VALORIZATION OF WASTE TRANSFORMER OIL AS ALTERNATIVE CARBON SUBSTRATE FOR POLYHYDROXYALKANOATE PRODUCTION BY TRANSFORMER OIL-DEGRADING BACTERIA

IDRIS SHEHU

UNIVERSITI SAINS MALAYSIA

2023

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by

IDRIS SHEHU

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

May 2023

ACKNOWLEDGEMENT

I would like to begin by expressing my gratitude to Allah SWT for sparing my life and the opportunity to accomplish this memorable academic journey. I would also like to thank my main supervisor, Professor Dato' Dr. Amirul Al-Ashraf Abdallah, for the excellent guidance throughout my PhD program. I will forever remain grateful for your kindness. To my co-supervisor, Associate Prof. (Dr.) Rashidah Bint Abdul-Rahim, I thank you very much for your support and encouragement. Likewise, I am also grateful for the assistance rendered by a senior colleague, Dr Kai Hee, and other Lab mates, including Noor Aida Omar, Jeremy Whong, Aiman Hakimi, Musa Ibn Abbas, Rozina Kakar, Noor Julia Akmar, and Priyanka. You people have made my stay in USM very memorable.

My sincere appreciation goes to the Government of the Federal Republic of Nigeria and specifically to the Management of Kaduna State University for the provision of sponsorship through TETFund AST&D intervention. I am indebted to all those that contributed in one way or the other. A special dedication to my late parents, may Allah SWT reward them for the moral training I received during their lifetime. I would like to also thank my wife Rukayya for the love, patience, and support throughout the program; you are indeed a motivator. To my lovely children Abdulmajid (Yaya), Nana-Fatima (Umma), Yusuf, Hafsat, and Aliyu (Haidar) who endured the absence of their father for quite a long time, I love you all. You people have brought great joy into my life. I am also indebted to my mentor, Prof. S. U. Abdullahi, thank you for all you have done for me. Lastly, I want to express my gratitude to the developers of the USM thesis template, whose product contributed immensely to the formatting of this document.

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LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Degree Celsius
~	Approximately
C_L	Dissolved oxygen concentration
γ	Gamma
g/L	Gram per litre
g/mL	Gram per millilitre
ΔH_m	Heat of Fusion
H_2SO_4	Hydrogen tetraoxosulphate
J/g	Joule per gram
$MgSO_4$	Magnesium Sulphate
µg	Microgram
µL	Microliter
mol%	Mol Percent
%	Percent
M_w/M_n	Polydispersity index
v/v	Volume per volume
w/v	Weight per volume
H_2O	Water
w/w	Weight per weight
wt%	Weight Percent

LIST OF ABBREVIATIONS

ABI	Application binary interface
ANOVA	Analysis of variance
ASTM	American society for testing and materials
bp	Base pair
BLAST	Basic local alignment tool
C	Carbon
CO_2	Carbon dioxide
CDW	Cell dry weight
cm	Centimeter
CME	Caprylic methyl ester
CFU	Colony-forming unit
CoA	Coenzyme A
Da	Dalton
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
DSC	Differential scanning calorimeter
DTA	Differential thermal analysis
EDTA	Ethylene diamine tetra acetic acid
FID	Flame ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography with mass spectrometry
g	Gram
GPa	Gigapascal
GPC	Gel permeation chromatography
H	Hydrogen
hr	Hour
HMDS	Hexamethyldisilazane
3HHD	3-Hydroxyhexadecanoate
3HOD	3-Hydroxyoctadecanoate
IS	Internal Standard

kbp	Kilo base pairs
kDa	Kilo Dalton
kg	Kilo gram
L	Litre
Ltd.	Limited
LCFAs	Long-chain fatty acids
lcl-PHA	Long-chain length polyhydroxyalkanoates
scl-PHA	Medium-chain length polyhydroxyalkanoates
min	Minutes
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimetre
*M*n	Number-average molecular weight
Molar	Molar
MPa	Megapascal
MSM	Mineral salt medium
M_w	Average molecular weight
NA	Nutrient agar
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NaOH	Sodium hydroxide
NB	Nutrient broth
NCBI	National center for biotechnology information
ng	Nanogram
NR	Nutrient-rich
OA	Oleic acid
OD	Optical density
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
phaA	gene encoding *β*-ketothiolase
phaB	gene encoding acetoacetyl-CoA dehydrogenase
PhaC	gene encoding PHA synthetase

PLA	Polylactic acid
PO	Palm oil
ppb	Part per billion
ppm	Part per millions
psi	Pound per square inch
PTFE	Polytetrafluoroethylene
rpm	Revolutions Per Minute
RCDW	Residual cell dry weight
rcf (*xg*)	Rotation centrifugation force
RNA	Ribonucleic acid
RSM	Response surface methodology
sec	Seconds
SD	Standard deviation
SEM	Scanning electron microscope
sp.	Species
TAE	Tris acetate EDTA
T_d	Decomposition temperature
T_g	Glass Transition temperature
TGA	Thermal gravimetry analysis
TE	Trace element
TEM	Transmission electron microscope
UV	Ultraviolet
vvm	Volume per volume per minute
WTO	Waste transformer oil

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PENGGUNAAN SISA MINYAK TRANSFORMER SEBAGAI ALTERNATIF KARBON SUBSTRATE UNTUK PENGHSILAN POLIHIDROKSIALKANOAT OLEH BAKTERIA PENGHURAI-MINYAK TRANSFORMER

ABSTRAK

Masalah pencemaran alam sekitar yang semakin meningkat akibat pengumpulan sisa plastik sintetik telah menimbulkan kebimbangan yang besar. Oleh itu, usaha berterusan diperlukan untuk menyediakan alternatif yang lebih baik kepada plastik tidak terbiodegradasi. Walau bagaimanapun, kos pengeluaran pengganti terbiodegradasi, mesra alam dan lebih baik seperti polihidroksialkanoat kekal tinggi. Penyelidikan ini dilakukan bertujuan untuk menilai kesesuaian sisa minyak transformer sebagai alternatif substrat karbon untuk pengeluaran polihidroksialkanoat. Sejumlah dua belas (12) sample terdiri dari tanah, air sisa dan sedimen dikumpulkan secara aseptic diambil dari pelbagai lokasi sekitar Pulau Pinang, Malaysia untuk mengasingkan bakteria untuk kajian ini. Sebanyak enam puluh dua (62) koloni tulen bakteria pengurai-minyak transformer berjaya dipencilkankan dari sampel menggunakan medium garam mineral dengan tambahan 1%(v/v) sisa minyak transformer sebagai satu-satunya sumber karbon. Mikroorganisma terpencil pada mulanya disaring untuk melihat kemampuan penghasilan polihidroksialkanoat menggunakan pewarnaan *Nile red*, mikroskop *fluorescence*, dan analisis kromatografi gas. Pengumpulan granul PHA di dalam sel disahkan menggunakan mikroskop transmisi elektron (TEM). Penguraian minyak oleh bakteria dinilai menggunakan kaedah pengekstrakan pelarut dan gravimetri. Dari dapatan kajian, enam belas 16 (26%) isolat menunjukkan reaksi terhadap pewarnaan *Nile red* dan mikroskop

