Bioconversion of Lignin and Glycerol to Polyhydroxyalkanoates Using *Pseudomonas putida* KT2440 Under Nutrient Limited Conditions

by

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Abstract

Polyhydroxyalkanoates (PHAs) are a type of bioplastic synthesized by several bacterial species using various carbon sources. PHAs are biocompatible, sustainable, and biodegradable, making them attractive alternatives to traditional petroleum-based plastics. However, the price of PHAs is currently higher than that of petroleum-based plastics due to the expensive carbon sources required, and the yields of PHAs are often low, necessitating large quantities of substrates for relatively small amounts of PHAs. The type of carbon sources and bacterial strains and fermentation condition used can significantly affect PHA yield. To reduce production costs, low-cost and abundant carbon sources such as crude glycerol and lignin have been the focus of much attention in recent years as potential feedstocks for PHA production. Production of PHA from these sources typically requires excess carbon sources and limiting essential nutrients such as nitrogen or phosphorus. This study aimed to investigate how nutrient limitation and lignin concentration affect cell growth and PHA yield in *Pseudomonas putida* KT2440.

In the initial stage, a study was conducted to determine the optimal mixtures of lignin monomers from various lignin monomeric units such as coniferyl alcohol derivative (vanillin and ferulic acid), p-coumaryl alcohol derivative (p-coumaric acid and 4-hydroxy methyl benzoate), and sinapyl alcohol derivative (syringic acid and syringol), during co-feeding glycerol and mixtures of lignin monomers under nitrogen-limited conditions. The findings revealed that the combination of glycerol with coniferyl alcohol derivatives, specifically vanillin and ferulic acid, resulted in increased cell growth and PHA yield when compared to using glycerol alone or co-feeding glycerol with other derivatives.

In a second study, the impact of the sulfur limitation on PHA yield and cell growth was examined by varying the carbon-to-sulfur molar ratio of the media using coniferyl alcohol derivatives

(vanillin and ferulic acid) and glycerol. The results showed that the carbon-to-sulfur molar ratio highly influenced both PHA yield and cell growth. The PHA yield increased significantly when *Pseudomonas putida* KT2440 was cultivated in limited sulfur conditions, and the highest PHA content was achieved when the carbon-to-sulfur molar ratio was 3000.

The third study investigated the impact of different lignin concentrations on PHA production by varying the molar ratio of lignin in the media under a carbon to sulfur molar ratio of 3000. Results revealed that under sulfur-limited conditions, the addition of small amounts of lignin derivatives (10% mol/mol) significantly increased both PHA content and cell growth. However, increasing the lignin monomer level further caused a decline in PHA yield.

In conclusion, the findings suggested that a combination of glycerol with coniferyl alcohol derivatives could enhance PHA yield and cell growth. Additionally, the study indicated that the sulfur limitation condition is another promising strategy to enhance PHA production using *Pseudomonas putida* KT2440, as it leads to a significant increase in PHA yield. These findings have important implications for the development of efficient and sustainable PHA production methods from abundant industrial by-products, which could help facilitate the wider application of PHA in various industries.

Preface

The thesis is an original work by Fantahun Bitew under the supervision of Professor David C. Bressler, including the development of methodology, validation of results, data analysis, and the writing of the original draft. Professor David C. Bressler contributed the thesis proofreading, edits, and revisions while also providing constructive feedback. All experiments regarding this thesis were conducted by Fantahun Bitew in the Biorefining Conversion and Fermentation laboratory within the Department of Agricultural, Food and Nutritional Science (AFNS). In Section 4.1, 4.2, and 4.3, Fantahun Bitew was responsible for designing, performing, and analyzing the experimental data. Dr. Michael Chae contributed to the experimental design and discussion in Section 4.1. Additionally, Dr. Justice Asomaning involved in designing analytical experiments for all sections of the thesis and in proofreading and editing in Section 4.1.

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List of abbreviations

ACP	Acyl carrier protein
ADP	Adenosine Diphosphate
ATP	Adenosine triphosphate
C/N	Carbon to nitrogen molar ratio
C/S	Carbon to sulfur molar ratio
C-5	5-carbon sugar
CDW	Cell dry weight
CO2	Carbon dioxide
CoA	Coenzyme A
CoA-SH	Coenzyme A with sulfhydryl functional group
ED	Entner-Doudoroff
EMP	Embden-Meyerhof-Parnas pathway
FAD	Flavin Adenine Dinucleotide
FADH	Flavin Adenine Dinucleotide (reduced)
FAO	Food and Agriculture Organization
FDA	U.S. Food and Drug Administration
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
lcl-PHA	Long chain length PHAs
LTH	Lipid to hydrocarbon technology

mcl	Medium chain length
MS	Mass spectrometry
MSM	Mineral salt medium
NAD+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
P(4HB)	Poly-4-hydroxybutyrate
P3HB or PHB	Poly -3-hydroxybutyrate
P3HD or PHD	Polyhydroxydecanoate
P3HDD or PHDD	Polyhydroxydodecanaote
P3HO or PHO	Polyhydroxyoctanoate
P3HV or PHV	Poly-3-hydroxy valerate
PHA	Polyhydroxyalkanoates
PHB or P3HB	Poly-3-hydroxybutyrate
PHHx or P3HHx	Polyhydroxyhexanote
PHP	Polyhydroxypropionate
PP	Pentose Phosphate
Scl	Short chain length
TCA	Tricarboxylic acid
WHO	World Health Organization

1 Introduction

1.1 Background

Nowadays, everyday life activities would be almost unimaginable without the use of plastics. However, petroleum-based plastics significantly contribute to environmental pollution since they cannot be broken down in nature (Haider et al., 2019). Over the past decade, there has been an increased focus on sustainable material including renewable and biodegradable options such as bioplastics. While these materials have been studied for over four decades, there has been a renewed interest in the research and production of bioplastics that break down quickly in the environment. Polyhydroxyalkanoates (PHAs) are bioplastic materials that are biocompatible, sustainable, and biodegradable. Several bacterial species are responsible for the production of PHAs from different substrates via fermentation process (Xu et al., 2021). Due to their desired properties, PHAs are considered possible alternatives to substitute fossil-based plastic in various packaging, medical, and agricultural applications (Sharma et al., 2021).

According to European Bioplastics, worldwide production capacity for bioplastics (PHA and other bioplastics) is expected to expand to around 6.3 million tonnes by 2027 from 2.2 million tonnes by 2022 (European Bioplastics, nova-institute (2022). Nevertheless, significant process advancement efforts are necessary to enable high PHA productivity and to expand the PHA market to expected quantities. High production cost associated with the relatively high price of carbon sources is one of the challenges in the PHA market to be competitive with fossil-based plastics (Choi & Lee, 1999). As result, in recent studies, industrial wastes and by-product streams have been used as possible substrate for microbial PHA production.

