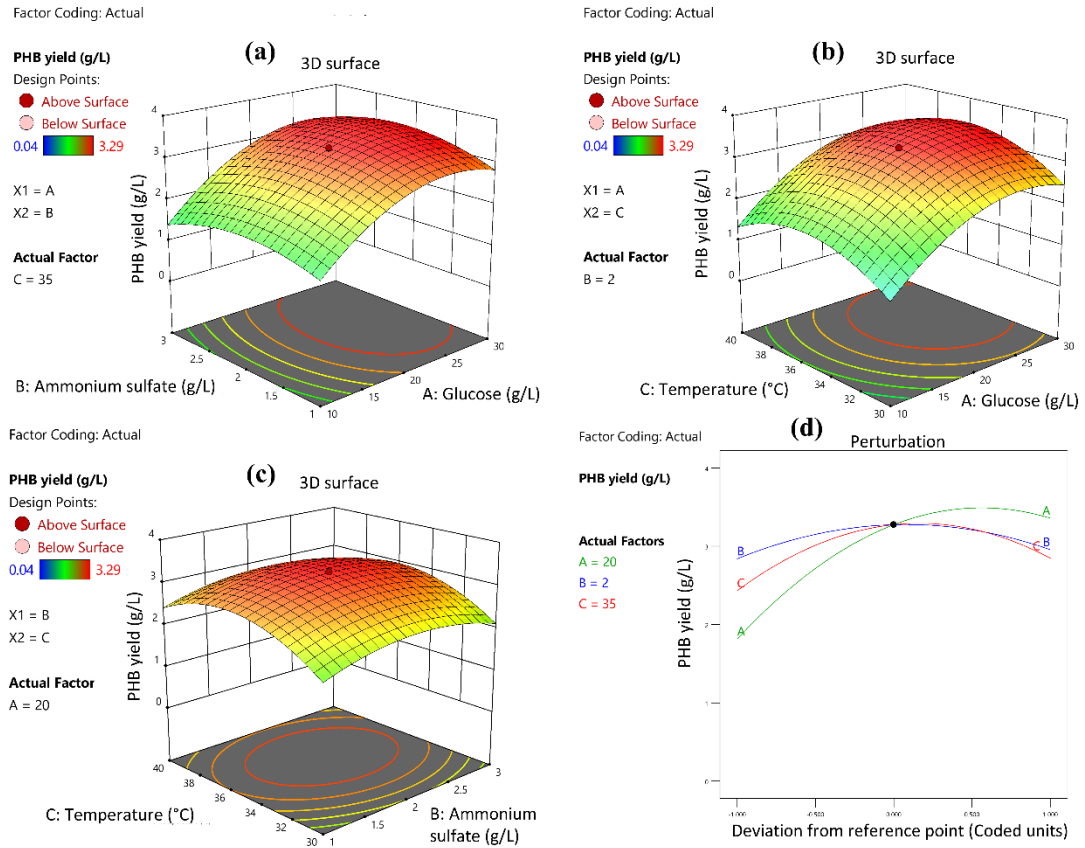


Fig. 3.8. 3D Surface plots showing the interaction between (a) AB, (b) AC, and (c) BC while the other factor is constant for PHB yield, (d) Perturbation plot illustrating the independent effect of each factor on PHB yield. A, B, and C represent the variables namely glucose, ammonium sulfate, and temperature, respectively

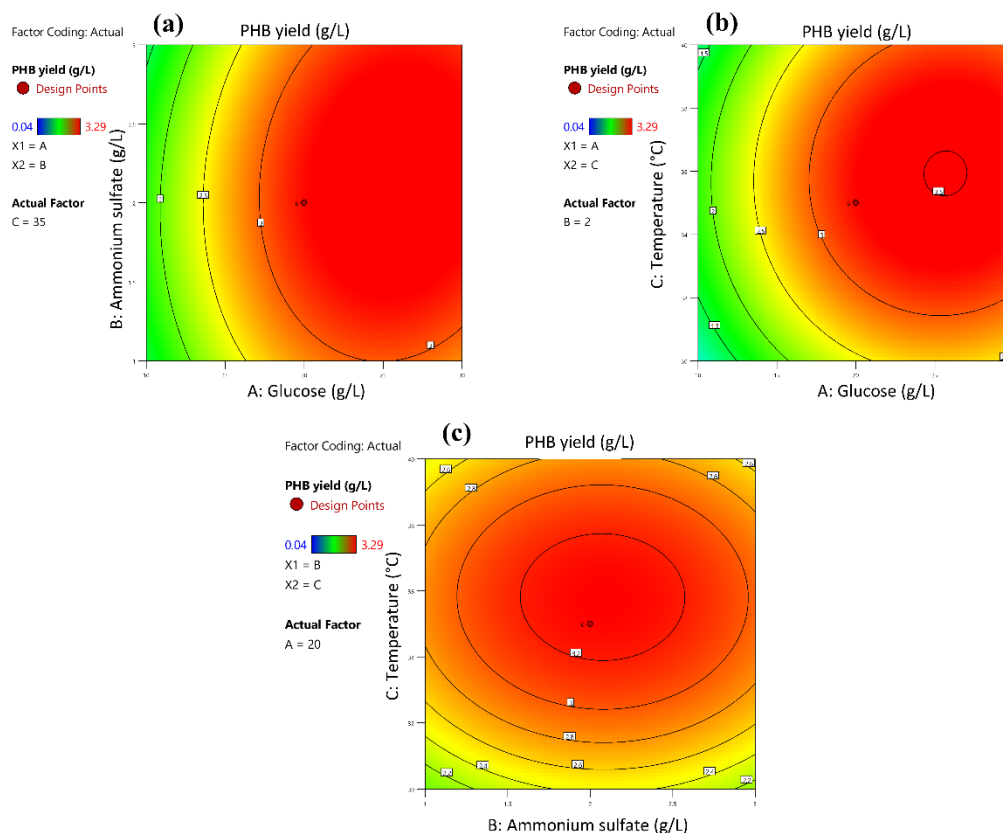


Fig. 3.9. 2D contour plots illustrating 2-way interaction among (a) AB, (b) AC, and (c) BC of PHB yield

3.4.5. Validation of the model

The statistical model was validated using the point prediction tool of RSM, which provided optimal values for the three variables: glucose (A), ammonium sulfate (B), and temperature (C). The experiment was carried out in triplicate to assess the precision of the projected model. This study specifies predicted values for carbon, nitrogen, and temperature as 25.28 g/L, 1.97 g/L, and 37 °C, respectively, as detailed in Table 3.11. These values indicate that enhancing biomass and PHB yield is attainable under the provided conditions. As depicted in Table 3.12, the observed biomass DCW yield (4.81 g/L) closely approximated the predicted yield (4.88 g/L), while the observed PHB yield (3.48 g/L) was similarly near the predicted yield (3.51 g/L), so confirming the model's validity. The ideal C:N ratio for optimal biomass and PHB yield is established at 28.2:1, resulting in a maximum biomass DCW yield of 4.81 g/L, a PHB yield of 3.48 g/L, and a PHB content of 72.34 %, showing a slight increase over previous benchmark published

earlier by Acharjee et al. (2024). The yield of PHB can be represented in either g/L or g/g to facilitate a thorough comprehension of its production. The PHB yield reported in g/L indicates the concentration of PHB in the production media, whereas the PHB yield expressed in g/g reflects the quantity of PHB produced relative to the total substrate consumed. The PHB yield, expressed in g/g, is determined to be 0.14 g/g.

Table 3.11. Point predicted table for best PHB production

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	Glucose	25.28	10.00	30.00	0.0000	Actual
B	Ammonium sulfate	1.97	1.0000	3.00	0.0000	Actual
C	Temperature	37	30.00	40.00	0.0000	Actual

Table 3.12. Constraints, criteria for optimization, solution along with observed and predicted response values

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance	Solution	Observed response ^b	Predicted response
A: Glucose	is in range	10	30	1	1	3	25.28	-	-
B: Ammonium sulfate	is in range	1	3	1	1	3	1.97	-	-
C: Temperature	is in range	30	40	1	1	3	37	-	-
Biomass DCW	maximize	0.81	4.56	1	1	3	4.8823	4.81	4.8823
PHB yield	maximize	0.04	3.29	1	1	3	3.51574	3.48333	3.51574

^b Average of triplicate experiments

3.5. Conclusion

The study primarily aimed to evaluate various organic wastes and glucose as carbon sources for PHB synthesis, while optimizing the production processes to determine the scalability of PHB production utilizing the RSM model. Among the different carbon sources analysed, glucose proved to be the most preferred substrate for PHB production. This work utilized PBD to evaluate the medium components influencing PHB formation by the previously isolated *Bacillus* sp. KE4 strain. The experimental results indicated that three of the eleven variables examined significantly influenced PHB production at a 95 % confidence level. The factors included glucose as the sole carbon source, ammonium sulfate as the nitrogen source, and temperature. These factors were further optimized using CCD, which involved a thorough examination of their interactions, culminating in a final optimized medium that maximized biomass DCW and PHB yield. The statistical model indicated that optimal conditions for the efficient production of PHB from *Bacillus* sp. KE4 include a glucose concentration of 25.28 g, an ammonium sulfate concentration of 1.97 g, and a constant temperature of 37 °C. Consequently, the optimized medium yielded a maximum biomass DCW of 4.81 g/L and a PHB yield of 3.48 g/L, which closely corresponded with the predicted values from the RSM model of 4.88 g/L and 3.51 g/L, respectively. The ideal C:N ratio for optimal biomass DCW and PHB yield is established at 28.2:1. The findings are beneficial and signify a significant advancement in optimizing culture media conditions using glucose as a carbon source for the scalability of PHB production.

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CHAPTER-4

Bio-fabrication of Polyhydroxybutyrate (PHB)-Based Composites and Their Applications

Section 4.1 of this chapter has been published in:

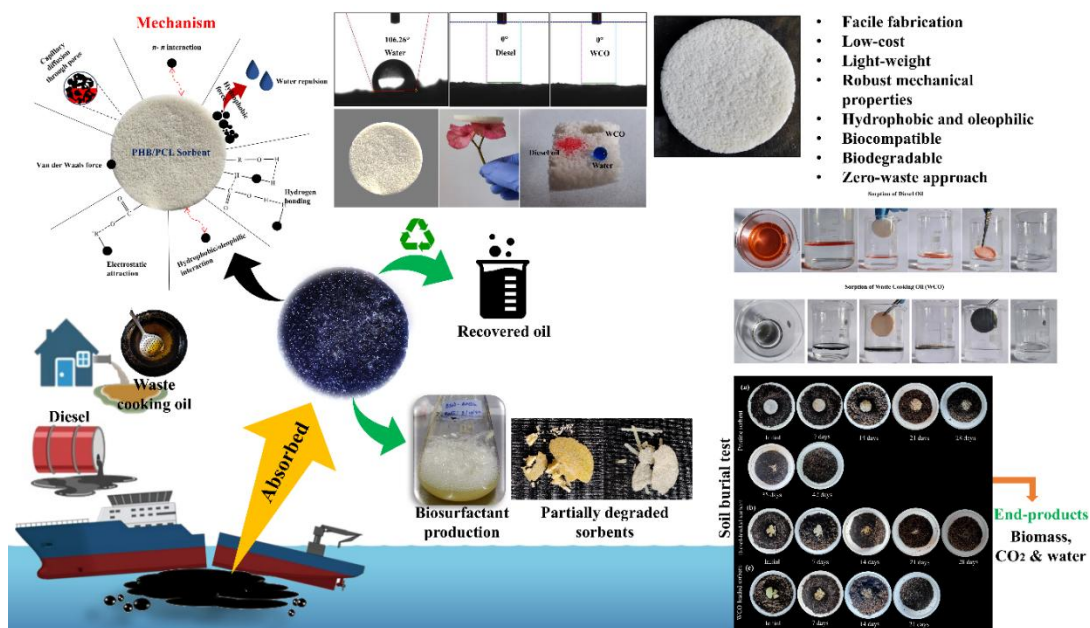
Acharjee, S. A., Bharali, P., Ramachandran, D., Hazarika, S., Dutta, N., & Manivannan, J. (2025). Bio-fabrication of biodegradable polyhydroxybutyrate/poly-caprolactone based sorbent: a net zero-waste approach for sustainable management of intricate hydrophobic pollutants. *International Journal of Biological Macromolecules*, 145825.

4.1. Bio-fabrication of biodegradable Polyhydroxybutyrate/Polycaprolactone based sorbent: a net zero-waste approach for sustainable management of intricate hydrophobic pollutants

4.1.1. Abstract

Generation of secondary contaminants, insufficient sorption and recovery, and degradability issues associated with the existing solutions render the management of hydrophobic pollutants very challenging. Hereby, a cost-effective, energy-efficient, non-cytotoxic, biocompatible, and biodegradable sorbent is fabricated employing a blend of Polyhydroxybutyrate (PHB) and Polycaprolactone (PCL) biopolymers using a facile salt leaching technique. The prepared PHB/PCL sorbent was able to swiftly absorb oil from an immiscible oil/water mixture because of its interconnected hierarchical pore structure, hydrophobicity and lipophilicity. The sorbent absorbed oil preferentially over water, achieving a saturated oil sorption capacity of 6.02 g/g for waste cooking oil (WCO) and 5.09 g/g for diesel oil, with recovery rates of 86.5 % for WCO and 85.7 % for diesel. Subsequent to the sorption of oils, the oil-saturated sorbents were further valorised through the production of biosurfactant utilizing the strain *Pseudomonas aeruginosa* AMS1a. Notably, the sorbents exhibited partial degradation during the valorisation process. The residual sorbents were buried in compost soil for further degradation, and were entirely decomposed within 28 days. The findings demonstrated an efficient biomaterial for addressing oil spill disasters while minimizing secondary contamination. Additionally, the sorbent also fulfilled the zero-waste notion of the Sustainable Development Goals (SDGs) defined by the United Nations.

Keywords: Bio-fabrication, porous sorbent, Hydrophobicity, Oil/water separation, biodegradability, zero-waste



4.1.2. Introduction

Pollution caused by hydrophobic pollutants has been rising recently due to the unrelenting expansion of agricultural and industrial operations, severely polluting different ecological systems. The primary sources of such pollutants are mostly associated with various anthropogenic and natural processes (Dewangan et al., 2021). The two most common pollutants emitted by anthropogenic activities are polycyclic aromatic hydrocarbons (PAHs) and petroleum hydrocarbons. Additionally, Waste Cooking Oil (WCO), classified as a hydrocarbon compound, is among the most common pollutants released into sewer systems by human activities (Zahri et al., 2021). Refined petroleum hydrocarbons, like diesel oil, are produced by distilling crude oil and consist of polycyclic aromatic hydrocarbons and low molecular weight alkanes. Diesel spills that occur during the usage and transportation of diesel fuel over rivers, lakes, and the ocean are among the causes of pollution from hydrocarbons in aquatic bodies. With regard to its hydrocarbon content and extensive use for industrial and transportation processes, diesel fuel is among the most potentially hazardous hydrocarbon pollutant and have the capacity to disturb ecological equilibrium and threatens the aquatic organisms (Urgel et al., 2024).

WCO is a by-product produced locally when edible vegetable oils are used to cook food stuffs in homes, hotels, restaurants, and other catering establishments.

WCO is predominantly produced and released into the environment, particularly in China and India, which makes it one of the most significant environmental contaminants. WCO is often discharged into sewers, drains, rivers, open areas, and forests in several urban areas. The improper management of large quantities of WCO leads to several harmful environmental issues, such as the production of foam, obstructions in the sewer system, impeding the pre-treatment of sewage at wastewater treatment facilities, escalating the organic burden on water sources, decreasing the concentration of dissolved oxygen, disrupting ecological equilibrium, and causing pollution of surface water and soil (Lopes et al., 2020).

The traditional clean-up procedures employed for oil spills include *in-situ* burning, mechanical processes (viz. booms, skimming, and vacuums), solidifiers, chemical dispersants, bioremediation, air floatation, gravitational and sedimentation. Each approach has its own set of benefits. However, numerous variables, including water temperature, proximity to the coastline, spill volume, oil kind and density, waves, weather, currents, and reaction time, affects the methods and efforts for clean-up operations. Techniques frequently preferred for the separation of WCO from water systems includes gravity separation, floatation, flocculation, filtering, and evaporation. Nevertheless, these techniques are ineffective when used alone, and they are limited to situations in which the oil is either poorly dispersed or not emulsified (Medeiros et al., 2022).

The utilization of porous sorbent materials with oleophilic and hydrophobic surfaces to eliminate oil from the surface of a water body is an another effective, economical, and adaptable approach. The efficacy of a sorbent material is determined by its porosity, hydrophobicity, high capacity for oil sorption, rapid rate of oil sorption, low density, affordability, easy operation, and adaptability for reuse (Hersey et al., 2015). A diverse range of sophisticated and innovative absorbent materials (fiber sheets, zeolites, alumina, and silica) with oleophilic and hydrophobic surfaces, exhibiting exceptional adsorption capabilities, have been synthesised for the purpose of separating and recovering oil from water. The absorption effectiveness of such sorbents is limited by several drawbacks, such as their truncated sorption capacities, low recovery efficiencies, generation of hazardous wastes, and inadequate buoyant properties. Moreover, bio-fabrication of

lightweight materials that are chemically inert, environmentally benign, and have a high sorption capacity is essential for dealing with oil spill related operations (Elhenawy et al., 2021).

Recently, synthetic polymer-based sorbent substrates like polydimethylsiloxane (PDMS), polyethylene (PE), polypropylene (PP), polystyrene (PS), polyurethane (PU), polyvinyl chloride (PVC), poly(vinylidene fluoride) (PVDF), or even their blends have emerged as a high-performing candidates, providing superior oil/water separation selectivity, rapid oil sorption capacity, and quick oil removal rates from water surfaces (Eom et al., 2022). However, petroleum-based polymers, despite their excellent performance, are both non-disposable and non-biodegradable, therefore presenting significant and undesired secondary environmental contamination (Zhang et al., 2019).

Natural adsorbents such as cellulose-based materials, clays, biopolymers, and waste from industry and agriculture, on the other hand, are inexpensive and safe for the environment. Their attributes provide them appealing replacements for synthetic materials, provided they demonstrate the suitable adsorption capability (Liu et al., 2024). Despite of their biodegradable nature several limitations that include difficulties in recovering and reusing, brittleness, and necessity of high price equipment and chemical additives (Hersey et al., 2015). For usage in large-scale oil spill clean-up operations, biodegradable synthetic polymers such as polyhydroxybutyrate (PHB), polycaprolactone (PCL), polylactic acid (PLA), chitosan, and cellulose, and other blended bio-derived materials are eco-friendly alternatives (Eom et al., 2022). Of these biopolymers, PHB is considered one of the most promising environmentally friendly biomaterials for the preparation of sorbent materials in various fields due to its outstanding properties, including non-toxicity, comparatively high melting point, easy blending with other polymers, solubility in a wide range of solvents, biocompatibility and biodegradability (Acharjee et al., 2023). Like PHB, PCL exhibits considerable promise in the development of hydrophobic materials owing to its exceptional mechanical and thermal stability, resistant to UV and chemical degradation, minimal water absorption, readily forms blends with other polymers and dissolves in a broad spectrum of solvents, biocompatibility and biodegradability (Semiromi et al.,

2019). PCL can be employed in oil-water separation applications by integrating diverse additives such as silica nanoparticles or by formulating blends with other polymers (Zhang et al., 2019). As of yet, there have been few reports on the preparation of hydrophobic material for oil/water separation utilizing biopolymers. There is currently no known report on the development of porous three-dimensional (3D) sorbent employing PHB and PCL biopolymers by salt leaching technique for the sorption of hydrophobic pollutants from water.

Therefore, the present work involved the bio-fabrication of a porous PHB/PCL sorbent using the salt leaching technique. Herein, we report the: i) bio-fabrication of porous PHB/PCL sorbent; ii) selective sorption of hydrophobic pollutants (diesel and waste cooking oil) from immiscible oil/water mixtures; iii) management of recovered hydrophobic pollutants and its valorisation; iv) reusability of the sorbent for retrieval experiments; v) cytotoxicity assay of the fabricated sorbent; and vi) complete biodegradation of sorbents in the soil.

4.1.3. Materials and Methods

4.1.3.1. Materials

Polyhydroxybutyrate (PHB, Mw = 1,55,436 g/mol, Polydispersity index 1.08) was successfully produced in Nagaland University (Department of Environmental Science) by microbial biosynthesis using *Bacillus* sp. KE4 strain (Acharjee et al., 2024). Polycaprolactone (PCL, Mw = 80,000 g/mol) were acquired from Sigma-Aldrich, USA. Sodium Chloride (NaCl, porogen), Chloroform (99 %), and Diethyl ether (99 %) was purchased from SRL, India. Sudan III, Sudan Black B, and methylene blue dye were acquired from HiMedia, India. Diesel oil was purchased from the fuel station of Mokokchung, India and WCO was procured from Men's hostel, Nagaland University, India.

4.1.3.2. Preparation of PHB/PCL sorbent

PHB biopolymer was obtained from *Bacillus* sp. KE4 strain as reported earlier by Acharjee et al. (2024). A 3D porous sorbent was fabricated (figure. 4.1.1) using PHB and PCL biopolymers *via* the salt leaching approach. Sodium chloride (NaCl) particles were employed as a porogen, while maintaining a weight ratio of 9:1 between the porogen and biopolymer. A PHB/PCL sorbent was produced with a weight-to-weight ratio of 1:3. In brief, 0.25 g of PHB and 0.75 g of PCL were

dissolved in 10 mL of chloroform and the resulting solution was allowed to stand for 3 h while being stirred periodically. Next, 9 g of precisely sieved NaCl was introduced into the solution. The paste-like biopolymer/particle combination was briskly agitated at 700 rpm to achieve uniform dispersion of the particles. Subsequently, the solution was poured into a 50 mm diameter glass petri dish and dried overnight in a fume hood for complete evaporation of the solvent. The resulting porous sorbent was submerged in deionized (DI) water for 48 h, with frequent changes of the DI water every 8 h ensuring thorough leaching of the porogen. The porous sorbent obtained was dried at 30 °C in an oven until it reached a consistent weight.

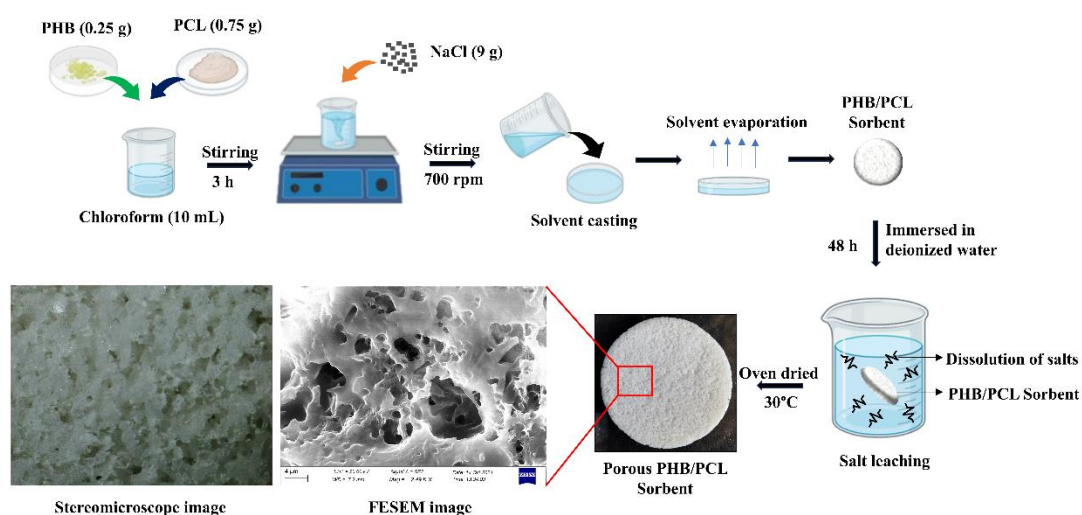


Fig. 4.1.1. Schematic illustration of the preparation of porous PHB/PCL sorbent

4.1.3.3. Characterization of PHB/PCL sorbent

To identify the presence of different characteristic functional groups in PHB, PCL and PHB/PCL blend, Fourier-transform infrared (FTIR) spectrophotometer (Shimadzu, Japan) was utilized. The spectra have been recorded from 4000 to 500 cm^{-1} by mixing the samples with the KBr powder in a certain ratio to form a thin disc. The Raman spectroscopy was also performed using inVia spectrometer system (Renishaw) coupled with a Confocal Raman microscope. The spectra were recorded with an excitation laser source of 785 nm, spectral range of 3500-500 cm^{-1} , gratings of 1200 l/mm, and exposure time of 15000 ms. To generate

the Raman spectra, Renishaw's WiRE version 5.5 software was used. To assess the crystalline pattern of the fabricated sorbent, X-ray diffraction (XRD) system (Rigaku, Japan) was utilized. The system used Copper K- α radiation (wavelength 1.54 Å), and scanning was performed in $2\theta = 10-50^\circ$ range at a scanning rate of $3^\circ/\text{minute}$. Here, θ represents the angle of incidence on the sorbent. The crystallinity index (%) was determined using the following equation (Acharjee et al., 2024).

$$CI (\%) = \frac{\sum A_c}{\sum (A_c + A_a)} \quad (4.1.1)$$

Where, CI represents crystallinity percentage, A_c represents the total area under the crystalline peaks, and A_a represents the total area of the amorphous halo.

The pore volume, surface area and pore diameter of the fabricated sorbent were determined using nitrogen (N_2) adsorption-desorption isotherms at 77.35 K with Autosorb iQ Station 1 Quantachrome Instruments, version 5.21. Brunauer-Emmett-Teller theory (BET) isotherms and Barrett-Joyner-Halenda (BJH) method were used to estimate the surface areas, pore volume, and pore size distributions. To prevent inaccurate results, the samples were degassed prior to the comprehensive examination. The thermal stability of the fabricated sorbent was assessed using a Thermogravimetric analyzer (STA 2500 Regulus). To avoid sample oxidation, the sample was heated between 30°C and 600°C at a rate of $10^\circ\text{C}/\text{minute}$ while nitrogen was present at a flow rate of $40\text{ mL}/\text{minute}$. The PHB/PCL sorbent's melting temperature (T_m) and degree of crystallinity (X_c) were ascertained using DSC. DSC measurements were carried out on a Thermal analyzer (Shimadzu, DSC-60) that had a cooling attachment. The sample was put in an aluminium pan and heated between 20°C to 300°C at a scanning speed of $10^\circ\text{C}/\text{minute}$ in a nitrogen environment. Melting enthalpy (ΔH_m) and T_m were computed from DSC endothermal peaks. After annealing for 1 minute, the sample was cooled to 20°C . The following equation as stated by Garcia-Garcia et al. (2016) was utilized to determine the X_c of PCL and PHB in the blends.

$$X_c = \left[\frac{\Delta H_m}{\Delta H_0 \Delta w} \right] \times 100 \quad (4.1.2)$$

where, ΔH_m represents the fusion enthalpy and ΔH_0 represents the enthalpy associated with the corresponding 100 % crystallinity polymer. Lastly, "w" denotes the weight fraction of PHB and PCL in each PHB/PCL blend. The polymer's

crystallinity indices were determined using the melting endotherms of the original samples by fusion and then normalized to the blend's constituent composition. A universal testing machine was used to assess the compressive mechanical property of the disc-shaped sorbent, which had a diameter of 50 mm and thickness of 6 mm. The speed of the crosshead was 1 mm/minute. Stereoscopic image of the sorbent *i.e.*, before absorption, after absorption and after regeneration were captured by Stereo-zoom (Euromex, China) in a magnification of 10X to visualise the surface of the sorbent. Additionally, a field emission scanning electron microscope (FESEM) (Carl zeiss supra-55, Germany) operating at an accelerating voltage of 10 kV was used to observe the cross section of the porous PHB/PCL sorbent after it had been shattered in liquid nitrogen. To assess the wettability of the sorbent, the water contact angle (WCA) and oil contact angle (OCA) of the sorbent was measured using the sessile drop method and observed in a contact angle goniometer (DSA25 Kruss, Germany). A drop of 4 μ L of the liquids (water and oils) were gently positioned on the surface of the sorbent at room temperature. All contact angles were measured at three different positions, and the average results were reported. A Charged-coupled Device video camera attached to the apparatus captured the sorbent's contact angle. The equipment's software (Image J) automatically determined the contact angles of the liquids on the sorbent.

4.1.3.4. Oil sorption test

The absorptive capacity of the fabricated sorbent was assessed to estimate its ability to absorb oils per 1 g of its dry weight. To evaluate the oil sorption capability of the porous sorbent, a certain quantity of sorbents was measured and then added to a beaker containing a fluid mixture of oil and water in a weight ratio of 1:20. In this work, the sorption capability of a hydrophobic sorbent was demonstrated using diesel and WCO. In an effort to enhance visual observation, diesel was dyed with Sudan III and WCO was dyed with Sudan Black B prior to sorption. The oil sorption reaction began immediately upon contact with the sorbents and the oil present on the water surface. Following sorption, the sorbents were weighed. The oil absorption capacity of the porous sorbent was assessed using the equation stated by Zhang et al. (2019):

$$Q = \frac{m_f - m_0}{m_0} \quad (4.1.3)$$

where, Q represents the sorption capacity [g (oil)/g (sorbent)], m_0 and m_f represents the weights of sorbents before and after sorption (g), respectively.

4.1.3.5. Oil retention profile test

The oil retention test was conducted using the methodology outlined by Rotaru et al. (2014). 1 g sorbent sample has been positioned on a 90 mm glass petri dish containing a 12 mm layer of oil (diesel and waste cooking oil). The sample was allowed to saturate with the oily liquid for a designated duration. The sorbent was subsequently extracted from the liquid phase and weighed on a digital balance, at a point where the absorbed oil commenced to drip. The weight of the oil loaded sorbent was measured during the free drainage of liquid at various time intervals (5, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300 secs). The retention capacity function of time was ascertained using the gravimetric method and denotes the ratio of sorption capacity at drainage time (t) to the initial (maximum) sorption capacity. The oil retention capacity (g/g) was calculated using equation 4.1.3 as mentioned above.

4.1.3.6. Immiscible oil/Water mixture Separation flux Experiment

The oil and deionized water were mixed in a 1:1 volume ratio. To enhance visual differentiation, deionized water was dyed with methylene blue, diesel was dyed with Sudan III, and waste cooking oil was dyed with Sudan black B. The prepared sorbent has been fixed in an aslant separation apparatus, having an inner diameter of 4 cm. The oil/water mixture was poured into aslant separation apparatus and filtered solely by gravity. The formula for calculating separation flux J (L/m²/h) is as follows (Zhang et al., 2023):

$$J = \frac{V}{A \times t} \quad (4.1.4)$$

V(L) signifies the filtered solution's volume, A(m²) signifies the area of the sorbent, and t(h) signifies the separation time.

4.1.3.7. Reusability and recovery

In order to assess the PHB/PCL sorbents' reusability, the oils were removed from the sorbent by rinsing it twice with 75 mL of diethyl ether over the course of

30 minutes. The diethyl ether was then eliminated from the sorbents by drying it in a fume hood for 5-6 h. To evaluate their usability, the dried sorbents were once again introduced to oils. The tests were conducted for six consecutive cycles of the sorption process. The mixture of oil and diethyl ether obtained after the rinsing procedure is separated *via* solvent evaporation using a rotary evaporator. The quantity of recovered oil was determined using equation stated by Patowary et al. (2015).

$$\text{Oil recovery} = \frac{W_{\text{OR}}}{W_{\text{OI}}} \times 100 \quad (4.1.5)$$

where, W_{OR} represents the weight of recovered oil and W_{OI} represents the initial weight of sorbed oil.

4.1.3.8. Management of recovered hydrophobic pollutants and its valorisation

After the sorption of oils, the oil-loaded sorbents were used as carbon source for the production of biosurfactant. Briefly, 2 mL of saturated *P. aeruginosa* AMS1a culture broth was taken out from the seed culture and inoculated into 100 mL of mineral salt medium (MSM) (Appendix II) infused with diesel and WCO-loaded matrices separately, then incubated in an orbital shaker at 30 °C and 140 rpm for 10 days. During a 10-day period, the growth behaviour of the chosen bacterial strain in MSM was determined by measuring the optical density of the culture medium at 600 nm and evaluating biosurfactant synthesis *via* an orcinol test (Appendix II) (Itoh et al., 1971) at 24-h intervals. Upon maturation, the culture was centrifuged at 8000 rpm for 15 minutes, and the bacterial pellets were removed. The cell-free culture supernatant was subsequently acidified with 6 N HCl, adjusting the pH to 2, and was maintained undisturbed overnight at 4 °C. The precipitate was isolated following centrifugation, subjected to two extractions with PBS (Appendix II) to eliminate excess acid, and subsequently dehydrated in a hot air oven until completely dried. The precipitated dried crude biosurfactant was then weighed and stored in a glass vial.

4.1.3.9. Soil burial test

The biodegradability of pristine PHB/PCL, and partially degraded diesel-loaded PHB/PCL and WCO-loaded PHB/PCL leftover sorbent of valorisation process were evaluated by quantifying the mass loss of the samples buried in

compost soil for 42 days. The samples were buried in the soil at a depth of 3-4 cm into an open circular plastic container (diameter: 95 mm; height: 80 mm) to facilitate aerobic conditions, and kept at a temperature of 28 °C. The soil's pH and moisture content were 6.98 and 55.8 %, respectively. The soil moisture was maintained by spraying water periodically. After every 14 days, buried samples were carefully excavated. Soil particles were cautiously brushed away using small paint brush, and then oven-dried at 45 °C to eliminate moisture prior to weight measurement. The mass loss percentage was utilized to evaluate biodegradation behaviours. The specimen's mass was measured before and after exposure using a precision electronic balance (Mettler Toledo, ME203E). The mass and the degradation percentage (%) were assessed using the procedure outlined by Acharjee et al. (2024).

$$\text{Degradation \%} = \frac{W_1 - W_2}{W_1} \times 100 \quad (4.1.6)$$

Here, W1 signifies the sorbent's initial mass and W2 its final mass following degradation.

