

**BIOSYNTHESIS AND CHARACTERIZATION OF
POLY(3-HYDROXYBUTYRATE-*co*-3-
HYDROXYVALERATE) FROM *Cupriavidus
malaysiensis* USMAA1020
USING GLYCERIN PITCH AS MAIN CARBON
SOURCE**

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UNIVERSITI SAINS MALAYSIA

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by

ROZINA

**Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science**

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This thesis is dedicated to the memory of my beloved brother-in-law Mr. Zia ul haq kakar, who has left this temporary world while battling with Covid 19.

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LIST OF SYMBOLS

%	Percentage
β	Beta
$^{\circ}\text{C}$	Degree Celsius
ΔH_m	Heat of fusion
μm	Micrometre
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxybutyryl Coenzyme A
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
C	Carbon atom
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	Calcium chloride dihydrate
CO_2	Carbon dioxide
CoA	Coenzyme A
$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	Cobalt (II) sulphate heptahydrate
$\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$	Chromium chloride hexahydrate
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	Copper (II) chloride dihydrate
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Iron (II) sulphate heptahydrate
g	Gram
H	Hour
H	Hydrogen atom
H_2O	Water
H_2SO_4	Sulphuric acid
J/g	Joule per gram
kg	Kilogram
K_2HPO_4	Dipotassium hydrogen phosphate

KH_2PO_4	Potassium dihydrogen phosphate
L	Litre
M	Molar
mcl	Medium chain length
min	Minute
mg	Milligram
Mg	Magnesium atom
mg/L	Milligram per liter
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	Manganese (II) chloride tetrahydrate
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Magnesium sulphate heptahydrate
mL	Millilitre
mm	Millimetre
mM	Millimolar
mol%	Mole percent
MPa	Megapascal
N	Newton
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
$(\text{NH}_4)_2\text{SO}_4$	Ammonium sulphate
nm	Nanometer
NR	Nutrient rich
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB- <i>co</i> -4HB)	Poly(3-hydroxybutyrate- <i>co</i> -4-hydroxybutyrate)
P(3HB- <i>co</i> -3HV)	Poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyvalerate)
PHA	Polyhydroxyalkanoate
PhaA; <i>phaA</i>	β -ketothiolase; gene encoding β -ketothiolase

PhaB; <i>phaB</i>	NADPH-dependent acetoacetyl-CoA dehydrogenase; gene encoding NADPH-dependent acetoacetyl-CoA dehydrogenase
PhaC; <i>phaC</i>	PHA synthase; gene encoding PHA synthase
RCDW	Residual cell dry weight
rcf	Rotation centrifugational force
rpm	Rotation per minute
scl	Short chain length
sd	Standard deviation
sp.	Species
<i>T_c</i>	Crystallization temperature
<i>T_g</i>	Glass transition temperature
<i>T_m</i>	Melting temperature
v/v	Volume per volume
wt%	Weight percent
w/v	Weight per volume
w/w	Weight per weight
ZnSO ₄ .7H ₂ O	Zinc sulphate heptahydrate

LIST OF ABBREVIATIONS

CDW	Cell dry weight
CME	Caprylic methyl ester
DSC	Differential scanning calorimetry
GC	Gas chromatography
ICI	Imperial Chemical Industries
IS	Internal standard
MSM	Mineral salt medium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
TEM	Transmission electron microscope
UV	Ultraviolet
NMR	Nuclear Magnetic Resonance

BIOSINTESIS DAN PENCIRIAN POLI(3-HIDROKSIBUTIRAT-*ko*-3-HIDROKSIVALERAT) DARIPADA *Cupriavidus malaysiensis* USMAA1020 MENGGUNAKAN SISA GLISERIN SEBAGAI SUMBER KARBON UTAMA

ABSTRAK

Plastik digunakan hampir di setiap genap kehidupan moden, dari pembuatan kapal terbang, kereta, bangunan, pakaian, kasut, bungkusan makanan, sehinggalah alat-alat perubatan. Kesan penggunaan plastik secara meluas telah menyebabkan banyak lambakan sisa buangan di peringkat global yang diketahui memberi kesan kepada ekosistem. Ini kerana plastik sintetik/ konvensional mengambil masa bertahun-tahun untuk terurai secara semula jadi. Pembakaran plastik juga menghasilkan bahan toksik dan meningkatkan CO₂ di dalam atmosfera, sekaligus menyebabkan pemanasan global. Disebabkan itu, usaha mencari alternatif kepada polimer yang berasaskan petrol sering dipergiatkan. Polihidroksialkanoat (PHA) telah disarankan sebagai pengganti bagi polimer yang berasaskan petrokimia kerana sifatnya yang boleh terbiodegradasi. Dalam kajian ini, *Cupriavidus malaysiensis* USMAA1020, sejenis bakteria Gram-negatif didapati mampu menghasilkan polimer terbiodegradasi P(3HB-*ko*-3HV) dengan menggunakan sisa gliserin. Sisa gliserin merupakan hasil sampingan daripada sintesis biodiesel yang diperolehi semasa proses transesterifikasi sebagai bahan mentah yang berkos rendah. Objektif kajian ini adalah untuk mengkaji beberapa parameter dengan kepekatan sisa gliserin (5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L), 1-pentanol (0.03 wt% C, 0.06 wt% C, 0.09 wt% C, 0.12 wt% C), ammonium sulfat (0.8 g/L, 1.1 g/L, 1.4 g/L, 1.7 g/L, 2.0 g/L), dan beberapa prekursor yang lain seperti asid nonaoik, asid valerik, asid propionik, seiring dengan kepekatan 1-pentanol bermula dengan 0.06 wt% C. Bagi meningkatkan penghasilan PHA, beberapa

kepekatan asid oleik telah digunakan sebagai sumber karbon sekunder. Kromatografi gas telah digunakan bagi mengesan kehadiran monomer 3HB dan 3HV. Keseluruhan jumlah kandungan PHA yang telah dikesan ialah 65%, 3HB (95 mol%), 3HV (4 mol%), berat sisa sel kering (4 g/l), dan PHA berkepekatan (6 g/l) dengan pelbagai campuran 2% sisa gliserin, 1.1 g/l ammonium sulfat, 1-pentanol 0.06 wt% C dan oleik 0.5 wt% C. Untuk pengoptimuman, metodologi tindak balas permukaan (RSM) telah diperkenalkan bagi menentukan kepekatan substrat yang optimum bagi menghasilkan PHA secara efektif. Kepekatan PHA didapati telah meningkat kepada 77% dari (65%) awalnya dan kepekatan PHA dari 8g/l dari (4 g/l). Monomer 3HV telah meningkat kepada 17.8 mol% dengan pertumbuhan 5.5 g/l. Dengan menggunakan pelbagai parameter, tiga kombinasi HV yang berbeza telah diperolehi iaitu, P(HB-ko-4%HV), P(HB-ko-10%HV), P(HB-ko-17%HV). Kopolimer yang mengandungi beberapa kombinasi unit monomer yang berbeza telah dicirikan melalui kalorimeter pengimbasan pembezaan (DSC), ujian tegangan, NMR dan TEM. TEM telah disahkan dengan kehadiran beberapa granula PHA. Secara amnya, kopolimer yang telah dihasilkan menunjukkan fleksibiliti (Young's modulus 162 MPa) dan pemanjangan semasa putus (9.6%). Apabila kandungan HV meningkat, suhu lebur kopolimer telah menurun daripada 160.39°C kepada 157.62°C. Hasil daripada analisis NMR, nilai yang telah diperolehi ialah 4.5, 5.5, dan 7.2. Kesemua ini mempunyai nilai yang lebih besar daripada nilai D (1) yang menyarankan bahawa kopolimer ini berbentuk blok, atau kopolimer bercampur. Ciri sisa buangan gliserin bersama produk sampingan 1-pentanol, prekursor kepada kopolimer P(3HB-ko-3HV) menunjukkan potensi sebagai plastik terbiodegenerasi.

