PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*CO*-3-HYDROXYVALERATE) BY *Cupriavidus malaysiensis* USMAA2-4$_{ABH16}$ HARBOURING *Cupriavidus necator* H16 LIPASE GENE

JEREMY WONG HAU SEUNG

UNIVERSITI SAINS MALAYSIA

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JEREMY WONG HAU SEUNG

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LIST OF SYMBOLS AND ABBREVIATIONS

ΔH_m	Enthalpy of Fusion
μ_{max}	Maximum Specific Growth Rate
3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
3HHD	3-hydroxyhexadecanoate
3HHp	3-hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3HN	3-hydoxynonanoate
3HO	3-hydroxyoctanoate
3HPD	3-hydroxypentadecanoate
3HTD	3-hydroxytridecanoate
3HTTD	3-hydroxytetradecanoate
3HUD	3-hydroxyundecanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
A_{3HB}	The area of 3HB
A_{3HV}	The area of 3HV
A_a	The areas under the amorphous hump
A_c	The areas under the crystalline peaks
A_{CME}	The area of methyl caprylate
A_{FAME}	The area of a specific fatty acid methyl ester
$A_{FAME\ total}$	The area of total fatty acid methyl ester detected
AR	Analytical Reagents
B. subtilis	*Bacillus subtilis*
C. malaysiensis	*Cupriavidus malaysiensis*
C. necator	*Cupriavidus necator*
C/N	Carbon/Nitrogen
CAGR	Compound Annual Growth Rate
CDW	Cell Dry Weight

CME	Methyl Caprylate
C_{MM}	The molar mass of a carbon atom
C_{No}	The number of carbon atoms
CS_{MW}	The molecular weight of the carbon source
CS_{wt}	The weight of the carbon source in g per 1000 mL
D	Dilution Rate
DMSO	Dimethyl Sulphoxide
DO	Dissolved Oxygen
E. coli	*Escherichia coli*
E_m	Young's Modulus
et al.	Et Aliī
FAME	Fatty Acid Methyl Ester
GC	Gas Chromatography
GPC	Gas Permeation Chromatography
H. pseudoflava	*Hydrogenophaga pseudoflava*
HA	(*R*)-hydroxyalkanoic acid
HA_{MCL}	Medium-chain-length HA
HA_{SCL}	Short-chain-length HA
HCl	Hydrochloric Acid
HDPE	High-density Polyethylene
IPTG	Isopropyl-β-D-1-thiogalactoside
K	The content constant of PHA
k_{3HB}	The composition constant of 3HB
k_{3HV}	The composition constant of 3HV
K_La	Volumetric Mass Transfer Coefficient
LDPE	Low-density Polyethylene
LLDPE	Linear Low-density Polyethylene
MCL-PHA	Medium-chain-length-PHA
MSM	Minimal Salt Medium
N_{MM}	The molar mass of a nitrogen atom
N_{No}	The number of nitrogen atoms
NS_{MW}	The molecular weight of the nitrogen source

NS_{wt}	The weight of the nitrogen source in g per 1000 mL
OD	Optical Density
OTR	Oxygen Transfer Rate
OUR	Oxygen Uptake Rate
P(3HB)	Poly(3HB)
P(3HB-*co*-3HHx)	Poly(3HB-*co*-3HHx)
P(3HB-*co*-3HV)	Poly(3HB-*co*-3-HV)
P(3HB-*co*-3HV-*co*-3HB)	Poly(3HB-*co*-3HV-*co*-3HB)
P(3HB-*co*-4HB)	Poly(3HB-*co*-4HB)
P(3HV)	Poly(3HV)
P. putida	*Pseudomonas putida*
PCR	Polymerase Chain reaction
PGA	Polyglycolic acid
PHA	Polyhydroxyalkanoates
PLGA	Polylactide-c*o*-glycolid
*p*NP	*p*-nitrophenol
*p*NPP	*p*-nitrophenyl Palmitate
pO_2	Dissolved Oxygen Tension
PPi	Pyrophosphate
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl Alcohol
q_p	Productivity
SCL-PHA	Short-chain-length-PHA
$\mathcal{T}$	Retention Time
T_c	Crystallization temperature
TCA	Tricarboxylic Acid
T_g	Glass transition temperature
T_m	Melting temperature
UK	United Kingdom
USA	United States America
USD	United States Dollar
X_1	Palm olein concentration

X_2	1-pentanol concentration
X_3	Ammonium sulfate concentration
X_c	Crystallinity Index
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
δ	Elongation at Break
σ	Tensile Strength

PENGHASILAN POLI(3-HIDROKSIBUTIRAT-*KO*-3-HIDROKSIVALERAT) OLEH *Cupriavidus malaysiensis* USMAA2-4$_{ABH16}$ PEMBAWA GEN LIPASE *Cupriavidus necator* H16

ABSTRAK

Polyhydroxyalkanoates (PHA) ialah biopoliester dengan sifat termoplastik yang biokompatibel dan terbiodegradasi. Poli(3-hidroksibutirat) [P(3HB)] ialah PHA yang paling biasa dihasilkan oleh pelbagai mikroorganisma, tetapi penggunaannya terhad kerana kekakuannya. Menggabungkan monomer sekunder seperti 3-hydroxyvalerate (3HV) menyumbang kepada beberapa penambahbaikan sifat PHA, termasuk fleksibiliti, keanjalan dan kebolehbiodegradasian. Asid oleik dan asid organik kos tinggi merupakan pilihan kesukaan kebanyakan bakteria penghasil PHA sebagai sumber karbon untuk penghasilan poli(3-hydroksibutirat-*ko*-3-hydroksivalerat) [P(3HB-*ko*-3HV)]. Kajian ini menunjukkan penilaian transforman pembawa gen lipase untuk penghasilan PHA daripada olein sawit dan 1-pentanol sebagai alternatif kepada asid oleik dan asid valerik. Dalam kajian ini, klon, *Cupriavidus malaysiensis* USMAA2-4$_{ABH16}$ yang merupakan transforman *C. malaysiensis* USMAA2-4 telah berjaya dibina. *C. malaysiensis* USMAA2-4$_{ABH16}$ pembawa gen *lipAB Cupriavidus necator* H16 yang boleh ekspresi mengumpul lebih daripada 60% berat P(3HB-*ko*-3HV) menggunakan olein sawit dan 1-pentanol sebagai sumber karbon. Penjajaran jujukan nukleotida sisipan *lipAB* menunjukkan 100% identiti dengan gen *lipAB C. necator* H16 dan jangkaan nilai 0.0. *C. malaysiensis* USMAA2-4$_{ABH16}$ menunjukkan pertumbuhan 4 kali ganda lebih tinggi dan aktiviti lipase 8 kali ganda lebih tinggi berbanding *C. necator* H16 di bawah tekanan kobalt dan alkohol. Olein sawit dengan