4.1.3.10. Bioactivity of the prepared PHB/PCL sorbent

The antibacterial effect of the fabricated sorbent was assessed through the disc diffusion method on Mueller Hinton agar (Appendix II), focusing on both gram-negative bacteria (*Salmonella enterica*, *Ralstonia eutropha*, and *Pseudomonas aeruginosa*) and gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus subtilis*). The sorbents were dissolved in chloroform and casted on a glass petri dish to form a thin film for the experiment. The films were subsequently sliced into small rectangular shapes and subjected to UV radiation for sterilizing. They were then positioned on agar plates that had been already inoculated with the corresponding bacteria. Readymade streptomycin (10 µg) paper disc was additionally placed on agar plates as a positive control. To facilitate bacterial growth, the plates were left to incubate overnight at 37 °C. The antibacterial activity was assessed by examining the emergence of inhibitory zones surrounding the rectangular test films.

4.1.3.11. Biocompatibility Test

4.1.3.11.1. Cell culture and PHB/PCL sorbent exposure

The HepG2 cells (human hepatoblastoma cell line) were acquired from NCCS, Pune, India. Initially, the cells were cultured in Dulbecco's Modified Eagle medium (Appendix II) along with fetal bovine serum (10 %), streptomycin (100 µg/mL), and penicillin (100 units/mL). The cell culture flasks were maintained in a 5 % CO₂ environment at 37°C. Following confluence, the viable cells had been shifted to 96-well plates for exposure and microplate-based fluorescence experiments. The PHB/PCL sorbent samples were then subjected to HepG2 cells at several concentrations (10, 20, 40 and 80 µg/mL) as formerly mentioned by Abdo et al. (2023). Subsequently, cells were subjected to samples in 96-well plates and incubated for 24 h in a 5 % CO₂ environment at 37 °C. Following the treatment regimen, the cells underwent a series of assays.

4.1.3.11.2. Cell viability

Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as outlined by Li et al. (2015), which involves the alteration of MTT into formazan crystals. Cells were seeded in 96-well plates at a density of 2×10^3 cells per well. Thereafter, MTT medium was introduced into the culture medium at a final concentration of 0.45 mg/mL. The solution was subsequently retrieved following 4 h of incubation at 37 °C, and the formazan was solubilized in 100 µL of dimethyl sulfoxide. The absorbance was obtained at 570 nm using a multimode microplate reader. The viability of the cells was assessed through the comparison of the results to control cells deemed 100 % viable.

4.1.3.11.3. Cellular oxidative stress

The amount of intracellular reactive oxygen species (ROS) generation was quantified in cells utilizing the universal ROS indicator CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate). Following deacetylation by intracellular esterases, free radicals oxidize H₂DCF, resulting in the formation of green fluorescent DCF (Dichlorofluorescein) (Ng and Ooi, 2021). In brief, treated cells were incubated in a CO₂ incubator with 10 µM CM-H₂DCFDA for 45 minutes at 37 °C. Subsequently, DCF was rinsed twice with PBS (Appendix II), and the relative intensity of DCF was measured (Excitation: 485 nm and

Emission: 530 nm) utilizing a multimode microplate reader, represented in arbitrary units (a.u.) as previously specified by Maadurshni et al. (2022).

4.1.3.11.4. Cellular superoxide generation

The present investigation quantified the generation of cellular superoxide in live cells utilizing the Dihydroethidium (DHE) fluorescence technique, as mentioned earlier by Nagarajan et al. (2024). Subsequently, following the treatment regimen, cells were rinsed with PBS and maintained at 37 °C in the absence of light with 5 µM DHE for 30 minutes in an incubator. Following incubation, cells were rinsed twice with PBS to remove surplus staining solution. The fluorescence intensity of DHE was measured (Excitation: 510 nm and Emission: 590 nm) with a multimode microplate reader and presented in a.u.

4.1.3.11.5. Cellular apoptosis assay using Flow cytometry

In the current study, early- and late-phase apoptosis in cells was enumerated by flow cytometry method using Annexin-V/Dead cell apoptosis kit (BD Biosciences). Briefly, the treated cells were collected and dispersed in 100 µL of 1X binding buffer and were stained with 5 µL of both Annexin V/FITC (fluorescein isothiocyanate) and propidium iodide at ambient temperature for 15 minutes. After the incubation period, 400 µL binding buffer was introduced and cells were evaluated using a flow cytometer (FACSVerse, BD Biosciences).

4.1.3.12. Statistical analysis

Statistical analysis was carried out by GNU PSPP software. To assess the statistical significance between means of values observed from the cellular parameters, a one-way analysis of variance (ANOVA) was used. The p value of < 0.05 was defined as statistically significant in this study.

4.1.4. Results and Discussion

4.1.4.1. Fabrication and Characterization of PHB/PCL sorbent

Multiple methods, including electrospinning, melt blown spinning, gas jet spinning, centrifugal jet spinning, and solution blow spinning, are employed for the fabrication of porous materials. Notably, electrospinning has gained significant attention in recent years because of its capacity to generate fibres with a high surface-to-volume ratio, uniform structure, adjustable porosity, and flexibility to conform to various sizes and shapes. Nevertheless, electrospinning, despite its

exceptional characteristics, has several limitations including limited scalability and productivity (Khan et al., 2018), inconsistent quality, jet instability, equipment maintenance, susceptibility to the production environment (temperature and humidity), need for optimisation of separator properties (pore size and porosity), and reliance on a high-voltage device for operation (Zulkifli et al., 2023). Therefore, the present work involved the fabrication of a porous PHB/PCL sorbent using the salt leaching technique. The synthesis reaction of PHB/PCL diblock copolymer is presented in figure 4.1.2. Despite of its various advantages, the applications of PHB are still limited by some substantial drawbacks, including rigidity and fragility, thermal instability, and inadequate mechanical properties. In order to enhance its physical features, PHB has been blended with polycaprolactone (PCL) in the present investigation. PCL is a biodegradable, semi-crystalline polymer and exhibits desirable mechanical properties with exceptional ductility and low melting point (about 55 °C) (Antunes et al., 2005). Antunes et al. (2005) discussed about the impact-modifying effect of PCL on PHB, resulting in enhanced ductility and flexibility, accompanied by a significant increase in elongation at break and energy absorption while preserving its biodegradability and biocompatibility attributes. Despite the low miscibility of PHB and PCL as indicated in DSC analysis reported by Garcia-Garcia et al. (2014); PCL exhibits significantly greater thermal stability than PHB. Further, authors mentioned that blending of PHB with PCL demonstrate a modest elevation in the thermal onset degradation temperature, potentially attributable to interactions among the two components.

There is currently no known report on the development of porous three-dimensional (3D) sorbent employing PHB and PCL biopolymers by the salt leaching technique for oil sorption from water. The salt leaching technique is centred on the scientific principle that salt particles are distributed throughout the polymer matrix, resulting in a very porous structure. The formation of a gel pastes by the polymer-salt combination, following partial evaporation of the solvent, enables the creation of porous materials with diverse geometries and dimensions. This method is straightforward, energy-efficient, time-saving, practical, cost-effective, and crucially, do not need pressure or superfluous costly equipment.

spectra were observed that refers to the absence of strong interactions and bonding between PHB and PCL.

Raman shifts and assignments of Raman bands of PHB/PCL sorbent are displayed in figure 4.1.4. The Raman spectra of the fabricated sorbent indicated that the band centred at 2935 and 2879 cm^{-1} are assigned to asymmetric CH_3 stretch and symmetric CH_2 stretch, respectively. The sharp peak at 1728 cm^{-1} is assigned to symmetric $\text{C}=\text{O}$ stretching vibration. The bands at 1444 and 1421 cm^{-1} are assigned to CH_2 bend, while bands at 1309 and 1287 cm^{-1} are assigned to CH_2 twist. The bands at 1111, 1065, and 1042 cm^{-1} are assigned to $\text{C}-\text{O}-\text{C}$. The bands at 917 and 841 cm^{-1} are assigned to $\text{C}-\text{COO}$. The results of Raman spectroscopy are in accordance to FTIR analysis of PHB/PCL sorbent showing similar assignments.

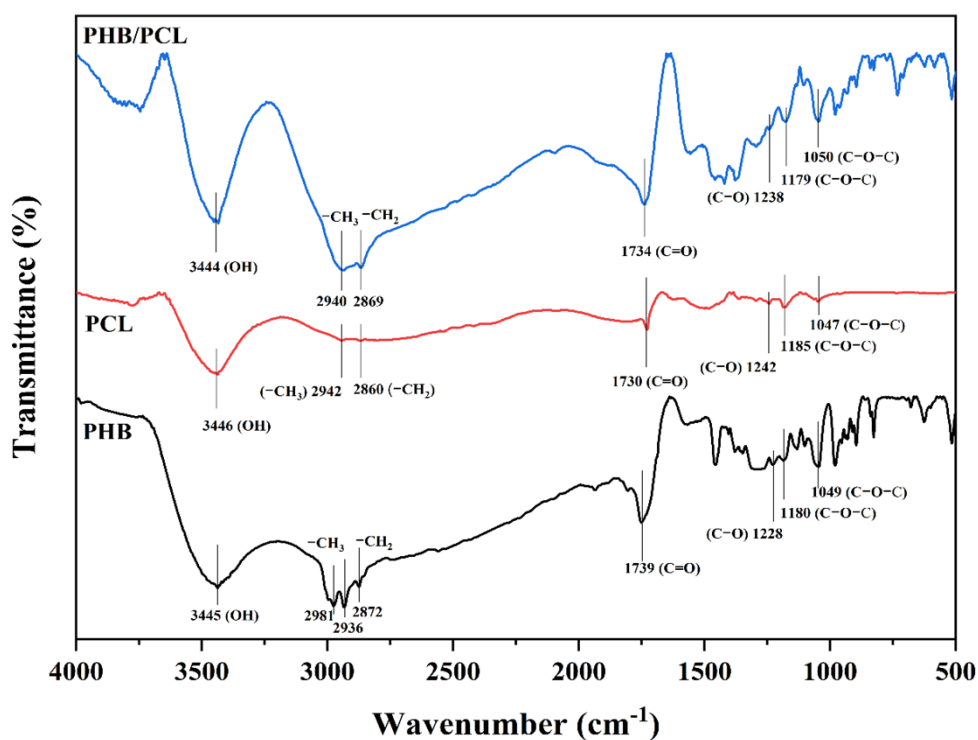


Fig 4.1.3. FTIR analysis of PHB, PCL and PHB/PCL blend

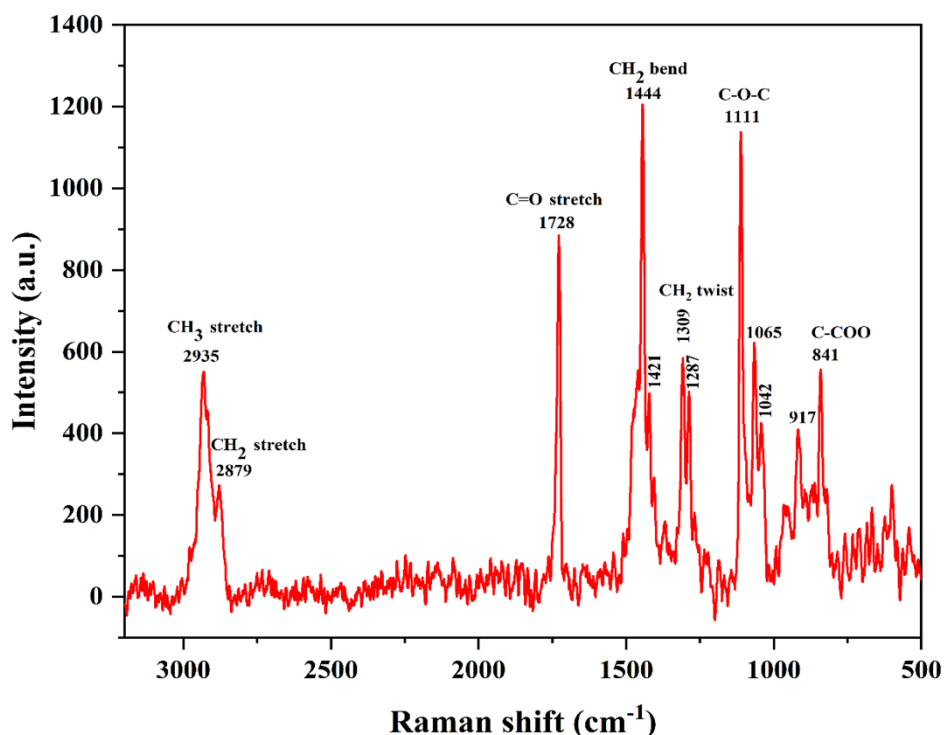


Fig 4.1.4. Raman spectrum of PHB/PCL sorbent

The XRD pattern of the PHB/PCL blend showed distinct diffraction peaks that were consistent with the findings of earlier investigations and could be ascribed to the XRD peak patterns of both constituent polymers. The diffraction peaks of PHB showed two distinct peaks at Bragg angles (2θ) = 13.41° and 16.87° , corresponding to the reflections (020) and (110), respectively, indicating orthorhombic crystal structure. In addition, two minor peaks at Bragg angles (2θ) = 25.2° and 27.01° corresponding to the reflections (121) and (040), respectively, indicating the partial crystalline nature of PHB (Pradhan et al., 2018). In contrast, the diffraction peaks of PCL showed two distinct peaks at Bragg angles (2θ) = 21.1° and 23.3° corresponding to the reflections (110) and (200), respectively, indicating the semi-crystalline nature of PCL (Chernozem et al., 2020). Figure 4.1.5 shows the XRD pattern of PHB/PCL sorbent. The crystallinity index (CI) of the sorbent was assessed using equation 4.1.1 and was calculated to be 29.4 %, indicating that the sorbent is semi-crystalline in nature. Semi-crystalline polymers display structured and densely packed molecular chains, resulting in mechanical properties

that are significantly more rigid and stiff than those of conventional polymer systems (Murmu et al., 2022).

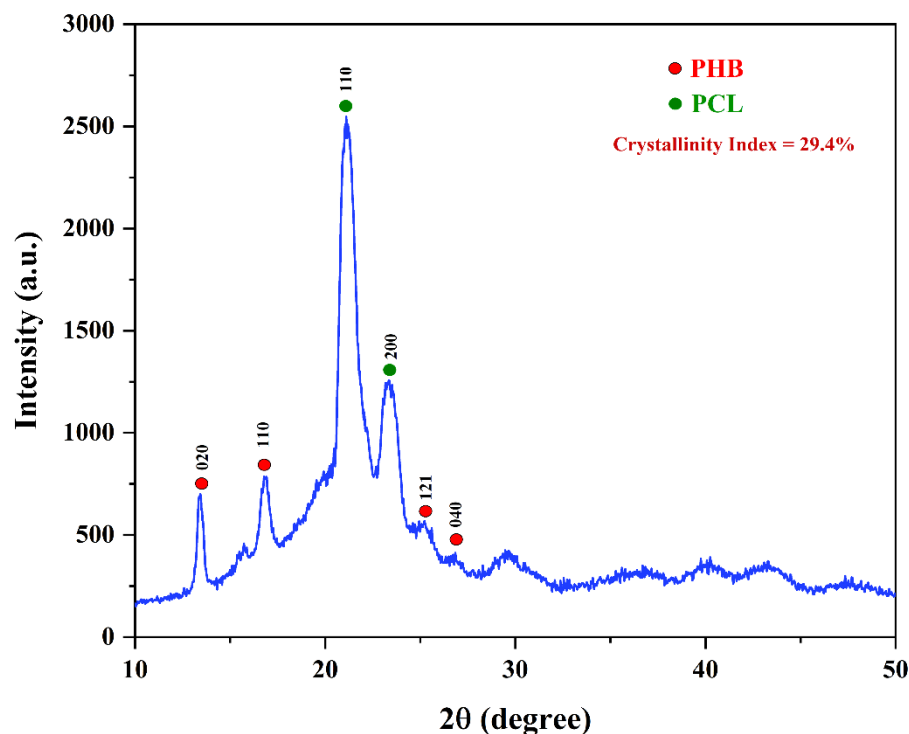


Fig 4.1.5. XRD curve of PHB/PCL sorbent

The thermostability of the porous PHB/PCL sorbent was assessed using thermal analysis, including TGA, DTG, DTA, and DSC as illustrated in figure 4.1.6 and 4.1.7. TGA analysis was specifically achieved to assess the mass loss of the sorbent over the desired temperature range. The TGA curves of PHB/PCL sorbent, as shown in the figure, exhibit two distinct stages, corresponding to PHB and PCL. The initial degradation phase pertains to PHB degradation (225 °C-320 °C), resulting in approximately 24.1 % mass loss, whereas the subsequent phase, occurring at elevated temperatures, is associated with PCL degradation (340 °C-450 °C), resulting about 64.7 % mass loss. Lin et al. (2019) also reported two deterioration phases of the PHB/PCL blend in the TGA curve. PHB initially degraded with a mass loss temperature between 250 °C and 300 °C, followed by the degradation of PCL, which occurred at a mass loss temperature ranging from 350 °C to 450 °C. The residual mass of the PHB/PCL sorbent at 799.7 °C was 6.64 %, consisting only of carbon. It is significant to note that PCL was significantly

more thermally stable than PHB despite having a lower melting temperature (T_m) of 64.2 °C; its onset degradation temperature was 340 °C, and its maximum degradation temperature (T_d) was 399.2 °C, exhibiting a good thermal stability. Zhang et al. (2020) prepared a fibrous Polylactic acid (PLA) membrane for oil adsorption using solution blow spinning. The maximum degradation temperature (T_d) of the membrane was 353.4 °C, which is comparatively lower than PHB/PCL sorbent.

DTG analysis is conducted to ascertain the peak temperatures associated with the maximum mass loss rate of the PHB/PCL sorbent. The DTG curves of the PHB/PCL sorbent exhibited two primary peaks assigned to the degradation of PHB at lower temperatures and PCL at higher temperatures. The maximum degradation temperature (T_d) of PHB was determined to be 299.2 °C, exhibiting a maximal mass-loss rate of 11.32 %/minute, whereas the T_d of PCL was determined at 399.2 °C, with a maximal mass-loss rate of 15.09 %/minute.

DTA is conducted to determine the phase transitions of the PHB/PCL sorbent. A DTA curves exhibit an exothermic peak if cross-linking mechanisms were implicated in the degradation of biopolymer (Acharjee et al., 2024). The DTA curve exhibited four different exothermic peaks. The exothermic peaks at 64.2 °C and 399.2 °C corresponds to melting temperature (T_m) and degradation temperature (T_d) of PCL, while exothermic peaks at 169.2 °C and 299.2 °C corresponds to T_m and T_d of PHB. The result of DTA is in accordance to the result of DTG of PHB/PCL sorbent.

The DSC analysis of the PHB/PCL sorbent reveals distinct evidence of immiscibility between the two biopolymers, evidenced by the presence of two separate peaks at their respective melting temperatures: one peak at 62.86 °C corresponding to PCL and another at 173.5 °C associated with the melting of PHB. The degree of crystallinity of each biopolymer in the blend was determined using the melt enthalpy (ΔH_m) for both biopolymers. The degrees of crystallinity (X_c) of PHB and PCL segments in the sorbent were found to be 22.8 % and 30.8 %, respectively.

DTA and DSC are both thermal analysis techniques that offer similar fundamental information but vary in their measurement methods. The former relies

on the temperature differential, whereas the latter depends on the heat flow differential (Schick et al., 2012). The DTA involves the direct measurement of the temperature differential between a sample and an inert reference material. Nevertheless, the DSC depends on the difference in energy necessary for maintaining the sample and reference at an equivalent temperature. Owing to the unpredictability of fusion enthalpies and transition temperatures derived from DTA, DSC is extensively employed across various fields. The current study established that the T_m for PHB, as evaluated by DTA, is 169.2 °C, while for PCL, it is 64.2 °C. The T_m measured by DSC for PHB was found to be 173.5 °C, while for PCL it was 62.86 °C. Although the measuring principles varies, the value of T_m for both PHB and PCL provided by the DTA and DSC analysis are comparable.

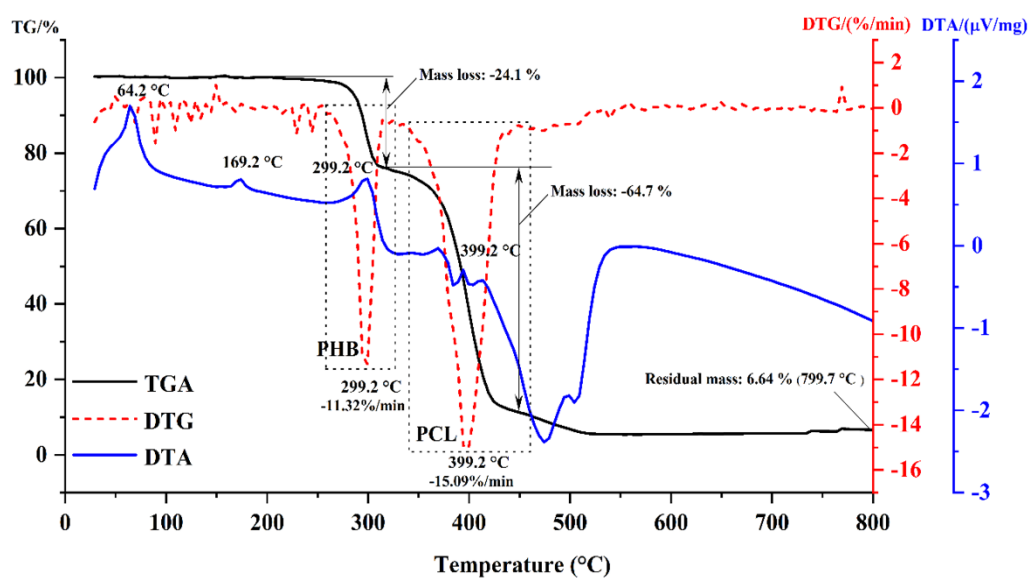


Fig. 4.1.6. TG/DTG/DTA analysis of PHB/PCL sorbent

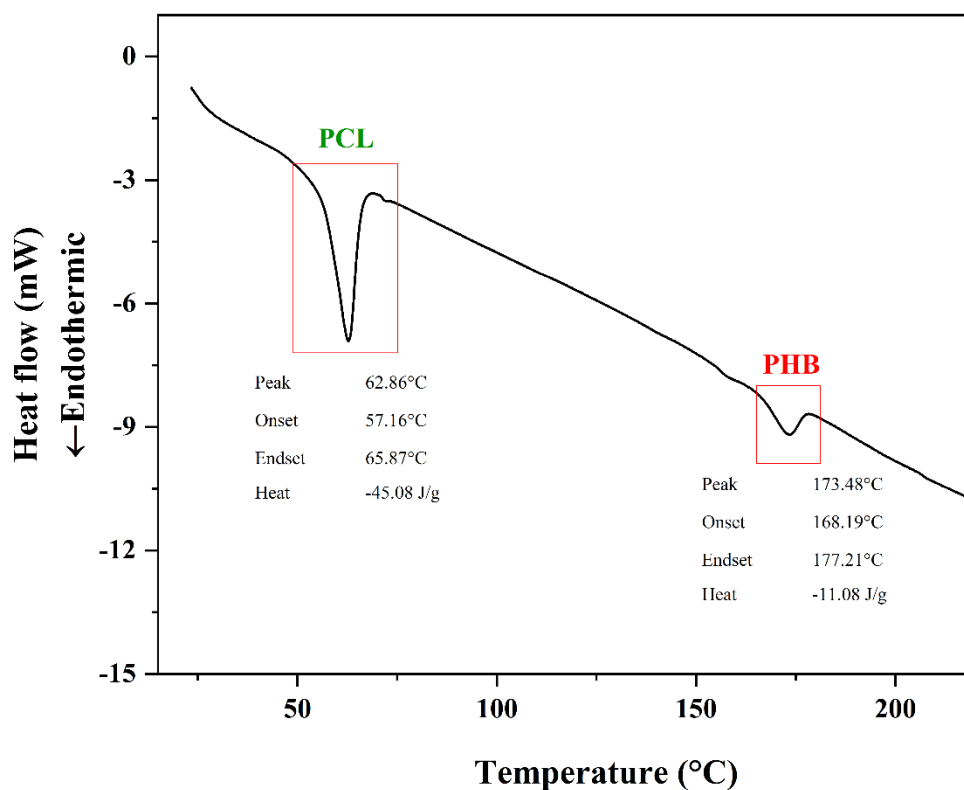


Fig. 4.1.7. DSC curve of PHB/PCL sorbent

The N₂ adsorption-desorption isotherm was assessed on the sorbent utilizing the BET instrument to acquire important insights on specific surface area, pore volume, and average pore diameter (figure. 4.1.8). The IUPAC categorization identifies the Type IV adsorption isotherm behaviour, as seen by the N₂ adsorption-desorption isotherm of the sorbent, indicating its mesoporous structure. The association of strong Type IV isotherm characteristics with the adsorption of sorbate molecules by monolayer-multilayer adsorption suggests a stable interaction between the adsorbent and adsorbate, consistent with previously reported result (Raj et al., 2021). The specific surface area, pore volume, and pore diameter were determined to be 81.642 m²/g, 0.038 cc/g, and 3.62 nm, respectively. The IUPAC categorizes mesopores as having a diameter (d) between 2–50 nm, macropores as having a diameter (d > 50 nm), and micropores as having a diameter (d < 2 nm) (Raj et al., 2021). Rotaru et al. (2014) also reported the mean pore diameter of clay polymer aerogel (M4) to be 3.70 nm corresponding to mesopore size.

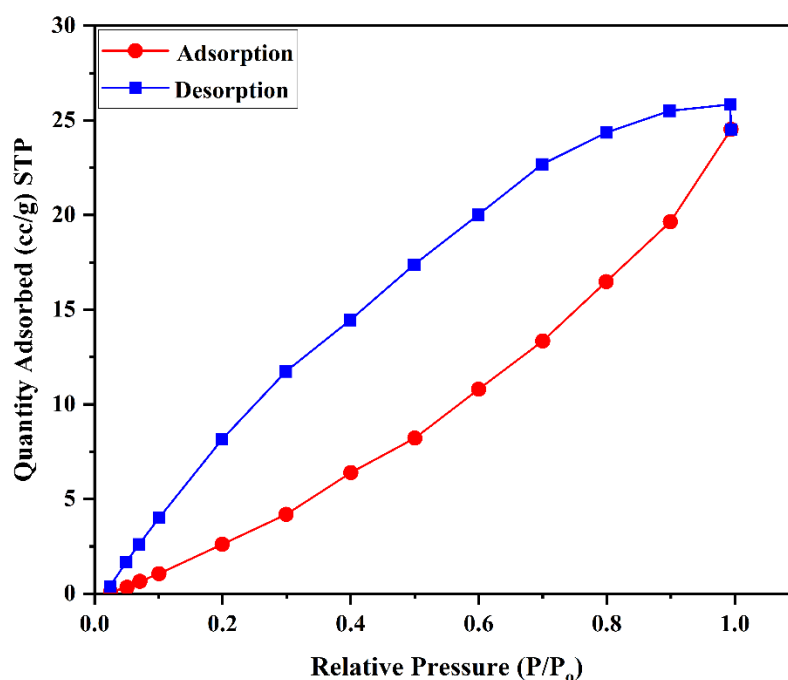


Fig. 4.1.8. N₂ adsorption-desorption isotherms of PHB/PCL sorbent

The BJH pore size distribution indicated pore sizes ranging from 3.62 nm to 301.57 nm. This range indicates that the pristine PHB/PCL sorbent is classified as a mesoporous–macroporous material. Macropores play an essential role in oil spill sorption (Rotaru et al., 2014). To explore macropores larger than 301.57 nm, we used FESEM analysis. Figure 4.1.9 illustrates the surface morphologies of the pristine PHB/PCL sorbent, the PHB/PCL sorbent following the sixth cycle regeneration of WCO and diesel oil. Figure 4.1.10 shows statistic histogram with a Gaussian fitting curve of the pore size distribution over the PHB/PCL sorbent. To construct the histogram, 96 counts of macropores were determined from FESEM images. FESEM analysis indicates that the PHB/PCL sorbent is a highly porous material with a macropore size distribution that extend from 0.23 μm to 7.96 μm . The mean macropore size for the PHB/PCL sorbent was found to be 0.90 ± 0.04 μm , as determined through statistical analysis of the histogram.

The pristine PHB/PCL sorbent's chemical composition revealed carbon and oxygen as the predominant constituents, comprising 63.13 wt % and 36.62 wt %, respectively. Furthermore, trace amounts of sodium at 0.25 wt % are detected as NaCl particles were trapped within the polymer matrix despite leaching. The

FESEM images (figure 4.1.9a) revealed significant interconnecting hierarchical pores created by the leaching of NaCl particles from the polymer matrix. This signifies a low interfacial attraction between NaCl and the polymer matrix. The porous structure of the sorbent is crucial for oil storage, facilitating oil sorption. Additionally, small pores can lower the mass transfer resistance of the sorbents, which improves the rate of oil absorption (Pinto et al., 2016).

Figure 4.1.7b illustrates the FESEM image of the PHB/PCL sorbent following the regeneration process of WCO. The imaging results indicated that after six cycles of regeneration, the porous structure of the sorbent was shrunk or collapsed. The EDS examination indicated an increase in carbon weight % from 63.13 to 86.54 wt %. However, the weight % of oxygen has drastically decreased from 36.62 to 13.40 wt %. Similarly, the PHB/PCL sorbent image (figure 4.1.9c) during diesel oil regeneration revealed that almost all of the pores had collapsed, leaving only a small number of open pores. The EDS examination indicated an increase in carbon weight % from 63.13 to 68.99 wt %, while the weight % of oxygen reduced from 36.62 to 30.96 wt %. In addition, trace amount of chlorine was detected with 0.06 wt % (figure 4.1.9b) and 0.05 wt % (figure 4.1.9c), showing that residual NaCl has remained after blending. But upon regeneration, the sample had no traces of sodium, which could have been washed out.

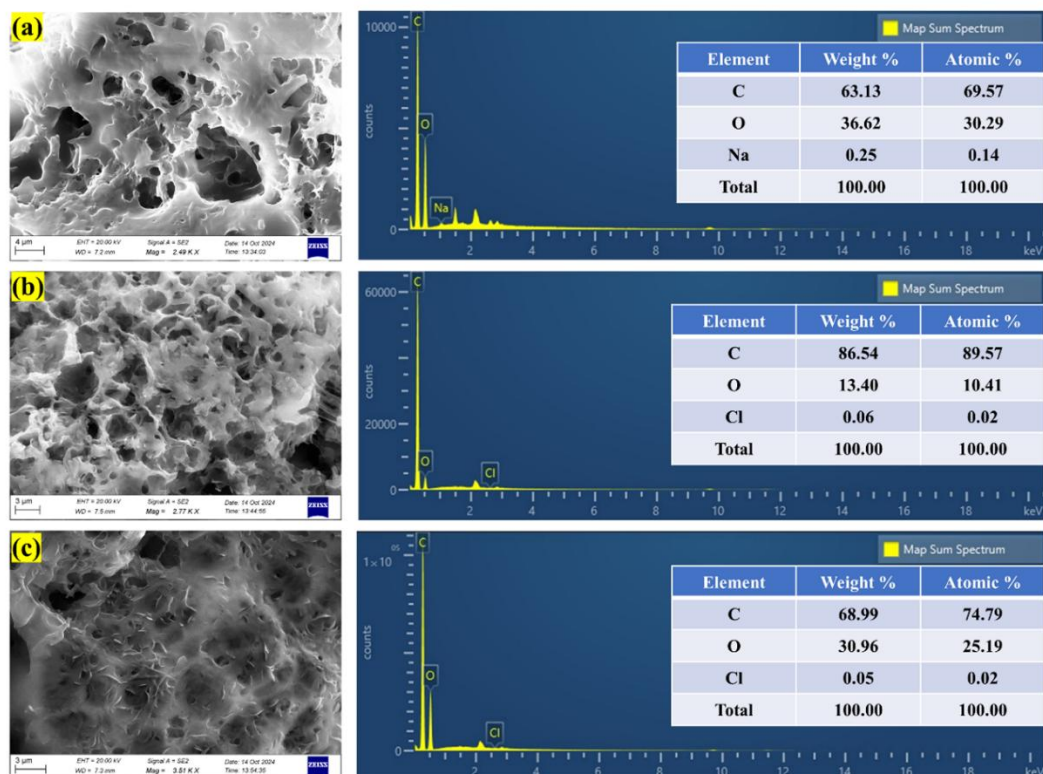


Fig. 4.1.9. FESEM image of the sorbent (a) Image of pristine PHB/PCL sorbent before absorption of oil and its map sum spectrum, (b) Image of PHB/PCL sorbent after regeneration (6th cycle) of WCO and its map sum spectrum, and (c) Image of PHB/PCL sorbent after regeneration (6th cycle) of diesel oil and its map sum spectrum

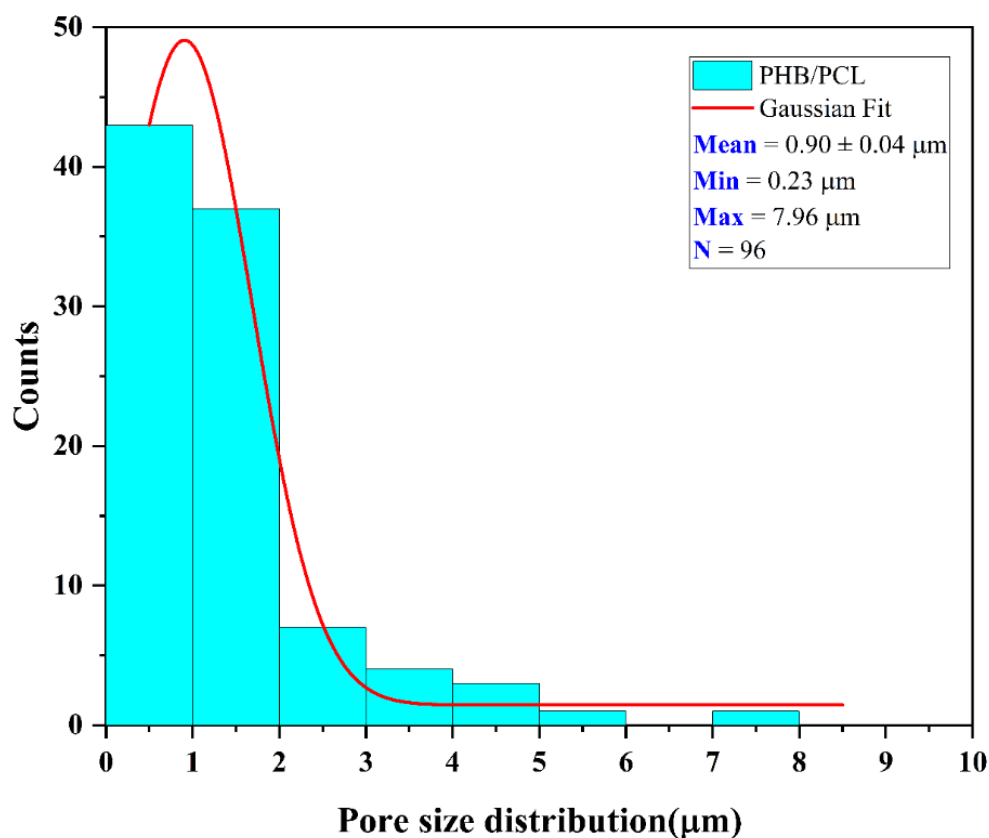


Fig. 4.1.10. Statistic histogram with a Gaussian fitting curve of the pore size distribution of the PHB/PCL sorbent

Stereoscope microscope was used for visual examination of the PHB/PCL sorbent. The stereomicroscope is an essential tool for examining diverse specimens and facilitates the thorough analysis of their surface characteristics. It is intended for the observation of small, 3D objects under transmitted and reflected light. The stereomicroscopic images of the sorbent before absorption, after absorption and after regeneration (6th cycle) for both diesel and WCO were captured and are illustrated in figure. 4.1.11. The image of the sorbent after regeneration for 6 times for both diesel and WCO shows a variation in sizes of the available pores over the surface as compared to the images of the sorbent before absorption.