**BIOSYNTHESIS AND CHARACTERIZATION OF POLY(3-
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USING GLYCERIN PITCH AS MAIN CARBON SOURCE**

ABSTRACT

Plastics are used in almost every part of modern life, from making aeroplanes, cars, and buildings to making clothes, shoes, packaging for food and drinks, and medical devices. As a result, plastic waste is gathered globally, worsening the effects on the ecosystem. Synthetic plastics/conventional plastics takes many years to disintegrate in nature, and incineration produces toxic substances and raises CO₂ levels in the atmosphere, causing issues like global warming. As a result, alternatives to petrol-based polymers are being sought. Polyhydroxyalkanoates (PHAs) have been proposed as a feasible substitute for petrochemical-based polymers because of their biodegradable nature. In the present study, *Cupriavidus malaysiensis* USMAA1020, a Gram-negative bacterium was able to produce biodegradable polymer P(3HB-co-3HV) by utilizing glycerin pitch which is a by-product of the biodiesel synthesis obtained during the transesterification as low-cost feedstock. The aim of the research was to screen various concentrations of glycerin pitch (5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L), 1-pentanol (0.03 wt% C, 0.06 wt% C, 0.09 wt% C, 0.12 wt% C), ammonium sulfate (0.8 g/L, 1.1 g/L, 1.4 g/L, 1.7 g/L, 2.0 g/L), and different precursors nonaolic acid, valeric acid, propionic acid, with respect to 1-pentanol at concentration of 0.06 wt% C. To enhance the production of PHA, oleic acid concentrations was used as a secondary carbon source. Gas chromatography was used to confirm the presence of 3HB and 3HV. The overall PHA content during preliminary synthesis was found to be

65%, 3HB (95 mol%), 3HV (4 mol%), residual cell dry weight (4 g/l), and PHA concentration (6 g/l) at 2% glycerin pitch, 1.1 g/l ammonium sulphate, 1-pentanol 0.06 wt% C and oleic acid 0.5 wt% C respectively. For optimization the response surface methodology (RSM) has been introduced which is the standard statistical and mathematical approach used to create an experimental design, analyse the interaction impact between factors, and determine the optimum conditions for variables to produce effective response in different biochemical processes. The PHA content increased to 77% from (65%) earlier and PHA concentration to 8g/l from (6 g/l). The 3HV monomer increased to 17.8 mol% from (4 mol%) with the growth 5.5 g/l from (4 g/l). Through various parameters the three combinations of different HV were obtained i.e. P(3HB-co-4%3HV), P(3HB-co-10%3HV), P(3HB-co-17%3HV). The copolymer containing different combinations of monomer units were characterized through differential scanning calorimeter (DSC), tensile test, NMR and TEM. Transmission electron microscopy is important to visualise the PHA granules inside the bacteria cytoplasm, hence the TEM images confirmed the presence of PHA granules. In general, the copolymer produced exhibited flexibility (Young's modulus 162 MPa) and elongation at break (9.6 %). As the HV content increased, the melting temperature of copolymers was decreased from 160.39°C to 157.62°C. In NMR analysis the values obtained were 5.5, 7.2, and 4.5 which are larger than the D value (1) suggested that may be the copolymers are blocky, or blends copolymers. This suggests that the glycerin pitch the by-product along with 1-pentanol the precursor are potential sources to produce copolymer P(3HB-co-3HV) which can offer variable properties as biodegradable plastic.

CHAPTER 1 INTRODUCTION

The substantial quantities and accumulation of plastics causing serious ecological air pollution due to landfills in natural environments over the last couple of years. The persistent and non-biodegradable nature of plastics, which ends up being one of the factors exacerbating the environmental impacts. The industrial world is now heavily reliant on fossil fuels for industries and the manufacturing of structural materials like foams, plastics, adhesives, and coatings. However, fossil fuels are a limited resource that has become a worldwide issue as our economy becomes increasingly dependent on petrochemical based plastics. To address these issues, there has been a significant development and a production of an eco-friendly polymer. Polyhydroxyalkanoate are biological macromolecules that come from a group of polyester, composed of units of R-3-hydroxy alkanolic acid monomers. The molecular weight of PHA varies from 2×10^5 to 3×10^6 Dalton which is comparable to traditional polyethylene. PHA and its copolymers as one of the most appealing biological macromolecules, which can be utilized as bioplastic because of its biodegradability and thermophysical properties. Due to its biocompatible nature, it has amazing applications in lots of areas biomedical applications such as drug delivery, tissue engineering, bio implantation, as well as food packaging, plastic utensils, manufacturing of bottles, fibbers. PHA and its copolymers have been found to be produced by several bacteria under nutritional stress (Ojha & Das, 2018; Ramachandran et al., 2011; Verlinden et al., 2007; Pernicova et al., 2019).

PHAs have gotten a lot of attention in recent years for their potential use in the production of tissue-engineering biomaterials since they feature properties like biocompatibility, biodegradability, that aren't found in existing synthetic polymers.

Poly(3-hydroxybutyrate), or PHB, is one of the PHAs that has been studied the most since D,L-b-hydroxybutyrate is molecule naturally present as ketone bodies in blood and tissue (Volova et al., 2021).

When an odd number of carbon substrates are utilised in the culture medium, P(3HB-*co*-3HV) is biosynthesized (Das et al., 2018). As valeric acid is introduced to a glucose-containing growth medium, a copolymer consisting of 3HB and 3HV is formed (Akaraonye et al., 2010). It was preferred to employ 1-pentanol as a 3HV precursor primarily to avoid some extent of inhibition in cell growth caused by other organic acids like valeric acid and propanol etc (Salim et al., 2011).