kandungan asid oleik yang tinggi dan ketepuan minyak yang rendah mengatasi prestasi minyak sawit mentah, minyak isirong sawit mentah dan minyak kacang soya dalam menghasilkan P(3HB-*ko*-3HV) kerana pemilihan kesukaan bakteria untuk asid oleik. *C. malaysiensis* USMAA2-4$_{ABH16}$ menunjukkan berat kering sel 6 kali ganda lebih tinggi berbanding *C. necator* H16 apabila dibekalkan dengan 1-pentanol dan bermandiri sehingga 0.15 wt% C 1-pentanol. Komposisi 3HV khusus (6, 9, 12, 15 mol%) P(3HB-*ko*-3HV) dicapai menggunakan metodologi permukaan tindak balas dan kepekatan PHA meningkat sebanyak 34% selepas pengoptimuman. Komposisi 3HV, kepekatan 3HV, hasil 3HV, kepekatan PHA dan hasil PHA yang dioptimumkan untuk skala pengeluaran 50 mL boleh dihasilkan semula pada skala pengeluaran 6 L. Strategi suap kelompok berpecah meningkatkan komposisi 3HV (12 mol% kepada 31 mol%), kepekatan 3HV (0.5 g/L kepada 1.6 g/L), hasil 3HV (0.22 g/g kepada 0.49 g/g), kepekatan PHA (4.1 g/L kepada 5.2 g/L), dan hasil PHA (0.45 g/g kepada 0.51 g/g). Pengeluaran suap kelompok berpecah pada pH 6.8−7.1 menyebabkan penggunaan keseluruhan sumber karbon dan hasil PHA yang tinggi sebanyak 0.78 g/g. P(3HB-*ko*-3HV) yang dihasilkan oleh *C. malaysiensis* USMAA2-4$_{ABH16}$ menunjukkan fleksibiliti yang lebih tinggi berbanding P(3HB-*ko*-3HV) komersial, polietilena berketumpatan tinggi dan polipropilena. Selain itu, P(3HB-*ko*-3HV) terbiosintesis menunjukkan kehabluran yang lebih rendah berbanding plastik sintetik biasa. *C. malaysiensis* USMAA2-4$_{ABH16}$ telah terbukti sebagai strain yang menjanjikan untuk menghasilkan PHA daripada olein kelapa sawit dan 1-pentanol.

PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*CO*-3-HYDROXYVALERATE) BY *Cupriavidus malaysiensis* USMAA2-4$_{ABH16}$ HARBOURING *Cupriavidus necator* H16 LIPASE GENE

ABSTRACT

Polyhydroxyalkanoates (PHA) are biopolyesters with thermoplastic properties that are biocompatible and biodegradable. Poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA produced by various microorganisms, but its application is limited due to its stiffness. Incorporating a secondary monomer such as 3-hydroxyvalerate (3HV) contributes to several PHA properties improvement, including flexibility, elasticity, and biodegradability. High-cost oleic acid and organic acids are preferred by most PHA-producing bacteria as the carbon sources for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] production. This study demonstrated the evaluation of a lipase genes-harbouring transformant for PHA production from palm olein and 1-pentanol as alternatives to oleic acid and valeric acid. In this study, a clone, *Cupriavidus malaysiensis* USMAA2-4$_{ABH16}$ which is the transformant of *C. malaysiensis* USMAA2-4 was successfully constructed. *C. malaysiensis* USMAA2-4$_{ABH16}$ harbouring expressible *Cupriavidus necator* H16 *lipAB* genes accumulated more than 60 wt% of P(3HB-*co*-3HV) using palm olein and 1-pentanol as the carbon sources. Nucleotide sequence alignment of the *lipAB* insert showed 100% identities with *C. necator* H16 *lipAB* genes and expect value of 0.0. *C. malaysiensis* USMAA2-4$_{ABH16}$ showed 4-fold higher growth and 8-fold higher lipase activity compared to *C. necator* H16 under cobalt and alcohol stress. Palm olein with high oleic acid content and low oil saturation outperformed crude palm oil, crude palm kernel oil, and soybean

oil in producing P(3HB-*co*-3HV) due to the preference of the bacteria for oleic acid. *C. malaysiensis* USMAA2-4$_{ABH16}$ showed 6-fold higher cell dry weight compared to *C. necator* H16 when supplied with 1-pentanol and survived up to 0.15 wt% C 1-pentanol. Specific 3HV composition (6, 9, 12, 15 mol%) of P(3HB-*co*-3HV) was achieved using response surface methodology and the PHA concentration increased by 34% post optimization. The 3HV composition, 3HV concentration, 3HV yield, PHA concentration, and PHA yield optimized for 50 mL production scale were reproducible at a 6 L production scale. The split fed-batch strategy increased 3HV composition (12 mol% to 31 mol%), 3HV concentration (0.5g/L to 1.6 g/L), 3HV yield (0.22 g/L to 0.49 g/g), PHA concentration (4.1 g/L to 5.2 g/L), and PHA yield (0.45 g/L to 0.51 g/g). Split fed-batch production at pH 6.8−7.1 caused complete carbon source consumption and a high PHA yield of 0.78 g/g. P(3HB-*co*-3HV) produced by *C. malaysiensis* USMAA2-4$_{ABH16}$ showed higher flexibility compared to the commercial P(3HB-*co*-3HV), high-density polyethylene, and polypropylene. Besides, the biosynthesized P(3HB-*co*-3HV) showed lower crystallinity compared to common synthetic plastics. *C. malaysiensis* USMAA2-4$_{ABH16}$ was proven as a promising strain to produce PHA from palm olein and 1-pentanol.

CHAPTER 1

INTRODUCTION

1.1 Research background

Plastic wastes have caused pervasive environmental pollution other than providing great convenience. According to The World Bank's What a Waste 2.0 report released in 2018, rapid urbanization and growing populations are expected to increase global annual waste generation by 70%, which will be 3.40 billion tonnes by 2050 from 2.01 billion tonnes in 2016. In 2016, 242 million tonnes of plastic waste were generated globally, which was 12% of global solid waste generated. To treat and dispose the plastic waste, 1.6 billion tonnes of carbon dioxide equivalent were generated in that particular year (Kaza et al., 2018).

The emergence of the COVID-19 pandemic in 2019 has caused massive use of face masks as a protection against the transmission of the virus worldwide. The disposal of the huge amount of used face masks has led to another solid waste problem. As polypropylene is one of the materials used to fabricate face masks, mass disposal of face masks leads to microplastic pollution (Aragaw, 2020). Due to high degradation stability, complete degradation of the plastic wastes cannot be achieved naturally and microplastic fragments which are common with sizes less than 5 mm^2 are becoming abundant in the environment at an alarming rate (Schmidt et al., 2018; Singh & Sharma, 2008).