Several industrial by-product streams, such as those produced from lignocellulosic, dairy-based, biodiesel, and food industries, are available carbon sources to produce polyhydroxyalkanoates

(PHAs) (Jiang et al., 2016). Lignin is a widely available by-product generated from the pulp and paper industry and the lignocellulosic bioethanol industry and is considered the most underutilized component of lignocellulosic biomass (Sun, 2020). As a result, recent studies have focused on converting lignin or lignin derivative compounds into value-added products. Lignin is an aromatic polymer composed of three monomeric units: sinapyl alcohol, coniferyl alcohol, and *p*-coumaryl alcohol (Figure 1.1). As shown in Table 1.1, the composition of lignin monomers varies significantly based on plant material or source from which it is derived. For example, softwood lignin mainly consists of coniferyl alcohol, while hardwood lignin mainly contains coniferyl and sinapyl alcohol. In addition, significant amounts of p-coumaryl alcohol are found in herbaceous such as grass lignin (Yinghuai et al., 2013). Due to its diverse metabolic pathways, *Pseudomonas putida* has been considered a promising biological host for lignin degradation and PHA production (Lin et al., 2016). During bacterial catabolism of lignin, lignin monomers are converted to catechol, protocatechuic acid, and gallic acid, depending on the lignin monomer units (Tang et al., 2022). These intermediates are converted into succinyl-CoA and acetyl-CoA via the β-ketoadipate pathway. Then, acetyl-CoA enters the PHA synthesis pathway or the Tricarboxylic acid (TCA) cycle for energy generation. (Xu et al., 2021; Xu et al., 2019).

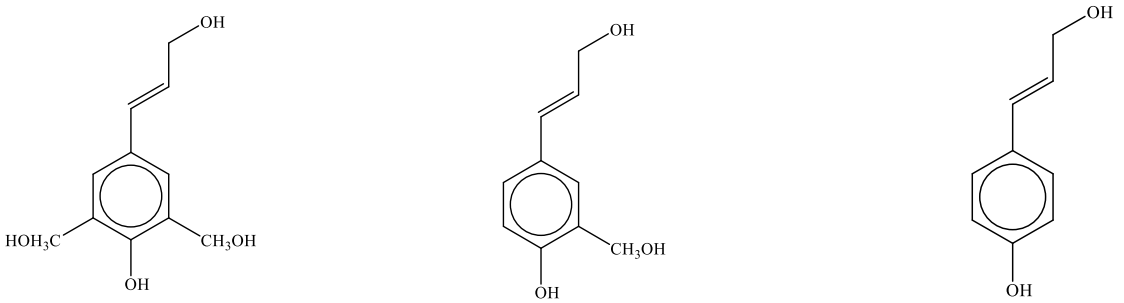

Figure 1.1. Model lignin monomers

Table 1.1. Lignin monomer composition in different lignin sources

Plant material	**Percentage**		
	p-Coumaryl alcohol	Coniferyl alcohol	Sinapyl alcohol
Softwood	<5	<95	Trace amount
Hardwood	0-8	25-50	46-75
Herbaceous	5-33	33-80	20-54

In addition, the biodiesel industry produces a massive amount of crude glycerol as a by-product (around 10 % w/w per biodiesel produced), which provides further opportunity. The structure of glycerol molecule is depicted in Figure 1.2. During glycerol metabolism in bacteria, glycerol is converted into acetyl-CoA via the Embden-Meyerhof-Parnas (EMP) pathway, Pentose Phosphate (PP) pathway or the Entner-Doudoroff (ED) pathway, and then subsequently to PHA and microbial biomass (Beckers et al., 2016). Therefore, in this research, we chose lignin- based aromatic compounds derived from three monomeric units and glycerol as the carbon sources for the production of PHA.

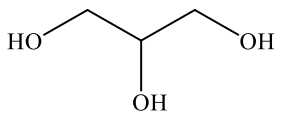

Figure 1.2. Glycerol molecule structure

PHA formation by various bacteria from structurally unrelated substrates (i.e., glucose, glycerol, lignin) is usually promoted in a growth medium that contains excess carbon sources and limited essential nutrients, such as nitrogen and phosphorus. Under nutrient-limited conditions, the presence of high nicotinamide adenine dinucleotide phosphate (NADPH) levels promotes the formation of PHA as it is required for the reduction of the precursor molecules used to synthesize

PHAs from unrelated carbon sources and inhibited the TCA cycle, consequently inhibiting the synthesis of microbial protein and cell growth. As a result, nitrogen limitation has been identified as a critical factor for polyhydroxyalkanoates production by *Pseudomonas putida* KT2440, leading researchers to further investigate the role of nutrient limitations (Dabrowska et al., 2021; Możejko-Ciesielska & Serafim, 2019; Ramírez-Morales et al., 2021).

In addition to nitrogen limitation, sulfur limitation has been found to influence PHA accumulation in microbes, particularly in photosynthetic bacteria. Studies have shown that sulfur limitation can trigger PHA production when using carbon sources, including sodium succinate, lactate, acetate, and butyrate (Carlozzi et al., 2019; Melnicki et al., 2009). However, these studies have mainly focused on the production of short-chain length PHAs (i.e., polyhydroxybutyrate, PHB,). In a separate study, Legendre et al., 2020 investigated the oxidative stress induced when *Pseudomonas fluorescens* is subjected to a sulfur-stress environment. They discovered that a decrease in the availability of sulfur leads to a reduction in the formation of NADH, while simultaneously leading to an increase in the synthesis of NADPH. The TCA cycle and oxidative phosphorylation also experienced a significant slowdown during sulfur deficiency (Legendre et al., 2020). Despite these insights, the effect of sulfur limitation on the production of polyhydroxyalkanoates (mcl-PHA) by *Pseudomonas putida* remains unknown, highlighting the need for continued exploration in this area.

Another possible approach that has been proposed to increase PHA yield is implementing co-feeding of substrates during fermentation. A study showed that cell growth and PHA yields by *Pseudomonas putida* KT2440 were enhanced through co-feeding glycerol with benzoate, vanillin, and vanillic acid, compared to glycerol alone. The addition of lignin-derived compounds raised oxidative stress, leading to a higher NADPH level in the microbial cell (Xu et al., 2021). However,

no study has been reported using glycerol with mixtures of lignin-based compounds derived from the different monomeric units.