Glycerol or 1,2,3 propanetriol, often known as glycerin, is the main by-product of the transesterification of animal fats and vegetable oils. The huge rise of the biodiesel sector has created a glycerol surplus on the market, as a by-product of biodiesel the chemical is manufactured continuously (Chilakamarry et al., 2021). The glycerine pitch is a viscous, grey, or medium brown liquid that is typically very alkaline. The description and composition of glycerol pitch have been determined largely by the processing plant. Fatty acids, glycerol, diglycerol, and inorganic salts are commonly found in pitch (Teoh et al., 2021). In the production of biodiesel, the exact value of the glycerol residual is defined by the transesterification process and the separation phase. Glycerol levels generally range from 30 to 60% by weight. Extensive studies have been done exploring various inventions for glycerol to reduce a market surplus of this product and to exploit the profitability of production of biodiesel. Glycerol has the capability to be introduced as a medium for microbial growth and the synthesis of microbial products in industrial fermentation (Kakar et al., 2021).

Optimization of PHA production using response surface methodology (RSM) has been studied when it comes to bacterial species. The primary advantage of using

RSM is to decrease the variety of speculative tests which are called for to compute the number of variables and their interactions (Ojha & Das, 2018).

In this study glycerine pitch the by-product of biodiesel was used as a carbon source to produce biodegradable copolymer P(3HB-*co*-3HV) from *Cupriavidus malaysiensis* USMAA1020. Preliminary synthesis was done for different concentration of glycerin pitch, 1-pentanol along with other precursors, oleic acid, ammonium sulphate. Through shake flask fermentation optimized method of RSM was used to improve the copolymer P(3HB-*co*-3HV) productivity. Three different compositions of the copolymer P(3HB-*co*-3HV) produced from the use of various concentrations of glycerin pitch and 1-pentanol were characterized through physical, mechanical, and thermal properties on the basis of their HV content.

1.1 Problem Statement

According to the Watch World Institute, the global level of petroleum-based polymers has increased over the last five decades, resulting in a gross value of 299 tons of trash that requires effective disposal techniques. However, plastics made mostly from petroleum extracts are rarely biodegradable, resulting in trash pyramids that cause severe environmental damage. The build-up of petroleum-based plastics in the environment, particularly in the marine environment, poses the greatest threat to the ecosystem's vitality (Folino et al., 2020). International organizations, scientists, and government authorities have identified several strategies for successfully managing plastic garbage. Governmental incentives supported recycling, unauthorized dumping, and litter control as one of their management tactics. Although this method has substantially decreased the environmental effect of these plastic wastes, particularly in Europe, the practice is not viable in emerging economies such as Africa, where recycling is done little or not at all. There is a need for a strategy that can be

used across all industries, economies, and societies to tackle the environmental challenges like global warming, different kind of pollution (Teoh et al., 2021). Producing biodegradable polymers that are not only ecologically benign but also cost-effective when compared to traditional plastics is one approach. The world's energy use is steadily increasing, with manufacturers accounting for half of all worldwide energy consumption (Akinmulewo & Nwinyi, 2019; Merapan, 2015).

PHA manufacturers face challenges of reducing their production costs to remain competitive with petrochemical plastics. PHA research that involves the utilisation of industrial wastes and by-products (such as waste oil, molasses, whey, crude glycerol, sweetwater, and glycerine pitch) was significantly promoted because the carbon source alone reported for half of the entire PHA manufacturing expenses (Jiang et al., 2016). PHA's high manufacturing costs couldn't compete with mass-produced petrochemical plastics, therefore it was replaced with more inexpensive feedstocks including waste material, glycerol, glycerine pitch, and cellulose. The use of biodiesel by-products (such as glycerine pitch and glycerol) in PHA manufacturing can help Convert the waste from the biodiesel industry to value added product such as PHA. At the same time, PHA can be a good substitute to the synthetic plastics (Cavalheiro et al., 2012).

1.2 Objectives

- i. To synthesize and optimize the production of copolymer P(3HB-*co*-3HV) through shake flask fermentation from *Cupriavidus malaysiensis* USMAA1020 using glycerine pitch.
- ii. To characterize the copolymer P(3HB-*co*-3HV) produced.

CHAPTER 2 LITERATURE REVIEW

2.1 Biodegradable Plastics

Biodegradable polymers are classified into two types based on their origin: natural and synthetic. Polymers of synthetic origin have several advantages over their natural counterparts, including greater adaptability in terms of possible uses, the capacity to modify mechanical properties and degradation rates, and greater control over the degradation process. However, even though natural polymers have high biocompatibility, they have not been thoroughly studied due to their unfavourable characteristics, such as antigenicity and batch-to-batch fluctuation (Doppalapudi et al., 2014). Table 2.1 represents the different types of biodegradable polymer with their sources.

Many countries are now incorporating various initiatives for the monitoring of waste products. Involving plastic waste reduction by means of the production of naturally degradable plastics. As a result of the expanding problem about the damaging results of plastic components extracted coming from petrochemicals on the natural environment. Such biodegradable plastic products need to keep the same component homes of typical artificial plastics and ought to be totally broken down when thrown away without releasing any kind of toxins to the environment (Joseph et al., 2022). But another problem developing checking out that biodegradation with suited techniques. For an effortless way of life plastic component have vast applications for each nearby and manufacturing region. In reaction to these plastics produced from fossil fuels have major effects on human well-being, and also on our environment too and likewise for they are basically unaffected by microbial degradation (Raza et al., 2018; Rodriguez-Perez et al., 2018).

Table 2.1 Different types of biodegradable polymers and their sources.

Synthetic polymers	Sources	Properties	References
Polyesters	Synthesized by ring opening or condensation polymerization depending on the starting monomer units.	biocompatibility and tunable degradation properties.	(Doppalapudi et al., 2014)
Polyanhydrides	Diacids are the most prevalent monomers for acetic anhydride-activated polyanhydride condensation.	low hydrolytic stability	(Bien-aimé & Uhrich, 2020)
Polyurethanes	Synthesized by using three monomers like a diisocyanate, a diol or diamine chain extender and a long-chain diol.	biocompatible, biological performances,	(González-García et al., 2018)
Polyphosphazenes	Poly (dichlorophosphazene) was the first successfully synthesized polyphosphazene which has been used as intermediate for synthesis of different polyphosphazenes by using substitution reaction.		(Lakshmi et al., 2003)
Poly alkyl cyano acrylates	Knoevenagel condensation reaction is used in the synthesis of poly alkyl cyano acrylates (PACAs).	biocompatible, biodegradable and low toxic	(Nicolas & Couvreur, 2009)
Poly (amino acids)	Amino acid-derived polymers were obtained by grafting amino acids on synthetic polymers, copolymerization of amino acids with other monomers, derivation of block copolymers with amino acid sequences and PEG, and by		(Doppalapudi et al., 2014)

	development of pseudo poly (amino acids).		
Natural polymers	(Polysaccharides), (Proteins)		(Doppalapudi et al., 2014)
Polymers of microbial origin (poly hydroxy alkanoates)	Biopolymers synthesized by many bacteria and act as carbon and energy storage granules.	Biocompatible, biodegradable.	(Mathuriya & Yakhmi, 2019)

Because of the cost of production and product structures, replacing a common plastic with biodegradable polymers is still exceedingly difficult. To address these concerns, researchers have been looking for suitable alternatives that have similar material functions but are less expensive to produce. Global warming and solid waste management are currently the most important problems that pose a significant threat to our world; polyhydroxyalkanoate (PHA) is the most significant contributor to both issues. It is synthesized through microbial fermentation and possesses features that are like photochemical polymers. PHA's production cost has been reduced by several efforts to improve fermentation and recovery methods through the isolation of better microbial strains, which could be produce from naturally occurring bacteria such as several forms of photosynthetic bacteria, *Cupriavidus*, *Bacillus*, *Alcaligenes*, *Rhodococcus*, *Pseudomonas* when nutrients are insufficient for their development (Lee et al., 1999; Sangkharak & Prasertsan, 2012; Shantini et al., 2013).