Polyethylene and polypropylene are the two most-produced low-density polymers. Accumulation of these synthetic plastics in freshwater shorelines and marine environments leads to microplastic pollution due to the breakdown of those plastic through weathering and exposure to wave action, wind abrasion, or ultraviolet radiation from sunlight. Microplastics absorbed by aquatic animals could be absorbed by humans through their daily diet (Schnurr et al., 2018; Zbyszewski et al., 2014). The substitution of conventional petroleum-based plastics with biodegradable polyesters has been a global effort to reduce global plastic waste generation. Polyhydroxyalkanoates (PHA) are biopolyesters that resemble the properties of conventional plastics with better renewability, biodegradability, and biocompatibility (Możejko-Ciesielska & Kiewisz, 2016; Pellis et al., 2021). They are accumulated as a reserved energy source by various microorganisms under nitrogen-limiting conditions with excess carbon and are producible through fermentation on large scales (Beckers et al., 2016; Impallomeni et al., 2018; López et al., 1998; Verlinden et al., 2011). Due to its biodegradability and biocompatibility, PHA are widely studied for agricultural, aquacultural, medical and commodity applications (Grande et al., 2017; Kwiecień et al., 2016; Meereboer et al., 2021; Shantini et al., 2015a).

Poly(3-hydroxybutyrate) [P(3HB)] is the most common short-chain-length PHA found in nature with its mechanical properties reported to be comparable to polypropylene despite its poorer elongation at break compared to polypropylene (Iwata et al., 2003; Sirohi et al., 2021). However, the application of P(3HB) is limited due to its stiffness and brittleness. The incorporation of 3-hydroxyvalerate (3HV) monomer results in poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] which is a biopolyester with thermoplastic properties and resolves the property weakness of P(3HB) (Amini et al., 2020; Kim et al., 2009).

1.2 Problem statement

High production cost caused mainly by the cost of the substrate is a major concern that leads to PHA application drawbacks. Production cost reduction is possible by substituting the substrates with low-cost waste from agricultural or food industries (Wang et al., 2021). The polymerization of P(3HB-*co*-3HV) requires 3-hydroxybutyrate (3HB) and 3HV monomers contributed by main and precursor carbon sources respectively (Byrom, 1992; Policastro et al., 2021). Due to the commercial value of oleic acid, using oleic acid as the main carbon source for P(3HB-*co*-3HV) production imposes high production cost, despite the preference of most PHA-producing bacteria towards oleic acid (Eggink et al., 1992; Zhila et al., 2020). Besides, choices of 3HV precursor are narrow due to the preference of most PHA-producing bacteria such as *C. necator*, *Herbaspirillum seropedicae*, and *Corynebacterium glutamicum* towards organic acids such as propionic acid, valeric acid, and their respective salts (Catalán et al., 2018; Gahlawat & Soni, 2017; Matsumoto et al., 2011). Therefore, it is necessary to diversify 3HV precursors selection to explore the potential of other 3HV precursors by using suitable PHA-producing bacteria.

Palm olein is a resulting liquid fraction from the fractionation of palm oil with a high oleic acid content of about 41.5–47.7%. A high proportion of palm olein is obtainable in one single step of fractionation (Derawi et al., 2014; Gibon, 2012; Padial-Jaudenes et al., 2020; Tarmizi & Ismail, 2008). In addition, the content of oleic acid in palm olein remains unchanged even after repeated exposure to deep-frying temperature (Bansal et al., 2010). This property of palm olein is an added value as used palm olein which is abundant in hot tropical countries is a potential low-cost alternative carbon source for oleic acid (Ishola et al., 2020; Lai et al., 2012).

Alkyl alcohols are rarely used as 3HV precursors for P(3HB-*co*-3HV) production due to the disability of most PHA-producing bacteria to survive alkyl alcohols. The ability to convert alkyl alcohols to 3HV has been reported only for several uncommon bacteria since reported for *Paracoccus denitrificans* (Cal et al., 2016; Ezhov et al., 2013; Galuzina et al., 2015; Kuin et al., 2019; Majid et al., 1999; Shantini et al., 2015b; Yamane et al., 1996). *C. malaysiensis* USMAA2-4 is one of those bacteria and it prefers 1-pentanol over valeric acid. A previous study showed that 40% of 1-pentanol supplied to *C. malaysiensis* USMAA2-4 was converted into 3HV. The supplemented 1-pentanol was converted to pentanal before being converted to valeric acid which was subsequently converted to 3HV. The percentage of conversion was higher than the 10% reported for valeric acid in the same study (Majid et al., 1999; Shantini et al., 2015b). On the other hand, poor viability was reported for *C. necator* supplemented with 1-pentanol (Berezina, 2012; Novackova et al., 2019).

Cupriavidus necator H16 is one of the common strains used for PHA production and has been studied extensively for PHA production from various plant oils owing to the ability to secrete a non-specific extracellular lipase (Kimura et al., 1999; Lee et al., 2008; Lu et al., 2013; Mifune et al., 2008; Ng et al., 2010; Purama et al., 2018). On the other hand, *C. malaysiensis* USMAA2-4 was unable to utilize plant oils for bacterial growth and PHA accumulation even though two lipase genes were predicted from its whole genome sequence via protein homology (Md. Iqbal & Amirul, 2014; Shafie et al., 2017). The disability of *C. malaysiensis* USMAA2-4 to utilize plant oils could be attributed to substrate specificity and selectivity of the predicted lipases (Albayati et al., 2020; Jensen et al., 1983). The GDSL family lipase predicted could exhibit stereo-specificity for acyl-coA and alcohol substrates whereas the other lipase

predicted is not categorised as triacylglycerol lipase (Shafie et al., 2017; Sugisaka et al., 2022).

Genetic incorporation of oil-utilizing ability into *C. malaysiensis* USMAA2-4 is necessary for this bacterium to utilize plant oils for bacterial growth and PHA accumulation. *C. necator* H16 is a suitable parental strain as it is also a PHA-producing bacterium. In addition, *C. malaysiensis* USMAA2-4 and *C. necator* H16 display low genetic variation as they are both Gram-negative bacteria and belong to the same genus (Lees et al., 2019; Martino et al., 2014; Verlinden et al., 2011). *C. necator* H16 *lipAB* genes were the genes of interest owing to extensive studies carried out with different plant oils to warrant the conversion of triacylglycerols into PHA. Besides, *lipAB* genes code for a non-specific extracellular lipase that is not substrate selective and causes complete hydrolysis of triacylglycerols into free fatty acids (Lu et al., 2013; Verma et al., 2021).

In this study, *C. malaysiensis* USMAA2-4 transformant harbouring *C. necator* H16 lipase genes was constructed. The performance of the transformant on bacterial growth and PHA accumulation when supplemented with different nutrients was evaluated before small-scale optimization through response surface methodology. The optimized small-scale P(3HB-*co*-3HV) production was scaled-up and the production kinetics of various fermentation strategies were studied. Finally, P(3HB-*co*-3HV) was extracted and the physicochemical properties of the extracted P(3HB-*co*-3HV) with different 3HV compositions were characterized and compared.