fluorescence. Analisis turutan fenotip dan gen 16S rRNA terhadap isolat ini menunjukkan bahawa mereka tergolong dalam empat genera bakteria yang berbeza iaitu *Acinetobacter* (10), *Serratia* (3), *Proteus* (1) and *Bacillus* (2). Peratusan penguraian minyak bagi mikroorganisma ini berjulat 19.58 ± 1.73 hingga 57.51 ± 2.06. Analisis GC menunjukkan kandungan PHA bagi mikroorganisma terpencil berkisar antara 7 hingga 33% b/b. Manakala analisis GC/MS dan NMR polimer yang ditulenkan menunjukkan kehadiran polihidroksialkanoat rantai sederhana (*mcl*-PHA); dengan 3-hidroksiheksadekanoat (3HHD) dan *3-hidroksioktadekanoat* (3HOD) sebagai komponen utama. Biosintesis PHA oleh bakteria terpilih (*Acinetobacter* sp.strain AAAID-15) diperhatikan telah meningkat dari 0.37 ± 0.03 g/l to 0.72 ± 0.24 g/l pada kepekatan sumber karbon masing-masing iaitu 0.5%v/v and 2.0%v/v. Penambahan ekstrak yis ke medium fermentasi pada kepekatan 0.5 g/l didapati telah meningkatkan pengumpulan PHA daripada 0.72 ± 0.24 g/l kepada 1.17 ± 0.15 g/l pada kepekatan sumber karbon yang tetap. Begitu juga, penambahan asid oleik pada kepekatan 0.74%(v/v) meningkatkan kepekatan PHA daripada 0.72 ± 0.24 g/l to 2.37 ± 0.39 g/l. Begitu juga, penambahan minyak sawit sebagai sumber asid lemak telah mempengaruhi pengumpulan PHA secara positif. Proses pengoptimuman telah meningkatkan pengumpulan PHA hingga kepekatan 2.59 ± 0.07 g/l. Berat molekul purata polimer dan polidispersiti index (PDI) masing-masing pada 110 kDa and 2.01. Manakala suhu lebur sekitar 88 ° C. Hasil kajian ini menunjukkan bahawa sisa minyak transformer boleh berfungsi sebagai substrat karbon alternatif untuk biosintesis polihidroksialkanoat dan juga menonjolkan kepelbagaian bakteria penghasil PHA yang mampu menggunakan aliran sisa yang sangat penting untuk kemajuan penyelidikan biosintesis PHA.

VALORIZATION OF WASTE TRANSFORMER OIL AS ALTERNATIVE CARBON SUBSTRATE FOR POLYHYDROXYALKANOATE PRODUCTION BY TRANSFORMER OIL-DEGRADING BACTERIA

ABSTRACT

The growing problems of environmental pollution resulting from accumulation of synthetic plastic wastes have been generating great concern. Consequently, a sustained effort is needed to provide better alternatives to the non-biodegradable plastics. However, the production cost of biodegradable, eco-friendly and better substitutes such as polyhydroxyalkanoates remain high. This research aimed to assess the suitability of waste transformer oil as an alternative carbon substrate for polyhydroxyalkanoates production. A total of twelve (12) samples comprising soil, wastewater, and sediment were aseptically collected from different locations around Pulau Pinang, Malaysia, to isolate bacteria for the work. Sixty-two (62) pure colonies of used transformer oil-degrading bacteria were successfully isolated from the samples using mineral salt medium (MSM) supplemented with 1%(v/v) waste transformer oil as the sole carbon source. The isolated organisms were initially screened for polyhydroxyalkanoates accumulation using Nile red staining, fluorescence microscopy, and gas chromatographic analysis. Accumulation of PHA granules within the cells was confirmed using transmission electron microscopy (TEM). The waste oil degradation potential of the bacteria was assessed using solvent extraction and gravimetric methods. From the results, sixteen 16(26%) of the isolates showed positive reactions to Nile red staining and fluorescence microscopy. Phenotypic and 16S rRNA gene sequence analyses of these isolates indicated that they belong to four

different bacterial genera of *Acinetobacter* (10), *Serratia* (3), *Proteus* (1), and *Bacillus* (2). The percentage of oil degradation of these organisms ranged between 19.58 ± 1.73 to 57.51 ± 2.06. The GC analysis revealed that the PHA content among the isolated organisms ranged between 7 to 33% of their CDW. The data obtained from GC/MS and NMR analyses of the purified polymer indicated that the polymer might have been composed of 3-hydroxyhexadecanoate (3HHD) and 3-hydroxyoctadecanoate (3HOD) as the major monomer constituents. The PHA biosynthesis by the selected bacterium (*Acinetobacter* sp. strain AAAID-15) was observed to have increased from 0.37 ± 0.03 g/l to 0.72 ± 0.24 g/l at a carbon source concentration of 0.5%v/v and 2.0%v/v, respectively. The addition of yeast extract to the fermentation medium at 0.5 g/l concentration was found to have improved the PHA accumulation from 0.72 ± 0.24 g/l to 1.17 ± 0.15 g/l at a fixed concentration of carbon source. Likewise, the addition of oleic acid at a concentration of 0.74%(v/v) increased the PHA concentration from 0.72 ± 0.24 g/l to 2.37 ± 0.39 g/l. Similarly, adding palm oil as a source of fatty acids positively influenced the PHA accumulation. The optimization process improved the PHA accumulation up to a concentration of 2.59 ± 0.07 g/l. The polymer's average molecular weight and polydispersity index (PDI) were 110kDa and 2.01, respectively. The melting temperature was about 88 °C. The findings of this work indicated that waste transformer oil could serve as an alternative carbon substrate for the biosynthesis of polyhydroxyalkanoates and also highlighted the diversity of PHA-producing bacteria capable of utilizing waste streams which is very critical to the advancement of research on PHA biosynthesis.

CHAPTER 1 INTRODUCTION

1.1 Background

Overdependence on conventional plastics has brought about waste accumulation and greenhouse gas emissions. Consequently, recent technologies are directed towards developing bio-green materials that exert significant side effects on the environment (Chee *et al.,* 2010). Intensive research during the past three decades has been focused on developing new materials with a view to overcoming the environmenta1 prob1ems linked with synthetic plastic waste. Microorganisms, especially bacteria, play a significant role in the bioconversion of several wastes to value-added products. The need to develop technologies for producing biopolymers from cheap and renewable resources is well recognized (Cerrone *et al.*, 2014). The utilization of organic and mineral wastes that do not compete with food is critical to the sustainable production and commercialization of biopolymers. The utilization of petrochemical-derived plastics has dominated almost every manufacturing industry ranging from automobile to medicine. Plastics as synthetic polymers are advantageous because their structure can be manipulated to produce a wide range of shapes with considerable strengths. Synthetic polyethene, polystyrene, and polyvinyl chloride are mostly used in the manufacture of plastics. These plastics being xenobiotic, are recalcitrant to microbial degradation (Reddy *et al*., 2003).

As the 21st century approaches, the plastics industry may be experiencing a paradigm shift-a change from an all-petroleum-based industrial economy to one that covers a wider base of materials that include fermentation by-products and plant-derived materials (Rosseto *et al.*, 2020). In the past few years, the choice of bioplastics as a viable substitute for non-biodegradable petroleum-derived plastics has been promulgated to be a promising solution for the problems of chemically synthesized

plastics and their catastrophic impact on the environment. Scientific discoveries over the past few years in the field of bacterial polymer have opened up new opportunities for the rational manipulation of bacteria towards the production of tailor-made biopolymers suitable for industrial applications (Rehm, 2010). Plastics with substantial mechanical integrity as well as excellent durability have been one of the critical issues of the rapid progress in the technology of material science. Petrochemical-based plastics production has increased up to two hundred-fold from one and half million tons around 1950 to about three hundred (300) million tons with an annual growth rate of up to 9% in 2013 (Chanprateep, 2010). However, typical petroleum-derived plastics are non-biodegradable, which mostly gather or aggregate around our environment, a problem that calls for great concern among communities, waste management agencies, and policy makers. Managing such solid waste is a global concern. Although it is hard to completely stop the use of petroleum-based plastics due to their versatile utility, it is possible to substitute or reduce their usage with a better alternative by promoting the production and application of biodegradable polymers with similar material properties (Mohapatra *et al.*, 2017).

Fortunately, several microbes store excess carbon sources as intracellular polyhydroxyalkanoates (PHAs) granules, especially when exposed to stress, such as nutrient limitation (Song *et al.*, 2008). PHAs are nonpersistent polymers that are completely produced by biotechnological processes. Bacterial polyhydroxyalkanoates possess unique properties that can lunch a range of new commercial opportunities (Freitas *et al.*, 2011). These special biopolymers exhibit a wider range of physicochemical properties that arise from the structural variation of their side chains. Consequently, they have been showing outstanding biocompatibility (Pérez-Rivero *et al.*, 2019). The potential use of PHAs as bioplastics is due to their desirable features

such as chiral polymeric structure, high molecular weight, and biodegradable aliphatic esters constituents. PHAs also have properties like those of synthetic plastic in terms of thermal stability and elasticity.