Biobased plastics but on the other hand, employ biomass as a raw material instead of oil, and their structure, chemical composition and biodegraded environment are all elements that impact their biodegradability. Biodegradability is a criterion that certain biobased polymers meet, while others may not. Polyhydroxyalkanoates (PHA) and polyhydroxybutyrate (PHB) are produced by microbial fermentation. Synthetic biopolymers made from biomass poly (lactic acid) (PLA) or petrochemicals (such as

poly (glycolic acid) (PGA), poly (butylene succinate-co-adipate) (PBSA)) polycaprolactone (PCL), and along with those made directly from biomass are shown in figure 2.1 (proteins and polysaccharides). Researchers are constantly on the lookout for new species that may produce large amounts of PHA. Identification and genetic modification are playing a large part in the bioplastics' industrialization of microorganisms that are naturally present and can produce polymers after being isolated. Bio polymers are major pollutant wastes in the industrial and agricultural sectors, even though they are abundant in nature. They are biodegradable, biocompatible, and cost-effective alternative materials produced from natural resources including proteins, starch, cellulose, and a number of other polysaccharides (Bayón et al., 2018; Koshti et al., 2018; Rujnić-Sokele & Pilipović, 2017; Sangkharak & Prasertsan, 2012; Zhong et al., 2020).

Bioplastics are produced at a rate of about 750,000 tons per year, compared to 200 million tons for synthetic plastics. Approximately 280 million tons of petrochemical-based polymers were produced in 2011, with 4% a projected annual growth until 2016. By 2050 synthetic polymer output is expected to increase to over 810 million tons. There is rising interest in developing biological alternatives to petrochemical-derived polymers (Gironi & Piemonte, 2011; Gumel et al., 2013).

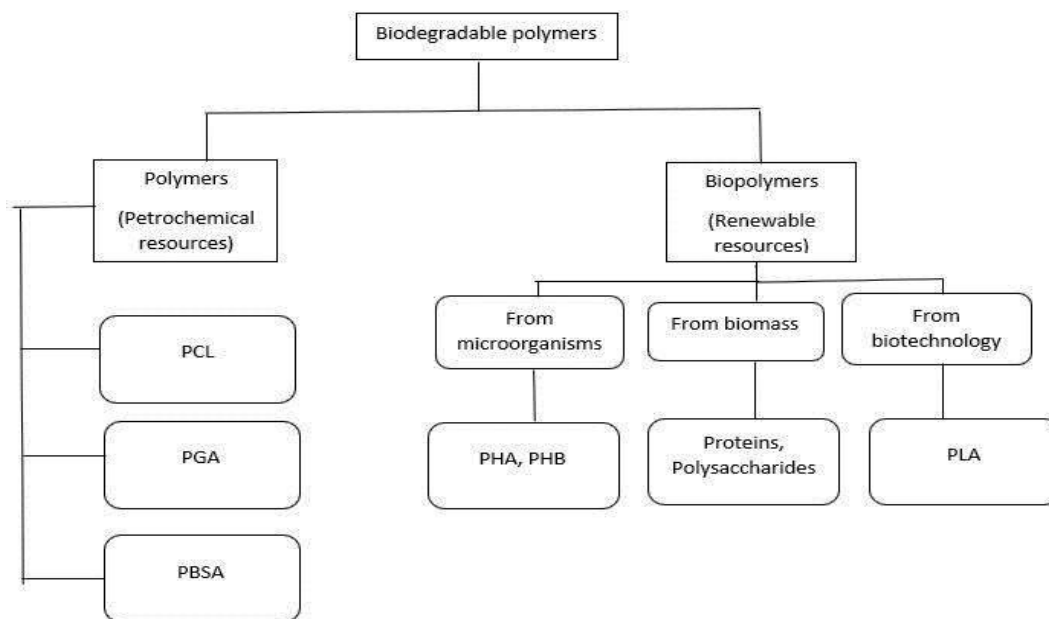


Figure 2.1 Classification of biodegradable polymers (modified from Zhong et al., 2020)

2.2 Polyhydroxyalkanoate (PHA)

Through fermentation methods, biocompatible and biodegradable polyesters polyhydroxyalkanoates (PHA), are manufactured on various scales such as in industries and laboratories as sustainable and environmentally friendly polymers. The production of PHA has no negative environmental consequences. Researchers have been more interested in these linear polyesters because they are biocompatible and biodegradable, commonly known as "green plastics." They're also thought to be a new kind of biofuel. Polyhydroxyalkanoates (PHAs) are a class of structurally varied microbial polyesters produced by a wide range of bacteria. Furthermore, because of their structural diversity, PHAs can easily undergo methyl esterification and be used as biofuels, expanding their application value (Riaz et al., 2021). PHA-based biofuels are comparable to biodiesel, with the exception that they contain more oxygen and neither nitrogen nor sulphur. The production of PHA is largely dependent on the regulation of cellular metabolism, which is concerned with carbon and energy. With

the introduction of various chemical compounds, it is possible to synthesize a high quantity of PHA without affecting cellular metabolism. This can also result in the fermentation process (Li et al., 2017; Wang et al., 2014).

The ultimate commercialization of PHA took years, several industries have experimented with various PHAs since the 1980s. People's interest in bioplastics, green plastics, and eco-friendly plastics, all of which are ecologically beneficial, was something they expected to see. PHAs have been reported in bacteria since the 1920s. PHA poly(3-hydroxybutyrate) (PHB) was discovered in *Bacillus megaterium* by Maurice Limoges a French scientist. Thus, it became the most studied PHA by the scientist for the fact to be found first. During metabolism, bacteria produce acetyl-coenzyme-A (acetyl-CoA), which is transferred to PHB by three biosynthetic enzymes. UV irradiation, osmotic shock, and heat these all are the stress conditions that PHA-accumulating organisms can withstand. The biodegradability of PHB synthesized by *Bacillus megaterium* and *Bacillus cereus* was originally demonstrated by Macrae and Wilkinson in 1958 (Akinmulewo & Nwinyi, 2019; Neureiter et al., 2022).

Polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides, and polyphenols are examples of organic compounds. These are potential candidates for substitution of synthetic plastics (Elmowafy et al., 2019). Among them polyhydroxyalkanoate (PHA), has received a great attention due to its biodegradable thermoplastic characteristics. Because of its hydrophobic nature within the bacterial cytoplasm, PHA is an efficient storage molecule with a little rise in osmotic pressure (Shantini et al., 2013). A regulatory structural protein termed Phasins and many other proteins including PHA synthase and depolymerizing enzymes, these PHA granules are linked to and surrounded by them. *Archaeobacteria*, *aerobes*,

anaerobes, and photosynthetic bacteria are among the more than 90 species and 300 microorganisms that make PHA (López et al., 2015; Salim et al., 2011). According to the researchers, for bacteria to manufacture polyhydroxyalkanoates, they need the presence of macro and microelements, the right temperature and pH, the right ratio of carbon to nitrogen, and the right amount of inoculum. In addition, unbalanced growth environments are better for PHA generation and accumulation. The fact that the PHAs were produced by a bacterial species using biotechnological techniques is the most important factor (Marciniak & Mo, 2021).

PHA synthesis in several microorganisms (*Cupriavidus necator*, *Wautersia eutropha*, *Alcaligenes latus*, *Pseudomonas oleovorans* etc.) has been studied, but only a few have showed promising characteristics in generating these bio polyesters. Many bacteria were unable to meet essential parameters as well as the capacity to absorb carbon sources at a proper growth rate and to produce considerable PHA (Zhang et al., 2022). PHA-producing organisms must meet several requirements, including a fast-increasing population, the ability to utilise affordable carbon sources, and a greater rate of production. Isolating them from the natural environment and developing strains for genetic recombination are two methods for obtaining new bacterial species to fulfil the demands. Both may be able to obtain suitable species to produce PHA (Surendran et al., 2020). Figure 2.2 shows the concept of economic sustainability in the PHA industry.

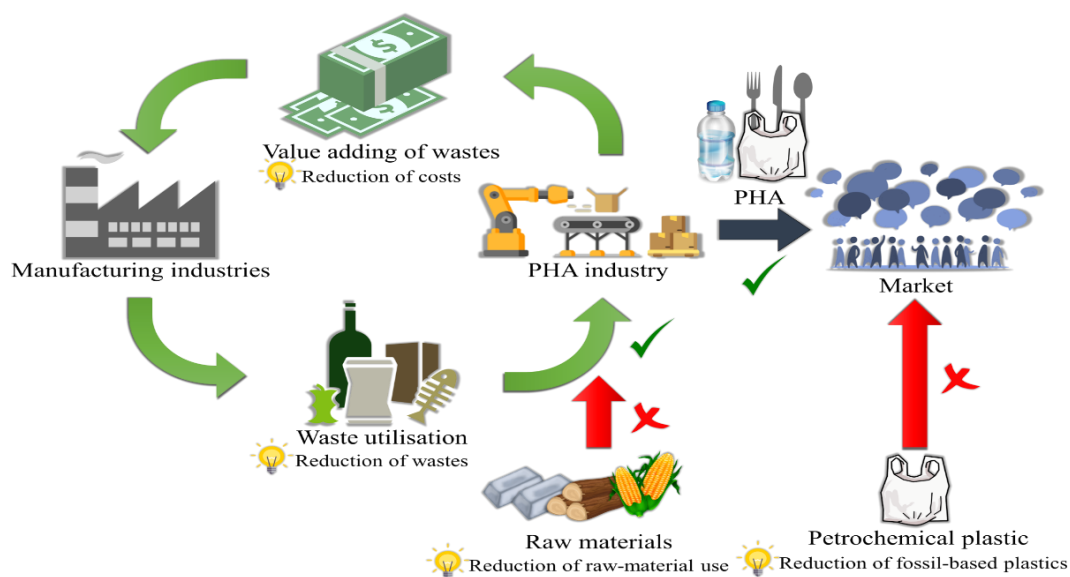


Figure 2.2 An illustration of the economic sustainability concept in the PHA industry

2.3 Different types of PHA

PHAs have (R)-configured monomeric units that are pure in terms of enantiomers. Due to the stereospecificity of the polymerizing enzyme, PHA synthase, the monomers are all in the R configuration. PHAs' alkyl side chains might be saturated, unsaturated, straight, branched, halogenated, epoxidized, aliphatic, aromatic, or include nitrile side chains. The length of the side chain and the type of functional group have a direct impact on polymer properties such as crystallinity and melting point (Basnett et al., 2018; Choi et al., 2020). Figure 2.3 shows the chemical structure of the PHA.

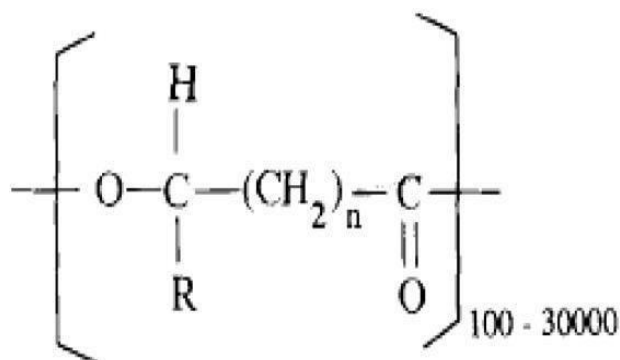


Figure 2.3 Chemical structure of PHA
(R refer to side chain and n refers to the number of repeating units)

PHA has over 150 monomer variations. As a result, PHA has a broader range of substance characteristics. Bacteria can usually manufacture PHAs with monomers ranging from 3 to 20 carbon atoms in length. PHAs with a chain length of C₃ to C₅ are known as short-chain length PHAs, while those with a chain length of C₆ to C₁₄ are known as medium-chain length PHAs, and those with a chain length of >C₁₄ are known as long-chain length PHAs. These may be used to make random copolymers, homopolymers, and block copolymers which have a variety of characteristics (Amirul et al., 2008; López et al., 2015; Singh et al., 2015; Urtuvia et al., 2014; Wang et al., 2014).

3-hydroxybutyrate (3HB or C₄), 3-hydroxydecanoate (3HV or C₅), 3-hydroxyhexanoate (3HHx or C₆), 3-hydroxydecanoate (3HD or C₁₀), 4-hydroxybutyrate, 3HD, or C₁₂ (4HB) are the most common PHA monomers (Singh et al., 2015). Different types of PHA and their functions are illustrated in Table 2.2.

Additionally, several PHA that are commonly synthesized such as PHB, P(3HB:3HV), P(3HB:3HHx), and P(3HB:4HB:3HHx), P(3HB:3HHx:3HO:3HD), P(3HB:3HV:3HHx), etc. various materials are produced using homopolymers, random copolymers, or block copolymers (Urtuvia et al., 2014; Wang et al., 2014).

Table 2.2 Different types of PHA and their functions.