1.3 Research objectives

The objectives of this study are:

1. To construct *C. malaysiensis* USMAA2-4 transformant harbouring *C. necator* H16 lipase genes.
2. To evaluate the performance of *C. malaysiensis* USMAA2-4 transformant, *C. malaysiensis* USMAA2-4_{ABH16} on bacterial growth and P(3HB-*co*-3HV) accumulation when supplemented with different nutrients.
3. To optimize small-scale P(3HB-*co*-3HV) production by *C. malaysiensis* USMAA2-4_{ABH16} through response surface methodology.
4. To study the P(3HB-*co*-3HV) production kinetics of various production strategies at a larger production scale.
5. To extract P(3HB-*co*-3HV) with different 3HV compositions from *C. malaysiensis* USMAA2-4_{ABH16}t and characterize the physicochemical properties of the extracted P(3HB-*co*-3HV).

CHAPTER 2

LITERATURE REVIEW

2.1 Polyhydroxyalkanoates, the Biodegradable Plastics

2.1.1 General introduction polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are biopolyesters produced by microorganisms under nutrient-limiting conditions with excess carbon source and are stored in polymerized form as inclusion bodies or granules (Anderson & Dawes, 1990; López et al., 1998; Obruca et al., 2020; Reis et al., 2003). PHA stored inside microorganisms can be utilized to survive harsh conditions which are not favourable for normal cell growth (López-Cortés et al., 2008; Obruca et al., 2020). PHA resemble the properties of conventional plastics such as water insolubility, relative hydrolytic resistance, and relative elasticity. PHA have several advantages over traditional polymers whereby they possess good ultraviolet resistance, high density, and low "stickiness" when melted. However, they have poor resistance to acids and bases (Bugnicourt et al., 2014; Ilyas et al., 2020). PHA are recognized as "biodegradable plastics" as they are biodegradable by depolymerase (Lee & Choi, 1999; Morohoshi et al., 2020). Besides, they are biocompatible owing to their low toxicity and are degradable *in vivo* to *D*-3-hydroxybutyrate, which is a normal constituent of human blood towards mammalian cells (Choi et al., 2005; Mohandas et al., 2021).

2.1.2 Classification of PHA

Chemically, PHA are classified by their functional alkyl group as shown in Figure 2.1 and Table 2.1.

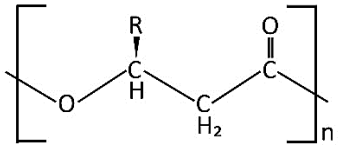

Figure 2.1 Chemical structure of PHA (PubChem SID: 405238958).

Table 2.1 Nomenclatures of PHA by their functional alkyl group

***R* group**	**Carbon number**	**PHA monomer**	**References**
Methyl	C_4	3-hydroxybutyrate (3HB)	Shantini et al., 2013
Ethyl	C_5	3-hydroxyvalerate (3HV)	Shantini et al., 2013
Propyl	C_6	3-hydroxyhexanoate (3HHx)	Xu et al., 2019
Butyl	C_7	3-hydroxyheptanoate (3HHp)	Wang et al., 2017
Pentyl	C_8	3-hydroxyoctanoate (3HO)	Xu et al., 2019
Hexyl	C_9	3-hydoxynonanoate (3HN)	Wang et al., 2017
Heptyl	C_{10}	3-hydroxydecanoate (3HD)	Xu et al., 2019
Octyl	C_{11}	3-hydroxyundecanoate (3HUD)	Razaif-Mazinah et al., 2017
Nonyl	C_{12}	3-hydroxydodecanoate (3HDD)	Wang et al., 2017
Decyl	C_{13}	3-hydroxytridecanoate (3HTD)	Wang et al., 2017
Undecyl	C_{14}	3-hydroxytetradecanoate (3HTTD)	Wang et al., 2017
Dodecyl	C_{15}	3-hydroxypentadecanoate (3HPD)	Barbuzzi et al., 2002
Tridecyl	C_{16}	3-hydroxyhexadecanoate (3HHD)	Xu et al., 2019

Structurally, PHA are classified into short-chain-length (SCL) PHA which consist of 3–5 carbons, and medium-chain-length (MCL) PHA which consist of 6–14 carbons. Another structural classification of PHA is SCL/MCL-PHA which are made up of SCL-PHA and MCL-PHA with carbon numbers ranging from 3–14. However, the prevalence of SCL/MCL-PHA is relatively rare in nature and extraordinary broad substrate specificity is needed for PHA synthase in the organisms to synthesise these hybrid PHA (Sharma et al., 2021; Steinbüchel & Lütke-Eversloh, 2003).

SCL-PHA are highly hydrophobic, rigid thermoplastics with high crystallinity which is typically 55–80% (Chen et al., 2006; Liu & Chen, 2007; Sirohi et al., 2021).

They have high melting temperatures which are slightly lower than their degrading temperatures and low glass transition temperatures. Their high crystallinity causes them to be brittle and stiff (Sirohi et al., 2021; Sujatha et al. 2007; Wu et al., 2003). P(3HB) is the most common SCL-PHA synthesized by bacteria. Although the mechanical properties of P(3HB) are comparable to polypropylene, the application of the polymer is limited due to its poor elongation at break with respect to polypropylene (Iwata et al., 2003; Sirohi et al., 2021).

MCL-PHA display higher elasticity compared to SCL-PHA, with low degrees of crystallinity and low melting temperatures (Nomura & Taguchi, 2007; Reddy et al., 2022). Their elastomeric properties allow them to be potentially suitable as biomaterials for biomedical applications (Sobieski et al., 2017; Zinn et al., 2001). Apart from its mechanical properties, some of the MCL-PHA functional groups can be modified by chemical reactions to produce polymers with the potential to extend their application as environmentally biodegradable polymers and functional biomaterials for biomedical usage (Hazer & Steinbüchel, 2007; Sharma et al., 2021). The physicochemical properties of SCL-PHA and MCL-PHA are summarized in Table 2.2 (Możejko-Ciesielska & Kiewisz, 2016).

Table 2.2 Physicochemical properties of SCL-PHA and MCL-PHA

Properties	**Homopolymer SCL-PHA**	**Homopolymer MCL-PHA**
Melting temperature (°C)	179	80
Glass transition temperature (°C)	4	−40
Young's modulus (GPa)	3.5	–
Elongation to break (%)	40	300
Tensile strength (Mpa)	5	20

Furthermore, PHA can also be classified into homopolymers and heteropolymers. P(3HB) is an example of a homopolymer whereby it is a linear polyester formed from the polymerization of 3HB through continuous linking of a

single 3HB monomer to another 3HB monomer via an ester bond. Heteropolymers, on the other hand, are copolymers or terpolymers formed as a result of the linkage between several different monomers. P(3HB-*co*-3HV) is a copolymer with two different monomers, 3HB and 3HV whereas poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) P(3HB-*co*-3HV-*co*-4HB) is a terpolymer with three different monomers, 3HB, 3HV and 4-hydroxybutyrate (4HB) (Kucera et al., 2019; Bugnicourt et al., 2014).