1.2 Thesis hypotheses

This thesis has three major hypotheses that were addressed in three chapters.

- It was hypothesized that glycerol with 10% of the lignin-derived aromatic mixtures would improve the cell growth of *P. putida* KT2440 and the yield of polyhydroxyalkanoates, compared to glycerol alone (Section 4.1).
- After assessing the literature, we found that there was a lack of fundamental understanding of how sulfur limitation affects the PHA synthesis and cell growth of *Pseudomonas* species. The sulfur-limited condition triggered the formation of NADPH and hindered the TCA cycle and oxidative phosphorylation. Therefore, it was hypothesized that under sulfur-limited conditions, carbon sources (glycerol with 10 % lignin monomers) would be readily channeled to the PHA synthesis (*de novo* fatty acid synthesis) pathway, which will improve the PHA yield and reduce the cell growth of *Pseudomonas putida* KT2440, compared to sulfur excess condition (Chapter 4.2).
- Aromatic compounds generate oxidative stress, which increases the NADPH content and NAD+ concentration. Consequently, it was hypothesized that increasing the ratio of carbon provided to the fermentation medium in the form of lignin monomers under sulfur limitation, would improve the PHA content of the cell (Chapter 4.3).

1.3 Thesis objectives

The overall objective of this thesis was to investigate the bioconversion of lignin and glycerol to polyhydroxyalkanoates (PHAs) using *Pseudomonas putida* KT2440 during shaking flask fermentation under different nutrient-limited conditions. The specific objectives were:

- To investigate the effect of lignin monomer mixtures with glycerol on the PHA yield and cell growth of *Pseudomonas putida* KT2440 under nitrogen-limited conditions,
- To explore the effect of sulfur concentration on both cell growth and PHA production by *Pseudomonas putida* KT2440 from glycerol and lignin derivatives and determine the PHA monomers distribution, and
- To investigate lignin monomer concentration on PHA yield and cell growth under limited sulfur conditions and determine the PHA monomer distribution.

2 Literature review

2.1 Introduction to polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHA) are a biopolymer produced by bacteria as an intracellular reserve for energy and carbon sources. PHA is formed in the cell cytoplasm as a hydrophobic granule with diameters ranging from 0.2 to 0.5 µm (Sudesh et al., 2000). PHA formation is usually promoted under the condition of excess carbon sources and limited essential nutrients in a growth medium, such as nitrogen and phosphorus. Approximately 150 PHA monomers have been identified and incorporated into PHAs, even though few are commercially available (Chen, 2009; Kourmentza et al., 2017).

2.2 Structure and classification of PHA

As shown in Figure 2.1, PHA polymers consist of hydroxy-fatty acid monomers, which are linked to one another through an ester bond, forming repeating polymer units. The monomeric units differ based on the type of side chain or R-group attached to them (Sharma et al., 2021). Depending on the number of carbon atoms in the PHA monomers, PHA is classified into three types, such as short chain length PHA (scl-PHA), medium chain length PHA (mcl-PHA), and long chain length PHAs (lcl-PHA). The monomer composition of PHAs depends on the carbon sources and the metabolic pathways available in the bacteria.

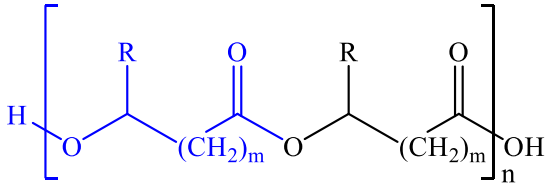

Figure 2.1. General chemical structure of PHA

Where n ≥ 100, m=1,2,3, R= H, saturated alkyl group or unsaturated alkyl groups, substituted alkyl groups, and branched alkyl groups.

2.2.1 Short chain length PHA (scl-PHA)

Short chain length PHA (scl-PHA) have monomers with ranging from 3-5 carbon atoms (C3-C5). As illustrated in Figure 2.2, poly -3-hydroxybutyrate (P3HB or PHB), poly-3-hydroxy valerate (P3HV or PHV), and poly-4-hydroxybutyrate P(4HB) are types of short chain length PHA.

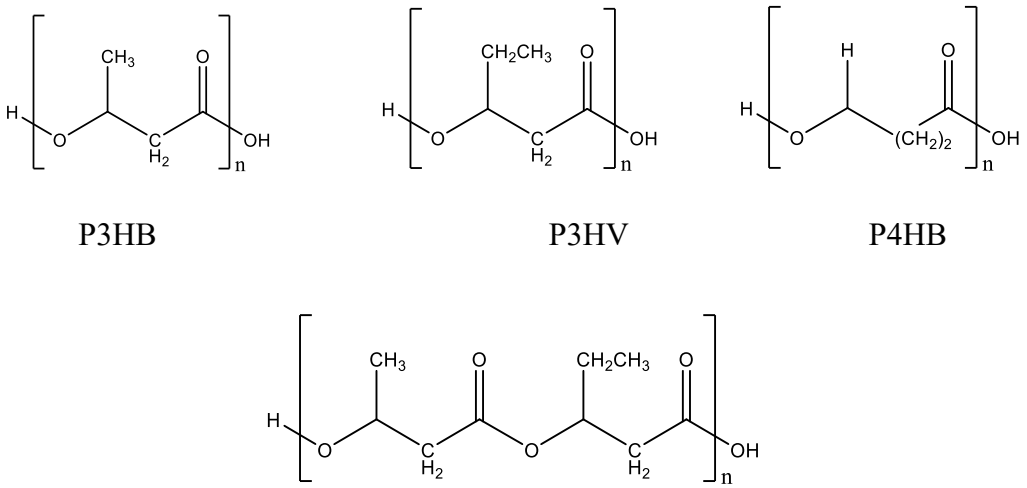

Figure 2.2. Types of short chain length PHA

P3HB is the most class of short-chain length PHA produced by a variety of microorganisms such as *Ralstonia eutropha* (Sandhya et al., 2013) and *Rhodococcus pyridinivorans* BSRT1-1 (Trakunjae et al., 2021) as well as the genera *Alcaligenes* (Steinbüchel & Schlegel, 1991), *Azotobacter* (Itzigsohn et al., 1995) , *Bacillus* , *Nocardia* (Matias et al., 2009) , *Pseudomonas* (Sabarinathan et al., 2018; Lee et al., 2021), and *Rhizobium* (Paganelli et al., 2011). However, most microbes produce P3HV and P4HB as a copolymer of P3HB, such as P(HBV) and P(3HB-co-P4HB). P(3HB-co-P4HB) is produced by a few wild-types microbial strains such as *Alcaligenes latus*, *Comamonas acidovorans*, *Hydrogenophaga pseudoflava*, *Burkholderia*

sacchari, *Cupriavidus necator* from structurally related carbon sources (such as 4-hydroxybutyric acid, γ-butyrolactone, 4-chlorobutyric acid, 1,4-butanediol). In addition, recombinant microbial strains*, such as E. coli,* have been used to produce P4HB commercially from structurally unrelated carbon sources (sugars) (Utsunomia et al., 2020). Co-polymer P(HBV) can be produced by *Cupriavidus necator* and *Alkaliphilus oremlandii* OhILA*s* strain. (Pramanik et al., 2014) (Samui & Kanai, 2019)