Types of PHAS	Functions
[P(3HB)] poly(3-hydroxybutyrate)	It can be combined with starch and organic plastics to partly degrade the latter
poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyvalerate [P(3HB- <i>co</i> -3HV)]	It has excellent processability and strength.
poly(3-hydroxybutyrate- <i>co</i> -4-hydroxybutyrate [P(3HB- <i>co</i> -4HB)]	It has higher mechanical properties and Bioabsorbability.
poly(3-hydroxybutyrate- <i>co</i> -3-hydroxyhexanoate [P(3HB- <i>co</i> -3HHx)]	It shows a close resemblance to polypropylene (PP) and low-density polyethylene (LDPE).

2.4 PHA-production

PHA-producing microorganisms may be found in nearly any biological niche, including estuary sediments, rhizosphere, aquatic microbial fields, freshwater sediments, treatment plants and wastewater. It may also be produced from a variety of prokaryotic bacteria. In fluids PHAs are insoluble, they are kept in intracellular granules within the cells. Bacteria benefit from storing extra nutrients in their cells since it does not impact their overall physiological fitness. By polymerizing soluble intermediates into insoluble molecules, the cell's osmotic condition does not change. It avoids the loss of these vital molecules from the cell and keeps nutrition supply secure and accessible at minimal prices. PHA bioplastics are biodegradable and biocompatible in biologically active settings. They are also found in nature. Depending on the metabolic capacities of the microbes, different carbon sources can be utilized to produce PHA. To manufacture a high-quality polymer using a mix of microbes, carbon

sources, several precursors, and a restricted substrate. Anoxic photosynthesis maintenance, exogenous stress protection, and the sulphur cycle in microbial mats fully reliant on photosynthetic carbon fixation all of which stimulate the sporulation process in *Bacilli*, are some of the most complicated physiological roles that PHA performs. Increasing the time by generating energy and oxidising NADH, diazotrophic nitrogen fixation regulates the redox capacity of nitrogen-fixing bacteria (Braunegg et al., 2004; Koller et al., 2017; Verlinden et al., 2007; Yang et al., 2012).

PHAs' high cost compared to polypropylene prevents them from gaining market share which is comparatively cheaper in price. Organic matter and carbon sources for growth account for 48% of production costs, while polymer accounts for 70%–80% of organic material expenses. Many additional industrial by-products have previously been tested for PHA synthesis (for example, molasses by product of the processing of sugar cane, cheese whey from food industry wastes, and waste glycerol from the biodiesel sector) (Cavalheiro et al., 2012). Different types of wasted used to synthesize PHA has been discussed in table 2.3.

Microorganisms, design their enzyme systems for naturally produced organics rather than for man-made polymers. Microorganisms must be able to destroy man-made polymers while adhering to certain restrictions. A proper nutritional environment is required for their growth, as well as efficient enzyme machinery for digesting molecules with similar structures to those found in nature (Aminabhavi et al., 1990; Tian et al., 2009).

PHAs are biodegradable, and microbes can convert them to carbon dioxide and water in aerobic settings, as well as methane and carbon dioxide in anaerobic conditions. Modifying the polymeric structure and monomeric composition can alter its mechanical, chemical, and thermal properties; these attributes can also be

accomplished by controlling growth conditions, feeding strategies, substrate kinds, and genetic makeup. Many approaches, such as bacterial selection, genetic material modification, proper feeding management, appropriate substrates, and physiological environment modification can be used to make PHA processes more feasible and simple to handle, resulting in lower production costs for large-scale availability (Cavalheiro et al., 2012; Singh et al., 2015).

Table 2.3 Different waste types (used as carbon sources to produce PHA).

Types of wastes	Examples	sources
Sugar wastes	fructose, saccharose, glucose, sucrose, or stachyose.	(Gómez Cardozo et al., 2016)
Fatty acids wastes	linolenic butyric, acetic or propionic acids, myritic, palmitic, stearic, oleic, linoleic.	(Gómez Cardozo et al., 2016)
Agro-alimentary wastes	animal farm waste, poultry litter, vegetal solid wastes, such as rice bran, pea-shells, apple pomace, onion peels, grape pomace chicory roots potato peels.	(Bhati & Mallick, 2016) (Kumar et al., 2016) (Hassan et al., 2013) (Patel et al., 2015) (Oh et al., 2015) (Follonier et al., 2015)
Industrial wastewater streams	(wastewater from the three-phase olive oil extraction process, leguminous processing wastewater and fruit processing wastewater, and candy bar factory wastewater, low and medium added value by-	(Valentino et al., 2015) (Pais et al., 2016) (Colombo et al., 2016) (Kourmentza et al., 2015) (Alsafadi & Al-Mashaqbeh, 2017)

	products such as cheese whey	
Food wastes	liquid wastes: cooked oil solid wastes: food waste composite (including boiled rice, cooked meat, cooked vegetables, uncooked vegetables, boiled spices, cooking oil, vegetable peelings) and spent coffee grounds.	(Cruz et al., 2014) (Amulya et al., 2016) (Gómez Cardozo et al., 2016)
Industrial wastes (Biodiesel)	Biodiesel fatty acid (by-product from glycerol purification), oil cake hydrolysate, Crude glycerol,	(Bera et al., 2015) (Ray et al., 2016) (Ribeiro et al., 2016)
Other wastes	municipal solid waste leachate and Activated wastewater sludge,	(Basset et al., 2016) (Korkakaki et al., 2016)

2.5 P(3HB-co-3HV)

Imperial Chemical Industries (ICI) produced poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] under the trade name Biopol™ in the 1980s using glucose produced by mutant *C. necator* (Akdoğan & Çelik, 2021). As microorganisms were grown on fatty acid substrate with odd carbon numbers, P(3HB-co-3HV) copolymers were formed (Bomrungnok et al., 2020). Valeric acid, propionic acid, and heptanoic acid are examples of fatty acids with an odd carbon number. PHB is a hard yet brittle substance, making it difficult to treat because it only decomposes at temperatures higher than its melting point of 177 °C. Incorporating HV into the

polymer, on the other hand, can improve its flexibility and lower its melting temperature (Koller & Mukherjee, 2022). The industry has taken notice of this copolymer, P(3HB-*co*-3HV), since it possesses characteristics like polypropylene and polyethylene. P(3HB-*co*-3HV) has a substantial advantage over petroleum-derived plastic because it can be biodegraded by bacteria that would use the copolymer as a source of energy (Baldera-Moreno et al., 2022). Microorganisms inhabit P(3HB-*co*-3HV) surface and develop enzymes that breakdown the copolymer into its monomer units, HB and HV (Melendez-Rodriguez et al., 2021). Petroleum-derived polymers, on the other hand, degrade relatively slowly in the environment (Wilkes & Aristilde, 2017). In natural settings like soil, compost, aerobic and anaerobic sewage sludge, fresh and sea water, estuarine sediments, and air PHBV breaks down in a variety of settings however, it is possible to break down P(3HB-*co*-3HV) into smaller particles.

Anaerobic sewage has the fastest degradation and seawater has the slowest degradation. In the presence of oxygen, carbon dioxide, water, and humus are produced, whereas methane is produced in the absence of oxygen. Furthermore, no hazardous chemicals are produced throughout the decomposition process (Carrasco et al., 2022; Volova, 2015). Figure 2.4 illustrates the chemical structure of 3HB and 3HV.