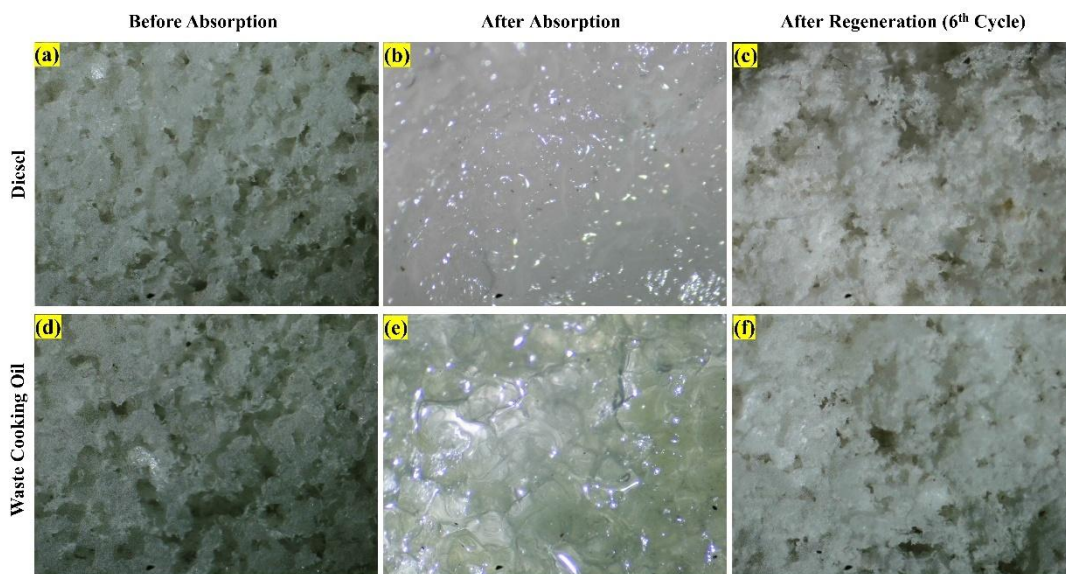


Fig. 4.1.11. Stereomicroscopic image (10X) of the sorbent (a) Pristine PHB/PCL sorbent before absorption of diesel oil, (b) Sorbent after absorption of diesel, (c) Sorbent after regeneration (6th cycle) of diesel oil, (d) Pristine PHB/PCL sorbent before absorption of WCO, (e) Sorbent after absorption of WCO, and (f) Sorbent after regeneration (6th cycle) of WCO

Hydrophobicity and lipophilicity are the two fundamental features of oil sorbents, which are crucial in oil sorption (Schick et al., 2012). A key aspect in evaluating a material's wettability properties is the WCA or OCA. Consequently, the sessile water droplet technique with a volume of 4 μL was employed to assess the wettability of the PHB/PCL sorbent. Using a glass syringe, vertical liquid droplets were applied to the surface of the sorbent. Three contact angle measurements were conducted at different locations, and the results were presented based on their average value. Figure 4.1.12a illustrates that the PHB/PCL sorbent exhibited a WCA of 106.26° and an OCA of 0° for both diesel and WCO.

The hydrophobicity and lipophilicity of the sorbent were also assessed by applying water and oil droplets onto sorbent's surface. Deionized water (stained with methylene blue), diesel oil (stained with Sudan III), and waste cooking oil (stained with Sudan black B) were selected to demonstrate the wettability of the sorbent. The water droplet formed a sphere on the sorbent's surface without sinking, as seen in figure 4.1.12d. In contrast, the sorbents were rapidly saturated by diesel

oil and waste cooking oil. These results further proved the good hydrophobicity of PHB/PCL sorbent. Figure 4.1.12b displays the top view of the PHB/PCL sorbent and figure 4.1.12c displays the light-weight nature of the sorbent.

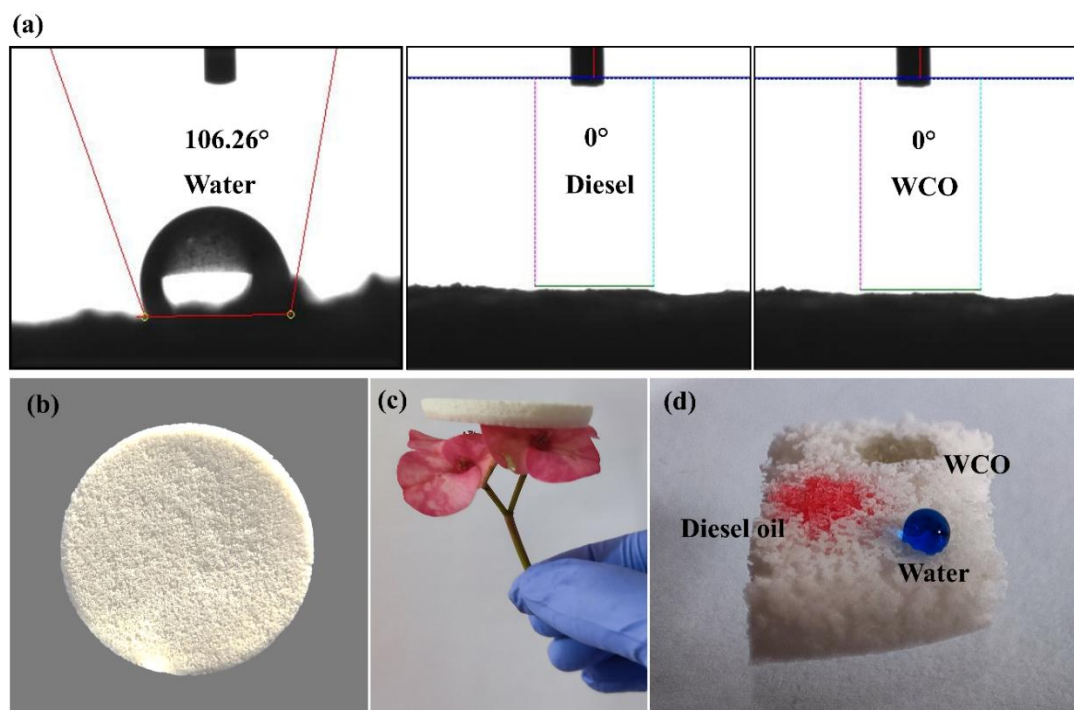


Fig. 4.1.12. (a) Contact angle of water, diesel and WCO, (b) Aerial view of the porous PHB/PCL sorbent, (c) Sorbent above flower petal showing its light-weight, and (d) Droplets of diesel oil (dyed with Sudan III), WCO (dyed with Sudan black B) and water (dyed with methylene blue)

4.1.4.2. Oil sorption process

To elucidate the oil sorption capacity of the sorbent, two oils (diesel oil and waste cooking oil) were used as the models. In an attempt to quantify the oil sorption potential of a sorbent from immiscible oil/water mixture, a volume of 3 mL of oil was introduced into a beaker filled with 60 mL of water. Floating on the water surface, a layer of oil was developed. Next, a disc-shaped PHB/PCL sorbent was inserted into the beaker comprising an oil/water mixture. The sorbent remained in the layer of an oil and the oil was absorbed completely within 30 secs. Additionally, the sorbent was placed in a beaker with just water for 30 secs, and there was no discernible change in the sorbent's weight. The sorption behaviour in water and an immiscible oil/water mixture indicated that the sorbent showed good

oil/water selectivity. The sorbent demonstrated good potential in the field of oil sorption as evidenced by its quick and highly selective oil sorption from oil/water mixture (figure 4.1.13). The sorbent was able to quickly absorb oil from an immiscible oil/water mixture because of its interconnected hierarchical pore structure (figure 4.1.9a), hydrophobicity and lipophilicity (figure 4.1.12a and 4.1.12d). The oil is driven through the pores, and subsequently retained in the void, in the presence of a capillary pressure (Cuong et al., 2024).

The sorption of diesel and WCO with PHB/PCL sorbent is further confirmed by FTIR analysis as shown in figure 4.1.14a and 4.1.14b. The FTIR spectra of PHB/PCL-diesel sorbent and pure diesel were found to be similar with comparable chemical functionality, which confirms the absorption of diesel by PHB/PCL sorbent. For instance, sharp peaks at 2956, 2924, 2854, 1638, 1460, 1377, and 722 cm^{-1} were only observed in the spectra of pure diesel and PHB/PCL-diesel sorbent, because these peaks are the characteristic absorption bands of diesel (Coronado et al., 2017). One small peak was observed in PHB/PCL-Diesel at 1747 cm^{-1} which corresponds to an ester carbonyl ($\text{C}=\text{O}$) group of PHB/PCL biopolymer. This peak was missing in the spectra of pure diesel. In addition, as compared to the spectra of pristine PHB/PCL, the $\text{C}=\text{O}$ group was present at peak 1734 cm^{-1} . This shifting of $\text{C}=\text{O}$ group from 1734 to 1747 cm^{-1} could be due to the interaction between the PHB/PCL biopolymer and diesel.

Similarly, the FTIR spectra of PHB/PCL-WCO sorbent and pure WCO were found to be similar with comparable chemical functionality, which confirms the absorption of WCO by PHB/PCL sorbent. For instance, sharp peaks at 3008, 2926, 2854, 1746, 1645, 1464, 1238, 1164, and 721 cm^{-1} were only observed in pure WCO and PHB/PCL-WCO sorbent, because these peaks are the characteristic absorption bands of WCO (Wahab et al., 2015). These peaks are corresponded to alkane groups, ester $\text{C}=\text{O}$ group of triglycerides, and alkene group found in the spectra of WCO.

Diesel oil and WCO had saturated oil sorption capacity of 5.09 g/g and 6.02 g/g, respectively, for PHB/PCL sorbent (figure 4.1.14c). This sorption capacity is higher than that of number of sorbents reported previously for similar kind of oils, including a polycarbonate (4.96 g/g) (Wang et al., 2019), Polyethylene polymeric

foam (0.49 g/g) (Patil et al., 2018), hydrophobic aerogel (2.8 g/g) (Wang et al., 2010), recycled textile fibre (4.4 g/g) (Sulyman et al., 2018), polyvinylidene fluoride (4.05 g/g) (Chen et al., 2017), Banana peel biochar (5.33 g/g) (Urgel et al., 2024), and magnetic biochar (4.01 g/g) (Sun et al., 2022). Nevertheless, the sorption capacity of the PHB/PCL sorbent is comparatively lower than that of other reported carbon-based sorbents (Carbon nanotubes and graphene) and polymer-based sorbents such as reduced-graphene oxide/polyvinylidene fluoride (Byun et al., 2024), Polyurethane (Santos et al., 2017), Polystyrene (Akanji et al., 2024), Polypyrrole/Melamine-formaldehyde (Dashairya et al., 2019). However, these sorbents necessitate expensive fabrication materials, specialized chemicals, costly equipment, and intricate, prolonged methods, hence constraining their scale of production. Moreover, petroleum-based polymers, despite their excellent performance, are both non-disposable and non-biodegradable, therefore presenting significant secondary pollution and ecotoxicological effects, further aggravating the environmental impact (Yeo et al., 2020). Comparison of PHB/PCL sorbent on oil sorption performance against other sorbents is presented in table 1. The preparation method for the PHB/PCL sorbent is a facile process that is easily scalable and cost-effective, requiring no expensive raw materials, specialized chemicals, nanoparticles, or expensive equipment. Consequently, in terms of methodology, affordability, and biodegradability, our sorbent has significant benefits over existing alternatives for the remediation of intricate hydrophobic pollutants such as leaks and oil spills.

Table 4.1.1. Comparison of PHB/PCL sorbent on oil sorption performance against other sorbents

Sorbents	Oil type	Sorpt	Hydrop	Biodegrad	Refere
		ion	hobicity		
		capa	hobicity	ability	nces
		city	(WCA°)		
		(g/g)			
Synthetic polymer-based oil sorbents					

Polypropylene fibers	Motor oil	8.96	129	Non-Biodegradable	(Jiang et al., 2023)
Polyvinylidene fluoride (PVDF) aerogel	Diesel	4.05	151	Non-Biodegradable	(Chen et al., 2017)
Non-woven polypropylene	Diesel	5.46	-	Non-Biodegradable	(Zaro et al., 2021)
Expanded polyethylene (EPE) polymeric foam	Engine oil	0.66	96	Non-Biodegradable	(Patil et al., 2018)
	Palm oil	0.78			
	Used engine oil	0.61			
	Used palm oil	0.58			
	Hair oil	0.62			
	Diesel	0.41			
Polycarbonate monolith	Kerosene oil	4.25	136.18	Non-Biodegradable	(Wang et al., 2019)
	Diesel	4.96			
	Soybean oil	33.56			
	Lubricating oil	38.90			
Polyurethane foams	Diesel	16.8	123	Non-Biodegradable	(Santos et al., 2017)
	Motor oil	20.7			
	Gasoline	15.7			
	Kerosene oil	13.01			
Metal organic framework doped	Kerosene oil	8.98	154.25	Non-Biodegradable	(Wang et al., 2019)
	Diesel	9.23			

polycarbonate (Z8/PC-2)	Diluted bitumen	5			
	Motor oil	1.41			
	Diesel	1.4			
Polystyrene nanofibers	Furnace oil	1.5	109	Non- Biodegrad able	(Ghiasv an et al., 2021)
	Crude oil	1.1			
	Lubricati ng oil	4.9			
Polypyrrole- encapsulated melamine formaldehyde	Diesel	60.30			
	Vegetabl e oil	94.13	170	Non- Biodegrad able	(Dashai rya et al., 2019)
	Hydrauli c oil	74.66			
Recycled textile fiber	Waste cooking oil	4.40	-	Non- Biodegrad able	(Sulym an et al., 2018)
	Crude oil	4.20			
Synthetic/bio-based oil sorbents					
	Soybean oil	9.83			
Polypropylene/ligni n sponge	Engine oil	7.88	113	Non- Biodegrad able	(Alasso d et al., 2020)
	Lubricati ng oil	5.5			
Chitin/halloysite nanotubes (C/HNTs) composites	Sunflowe r seed oil	~6	98	Biodegrad able	(Zhao et al., 2019)
Poly (methyl methacrylate) grafted wheat straw	Diesel	1.12	111	Non- Biodegrad able	(Suresh et al., 2022)
Silica-based oil sorbents					

Silica aerogel	Vegetable oil	14.6	-	Non-Biodegradable	(Wang et al., 2012)
	Crude oil	11.7			
	Motor oil	15.1			
Hydrophobic silica aerogels	Vegetable oil	2.8	130	Non-Biodegradable	(Wang et al., 2010)
Carbon-based oil sorbents					
Thermally reduced graphene	Diesel	1.55	-	Non-Biodegradable	(Diraki et al., 2018)
Graphene nanoplatelets	Diesel	0.805	-	Non-Biodegradable	(Diraki et al., 2018)
Reduced-graphene oxide nanoribbon-coated polyvinylidene fluoride (r-GONR-coated PVDF)	Motor oil	18.3	100	Non-Biodegradable	(Byun et al., 2024)
	Diesel	11.5			
CNT/ polyvinyl alcohol -recycled polyethylene terephthalate (CNT/PVA-rPET)	Kerosene oil	12	119	Non-Biodegradable	(Janqamsari et al., 2021)
Coffee grounds– sugarcane bagasse carbon aerogels	Soybean oil	10.02	101.6	Biodegradable	(Phat et al., 2024)
	Lubricating oil	5.804			
Phenyltrimethoxysilane-polyethylenimine-reduced graphene oxide-Polyurethane sponge	Bicycle chain oil	8.8	95.82	Non-Biodegradable	(Periasamy et al., 2017)
	Engine oil	8.4			

(PTMOS/PEI/rGO/PU)					
Vermiculite-carbon nanotube nanosponge	Vegetable oil	3.5	-	Non-Biodegradable	(Moura et al., 2009)
	Engine oil	3.7			
	Diesel	3.8			
Multi-walled carbon nanotube (MWCNT)-SiO ₂ /PVDF composite membranes	Pump oil	4.2	155	Non-Biodegradable	(Jiang et al., 2021)
	Machine oil	4.8			
	Lubricant oil	4.2			
	Soybean oil	4.5			
	Sunflower oil	4.6			
Polystyrene-reduced graphene oxide composite	Vegetable oil	98.86	-	Non-Biodegradable	(Akanji et al., 2024)
	Fresh engine oil	86.25			
	Used engine oil	83.47			
	Engine oil	4.44			
Banana peel biochar	Diesel	5.33	98.45	Non-Biodegradable	(Urgel et al., 2024)
Bio-based oil sorbents					
Peat moss	Crude oil	11.8	104.33	Biodegradable	(Narayan et al., 2018)

Raw cotton	Motor oil	10.33	110	Biodegradable	(Jiang et al., 2023)
Porous Polylactic acid film	Vegetable oil	5	151.6	Biodegradable	(Xue et al., 2013)
	Diesel	~4.5			
	Gasoline	5			
Polycaprolactone (PCL)/Poly-D, L-Lactic acid (PDLLA) modified melamine	Crude oil	6	162	Biodegradable	(Aruna giri et al., 2021)
	Soybean oil	~6.9			
Polyhydroxybutyrate/polycaprolactone (PHB/PCL)	Waste cooking oil	6.02	106.26	Biodegradable	This study
	Diesel	5.09			

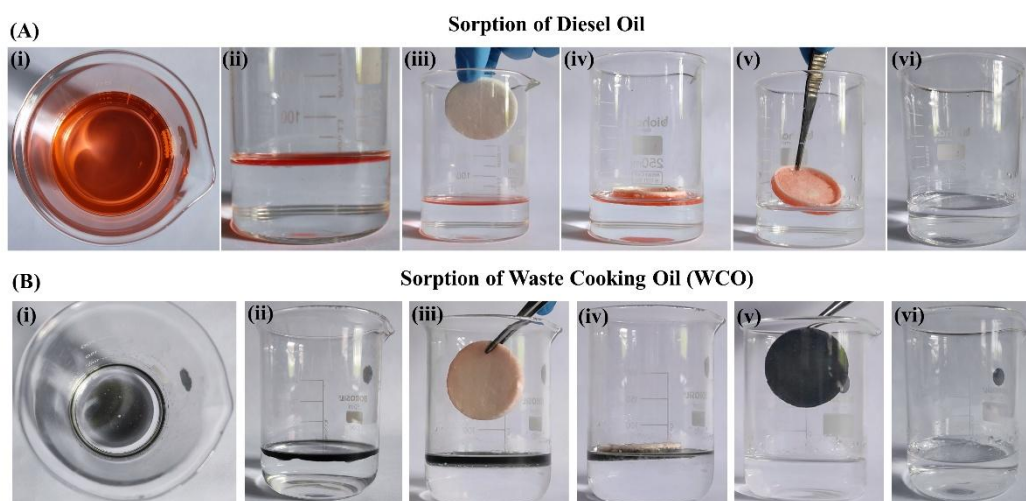


Fig. 4.1.13. (A) A series of photographs showing the sorption of diesel oil using PHB/PCL sorbent; (i) top view of diesel oil/water mixture (dyed with Sudan III), (ii) Diesel oil floating on the surface of water, (iii) Sorbent before sorption of diesel oil, (iv) sorbent showing its buoyancy effect while absorbing the diesel oil from water, (v) fully soaked sorbent placed in diesel oil/water mixture, and (vi) clear water after absorption of diesel oil, (B) A series of photographs showing the sorption of WCO oil using PHB/PCL sorbent (i)-(vi)

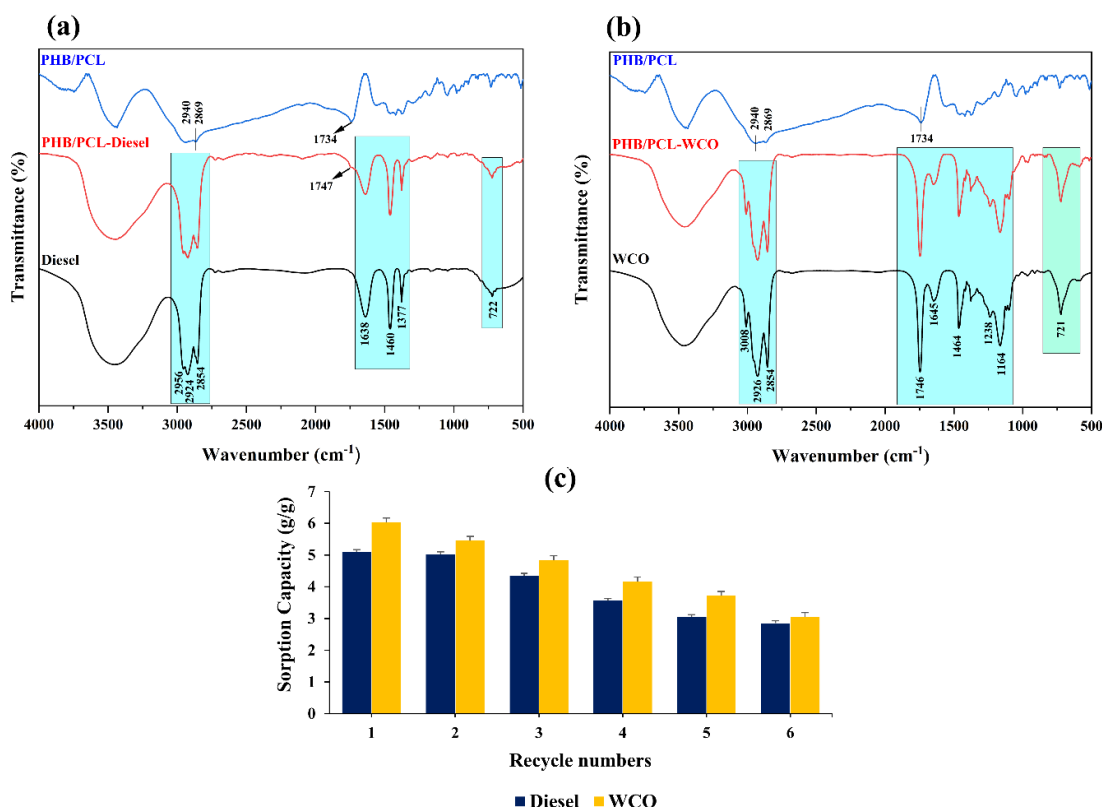


Fig. 4.1.14. (a) FTIR spectrum of diesel loaded PHB/PCL sorbent, (b) FTIR spectrum of WCO loaded PHB/PCL sorbent, and (c) Sorption capacity of diesel and WCO by PHB/PCL sorbent

4.1.4.3. Mechanical properties

The evaluation of the mechanical properties of the sorbent involved measuring the compressive load-elongation curve, which showed the structural integrity of the sorbents under compression. The compressive load-elongation curve exhibits three distinct phases: a linear elastic region, a gradually ascending plateau region, and a sharply increasing densification region. Duarte et al. (2018) showed that these three steps are a typical property of an open cellular material. The elastic region is defined as a reversible area in which the imposed deformation can be reversed, allowing the cell walls to bend but entirely reverting to their original shape upon removal of the applied load or stress. The plateau region is defined by the fracture of certain cells and a rupture of the porous structure. Finally, when a continuous force is further applied it results in the formation of a densification region, leading to the collapse of cell walls and irreversible deformation. The compression test results of

the sorbent up to 10 cycles is presented in table 4.1.2. Figure 4.1.15 illustrates the compressive load-elongation curves of porous sorbent for 0, 4, 8, and 10 cycles for WCO and diesel samples. The figure distinctly illustrates that the load and elongation progressively diminish from 0 to 8 cycles in both WCO and diesel samples. Following 10 cycles of oil sorption, the sorbent in both WCO and diesel samples shown a considerable reduction in load capacity; however, a minor increase in elongation was noted in both oil samples. The minor increase in elongation may result from the swelling and softening of the sorbent, attributable to repeated cycles of oil sorption, which contribute to material fatigue and deformation (Metze et al., 2023).

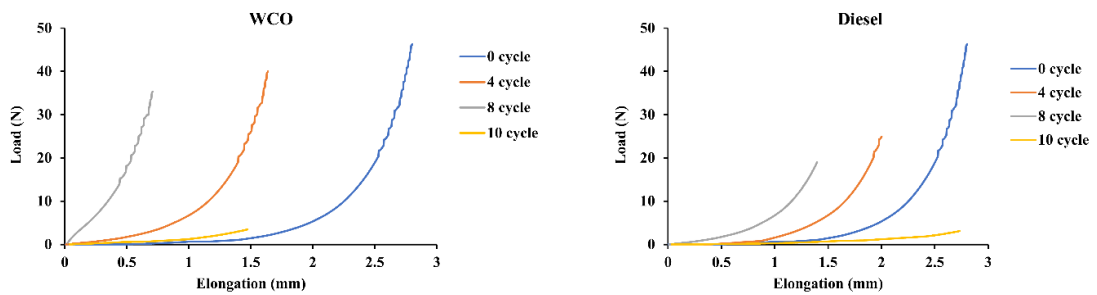


Fig. 4.1.15. Compressive load-elongation curves of PHB/PCL sorbent for WCO and diesel samples

Table 4.1.2. Compression test results of the sorbent up to 10 cycles

Oil absorbed	Cycles	Peak load (N)	Elongation (mm)	Compressive strength (N/mm ²)
WCO	0	46.24	2.803	0.094
	4	39.98	1.639	0.081
	8	35.3	0.708	0.071
	10	3.48	1.475	0.007
Diesel	0	46.24	2.803	0.094
	4	24.94	2.003	0.050
	8	19	1.398	0.038
	10	3.08	2.732	0.006

4.1.4.4. Oil retention profile test

It is essential to note that oil sorption and dripping occur at distinct phases of the oil removal process. The initial phase involves sorption occurring when the sorbent directly interacts with the oil. When these components come into contact, oil sorption manifests as a multifaceted process, encompassing the sorption of hydrocarbon vapours, followed by capillary condensation, and fluid entrapment by adhesive and cohesive forces (Condurache et al., 2022). Depending on the physical form of the sorbent and the characteristics of the oil, one phenomenon may dominate the other. The second phase, known as the separation stage, involves removing the saturated sorbent from the liquid bath so that the surplus oil can drip off the loaded sorbent. The surplus oil held by cohesive forces or poorly confined in extensive macro-pores will be drained by gravity, thereby influencing the retention capacity of the sorbent (Bazargan et al., 2015). Consequently, the dripping phenomenon is linked to the fluid-desorption process. The dripping signifies a partial fluid desorption from the saturated sorbent. Subtracting the dripped volume from the total collected oil reveals the retention capacity of the oil adhered to the sorbent. Consequently, the dripping kinetics provide insights into the dynamics of partial fluid desorption, while elucidating the retention dynamics of oil by the sorbent throughout the separation phase (Condurache et al., 2022).

According to the approach outlined in ASTM F726, the proposed dripping duration is 30 secs for light and medium viscosity oils, and 2 minutes for heavy or aged oils. Nevertheless, this duration is frequently insufficient and results in mistakes when interpreting the outcomes. The uptake capabilities reported using the ASTM F726 method are frequently inflated and significantly exceed the real practical uptake capacity of the sorbent (Bazargan et al., 2015). Upon immediate removal of the sorbent from the oil phase, with a dripping time $t = 0$, equation 4.1.3 provides the maximal sorption capacity $Q_{\max} = Q(t_0)$. In practice, it is challenging to estimate this, as the drainage of liquid from saturated sorbent begins immediately upon its removal from the liquid phase. Consequently, it was resolved to choose a brief dripping duration as a criterion for assessing the initial sorption capacity. In oil removal applications utilizing solid materials, the sorption capacity effectively denotes the retention capacity, which is contingent upon the system's exposure to the duration of dripping. When this duration is extended, equilibrium is achieved

between the retention and drainage of the liquid. The dripping effect ceases at this point. Thus, it is essential to record the calculated retention capacity of a specific sorbent-oil system at various drainage intervals (retention kinetics) (Condurache et al., 2022).

The dynamics of oil retention have been investigated by assessing the retention capacity at various dripping times (t), in accordance with equation 4.1.3. The retention kinetics profile was established for the time stretch of 0.5 to 300 secs. Figure 4.1.16 shows the dripping profile of the sorbent. The sorbent attained its equilibrium capacity within 180 secs of dripping, and no more dripping was noted thereafter. This factor is very beneficial for distinguishing between oil sorption and retention capacity.

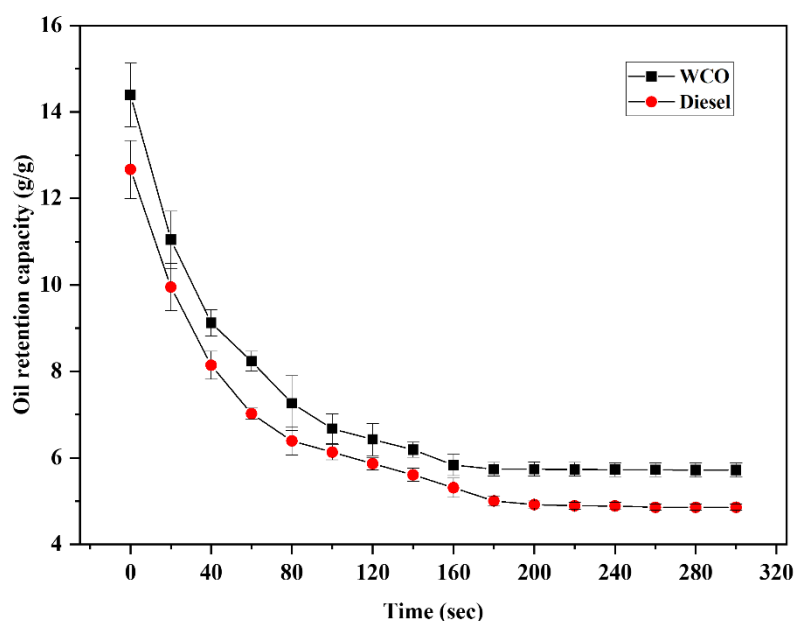


Fig 4.1.16. Dripping profile of the PHB/PCL sorbent

4.1.4.5. Immiscible oil/Water mixture Separation flux Experiment

Flux is an essential parameter that signifies the rate of separation of an immiscible oil-water mixture. Figure 4.1.17 illustrates that when an oil/water mixture was introduced into an aslant separation apparatus, the water retained in the upper glass funnel of the aslant separation apparatus while the oil phase permeated through the sorbent under the influence of gravity and collected in the beaker placed underneath. The separation flux for the diesel oil/water mixture was determined to be 159.18 L/m²/h, whereas for the WCO/water mixture, it was 119.33 L/m²/h.