Furthermore, they are biocompatible and not toxic to the environment (Chen and Wu, 2005). However, according to Możejko-Ciesielska and Kiewisz (2016), the cost of carbon sources in producing biodegradable plastics could constitute about 50% of the final production cost. For instance, analysis and economic evaluation confirmed that commercial production of PHAs from 10 carbon atoms alkanes (octane) would cost about US$ 5–10 Kg^{-1} (Singh and Chandel, 2018). Incidentally, microbes, especially bacteria, can produce PHAs from different carbon sources, from simple and inexpensive, complex waste effluents to plant oils, waste engine oil, and other waste oils (Surendran *et al.*, 2020). Simple carbohydrates, alkanes, and fatty acids constitute another suitable carbon feed to produce PHAs (Gong *et al*., 2007). In addition, waste materials generated and discharged from various agricultural and food processing industries form another potential renewable feedstock that researchers have currently explored across the globe. The utilization of such waste streams as carbon sources for PHA production reduces the substrate cost. It correspondingly saves the cost of managing waste, which is often very expensive with a negative impact on the environment. There is no doubt that PHA biosynthesis and its related technologies are creating an industrial value chain ranging from materials, fermentation, energy to medical fields (Chen, 2009).

PHAs and their composites have been reported to have wider applications in tissue engineering. Such applications include the production of suture fasteners, sutures, meniscus repair devices, tacks, rivets, staples, screws (including interference screws), bone plating systems, cardiovascular patches, surgical mesh, slings, repair

patches, orthopaedic pins (including bone filling augmentation material), stents, adhesion barriers, regeneration devices/guided tissue repair, articular cartilage, repair devices, nerve guides, atrial septal defect repair devices, tendon repair devices, pericardial patches, vein valves, bulking and filling agents, meniscus regeneration devices, tendon grafts and ligament, spinal fusion cages, ocular cell implants, skin substitutes, dural substitutes, bone graft substitutes, and hemostats. Changing PHAs compositions allows favourable mechanical properties, biocompatibility, as well as degradation times within required time frames under specific physiological conditions (Chen and Wu, 2005).

1.1.1 Problem statement

The growing land and water pollution problems resulting from the lack of degradability of synthetic plastics have been generating great concern about plastic waste. The capacity of landfill sites is being overstretched. Consequently, with the excessive usage of plastics as well as increasing pressure being mounted on the resources available for the disposal of plastic waste, the need for biodegradable plastics has assumed increasing importance in the last few decades (Shah *et al*., 2008). Petrochemical plastics are derived from non-renewable petroleum resources. These products are incompatible with carbon cycles in our environment. They are recalcitrant to microbial degradation; their excessive molecular size appears to be mainly responsible for the resistance to biodegradation as persistence in soil for a long time (Reddy *et al.*, 2003). Therefore, huge amounts of these synthetic polymers are accumulated as municipal and industrial wastes in the environment.

Furthermore, several aromatic rings, halogens, and unique chemical bonds associated with these compounds make them resistant to biodegradation by microbes. Therefore, these products can remain in the environment for up to 100 years, causing

serious air pollution due to hydrogen cyanide and dioxin from their basic substances, such as polyvinyl chloride and acrylonitrile (Motamedi *et al.,* 2015). Bacteria use numerous enzymes, such as PHA depolymerase, esterases, and lipases, to degrade PHAs in the environment (Kim *et al.,* 2007).

The production of petroleum-based plastic has the great disadvantage of creating non-degradable waste products that are difficult to handle. The non-biodegradable property of these products, particularly their high molecular mass and complex structure, impart a serious environmental problem as they can persist in the soil, water bodies, and landfills for several years. Concern over the damaging effects of such petrochemical-derived plastics in the public domain has increased. This awareness incited a global scientific initiative toward developing alternative eco-friendly biodegradable plastics (Raza *et al.,* 2018). On the other hand, urbanization and the ever-increasing world population require more and extended usage of electric power network; and, by extension, requires more transformers that utilize insulating oil and continuously generate waste oil over time. Also, there has been considerable interest in exploring the diverse approach to reducing biodegradable plastics' production cost that provides a better alternative to synthetic plastics. These approaches include but are not limited to: prospecting and characterizing new and more efficient PHA-producing microbes, exploration for cheap and readily available waste substrates for PHA production, developing cost-effective and reliable analytical techniques for identification and characterization and quantification of PHAs (Tan *et al*., 2014). Therefore, there is a need for a sustained effort to explore microbial resources and cheap substrates to solve the negative impact associated with the accumulation of synthetic plastic waste and waste oil.

1.1.2 Aim and Objectives

This research aims to assess waste transformer oil's suitability as an alternative carbon substrate for bioplastic production.

The specific objectives are:

i. To isolate and characterize PHA-producing bacteria using waste transformer oil.
ii. To optimize the PHA production process.
iii. To characterize the PHA produced by the selected isolate.

1.2 Significant Contribution of the Research

This research contributed to identifying the new and PHA-producing bacteria, and the suitability of waste transformer oil (WTO) as an alternative substrate for biopolymer production has been highlighted. It also provided insight that may help develop cost-effective production strategies for producing the PHAs by optimizing culture conditions to produce the novel PHA. Furthermore, it has provided an alternative means of managing the ever-increasing number of hydrocarbon-based wastes, particularly transformer oil, whose constituents may be toxic and can pose environmental pollution. Therefore, it provided a potential means to offset waste treatment costs through the valorization of the waste oil for PHA production. Above all, the research has contributed to understanding the basic steps involved in the biosynthesis of higher carbon polyhydroxyalkanoates, thereby creating the foundation that can be explored toward improving the economic-production efficiency of this important class of biopolymers with wider applicable material properties.

CHAPTER 2 LITERATURE REVIEW

2.1 Plastics

Historical records revealed that plastic was derived from the Greek word "plastikos," which means 'able to be molded into diverse shapes' (Shah *et al*., 2008). Plastics manufactured presently are made from inorganic and organic raw materials, such as carbon, hydrogen, silicon, nitrogen, chloride, and oxygen. The basic materials used in the production of synthetic plastics are mostly mined from coal, oil, and natural gases (Cabernard *et al*., 2022). Plastics are long-chain polymeric molecules considered practical innovations with enormous usage in daily life (Shah *et al*., 2008). In short, plastics generally have become a vital part of modern life. The materials gain worldwide attention due to their desirable properties that include, among others: flexibility, durability, impermeable to water, as well as low production cost (Aziz *et al*., 2017).

Moreover, the use of plastics is of considerable advantage; because, as synthetic polymers, their structure can be manipulated chemically to have a wide range of shapes and strengths (Reddy *et al*., 2003). Today, plastic materials have been unceasingly and systematically enhanced to make them more stable, resistant to chemical attack, mechanically stronger, and less affected by environmental conditions. In addition, their demand and popularity have increased due to their rust-proof characteristics. Other features that account for the increased usage of synthetic polymers in our daily life include their relatively low price, availability, and a wider range of colouration (Kalia *et al*., 2000).

Furthermore, most available plastics are manufactured synthetically and have better properties than naturally occurring ones. Crude oil and natural gas remain the basic raw materials for modern plastics (Kalia *et al*., 2000; Reddy *et al.*, 2003).

Thermoplastic resins constitute around two-thirds of the global oil-based commodity production and the global usage of these materials grows at about 5% per year (Proshad *et al*., 2018).

Plastics with substantial mechanical integrity and excellent durability have been one of the critical issues of the rapid progress in material science technology. Production of plastics has increased up to two hundred-fold from one and a half million tons around 1950 to about three hundred (300) million tons, with an annual growth rate of up to 9% in 2013 (Chanprateep, 2010). However, typical petrochemical-derived plastics are non-biodegradable and mostly gather or aggregate around our environment. Due to their durability and visibility, plastics have attracted more public and media attention than any other component of a solid waste stream. Due to their non-biodegradable nature, environmentalists campaigned against their production and usage (Kalia *et al*., 2000). As a result, managing waste has become a global concern. Although it is hard to completely stop the usage of plastics due to their versatile utility, it is possible to substitute or reduce their use with a better alternative by promoting the production and application of biodegradable polymers with similar material properties (Mohapatra *et al*., 2017). However, the substitution of these conventional plastics with promising eco-friendly alternatives is limited by their high manufacturing cost, particularly carbon feedstocks. Thus, the search for cheap and suitable feedstocks for PHAs is one of the key issues in their entire production chain (Favaro *et al*., 2019).