PHB and PHV belong to a group of PHAs known as short-chain-length PHAs (scl-PHAs). Medium-chain-length PHAs (mcl-PHAs), on the other hand, contain C₆ to C₁₆ 3-hydroxy fatty acids. It is proposed that the bacteria-produced PHB 'homopolymer' contains fewer than 1 mol% 3-hydroxyvalerate monomers. When mixed substrates such as, valerate and glucose are used copolymers of PHB are formed. The microorganisms convert the substrates into scl-PHAs such as poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) (3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB) (Joseph et al., 2022; Zhang et al., 2022).

Scientists have conducted a significant amount of research on the homopolymer known as P(3HB). Although it is a common type of PHA, its unfavourable properties, such as low tensile strength and high crystallinity have limited its usage as a thermoplastic. Because of its low elasticity, stiffness, and brittleness, making it difficult to make other products. Various studies have been carried out to improve its application, with one of the alternatives being the addition of a comonomer such as 3HV, to make P(3HB-*co*-3HV) copolymer. PHA synthase (PHAC) is the major enzyme involved in the integration of numerous monomers into to the PHA polymer chain. The ability of the same enzyme to detect the exact HA monomers and subsequent activities varies, and these differences influence the PHA metabolism. Secondary monomers can be added to modify the characteristics of this microbial polymer, which can be achieved by managing different carbon precursors wherein the bacteria are cultured (Huong et al., 2017; Loo et al., 2005).

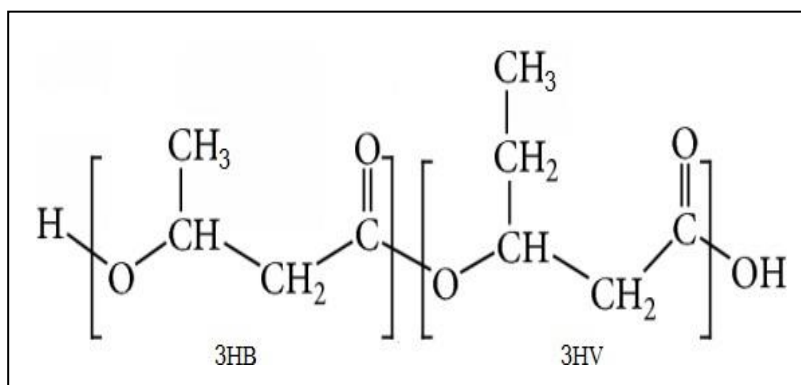


Figure 2.4 Chemical structure of P(3HB-*co*-3HV)

2.6 Biosynthesis pathway of P(3HB-*co*-3HV)

Poly(3-hydroxybutyrate) (PHB) was the first PHA reported and best described. However, due to its extremely crystalline structure, PHB has undesirable physical properties in terms of brittleness and processability. These residential characteristics can be improved by using PHA copolymers, one of the most prevalent of which being

poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV). The proportion of 3-hydroxyvalerate (3HV) in the copolyester may be used to tailor the polymer's suitable structures. For example, raising the 3HV web content to 30 mol % can reduce the degree of crystallinity of PHB by roughly 40%, lowering the melting temperature (T_m) and brittleness while expanding the processing window. With a higher 3HV material, the mechanical characteristics of the material are also improved in terms of ductility (Meléndez-Rodríguez et al., 2021).

Sodium propionate, Levulinic acid and pentanol were found to be effective for 3-hydroxyvalerate enhancers in Berezina's 2012 study. Furthermore, when rich inoculum was grown in mineral salt medium containing glucose and glutamate, the 3HV concentration was reduced. However, 1-pentanol had a detrimental impact on biomass output and copolymer content, according to the research. A copolymer's monomer makeup can also be affected by the pH of the culture medium (Berezina, 2012). Figure 2.5 shows the metabolic pathway of PHBV synthesis.

The copolymer P(3HB-*co*-3HV) been synthesized using two distinct routes, which includes the production of 3HB and 3HV monomers. The β -ketothiolase enzyme (PhaA) catalyses the condensation of two acetyl-CoA molecules or one acetyl-CoA and one propionyl-CoA to create acetoacetyl-CoA or 3-ketovaleryl-CoA when gene of phaA was transcribed and translated. NADPH-dependent acetoacetyl-CoA reductase (PhaB), which is generated by the phaB gene, then reduces acetoacetyl-CoA or 3-ketovaleryl-CoA to 3-hydroxybutyryl CoA (3HB-CoA) or 3-hydroxyvaleryl-CoA (3HV-CoA). PHA synthase (PhaC) randomly polymerized 3HB-CoA and 3HV-CoA into a P(3HB-*co*-3HV) polyesters chain (Liu et al., 2009).