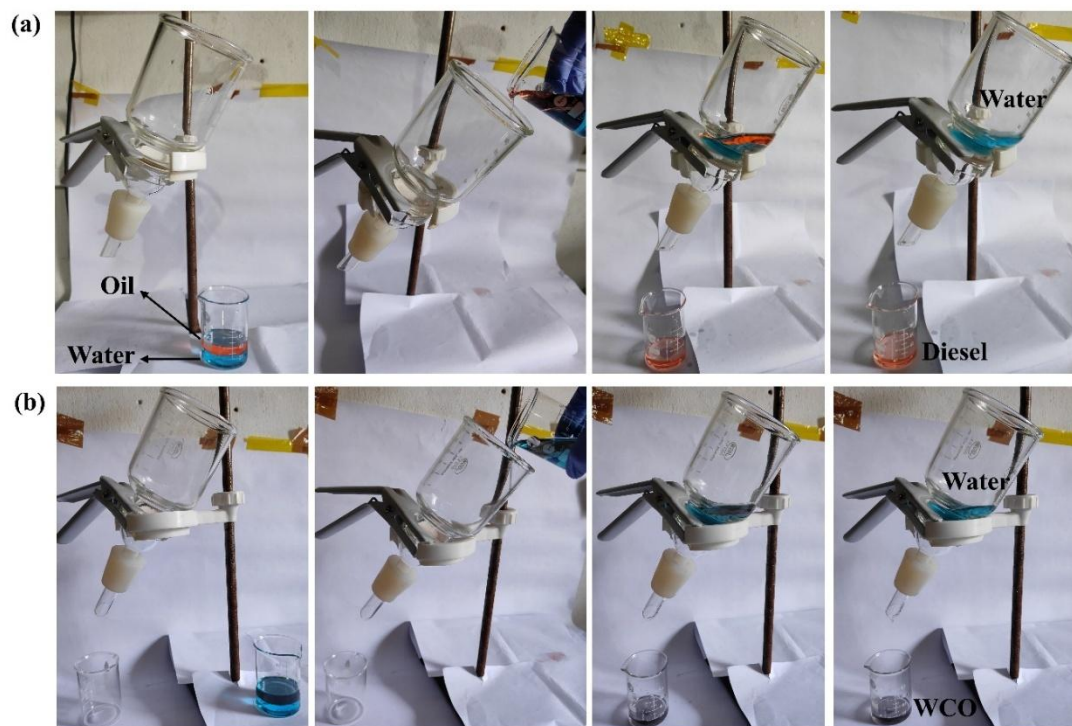


Fig 4.1.17. Photographs of immiscible oil/Water mixture separation flux experiment (a) Diesel oil/water separation and (b) WCO/water separation

4.1.4.6. Reusability and recovery

Reusability and oil recovery are essential factors in the design of materials for oil removal, necessitating environmental and economic sustainability. To assess the feasibility of recycling the sorbents, we sought a technique capable of extracting the absorbed oil without compromising the structural integrity of the sorbents. Considering the insolubility of PHB and PCL in several organic solvents, we selected diethyl ether as the model solvent for the clean-up and recycling of the sorbents, due to its inherent ability to dissolve oily substances while preserving the porous structure of the sorbents. In one study, Scaffaro et al. (2017) also recycled the PCL/PEG sorbents by rinsing the oil-saturated sorbent with diethyl ether. The current investigation revealed that the oil extracted from the oil-diethyl ether mixture by the solvent evaporation method with a rotary evaporator achieved recovery rates of 86.5 % for WCO and 85.7 % for diesel.

However, a reduction in sorption capacity for diesel and WCO is noted in the sixth cycle, measuring 2.9 g/g and 3.1 g/g, respectively. This reduction can be attributed to the saturation of the active sites on the sorbent's surface after each

application. As shown in figure 4.1.9b and 4.1.9c, the FESEM image of the sorbent after 6th cycle exhibited a shrinkage of porous structure, hence, limited sites may be accessible for the attachment of oil particles, resulting in diminished sorption capability. This issue was observed in several studies. Recently, Al-Muhtaseb et al. (2024) also mentioned about the significant decrease in the sorption capacity after the first cycle due to limited active sites present in the adsorbent.

To address this issue, the structural integrity and strength of PHB/PCL can be improved by incorporating metal oxide nanoparticles (Silicon dioxide, titanium dioxide, Zinc oxide), fillers (magnetite-reduced graphene oxide, chitosan, cellulose nanocrystals, hydroxyapatite), and carbon nanomaterials (graphene, graphene oxide, reduced graphene oxide, and carbon nanotubes). Organosilicons can also be incorporated during fabrication of PHB/PCL sorbent to increase the surface roughness and hydrophobicity of PHB/PCL sorbents (Zhang et al., 2020; Zhang et al., 2021).

4.1.4.7. Management of recovered hydrophobic pollutants and its valorisation

Due to the subordinate treatment efficiency and the production of secondary pollutants, conventional methods for treating oil-laden waste water are frequently insufficient (Abuhasel et al., 2021). For pollutants to be completely removed, cutting-edge approaches that incorporate many technologies must be implemented (Adetunji and Olaniran, 2021).

Once the tested hydrophobic substrates are separated out from the oil/water system or from the aqueous phase, there are two possible ways to manage it. First, recovery of the hydrophobic substrates from the adsorbent matrix using standard technique in order to reuse them. As discussed in the section 4.1.3.7, recovery rates achieved were 86.5 % for WCO and 85.7 % for diesel.

Second, if the hydrophobic substrates recovery is economically not profitable, then they could be utilized as feedstock for the production of value-added products. Among the established methods, one of the most environmentally benign approaches for utilizing waste hydrophobic substrates is the synthesis of biosurfactants employing hydrocarbonoclastic bacterial species. The hydrocarbonoclastic group of bacteria possesses an intrinsic ability to metabolize various hydrocarbons including petroleum and are recognized for secreting

biosurfactants that facilitate the pseudo-solubilization of hydrophobic substrates for their metabolic processes. In the present investigation, a hydrocarbonoclastic native strain of *Pseudomonas aeruginosa* AMS1a, previously isolated from Mokokchung, Nagaland, India with the NCBI accession number OR642782, was used to treat the sorbent matrices loaded with the test hydrophobic substrates, as depicted in figures 4.1.18a and 4.1.19a.

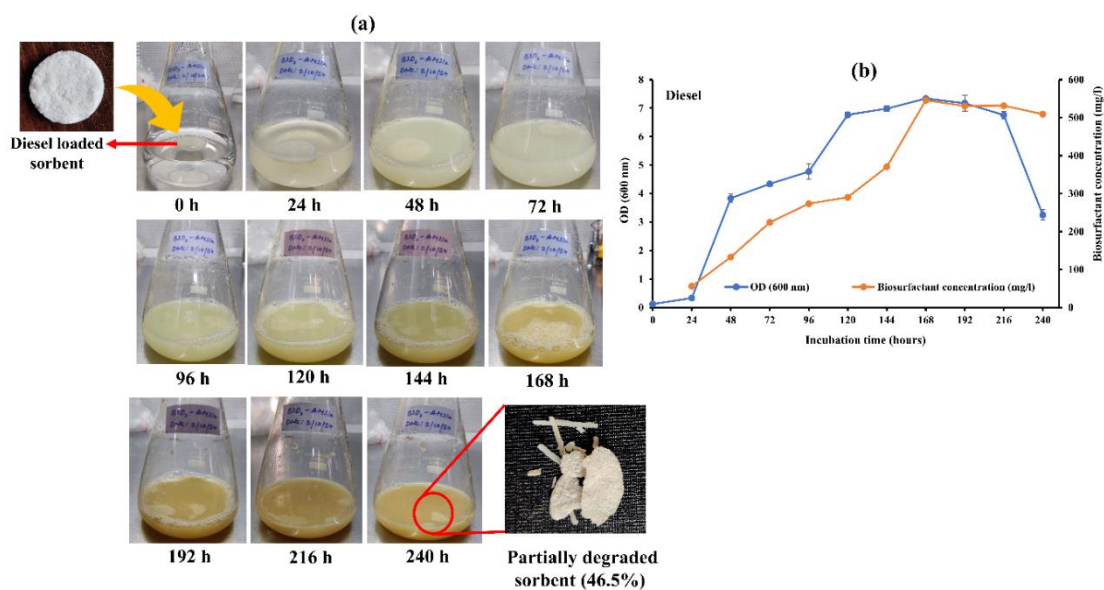


Fig. 4.1.18. (a) Culture broth of *P. aeruginosa* AMS1a immersed with diesel loaded sorbent captured after every 24 h for 240 h, and image of partially degraded diesel loaded sorbent after 240 h and (b) Growth kinetics and biosurfactant production by *P. aeruginosa* AMS1a.

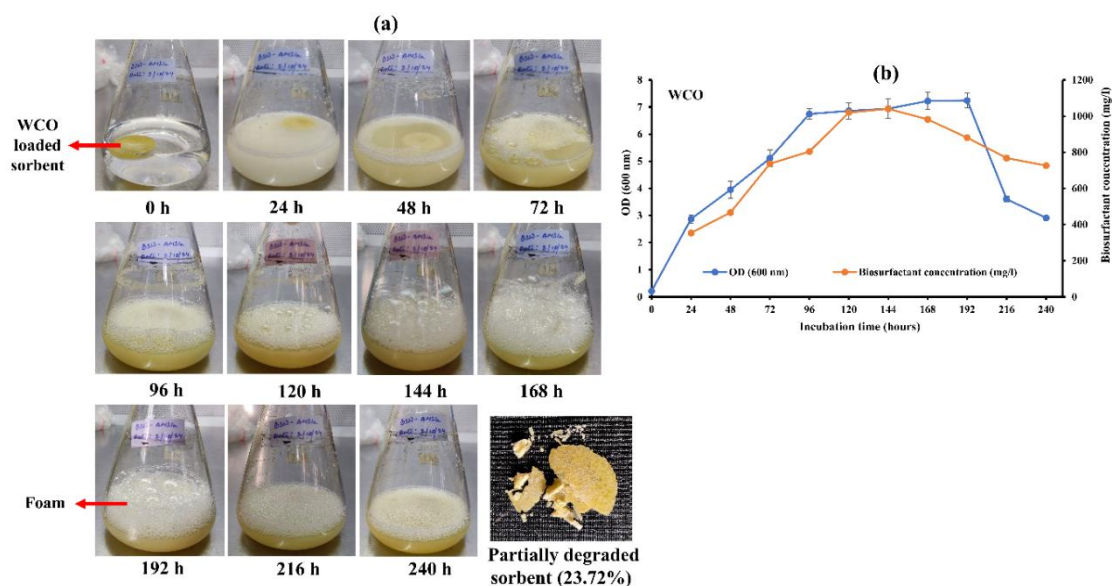


Fig. 4.1.19. (a) Culture broth of *P. aeruginosa* AMS1a immersed with WCO loaded sorbent captured after every 24 h for 240 h, and image of partially degraded WCO loaded sorbent after 240 h and (b) Growth kinetics and biosurfactant production by *P. aeruginosa* AMS1a.

The growth behaviour of the bacterial strain in the presence of matrix along with the tested hydrophobic substrates were determined individually at a constant temperature of 30 °C and agitation of 140 rpm for a period of 10 days. It is evident from the growth curve of diesel-loaded sorbent supplemented medium that the bacterial population were at lag phase till 24 h and went to exponential phase from 24 h indicating a rapid increase in the bacterial population and was continuous up to 168 h. The stationary phase was sustained till 216 h. Following 216 h of incubation, density of bacterial cells in the culture declined indicating its death phase. In the case of WCO loaded sorbent supplemented medium, bacterial cells entered into exponential phase within 24 h indicating a rapid increase in bacterial population with a minimal lag phase and was continuous up to 168 h. The stationary phase was continued till 192 h. Following 192 h of incubation, density of the bacterial cells in the culture declined at a steady rate till 240 h indicating its death phase.

It was observed that the biosurfactant production increased with the increase in bacterial growth in the culture medium and accelerated in the exponential phase

in both the cases. Figure 4.1.18b and 4.1.19b represents the pattern of biosurfactant production by the bacterial strain over the period of 10 days. In each case, the concentration of biosurfactant in the culture media grew up to the stationary phase and then remained inevitable until the bacteria reached the late stationary phase, after which the concentration of biosurfactant progressively dropped. The rhamnolipid attained concentrations of approximately 546 mg/L and 1040 mg/L in diesel loaded sorbent and WCO loaded sorbent supplemented culture media after 168 h and 144 h of incubation, respectively. The net yield of rhamnolipid from the diesel-loaded sorbent and WCO-loaded sorbent supplemented culture media after 10 days of treatment was 410 mg/L and 842 mg/L, respectively. The investigated hydrophobic pollutants in this study, which serve as carbon feedstock for *P. aeruginosa* AMS1, differ significantly in composition. Diesel contains a variety of hydrocarbons, primarily aliphatic (C9–C20) and aromatic hydrocarbons. Conversely, WCO contains a variety of fatty acid types, hydrocarbons, polymerized derivatives, and glyceride molecules (Kumar and Negi, 2015). Thus, it is clear that the type of hydrophobic substrate used affects the pattern of biosurfactant production. Additionally, the mass of the sorbent decreased by 46.5 % in the diesel loaded sorbent and 23.72 % in the WCO loaded sorbent supplemented culture media after a 10-day treatment period, indicating the degradation of the matrices during the treatment process.

The mentioned approach has several advantages. First, the recovery of both diesel and WCO from the sorbent matrix will not be required if it is economically not profitable; instead, they can be utilized collectively, along with the tested hydrophobic substrates and the sorbent matrix, for biosurfactant production. As described in section 4.1.3.10, the sorbent matrix is non-toxic and compatible with the *P. aeruginosa* AMS1a. Second, once the diesel or WCO is recovered, there won't be any concerns with waste generation or secondary pollution. Consequently, this method can also address the management of sorbent matrix to a greater extent once it has been used. Furthermore, after being treated with *P. aeruginosa* AMS1a for the production of biosurfactants, the unused portion of WCO and diesel-loaded matrices showed total disintegration in the soil burial assay findings (section 4.1.3.9), validating waste valorisation and zero waste concept. Additionally, the

current study examines the notion of valorisation, wherein pollutants and wastes are controlled efficiently alongside the generation of valuable microbial products, which can be widely applied, particularly in bioremediation technology.

4.1.4.8. Bioactivity of the prepared PHB/PCL sorbent

The antibacterial activity of the fabricated sorbent was assessed by observing the formation of inhibitory zones around the test rectangular films. The streptomycin discs (10 µg) exhibited zones of inhibition on all plates streaked with different gram-negative and gram-positive bacterial strains (figure 4.1.20). Nevertheless, the fabricated sorbent exhibited no inhibitory zone, indicating that this material lacks substantial antibacterial properties. The lack of antibacterial qualities in the sorbent may have enabled the bacteria to partially degrade the oil-loaded sorbent and fully degrade it when buried in compost soil.

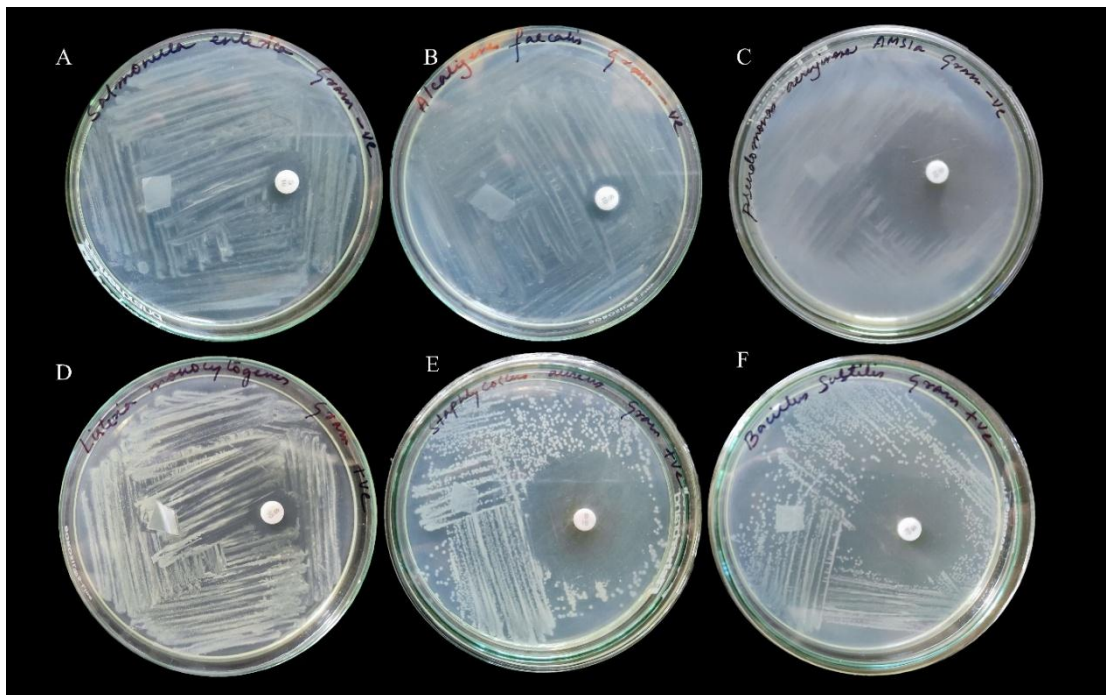


Fig. 4.1.20. Bioactivity of the prepared PHB/PCL sorbent against (a) *Salmonella enterica*, (b) *Alcaligenes faecalis*, (c) *Pseudomonas aeruginosa*, (d) *Listeria monocytogenes*, (e) *Staphylococcus aureus*, and (f) *Bacillus subtilis*

4.1.4.9. Soil burial test

Despite the tremendous advancement in the development of sorbent materials for oil/water separation, the disposal of utilized separation materials remains a significant challenge due to their non-biodegradable properties in the

natural environment (Gu et al., 2017). Consequently, it is essential to assess the biodegradability of the sorbent to prevent the formation of secondary pollutants. Figure 4.1.21a shows the gradual changes in the pristine PHB/PCL sorbent's appearance throughout the soil burial experiment. The initial mass of the pristine PHB/PCL sorbent prior to soil burial was 0.290 g. After 7 days, the mass of the sorbent reduced to 0.284 g with only 2.11 % biodegradation %, followed by 0.272 g after 14 days with 6.61 % biodegradation %, and after 21 days, the mass of the sorbent was 0.231 g with 25.5 % biodegradation %. Following 28 days, the mass of the sorbent was 0.163 g with biodegradation % of 77.9 %. After 35 days, it was not possible to recover samples as a single piece, and is considered as a critical point at which the most dramatic deterioration was observed. Following a period of 42 days, there were no traces of sorbent evident in the soil beds, which indicated that the sorbent had been completely degraded by microorganisms. Figure 4.1.21b shows the gradual changes in the partially degraded diesel-loaded sorbent's appearance during the soil burial test. The initial mass of the partially degraded diesel-loaded sorbent prior to soil burial was 0.146 g. After 7 days, the mass of the sorbent reduced to 0.124 g with only 17.7 % biodegradation %, followed by 0.98 g after 14 days with 48.9 % biodegradation %. Following 21 days, the most pronounced degradation was seen, and samples could not be retrieved intact. After 28 days, no remnants of sorbent were visible in the soil beds, indicating its complete degradation by microorganisms. Likewise, figure 4.1.21c shows the gradual changes in the partially degraded WCO-loaded sorbent's appearance during the soil burial test. The initial mass of the partially degraded WCO-loaded sorbent prior to soil burial was 0.225 g. Following 7 days, the mass of the sorbent reduced to 0.181 g with only 24.3 % biodegradation %, followed by 0.129 g after 14 days with 74.4 % biodegradation %. Finally, after 21 days, the WCO-loaded sorbent was degraded completely, confirming the complete assimilation of sorbent by microorganisms.

The diversity of microbial communities in soil greatly impacts biodegradation; however, conditions such as temperature, pH, and soil moisture can also be crucial (Fernandes et al., 2020). Microorganisms' ability to biodegrade polymers is generally seen as an environmentally benign way for an effective management of the waste. Both abiotic hydrolysis and microbial ability to

hydrolyse the main chain of polymers cause PHB-based biopolymers to degrade. The smaller polymer molecules that are produced are then internalized and catabolized into biomass, CO₂, and water (Acharjee et al., 2023).

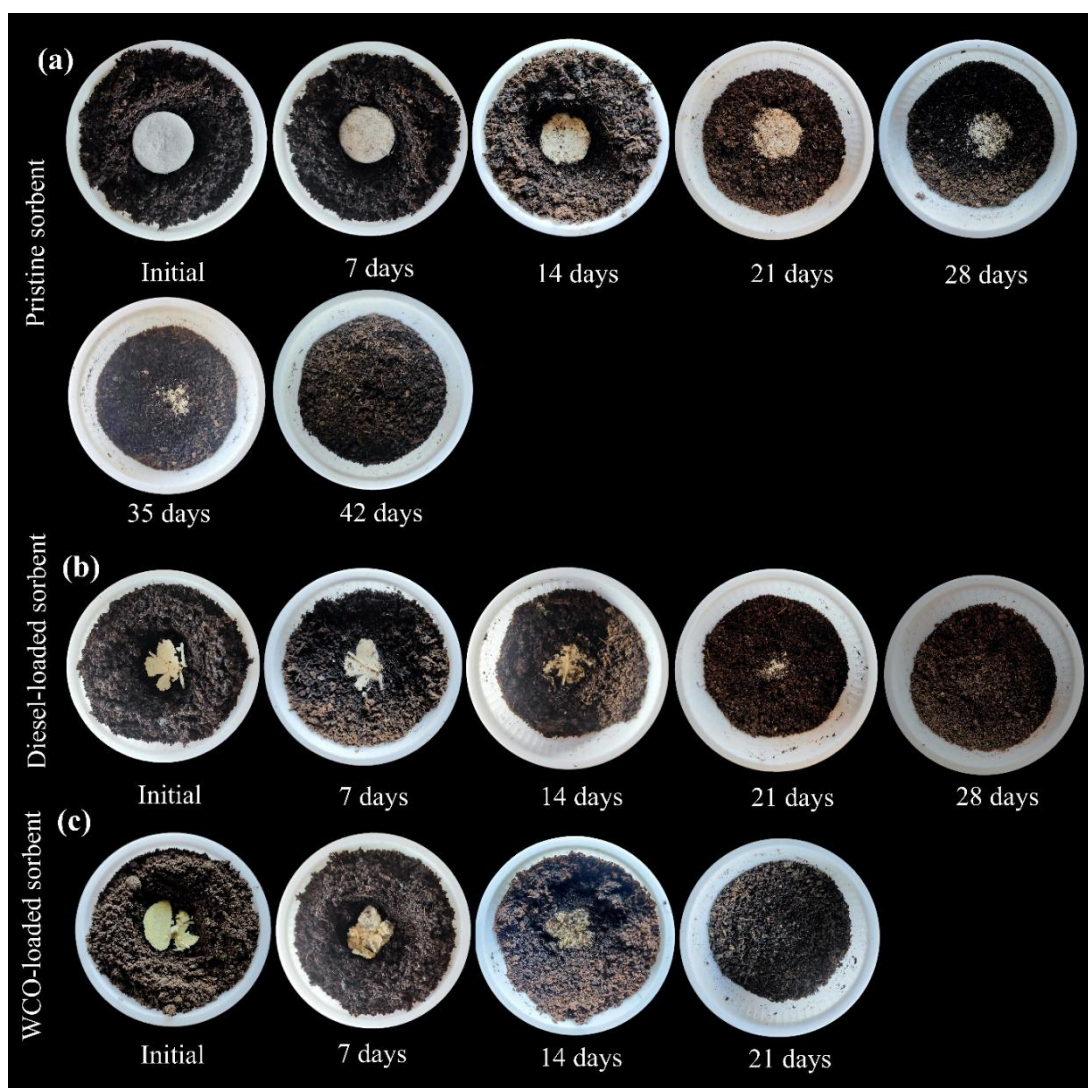


Fig. 4.1.21. Biodegradation study of (a) pristine sorbent, (b) partially degraded diesel-loaded sorbent, and (c) partially degraded WCO-loaded sorbent

4.1.4.10. Biocompatibility test

The effect of the PHB/PCL sorbent on HepG2 cells was assessed to evaluate their non-toxic and biocompatibility nature. Cell viability is a vital parameter for evaluating the biological compatibility of biopolymer-based materials, with higher viability values typically signifying improved cell proliferation (Zhuikov et al., 2020). The dose-dependent cytotoxicity of PHB/PCL is depicted in figure 4.1.22A. Results indicated that there is no significant ($p < 0.05$) difference in cell viability

between control and treated (10, 20, 40 and 80 $\mu\text{g/mL}$) groups. This confirms that the prepared sorbent did not exhibit cytotoxicity to the cells. Cell viability $< 50\%$ in comparison to control cells indicates significant toxicity of the substance (Abdo et al., 2023). However, in our findings, cell viability exceeded 90% , suggesting that examined sorbent is safe and may serve as potential candidates for sorption applications.

ROS function as signalling molecules in normal biological processes. Nevertheless, the excess generation of ROS may trigger damage to many cellular organelles and processes, which may eventually disturb normal physiology (Auten and Davis, 2009). Excessive generation of ROS is linked with the emergence of numerous human diseases *viz.*, cancer, cardiovascular, neurological and metabolic disorders, inflammation, and aging (Snezhkina et al., 2019). Therefore, the total ROS generation assay was carried out to evaluate the amount of ROS activity in the examined cell following sorbent exposure and ascertain whether the sorbent is causing any stress on the cells. Figure 4.1.22B illustrates the dose-dependent effect of PHB/PCL on total ROS generation, showing no significant ($p < 0.05$) elevation in treated cells (10, 20, 40 and 80 $\mu\text{g/mL}$) compared to control.

The generation of superoxide anion radical ($\text{O}_2^{\bullet-}$) is crucial for the survival of aerobic microorganisms. This free radical works as a signalling molecule, controlling several biological processes which includes apoptosis, aging, and senescence. However, when excessive production of $\text{O}_2^{\bullet-}$ happens and/or antioxidant defences are insufficient, oxidative stress can arise, depleting essential biomolecules and affecting their physiological function. These impacts have been linked to the development of numerous diseases (Campos Chiste et al., 2015). The $\text{O}_2^{\bullet-}$ is the primary ROS (Snezhkina et al., 2019). The results indicated that there is no significant ($p < 0.05$) elevation in superoxide levels between control and treated (10, 20, 40 and 80 $\mu\text{g/mL}$) groups (figure 4.1.22C).

Apoptosis is a distinct type of programmed cell death that arises in multicellular organisms under typical physiological conditions to facilitate several biological processes, including normal cell turnover, immunological function, tissue homeostasis, and embryonic and cerebral development (Renehan et al., 2001). To study the mode of cell death induced by PHB/PCL sorbents, cellular

apoptosis assay was achieved by flow cytometry method using Annexin-V/Dead cell apoptosis kit. The Annexin V (phospholipid-binding protein) has a high affinity to phosphatidylserine (PS) which binds to PS that is translocated to the outer leaflet of the plasma membrane during apoptosis. On the other hand, the fluorescent DNA binding dye, propidium iodide (PI) stains necrotic, late apoptotic, and dead cells, but is impermeant to live and early apoptotic cells (Kari et al., 2022). The Annexin/PI stained flowcytometry observation of control (figure 4.1.22D) and PHB/PCL exposure (figure 4.1.22E) was illustrated as scatter plots. There was no prominent level of apoptotic cells (early/late apoptosis) and necrosis observed during the high dose of PHB/PCL exposure (80 $\mu\text{g/mL}$). This clearly indicates the non-toxic nature of the sorbents with good biocompatibility.

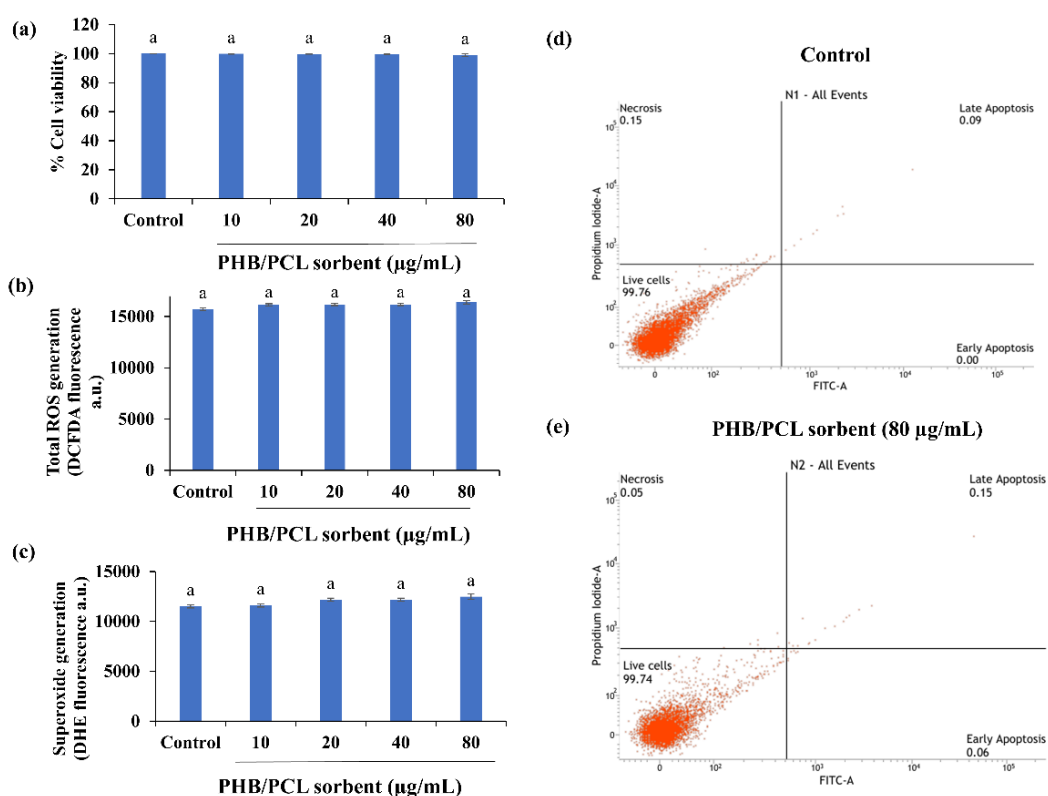


Fig. 4.1.22. Biocompatibility tests (A) dose-dependent effect of PHB/PCL sorbent on cell viability, (B) total ROS generation, (C) Cellular superoxide generation, (D) Annexin/PI-stained flow cytometry observation of control, and (E) Annexin/PI-stained flow cytometry observation of PHB/PCL sorbent

4.1.5. Conclusion

The present study revealed a straightforward production of a hydrophobic and oleophilic porous PHB/PCL blend by an effective and eco-friendly salt-leaching approach. This bio-fabrication approach is an easy, energy-efficient, time-saving, practical, cost-effective, and significantly do not need superfluous costly equipment. The prepared sorbent attained a saturated oil sorption capacity of 6.02 g/g for WCO and 5.09 g/g for diesel oil, with recovery rates of 86.5 % for WCO and 85.7 % for diesel. Remarkably, the fabricated sorbent is efficient in managing the recovered hydrophobic pollutants by valorising the oil-loaded sorbents for the production of biosurfactant. The sorbent displays crucial features of easy washability and reusability because the oil absorbed in the sorbent matrices could simply be rinsed off by solvent and then air dried at room temperature, displaying 6 cycles of the adsorption-desorption process. To examine the biocompatibility of the sorbent; cell viability, ROS generation, superoxide anion radical ($O_2^{\bullet-}$) generation, and apoptosis assays were investigated on human hepatoblastoma cell line (HepG2 cells). Interestingly, the test found that the sorbent is biocompatible, demonstrating its non-toxic nature. In addition, the sorbents displayed total disintegration in soil burial test. This non-toxic, biocompatible, and biodegradable PHB/PCL sorbent possesses critical features to furnish a fascinating glimpse into the future advancement of reusable and effective eco-friendly sorbents to manifest as a possible clean and green solutions for future hydrophobic pollutants. The possibility of developing 3D porous sorbent with a low-cost and facile procedure and to employ them successfully in the sorption of oils makes them a suitable option for the remediation of hydrophobic pollutants present in water. This work proposes a practical and sustainable design strategy for biodegradable PHB/PCL sorbent with zero-waste approach for eliminating secondary waste following the remediation processes.