Presently, most of the available plastics are produced synthetically, and they have much better properties than naturally occurring ones. The basic raw materials for all modern plastics are crude oil and natural gas. Crude oil is obtained from underground oil deposits or the seabed by offshore drilling. The oil is a blend of heavy hydrocarbons. When subjected to heating under pressure and in the presence of a catalyst, the heavy

oil molecules eventually break down into smaller molecules in a process called catalytic cracking. The mixture of hydrocarbons obtained in the process is subsequently subjected to fractional distillation. The fractions are normally evaporated at different temperatures and can be separately condensed. Naphtha, which closely resembles the petrol used in automobiles, is a fraction mainly used to make various kinds of plastics. Sometimes, the Naphtha is further cracked to get lighter fractions, which serve as raw materials for most common plastics. Polyethene and PVC are the two main plastics manufactured from ethylene gas obtained from the cracking of naphtha.

On the other hand, natural gas, which comes from oil fields, is a source of another important raw material for plastics, called formaldehyde. The formaldehyde used for making rigid types of plastics is made from methanol produced from natural gas. There are numerous other chemicals which also serve as starting materials for many types of polymers and plastics, all of which are derived from crude oil. A typical example is butylene used in making synthetic rubber (Kalia *et al*., 2000). Owing to their recalcitrant nature, the disposal of synthetic plastic materials is becoming increasingly difficult. This problem necessitates the enactment of laws in many developed countries to discourage the usage of synthetic plastic and encourage the use of biodegradable plastics in certain applications. Therefore, it is estimated that bioplastics will account for around 3% of all plastic waste (Kalia *et al.*, 2000).

Considerable effort is being directed toward minimizing the environmental impact brought about by the excessive use of synthetic plastics. Incineration and burial in landfills are typical examples of the current waste management approaches to handle the ever-increasing amount of plastic waste in the environment Although incineration can convert a large fraction of polymer refuse dumped in the landfill, it generate toxic molecules such as dioxins which can do more harm than plastic waste. Another

disadvantage is that the cost of incineration is too high and thus limiting its application (Loo and Sudesh, 2007). These are problems that call for great concern among communities and waste management agencies as well as policymakers.

2.2 Environmental issues and management of transformer oil

Urbanization requires more and extended usage of electric power networks and, by extension, requires more transformers that utilize insulating oil and continuously generate waste oil over time. For a long time, petroleum-based mineral oils have been used in electrical transformers, primarily for insulating purposes; the oil also serves to cool the transformers. However, the long-term usage of transformer oil results in some changes in its physical and chemical characteristics, making it unfit for cooling and insulating purposes. Thus, after being used up, the disposal of waste transformer oil (WTO) from electrical power stations, as well as a large number of electrical transformers located in populated areas and shopping centres throughout the world, is becoming increasingly complex; this is so because it could contaminate waterways and soil if serious spills happen. This problem necessitates the need to look for an immediate solution (Raj *et al.*, 2015). For nearly ten decades, transformer oils have been a source of environmental concern due to their high content in polychlorinated biphenyls (PCBs), which are a blend of theoretically 209 possible isomers. For instance, it was reported that up to 26% of PCBs produced between the year 1929 and 1970 was used for transformer oil formulation else elsewhere (Rojas-Avelizapa *et al*., 1999a). In recent times, many industries have made an effort to exclude PCBs from the transformer oil they produce due to strict environmental regulations. However, the lack of proper storage and disposal of the used oils, inadequate use of these oils, and explosions of transformers can cause a serious environmental pollution problem. Furthermore, toxic,

carcinogenic, and reproductive consequences of PCBs on both humans and animals have been established. (Pelitli *et al*., 2015)

Coincidentally, there is a continuous search for cheaper substrates to produce biodegradable plastics to reduce the existing high production cost that remains a big challenge to the commercialization of this eco-friendly alternative product. There were several attempts to produce bioplastics using oils and waste oils. For instance, waste glycerol (Cavalheiro *et al*., 2009,; Teeka *et al*., 2010), palm oil (Loo *et al*., 2005), crude glycerol (Teeka *et al*., 2012; Posada *et al*., 2011). Synthetic plastics have become an important commodity considered to have improved the quality of human life, replacing packaging materials like glasses and paper (Możejko-Ciesielska and Kiewisz, 2016). Typically, the oil used in electric transformers for insulating purposes consists of a complex blend of over three thousand (3000) hydrocarbons, mostly branched aliphatic, paraffinic, or naphthenic crudes. Considering the harmful environmental effects that this waste oil may cause and the attempt to manage it effectively, researchers have started viewing it as a viable alternative fuel for diesel engines (Prasanna *et al*., 2015) and, recently, as an alternative carbon feed for bioplastic production.

Both aromatic hydrocarbons and polar compounds with high resistance to microbial degradation have been widely used constituents of transformer oils (Furukawa and Fujihara, 2008). A major concern associated with the used transformer oils is their handling and disposal. This issue is critical because of the chemical composition of these oils, most of which are hazardous. These components may include polychlorinated biphenyls (PCBs) such as clothes, aroclors, phenaclors, and kanechlors (Kaanagbara, 2005). Rojas-Avelizapa *et al*. (1999) reported that PCB is a mixture of over two hundred isomers. The legal meaning and inclusion range from chlorinated diphenyl to terphenyl compounds (Kim *et al*., 2000). Therefore, diligence is needed in

their evaluation to avoid violating the existing regulations and laws. PCBs in transformer oils were accepted due to their chemical inertness, heat resistance properties, non-flammability, considerably low vapor pressure, and dielectric properties. However, due to the growing number of reports on detecting PCBs in environmental samples in the early seventies, some countries enacted laws regulating the usage to minimize the release into the environment (Kaanagbara, 2005).

Reuse and recycling are better options for deriving value-added products or energy from waste streams and minimizing management and disposal problems. Various waste oil streams have been valorized to convert waste to wealth. For instance, vegetable oils, palm oil mill effluent, waste glycerol, plastic pyrolysis oil, waste frying oil, etc., have all been tested as a carbon source for the production of biodegradable plastics (Cavalheiro *et al*., 2009; Obruca *et al*., 2010; Teeka *et al*., 2010; Verlinden *et al*., 2011). Likewise, used transformer oil has recently been studied as an alternative fuel (Nabi *et al*., 2013). The effort to harness its usage as an alternative carbon source to produce biodegradable plastics will undoubtedly assist in reducing the problem of its handling and disposal.

2.3 Bioplastics

Bioplastics are polyesters produced from bacterial fermentation that are biodegradable and non-hazardous. These polymers can be produced by a wide variety of bacteria and are made only when stress conditions are established, especially when nutrient levels are low, and more specifically, low levels of nitrogen and oxygen (Nath *et al*., 2008). Bioplastics are an important class of biomaterials valued for their characteristics of quick biodegradation, synthesis using diverse feedstocks, and use of bioprocesses in their production (Stephen *et al.*, 2014). The recognition and better

understanding of the impact of waste problems on the environment have awakened a new interest in the area of degradable plastics (Shah *et al.*, 2008). In response to increasing public concern over environmental threats caused by plastics, several countries around the globe are developing various solid waste management programs aimed at reducing plastic waste through the development of biodegradable plastic materials. This effort is complemented by intense research on biodegradable plastic material. To date, some biodegradable plastic materials under development include Polyhydroxyalkanoates (PHAs), aliphatic polyesters, polylactides, polysaccharides, polycaprolactone, copolymers, and blends of the ones earlier listed (Kalia et al., 2000; C. S. K. Reddy *et al*., 2003). Biodegradable plastics, such as PHAs, etc., have opened the way for a new approach to waste management because these materials are designed to degrade under environmental conditions in waste treatment facilities (Shah *et al.*, 2008; Witt *et al*., 1997). With the looming fossil fuel crisis, the search for substitutes for conventional plastics is crucial in reducing mankind's dependency on fossil resources. Incidentally, one of the potential substitutes is polyhydroxyalkanoates (Tan *et al.*, 2014).