2.1.3 Formation of PHA

PHA synthases are the key enzymes in PHA polymerization. There are more than 60 PHA synthase genes from eubacteria that have been cloned, sequenced and studied based on the homology in prokaryotic genome sequence data banks (Chen et al., 2020; Rehm & Steinbüchel, 1999; Steinbüchel & Hein, 2001). The substrates of PHA synthases are coenzyme A thioesters of (*R*)-hydroxyalkanoic acid (HA). The HA can be of various carbon chain lengths and substituents with a hydroxyl group at C3, C4, C5, or C6 positions (Sharma et al., 2021; Steinbüchel & Valentin, 1995).

In PHA-accumulating microbial cells, PHA synthases are bound to the surface of the PHA granules together with the granule-associated protein, phasins (PhaP and PhaF) and specific regulator proteins (PhaR) which affect the number, size, and distribution of the granules (Haywood et al., 1989; Long et al., 2018; Maehara et al., 2001; Mato et al., 2020; Pieper-Fürst et al., 1994; Pötter et al., 2002; Steinbüchel et al., 1995; York et al., 2002). PHA synthases are classified into four classes according to their subunit composition and substrate specificity as summarized in Table 2.3.

Table 2.3 Classes of PHA synthase and their resulting PHA

Substrate specificity	PHA synthase			Accumulated PHA	References
	Class	Subunit(s)	Microorganisms		
HA_{SCL} (C3-C5)	I	PhaC	*Cupriavidus necator*	SCL-PHA	Tang et al., 2022
	III	PhaC, PhaE	*Allochromatium vinosum*		Neoh et al., 2022
	IV	PhaC, PhaR	*Bacillus megaterium*		Neoh et al., 2022
HA_{MCL} (C6-C14)	II	PhaC	*Pseudomonas oleovorans*	MCL-PHA	Prieto et al., 1999
			Pseudomonas putida		Borrero-de Acuña et al., 2019
			Pseudomonas aeruginosa		Javed & Jamil, 2021
$HA_{SCL-MCL}$ (C3-C14)	I	PhaC	*Aeromonas caviae* FA440	SCL-MCL-PHA	Preusting et al., 1993
	II	PhaC	*Pseudomonas* sp. 61-3		Li et al., 2019a

Class I, II, III, and IV PHA synthases are represented by that of *C. necator*, *Pseudomonas aeruginosa*, *Allochromatium vinosum,* and *B. megaterium* respectively (Neoh et al., 2022; Tang et al., 2022). Class I PHA synthase involves in the accumulation of SCL-PHA from SCL hydroxyalkanoic acid (HA_{SCL}) whereas Class II PHA synthase involves the accumulation of MCL-PHA from MCL hydroxyalkanoic acid (HA_{MCL}). Unlike Class I and Class II PHA synthases with only PhaC subunit (Borrero-de Acuña et al., 2019; Javed & Jamil, 2021; Prieto et al., 1999). Class III and Class IV PHA synthases consist of additional PhaE and PhaR subunits respectively. Both Class III and Class IV PHA synthases involve in SCL-PHA accumulation. Under some specific conditions, microorganisms such as *Aeromonas* sp. and *Pseudomonas* sp. can accumulate SCL/MCL-PHA in the presence of Class I and Class II PHA synthases (Li et al., 2019a; Preusting et al., 1993).

2.1.4 Intracellular degradation of PHA

Intracellular PHA exist as mobile amorphous elastomeric membrane-enclosed inclusion. The PHA inclusions (also known as PHA granules) inside bacterial cells are neither solid nor liquid and are not in crystalline structures (Barnard & Sanders, 1989; Obruca et al., 2020). PHA are known as biodegradable plastics because it is degradable by depolymerase enzymes. PHA depolymerase enzymes are secreted by microorganisms intracellularly and extracellularly, which enable the degradation of PHA to occur in either way.

Generally, PHA depolymerase acts to hydrolyse water-insoluble PHA into water-soluble forms to be utilizable by the microorganisms. PHA depolymerase consists of a catalytic domain and a substrate-binding domain connected by a linker domain. The substrate-binding domain binds to the crystalline PHA and triggers the subsequent cleavage of the polymer chain by the catalytic domain (Mohanan et al., 2020; Numata et al., 2009; Sudesh et al., 2000). As PHA depolymerase enzymes are secreted by various microorganisms, PHA can be found degradable in environments with high microbial activity such as soil, sewage sludge, lake water, and marine environment (Lee & Choi, 1999; Mergaert et al., 1993; Hung et al., 2022; Mohanan et al., 2020; Syahirah et al., 2020).

Intracellular degradation of PHA takes place when microorganisms experience stress under carbon-limiting conditions. To survive, accumulated PHA granules are hydrolysed to provide carbon and energy (Luengo et al., 2003; Madison & Huisman, 1999; Obruca et al., 2020). PHA is first hydrolysed to a monomeric component, 3-hydroxyalkanoic acid by PHA depolymerase and oligomer hydrolase (Kobayashi et al., 2005; Morohoshi et al., 2020). By taking P(3HB) as an example, its intracellular

degradation resulted in the liberation of 3-hydroxybutyric acid which is subsequently oxidized by a dehydrogenase to acetoacetyl-CoA. The resulting acetoacetyl-CoA is converted to acetyl-CoA by *β*-ketothiolase to be metabolised (Eggers & Steinbüchel, 2013; Lemes et al., 2015). Upon hydrolysis, PHA granules will decrease in size with no changes in granule number or vary for both granule size and number. As the breakdown products of PHA are non-toxic and can be found abundantly and naturally in animals, it is also categorized as biocompatible (Low, 2003; Mohanan et al., 2020).

There are two enzymes that are important for PHA-producing microorganisms to survive harsh environments. Bacterial PHA synthase contributes to the formation of PHA whereas PHA depolymerase causes the degradation of PHA. Therefore, it is crucial to determine the rate of PHA hydrolysis and the rate of PHA synthesis to avoid PHA hydrolysis in PHA production processes. By referring to *C. necator*, the rate of PHA hydrolysis is ten times slower than the rate of PHA synthesis in a nitrogen-free medium. PHA depolymerase exerts the highest activity at the early P(3HB) synthesis stage but subsequently decreases and remains at a lower level before decreasing further at the stationary phase. However, its specific enzyme activity increases with the addition of nitrogen or the depletion of carbon sources in the medium (Doi et al., 1992; Obruca et al., 2020). The finding provides an important insight into why PHA is not accumulated due to the demand for growth and survival.

2.1.5 Extracellular degradation of PHA

Extracellular degradation of PHA occurs when extracellular PHA depolymerase enzymes are secreted by microorganisms to hydrolyse solid PHA in their surrounding environment. Unlike intracellular PHA depolymerase enzymes, extracellular

depolymerase enzymes hydrolyse PHA with crystalline structures and their enzymatic structures are more complicated compared to the former ones (Handrick et al., 2001; Tseng et al., 2006; Zaheer & Kuddus, 2018).