2.2.2 Medium Chain length PHAs (mcl-PHAs)

As shown in Table 2.1, mcl-PHAs have monomer subunits with chain lengths of 6–14 carbon atoms (C6-C14). They are produced mainly by *Pseudomonas* species (Ciesielski et al., 2015). The side chain of mcl-PHAs can contain various straight, branched, saturated, unsaturated, and aromatic chains. Around 93% of PHA monomers out of more than 150 identified PHA monomers are accounted for mcl-PHAs (Choi et al., 2020). The medium-length PHAs have a variety of mechanical and physical characteristics, which makes them suitable biopolymers for many industrial applications.

2.2.3 Long chain length PHA (lcl-PHA)

Also shown in Table 2.1, long chain-length PHAs consist of monomer subunits with chain lengths of 15 or more carbon atoms (Lee & Choi, 1995). Currently, limited information is available about lcl-PHAs. A study conducted by Singh & Mallick (2008) showed that *Pseudomonas aeruginosa* produces a long chain length PHA that contains 3-hydroxypentadecanoate (C15) monomeric units (Singh & Mallick, 2008). Further investigation is required about lcl-PHAs and their production by various microorganisms, as it could reveal novel applications and insights into the potential of these biopolymers.

Table 2.1. List of common mcl-PHAs and lcl-PHAs and their nomenclatures

R group	Total carbon number	Name of PHA	Type of PHA
C_3H_7	6	Poly(3-hydroxyhexanaote)	
C_4H_9	7	Poly(3-hydroxyheptanoate)	
C_5H_{11}	8	Poly(3-hydroxyoctanoate)	
C_6H_{13}	9	Poly(3-hydroxynonanoate)	
C_7H_{15}	10	Poly(3-hydroxydecanaote)	mcl-PHA
C_8H_{17}	11	Poly(3-hydroxyaundecanaote)	
C_9H_{19}	12	Poly(3-hydroxydodecanaote)	
$C_{10}H_{21}$	13	Poly(3-hydroxytridecanaote)	
$C_{11}H_{23}$	14	Poly(3-hydroxytetradecanaote)	
$C_{12}H_{25}$	15	Poly(3-hydroxypentadecanaote)	
$C_{13}H_{27}$	16	Poly(3-hydroxyhexadecanaote)	lcl-PHA
$C_{14}H_{31}$	17	Poly(3-hydroxyheptadecanaote)	

2.3 Properties of PHAs

Much interest has recently been placed on polyhydroxyalkanoates (PHAs) since they share many mechanical properties with petrochemical-based polymers such as polypropylene and polystyrene. Properties that affect the quality and potential applications of PHA polymers include melting temperature, glass transition temperature, crystalline structure, tensile strength, and elongation to break percentage. The physical and mechanical properties of PHA are highly dependent on its specific molecular composition. For example, short chain length PHAs (scl-PHAs) such as PHB are highly crystalline, rigid, brittle, and have a low elongation to break with a melting temperature

of 180 °C and a glass transition temperature of 4 °C (Muneer et al., 2020). In contrast, P4HB has more elastic properties with a melting temperature of 54 °C, a glass transition temperature of -49 °C, and elongation to break of 1000% (Rai et al., 2011). Copolymers such as PHBV and PHB4B demonstrate a low glass transition temperature (Tg), a low degree of crystallinity, a low tensile strength, and a high elongation to break compared to homopolymers (PHB) (Balaji et al., 2013). Medium chain length polyhydroxyalkanoates (mcl-PHAs) differ from scl-PHAs in their properties. Medium chain length polyhydroxyalkanoates have a low degree of crystallinity, a low strength, and a high elongation to break. Mcl-PHAs have melting temperature values ranging between 40 and 60 °C and glass transition temperature values between −50 and −25 °C (Simon-Colin et al., 2008; Rai et al., 2011) However, the type of carbon sources that were employed during fermentation have a significant impact on the physical qualities of the products. For instance, the tensile strength of mcl-PHAs made from octanoic acid was greater than that of polymers derived from nonanoic or decanoic acid polymers, and the percentage of elongation to break was lower for octanoic acid polymers. In contrast, Mcl-PHAs that are generated from carbohydrates tend to have poor tensile strength and low elongation-at-break values (Dartiailh et al., 2021). Due to a wide range of physical and mechanical properties, PHAs have the potential to compete with petroleum-based plastic materials, such as polypropylene and polyethylene (Rai et al., 2011).

PHAs offer superior characteristics to petroleum-based plastics including biodegradability, renewability, and biocompatibility. Biodegradability is a vital property that distinguishes PHA from fossil-based plastics such as polypropylene. Unlike fossil-based plastics, PHAs are biodegraded by microbes present in most natural environments (Follain et al., 2014). The biodegradation products of PHAs are carbon dioxide and water in the presence of oxygen, whereas methane and carbon dioxide are released in the absence of oxygen. However, the biodegradation

rate depends on the monomeric composition and environmental conditions (Dilkes-Hoffman et al., 2019). Another common benefit of PHA is its sustainability as PHAs are produced by bacteria using different carbon sources including waste or industrial by-products. Because of their advantageous properties and benefits (i.e., biodegradability and sustainability), PHAs are increasingly considered promising alternatives to replace petrochemical polymers such as polypropylene and polystyrene (Możejko-Ciesielska & Kiewisz, 2016).

2.4 Substrates for PHA production

Carbon source is important during PHA production since it may account for more than 50% of total production expenses (Choi et al., 2020; Singh & Mallick, 2008). So far, various substrates have been used as carbon sources for PHA production and their selection can affect the final composition and types of PHA produced. Therefore, carefully considering and optimizing carbon sources are essential for efficient and cost-effective PHA production processes.