Bioplastics, as a group of biodegradable plastics, can be categorized into three classes that include: photodegradable, semi-biodegradable, and completely biodegradable (Reddy *et al*., 2003). Photodegradable plastics have light-sensitive groups incorporated into the backbone of the polymer as additives. Extensive ultraviolet radiation can disintegrate their polymeric structure within a few months, thereby rendering them open to further degradation by microbial agents (Kalia *et al*., 2000). The second category, semi-biodegradable, is plastics in which starch molecules are incorporated to hold together the short polyethylene fragments. Once discarded, the microbes in the environment can attack the starch, releasing the polymer fragments that

other microbes can subsequently degrade. Microorganisms, particularly bacteria, attack the starch but are turned off by the polyethylene fragments, which may persist for quite a long time as non-degradable (Reddy *et al*., 2003). The third category of biodegradable plastic is rather new and promising due to its susceptibility to microbial mineralization. Such plastics include polyhydroxyalkanoates (PHA), polylactides (PLA), polysaccharides, aliphatic polyesters, copolymers, and blends of these.

According to the European bioplastics data report, the global bioplastics production capacity is anticipated to rise from around 2.1 million tons in 2020 to around 2.8 million tons in 2025. Novel biopolymers, such as bio-based polypropylene (PP) and particularly PHAs (polyhydroxyalkanoates), would continue to drive this growth. The manufacture of polylactic acid (PLA) will also continue to increase due to new investments in its production sites in some world-leading economies such as China, the US, and Europe. The global production capacity of bioplastics 2020 by material type is shown in Figure 2.1

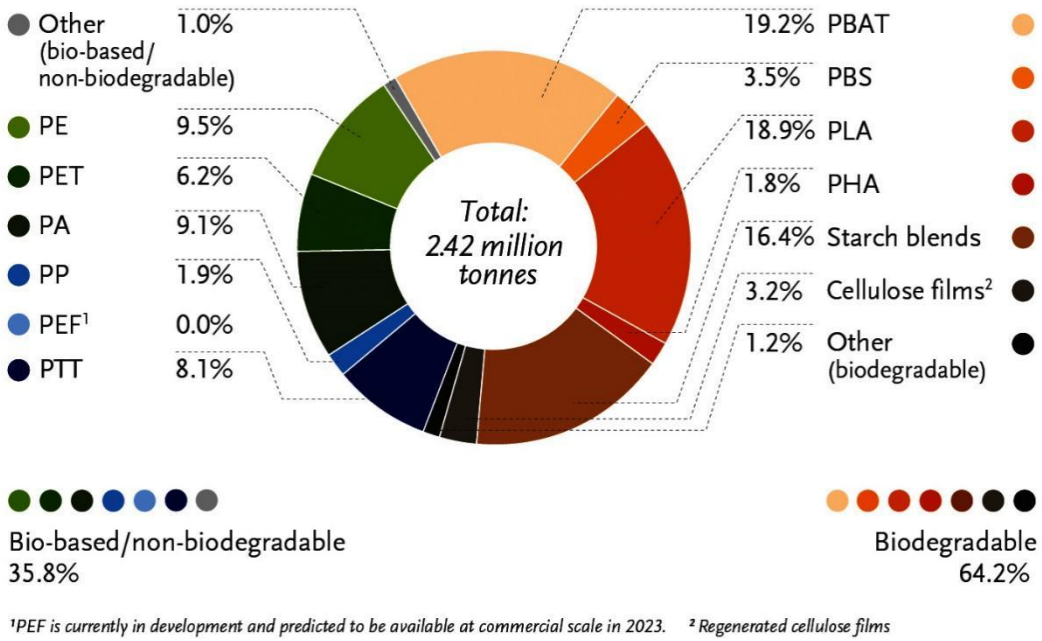


Figure 2.1 Global production capacity of bioplastics 2021 by material type. Source: (European Bioplastics, 2021).

2.4 Historical development of PHAs

Polyhydroxyalkanoates (PHAs) represent a versatile group of prokaryotic reserve materials that display high potential for application in numerous fields of the plastic market, partly due to their plastic-like properties (Koller and Rodríguez-Contreras, 2015). PHAs are synthesized inside the bacterial cell, which exists as granules by polymerizing 3-hydroxy fatty acids through an enzyme PHA synthase (*Pha*C). The granules are formed to serve as carbon and energy storage compounds (Rigouin *et al*., 2019). Although the observation of cellular inclusions in prokaryotic cells such as bacteria was documented as early as 1888 by Beijerinck, PHAs eresaid to have been discovered by a French scientist (Maurice Lemogine) at the Pasteur Institute in the year 1926 while working on a culture of *Bacillus megaterium* (Chee *et al*., 2010; Cheema, 2011; Steinbüchel, 2002; Tan *et al*., 2014; Yellore, 2000). However, even as early as before this discovery, the presence of sudanophilic, lipid-like inclusions that are readily soluble in chloroform was observed in bacteria described as *Azotobacter chroococcum* (Sudesh *et al*., 2000). The inclusions observed in *Bacillus megaterium* were later confirmed to be poly-3-hydroxybutyrate (P3HB). Several reports emerged following the discovery of 3HB that indicate the diversity of microorganisms capable of accumulating this interesting biomolecule. Until around 1974, (*R)*-3-hydroxybutyrate (3HB) was the only PHA constituent of microbial PHA documented; this milestone changed by the discovery of (*R)*-3-hydroxyvalerate (3HV) by Wallen and Rohwedder in bacteria isolated from activated sludge in 1974 (Wallen and Rohwedder, 1972). Members of Gram-positive, Gram-negative, photosynthetic bacteria, archaebacteria, and cyanobacteria have all been reported to accumulate poly-3HB both aerobically and anaerobically (Jau *et al*., 2005; Pantazaki *et al*., 2009). It was not until roughly six decades later that *Pseudomonas putida* GPo1, a bacterium previously

known as *Pseudomonas oleovorans* GPo1, was discovered to produce basic PHBs PHBs with different chemical compositions. Bacterial-produced plastics exhibit different strengths depending on their composition (Alexander, 2001).

Although constituents of PHAs other than 3-hydroxybutyrate (3HB) were reported since in the sixties, such reports did not attract the needed attention until the early eighties when constituents such as 3-hydroxyvalerate (3HV), 3-hydroxyhexanoates (3HHx), and 3-hydroxyoctanoate (3HO) were incidentally detected in axenic cultures of some bacteria isolated from the environment (Steinbüchel, 2002). By the year 1973, the role of Poly-3HB as a bacterial storage polymer with a similar function as glycogen and starch was recognized (Gong *et al*., 2007). In the late 1980s, the first achiral PHA-building block was described by Doi *et al*. Soon, it was realized that the exact composition of PHAs is crucial for their physicochemical properties and degradability, and so for their applicability as "green plastics" (Koller and Rodríguez-Contreras, 2015).

There is no doubt that new PHA constituents will continue to be detected using diverse substrates or other bacterial species. Among the bacteria well studied on their PHA production is *Cupriavidus necator* (formerly known as *Alcaligenes eutrophus*). This bacterium can produce *scl*-PHA consisting of 3HB, 3HV, and 4HB monomers (Donaruma, 1991; Kunioka *et al*., 1989; Saito *et al*., 1996). *Pseudomonas oleovorans* and *P. putida* are also known for their *mcl*-PHA-producing ability, consisting of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) monomer units representing the major components. The most recent approach in this area is the site-directed mutagenesis of genes coding for PHA synthesis. Another approach is 'metabolic design' (Steinbüchel, 2002). This important material (PHA) has attracted a considerable commercial and research interest mainly due to its biocompatibility, biodegradability,

chemical diversity, and its production from renewable carbon resources (Shah *et al*., 2008; Tan *et al*., 2014). Currently, the production of *scl*-PHA at a commercial scale has been established by several companies, such as Monsanto (Kellerhals *et al.*, 2000). On the other hand, *mcl*-PHAs are yet to be fully commercialized due to their relatively low yield compared to *scl*-PHAs (Lee *et al*., 2000).