Extracellular depolymerase enzymes consist of signal peptides (22–58 amino acids) for the translocation of the enzyme. It has three functional domains, which are the catalytic domain with 320–400 amino acids, the linker domain with 50–100 amino acids, and the substrate-binding domain with 40–60 amino acids from the N-terminus to the C-terminus. Extracellular depolymerase enzymes are further categorized into Type I and Type II depolymerase based on their catalytic domain. The difference between them is the sequential order of active amino acids which formed the catalytic triad and their specific locations. The catalytic triad of Type I extracellular PHA depolymerase starts from the N-terminus to the C-terminus whereas the catalytic triad of Type II extracellular PHA depolymerase is located at the N-terminus (Jaeger et al., 1995; Jendrossek & Handrick, 2002; Zaheer & Kuddus, 2018).

Interestingly, several lipases are capable of hydrolysing PHA and may share a similar substrate hydrolysis mechanism as PHA depolymerase enzymes. These lipases are specifically prone to polymers without side chains in the carbon backbone, for example, poly(6-hydroxyhexanoate) and poly(4-hydroxybutyrate) (Jaeger et al., 1995; Sharma et al., 2019).

In general, PHA tend to degrade more rapidly in a region with abundant PHA-degrading microorganisms. Despite the abundance of microbial population, colonization of the PHA-degrading microorganisms must take place before the secretion and release of PHA depolymerase enzymes can occur (Sang et al., 2000; Zaheer & Kuddus, 2018). The availability of oxygen is also a key factor as the PHA

degradation rate is faster under aerobic conditions (Martínez-Tobón et al., 2018; Voinova et al., 2008). Furthermore, PHA degradability increases in parallel with increasing temperature (Martínez-Tobón et al., 2018; Volova et al., 2010). However, PHA degradation is dependent on a lot of other factors such as the type of microorganisms, environments, and climatic conditions (Boyandi et al., 2013; Doi et al., 1992; Fernandes et al., 2020; Martínez-Tobón et al., 2018; Quinteros et al., 1999; Zaheer & Kuddus, 2018). Some of the discovered PHA-degrading microorganisms and their respective environments are summarized in Table 2.4.

Table 2.4 PHA-degrading microorganisms from different environments

Environment	**PHA-degrading microbe**	**References**
Soil	*Acidovorax* sp., *Aspergillus fumigatus*, *Comamonas* sp., *Cupriavidus* sp., *Pseudomonas lemoignei*	Hori et al., 2020; Jung et al., 2018; Syahirah et al., 2020;
Activated sludge	*Cupriavidus* sp., *Pseudomonas* sp.	Hung et al., 2022; Tan et al., 2015
Seawater	*Comamonas testosterone*	Mukai et al., 1993
Marine soil	*Bacillus* sp.	Cho et al., 2021
Anaerobic sludge	*Ilyobacter delafieldii*	Janssen & Harfoot, 1990
Lake water	*Alcaligenes faecalis*, *Cupriavidus* sp., *Ideonella* sp., *Pseudomonas stutzeri*,, *Streptomyces* sp.	Mukai et al., 1994; Syahirah et al., 2020

There are some interesting findings worth discussing regarding extracellular PHA biodegradation. First and foremost, a copolymer is easier to be disintegrated by microorganisms compared to a homopolymer. This is because of the nature of homopolymer which is highly crystalline. The presence of another monomer decreases the polymer crystallinity and thus increases the degradation rate (Iwata et al., 1999; Mergaert et al., 1993).

In addition, PHA with longer side chains were found to show better biodegradability and the same explanation is assumed (Li et al., 2007; Zaheer & Kuddus, 2018). Besides, high molecular weight ceases PHA degradation as high molecular weight causes a sharp decrease in solubility and thus making the polymer hostile to microbial enzymatic attack (Doi, 1990; Gu et al., 2000; Zaheer & Kuddus, 2018). PHA degradation is also affected by its shape where the biodegradation rate of polymer films is higher than that of PHA pellets (Fernandes et al., 2020; Volova et al., 2010).

2.1.6 Biocompatibility of PHA

PHA are *in vivo* biocompatible due to the presence of their breakdown products in a wide range of organisms, from bacteria to higher mammals. P(3HB) or 3-hydroxybutyric acids is a ketone body that is synthesized from acetyl-CoA in the human liver and can be used as an energy source by the human brain when the blood glucose level is low. Poly(3-hydroxyvalerate) [P(3HV)] or 3-hydroxypentanoic acid is a ketone body that resulted from the condensation of propionyl-CoA with acetyl-CoA by thiolase enzymes and acts as an indicator for methylmalonic acidemia and propionic acidemia (Nyhan et al., 2020; Sweetman et al., 1978; Zhou et al., 2018). P(3HB) and P(3HB-*co*-3HV) do not affect platelet responses and do not activate the complement system when in contact with blood, thus are hemocompatible (Jirage et al., 2013; Mohandas et al., 2021). Besides, the local pH of P(3HB) scaffolds local remains unchanged during degradation, thus they do not trigger immune response such as that reported for polylactide-*co*-glycolid (PLGA), polyglycolic acid (PGA), and polylactic acid (PLA) (Koller, 2018b).

PHA films possess surface properties that favour cell proliferation and attachment, enabling their applications as scaffolding materials in tissue engineering (Chang et al., 2014; Pecorini et al., 2022; Shishatskaya & Volova, 2004). PHA open porous microspheres of 300−600 μm diameter with surface pores of 10–60 μm and average interconnected passages size of 8.8 μm show high *in vitro* cell adhesion of 93.4%, encourages continuous cell proliferation and migration of more living cells to the site with damaged tissues (Wei et al., 2018). Subcutaneous implantation of P(3HB) film of different molecular weights from 300 to 1500 kDa shows relatively low tissue reaction to films which denotes high biocompatibility *in vivo* (Jirage et al., 2013; Koller, 2018b; Sevastianov et al., 2003). P(3HB) natural oligomers are present in animal tissues at normal conditions and are absent for chemically synthesized biodegradable polymers such as polylactides and polyglycolides. Owing to its biocompatibility, P(3HB) has been approved by the Toxicological certificate of the Institute of Medical Technique (Ministry of Health, Russia) for medical application as a non-toxic and biocompatible mate (Jirage et al, 2013).

Although PHA are demonstrated to be biocompatible, prior purification and careful processing are necessary to meet the requirements for biomedical usage. Microbial components such as cell debris and metabolites must be removed through purification before PHA processing. Purification is crucial, especially for Gram-negative bacteria with endotoxins constitute lipopolysaccharides (LPS). LPS are heat-resistant components located at the outer cell membrane of Gram-negative bacteria and are liberated during PHA extraction steps, where the bacterial cells are lysed (Sampath, 2018). PHA contaminated by LPS trigger an inflammatory reaction, activates blood coagulation and the complement reaction (Koller, 2018b; Ran et al.,

2019; Sampath, 2018). Therefore, endotoxin removal, repeated dissolving and precipitation of PHA are needed to guarantee the high purity of PHA.