2.4.1 Carbon sources for short-chain length (scl-PHAs)

Polyhydroxybutyrate (PHB) can be effectively produced from various carbon sources using bacterial strains including *Cupriavidus necator*, *Paraburkholderia sacchari, and Azohydromonas lata* (Koller, 2018). Commercial PHB processes often employ carbon sources such as carbohydrates from first-generation biomass *(*derived from edible crops). One factor contributing to the high production costs is the cost of these purified carbon sources (Vlaeminck et al., 2022). As a result, by-products and wastes from industrial processes appear to be desirable carbon sources for the production of PHA. Lignocellulosic biomass hydrolysate, which includes simple sugars including glucose, galactose, xylose, and mannose, has been proposed as a sustainable and cost-effective feedstock for the production of PHA. The bacterium *Paraburkholderia sacchari* IPT 101

LMG 19450 was grown in a medium supplemented with hardwood hydrolysate that contains glucose, xylose, and acetate. They found that a maximum PHB yield (34.5 g/L) is significantly higher than from a synthetic hydrolysate (22.0 g/L) (Dietrich et al., 2020). While the bioconversion of sugar component of lignocellulosic biomass into PHB is well studied, research on lignin conversion remains limited. To address this gap, Tomizawa et al., investigated the growth 11 PHA-producing strains and their PHB accumulation in a lignin derivatives-containing mineral salt medium. Most of the studied strains exhibited poor growth, while *Ralstonia eutropha* H16 showed good growth and produced scl-PHA (0.6 g/L) (Tomizawa et al., 2014). Another effective carbon source for PHB production due to its lower cost and higher sugar content, is sugar industry by-products. For instance, Acosta-Cárdenas et al., 2018 cultivated *R. eutropha* on a medium containing molasses and vinasse, achieving a PHB content of 86% per CDW (Acosta-Cárdenas et al., 2018). Another promising substrate for scl-PHAs is cheese whey. Peña-Jurado et al., (2019) investigated this by cultivating *Bacillus subtilis* EPAH18 on a medium with whey as a carbon source. They optimized the fermentation process to increase the yield of PHB and estimated the cost of production. They concluded that cheese whey is a potential low-cost substrate for the PHB. The estimated cost at the optimized process parameters was 10.2 USD/kg for PHB (Peña-Jurado et al., 2019).

Expanding on other possible carbon sources for PHB production, several bacterial species have been found to utilize glycerol effectively. These species include *Cupriavidus necator*, *Burkhoderia sp*., *Haloarchaea* strains, and other gram negative or positive bacterial strains (Koller & Obruča, 2022). Additionally, following fermentation process optimization, the bacterial strain *Bacillus* sp. ISTVK1 was found to accumulate polyhydroxyvalerate (PHV) at a yield of up to 85% per CDW (Morya et al., 2018) from glycerol. Substrates containing propionic acid, levulinic acid, and

pyruvic acid are also promising propionyl-CoA sources, which is the responsible precursor for the biosynthesis of poly-3-hydroxyvalerate (P3HV). Whereas 4-hydroxybutyric acid is a good candidate for poly-4-hydroxybutyrate (P4HB) (Steinbüchel & Lütke-Eversloh, 2003).

2.4.2 Substrates for medium chain length PHAs (mcl-PHAs)

Mcl-PHAs are synthesized by a wide range of *Pseudomonas* species (Ciesielski et al., 2015). *Pseudomonas* species, known for their versatility and adaptability, can utilize a wide range of carbon sources for growth and PHA production. When these bacteria metabolize different carbon sources, they activate specific metabolic pathways, such as the fatty acid de novo biosynthesis and fatty acid β-oxidation pathways. These pathways result in the production of mcl-PHAs with varying monomer compositions, molecular weights, and properties.

2.4.2.1 Aliphatic fatty acids and alkanes

Fatty acid mixtures, which are the main product of hydrolysis of widely available inedible feedstocks, such as used cooking oils and fats, have attracted much interest as a potential source of feedstock for polyhydroxyalkanoates (PHAs) using beta oxidation pathways. Consequently, various *Pseudomonas* species have used different pure fatty acids as a carbon source to produce mcl-PHA. For example, *Pseudomonas putida* KT2440 employed nonanoic acid plus undecanoic acid (Z. Sun et al., 2009), *Pseudomonas putida* LS46 used C6 to C14 fatty acid (Dartiailh et al., 2020), *Pseudomonas oleovorans* used octanoic and undecanoic acid (Hazer et al., 2009). In another study, the thermotolerant bacterium *Bacillus thermoamylovorans* strain PHA005 utilized sodium octanoate as a carbon source, producing 50.77% CDW of mcl-PHAs (Choonut et al., 2020). In addition, researchers studied the production of PHA from the mixture of fatty acids from sources such as waste cooking oil, vegetable-based free fatty acids, animal-based free fatty

acids, and biodiesel wastes (Borrero-de Acuña et al., 2021; Kellerhals et al., 2000; Ruiz et al., 2019). During fatty acid metabolism, the fatty acids are changed into acyl-CoA, which is then successively oxidized into trans-2-enoyl-CoA, (S)-3-hydroxy acyl-CoA, and 3-ketoacyl-CoA intermediates via fatty acid β-oxidation. All these beta oxidation intermediates are converted into (R)-3-hydroxy acyl-CoA, which is then polymerized into mcl-PHA (Steinbüchel & Lütke-Eversloh, 2003).

In addition, *Pseudomonas oleovorans* utilized carbon sources from C6 to C12 n-alkanes and 1-alkenes to produce PHA. These carbon sources are metabolized into their equivalent fatty acids, which are then oxidized into (R)-3-hydroxy acyl-CoA through β-oxidation pathways. Finally, these intermediate molecules polymerized into intracellular poly (3-hydroxyalkanoates) (PHAs). The length of the PHA monomers is reduced by one or two carbon units (Lageveen et al., 1988). Mixture of aliphatic alkanes, such as n-octane, n-decane, and n-dodecane, are also used by *Pseudomonas resinovorans* as a carbon source for PHA synthesis (Jeon et al., 2022). However, producing PHAs from alkanes or pure fatty acids on a large scale is not practical due to the high cost of carbon sources. Therefore, these carbon sources are co-fed with carbohydrates, such as glucose. A higher PHA content (75% CDW) was reported when *Pseudomonas putida* KT2440 was grown on glucose with nonanoic acid and acrylic acid using fed-batch fermentation strategy (X. J. Jiang et al., 2013). In addition, Le Meur et al. observed that when the recombinant *Pseudomonas putida* KT2440 utilized octanoic acid and xylose, it accumulated 20% CDW PHA (Le Meur et al., 2012). A copolymer, poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHB-cofi-PHHx) accumulated by recombinant *Cupriavidus necator* H16 from palm kernel oil. The cell accumulated a copolymer up to 76.5% CDW (Riedel et al., 2012).