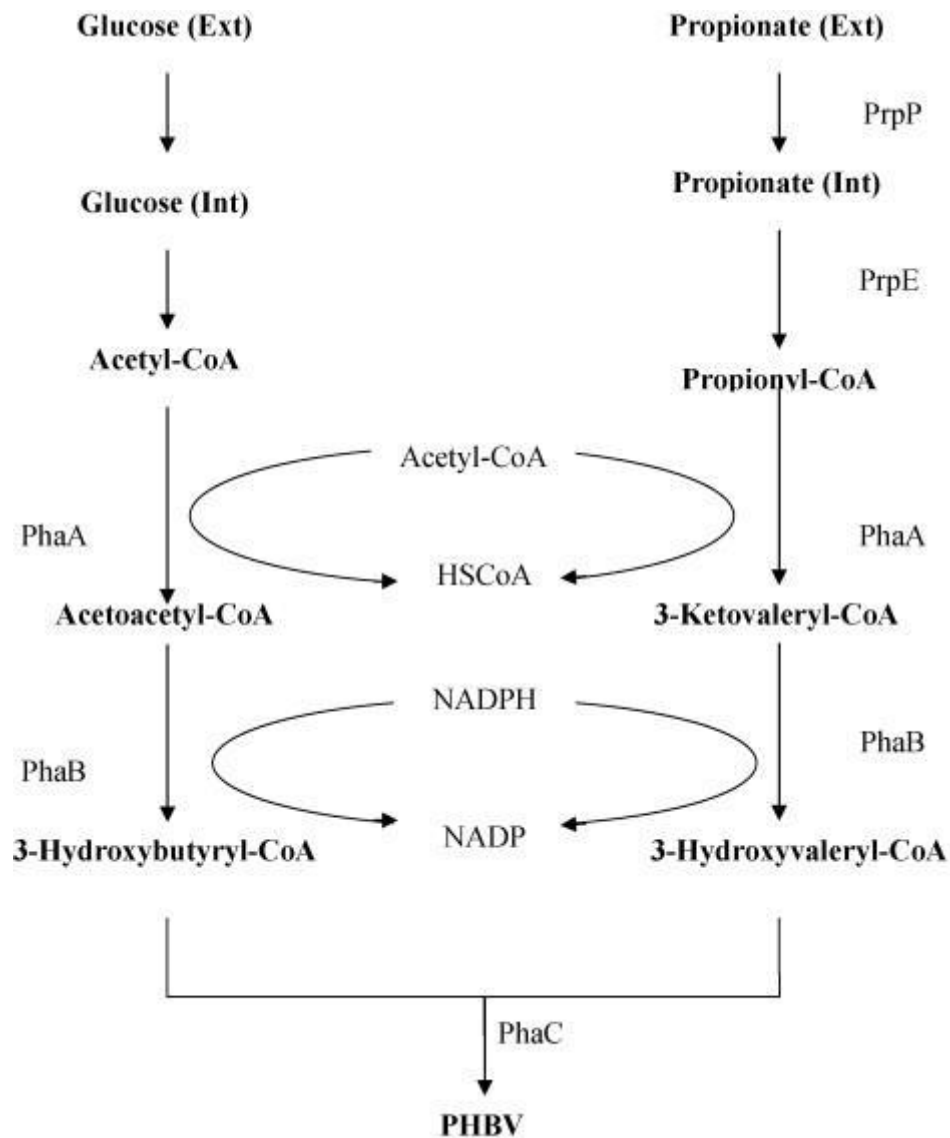


Figure 2.5 Showed the metabolic pathway for PHBV synthesis. Ext: extracellular; Int: intracellular (Liu et al., 2009).

Even though PHAs are still pricey, some businesses that manufacture PHB and PHBV remain the key members of commercially manufactured PHAs. PHA products like as Mirel, Nodax and Biopol are available on the market. Every year, majority of firms generate only few hundred tons of PHA. Some companies are starting to expand their manufacturing capacity (Mumtaz et al., 2010; Tian et al., 2009).

2.7 By product from biodiesel industry

Biodiesel is a diesel fuel replacement that may be used in vehicles or businesses. It's created with natural ingredients including animal fats, and vegetable oils. It is non-toxic and quickly destroyed by a variety of microbes, making it eco-friendly. Biodiesel production is in increasing demand as a renewable fuel. The by-product is crude glycerol, which has several essential uses. Though it was predicted that biodiesel production capacity would expand, it is still less than a goal level, and it is increasing at a moderate rate. The major reason for this is the high cost of manufacturing. However, it can be decreased by using its by-product. Biofuel refers to liquid gas, whereas biomass refers to solid fuels. Biofuel production will expand since it contains components that are environmentally safe and can be made from both edible and non-edible oils. Biodiesel, bioethanol, biohydrogen, and biomethanol, are all examples of biofuels. Biodiesel is a key component of biofuels and is described as the monoalkyl esters of vegetable oils or animal fats. Biofuels in the form of biodiesel or bioethanol are currently being utilized in nations such as the United States, Brazil, Germany, Italy, Australia, and Austria, with many more countries to follow. Biodiesel, more than any other type of biofuel, has gained widespread approval for its chemical composition and energy content when compared to traditional diesel. Malaysia, Germany, and Italy are among the countries that use commercially mixed diesel as a transportation fuel (Hanna & Fangrui, 1999; Yang et al., 2012; Yusuf et al., 2011; Zhang et al., 2022).

Biodiesel has similar characteristics to diesel, such as nontoxic, being biodegradable, and less expensive to manufacture than petroleum, as well as being carbon neutral. Biodiesel wastes have been accumulating in significant quantities as the need for biodiesel manufacturing has increased dramatically. The yearly waste

generated by the manufacturing of 150 million gallons of biodiesel is estimated to be between 23 and 35 million gallons. The three wastes from biodiesel are wastewater, glycerol, and methanol. All these wastes may be utilized as carbon sources in the fermentation process to produce environmentally friendly biopolymer, various products such ethanol, succinate, hydrogen, acetate, and 1,3-propanediol. The transesterification process produces the majority of biodiesel. The global biodiesel production yearly growth rate system is being improved. The output reached 1.3 billion liters in 2011, with a growth rate of 26.28 %. The synthesis of PHAs from biodiesel wastes has been estimated to be between 30 to 62 %. According to one study, for every gallon of biodiesel generated, around 1.05 pounds of glycerol is created. This also revealed that a 30-million-gallon-per-year factory produced 11,500 tons of 99.9% pure glycerin. The biodiesel industry was expected to reach 37 billion gallons by 2016, with crude glycerol accounting for roughly 4 billion gallons. Furthermore, because of the high production rate of refined glycerol, it is required to utilize this raw material to make it sustainable for key applications (Javaid et al., 2020; Patil et al., 2014; Sangkharak & Prasertsan, 2011; Yang et al., 2012).

2.8 Glycerine pitch

All glycerol in present era derives from biological sources. Glycerol is widely used in a variety of sectors, including chemicals, synthetic materials, beverages, food, and pharmaceuticals. From sweeteners, cosmetics, paints, toiletries, cough syrups, softening agents, solvents, surface coatings, and a variety of other items all include this ingredient. However, for these uses, high-quality glycerol must be manufactured. Glycerol has been employed in numerous investigations to produce microbial PHA utilizing pure cultures of diverse natural species, mostly Gram-negative eubacteria. According to the researchers the composition of crude glycerol, affects the percentage

of monomer in extracted biopolymers, as well as their thermal characteristics. Certain pollutants, such as organic and inorganic salts, alcohol, heavy metals, polyol, or ash may affect the rate of microbial growth in microorganisms cultivated on biodiesel-derived glycerol. The high concentration of sodium chloride in crude glycerol has been demonstrated to inhibit bacterial growth and have a significant influence on PHA generation and ultimate bioproduct output (Koller & Marsalek, 2015a; Marciniak & Mo, 2021; Strahan, 2011).

Typically, 1 kilogram of crude glycerol is synthesized for every 10 kg of biodiesel produced. Purification of glycerin is important for both economic and environmental reasons. To purify and identify the composition of glycerol pitch like ash content, moisture content, acidity, several methods are used which includes, fourier transform infrared (FTIR) and high-performance liquid chromatography (HPLC). Glycerol pitch was found to include 15 % to 25 % diglycerol, 55 % to 65 % glycerol, and less than 10 % fatty acids and inorganic salts in most investigations (Hazimah et al., 2003; Patil et al., 2014).

As an alternative product, industries are currently manufacturing bio-based plastics made from nontoxic and healthy components. Because they are renewable and recyclable. However, because of their high cost, PHA manufacture is unable to compete with petrochemical plastics. Researchers are attempting to reduce the cost of PHA manufacturing by using waste materials and inexpensive substrates like as glycerol. They are, nevertheless, unable to reduce production costs. Glycerol has been used to make bioplastics by bacterial fermentation for a variety of reasons, including its inexpensive cost, reduced substrate, and universality. The cost of generating PHA can be reduced by employing a low-cost carbon source. The co-product of biodiesel synthesis, glycerol, is an intriguing substance to employ as a carbon source for