Besides, extracted PHA are also prone to contamination by various organic solvents used in PHA extraction and precipitation. Residues of chloroform for PHA extraction and methanol for PHA precipitation could be cytotoxic if the contaminated PHA are used for *in vivo* applications. Residual chloroform could modify the properties of the cell membrane lipid matrix that may lead to cell death and residual methanol could exert an inhibitory effect on cell proliferation at a concentration of more than 10% (Miller & Pang, 1976; Nguyen et al., 2020; Turkyilmaz et al., 2009). Complete removal can be achieved by ensuring the complete evaporation of residual solvents from the PHA pellets, considering the volatile nature of these solvents, followed by proper washing.

2.1.7 Applications of PHA

The biodegradability of PHA is important for agricultural, medical, and commodity applications. Mulching contributes to higher crop productivity, better horticulture products, minimized water evaporation from the soil, lower risk of soil erosion, reduced water consumption, and weed control (Espi et al., 2006). PHA-based mulch films overcome the negative environmental impacts caused by low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), and high-density polyethylene (HDPE) with poor biodegradability (Sarkar et al., 2018). Herbicides and insecticides can also be integrated into PHA-based pellets and sown along the plantation to be released upon degradation from the pellets depending on the level of pest activity (Anunciado et al., 2021; Philip et al., 2007; Yogesh et al., 2012).

Besides, PHA provide gradual *in vivo* biodegradation that corresponds to tissues regeneration or drug release for applications such as implants for as implants to support bones, stents to support arteries in angioplasty, and drug delivery carriers due to enzymatic attack on the amorphous regions without triggering host immune response (Naser et al., 2021). The 3HV fraction of P(3HB-*co*-3HV) contributes to a greater amorphous region for enzymatic attacks that leads to enhanced and adjustable biodegradability for applications such as implants for bone support, stents for artery support in angioplasty and drug delivery carriers. As compared to poly(lactic acid) which is another biodegradable aliphatic polyester of great biotechnological importance, P(3HB-*co*-3HV) has a 2-fold lower maximum water permeability, causing lower hydrolytic degradation due to lower water uptake. However, the degradation rate of P(3HB-*co*-3HV)-based biomedical devices are adjustable with the molar fraction of 3HV (Ali & Jamil, 2016; Laycock et al., 2013; Naser et al., 2021). Hydrophilic poly(ethylene glycol) and ascorbic acid can also be incorporated into P(3HB-*co*-3HV) to form materials with a hydrophilic outer layer and hydrophobic inner layer to improve chemical functionalization and compatibility with therapeutic drugs besides benefiting drug release control (Catoni et al., 2013; Bhatia et al., 2019).

Furthermore, PHA are applicable as packaging materials. P(3HB-*co*-3HV) is a potential substitute for polypropylene owing to its comparable melting temperature, glass transition temperature, Young's modulus, elongation at break and tensile strength to those of polypropylene (Możejko-Ciesielska & Kiewisz, 2016). P(3HB-*co*-3HV) also displays high water and aroma (limonene and linalool) barrier properties which make it suitable for food packaging (Cava et al., 2006; Chen et al., 2011; Cherpinski et al., 2018). As polypropylene and LDPE are applied extensively for packaging and consumables, which are highly disposable, the substitution with PHA

can contribute to reduced stable solid waste creation of petroleum-based plastics (Alsabri et al., 2022; Sen & Raut, 2015). Poly(butylene succinate), poly(butylene adipate-co-terephthalate), natural rubber, or other polymers with plasticizer or toughness properties can be incorporated to overcome the limitations and extend their application as packaging materials (Javadi et al., 2010; Meereboer et al., 2021; Tao et al., 2009; Zhao et al., 2013; Zhao et al., 2019).

Besides, endogenous P(3HB-*co*-3HV) can act as the electron donor for microbial denitrification of wastewater in the aquaculture industry where biomass with PHA-accumulating ability, generally P(3HB) and P(3HV), from activated sludge can be employed to remove resulting ammonia from fish excretion and dead animal bodies in circulating water. As the biomass is precultured for PHA accumulation, the addition of acetate and ethanol in conventional techniques was not required to promote microbial activity. The denitrification using endogenous PHA can be carried out accurately, coupling with slow metabolic activity in the absence of an exogenous carbon source and the presence of nitrogen (Krasnits et al., 2013; Santorio et al., 2019). Without the addition of volatile fatty acids during the denitrification process, contamination with the dissolved organic carbon that lowers the effluent water quality can be prevented. The application of endogenous PHA as the carbon source for denitrifying bacteria is also more cost-effective compared to extracted PHA (Hiraishi & Khan, 2003; Liu et al., 2022). Furthermore, applications of P(3HB-*co*-3HV) incorporated with other desired properties are shown in Table 2.5.

Table 2.5 P(3HB-*co*-3HV)-based biomaterials and their potential applications

3HV fraction (mol%)	Incorporated components	Incorporation method	Potential applications	References
10	*α*-P(3HB)	Solvent casting	Packaging material	Scandola et al., 1997.
8–24	Hydroxyapatite	Melt-pressing	Bone implant	Galego et al., 2000
7	Poly(*ε*-caprolactone)	Solvent casting	Packaging material	Chun & Kim, 2000.
14	Poly(butylene succinate)	Solvent casting	Packaging material	Qiu et al., 2003.
1	Poly(d,l-lactide)	Compression molding	Biomedical, agricultural and packaging material	Wang et al., 2008.
5	Poly(propylene carbonate)	Solvent casting	Packaging material	Tao et al., 2009.
–	Poly(butylene adipate-*co*-terephthalate)	Conventional injection molding or microcellular injection molding	Packaging material	Javadi et al., 2010.
12 & 33	Monomethoxy poly(ethylene glycol)	Transesterification	Drug delivery carrier	Shah et al., 2010
4	Organophilic attapulgite	Solvent casting	Packaging material	Thiré et al., 2011
13	Poly(butylene succinate) & dicumyl peroxide	Compression molding	Packaging material	Ma et al., 2012.
4	Poly(ethylene glycol)	Solvent casting	Drug delivery carrier	Catoni et al., 2013
–	Poly(lactic acid) & nanoclay	Twin screw extrusion	Packaging material	Zhao et al., 2013
12	Poly(2-hydroxyl ethyl methacrylate) & collagen	Solvent casting followed by solute leaching technique	Bone implant	Bakare et al., 2014
3 & 18	Zinc oxide	Melt-mixing, electrospinning, or coating	Active food packaging and food contact surface applications	Castro-Mayorga et al., 2017
12	Cellulose nanocrystals	Solvent casting	Packaging material	Malmir et al., 2017
3	2-methyl-4-chlorophenoxyacetic acid	Melt-blending and hot-pressing	Mulch	Kwiecie et al., 2018
59	Ascorbic acid	Solvent casting	Therapeutic implant	Bhatia et al., 2019
3	Natural rubber	Twin screw extrusion	Packaging material	Zhao et al., 2019
4	Eugenol	Electrospinning	Antimicrobial food packaging	Figueroa-Lopez et al., 2020
–	Poly(ethylene glycol)	Solvent casting	Skin grafting	Pillai et al., 2020
6	Starch, cellulose, or alginate	Solvent casting	Mulch	Syahirah et al., 2020
–	Zinc oxide	Laser 3D molding	Bone repair	Shuai et al., 2020
5	Distillers' dried grains with solubles or *Miscanthus*	Twin screw extrusion	Packaging material	Meereboer et al., 2021
2	Poly(lactic acid) & carbon nanotubes	High-speed spinning	Electrical and electromagnetic	Silva et al., 2021