2.4.2.2 Carbohydrates and other non-fatty acid carbon sources

Since the price of carbon sources accounts for more than half of the total cost of PHA production, several studies have looked at identifying low-cost carbon sources, such as lignocellulosic wastes, diary-based wastes, biodiesel wastes, and food wastes (Ganesh Saratale et al., 2021). Depending on the kinds of carbon sources, several metabolic pathways are used to convert those carbon sources into acetyl-CoA. Then, acetyl-CoA enters the PHA synthesis pathway or the TCA cycle for energy generation. The fatty acid de novo synthesis pathway is responsible in the biosynthesis of MCL-PHA from carbon sources that are not structurally related to PHA monomers, such as carbohydrates and other non-fatty acid substrates.

- **Lignocellulosic wastes**

The lignocellulosic waste consists of cellulosic components such as cellulose, hemicellulose, and lignin. Glucose and C5 sugars (mannose and xylose) are significant products released during the hydrolysis of cellulose and hemicellulose (Laca et al., 2019). In addition, molasses, a by-product of the sugar industry, also contains a significant amount of sucrose (Palmonari et al., 2020). Consequently, hydrolysis of sucrose releases glucose and fructose as a monomer. As a result, *P. putida* IPT 046 was cultivated on glucose and fructose to produce high cell density and PHA content. A PHA content of 63% CDW was accumulated by *P. putida* IPT 046 using an equimolar mixture of fructose and glucose (Diniz et al., 2004). However, *Pseudomonas* species, in general, produce minimal levels of mcl-PHA from carbohydrates and other sources of carbon that are unrelated (Oliveira et al., 2020). Therefore, adding fatty acid as a co-substrate with glucose highly improved PHA yield (Z. Sun et al., 2009). In a recent study, both wild *Pseudomonas putida* KT2440 and its mutant strain were cultivated on the media containing hydrolyzed sucrose and decanoic acid. In this result, mcl-PHA with 99 mol% of 3-hydroxydecanoate was produced in the

mutant strain. However, PHA with heterogeneous monomeric composition was observed in the wild strain (Oliveira et al., 2020).

In the study conducted by Hossain et al., 2022 *Pseudomonas putida* KT2440 was shown to use glucose and xylose, both of which were generated after the alkaline pre-treatment and enzymatic hydrolysis of rice straw and hemp hurd, to create mcl-PHAs. Under nitrogen-deficient conditions, 0.47–0.61 g/L of mcl-PHAs were obtained from the hydrolysates of pretreated rice straw and hemp hurd samples, which provided a new insight on lignocellulose upgrading to mcl-PHAs (Hossain et al., 2022). A copolymer of scl-PHA and mcl-PHA (P3HB-co-P3HHx) was produced by *Cupriavidus necator* DSM 428 from molasses and olive oil. Cultivation of the strain on the mixture of molasses and olive oil yielded significant PHA content (40% CDW) compared to molasses alone (24.33% CDW) and olive oil alone (18.66% CDW)(Razzaq et al., 2022).

Lignin is one of the most underutilized components of lignocellulosic biomass in the lignocellulosic biofuel, paper, and pulp industries (R.-C. Sun, 2020). As a result, recent studies have focused on converting lignin or lignin derivative compounds to value-added products. Due to its diverse metabolic pathways, *Pseudomonas putida* has been considered as a promising biological host for lignin degradation and PHA production (Lin et al., 2016). During bacterial catabolism of lignin, lignin monomers are converted to catechol, protocatechuic acid, and gallic acid, depending on the lignin monomer units (Tang et al., 2022). These intermediates are converted into succinic-CoA and acetyl-CoA via the β-ketoadipate pathway. Then, acetyl-CoA enters the PHA synthesis pathway or the TCA cycle for energy generation. (Zhaoxian Xu et al., 2021) (Zhaoxian Xu et al., 2019).*Pseudomonas putida* NX-1 has been cultivated on media containing Kraft lignin as the only carbon source. After 144 h of cultivation, the PHA content reached 37.64%

per CDW, and the ultimate PHA yield was 114.16 mg/L. However, the ultimate optical density was lower than 0.6, indicating low cell growth (Zhaoxian Xu et al., 2021).

In research conducted by Liu et al., fermentable sugars were generated from corn stover by alkaline treatment followed by enzymatic hydrolysis. The remaining residue that contains both lignin and residual sugars was used as a carbon substrate for PHA production by *Pseudomonas putida* KT2440, which produced 1.5 g/L of PHA. It was concluded that the co-production of fermentable sugar and PHA has potential advantages for biorefinery sustainability (Z.-H. Liu et al., 2019). Furthermore, alkaline pretreated lignin (APL) obtained from corn stover was used as a carbon source. This APL fraction consists mainly of lignin, glucose (0.13 g/L), and acetate. In addition, model compounds such as p-coumaric acid, ferulic acid, glucose, acetate, and their mixture were used for PHA production. The PHA yield was 0.252 g/L with 32% CDW PHA content, which is comparable to PHA obtained from model compounds (Linger et al., 2014).

- **Dairy by-products**

Whey, which accounts for 85–95% of the milk volume used in cheese manufacturing, is another inexpensive carbon source (Guimarães et al., 2010). Lactose, which is found in whey, can be hydrolyzed into glucose and galactose, which are the substrates for producing PHAs. For instance, after 24 hours of growing on whey, *Thermus thermophilus* HB8 accumulated PHA at a level of up to 35% CDW. The produced polymer had both short-chain length PHA and medium-chain length PHAs (Pantazaki et al., 2009).

- **Biodiesel wastes**

Crude glycerol is the primary by-product of the biodiesel producing industries, which results in the release of 1 kg of glycerol for every 10 kg of biodiesel (Monteiro et al., 2018). Therefore, it is

vital to develop processes to utilize glycerol to guarantee the sustainability of the biodiesel sector on a global scale (Monteiro et al., 2018). Different *Pseudomonas* species have been grown on media containing glycerol for producing mcl-PHAs (Pereira et al., 2019; Sathiyanarayanan et al., 2017; P. K. Sharma et al., 2012). According to Poblete-Castro et al., among the several *Pseudomonas putida* strains studied, *Pseudomonas putida* KT2440 is the most effective PHA producer compared to *Pseudomonas putida* KT2442, S12, and F1 (Poblete-Castro et al., 2014b). Beside *Pseudomonas* species, *Bacillus megaterium* DSM 509 produces a mixture of scl-PHA and mcl-PHA using glycerol as a carbon source (Shahid et al., 2021). In another study, the PHA copolymer (SCL-co-MCL) was also synthesized from glucose or glycerol under non-sterile conditions by the halophilic bacteria *H. cupida* J9, which was isolated from highly saline wastewater (Y. Liu et al., 2022).