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4.2. Fabrication of Essential oil fortified Polyhydroxybutyrate (PHB) based green antimicrobial bio-packaging material for food safety

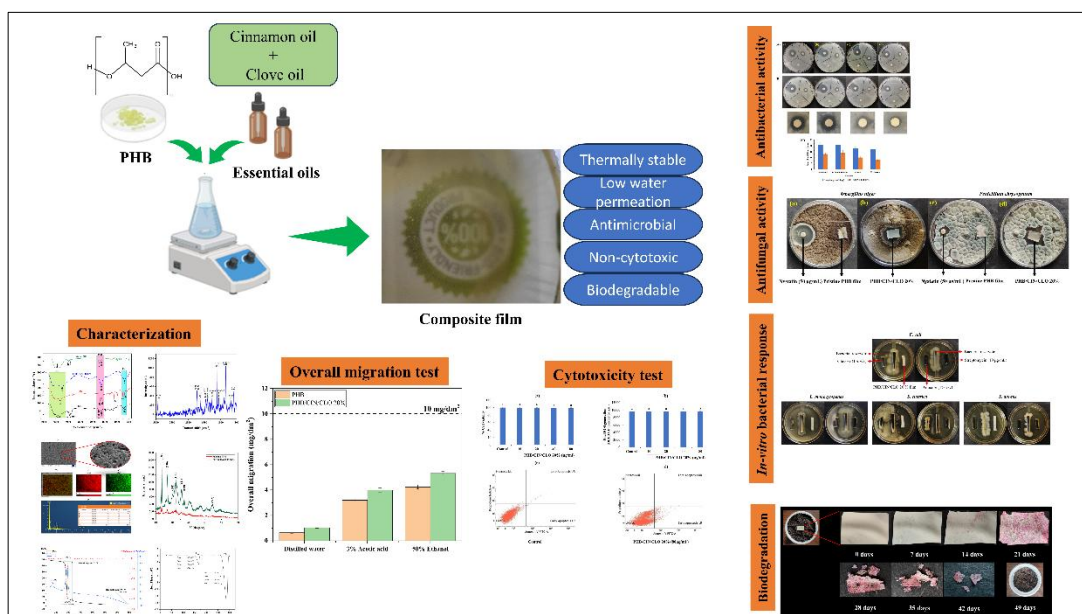
4.2.1. Abstract

The aim of the study was to fabricate an active bio-packaging material suitable for food packaging applications based on polyhydroxybutyrate (PHB) composite, since PHB represents as one of the important alternatives to petroleum-based plastics in the frame of circular economy. A combination of *Cinnamomum verum* and *Syzygium aromaticum* essential oil (EO) as an antimicrobial agent was fortified into PHB matrix by solvent-casting method to fabricate composite film (CF). Results from FTIR and TGA studies revealed that EO fortification altered molecular order and intermolecular interactions and improved the thermal stability of the PHB matrix. FE-SEM investigation of CF revealed minute pores of varying diameters, indicating uniform EO dispersion in the PHB matrix. The CF showed 36.7 % crystallinity index and 19.9 % crystallinity degree with a water contact angle of 91° and a water vapour permeability of 0.162 g.mm/kPa.h.m². Additionally, overall migration values of the CF for diverse food simulants were found to be within 10 mg/dm², indicating its suitability for food packaging. Against various food-spoiling pathogen tested, the CF demonstrated strong antibacterial and antifungal properties. Primary rat hepatocytes were used to test the biocompatibility of the CF by determining cell viability, ROS generation, and apoptosis. The results showed that the CF was non-toxic and biocompatible with tested hepatocytes. Moreover, the CF was found to degrade completely in 49 days in soil burial test, confirming its

biodegradable nature. Hence, present study promotes sustainable consumption and production, aligning with Sustainable Development Goal (SDG) 12 by minimizing waste and fostering a circular economy.

Keywords: Polyhydroxybutyrate (PHB), Essential oils, Food packaging, Antimicrobial, Biocompatible, Biodegradable

Graphical Abstract



4.2.2. Introduction

Food packaging continues to be vital and a critical component of the food industries. Currently, the ready-to-eat (RTE) sector is among the fastest-growing segments of the food industry. The RTE products that generally undergo mild treatments are known to accelerate physiological deterioration, biochemical alterations, and microbiological degradation, particularly in fruits due to their high sugar and water content. This can result in economic losses due to food spoilage and result in outbreaks, causing illness and fatalities from foodborne pathogens (Iseppi et al., 2024). Based on the previous reports a significant group of bacterial species associated with contaminated food and are known to be responsible for a

number of health ailments. *Escherichia coli* is the most significant foodborne pathogenic bacterium. It is transferred *via* the faecal-oral pathway and must, under no circumstances, be found in any food stuff. The primary symptom induced by this bacterium is its diarrheagenic consequences. Additionally, it is recognized for causing sepsis, meningitis, and several gastrointestinal disorders (Ekici & Dümen, 2019). Staphylococcal food-borne disease (SFD) is another globally prevalent foodborne disease, caused by the contamination of food with *Staphylococcus aureus* enterotoxins. It is a predominant cause of reported foodborne diseases in the United States, resulting in around 2,41,000 cases annually (Kadariya et al., 2014). *Listeria monocytogenes*, a well-known foodborne pathogen, can induce an invasive infection that causes sepsis, listeriosis, and meningitis in immunosuppressed individuals, albeit infrequently in healthy individuals and spontaneous miscarriages in pregnant individuals. It may also induce gastroenteritis in healthy individuals (Belias et al., 2024). Salmonellosis is a frequently documented foodborne disease globally, induced by *Salmonella* species. In China, *Salmonella enterica* accounts for 70 % of diarrheal cases attributed to foodborne diseases. Moreover, *S. enterica* constitutes one of the four primary global reasons of diarrheal diseases (Zhang et al., 2024).

Many fungal species that can generate mycotoxins are also common contaminants of food and agricultural products. Molds belonging to the genera *Aspergillus* and *Penicillium* are important in food sector because they have the ability to produce mycotoxin. These organisms may thrive on various substrates and under diverse conditions of moisture, pH, and temperature. Consequently, the majority of foods are vulnerable to fungal contamination at various phases of production, processing, transportation, or storage. The occurrence of mold development invariably presents the potential for mycotoxin production (Bullerman, 1979). Furthermore, the ingestion of harmful mycotoxins in contaminated food products impacts human and animal health, even at nanomolar (nM) concentrations. *Aspergillus* is the most prevalent environmental contaminant, directly compromising life with a 40-90 % mortality risk in immunocompromised individuals (Navale et al., 2021).

The conventional technique for packaging perishable food items uses firmly established plastic films composed of petrochemical derived polymers (Bhat et al., 2022). The predominantly utilized petrochemical-based polymers include polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) (Kumari et al., 2024; Acharjee et al., 2023). Together, they constitute over 90 % of the total volume of plastics utilized in the industrial sector and roughly 60 % of the net plastic wastes that are generated from these materials (Zhang et al., 2021). The release of these materials into the environment results in significant threats, including land and water contamination, greenhouse gas emissions, and the generation of microplastics (Acharjee et al., 2023). Realising such environmental concerns, worldwide numerous governments and non-governmental organizations have advocated the limitations or alternatives to non-degradable packaging (Singh et al., 2017). The advancement of bio-based degradable materials or bio-packaging material that offer "active" protection against contaminating microbes and oxidative deterioration of food items is a leading focus of current research in food packaging.

Polyhydroxybutyrate (PHB) has garnered significant interest in the food packaging sector in recent years due to its renewable source from waste and non-food crops, moderate to high mechanical strength, medium-to-high barrier properties, biodegradability, biocompatibility, and physical characteristics comparable to conventional plastics (Rech et al., 2021). Despite these benefits, PHB exhibits a limited processing window and significant brittleness due to its low heat stability and high crystallinity, constraining its industrial applicability (Garcia-Garcia et al., 2022). Various techniques, namely the use of plasticizers, nanofillers, and blending with biopolymers, were investigated to address the limitations of PHB in food packaging. Nonetheless, the outcomes of PHB plasticization have thus far been unsuccessful (Kumari et al., 2023). A study revealed that incorporating 30 wt % (weight percentage) triacetyl glycerol into PHB resulted in a two-fold enhancement in elongation at break (EB), but significantly diminishing the tensile strength (TS) and crystallinity of the composite films. No substantial enhancement in EB was observed with the incorporation of 30 wt % of plasticizers such as polyadipate, dioctyl phthalate, and dioctyl adipate, in PHB (Baltieri et al., 2003).

Kurusu et al. (2015) examined the impact of incorporating 10 wt % of industrial plasticizers, specifically tributyl citrate, triethyl citrate, and tri (ethylene glycol) bis (2-ethyl hexanoate), into PHB using the melt mixing technique. Nonetheless, an enhancement in EB from 6.8 % to 7.2 % was noted solely for PHB integrated with tri (ethylene glycol) bis (2-ethyl hexanoate).

The majority of plasticizers are synthetic and difficult for the environment to mineralise since the enzymes necessary for breaking down the chemical bonds in the plasticizer molecules are either lacking or ineffective. Their high concentration in the environment may therefore have a detrimental effect on microorganisms, which in turn may have a ripple effect on the processes of biodegradation. Furthermore, the microflora involved in the biodegradation process is adversely affected when chemically stable plasticizers are added to the biopolymer to improve its physico-chemical characteristics, potentially reducing the advantages of employing biopolymer (Cho et al., 2022). Likewise, the application of synthetic molecules for controlling food deterioration and growth of pathogenic bacteria has been a contentious matter. Moreover, concerns about the migration of chemical plasticizers from packaging to food and their detrimental effects on human health urge the development of novel and safe natural products to extend the shelf life of food products (Zhang et al., 2016; Moeini et al., 2021).

Essential oils (EOs) are plant derived bioactive products that are extensively utilized in the food industry for their inherent antibacterial, antioxidant, and bio-preservative properties, which aid in extending the shelf life of food products (Sharma et al., 2021). Besides offering antibacterial and antioxidant qualities, it can enhance the flexibility of biopolymer films. The research conducted by Ojagh et al. (2010) demonstrated that the integration of Cinnamon EO into chitosan films enhances its structural and physical attributes, including tensile strength, elongation at break, surface hydrophobicity, and flexibility. Furthermore, the U.S. Food and Drug Administration (FDA) designated EOs as “Generally recognized as safe” (Atarés and Chiralt, 2016). Regardless of the intriguing potential of EOs in the food industry, their strong fragrance limits their practical application in food preservation. A potential solution for such specific issue could be developing blends of different EOs. Without compromising the antimicrobial properties, combination

of EOs could reduce the concentration and thereby decrease the negative sensory impact through their synergistic activities (Hyldgaard et al., 2012). Moreover, despite being individually active, EO combinations may provide the films with a synergistic antimicrobial activity (Rech et al., 2021; Iseppi et al., 2024; Zeng et al., 2025).

In light of the previously mentioned facts, the current study investigated the appropriateness of integrating two well-known EOs *i.e.* clove and cinnamon into PHB to fabricate a film material that can be used in food packaging. To assess its potential as a food packaging material, the developed PHB-EOs composite film was examined for its physico-chemical, packaging and biological potentials. The safety of employing the developed composite film for food packaging was assessed using a cytotoxicity studies using primary rat hepatocytes in addition to its antibacterial efficacy against the selected foodborne pathogen. Lastly, biodegradation tests were conducted to determine the degradation kinetics of the fabricated composite film material in order to address the concerns related to solid waste management.

4.2.3. Materials and methods

4.2.3.1. Chemicals and reagents

Acetone, ethanol, acetic acid, chloroform, Sabouraud Dextrose Agar (SDA), Mueller Hinton agar (MHA), peptone, Beef extract, sodium chloride (NaCl), streptomycin (10 µg/mL), and nystatin (50 µg/mL) were purchased from HiMedia, Mumbai, India. Essential oils (EOs) *i.e.*, Cinnamon oil (CIN) and Clove oil (CLO) were purchased from KS essentials, India. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and molecular biology grade water were obtained from Sigma-Aldrich, USA. In all the experiments performed, ultrapure water was used from the Arium pro UV, Sartorius ultrapure water system. Test organisms including bacterial strains namely *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, and *Listeria monocytogenes* and fungal strains namely *Aspergillus niger* and *Penicillium chrysogenum* were used in the present study.

4.2.3.2. Fabrication of active PHB/EOs composite films

Initially, different concentrations of combined EOs (CIN/CLO 20 %, CIN/CLO 25 %, CIN 15 % + CLO 20 %, CIN 15 % + CLO 25 %) were screened for their efficient antimicrobial activity. The combination of CIN and CLO EOs were then added in a volume ratio of 7:3 as it was determined to have synergistically better bacteriostatic effects as reported by Zeng et al. (2025). PHB/EOs composite film was fabricated by simple solution casting technique. Initially, 1 g of PHB was solubilized in 20 mL of chloroform by continuous mixing at 60 °C for 2 h. The best concentration of EOs was added to PHB chloroform solution as wt % with respect to the weight of PHB biopolymer, and the mixture was stirred at 300 rpm for 2 h in ambient temperature. The beaker containing the polymeric solution was sealed to prevent solvent evaporation. The solution was subsequently placed in a Petri dish with a diameter of 15 cm until complete evaporation of the solvent occurred. PHB film without any added EOs was taken as a control in present study and is referred to as pristine PHB.

4.2.3.3. Characterization

4.2.3.3.1. Physicochemical characteristics

Fourier transform infrared (FTIR) spectroscopy (PerkinElmer, Spectrum Two, 104,140) was conducted to identify the functional groups in the EOs (CIN, CLO), pristine PHB, and active PHB/CIN/CLO 20 % composite films. The FTIR spectra were obtained within a wavenumber range of 400 to 4000 cm^{-1} , averaging 64 scans. Raman spectroscopy was conducted with a inVia spectrometer system (Renishaw) combined with a Confocal Raman microscope. The spectra were obtained using a 785 nm excitation laser source, a spectral range of 3500-500 cm^{-1} , gratings of 1200 1/mm, and an exposure duration of 15,000 ms. Raman spectra were generated using Renishaw's WiRE version 5.5 software. X-ray diffraction (XRD) examination was conducted to characterize active PHB/CIN/CLO 20 % composite films utilizing a sophisticated X-ray diffractometer (Miniflex Benchtop, RIGAKU) operated at 40 kV and 30 mA with Cu $K\alpha$ radiation at a wavelength of 1.54 Å. Each scan was conducted between the 10 – 45° (2 θ degree) range at a velocity of 3°/minute, where

θ denotes the angle of incidence of the X-ray beam on the sample. The morphology of the film samples was analysed using a Field Emission Scanning Electron Microscope (FESEM). The samples were positioned on aluminium stumps, cleansed in a buffer solution, subsequently coated with gold in a sputter coater, and then analysed under a microscope. Subsequently, imaging was performed utilizing a FE-SEM (JEOL, JSM7610F) at an accelerating voltage of 15 kV (Maity et al., 2020).

4.2.3.3.2. Thermal properties

The thermal stability of the PHB/CIN/CLO 20 % composite film was evaluated using a Thermogravimetric Analyzer (STA 2500 Regulus). To prevent sample oxidation, the sample was heated from 30 °C to 600 °C at a rate of 10 °C/minute in the presence of nitrogen at a flow rate of 40 mL/ minute. The melting temperature (T_m) and degree of crystallinity (X_c) of the composite film were determined using differential scanning calorimetry (DSC). DSC measurements were conducted using a thermal analyser (Shimadzu, DSC-60) equipped with a cooling attachment. The sample was placed in an aluminium pan and heated from 20 °C to 300 °C at a scanning rate of 10 °C/minute in a nitrogen atmosphere. The melting enthalpy (ΔH_m) and melting temperature (T_m) were determined from the endothermic peaks observed in differential scanning calorimetry (DSC). Following a 1-minute annealing process, the sample was cooled to 20 °C. The crystallinity (X_c) of the active composite film is determined using the equation outlined by Hernández-Núñez et al. (2019).

$$X_c = \left[\frac{\Delta H_m}{\Delta H^\circ} \right] \times 100 \quad (4.2.1)$$

Here, ΔH_m denotes the melting enthalpy of the sample (J/g). ΔH° is the expected melting enthalpy of 100 % crystalline PHB, quantified at 146 J/g.

4.2.3.3.3. Static contact angle measurements

To evaluate the wettability of the composite films, the water contact angle (WCA) was determined using the sessile drop method and analysed with a contact angle meter (Kyowa, FAMAS, Japan). A 4 μ L droplet of water was carefully placed on the surface of composite film at ambient temperature. Contact angles were evaluated at three distinct sites, and the average results were documented. A CCD

(Charge-coupled device) video camera connected to the equipment recorded the water contact angle of the composite film. The software of the equipment (Image J) autonomously calculated the contact angles of water on the composite film.

4.2.3.4. Water vapor permeation (WVP) test

The WVP test was conducted according to the ASTM E96 standard with the desiccant method (Mittal et al., 2023). Initially, glass vials were filled with 15 mL of water [100 % relative humidity (RH)] and meticulously sealed with the prepared films. The vials were originally weighed and thereafter placed at 30 °C in a desiccator with calcium chloride as a desiccant (0 % RH). The weights of these vials were measured again after every 24 h. The test was conducted in duplicate to obtain the precise permeation value. The WVP (g.mm/kPa.h.m²) was determined using the following formula:

$$WVP = \frac{WVTR \times t}{\Delta P} \quad (4.2.2)$$

Where, WVTR = water vapour transmission rate, *t* is the thickness of the film, ΔP is the water vapour partial pressure difference (kPa) between the two sides of the film.

4.2.3.5. Overall Migration (OM) in Food Simulants

The migration of components from PHB control films and PHB/CIN/CLO 20 % composite films into various food simulants was analysed according to Bureau of Indian Standards: 9845–1998 criteria (IS: 1981, Commission 2011). The films were subjected to food simulants, including distilled water, 50 % ethanol, and 3 % acetic acid representing hydrophilic, lipophilic, and acidic food substances, respectively, maintained at 40 °C for 10 days to simulate filling and storage conditions. The extracted simulant was evaporated, and the migrating material was quantified gravimetrically. The outcomes were articulated in milligrams per square decimetre (mg/dm²) (Bhat et al., 2022). The residual weight per film surface was used to calculate the overall migration:

$$OM = \frac{m_2 - m_1}{S} \quad (4.2.3)$$

Here, OM represents the overall migration in mg/dm², *m*₁ and *m*₂ represents the weight of the beaker prior to and subsequent to OM, respectively, and *S* represents the area of the film.

4.2.3.6. Antibacterial activities

The antibacterial efficacy of pristine PHB and PHB/CIN/CLO 20 % composite films was evaluated using the disc diffusion method on Mueller Hinton agar (Appendix II), targeting both gram-negative (*Escherichia coli* and *Salmonella enterica*) and gram-positive bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*). The films were subsequently placed on agar plates that had been previously inoculated with the appropriate bacteria. A readymade streptomycin (10 µg/mL) paper disc was placed onto the agar plates as a positive control. The plates were incubated overnight at 37 °C to promote bacterial growth. The antibacterial activity was initially evaluated by observing the formation of inhibitory zones around the rectangular test films. To determine the zones of inhibition (mm), the tested films were cut into disc shapes (6 mm) and placed on agar plates.

The *in-vitro* bacterial response towards PHB/CIN/CLO 20 % composite films was assessed utilizing the chemical gradient motility agar (CGMA) method as described by Garg and Kanitkar (2006), with minor modifications. CGMA medium (Appendix II) was formulated by mixing Peptone (5g), Beef extract (3 g) and NaCl (5 g) in 1 L of distilled water, with the pH adjusted to 7. Subsequently, 7 g of agar powder was added and autoclaved for 20 minutes. The bacterial strains under examination were cultivated in nutrient broth overnight at 37 °C. Following the solidification of CGMA medium, rectangular wells were made as depicted in figure 4.2.9.

In this experiment, two petri dishes were utilized individually for each bacterial strain. In petri dish 1, the elongated central well contains the corresponding bacterial broth; the left well contains glucose (1 % v/v), and a composite film measuring 3 × 0.5 cm is positioned on the right side. In Petri dish 2, the elongated central well contains the corresponding bacterial broth; the left well has glucose (1 % v/v), while the right well contains streptomycin (10 µg/mL). Petri dish 2 serves as a control for each bacterium examined in the experiment. The dimensions of the central long rectangular well are 4 × 1 cm, whilst the wells on either side are 3 × 1 cm. Firstly, wells on the left and right sides were filled with glucose (1 % v/v) and streptomycin (10 µg/mL) solution, respectively and kept undisturbed for 60 minutes. The central well was thereafter filled with 200 µL of

freshly grown tested bacterial culture broth. The plates were incubated at 37 °C for 24 h. In the control plates (petri dish 2), glucose (1 % v/v) served as the positive control, whereas streptomycin (10 µg/mL) functioned as the negative control.

4.2.3.7. Antifungal activities

The antifungal activity of pristine PHB and PHB/CIN/CLO 20 % composite films was assessed using the dual culture overlay method (Mandal and Mandal, 2023) on Sabouraud Dextrose Agar (SDA) (Appendix II) against *Aspergillus niger* and *Penicillium chrysogenum*. SDA plates were initially spread with a 48-h grown old fungal broth culture, followed by the placement of pristine PHB and PHB/CIN/CLO 20 % composite films on the SDA plates. A pre-prepared nystatin (50 µg/mL) paper disc was placed on the SDA plates as a positive control. The plates were incubated at 28 °C for 3-5 days to facilitate fungal spore development. The antifungal activity of PHB/CIN/CLO 20 % composite films was determined against the tested fungal strain *via* zones of inhibition.

4.2.3.8. Cytotoxicity assay of composite film on primary rat hepatocytes

4.2.3.8.1. Cell culture and PHB/CIN/CLO 20 % composite film exposure

The primary rat hepatocytes were sourced from Bharathiar University, India. The DMEM-F12 medium (Appendix II), added with 10 % FBS and 1 % antibiotics (penicillin and streptomycin), was used to sustain cells in a humidified environment comprising 95 % air, at 37 °C and 5 % CO₂, within an incubator (Nectarnova, Syngene). The passaging proceeded at 80-90 % confluence, permitting attachment to the surface with suitable control measures. The cells were inoculated in 96-well plates and subjected to PHB/CIN/CLO 20 % composite films at concentrations of 10, 20, 40, and 80 µg/mL. All subsequent cell culture-based fluorescence studies were conducted in tissue culture-grade clear-bottom black plates (Invitrogen, USA), with a minimum of three repetitions and three wells per experimental group.

4.2.3.8.2. Cell viability/ cytotoxicity – Resazurin assay

The evaluation of cell viability and cytotoxicity was conducted using the resazurin assay as previously described by Maadurshni et al. (2025). Cell viability is determined by the conversion of oxidized non-fluorescent blue resazurin by mitochondrial activity in living cells to a red fluorescent dye (resorufin). In the

current investigation, following the treatment regimen, resazurin solution (Sigma-Aldrich, USA) was added to the cell culture media at a concentration of 80 μ M, and the cells were incubated for 4 h at 37 °C. The fluorescence intensity was evaluated (Excitation/Emission: 540/590 nm) with a multimode microplate reader. The relative percentage of cell viability was determined from the fluorescence intensity (a.u.) data, with control cells regarded as 100 % viable.

4.2.3.8.3. Cellular total ROS generation - CMH₂DCFDA assay

The intracellular production level of reactive oxygen species (ROS) in cells was evaluated using the universal ROS indicator CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) (Invitrogen, USA). This probe readily penetrates cells, demonstrating superior retention in living cells compared to H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate). After deacetylation by intracellular esterases, H₂DCF (2',7'-dichlorodihydrofluorescein) is oxidized into the green fluorescent compound DCF (Dichlorofluorescein) by hydroxyl, carbonate, nitrogen, or thiyl radicals (Ng and Ooi, 2021). Treated cells were incubated with 10 μ M CM-H₂DCFDA for 45 minutes at 37 °C in a CO₂ incubator. Following two rinses of the cells with phosphate buffer saline (PBS) solution, the relative DCF signal intensity was quantified (Excitation/Emission: 485/530 nm) utilizing a multimode microplate reader and reported in arbitrary units (a.u.) (Asensio-López et al., 2017).

4.2.3.8.4. Cellular apoptosis assay using flow cytometry

The present work quantified early- and late-phase apoptosis in cells utilizing flow cytometry with the Annexin-V/Dead cell apoptosis kit. The treated cells were collected and suspended in 100 μ L of 1 $\times$ binding buffer, then stained with 5 μ L each of Annexin V/FITC (Fluorescein isothiocyanate) and propidium iodide (PI) at room temperature for 15 minutes. Following the incubation period, 400 μ L of binding buffer was added, and the cells were analysed using a flow cytometer (FACSVerse, BD Biosciences).

4.2.3.9. Statistical analysis

Statistical analysis was conducted using GNU PSPP software. The experimental values were reported as mean $\pm$ standard deviation for illustrative purposes. This study employed one-way analysis of variance (ANOVA) and a Tukey HSD test to

assess the statistical significance of the means of the observed cellular parameters. In this investigation, statistical significance was established as a p value < 0.05 .

4.2.3.10. Biodegradation study

The biodegradability of PHB/CIN/CLO 20 % composite films was assessed by measuring the mass loss of films buried in compost soil for 49 days. The samples were embedded in the soil at a depth of 3-4 cm within an open circular plastic container (diameter: 95 mm; height: 80 mm) to promote aerobic conditions and maintained at a temperature of 37 °C. The pH of the soil was 7.1, and the moisture content was 52.5 %. Soil moisture was preserved with occasional water spraying. Samples were meticulously excavated every 7 days. Soil particles were cautiously removed with the help of a small paintbrush and subsequently oven-dried at 50 °C to eradicate moisture before weighing. The mass loss % was employed to assess biodegradation behaviours. The film's mass was quantified prior and subsequent to exposure utilizing a precision electronic balance (Mettler Toledo, ME204). The mass and degradation percentage (%) were evaluated according to the methodology described by Acharjee et al. (2024).

$$\text{Degradation \%} = \frac{W_1 - W_2}{W_1} \times 100 \quad (4.2.4)$$

Here, W_1 denotes the original mass of the composite film, while W_2 represents its final mass after degradation.

4.2.4. Results and Discussions

4.2.4.1. Physicochemical characteristics

FTIR is a dependable and efficient method for examining distinct functional groups in polymers and for elucidating interactions among the constituents of active composite films (Bhat et al., 2022). Figure 4.2.1 displays the FTIR spectra of pristine PHB film, PHB/CIN/CLO 20 % composite film, CIN, and CLO. The absorption peaks characteristics for pristine PHB film are as follows: The extensive absorption band at 3446 cm^{-1} corresponds to the hydroxyl stretch (O–H). The bands detected in the 2900 cm^{-1} spectrum range are ascribed to the C–H stretching bond, an attribute of PHB polymers. The absorption bands at 1720 cm^{-1} and 1641 cm^{-1} correspond to the carbonyl (C=O) stretching of an ester group and the C=O bond associated with a conjugated carbonyl or amide group, respectively. The absorption

peaks characteristic of CIN are as follows: 3314 cm^{-1} (O–H stretching of hydroxyl groups), typically linked to phenolic and alcoholic moieties; 1672 cm^{-1} (C=O stretching of trans-cinnamaldehyde); 1626 cm^{-1} (C=C stretching vibration of cinnamaldehyde); 1124 cm^{-1} (C–O stretching vibration); 689 and 748 cm^{-1} (C–H vibrations of the benzene ring and olefin). The absorption peak properties of CLO are as follows: 3440 cm^{-1} (O–H stretching of hydroxyl groups of eugenol) characterized by an O–H group linked to an aromatic ring, resulting in a peak that is typical for such compounds; 2926 and 2854 cm^{-1} (C–H stretching from the methylene group), 1744 cm^{-1} (C=O stretching vibrations), 1631 cm^{-1} (C=C stretching vibrations in the aromatic rings of eugenol), and 735 and 698 cm^{-1} (C–H vibrations of the benzene ring), thereby affirming the presence of the phenyl group. The FTIR spectra of the PHB/CIN/CLO 20 % composite film exhibited transmittance peaks at 3227 cm^{-1} , 1640 cm^{-1} , and 694 cm^{-1} , corresponding to the hydroxyl stretch (O–H), C=O stretching group, and C–H vibration of the benzene ring, respectively. These peaks are indicative of numerous primary components of essential oils, including cinnamaldehyde, the principal constituent of cinnamon oil, and eugenol, the principal constituent of clove oil. The inclusion of several constituents has resulted in the broadening and shifting of peaks due to intermolecular interactions and alterations in the local chemical environment, as perceived from the FTIR spectra of the PHB/CIN/CLO 20 % composite film. This analysis suggests that the essential oils were well integrated into the polymeric matrix of PHB following the complete evaporation of chloroform.

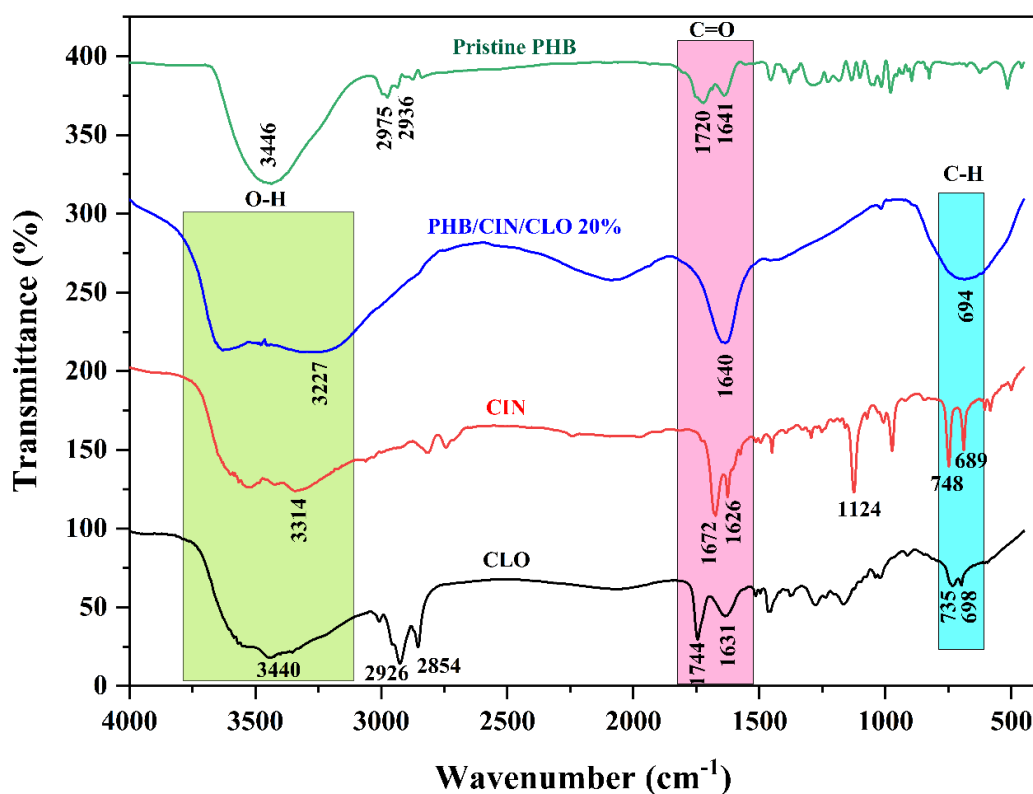


Fig. 4.2.1. FTIR spectra of pristine PHB film, PHB/CIN/CLO 20 % composite film, CIN, and CLO

The Raman shifts and assignments of the Raman bands for the PHB/CIN/CLO 20 % composite film are presented in figure 4.2.2. The Raman spectra of the composite film revealed that the band at 2937 cm^{-1} corresponds to the C–H stretch. The pronounced peak at 1731 cm^{-1} corresponds to the C=O stretching of an ester group, recognized as markers of PHB. The peak at 1631 cm^{-1} corresponds to C=C stretching vibrations of aromatic and olefinic groups from the components of cinnamaldehyde and eugenol. The peaks at 1459 and 1405 cm^{-1} correspond to the bending or deformation of –CH_3 (methyl) and –CH_2 (methylene) groups (Vargas Jentzsch et al., 2018; Anwar et al., 2024). The peaks at 1261 and 1059 cm^{-1} correspond to the C–O–C ether group. The distinct peaks at 1101 cm^{-1} and 841 cm^{-1} correspond to the C–O stretching vibration and the C–COO ester group, respectively. The peaks at 672 and 601 cm^{-1} correspond to the vibrational modes of phenolic chemicals found in essential oils, including eugenol and cinnamaldehyde. These low-frequency vibrations are indicative of ring deformation

and C–C stretching within the aromatic rings of these compounds (Anwar et al., 2024).

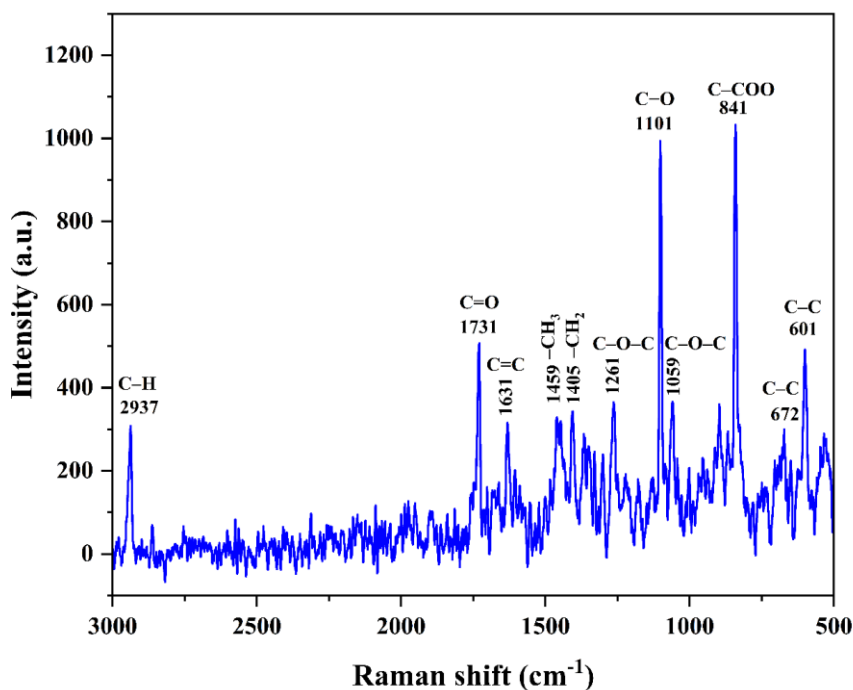


Fig. 4.2.2. Raman spectra of PHB/CIN/CLO 20 % composite film

X-ray diffraction analysis is an effective method for examining the film morphology and crystallinity of active films utilized in the development of packaging materials. It provides details about crystal structure and chemical makeup. The XRD results of pristine PHB film and PHB/CIN/CLO 20 % composite film are shown in figure 4.2.3. Pristine PHB film exhibits a characteristic XRD diffraction pattern at $2\theta = 13.59^\circ$, 16.89° , 20.2° , 22.42° , 25.81° and 27.19° , which attributes to the reflections (020), (110), (101) (111), (121) and (040), respectively. The peaks at 13.59° and 16.89° are corresponded to the crystalline nature of the PHB. The aforementioned peaks were observed for PHB/CIN/CLO 20 % composite film, but with varying intensities. In addition, intense peak at 44.6° was observed PHB/CIN/CLO 20 % composite film that corresponds to the reflection (222) (Kumari et al., 2023). The emergence of this peak could be due to the addition of EOs into the PHB matrix. The crystallinity of both pristine PHB and PHB/CIN/CLO 20 % composite film was evaluated by dividing the area of crystalline peaks by the cumulative area of all the peaks observed in the XRD

spectra (Frone et al., 2020). The crystallinity index values of pristine PHB and PHB/CIN/CLO 20 % composite film are 25.7 and 36.7 %, respectively. A similar observation corresponding to an increase in crystallinity with EO loading is reported for PHB/grapeseed oil, PHB/bergamot oil, and PHB/ginger oil (Kumari et al., 2023), PHB/tea tree oil/citronella composites (Rech et al., 2021), and PLA/rosemary (Yahyaoui et al., 2016). The increased crystallinity of composite films is due to increased free volume and polymer chain mobility caused by the plasticizing effect of essential oils, which facilitates the formation of packaged structures (Garcia-Garcia et al., 2020).