2.1.8 PHA as the next generation plastics

The urge to search for alternatives to petroleum-based plastics which caused a huge burden on solid waste management has led to increased attention on PHA. Markets and Markets released a report in 2019 that estimated the global PHA market size is projected to reach USD 98 million by 2024 from USD 57 million in 2019. The growing compound annual growth rate (CAGR) of 11.2% indicates the increasing demand for biodegradable plastics for sustainable development and a circular economy. The PHA industry is projected to succumb to the global demand for food packaging and services by producing more biodegradable plastic bags, sheets, and disposable cutlery to reduce mass waste creation. SCL-PHA is estimated to lead the PHA market in terms of value and volume due to extensive studies conducted and easy availability compared to MCL-PHA (Markets and Markets, 2019). Several PHA manufacturers and their main manufacturing products are summarized in Table 2.6.

Table 2.6 PHA manufacturers over the world

Manufacturers	**Trademarks**	**Feedstock**	**PHA producers**	**PHA**	**References**
Biomer, Germany	Biomer	Renewable resources	*Alcaligenes latus*	P(3HB)^{S}	Hänggi, 2009
PHB Industrial S.A., Brazil	Biocycle	Saccharose	*Bacillus* sp.	P(3HB)^{S}	Biocycle (n.d.)
Jiang Su Nan Tian, China	Jiangsu Nantian	Glucose	*Escherichia coli*	P(3HB)^{S}	Riaz et al., 2021
TianAn Biopolymer, China	Enmat	Dextrose or glucose and propionic acid	*Cupriavidus necator*	P(3HB-*co*-3HV)^{S}	Enmat (n.d.)
Kaneka Corporation, Japan	Kaneka PHBH	Plant oils	*Cupriavidus necator*	P(3HB-*co*-HHx)^{M}	Kaneka. (n.d.).
Tianjin GreenBio Materials, China	GreenBio	Satrch and glucose	*Escherichia coli*	P(3HB-*co*-4HB)^{S}	Riaz et al., 2021
ETH, Switzerland	PHA	Fatty acids	*Pseudomonas putida*	MCL-PHA	Riaz et al., 2021

Poly(3-hydroxubutyrate) [P(3HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-HHx)], poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-*co*-4HB)]
^{S}SCL-PHA, ^{M}MCL-PHA

2.2 Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)]

2.2.1 P(3HB-*co*-3HV) structure and properties

P(3HB) is a SCL homopolymer that is relatively stiff (*Em* above 1 GPa) and brittle due to poor elongation at break (below 10%) (Amini et al., 2020; El-Hadi et al., 2002). P(3HB) is a fragile material and its mechanical properties deteriorate with time due to secondary crystallization accompanied by ageing at room temperature and this phenomenon has been described as the major cause for its brittleness (De Koning & Lemstra, 1993; Sirohi et al., 2021). P(3HB-*co*-3HV) is a SCL copolymer consisting of a C_4 3HB monomer and a C_5 3HV monomer. The properties of P(3HB-*co*-3HV) are determined by the ratio of the monomers where the 3HB monomer contributes stiffness and the 3HV monomer contributes flexibility. The incorporation of the 3HV monomer leads to the disruption of P(3HB) crystallinity by causing defection of the P(3HB) lamellae crystals, thus P(3HB-*co*-3HV) improved polymer flexibility compared to P(3HB). With decreased crystallinity, the copolymer displays decreased stiffness, decreased brittleness, and enhanced biodegradability compared to that of P(3HB) (Laycock et al., 2013; Naser et al., 2021). The higher degradation rate of P(3HB-*co*-3HV) that is directly proportional to the molar fraction of 3HV is related to its lower degree of crystallinity and melting point compared to that of P(3HB) (Naser et al., 2021). The chemical structure of P(3HB-*co*-3HV) is illustrated in Figure 2.2.

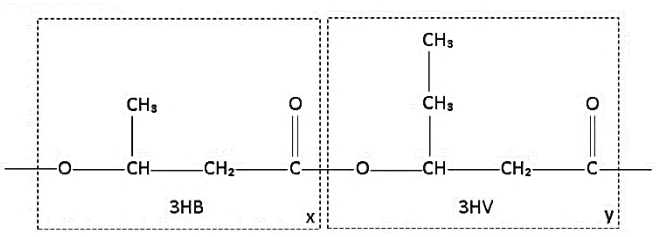

Figure 2.2 Chemical structure of P(3HB-*co*-3HV) (PubChem CID: 107801)

P(3HB-*co*-3HV) has several physicochemical properties that are comparable to polypropylene. Its melting point, glass transition temperature, Young's modulus, and elongation at break which is close to that of polypropylene make it a potential substitute for polypropylene. As polypropylene is applied widely for packaging and consumables which are highly disposable, the substitution by P(3HB-*co*-3HV) can contribute to reduced stable solid waste creation by petroleum-based plastics. The physicochemical properties of P(3HB-*co*-3HV) and polypropylene are summarized in Table 2.7 (Możejko-Ciesielska & Kiewisz, 2016).

Table 2.7 Physicochemical properties of P(3HB-*co*-3HV) and polypropylene

Properties	**P(3HB-*co*-3HV)**	**Polypropylene**
Melting temperature (°C)	137–170	176
Glass transition temperature (°C)	10 – –6	–10
Young's modulus (GPa)	0.7–2.9	1.7
Elongation to break (%)	30–38	38
Tensile strength (Mpa)	up to 690	400

2.2.2 Microbial biosynthesis of P(3HB-*co*-3HV)

The main carbon source and precursor carbon source must be supplied to the bacteria to produce P(3HB-*co*-3HV). The main carbon source contributes to the 3HB fraction of the copolymer in addition to biomass generation from resulting acetyl-CoA. Under normal growth conditions, acetyl-CoA resulting from main carbon source utilization enters the tricarboxylic acid (TCA) cycle for cellular growth. However, acetyl-CoA accumulates inside the bacterial cell under nitrogen-limiting conditions and the excess acetyl-CoA triggers the enzymes responsible for P(3HB) production (Majid et al., 1999; Policastro et al., 2021).

Acetyl-CoA needed for both bacterial growth and 3HB monomer formation is generated from glycolysis of carbohydrates such as sugar and β-oxidation of fatty acids (Huijberts et al., 1992; Policastro et al., 2021). Unlike directly utilizable sugars, lipase