2.5 Polyhydroxyalkanoates

Among the various forms of biodegradable polymers, polyhydroxyalkanoates is a class that draws considerable attention (Mochizuki, 2005). Polyhydroxyalkanoates are considered biobased and biodegradable materials used as bioplastics for environmental, agricultural, biomedical, and numerous other applications. PHAs are polymers produced by a completely biological process in which carbon substrates are directly converted into PHAs by microbial fermentation (Park *et al.*, 2012a). They are deposited within the cell up to the level of 90% of the cell's dry weight (Jendrossek, 2001). Polyhydroxyalkanoates are often described as naturally occurring and commercially interesting thermoplastics (Tyo *et al*., 2006b). PHAs are a family of linear polyesters of 3, 4, 5, and 6-hydroxy acids synthesized by a diverse group of microbes, especially bacteria, as intracellular carbon and energy reserves; through the fermentation of sugar, lipid, alkanes, alkenes, and alkanoic acids. The production of polyhydroxyalkanoates (PHAs), particularly at a laboratory scale, has been studied for more than eight decades. Recently, some notable driving forces, such as crude oil prices and public awareness of various environmental issues, have inspired extended research on biopolymers, particularly polyhydroxyalkanoates (Keshavarz and Roy, 2010).

To date, the PHA-producing trait is widespread among many bacteria and some other microbes occurring in nearly 100 bacterial and archaeal genera, according to Lu

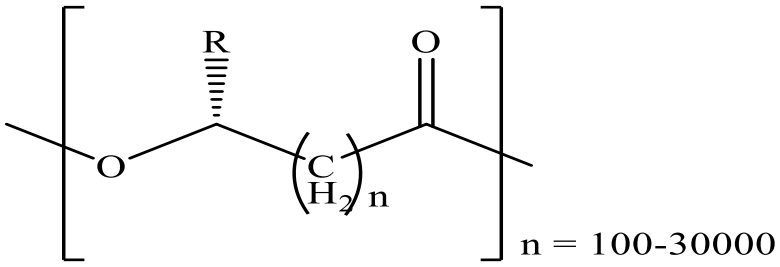
et al. (2009); Poli *et al*. (2011), and Tan *et al*. (2014). The synthesis of these polymers is mostly accomplished under nutrient-limiting conditions with an excess carbon source (Khanna and Srivastava, 2005a; Park *et al*., 2012a; Rehm, 2010; Tian *et al*., 2009). When the supply of the limiting nutrients is re-established, the PHA can be degraded by the intracellular enzyme (depolymerizes) and subsequently utilized as a carbon and energy source (Shah and Alshehrei, 2017). Interestingly, they can be produced using renewable resources, in addition to being biodegradable. PHAs granules are found as discrete cytoplasmic inclusions in the cells. PHA granules within the cells are proposed to be referred to as "carbonosomes" by some leading scientists with the view to underline their complex biological functions. The granules are deposited as spherical inclusions with an amorphous, hydrophobic core surrounded by PHA metabolic proteins (Rehm, 2010). PHAs granules in microbial cells confer stress resistance to microorganisms under famine and environmentally challenging circumstances (Jendrossek, 2009; Koller, 2018b; Obruca *et al*., 2018; Slaninova *et al*., 2018). Upon extraction from the cells, PHAs are known to exhibit thermoplastic and elastomeric properties. They are recyclable natural materials and can easily be degraded. Hence, they can serve as an excellent replacement for petroleum-derived plastics in terms of physical characteristics, processability, as well as biodegradability. In addition, they are biocompatible and therefore have several medical applications (Mochizuki, 2005; Steinbüchel, 2002). PHA complies with the rising global concept of sustainable development and is therefore considered environmentally friendly material. PHAs are valuable materials that can be produced from several renewable carbon sources by microbes, making them sustainable and environmental-friendly materials (Tan *et al*., 2014). PHAs are synthesized via a completely biological process in which the carbon source is directly transformed into PHAs by microbial fermentation. Many bacteria

accumulate PHAs within their cells as energy and carbon storage materials when they encounter limited growth conditions in the presence of excess carbon sources (Park *et al.*, 2012). At a global scale, the polyhydroxyalkanoates (PHAs) market size is predicted to reach up to 23,734.65 metric tons by 2021, at a compound annual growth rate of 6.27%, between 2016 and 2021 (Pérez-Rivero *et al*., 2019). Though PHAs have been known as a good substitute for conventional petrochemical plastics, the high cost of production is a major factor that has limited the wider application of PHAs as commodity plastic. Improving PHAs production strategies, especially through the utilization of appropriate carbon and nitrogen sources at suitable concentrations, can reduce the cost of the final product, suggesting broader use of PHAs in daily life (Kulpreecha *et al*., 2009; Li *et al*., 2007). Choi and Lee (1997) reported that 40–48% of the overall production cost is attributed to raw materials, whereas the carbon source could account for up to 70–80% of the total expense. A production process based on waste carbon sources such as agricultural wastes, and by extension waste transformer oil, etc., are the critical requirement of the day, instead of noble ones. Recently, there has been a dramatic increase in the number of research publications on biosynthesis, characterization, and modification of the PHA family of biopolymers (Philip *et al*., 2007). New developments in PHA biosynthesis indicate that PHA is likely to become the preferred next-generation bioplastic. Nevertheless, to date, the market penetration of this eco-friendly material is still limited (Kunasundari and Sudesh, 2011).

2.5.1 PHAs structure and composition

PHAs are polyesters. A PHA molecule typically comprises 600 to 35,000 (R)-hydroxy fatty acid monomer units. Each monomer unit harbours a side chain R group, which is mostly a saturated alkyl group. However, the side chain can occasionally take the form of unsaturated alkyl groups, substituted alkyl groups, and branched alkyl

groups (Khanna and Srivastava, 2005a; Lu *et al*., 2009; Tan *et al*., 2014). The R functional groups on the PHAs have the ability to switch designation based on the carbon/energy source used during fermentation (Figure 1). The average molecular weight of up to ~5 x 10^9 Da has been documented (Yellore, 2000). Figure 2.2 shows the general structure of PHA molecule.



Value of n	R group	Number of carbons	Resulting polymer
1	Hydrogen	3	Poly(3-Hydroxypropionate)
	Methyl	4	Poly(3-Hydroxybutyrate)
	Ethyl	5	Poly(3-Hydroxyvalerate)
	Propyl	6	Poly(3-Hydroxyhexanoate)
	Butyl	7	Poly(3-Hydroxyheptanoate)
	Pentyl	8	Poly(3-Hydroxyoctanoate)
	Hexyl	9	Poly(3-Hydroxynonanoate)
	Heptyl	10	Poly(3-Hydroxydecanoate)
	Octyl	11	Poly(3-Hydroxyundecanoate)
	Nonyl	12	Poly(3-Hydroxydodecanaote)
	Decyl	13	Poly(3-Hydroxytridecanoate)
	Undecyl	14	Poly(3-Hydroxytetradecanoate)
	Dodecyl	15	Poly(3-Hydroxypentadecanoate)
	Tridecyl	16	Poly(3-Hydroxyhexadecanoate)
2	Hydrogen	4	Poly(4-Hydroxybutarate)
3	Hydrogen	5	Poly(5-Hydroxyvalerate)

Figure 2.2 General structure of PHAs molecules

2.5.2 Nomenclature and classification of PHAs

The naming of PHAs is based on the monomer arrangements in the polymer chains (Rigouin *et al*., 2019). The molecular structure shows that PHAs are composed of repeating units of (R)-3-hydroxy alkanoic acid with varying carbon lengths (Tan *et al*., 2014). Although several criteria are used to classify chemical compounds, PHAs are mostly classified based on the number of carbon atoms in the monomer unit. Based on this criterion, two (2) major classes are short-chain length [*scl*-PHAs] (with repeating units of three to five carbons in length) and medium-chain length [*mcl*-PHAs] (those having repeating units of six to fourteen carbons in length), (Pérez-Rivero *et al*., 2019). In other classifications, a third group is also considered long-chain length polyhydroxyalkanoates (*lcl*-PHAs) comprising polymers whose monomers have 15 or more carbon atoms (Kalia *et al*., 2000; Kato *et al*., 1996; Khanna and Srivastava, 2005a; Lu *et al*., 2009). However, Laycock *et al*. (2013) included PHAs with up to 18 carbon numbers as medium-chain length polymers.

Another classification is based on the kind of monomer present; for this criterion, PHAs are classified into homopolymer and heteropolymer. The former contains only one type of hydroxy alkanoates as the monomer unit, examples of which include poly(3-hydroxybutyrate) and poly(3-hydroxyhexanoate). The latter contains more than one kind of hydroxy alkanoate as monomer units, for example, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate), and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (Asrar *et al*., 2002; Rai *et al.*, 2011). Around 150 different PHA monomers have been discovered and identified. Undoubtedly, more will be added to the list by discovering new PHAs via chemical or physical modification of naturally-occurring ones (Zinn and Hany, 2005). The genetic modification approach has been contributing to and certainly will continue

to contribute toward the successful production of PHAs with specialized functional groups (Escapa *et al*., 2011).