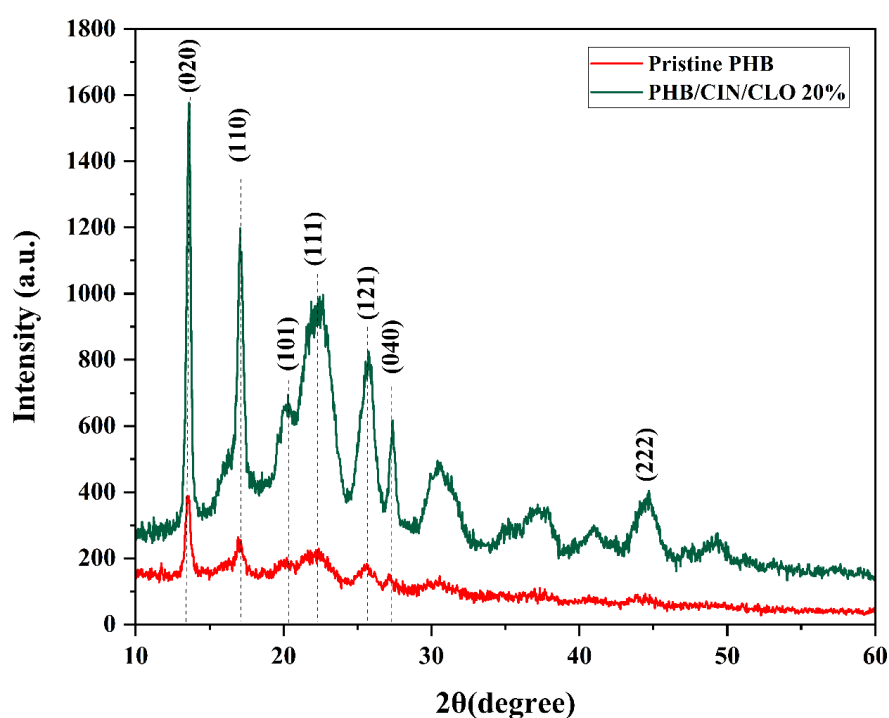


Fig. 4.2.3. XRD spectra of pristine PHB and PHB/CIN/CLO 20 % composite film

The analysis of surface morphology of PHB/CIN/CLO 20 % composite film was analysed by FESEM-EDS as shown in figure 4.2.4. The composite film exhibited some small pores with varied sizes. Similar observations were observed for the chitosan/thyme oil blend (Hosseini et al., 2009), and PHB/PEG/CEO blend (Mittal et al., 2022). The pores observed in the cross-sections of the composite film indicate a homogeneous dispersion of EOs inside the PHB matrix, which either evaporate during the FESEM test or are gradually released from the film. An EDS analysis

was conducted to examine the chemical composition of the composite film. This study reveals that carbon and oxygen are the predominant constituents, with 80.93 wt % and 18.72 wt %, respectively.

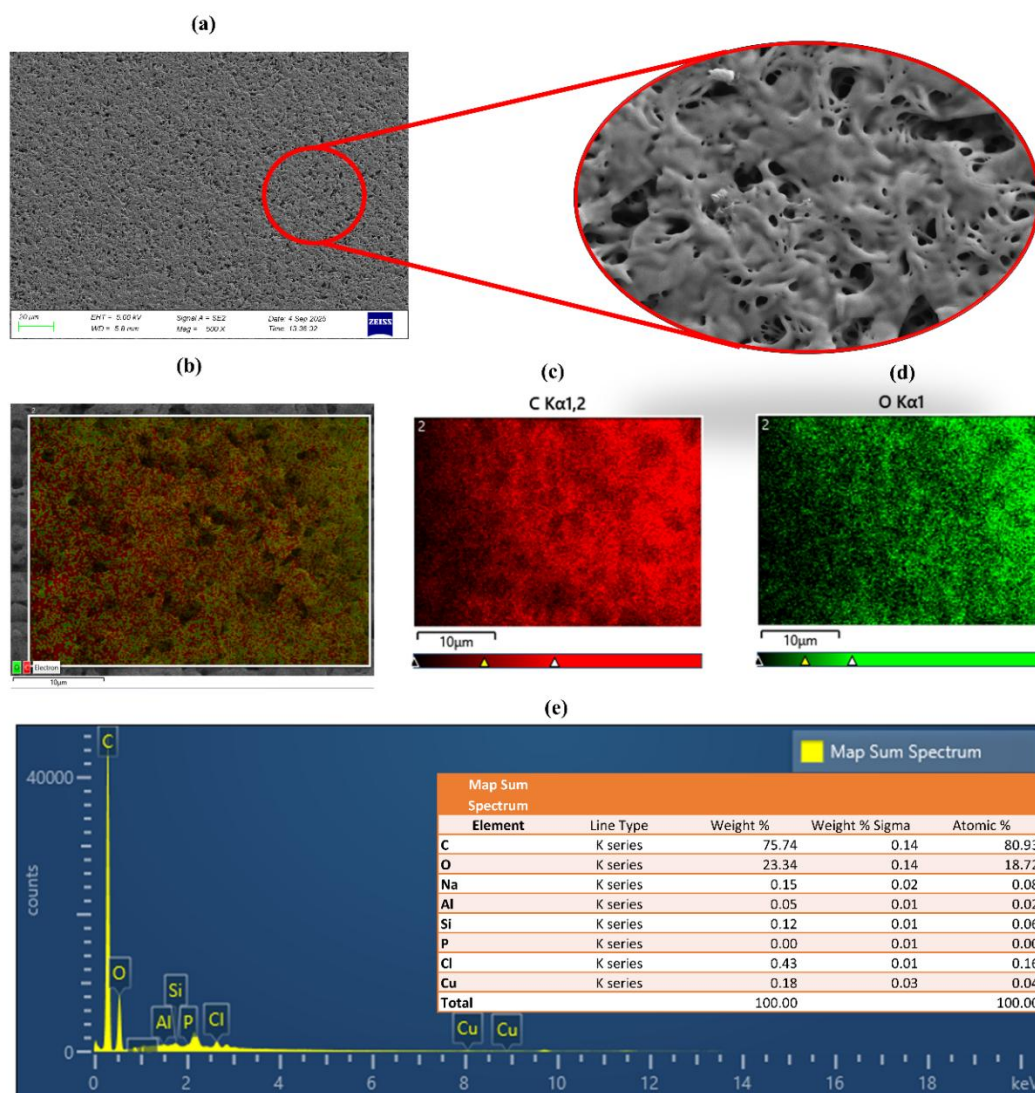


Fig. 4.2.4. FESEM micrograph of PHB/CIN/CLO 20 % composite film a) 5.00 X magnification, (b) EDS spectrum of composite film showing the homogenous distribution of carbon and oxygen in the area, (c) Elemental mapping of composite film attributed to carbon, (d) Elemental mapping of composite film attributed to oxygen, and (e) Map sum spectrum of composite film

4.2.4.2. Thermal analysis

The thermostability of the PHB/CIN/CLO 20 % composite film was evaluated by thermal analytic techniques, including TGA, DTG, DTA, and DSC, as depicted in

figures. 4.2.5 and 4.2.6. TGA study was performed to evaluate the mass loss of the composite film across the specified temperature range. The TGA curves of the composite film, as depicted in the figure 4.2.5, demonstrate a unidimensional process. A single-step composite decomposition initiates at 232 °C, subsequent to the melting point of PHB (Abdelmalek et al., 2022). The maximum degradation temperature of the composite film is identified to range from 232 °C to 328 °C, resulting in an approximate mass loss of 69.75 %. The resultant composite film exhibits enhanced thermal stability, making it suitable for various applications, including the food, biomedical, and agricultural sectors.

The DTG analysis is conducted to ascertain the rate of mass loss of the composite film in relation to temperature. The maximum degradation temperature (T_d), a critical factor for polymer composites, is ascertained from the peak temperature in DTG profiles. The composite film exhibits a T_d of approximately 286 °C and a peak mass-loss rate of 41.67 %/minute.

DTA is conducted to ascertain the disintegration heat. A DTA thermogram would have an exothermic peak if cross-linking mechanisms were implicated in the degradation of the composite film (Saranya and Shenbagarathai, 2011). The DTA curve displayed a minor exothermic peak at 166 °C, succeeded by a pronounced exothermic peak at 286 °C, indicating a phase transition and the combustion of hydrocarbons in the PHB/CIN/CLO 20 % composite film structure.

The melting temperature (T_m), melting enthalpy change (ΔH_m), and degree of crystallinity (X_c) of the PHB/CIN/CLO 20 % composite film were determined using DSC. The X_c of the polymer is a crucial factor in characterizing the material's mechanical properties. Polymers exhibiting high crystallinity are generally more brittle and possess a limited range of applications. The composite film exhibited an endothermic peak on the DSC thermogram, corresponding to T_m (167.8 °C) with ΔH_m of 29.05 J/g. An endothermic peak is found at an elevated temperature of 287.01 °C, attributed to the degradation of the composite film. The melting temperature (T_m) and enthalpy of fusion (ΔH_m) of pristine PHB that has been previously published (Acharjee et al., 2024) are 168.7 °C and 63.21 J/g, respectively. The crystallinity degree (X_c) of the pristine PHB was previously reported as 43.2 %, whereas the X_c of the PHB/CIN/CLO 20 % composite film was

found to be 19.9 %. The X_c value of the PHB/CIN/CLO 20 % composite film is lower than that of pristine PHB, indicating a reduction in crystallinity, which is a significant characteristic for the processing of PHB. The reduction in crystallinity affects the mechanical properties of polymers; specifically, a lower degree of crystallinity correlates with increased material elasticity. Additionally, higher crystallinity is associated with elevated melting temperatures, necessitating greater thermal energy for material melting (Silva et al., 2020). Sharma et al. (2022) reported comparable findings from the DSC analysis, indicating that the X_c of the neat material was 29.24 %. However, upon the incorporation of 20 % thyme oil into the polymer matrix, the X_c decreased to 19.39 %. Silva et al. (2020) also reported a decrease in X_c from 48.09 % for pristine material to 21.51 % following the addition of 15 % clove essential oil.

The crystallinity values of the composite film obtained from XRD (36.7 %) and DSC (19.9 %) differ due to the application of distinct methodologies; XRD highlights surface crystallinity, while DSC illustrates bulk behaviour (Frone et al., 2020; de Sousa Junior et al., 2022). In contrast to DSC, which relies on the melting or crystallization enthalpy for its assessment, XRD conducts its analysis by comparing the integrated intensities of characteristics associated with the crystalline and amorphous phases (Tarani et al., 2023).

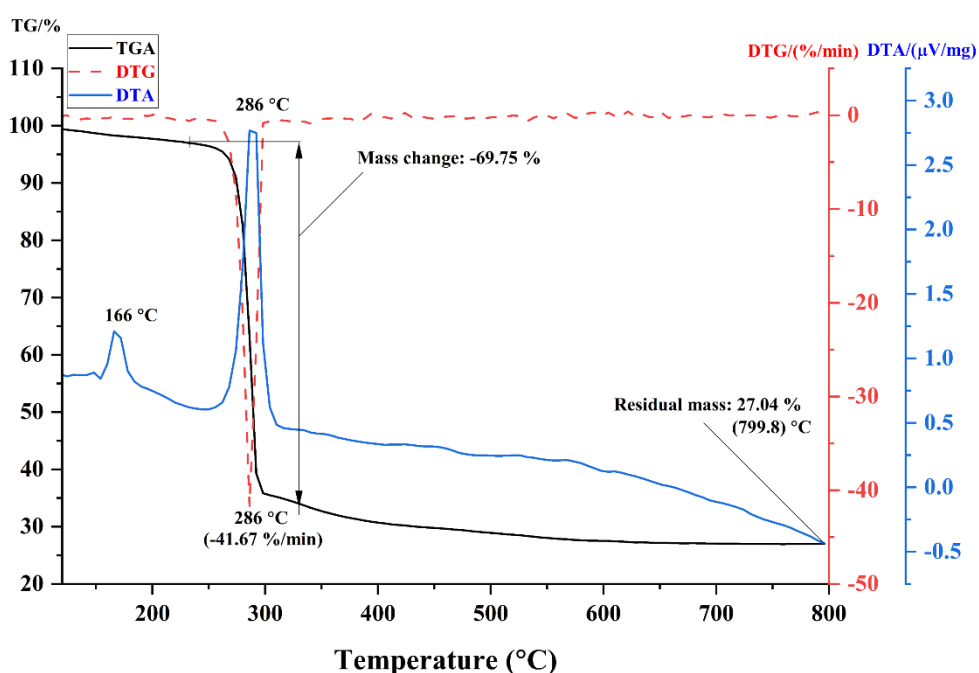


Fig. 4.2.5. TG/DTG/DTA curves of PHB/CIN/CLO 20 % composite film

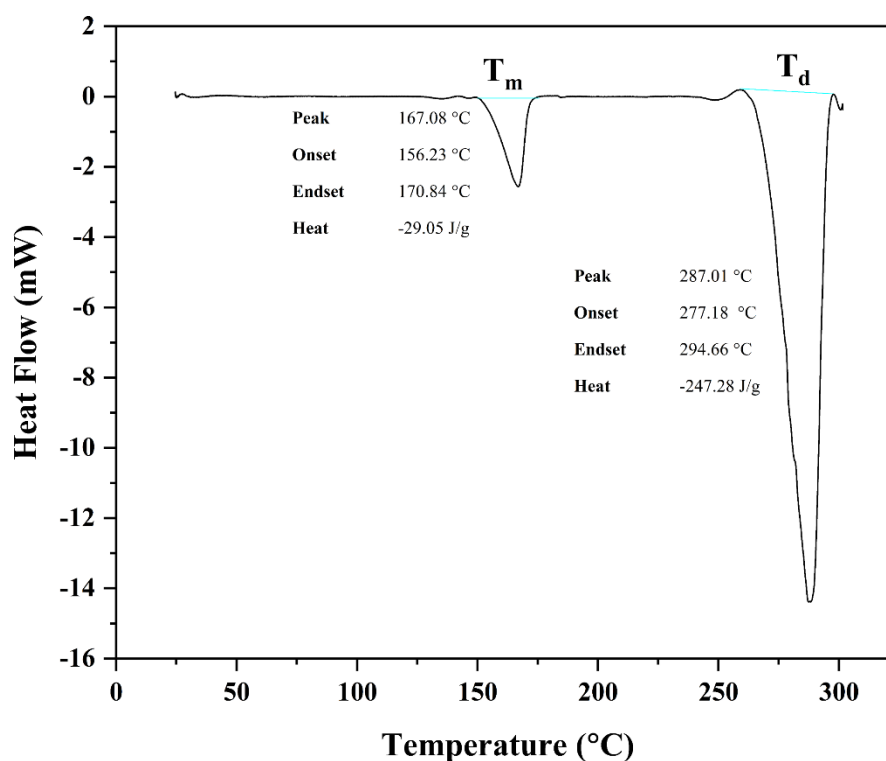


Fig. 4.2.6. DSC thermogram of PHB/CIN/CLO 20 % composite film

4.2.4.1. Water contact angle (WCA)

The WCA serves as a critical metric for assessing a surface's resistance to water and the wettability of films, where a contact angle $\theta > 65^\circ$ denotes a "hydrophobic surface" and $\theta < 65^\circ$ signifies a "hydrophilic surface" (Vogler, 1998; Zhang et al., 2022). In general, hydrophobic films exhibit WCA values over 90° , however those with a surface WCA greater than 65° are also classified as hydrophobic (Cui et al., 2023; Kalita et al., 2025). The active composite films exhibited a WCA of 91° , signifying their low affinity for water and suggesting their possible applicability in food packaging (figure 4.2.7). Kumari et al. (2023) developed PHB/essential oil composites for prospective active food packaging applications, revealing a WCA of $> 65^\circ$, consistent with our findings. Similarly, Sharma et al. (2022) developed an active P(3HB-co-4HB)/thyme oil 30 % composite film for food packaging applications, with a WCA of 70.4° .



Fig. 4.2.7. Water contact angle of the PHB/CIN/CLO 20 % composite film

4.2.5. Water vapor permeation (WVP) test

The water vapor barrier is a crucial attribute of food packaging materials that prevents moisture transfer from the environment to the food inside. Food packaging materials must have minimal water vapor permeability to preserve food quality. The water vapor permeability of the PHB/CIN/CLO 20 % composite film was measured at 0.162 g.mm/kPa.h.m². The water vapor permeability of various packaging composite films, including pectin-k-carrageenan/peppermint oil (0.3 %) (Bhatia et al., 2024a), pectin/xanthan gum/grapefruit essential oil (Bhatia et al., 2024b), and guar gum-calcium caseinate/Clary sage essential oil (Bhatia et al., 2024c), has been reported as 0.64, 0.56, and 0.21 g.mm/kPa.h.m², respectively. Lu et al. (2021) measured the water vapor permeability of the PLA/silica nanoparticles/clove oil composite film as 2.14 g.mm/kPa.h.m². Sharma et al. (2022) measured the water vapor permeability of P(3HB-co-4HB) with thyme oil (30 % v/w) as 0.36 g·mm/m²·kPa·day. Mittal et al. (2023) reported the water vapor permeability of PHB/CEO as 1.89 g·mm/m²·kPa·day. The assessed permeability values demonstrate the water barrier characteristics of the PHB/CIN/CLO 20 % composite film, highlighting its potential uses in food packaging.

4.2.6. Overall Migration (OM) in Food Simulants

The packaging material must exhibit minimum interactions with the food product and must not leach additives into the food in unacceptable quantities (Bureau of

Indian Standards, IS 1981). Therefore, to enhance its efficacy as packaging material, the migration into food substances must be assessed. The migration of components in film samples is influenced by the characteristics of food simulants, the solubility of the active films, and the interactions among biopolymers and additives. The OM values for each simulant (distilled water, 3 % acetic acid, and 50 % ethanol) were examined for pristine PHB and the PHB/CIN/CLO 20 % composite films, with the results summarized in figure 4.2.8. According to regulation 10/2011/EC, the OM of packaging materials must not surpass 10 mg/dm² of food contact surface (Requena et al., 2017). However, according to standards for active food contact products, the active compounds discharged must not be considered into the OM. Furthermore, the OM of volatile chemicals, such as EOs utilized in food packaging, is insignificant as they likely migrate with solvents and do not affect the total residual weight. This is especially accurate when employing aqueous solvents, as chemical evaporation emerges due to the steam drag effect (Requena et al., 2017; Kumari et al., 2023). The OM values of PHB control films and PHB/CIN/CLO 20 % composite films for all the used food simulants were within the allowable range of 10 mg/dm². The OM of components of PHB control films and PHB/CIN/CLO 20 % composite films into distilled water was less in comparison to other food simulants.

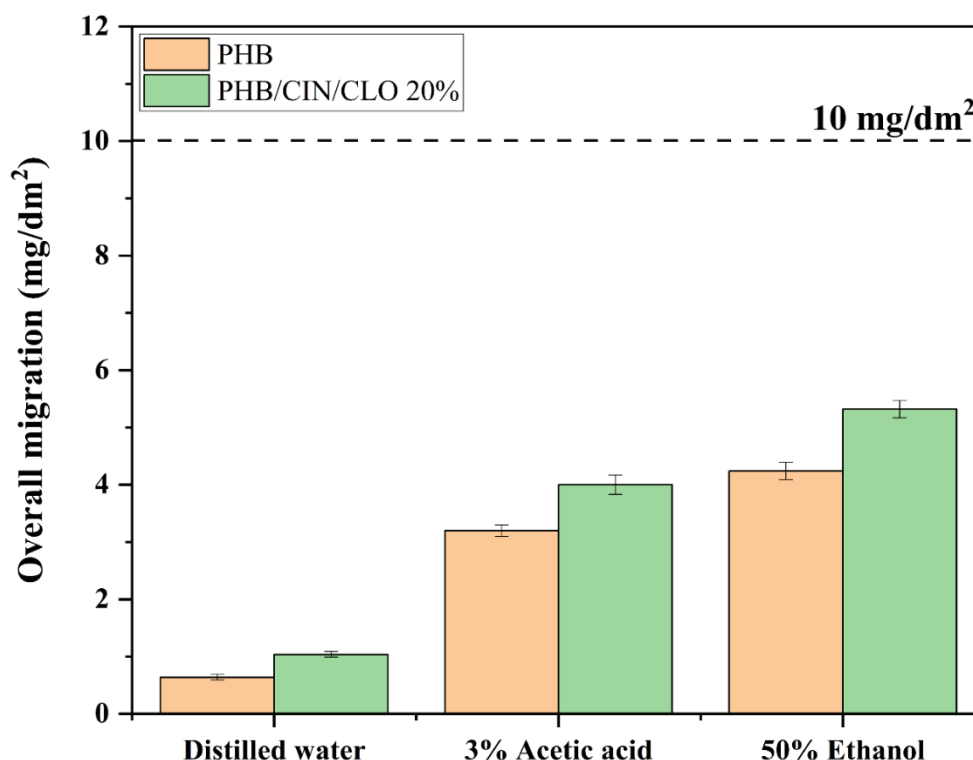


Fig. 4.2.8. Overall migration components of pristine PHB and PHB/CIN/CLO 20 % composite films into food simulants

4.2.7. Antibacterial activities

The screening of antibacterial efficacy of various concentrations of EOs against foodborne bacteria, namely *Staphylococcus aureus* (gram-positive), *Listeria monocytogenes* (gram-positive), *Escherichia coli* (gram-negative), and *Salmonella enterica* (gram-negative), are shown in Supplementary figure 4.2.1, while the table of inhibition zones is provided in Supplementary table 4.2.1. The CIN/CLO 20 % demonstrated efficacy against all foodborne pathogens. Consequently, CIN/CLO 20 % was integrated into PHB and designated as PHB/CIN/CLO 20 % composite films. The antibacterial test results of pristine PHB and PHB/CIN/CLO 20 % composite films against foodborne bacteria are illustrated in figure 4.2.9. The control film (PHB) exhibited minimal antibacterial action against the tested pathogens. The PHB/CIN/CLO 20 % composite films demonstrated significant inhibitory effects against *S. aureus* and *L. monocytogenes*, while exhibiting moderate inhibitory effects against *E. coli* and *S. enterica*. The antibacterial efficacy

of EOs is contingent upon their contents and the type of bacterium. EOs may impede bacterial proliferation through one or more of the following mechanisms: damage to the cell wall resulting in cytoplasmic leakage, coagulation of cytoplasm, depletion of proton motive force (PMF), and membrane protein disruption (Varghese et al., 2020). In comparison to *E. coli* and *S. enterica*, the inhibitory efficacy of EO-infused films was greater against *S. aureus* and *L. monocytogenes*. The cell walls of gram-positive bacteria, including *S. aureus* and *L. monocytogenes*, consist of peptidoglycan and provide minimal resistance to the infiltration of EOs. In contrast, the cell walls of gram-negative bacteria, including *E. coli* and *S. enterica*, are intricate, featuring a thin peptidoglycan layer situated between the cytoplasmic membrane and the outer membrane. Furthermore, the lipopolysaccharides (LPS) in the outer membrane of gram-negative bacteria impede the infiltration of EOs, rendering these bacteria more resistant than gram-positive bacteria (Nazzaro et al., 2013; Kumari et al., 2023).

The process by which EOs suppress bacteria is intricate and multifaceted. Cinnamaldehyde, the prime active component in cinnamon EO, efficiently suppresses bacterial proliferation by impairing the structural integrity of the bacterial cell membrane, enhancing its permeability, and resulting in cellular leakage (Zhang et al., 2016). Moreover, eugenol in clove EO can impede bacterial protein synthesis, and in conjunction with cinnamaldehyde, it may produce a more extensive antibacterial impact within cells (Wijesinghe et al., 2021). The synergistic effect may result from the increased concentration of cinnamon EO, which can more efficiently modulate cell membrane permeability and enhance the absorption and efficacy of the active compounds in clove EO (Zeng et al., 2025). The inhibitory impact of EOs on all examined foodborne pathogens results from the synergistic effects of their active constituents or interactions among their components.

The assessment of the *in-vitro* bacterial response of PHB/CIN/CLO 20 % composite films against the tested bacteria is carried out to evaluate the effectiveness of the composite films as antibacterial film. Following a 24 h incubation period, the organism's growth had been observed in both petri dishes 1 and 2 (figure 4.2.10). In petri dish 1, bacterial growth was seen in the direction of the glucose-loaded well, but not in the direction of the composite films. In petri dish

2, the bacterial growth was towards the well containing glucose, but no growth was seen towards the wells containing streptomycin (10 µg/mL). The results from Petri dish 1 indicate a positive chemotactic response (or movement) of *E. coli*, *L. monocytogenes*, and *S. enterica* towards glucose, while exhibiting a negative response towards the composite films. In petri dish 2, there is a clear positive chemotactic response (or movement) of *E. coli*, *L. monocytogenes*, and *S. enterica* towards glucose, and a negative response towards the antibiotic streptomycin. Motility is essential for bacteria to evade threats and seek nutrients in their environment, which are vital to their virulence and pathogenicity (Matilla and Krell, 2018). However, in the case of *S. aureus*, the findings in both petri dishes demonstrated that there was a nearly insignificant movement of bacteria toward glucose, composite film, and streptomycin. One possible explanation for this is that *S. aureus* does not possess pili or flagella, and is rendered non-motile (Liu and Lin, 2023). Nevertheless, enormous colonies were seen on the periphery of the well that is entirely filled with bacterial broth. This occurs due to the fact that the organism is able to migrate on the surface of soft agar *via* spreading. Its movement is facilitated by the accumulation of water within a colony as well as the formation of biosurfactants known as phenol soluble modulins (PSMs). These biosurfactants reduce the surface tension of the water, which in turn enables water to flood over the surface of the agar and disseminate the organism (Lin et al., 2016).

4.2.8. Antifungal activities

The antifungal efficacy of both pristine PHB and PHB/CIN/CLO 20 % composite film against *Aspergillus niger* and *Penicillium chrysogenum* was evaluated by examining the formation of inhibitory zones surrounding the test rectangular films (figure 4.2.11). Two distinct petri dishes were utilized for each fungal strain. In petri dish 1, antifungal nystatin (50 µg/mL) and pristine PHB film were positioned at opposing ends, while in petri dish 2, a PHB/CIN/CLO 20 % composite film was placed in the centre. The findings from petri dish 1, inoculated with *A. niger*, indicated that the antifungal medication nystatin formed a clear zone of inhibition, but the pristine PHB film was engulfed by mycelial growth, demonstrating its lack of antifungal properties. The findings from petri dish 2 indicated that the PHB/CIN/CLO 20 % composite film possessed apparent inhibitory effects on the

mycelial growth, exhibiting a clear zone of inhibition. Comparable outcomes were observed with *P. chrysogenum*, where the antifungal medication nystatin showed a clear zone of inhibition, but pristine PHB was engulfed by mycelial proliferation. In Petri dish 2, the PHB/CIN/CLO 20 % composite film effectively inhibited mycelial growth, forming a clear zone of inhibition.

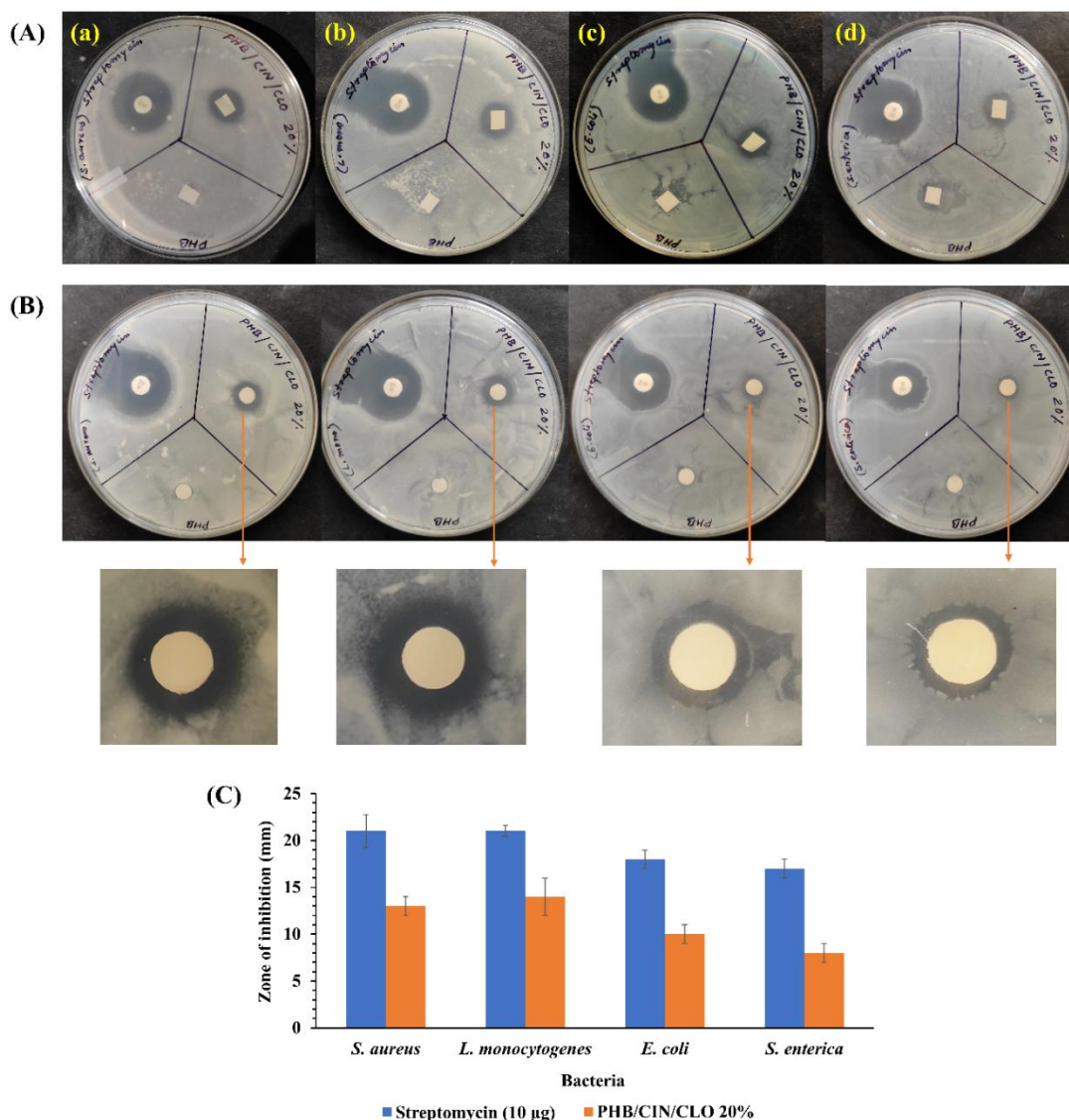


Fig. 4.2.9: (A) Antibacterial activity of PHB/CIN/CLO 20 % composite films cut into rectangular shaped against food pathogens (a) *Staphylococcus aureus* (gram-positive), (b) *Listeria monocytogenes* (gram-positive), (c) *Escherichia coli* (gram-negative), and (d) *Salmonella enterica* (gram-negative) (B) Antibacterial activity of PHB/CIN/CLO 20 % composite films cut into disc shaped against food pathogens

while showing the inhibition zones more clearly for better visualization, and (C) Zone of inhibition (mm) shown by the tested bacteria

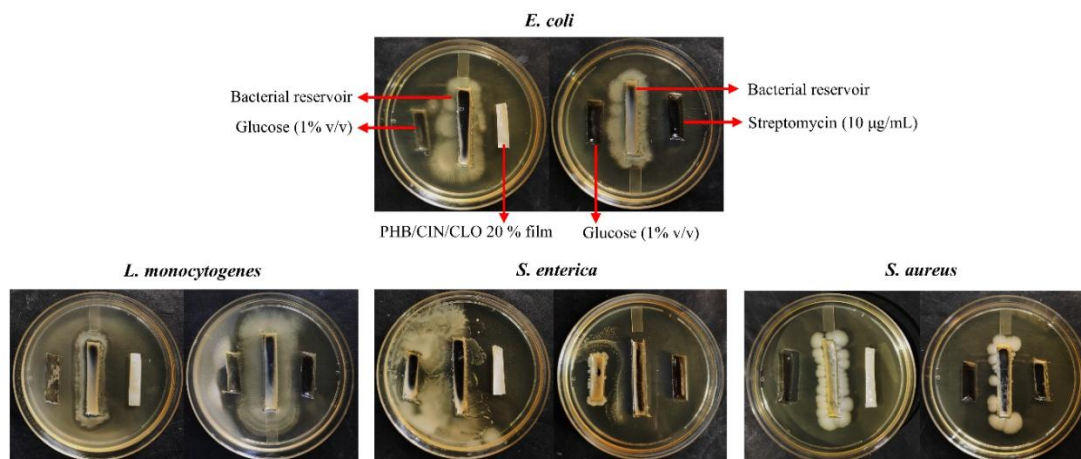


Fig. 4.2.10: *In-vitro* bacterial response of PHB/CIN/CLO 20 % composite film and glucose towards tested bacteria. Streptomycin (10 µg/mL) was used as positive control and glucose (1 % v/v) was used as negative control

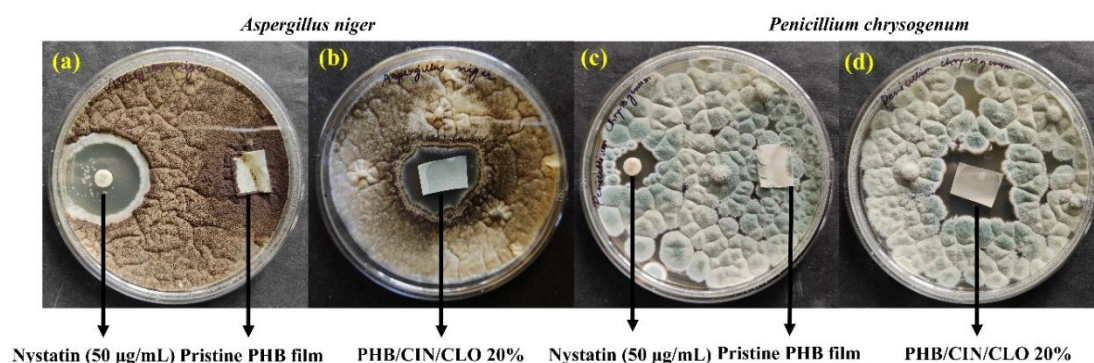


Fig. 4.2.11. Antifungal activity of pristine PHB film and PHB/CIN/CLO 20 % composite film against *Aspergillus niger* and *Penicillium chrysogenum*

4.2.9. Cytotoxicity assay

4.2.9.1. Cell viability and cytotoxicity

An *in-vitro* cytotoxicity test was conducted to ascertain the quantitative biocompatibility of the PHB/CIN/CLO 20 % composite film. A crucial factor in assessing the biocompatibility of a composite film is cell viability; higher viability value often indicates enhanced cell proliferation (Zhuikov et al., 2020). Figure 4.2.12a depicts the dose-dependent cytotoxicity of the PHB/CIN/CLO 20 % composite film in primary hepatocytes. The results indicate that composite film exposure does not significantly ($p > 0.05$) influence cell viability up to 80 µg/mL.