Besides, the co-feeding of glycerol with either fatty acid mixtures or other unrelated carbon sources has been investigated for producing PHAs. For example, *Pseudomonas putida* mt-2 utilizes glycerol with octanoate or long-chain fatty acids (Fontaine et al., 2017). These findings indicated that biodiesel waste, a mixture of glycerol and fatty acids, could be a promising carbon source for PHA production. In addition, PHA yield and content in *Pseudomonas putida* KT2440 have been enhanced when glycerol is co-fed with lignin derivatives such as benzoate, vanillin, and vanillic acid, indicating major wastes from current biorefinery industries could be used as carbon sources for PHA production, which would reduce costs related to carbon sources (Zhangyang Xu et al., 2021).

- **Glycerol and lignin as a promising substrate for PHA production**

The production of biodiesel throughout the world has increased in recent years. According to U.S. Energy Information Administration (EIA), the total biodiesel produced in the United States

reached 2.3 billion gallons per year in 2021, and it is predicted to reach approximately 5 billion gallons per year by 2024. The FAO report (OECD et al., 2022) indicated that around 15 % of vegetable oil is used as a feedstock for biodiesel production. However, over half of the total manufacturing costs are attributable to the cost of these feedstocks. Therefore, biodiesel manufacturing needs government subsidies to be cost-competitive with fossil fuel-derived fuels (Anuar & Abdullah, 2016). In addition, the biodiesel industry experiences the uncontrollable release of glycerol as a by-product. Utilizing this abundant by-product as a feedstock for the production of various chemicals and materials can benefit the biodiesel industry in several ways, including generating revenue streams and improving resource efficiency, which are essential for achieving a sustainable energy future (Cornejo et al., 2017, Cespi et al., 2015). In addition, Dr. Bressler's research team created the patented lipid-to-hydrocarbon Technology (LTH), a method for converting fats and oils into drop-in fuels in a two step process (U.S. Patent No. 8,067,653 B2). In the first step, thermal hydrolysis, triacylglycerols are hydrolyzed into fatty acids, which are then pyrolyzed into drop-in fuels in the second step. In addition, the hydrolysis process releases glycerol as a by-product (Espinosa-Gonzalez et al., 2014). Unlike biodiesel-based crude glycerol, this by-product is free from methanol and salt residual which is suitable for microbial growth. Therefore, glycerol generated from LTH technology and biodiesel industry, is a promising feedstock for bacterial fermentation in value-added product synthesis such as PHA.

Lignocellulosic ethanol has been considered a more environmentally friendly alternative to fossil fuels. However, the commercialization of ethanol produced from lignocellulosic materials confronts many significant challenges, such as costs related to pre-treatment, effective sugar release, fermentation of sugars into ethanol, and distillation (Toor et al., 2020). The components cellulose and hemicelluloses may readily be hydrolyzed into sugars and then fermented into

ethanol. However, lignin, which naturally makes up to 30% of dry biomass, cannot easily be fermented into ethanol. Consequently, the pre-treatment process is necessary to remove lignin as a by-product, which increases the cost of the entire process (Espinosa-Gonzalez et al., 2014).

Similarly, lignin is removed from the biomass during the pulp and paper processing, and a significant amount of lignin is produced as a by-product. However, lignin is the most underutilized lignocellulosic biomass component, where only around 2% of lignin is utilized mainly to generate electricity and chemicals, indicating that further research is needed to fully exploit the advantages of this widely available and cheap biobased feedstock (Cao et al., 2018). In conclusion, it is vital to develop processes to use these by-products (lignin and glycerol) for bioplastic synthesis to guarantee the sustainability of biodiesel and lignocellulosic biorefineries.

2.5 Biosynthesis pathways of polyhydroxyalkanoates

2.5.1 Short chain length PHA (scl-PHA)

As illustrated in Figure 2.3, during the synthesis of P3HB from unrelated carbon sources, carbon sources are transformed into acetyl-CoA through distinct metabolic pathways depending on the types of carbon sources given. Acetyl-CoA is the primary intermediate of many microbes' metabolism. After that, acetyl-CoA is changed into acetoacetyl-CoA by β-ketothiolase, and then acetoacetyl-CoA is reduced into (R)-3-hydroxybutyryl-CoA via acetoacetyl-CoA reductase. PHA synthase is responsible for the last step in polymerizing the precursor ((R)-3-hydroxybutyryl-CoA) into PHB. The bacterium *Rhodospirillum rubrum* has two stereospecific enoyl-CoA hydratases instead of acetoacetyl-CoA reductase, which are responsible for the formation of 3-hydroxybutyryl-CoA from acetoacetyl-CoA (Steinbüchel & Füchtenbusch, 1998). PHB can be produced from structurally similar carbon sources without synthesizing an acetyl-CoA intermediate. For instance, butyric acid is directly converted into acetoacetyl-CoA, leading to the