Based on biosynthetic origin, PHAs can be grouped into natural and semisynthetic ones. Natural PHAs are those PHAs produced naturally by microbes from the general substrates, e.g., P(3HB). In contrast, the semisynthetic are those produced naturally but have specific functional groups incorporated through the addition of unusual precursors, e.g., poly(3-hydroxybutyrate-*co*-3-mercaptopropionic) [P(3HB-*co*-3MP)] (Loo and Sudesh, 2007). Table 2.1 shows the physical properties of different classes of PHAs as compared to the properties of synthetic plastics.

Table 2.1 Some physical properties of the different classes of PHAs as compared to synthetic plastic (polypropylene) (Zinn and Hany, 2005).

		PHAs		
	Poly(propylene)	*scl*-PHAs	*mcl*-PHAs	*lcl*-PHAs
Melting point ($^{\circ}$C)	176	80-180	30-80	-
Density (g/cm^3)		1.25	1.05	-
Crystallinity (%)	70	40-80	20-40	?
Extension at break (%)		6-10	300-450	-
UV light resistance	poor	good	Good	good
Solvent resistance	good	poor	poor	poor
Biodegradability	None	good	Good	good
Large scale production	++	+/-	+/-	-

2.5.3 Short-chain length polyhydroxyalkanoates

Based on the species of bacteria involved and the type of carbon source used during the fermentation process, different polymers can be produced that are majorly grouped into short-chain length PHAs (*scl*-PHAs), mostly having 3-hydroxy fatty acids of 3–5 carbon units and medium-chain length PHA (*mcl*-PHA) that contain more than

five carbon units (Ashby and Solaiman, 2008; Cruz *et al*., 2016a). *scl*-PHAs show a wider range of properties depending on the monomeric composition. For instance, P(3HB), with a T_m of 180 °C and a T_g of 4 °C is exceedingly crystalline, brittle, and stiff and usually has a tensile strength comparable with that of polypropylene. Another *scl*-PHA P(4HB) with a T_m of 54 °C and a T_g of 49 °C is a flexible thermoplastic material whose tensile strength is comparable to that of polyethylene (Martin and Williams, 2003).

2.5.4 Medium-chain length polyhydroxyalkanoates

Medium-chain length polyhydroxyalkanoates (*mcl*-PHAs) are elastomers having lower crystallinity and low glass transition temperature when compared with their short-chain length counterparts. Consequently, members of this class of PHAs have been drawing considerable interest due to their flexible properties desirable for a wide range of medical and biotechnological applications. Medium-chain length polyhydroxyalkanoates (*mcl*-PHAs) are polyesters produced and accumulated in a large variety of Gram-negative bacteria, mainly pseudomonads. Their constituents are typically monomers of the chain length of C6-C14. The *mcl*-PHA are promising materials for diverse applications partly due to their useful mechanical properties and are biocompatible and biodegradable. The versatile metabolic capacity of some strains of *Pseudomonas* species enables them to synthesize *mcl*-PHAs containing various functional substituents that are of great interest. The diverse functional groups present in these polymers can allow the creation of tailor-made products, thereby improving the physical properties of the polymers. Furthermore, some of the functional substituents can be chemically modified to get more user groups that can broaden the potential applications of these special environmentally friendly polymers, particularly as biomaterials for use in biomedical fields (Kim *et al*., 2007b).

Interestingly, the material properties are similar within one class. Medium-chain length polyhydroxyalkanoates (*mcl*-PHAs) are distinct from short-chain length PHAs due to structural differences. In the former, the length of side chains and the number of double bonds can be regulated to a certain extent by supplying the cells with appropriate fatty acids (alkenoic and alkanoic acids) during biosynthesis in a close system. Similarly, multiple-nutrient-limited growth conditions can precisely control the number of multiple bonds and side chain length in a chemostat (Wampfler *et al*., 2010). Concerning the physical features, *mcl*-PHAs are elastomeric, which is quite different from *scl*-PHAs, such as poly-3-hydroxybutyrate (PHB), which is highly crystalline and stiff (Simon-Colin *et al*., 2012). Owing to their composition, which is chiefly medium and long-chain fatty acids units, oil-containing carbon sources can act as precursors for different types of PHA, with specific properties leading to possible novel applications (Cruz *et al*., 2016a). Monomers obtained from the depolymerization of *mcl*-PHA could be used to synthesize some bioactive compounds, such as antibiotics (Jiang *et al*., 2006).

The *mcl*-PHAs are relatively less abundant and frequently produced and accumulated by bacterial species belonging to the genus pseudomonas, particularly fluorescent pseudomonas (De Eugenio *et al*., 2010). Many strains of these organisms, especially those that belong to the rRNA group I, produce *mcl*-PHAs as copolymers containing at least two types of 3-hydroxyalkanoate units when cultured in a medium supplied with alkanes and alkanoic acids with carbon chains longer than pentane and pentanoic acid (Kim *et al*., 2007a). Among the *Pseudomonas* species capable of producing *mcl*-PHAs, *P. oleovorans* and *P. putida* have been extensively investigated. These forms of polyhydroxyalkanoates are produced mostly under unbalanced growth conditions. Contrary to the case of short-chain length PHAs (*scl*-PHAs) accumulation by the producing organisms, in which the growth and product formation is coupled, the

requirements for biomass and *mcl*-PHA formation are demanding, which can be wide-ranging that do not necessarily correlate, thereby making efficient *mcl*-PHA production difficult as observed of *P. oleovorans* (Jung *et al*., 2001). Medium-chain length PHAs have low crystallinity, low melting temperature (T_m) values ranging between 40 and 60°C, and glass transition temperature (T_g) values between -50 and -25°C, low tensile strength, and relatively high elongation to break. *mcl*-PHAs and their copolymers are suitable for applications where flexible biomaterials are needed, such as heart valves and other cardiovascular applications. They are also the preferred choice for controlled drug delivery (Bassett, 1982; Rai *et al*., 2011). *mcl*-PHAs, compared to short-chain length PHAs, are more structurally diverse, hence being more readily tailored for specific applications. Several composites have successfully been fabricated using *mcl*-PHAs and their copolymers, such as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) in combination with hydroxyapatite, poly(3-hydroxyoctanoate) in combination with single-walled carbon nanotubes, etc. (Rai *et al*., 2011). The *mcl*-PHAs produced by many bacteria are in demand due to their flexibility and elastomeric properties which are considered superior qualities in the global market of bioplastics (Yasotha *et al*., 2006).

Even though *mcl*-PHAs are water-insoluble, hydrophobic polymers, they are degradable by microbes that produce mcl-PHA depolymerase. This extracellular enzyme breaks the polymer into its monomeric constituents. *Mcl*-PHA-degraders are relatively rare in natural environments, with only a limited number reported to date (Kim *et al*., 2007). Numerous *mcl*-PHAs having side chains containing functional groups have been produced by different microorganisms in experimental trials. For instance, carbon-to-carbon double and triple bonds, ketone and acetoxy, and aromatic groups. These PHAs normally are completely amorphous and soft-sticky material. One

of the interesting advantages of the functional groups in their structure is the provision of sites for chemical modifications, which facilitate the modification of the physical properties of the polymer (Hartmann *et al*., 2004). Table 2.2 provides a list of some bacteria capable of producing *mcl*-PHAs and the carbon substrates used.