A cell viability value of less than 50 % relative to control cells indicates significant toxicity of a compound (Abdo et al., 2023). However, the cell viability of the PHB/CIN/CLO 20 % composite film exceeded 90 % across all tested concentrations, indicating that these composite films are safe and suitable for benign packaging applications.

4.2.9.2. Cellular oxidative stress

Oxidative stress results from an increase in reactive oxygen species (ROS) levels, adversely affecting cellular homeostasis, structures, and functions (Snezhkina et al., 2019). An oxidative stress assay was conducted to ascertain if the PHB/CIN/CLO 20 % composite films generate cellular oxidative stress in primary hepatocytes. Figure 4.2.12b illustrates the dose-dependent effect of PHB/CIN/CLO 20 % composite films on total ROS in primary hepatocytes. The results suggest that no significant ($p > 0.05$) changes were found in total ROS generation during the exposure to the composite films.

4.2.9.3. Cellular apoptosis assay using flow cytometry

Apoptosis is a specific form of programmed cell death that occurs in multicellular organisms under standard physiological conditions to support various biological processes, including normal cell turnover, immune function, tissue homeostasis, and embryonic and cerebral development (Renahan et al., 2001). The manner of cell death generated by PHB/CIN/CLO 20 % composite films was investigated by a cellular apoptosis assay conducted *via* flow cytometry, utilizing an Annexin-V/Dead cell apoptosis kit. Annexin V is a calcium-dependent protein that binds phospholipids, with a strong affinity for phosphatidylserine (PS). A healthy cell surface is safeguarded by lipids arranged asymmetrically on the inner and outer leaflets of the plasma membrane. The Annexin V/PI procedure is a widely utilized method for the examination of apoptotic cells. Propidium iodide (PI) only stains necrotic and late apoptotic cells, as it penetrates only those cells that have damaged membrane integrity (Kari et al., 2022). The flow cytometry analysis of Annexin/PI staining for the control (figure 4.2.12c) and PHB/CIN/CLO 20 % composite films exposure (figure 4.2.12d) was presented as scatter plots. No significant levels of apoptotic cells (early/late apoptosis) or necrosis were seen following exposure to high doses of PHB/CIN/CLO 20 % composite films (80 $\mu\text{g/mL}$). This explicitly

demonstrates the non-toxic characteristics of the composite films, exhibiting excellent biocompatibility.

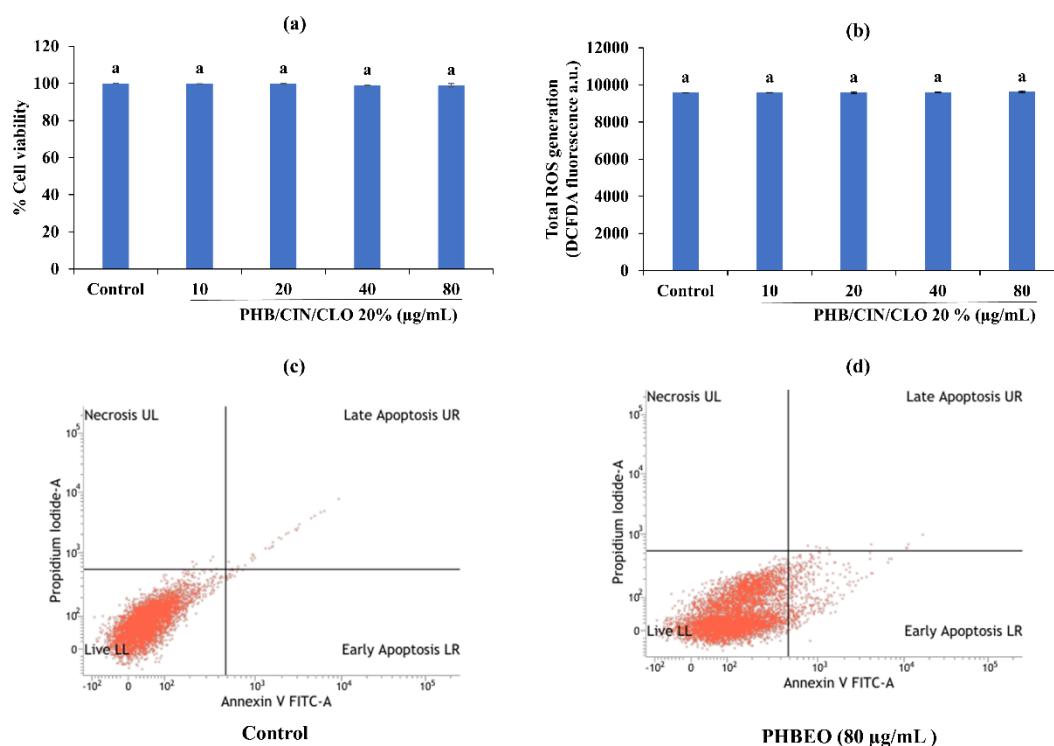


Fig. 4.2.12. Biocompatibility tests (A) dose-dependent effect of PHB/CIN/CLO 20 % composite films on cell viability, (B) total ROS generation, (C) Annexin/PI-stained flow cytometry observation of control, and (D) Annexin/PI-stained flow cytometry observation of PHB/CIN/CLO 20 % composite films

4.2.10. Biodegradation study

Figure 4.2.13a depicts the visual alterations in PHB/CIN/CLO 20 % composite films at different stages of deterioration. Alongside visual observations, the weight and degradation % of the composite films were quantified (Supplementary Table 4.2.2), and figure 4.2.13b illustrates the weight loss graphically. As the film deteriorated, its mass significantly diminished. The percentage of biodegradation increases concurrently with the duration of deterioration (Fernandes et al., 2020). The experiment revealed evident physical morphological changes on the film's surface, including holes, ruptures, breaches, and progressive disintegration of its components. The original mass of the composite films was 0.0413 g. After 7 days, the film's weight marginally decreased to 0.041 g, reflecting a degradation of 0.8

%; after 14 days, it further diminished to 0.038 g, corresponding to an 8.06 % degradation; after 21 days, the weight was recorded at 0.032 g, indicating a 22.58 % degradation; after 28 days, the weight fell to 0.0166 g, with a degradation of 59.67 %; after 35 days, the weight was 0.01 g, demonstrating a 75.8 % degradation; and after 42 days, the weight was 0.0066 g, with a degradation of 59.67 %. Ultimately, after 49 days, the weight of the composite films was observed to fully break down, achieving a biodegradation percentage of 100 %. The crystalline structure and uneven surface of the composite film, which promote microbial adhesion and subsequent micropore formation, could be the reason for the film's accelerated breakdown in natural conditions (Boyandin et al., 2012). The incorporation of essential oil accelerated the deterioration rate, likely due to the formation of voids, as elucidated in the FESEM-EDS analysis. This further induces fragility in the films, facilitating microbial attack and so enhancing the deterioration process (Nisar et al., 2018). A study conducted on starch-PVA films infused with oregano and neem EOs shown an increased biodegradation rate and simple microbial attack (Cano et al., 2016).

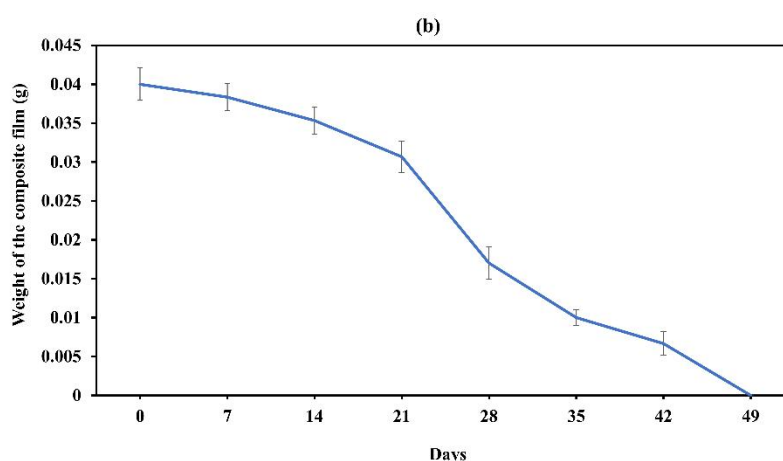
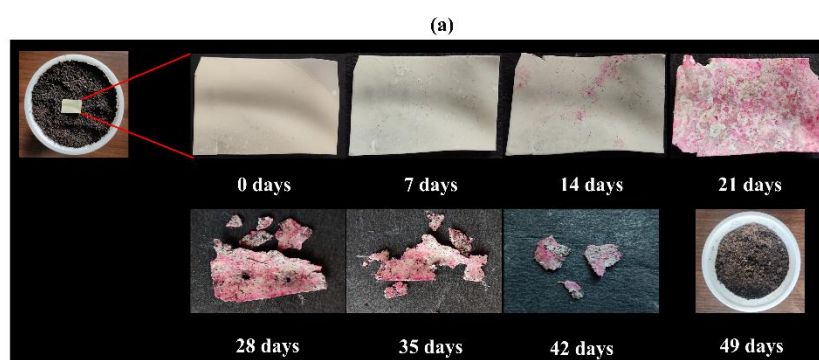


Fig. 4.2.13. (a) Biodegradation study of PHB/CIN/CLO 20 % composite films and (b) Reduction in mass of composite films (The error bars indicate the standard deviation from the mean. Three replicants are used for every data point)

4.2.11. Limitation and future scope of the study

PHB has demonstrated significant potential as a biopolymer for food packaging applications due to its derivation from non-food grade natural materials and the complete compostability of its composites. Nonetheless, the limited processing window and high brittleness of PHB materials presently restrict their use in food packaging. The application of melt-blending processing technologies, particularly those that incorporate plasticizers and/or more flexible biopolymers or utilize green plasticizers, appears to provide the most sustainable and readily scalable solutions. These formulations can be produced in existing industrial processing facilities, enhancing their processability, ductility, and overall mechanical performance. Nevertheless, additional research is required to enhance the processing and performance of the produced materials, as well as to identify more effective management strategies for future PHB-based packaging waste generated.

4.2.12. Conclusion

This work involved the development of an active packaging material by integrating a combination of EOs (cinnamon and clove oil) into a PHB matrix by the solvent casting technique. The study sought to employ natural biopolymers alongside other active natural substances, particularly PHB, since it presently represents a substantial alternative to petroleum-based plastics within the framework of the circular economy. FTIR analysis of the developed composite films indicated that the inclusion of EOs modified the molecular arrangement and intermolecular interactions within the PHB matrix. The DSC study revealed a decrease in melting temperature and crystallinity of the composite films. Furthermore, EO mixture induced a plasticizing effect by lowering the melting temperature of the biopolymer and improving film flexibility. The OM values for the active composite films across various food simulants remained under the allowable limit of 10 mg/dm². The integration of EOs into the PHB matrix has exhibited significant antibacterial and antifungal efficacy against all studied pathogenic microorganisms implicated in food spoilage. The biocompatibility of the composite films was assessed through

evaluations of cell viability, ROS production, and apoptosis in primary rat hepatocytes. The test revealed that the composite film is biocompatible, indicating its non-toxic nature. The biodegradation study of the composite film was also performed and exhibited complete degradation within 49 days during the soil burial test. All these findings indicated that the EOs integrated into PHB active films exhibited superior structural, physicochemical, mechanical, thermal, biocompatibility, and biodegradability properties, thus rendering them suitable for food packaging applications.

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CHAPTER-5

Summary, Limitations and Future Perspectives

5.1. Overall Summary

For the past 200 years, the world has observed the consumption of derivatives of fossil fuels and their impacts. Among the derivatives, widespread usage of petrochemical-based synthetic polymers is responsible for global ecological imbalance in a number of areas, including the stability of the Earth's atmosphere, terrestrial and aquatic life, food chains, and the plant realm. A replacement for synthetic polymer-based plastics with biodegradable polymers-based material derived from microorganisms, particularly of bacterial origin, has been considered as a means of reducing environmental pollution. Polyhydroxyalkanoates (PHAs) are seen as potential substitutes for conventional petrochemical plastics because of their appealing qualities that are comparable to those of thermoplastics derived from petroleum and their high level of biodegradability. The most well-known biopolymer among the many PHAs is polyhydroxybutyrate (PHB); and due to their biodegradability, biocompatibility, diversity in chemical structure, and ability to be produced from renewable carbon resources, PHB have drawn substantial research and commercial interests. Therefore, in present study, a potent PHB-producing indigenous bacterial strain was

successfully isolated from kitchen waste effluent site in Lumami, Zunheboto, Nagaland, India, and identified through 16S rRNA gene sequencing as *Bacillus* sp. KE4. The strain was able to produce a maximum dry cell weight (DCW) of 4.4 g/L with a PHB concentration of 3.1 g/L, PHB yield of 0.15 g/g, and the PHB content was 70.4 % of the DCW. The findings of TGA, DTG, DTA and DSC studies corresponded to PHB's excellent thermal stability. The weight average molar mass (M_w), number average molar mass (M_n), and polydispersity index (PDI) of the extracted PHB was 1,55,436 g/mol; 1,43,258 g/mol; and 1.08, respectively. The XRD analysis validated the material's partly crystalline nature. The Young's modulus, tensile strength and elongation at break showed rigid and brittle nature of PHB. Biodegradation experiments indicated significant environmental compatibility, with considerable breakdown noted after 28 days of soil burial, while biological assays validated its non-cytotoxic, biocompatible, and non-bactericidal characteristics.

A variety of organic wastes, such as fruit pulp of Bael, waste sugarcane molasses, jackfruit wastes, papaya peels, pumpkin wastes, and glucose individually, were evaluated for their viability as carbon sources for PHB production. Of the carbon sources examined, glucose emerged as the most effective substrate for PHB synthesis. Statistical methodologies such as Plackett-Burman Design (PBD) and Central Composite Design (CCD) within Response Surface Methodology (RSM) were employed to assess the significant variables affecting the yield of PHB. Initially, three parameters (glucose, ammonium sulphate, and temperature) were selected from PBD for further optimization using CCD, wherein the interactions among these factors were examined in depth, resulting in a final optimized medium for enhanced biomass and PHB yield. The ideal C:N ratio for optimal biomass and PHB yield is established at 28.2:1, resulting in a maximum biomass DCW yield of 4.81 g/L, a PHB yield of 3.48 g/L, and a PHB content of 72.34 %, showing a slight increase over PHB production before optimization (*i.e.*, 70.4 %). The maximum yield was attained under ideal conditions of 25.28 g glucose concentration, 1.97 g ammonium sulphate concentration, and a constant temperature of 37 °C.

However, a sustainable production and development of PHB for commercial purposes have been constrained by a lack of advanced features *viz.* poor physical and mechanical properties. Therefore, PHB need to be upgraded to enhance its inherent qualities. This upgradation can be primarily achieved by blending PHB either with natural or synthetic bio-based polymers and/or adding reinforcements to develop novel PHB-based biocomposites and blends. Therefore, a cost-effective, energy-efficient, non-cytotoxic, biocompatible, and biodegradable 3D porous sorbent is fabricated employing a blend of PHB and polycaprolactone (PCL) biopolymers using a facile salt leaching technique. The sorbent demonstrated good water repellence, achieving a saturated oil sorption capacity of 6.02 g/g for waste cooking oil (WCO) and 5.09 g/g for diesel oil, with recovery rates of 86.5 % for WCO and 85.7 % for diesel. Sub-sequent to the sorption of oils, the oil-saturated sorbents were further valorised through the production of biosurfactant utilizing the strain *Pseudomonas aeruginosa* AMS1a. The strain effectively produced biosurfactant with concentrations of 1040 mg/L and 546 mg/L utilizing WCO and diesel as carbon sources, respectively. Notably, the sorbents exhibited partial degradation during the valorisation process. The residual sorbents were buried in compost soil for further degradation, and were entirely decomposed within 28 days. The findings demonstrated an efficient biomaterial for addressing oil spill disasters while minimizing secondary contamination. The sorbent also fulfilled the zero-waste notion of the Sustainable Development Goals (SDGs) defined by the United Nations.

In the field of active packaging, a combination of essential oils (EOs) of Cinnamon and Clove as an antimicrobial agent was incorporated into PHB matrix by conventional solvent-casting method for food packaging application. Migration values for active composite films for diverse food simulants were well within 10 mg/dm². The water vapor permeability of the active composite film is measured at 0.162 g.mm/kPa.h.m², highlighting its potential uses in food packaging. EOs in PHB matrix have shown good antibacterial and antifungal effects against all tested food-spoiling pathogens. To test composite film's biocompatibility, primary hepatocytes were tested for cell viability, ROS production, and apoptosis. The composite film was found biocompatible, proving its non-toxicity. Additionally, the

composite film degraded completely within 49 days in soil burial test, confirming its highly biodegradable nature.

When taken as collectively, the findings of present study demonstrate that *Bacillus* sp. KE4 is effective and adaptable strain for the production of PHB. In addition to making a significant contribution to the development of green and circular bioeconomy endeavours, the successful development of PHB-based biocomposites sheds light on the potential of these biomaterials in a wide range of applications, including water remediation and active food packaging. In addition, present study encourages sustainable consumption and production, which is in line with Sustainable Development Goal (SDG) 12 because it reduces waste and encourages the development of a circular economy.

5.2. Limitations

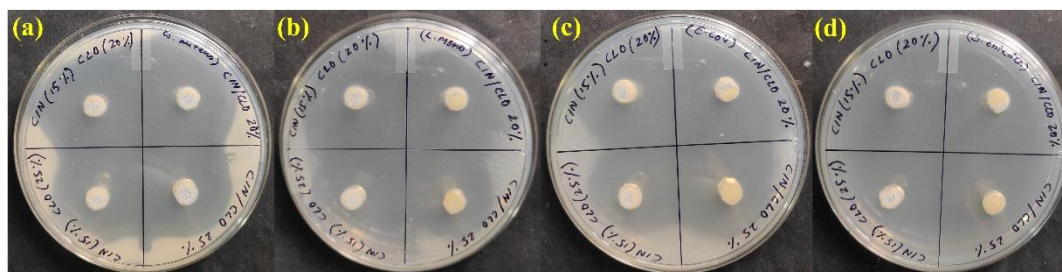
The findings of the investigation emphasize the promise of PHB as a versatile biomaterial applicable in water remediation and active food packaging. Despite the encouraging findings, numerous restrictions still that must be resolved prior to the implementation of such insights in real, large-scale applications. A significant hurdle is the scalability of PHB production. The existing method mostly depends on batch fermentation, which is intrinsically constrained by low productivity and elevated labour requirements. Furthermore, the yields of PHB obtained in laboratory settings may not immediately correspond to those in industrial-scale processes. Despite the advantages of PHB biopolymers, some limitations restrict their application, including high crystallinity, brittleness, and elevated cost. Consequently, PHB is frequently utilized not in its pure state, but as an integral component of multi-faceted materials. A further limitation pertains to the extent of performance assessment. The produced PHB-based composites, such as PHB/PCL and PHB/CIN/CLO 20 %, have intriguing multifunctional capabilities under controlled settings; nonetheless, their stability, durability, and efficacy in complicated, real-world contexts remain largely unexamined. The lack of long-term environmental exposure studies creates deficiencies in comprehending the behaviour and degradation of these materials in real-world applications.

5.3. Future perspectives

Overcoming such limitations grants numerous opportunities for subsequent research. Advancements are crucial for transitioning PHB applications from the laboratory to practical implementation. A critical area of emphasis is the advancement of scalable fermentation systems, namely through the implementation of continuous or bioreactor-based techniques, including stirred-tank, annular, airlift, or AI-driven bioreactors. An AI-driven bioreactor is a proposed, next-generation device that employs artificial intelligence and machine learning to automatically optimize the production process. This can attain higher, more uniform yields and reduce manual labour by perpetually adjusting the fermentation conditions for optimal efficacy. Currently, the fabrication of PHB blends and composites is receiving a lot of interest. Blended PHB have numerous uses, and could be use together with other polymers/additives effectively for packaging, agriculture and specifically in pharmaceuticals. These blends are employed in a number of drug delivery systems, including those used to treat neurological and metabolic disorders, targeted cancer therapy, antidiabetic drugs, and controlled malnutrition. Likewise, researchers are also focusing on the PHB-based blends for the development of cartilage and artificial bones, as well as other biomedical applications including cardiac, vascular, and muscle tissues. However, the myriad blend and composite options available with the diverse PHB have not yet been fully investigated. Therefore, for the foreseeable future, it will continue to be a productive area of study.

APPENDIX-I

SUPPLEMENTARY FIGURES AND TABLES



Supplementary Fig. 4.2.1. Screening of antibacterial efficacy of various concentrations of essential oils against tested foodborne bacteria

Supplementary Table. 2.1. Weight loss and degradation percentage of PHB film

Days	Weight of the PHB film (mg)	Biodegradation percentage (%)
Initial	7.06 ± 0.06	0
7	6.83 ± 0.07	3.25
14	5.79 ± 0.3	17.9
21	4.77 ± 0.38	32.4
28	2.73 ± 0.19	61.34

Supplementary Table. 4.2.1. Zone of inhibition observed for different concentrations of EOs (CIN/CLO) in a ratio of 7:3 for the tested bacteria

Bacteria	Zone of inhibition (mm)			
	CIN/CLO 20%	CIN 15% + CLO 20%	CIN/CLO 25%	CIN 15% + CLO 25%
<i>E. coli</i>	40 ± 1.00	33 ± 1.00	35 ± 1.52	32 ± 1.00
<i>L. monocytogenes</i>	39 ± 1.52	38 ± 0.577	29 ± 1.15	33 ± 0.57
<i>S. enterica</i>	39 ± 2.00	35 ± 1.52	30 ± 0.57	23 ± 0.57
<i>S. aureus</i>	40 ± 0.57	36 ± 1.00	33 ± 1.00	32 ± 1.52

Supplementary Table 4.2.2. Weight loss and degradation percentage of composite film

Days	Weight of the composite film (g)	Degradation percentage (%)
0	0.0413 ± 0.002	0
7	0.041 ± 0.001	0.8
14	0.038 ± 0.001	8.06
21	0.032 ± 0.002	22.58
28	0.0166 ± 0.002	59.67
35	0.01 ± 0.001	75.8
42	0.0066 ± 0.001	83.87
49	0	100

APPENDIX-II

MATERIALS USED IN THE STUDY

I. Carbon-enriched nutrient agar media

Sl. No.	Ingredients	g/L
1	Disodium hydrogen phosphate	6
2	KH ₂ PO ₄	3
3	NH ₄ Cl	1
4	NaCl	0.5
5	Yeast extract	0.05
6	Agar	17
7	Glucose	10

Preparation of the medium

Each component was dissolved in distilled water, and the final volume was set up to 1 L. The pH of the culture medium was adjusted to 7. The medium underwent sterilization *via* autoclaving at 121 °C for 20 minutes.

II. Nutrient broth medium

Sl. No.	Ingredients	g/L
1	Peptone	10
2	Beef extract	10
3	NaCl	5

Preparation of the medium

Each component was dissolved in distilled water, and the final volume was set up to 1 L. The pH of the culture medium was adjusted to 7. The medium underwent sterilization *via* autoclaving at 121 °C for 15 minutes.

III. PHB production medium

Sl. No.	Ingredients	g/L
1	(NH ₄) ₂ SO ₄	2
2	KH ₂ PO ₄	13.3
3	MgSO ₄	1.3
4	Citric acid	1.7
Trace elements (10 ml/L)		
1	FeSO ₄ .7H ₂ O	10
2	ZnSO ₄ .7H ₂ O	2.25
3	CuSO ₄ .5H ₂ O	1
4	MnSO ₄ .5H ₂ O	0.5
5	CaCl ₂ .2H ₂ O	2
6	Na ₂ B ₄ O ₇ .10H ₂ O	0.23
7	(NH ₄) ₆ MO ₇ O ₂₄	0.1
8	HCl	10 mL

Preparation of the medium

All the components *viz.*, (NH₄)₂SO₄, KH₂PO₄, MgSO₄, and citric acid were dissolved in distilled water. Subsequently, 10 mL of trace elements was added in distilled water and the final volume was set up to 1 L. The pH of the culture medium was adjusted to 7. The medium underwent sterilization *via* autoclaving at 121 °C for 20 minutes.

IV. Dulbecco's Modified Eagle Medium (DMEM)

Firstly, 0.67 g of DMEM was diluted in 25 mL of tissue culture-grade water with continuous stirring until fully dissolved. Subsequently, 0.187 g of NaHCO₃ (sodium bicarbonate) powder was introduced and agitated until fully dissolved. The pH was adjusted to 0.2-0.3 units below the intended pH (e.g., pH 7.0-7.6) utilizing 1 N HCl or 1 N NaOH, as the pH often increases during filtering. The final volume

was adjusted to 50 mL using tissue culture grade water. The medium was promptly sterilized by filtration using a sterile membrane filter with a pore size of 0.22 μm .

V. Mineral Salt Medium (MSM)

Sl. No.	Ingredients	g/L
1	Na_2HPO_4	2
2	KH_2PO_4	2
3	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
4	NaNO_3	2.5
5	NaCl	0.8
6	CaCl_2	0.2
7	KCl	0.8
8	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.001
Trace elements		
1	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.116
2	H_3BO_3	0.232
3	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.41
4	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	2.25
5	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.008
6	$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	0.008
7	$(\text{NH}_4)_6\text{MO}_7\text{O}_{24}$	0.02

Preparation of the medium

Each component was dissolved in distilled water, and the final volume was set up to 1 L. The pH of the culture medium was adjusted to 6.8. The medium underwent sterilization *via* autoclaving at 121 °C for 20 minutes.

VI. Muller-Hinton Agar (MHA)

Sl. No.	Ingredients	g/L
1	Beef extract	2
2	Acid hydrolysate of casein	17.5
3	Starch	1.5
4	Agar	16

Preparation of the medium

Each component was dissolved in distilled water, and the volume was adjusted to 1 L. The mixture was meticulously mixed and gently heated until it attained boiling point. Sterilization was performed using autoclaving at 121 °C for 15 minutes.

VII. Sabouraud Dextrose Agar (SDA)

65 g of SDA powder was dissolved in 1 L of distilled water and mixed thoroughly. The solution was then sterilized using autoclave at 121 °C for 15 minutes.

VIII. Chemical-gradient motility agar (CGMA)

Sl. No.	Ingredients	g/L
1	Peptone	5
2	Beef extract	3
3	NaCl	5
4	Agar	7

Preparation of the medium

CGMA medium was formulated by mixing Peptone (5g), Beef extract (3 g) and NaCl (5 g) in 1 L of distilled water, with the pH adjusted to 7. Subsequently, 7 g of agar powder was added and autoclaved for 20 minutes.

IX. Composition of Media and Reagents Used for Biochemical Tests

(a) Indole Test (Tryptone Broth)

Sl. No.	Ingredients	g/L
1	Tryptone	10
2	Sodium Chloride	5

Preparation of the medium

Each component was dissolved in distilled water, and the final volume was set up to 1 L. The medium underwent sterilization *via* autoclaving at 121 °C for 15 minutes. After incubating the test organisms, Kovac's reagent was introduced to identify indole production.

(b) Methyl Red (MR) Test

Sl. No.	Ingredients	g/L
1	Peptone	7
2	Glucose	5
3	Dipotassium phosphate	5

Preparation of the medium

Each component was solubilized in distilled water, and the final volume was set up to 1 L. The medium underwent sterilization *via* autoclaving at 121 °C for 15 minutes. After incubating the test organisms, methyl red indicator solution was introduced, which was made by dissolving 0.04 g of methyl red in 300 mL of 95 % ethanol and thereafter diluting to a final volume of 500 mL with distilled water.

(c) Voges–Proskauer (MR–VP) Medium

Sl. No.	Ingredients	g/L
1	Peptone	7
2	Glucose	5
3	Dipotassium phosphate	5

Preparation of the medium

Each component was solubilized in distilled water, and the final volume was set to 1 L. The medium underwent sterilization *via* autoclaving at 121 °C for 15 minutes. In the Voges–Proskauer (VP) test, after incubation, Barritt’s reagents were introduced: Reagent A, consisting of 5 g of α -naphthol dissolved in 100 mL of pure ethanol, and Reagent B, comprising 40 g of potassium hydroxide dissolved in 100 mL of distilled water.

(d) Citrate Utilization Medium (Simmons’ Citrate Agar)

Sl. No.	Ingredients	g/L
1	Sodium citrate	2
2	Ammonium dihydrogen phosphate	1
3	Dipotassium phosphate	1
4	Sodium chloride	5
5	Magnesium sulfate	0.2
6	Bromothymol blue	0.08
7	Agar	15

Preparation of the medium

Each component was solubilized in distilled water, and the final volume was set up to 1 L. The pH of the medium was calibrated to 6.9 ± 0.2 , and the media was sterilized using autoclaving at $121\text{ }^{\circ}\text{C}$ for 15 minutes. The liquid was thereafter dispensed into tubes and permitted to harden at an inclined angle.

(e) Motility Test Medium

Sl. No.	Ingredients	g/L
1	Peptone	10
2	Sodium Chloride	5
3	Agar	4
4	pH	± 0.2

Preparation of the medium

Each component was solubilized in distilled water, and the final volume was set up to 1 L. The pH of the media was modified to 7.3 ± 0.2 . The produced medium was subsequently sterilized *via* autoclaving at $121\text{ }^{\circ}\text{C}$ for 15 minutes. The medium was formulated as a semi-solid to enable the movement of motile bacteria.

(f) Hydrogen Sulfide (H₂S) Test — SIM Medium

Sl. No.	Ingredients	g/L
1	Peptone	30
2	Beef extract	3
3	Ferrous ammonium sulfate	0.2
4	Sodium thiosulfate	0.025
5	Agar	3

Preparation of the medium

Each component was solubilized in distilled water, and the final volume was set up to 1 L. The medium's pH was calibrated at 7.3 ± 0.2 and sterilized using autoclaving at 121 °C for 15 minutes. The SIM medium was formulated as a semi-solid to facilitate concurrent assessment of hydrogen sulfide generation, indole synthesis, and motility.

(g) Catalase Test

3 % hydrogen peroxide solution: 3 mL H₂O₂ (30 %) in 27 mL distilled water.

(h) Carbohydrate test (Molisch's reagent)

Molisch's test is a universal assay for all carbohydrates. Molisch's reagent is prepared by dissolving 3.75 g of α -naphthol in 99 % ethanol. Concentrated sulfuric acid converts the specified carbohydrate into furfural or its derivatives.

X. Reagents used in the study

(a) Orcinol reagent

Initially, 0.025 g of FeCl₃ was dissolved in 25 mL of Concentrated HCl. Subsequently, 875 μ L of 6 % Orcinol was added in ethanol.

(b) Phosphate Buffered Saline (PBS)

Sl. No.	Ingredients	g/L
1	Sodium chloride	8
2	Potassium Chloride	0.2
3	Disodium Phosphate	1.44
4	Monopotassium Phosphate	0.024

Preparation of the medium

Each component was solubilized in distilled water, and the final volume was set up to 1 L. The medium's pH was calibrated at 7.4 ± 0.2 and sterilized using autoclaving at 121 °C for 15 minutes. Finally, the PBS solution was stored at room temperature.

(c) MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)

MTT powder was incorporated into PBS to achieve a final concentration of 5 mg/mL. The solution was subsequently vortexed until the powder was entirely dissolved. The solution was sterilized by filtration through a 0.22 µm filter upon dissolving.