Table 2.2 List of some bacteria capable of synthesizing *mcl*-PHA from different carbon sources

Bacterial strain	**Carbon source**	**Monomers detected**	**Reference**
Pseudomonas mendocina NK-01	Glucose	HO, HD. HDD, HTD, HOD	(Guo *et al*., 2013)
P. citronellolis NRRL B-2504, *P. resinovorans* NRRL B-2649	Olive oil distillate & fatty acids by-product	HHx, HO, HD. HDD, HTD	(Cruz *et al*., 2016a)
P. oleovorans	*n*-Octane	-	(Jung *et al*., 2001)
Pseudomonas putida KT2440 (engineered)	Xylose & octanoic acid	-	(Le Meur *et al*., 2012)
P. putida KT2440, *P. putida* C-A3, *P. putida* GO16	Volatile fatty acids from anaerobic digestion of grass	HO, HN, HD, UD, HD, HTrD, HTD	(Cerrone *et al*., 2014)
P. putida mt-2, *Bacillus megaterium* DSM 509	Fatty acids and sugars	HHx HO, HN, HD, HDD, HTD	(Shahid *et al*., 2013)
P. putida strain F1, *P. putida* strain mt-2, *P. putida* strain CA-3,	Benzene, toluene, ethylbenzene, xylene (BTEX) and styrene	HO, HD, HDD	(Nikodinovic *et al*., 2008)
Pseudomonas mediterranea	Glycerol	HHx, HO, HD, HDD	(Pappalardo *et al*., 2014)
Pseudomonas putida IPT 046	Glucose and fructose	HHx, HO, HD	(Sánchez *et al*., 2003)
Acinetobacter sp. ASC1, *Pseudomonas sp.* ASC2 CDM, *Enterobacter sp.* ASC3 CDM *Bacillus sp.* ASC4 CDM	Crude glycerol	HDD	(Muangwong *et al*., 2016)
Pseudomonas chlororaphis subsp. Aurantiaca	Glycerol	HHx, HO, HD, HDD, HTD	(Pereira *et al*., 2019)

P. citronellolis	Apple pulp waste	HHx, HO, HD, HDD, HTD	(Rebocho *et al*., 2019)
Bacillus thermoamylovorans PHA005	Sodium octanoate	HO, HD, HTD, HHD, HOD	(Choonut *et al*., 2020)
Pseudomonas aeruginosa TISTR 1287	Palm oil	HHx, HO, H2O, H2D	(Tanikkul *et al*., 2020)
Escherichia coli	Glucose, molasses	HO, HD	(Narayanan et al., 2020)
Cupriavidus necator Re2058/pCB113	Spent bleaching clay	3HB-co-3HHx	(Hairudin *et al*., 2021)
Pseudomonas citronellolis SJTE-3	Drilling fluid and oily mud		(Koutinas *et al*., 2021)

Key: HHx = hydroxyhexanoate; HO = hydroxyoctanoate; H2O = hydroxy-2-octanoate, H2D = hydroxy-2-decenoate, HN = hydroxynonanoate; HD = hydroxydecanoate; HDD = hydroxydodecanoate; HTrD = hydroxytridecanoate HTD = hydroxytetradecanoate; HHD = hydroxyhexadecanoate; HOD = hydroxyoctanoate.

2.6 Biosynthetic pathways of polyhydroxyalkanoates

During the last few decades, basic and applied research has provided much information about the biochemical and molecular basis of the enzymatic processes involved in synthesizing PHAs in microbes. There are three distinct metabolic phases in the biosynthetic pathway of bacterial PHA (Steinbüchel, 2002). Preparatory to the synthesis, a specific transport system to enable entry of a carbon source suitable for PHA biosynthesis into the cytoplasm is needed. The carbon source's transport is often achieved by diffusion into the cell depending on the chemical nature and form of the carbon source (Steinbüchel, 2002). The synthesis begins with the initial condensation of two acetyl-CoA to acetoacetyl-CoA catalyzes by 3-ketothiolase (*pha*A). The second phase involves anabolic or catabolic reactions to convert the carbon source into a hydroxy acyl-coenzyme A thioester which normally serves as a substrate for PHA synthase. Reduction reaction catalyzes by acetoacetyl-CoA reductase (*pha*B) forms 3-hydroxybutyryl-CoA in the second phase (Chien Bong *et al*., 2021). In the third phase, which involves the polymerization step, PHA synthase (*pha*C), the key enzyme of the PHA biosynthetic pathway, uses the thioesters formed during the second phase as substrates catalyze the formation of the ester bond with the simultaneous release of coenzyme A. However, it cannot generally be excluded that the PHA synthases also use other thioesters of HA as substrates (Steinbüchel, 2002). Most other PHAs are only synthesized provided that pathways exist which mediate between central metabolic intermediates or special precursor substrates on one hand and coenzyme A thioesters of hydroxyalkanoic acids, which serve as the substrates of the PHA synthase catalyzing the polymerization of PHA (Steinbüchel, 2001).

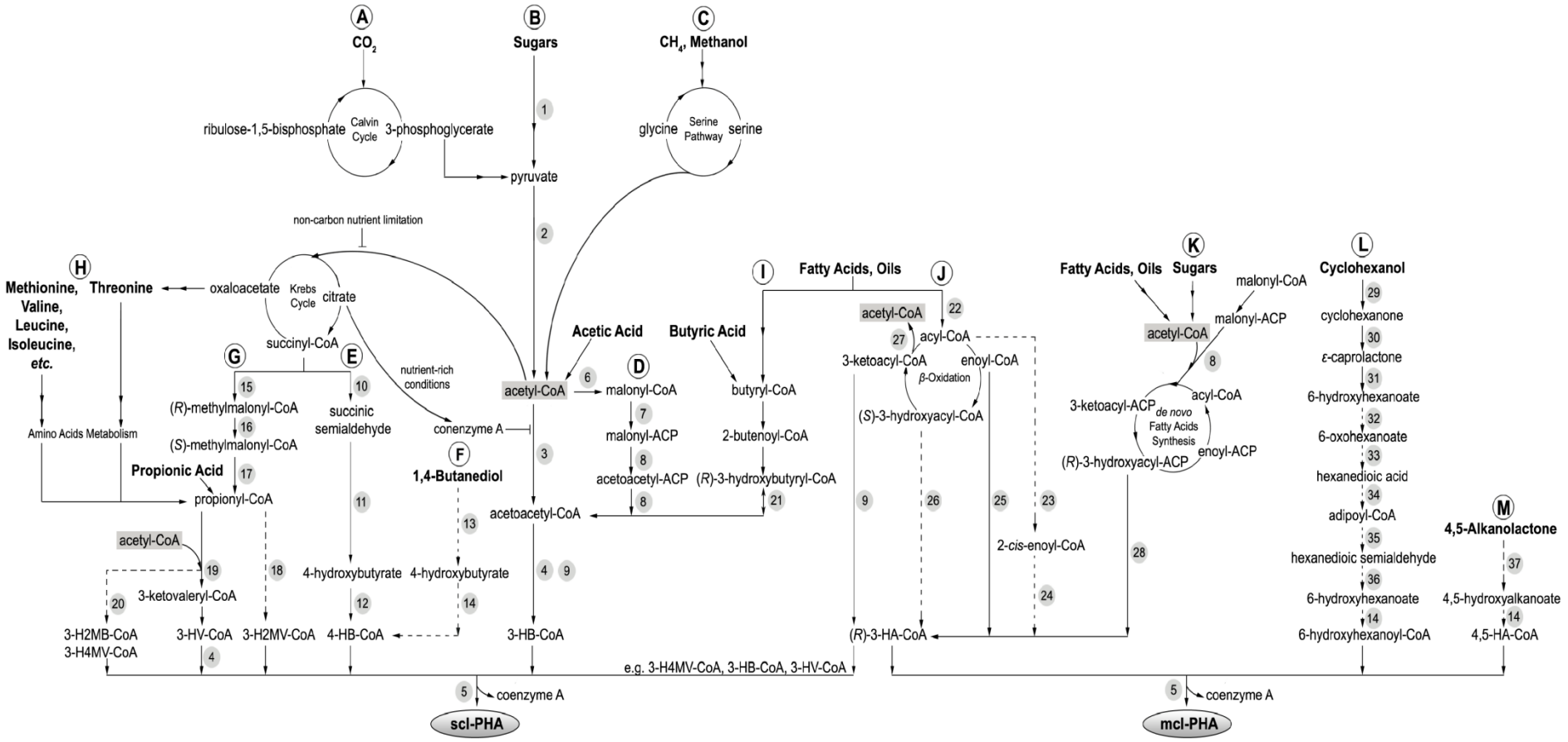


Figure 2.3 PHAs Biosynthetic pathways. Dotted lines represent putative pathways. Numbers signify enzymes involved in the chemical reactions, as listed in Tan *et al*. (2014).