(d) 0.02 % Alcoholic solution of Sudan Black-B (SBB)

0.02 g of the SBB dye was solubilized in 100 mL of 70% ethanol. The solution was thereafter stirred, ideally overnight, to ensure the complete dissolution of the dye.

(e) Nile Blue A solution (NBA)

Initially, a stock solution of 1000 µg/mL was prepared by dissolving 10 mg of NBA dye in 10 mL of DMSO (Dimethyl sulfoxide), and mixed vigorously to ensure the complete dissolution of the dye. Lastly, a concentration of 0.5 µg/mL was used in the study.

XI. Assembled FASTA sequence of *Bacillus* sp. KE4 (16s rRNA sequencing)

>KE4

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CGGGTGTACAACTGAAGAGGAGGGACGGGCGGTGTGTACAAGGCC
CGGGAACGTAAACACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
AGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTT
TATGGGATTGGCTTGACCTCGCGGTCTTGCAGCCCTTTGTACCATCCATT
GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCGATGATGATTTGACGTCA
TCCCCACCTTCCTCCGGTTTGTACCGGCAGTCACCTTAGAGTGCCCCAA
CTAAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTC
ACTCTGTCCCCCGAAGGGGAACGCTCTATCTCTAGTGTTGTCACAGGAT
GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC
TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGC
GACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAA
AGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGAC
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TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCG
 TCAGTTACAGACCAAAAAGCCGCCTTCGCCACTGGTGTTCCTCCACATC
 TCTACGCATTTACACGCTACACGTGGAATTCCGCTTTTTCTCTTCTGCAC
 TCAAGTTCCCCAGTTTCCAATGACCCTCCACGGGTTGAAGCCGTGGCTT
 TCAACATCAAACCTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAATAAT
 TTCCGGAATAACGCTTGCCACCTACGTATTACCGCGGGCTGCTGGGCAC
 GTAGTTAGCCGTGGGCTTTCTGTAAAGGTACCGTC

XII. Bacterial Strains Used in the Study

Sl. No.	Bacterial Strain	Type	Source	Reference No.
1	<i>Bacillus subtilis</i>	Gram-positive	MTCC	121
2	<i>Listeria monocytogenes</i>	Gram-positive	MTCC	657
3	<i>Staphylococcus aureus</i>	Gram-positive	MTCC	3160
4	<i>Escherichia coli</i>	Gram-negative	MTCC	40
5	<i>Pseudomonas aeruginosa</i>	Gram-negative	MTCC	6458
6	<i>Ralstonia eutropha</i>	Gram-negative	MTCC	8320
7	<i>Alcaligenes faecalis</i>	Gram-negative	MTCC	4305
8	<i>Salmonella enterica</i>	Gram-negative	MTCC	1164

XIII. Fungal Strains Used in the Study

Sl. No.	Fungal Strain	Source
1	<i>Aspergillus niger</i>	Department of Botany, Nagaland University, Lumami

2	<i>Penicillium chrysogenum</i>	Department of Botany, Nagaland University, Lumami

XIV. Source of human liver hepatocellular carcinoma cell line (HepG2)

Details	Description
Cell Type	HepG2 cell line
Provided by	Dr. Jeganathan Manivannan, Department of Environmental Sciences, Bharathiar University, Coimbatore, India
Source	National Centre for Cell Sciences (NCCS), Cell Repository, Pune, India.

XV. Source and Ethical Approval for Primary Rat Hepatocytes

Details	Description
Cell Type	Primary rat hepatocytes
Provided by	Dr. Jeganathan Manivannan, Department of Environmental Sciences, Bharathiar University, Coimbatore, India
Rat Strain	Male albino Wistar rats
Age & Weight	8–10 weeks old; 180–220 g
Source of Animals	Laboratory Animal Medicine Unit, TANUVAS, Chennai, India
Housing Conditions	3 rats per cage; temperature: 25 ± 3 °C; humidity: 40–55 %; light/dark cycle: 12 h/12 h
Feeding	Feed and water ad libitum
Ethical Approval	Animal Ethical Committee, Bharathiar University
Registration No.	722/Go/Re/S/02/CPCSEA
Proposal No.	06
Guidelines Followed	CPCSEA, New Delhi, India
Reference	Nagarajan et al. (2024)

XVI. List of databases, tools and software used

Sl. No.	Name of Tools/Software
1.	NCBI BLAST
2.	MEGA X
3.	ChemDraw 3D
4.	Origin 2021
5.	Microsoft Excel 2021
6.	Design Expert 13
7.	CasaXPS
8.	GNU PSPP software
9.	Image J

APPENDIX-III

LIST OF PUBLICATIONS

List of Paper Publications/Book Chapters

a) Paper published as first author

1. **Acharjee, S. A.**, Bharali, P., Gogoi, B., Sorhie, V., Walling, B., & Alemtoshi. (2023). PHA-based bioplastic: a potential alternative to address microplastic pollution. *Water, Air, & Soil Pollution*, 234(1), 21.
2. **Acharjee, S. A.**, Bharali, P., Ramachandran, D., Kanagasabai, V., Hazarika, S., & Vishwakarma, V. (2024). Polyhydroxybutyrate (PHB)-Based sustainable bioplastic derived from *Bacillus* sp. KE4 isolated from kitchen waste effluent. *Sustainable Chemistry and Pharmacy*, 39, 101507.
3. **Acharjee, S. A.**, Gogoi, B., Bharali, P., Sorhie, V., & Alemtoshi, B. W. (2024). Recent trends in the development of Polyhydroxyalkanoates (PHAs) based biocomposites by blending with different bio-based polymers. *Journal of Polymer Research*, 31(4), 98.
4. **Acharjee, S. A.**, Bharali, P., Ramachandran, D., Hazarika, S., Dutta, N., & Manivannan, J. (2025). Bio-fabrication of biodegradable polyhydroxybutyrate/polycaprolactone based sorbent: a net zero-waste approach for sustainable management of intricate hydrophobic pollutants. *International Journal of Biological Macromolecules*, 145825.
5. **Acharjee, S. A.**, Bharali, P., Ramachandran, D., Kanagasabai, V., Hazarika, S., & Vishwakarma, V. (2025). Bacterial cell factories for sustainable production of green polyesters using saccharides: unlocking the potential to mitigate microplastic pollution. *Journal of Polymer Research*, 32(7), 1-22.

b) Paper published as co-author

1. Gogoi, B., **Acharjee, S. A.**, Bharali, P., Sorhie, V., & Walling, B. (2024). A critical review on the ecotoxicity of heavy metal on multispecies in global context: A bibliometric analysis. *Environmental Research*, 248, 118280.

2. Kulnu, A. S., **Acharjee, S. A.**, Humtsoe, R. N., Kuotsu, R., Walling, B., & Sorhie, V. (2024). Ethnoecological insights on wild fodder bioresources and their geospatial perspectives on sustainable piggery in Wokha and Zunheboto districts of Nagaland, India. *Genetic Resources and Crop Evolution*, 71(2), 691-720.
3. Sorhie, V., Gogoi, B., Walling, B., **Acharjee, S. A.**, & Bharali, P. (2022). Role of micellar nanoreactors in organic chemistry: Green and synthetic surfactant review. *Sustainable Chemistry and Pharmacy*, 30, 100875.
4. Bharali, P., Gogoi, B., Sorhie, V., **Acharjee, S. A.**, Walling, B., Alemtoshi, & Shah, M. P. (2024). Autochthonous psychrophilic hydrocarbonoclastic bacteria and its ecological function in contaminated cold environments. *Bio-degradation*, 35(1), 1-46.
5. Debbarma, R., Kumari, S., **Acharjee, S. A.**, & Bharali, P. (2025). Investigating the anticancer properties of starter culture of Tripura: an in-silico study on non-small cell lung cancer. *In Silico Pharmacology*, 13(2), 64.
6. Walling, B., Bharali, P., Hazarika, S., Dutta, N., & **Acharjee, S. A.** (2023). In-situ biofabrication of bacterial nanocellulose (BNC)/graphene oxide (GO) nano-biocomposite and study of its cationic dyes adsorption properties. *International Journal of Biological Macromolecules*, 251, 126309.
7. Walling, B., Bharali, P., Ramachandran, D., **Acharjee, S. A.**, & Vishwakarma, V. (2024). Bacterial valorization of agricultural-waste into a nano-sized cellulosic matrix for mitigating emerging pharmaceutical pollutants: An eco-benign approach. *International Journal of Biological Macromolecules*, 277, 133684.
8. Walling, B., Borah, A., Hazarika, S., Bharali, P., **Acharjee, S. A.**, & Nath, P. D. (2025). Production of bacterial nanocellulose as green adsorbent matrix using distillery wastes for dye removal: a combined approach for waste management and pollution mitigation. *Biomass Conversion and Biorefinery*, 15(5), 7265-7281.

c) Book chapter published as first author

1. **Acharjee, S. A., & Bharali, P.** (2025). Microbial Biopolymer from Renewable Feedstock: A Sustainable Approach for the Production of Polyhydroxyalkanoates (PHAs). In *Advanced Green Technology for Environmental Sustainability and Circular Economy* (pp. 89-111). CRC Press. **(Taylor & Francis)**

d) Book chapter published as co-author

1. Gogoi, B., Bharali, P., Walling, B., **Acharjee, S. A.**, & Vishwakarma, V. (2025). An insight into the ecological toxicity triggered by anthropogenically driven heavy metal micropollutants on biotic community. *Development in Waste Water Treatment Research and Processes*, 615-632. **(Springer)**

e) Patent Granted

1. Patent Office, Government of India

Patent Title: Polyhydroxybutyrate (PHB)-based Sustainable Bioplastic derived from *Bacillus* sp. KE4 isolated from kitchen waste effluent

Patent no: 568503

Application no: 202431045242

Date of filing: 12/06/2024

Name of Inventor: Shiva Aley Acharjee, Pranjal Bharali, Bendangtula Walling, Bhagyudoy Gogoi, Viphezolite Sorhie, Alemtoshi

f) Patents Filed

1. Patent Application Publication: India

Application no: 202431045240 A

Date of filing: 12/06/2024

Publication date: 21/06/2024

Patent Title: Bacterial Nanocellulose Green Adsorbent matrix using Distillery wastes for Dye removal and Waste Management

Name of Inventor: Bendangtula Walling, Pranjal Bharali, **Shiva Aley Acharjee**, Bhagyudoy Gogoi, Alemtoshi, Viphezolite Sorhie

2. Patent Application Publication: India

Application no: 202431049557 A

Date of filing: 28/06/2024

Publication date: 05/07/2024

Patent Title: Bio-fabrication of Bacterial Nanocellulose (BNC)/ Graphene Oxide (GO) Nano- Biocomposite

Name of Inventor: Bendangtula Walling, Pranjal Bharali, **Shiva Aley Acharjee**, Bhagyudoy Gogoi, Alemtoshi, Viphrezolie Sorhie



PHA-Based Bioplastic: a Potential Alternative to Address Microplastic Pollution

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 Bhagyudoy Gogoi · Viphezol Sorhie ·
 Bendangtula Walling · Alemtoshi

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Abstract Petroleum-derived plastics are linked to a variety of growing environmental issues throughout their lifecycle, including emission of greenhouse gases, accumulation in terrestrial and marine habitats, pollution, among others. There has been a lot of attention over the last decade in industrial and research communities in developing and producing eco-friendly polymers to deal with the current environmental issues. Bioplastics preferably are a fast-developing family of polymeric substances that are frequently promoted as substitutes to petroleum-derived plastics. Polyhydroxyalkanoates (PHAs) have a number of appealing properties that make PHAs a feasible source material for bioplastics, either as a direct replacement of petroleum-derived plastics or as a blend with elements derived from natural origin, fabricated biodegradable polymers, and/or non-biodegradable polymers. Among the most promising PHAs, polyhydroxybutyrates (PHBs) are the most well-known and have a significant potential to replace traditional plastics. These biodegradable plastics decompose faster after decomposing into carbon dioxide, water, and inorganic chemicals. Bioplastics have been extensively utilized in several sectors such

as food-processing industry, medical, agriculture, automobile industry, etc. However, it is also associated with disadvantages like high cost, uneconomic feasibility, brittleness, and hydrophilic nature. A variety of tactics have been explored to improve the qualities of bioplastics, with the most prevalent being the development of gas and water barrier properties. The prime objective of this study is to review the current knowledge on PHAs and provide a brief introduction to PHAs, which have drawn attention as a possible potential alternative to conventional plastics due to their biological origin, biocompatibility, and biodegradability, thereby reducing the negative impact of microplastics in the environment. This review may help trigger further scientific interest to thoroughly research on PHAs as a sustainable option to greener bioplastics.

Keywords Microplastics · Bioplastics · Polyhydroxyalkanoates (PHAs) · Polyhydroxybutyrates (PHBs)

1 Introduction

Plastic products are incredibly versatile due to their low thermal and electric conductivity, lightweight, rigid, long-lasting and corrosion resistance, which enable them to serve as an oxygen and water barrier. Their low cost also contributes to their easy and widespread production, where they are utilized in

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Polyhydroxybutyrate (PHB)-Based sustainable bioplastic derived from *Bacillus* sp. KE4 isolated from kitchen waste effluent

Shiva Aley Acharjee^a, Pranjal Bharali^{a,*}, D. Ramachandran^b, Viswanathan Kanagasabai^b, Monti Gogoi^c, Swapnali Hazarika^c, Pranab Jyoti Koch^d, Nipu Dutta^e, Gobichettipalayam Balasubramaniam Maadurshni^f, Jeganathan Manivannan^f, Sony Kumari^d, Bendangtula Walling^a, Bhagyudoy Gogoi^a, Alemtoshi^a, Viphezoliz Sorhie^a, Vinita Vishwakarma^g

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ARTICLE INFO

Handling Editor: Fabio Aricò

Keywords:

Polyhydroxybutyrate (PHB)
Bacillus sp. KE4
Biopolymer
Biodegradable
Biocompatible

ABSTRACT

Petrochemical-derived plastics pose a major threat to the biosphere and the environment due to their acute or long-term toxicity. Therefore, to ensure sustainability, it is essential to find an effective replacement for synthetic plastics with natural and ecofriendly biomaterial. In this context, production of polyhydroxybutyrate (PHB) was produced by the *Bacillus* sp. KE4 using glucose as the carbon source, and a maximum dry cell weight (DCW) of 4.4 g/l with a PHB concentration of 3.1 g/l, PHB yield of 0.15 g/g was obtained, and the PHB content was 70.4% of the DCW. The extracted biopolymer was characterized using FTIR, GC-MS, ¹H NMR, and ¹³C NMR which confirmed the structure of the biopolymer as PHB. The findings of TGA, DTG, DTA and DSC studies corresponded to PHB's excellent thermal stability. PHB biopolymer has a melting temperature of 168.7 °C and a maximum degradation temperature of 273 °C. The weight average molar mass (Mw), number average molar mass (Mn), and polydispersity index (PDI) of the extracted PHB was 1,55,436 g mol⁻¹; 1,43,258 g mol⁻¹; and 1.08, respectively. The XRD analysis validated the material's partly crystalline nature. FESEM-EDS and XPS were employed to determine the elemental composition of the purified PHB film. The Young's modulus, tensile strength and elongation at break showed rigid and brittle nature of PHB. Using open windrow composting method, the extracted PHB biopolymer film was assessed to determine its biodegradability. Within 28 days of soil burial, it was found that the biopolymer film had degraded by 61.34%. Cytotoxicity assessment in HepG2 cells indicated that PHB is non-cytotoxic in nature. The results have showed that the biomaterial is environmentally benign, biodegradable, and biocompatible; therefore, it may find its diverse application covering packaging, medicine, agriculture and allied fields.

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Recent trends in the development of Polyhydroxyalkanoates (PHAs) based biocomposites by blending with different bio-based polymers

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Abstract

Currently, Polyhydroxyalkanoates (PHAs) especially Polyhydroxybutyrate (PHB) have gained much attention as an environmentally benign alternative to petroleum-derived plastics. However, path to a bright future of sustainable production and development has been hampered by their dubious quality. PHAs are biodegradable, biocompatible, and non-toxic which make them excellent choices for packaging, agriculture, and medicine. Despite having such great scope, PHAs have a number of disadvantages, including a high cost, brittleness, thermal instability, and poor mechanical properties. Thus, PHAs need to be upgraded to enhance its inherent qualities. This upgradation can be primarily achieved by blending PHAs either with natural or synthetic bio-based polymers and adding reinforcements to develop novel PHAs-based biocomposites and blends. Keeping the view of future PHAs multi-dimensional applications, the present study provides an extensive understanding of the blending of PHAs to enhance their physico-chemical traits. Additionally, a number of state-of-the-art processing methods are also discussed in the present study.

Keywords Polyhydroxyalkanoates (PHAs) · Polyhydroxybutyrate (PHB) · Blending · Biopolymer · Biocomposites

Introduction

Environmental issues have become insurmountable due to the unprecedented frequency of utilizing petroleum derived plastics. In the packaging sector, where the continued rise in the consumption of outdoor food has resulted in a huge increase in the manufacture and usage of packaging materials, these problems are noticeably more problematic. Additionally, the COVID-19 epidemic has hastened the disposal of plastics made from fossil fuels from the food and medical industries [1]. Due to the COVID-19 epidemic, there has been a huge surge in plastic pollution as a result of the influx of biomedical waste, the need for appropriate personal protective equipment (PPE), the increase in internet shopping and delivery of food, the ban on reusable bags, and many other factors [2]. Despite the efforts to stop the

endless cycle of plastic production and the harm it causes to the nature, it is predicted that every year more than 40 million tonnes of plastic enters the environment, 11 million tonnes of which ends up as macro- and microplastic waste in the ocean [3]. As a result, extensive research is being done on a global scale to develop more ecologically friendly and sustainable packaging materials that are made from bio-masses, microorganisms, and other renewable feedstocks. In this regard, biopolymers produced through bacterial fermentation, such as those in the Polyhydroxyalkanoates (PHAs) family, which includes poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) polymers, are viewed as prospective candidates for substituting the petroleum-derived polymeric materials and resolving the waste dumping issue [1, 4]. PHAs can be easily broken down both anaerobically and aerobically without the need of particular environmental conditions. Moreover, they are non-toxic and biocompatible, which makes them excellent choices for pharmaceutical and medical applications including drug delivery [5]. Despite having several benefits over synthetic polymers, their applications have been constrained by a lack of advanced features, poor physical and mechanical properties needed for commercial purposes, low

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Bio-fabrication of biodegradable polyhydroxybutyrate/polycaprolactone based sorbent: a net zero-waste approach for sustainable management of intricate hydrophobic pollutants

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ARTICLE INFO

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Porous sorbent
Hydrophobicity
Oil/water separation
Biodegradability
Zero-waste

ABSTRACT

Generation of secondary contaminants, insufficient sorption and recovery, and degradability issues associated with the existing solutions render the management of hydrophobic pollutants very challenging. Hereby, a cost-effective, energy-efficient, non-cytotoxic, biocompatible, and biodegradable sorbent is fabricated employing a blend of PHB and PCL biopolymers using a facile salt leaching technique. The sorbent demonstrated remarkable water repellence, achieving a saturated oil sorption capacity of 6.02 g/g for waste cooking oil (WCO) and 5.09 g/g for diesel oil, with recovery rates of 86.5 % for WCO and 85.7 % for diesel. Subsequent to the sorption of oils, the oil-saturated sorbents were further valorised through the production of biosurfactant utilizing the strain *Pseudomonas aeruginosa* AMS1a. The strain effectively produced biosurfactant with concentrations of 1040 mg/l and 546 mg/l utilizing WCO and diesel as carbon sources, respectively. Notably, 46.5 % of diesel-loaded sorbent and 23.72 % of WCO-loaded sorbent exhibited partial degradation during the valorisation process. The residual sorbents were buried in compost soil for further degradation, and were entirely decomposed within 28 days. The findings demonstrated an efficient biomaterial for addressing oil spill disasters while minimizing secondary contamination. Additionally, the sorbent also fulfilled the zero-waste notion of the Sustainable Development Goals (SDGs) defined by the United Nations.

1. Introduction

Pollution caused by hydrophobic pollutants has been rising recently due to the unrelenting expansion of agricultural and industrial operations, severely polluting different ecological systems. The primary sources of such pollutants are mostly associated with various anthropogenic and natural processes [1]. The two most common pollutants emitted by anthropogenic activities are polycyclic aromatic hydrocarbons (PAHs) and petroleum hydrocarbons. Additionally, Waste Cooking Oil (WCO), classified as a hydrocarbon compound, is among the most common pollutants released into sewer systems by human activities [2]. Refined petroleum hydrocarbons, like diesel oil, are produced by

distilling crude oil and consist of polycyclic aromatic hydrocarbons and low molecular weight alkanes. Diesel spills that occur during the usage and transportation of diesel fuel over rivers, lakes, and the ocean are among the causes of pollution from hydrocarbons in aquatic bodies. With regard to its hydrocarbon content and extensive use for industrial and transportation processes, diesel fuel is among the most potentially hazardous hydrocarbon pollutant and have the capacity to disturb ecological equilibrium and threatens the aquatic organisms [3].

WCO is a by-product produced locally when edible vegetable oils are used to cook food stuffs in homes, hotels, restaurants, and other catering establishments. WCO is predominantly produced and released into the environment, particularly in China and India, which makes it one of the

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Bacterial cell factories for sustainable production of green polyesters using saccharides: unlocking the potential to mitigate microplastic pollution

Shiva Aley Acharjee¹ · Pranjal Bharali¹ · D. Ramachandran² · Viswanathan Kanagasabai² · Swapnali Hazarika³ · Pranab Jyoti Koch⁴ · Nipu Dutta⁵ · Gobichettipalayam Balasubramaniam Maadurshni⁶ · Jeganathan Manivannan⁶ · Sony Kumari⁸ · Bhagyudoy Gogoi¹ · Bendangtula Walling¹ · Viphezolie Sorhie¹ · Alemtoshi¹ · Vinita Vishwakarma⁷

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Abstract

Microplastics, an upshot of petroleum-based synthetic polymers, have emerged as a global contaminant due to their persistence and accumulation in marine and terrestrial ecosystems. Polyhydroxybutyrate (PHB), a microbial-derived polyester, is an effective and sustainable alternative for synthetic polymers due to its pro-environmental qualities. In the present study, *Bacillus subtilis* FW1, isolated from a fish waste disposal site in Mokochung, Nagaland, India, was able to accumulate 69.2% of PHB as cell dry weight of 2.31 gm/L and yielded a PHB concentration of 1.6 gm/L with a molecular weight 1.9×10^5 g/mol when 20% (w/v) monosaccharides especially glucose was used as the most favorable carbon substrate. The extracted PHB exhibited exceptional thermal stability. The melting temperature of the extracted PHB is 165 °C, whereas its maximum degradation temperature is 280 °C. Carbon (C) and oxygen (O) were found to be the main elemental compositions of the extracted PHB, according to XPS and FESEM-EDS studies. After 28 days of soil burial using the open windrow composting method, it was found that the PHB film had degraded by 59.6%. Evaluating the cytotoxicity of the extracted PHB in HepG2 cells revealed its non-cytotoxic nature. The results suggest that *B. subtilis* FW1 is a viable option for PHB biopolyester synthesis in the industrial setting. Furthermore, it may effectively combat the prevalent issue of microplastic pollution while gaining wide uses in the packaging, biomedical, agricultural, and other related sectors because of its biodegradability, biocompatibility, and eco-benign nature.

Keywords Microplastic · Polyhydroxybutyrate (PHB) · Biopolyester · Non-cytotoxic · Biodegradable

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6 Microbial Biopolymer from Renewable Feedstock

A Sustainable Approach for the Production of Polyhydroxyalkanoates (PHAs)

Shiva Aley Acharjee and Pranjal Bharali

6.1 INTRODUCTION

For the past 200 years, the world has observed the consumption of derivatives of fossil fuels and their impacts. This widespread use of petrochemical-derived polymers is responsible for global ecological imbalance in a number of areas, including the stability of the Earth's atmosphere, terrestrial and aquatic life, food chains, and the plant kingdom (de Paula et al. 2018). These synthetic polymers are exceedingly tenacious in nature, and an alarming buildup of such plastics has created issues with safe disposal, recycling, trash landfilling, bio- or photo-degradation, and incineration. Consequently, there is a pressing need for biodegradable polymers right now (Pradhan et al. 2020). Researchers throughout the world are more encouraged than ever to look for innovative alternatives as a result of greater awareness of the adverse impacts of synthetic plastics on humans and the environment. A replacement for synthetic plastics with biodegradable polymers made by microorganisms, particularly bacteria, has been considered as a means of reducing environmental pollution (Krishnan et al. 2021). Biodegradable "green plastics" like polyhydroxyalkanoates (PHAs) provide bioeconomy notions, curtail emission of greenhouse gases (GHGs) (by about 200%), use fewer fossil fuel energy (by about 95%), and produce less toxic waste, according to recent life cycle assessment studies and environmental footprint tools (Dietrich et al. 2017). PHAs are therefore regarded as a better alternative to polymers made from petroleum (Andler et al. 2021). PHAs are a class of naturally occurring biopolyesters made of hydroxy fatty acids produced by various microorganisms. These PHAs are grouped into three categories based on the quantity of carbon atoms they contain: short-chain (C3–C5), medium-chain (C6–C14), and long-chain

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पत्र सं. SL No. 033138803




पेटेंट कार्यालय, भारत सरकार **The Patent Office, Government Of India**

पेटेंट प्रमाण पत्र | Patent Certificate
(पेटेंट विधिव्यवस्था की नियम 74) (Rule 74 of The Patents Rules)

पेटेंट सं. / Patent No.	: 568503
आवेदन सं. / Application No.	: 202431045242
घाड़ल करने की तारीख / Date of Filing	: 12/06/2024
पेटेंटी / Patentee	: Nagaland University
आविष्कारकों का नाम / Name of Inventor(s)	: 1. Shiva Aley Acharjee 2. Pranjal Bharali 3. Bendangtula Walling 4. Bhagyudoy Gogoi 5. Viphrezolie Sorhie 6. Alemtoshi

प्रमाणित किया जाता है कि पेटेंटी को, उपरोक्त आवेदन में बयांनकृत *Polyhydroxybutyrate (PHB)-based Sustainable Bioplastic derived from Bacillus sp. KE4 isolated from kitchen waste effluent* नामक आविष्कार के लिए, पेटेंट अधिनियम, 1970 के उपबंधों के अनुसार आज तारीख जून 2024 के बारहवें दिन से बीस वर्ष की अवधि के लिए पेटेंट अनुदान किया गया है।

It is hereby certified that a patent has been granted to the patentee for an invention entitled *Polyhydroxybutyrate (PHB)-based Sustainable Bioplastic derived from Bacillus sp. KE4 isolated from kitchen waste effluent* as disclosed in the above mentioned application for the term of 20 years from the 12th day of June 2024 in accordance with the provisions of the Patents Act, 1970.




अनुदान की तारीख : 08/07/2025
Date of Grant :

टिप्पणी - इस पेटेंट के नवीकरण के लिए फीस, यदि इसे बनाए रखा जाता है, जून 2026 के बारहवें दिन को और उसके परवर्तन उसके वर्ष के उन्नीसवें दिन तक होगी।

Note - The fees for renewal of this patent, if it is to be maintained, will fall / has fallen due on 12th day of June 2026 and on the same day in every year thereafter.

(12) PATENT APPLICATION PUBLICATION		(21) Application No.202431045240 A
(19) INDIA		
(22) Date of filing of Application :12/06/2024		(43) Publication Date : 21/06/2024
(54) Title of the invention : Bacterial Nanocellulose Green Adsorbent Matrix using Distillery Wastes for Dye Removal and Waste Management		
(51) International classification :C02F1/28, C12P19/04, B01J20/24, B01J20/30 (86) International Application No :NA Filing Date :NA (87) International Publication No : NA (61) Patent of Addition to Application Number :NA Filing Date :NA (62) Divisional to Application Number :NA Filing Date :NA		(71)Name of Applicant : 1)Nagaland University Address of Applicant :Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ***** Name of Applicant : NA Address of Applicant : NA (72)Name of Inventor : 1)Bendangtula Walling Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ***** 2)Pranjal Bharali Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ***** 3)Shiva Aley Acharjee Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ***** 4)Bhagyudoy Gogoi Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ***** 5)Alemtoshi Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ***** 6)Viphrzeolie Sorhie Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto *****
(57) Abstract : The invention is usage of rice beer stillage as a carbon source to produce bacterial nanocellulose (BNC). A method of production of Bacterial Nanocellulose (BNC) comprising the steps of: preparation of carbon source from rice beer stillage; and production and purification of BNC. The BNC made using rice beer stillage has excellent thermostability (up to 380 °C), a dense nano-fibrous network with cellulose I type properties, and a high crystallinity index (86.23%). It demonstrated a high water-holding capacity (97.53 g g ⁻¹) and a robust tensile strength (68.044 MPa). BNC also demonstrated its recyclable nature by going through several cycles of sorption and desorption. According to this invention, waste distillery stillage may be efficiently used as a sustainable raw material to produce BNC that is safe for the environment and has a wide range of uses.		
No. of Pages : 25 No. of Claims : 7		

(12) PATENT APPLICATION PUBLICATION	(21) Application No.202431049557 A
(19) INDIA	
(22) Date of filing of Application :28/06/2024	(43) Publication Date : 05/07/2024
(54) Title of the invention : Bio-fabrication of Bacterial Nano-Cellulose (BNC) /Graphene Oxide (GO) Nano-Biocomposite	
(51) International classification (86) International Application No Filing Date (87) International Publication No (61) Patent of Addition to Application Number Filing Date (62) Divisional to Application Number Filing Date	(71)Name of Applicant : 1)Nagaland University Address of Applicant :Nagaland University, Lumami Headquarters, Zunheboto district, Nagaland Zunheboto ----- Name of Applicant : NA Address of Applicant : NA (72)Name of Inventor : 1)Bendangtula Walling Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ----- 2)Pranjal Bharali Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ----- 3)Shiva Aley Acharjee Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ----- 4)Bhagyudoy Gogoi Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ----- 5)Alemtoshi Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ----- 6)Viphezolie Sorhie Address of Applicant :Applied Environmental Microbial Biotechnology Laboratory, Department of Environmental Science, Nagaland University, Lumami, Zunheboto-798627, Nagaland, India Zunheboto ----- (57) Abstract : In this invention, a time-dependent technique is used to synthesize Bacterial Nanocellulose/Graphene Oxide Nano-Biocomposites (BNC-GO-NBCs) by Komagataeibacter saccharivorans NUWB1. The dried BNC-GO-NBC exhibited great tensile strength (84.72 MPa), enhanced thermostability (up to 380°C), high crystallinity (74.21%), and a porous structure with embedded GO layers, according to physicochemical examination. A mesoporous structure with a significant adsorption capacity for cationic dyes is shown by the N₂ adsorption-desorption isotherm. Additionally, the substance demonstrated the ability to regenerate and shown non-cytotoxic qualities in tests for oxidative stress, cell cytotoxicity, and antibacterial activity. These results point to BNC-GO-NBC's intriguing potential for environmental applications, including dye adsorption. No. of Pages : 20 No. of Claims : 7

APPENDIX-IV

CONFERENCES/WORKSHOPS/SEMINARS

Lists of Conferences/Seminars/Webinars/Workshop Attended

1. Presented paper in the International Conference on Bioresources & Bioeconomy (ICBB-2022) held on 19-21 September, 2022 at Department of Botany, Nagaland University, India.
2. Presented paper in the International Conference on Challenges & Prospects of Bioresource Conservation in Eastern Himalaya- with special reference to UN-Sustainable Development Goals held on 3-4 May, 2023 at Department of Zoology, Gauhati University, India.
3. Presented paper in ICSSR- New Delhi sponsored National Seminar on “Anthropology in the 21st Century: Emergency areas of Research in Northeast India and its role in National Development” on 19th and 20th March, 2024, organized by Department of Anthropology, Nagaland University.
4. Participated in National Level workshop and Staff Development Program on Role of Molecular Docking and ADME in Drug Discovery using SeeSAR and StarDrop Organized by Royal School of Pharmacy (RSP), Royal Global University, Guwahati from 10th – 11th December, 2021.
5. Completed One Week online course on Remote Sensing and GIS Applications in Forestry and Ecology Organized by North Eastern Space Application Centre (NESAC) from 1st- 5th February, 2021.
6. Participated in Webinar on Covid 19: Necessary Reflections on the Disaster Management Act, 2005 Organized by Legal Cell, Nagaland University on 17th May, 2021.
7. Participated in One-Day Workshop on “Quality Improvement Parameters in Research Publication” organized by the department of Chemistry Department, Nagaland University, India.
8. Participated in Science Academies Lecture Workshop on “Traditional Knowledge, Medicinal Plants and Biodiversity Conservation” organized by the Department of Forestry, Nagaland University on 13th and 14th March 2024 sponsored by INSA, IASc and NASI.

9. Participated in the DST-SERB High-End Workshop on “Exposure to a Galaxy of Spectroscopic Techniques in Physics, Chemistry and Biological Sciences” organized by the Department of Physics, Nagaland University during March 20-26, 2024.
10. Participated in the “One-Day workshop on Role of Animals in Research” Organized by Department of Zoology, Nagaland University, India on 30th September, 2024.
11. Participated in Symposium on “Innovation and Entrepreneurship in Environmental Science for Sustainable Future” on 18th March, 2025, Organized by Department of Environmental Science, Nagaland University, India.
12. Participated in the Webinar on “AI Applications in Academia”, organized by the Department of Environmental Science, Nagaland University on the 25th April, 2025.
13. Completed One Week (Online) Faculty Development Programme on “Environmental Pollution and abatement” (04th - 09th September, 2023) organized by Nagaland University in collaboration with Mahatma Hansraj Faculty Development Centre Hansraj College, University of Delhi.