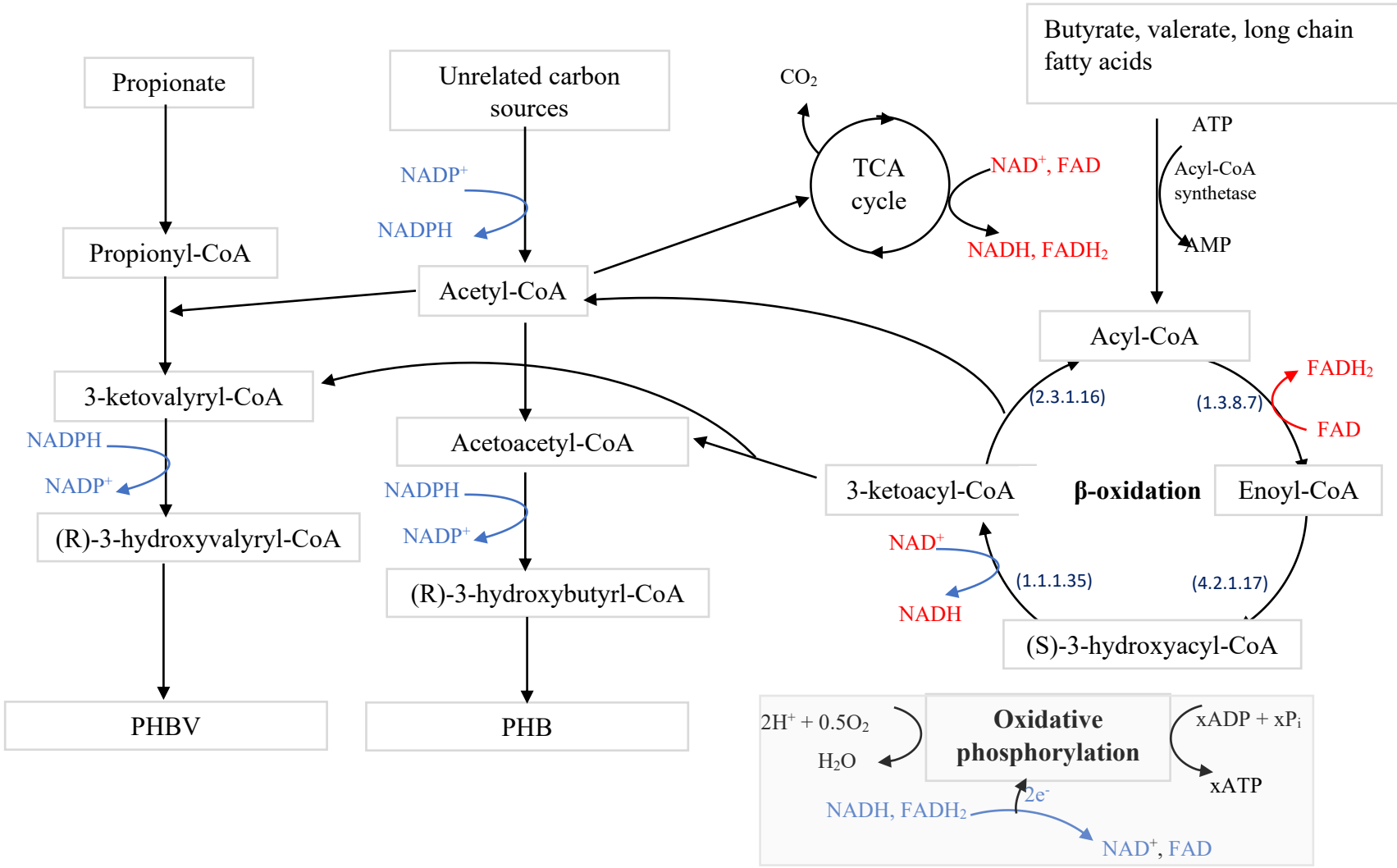

Figure 2.3. Pathway for synthesis of scl-PHA from unrelated substrates, volatile fatty acids, and long chain fatty acids

synthesis of PHB with a high potential product yield (0.94 Cmol PHA/Cmol substrate); this makes butyrate an intriguing carbon source for PHB synthesis (Shi et al., 1997). In the presence of propionic acid or other propionyl-CoA-producing substrates such as odd carbon chain fatty acid, 3-ketothiolase combines one propionyl-CoA with one acetyl-CoA to make 3-ketovaleryl-CoA. 3-ketovaleryl-CoA is then reduced to R-3-hydroxyvaleryl-CoA monomers and polymerized by PHA synthase into polyhydroxyvalerate (PHV) (Braunegg et al., 1998). When valeric acid is used as a substrate, like the PHB formation from butyric acid, valeric acid is converted into valeryl -CoA and then oxidized into 3-ketovaleryl-CoA without forming a shorter alkyl-CoA such as propionyl-CoA in this case (Braunegg et al., 1998).

Another scl-PHA, polyhydroxypropionate (PHP), is produced from glycerol using engineered *E. coli* and *Klebsiella pneumoniae* (Andreessen et al., 2010; Feng et al., 2015). 3-hydroxypropionate containing PHA such as P(HB-co-HP) is synthesized from carbon sources, including 3-hydroxypropionic acid, 1,5-pentanediol, or 1,7-heptanediol (Nakamura et al., 1991).

2.5.2 Medium chain length PHA (mcl-PHA)

Pseudomonas species are primarily responsible for the synthesis of mcl-PHAs because of their adaptable metabolism, fast growth, resistance to harsh environments, and inherent capacity to biosynthesize mcl-PHA. *P. putida* GPo1 was the bacterium that made the first discovery of mcl-PHAs (poly-hydroxyoctanoate) when grown on n-octane (de Smet et al., 1983). Since then, several *pseudomonas* species were identified for synthesizing mcl-PHAs. These polymers are also produced by other few bacterial species such as *C. necator* DSMz 428 and *Bacillus thermoamylovorans* strains (Choonut et al., 2020a; Razzaq et al., 2022). There are two major metabolic processes involved in the synthesis of mcl-PHA depending on the types of carbon

sources and types of microbial species 1) fatty *de novo* biosynthesis pathway, and 2) fatty acid β-oxidation pathway.

2.5.2.1 The de novo fatty acid biosynthesis pathway

As illustrated in Figure 2.4, mcl-PHAs are produced by the fatty de novo biosynthesis pathway when the microorganism is cultured on unrelated carbon sources like glucose, glycerol, acetate, and lignin. First, carbon sources are metabolized to acetyl-CoA through different metabolism pathways depending on the types of carbon sources. After that, acetyl-CoA is transformed into 3-ketoacyl-ACP (an intermediate molecule in de novo pathway) in a step-by-step process with the assistance of three enzymes, namely actyl-CoA carboxylase (AccA), malonyl-CoA-ACP transferase (FabD), and β-ketoacyl ACP synthase (FabH). As part of the *de novo* fatty acid biosynthesis pathway, 3-ketoacyl-ACP is converted to (R)-3-hydroxyaceyl-ACP by ketoacyl ACP reductase (FabG). This pathway is a reductive process that uses NADPH as the electron donor. Finally, the (R)-3-hydroxyaceyl-ACP intermediate is converted to R-3-hydroxyaceyl-CoA, the precursor for PHA formation, by the (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG) and then polymerized to PHA using PHA synthase (PhaC) (Rehm et al., 1998). It has been demonstrated that increasing the expression of PhaG in a cell increases the amount of PHA that can be derived from unrelated carbon sources such gluconate, citrate, and glycerol (Fiedler et al., 2000, Wang et al., 2012, Kato et al., 1996, Dabrowska et al., 2020). The composition of mcl-PHA is particularly dependent on the types of bacterial species or cultivation conditions (Zhangyang Xu et al., 2019b). The substrate does not significantly impact the final composition of the mcl-PHA that is produced through the de novo pathway. For instance, mcl-PHA containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) are produced by *Pseudomonas* sp.strain NCIMB 40135 from different unrelated carbon sources such as glycerol, glucose, acetate, fructose (Haywood et al., 1990;

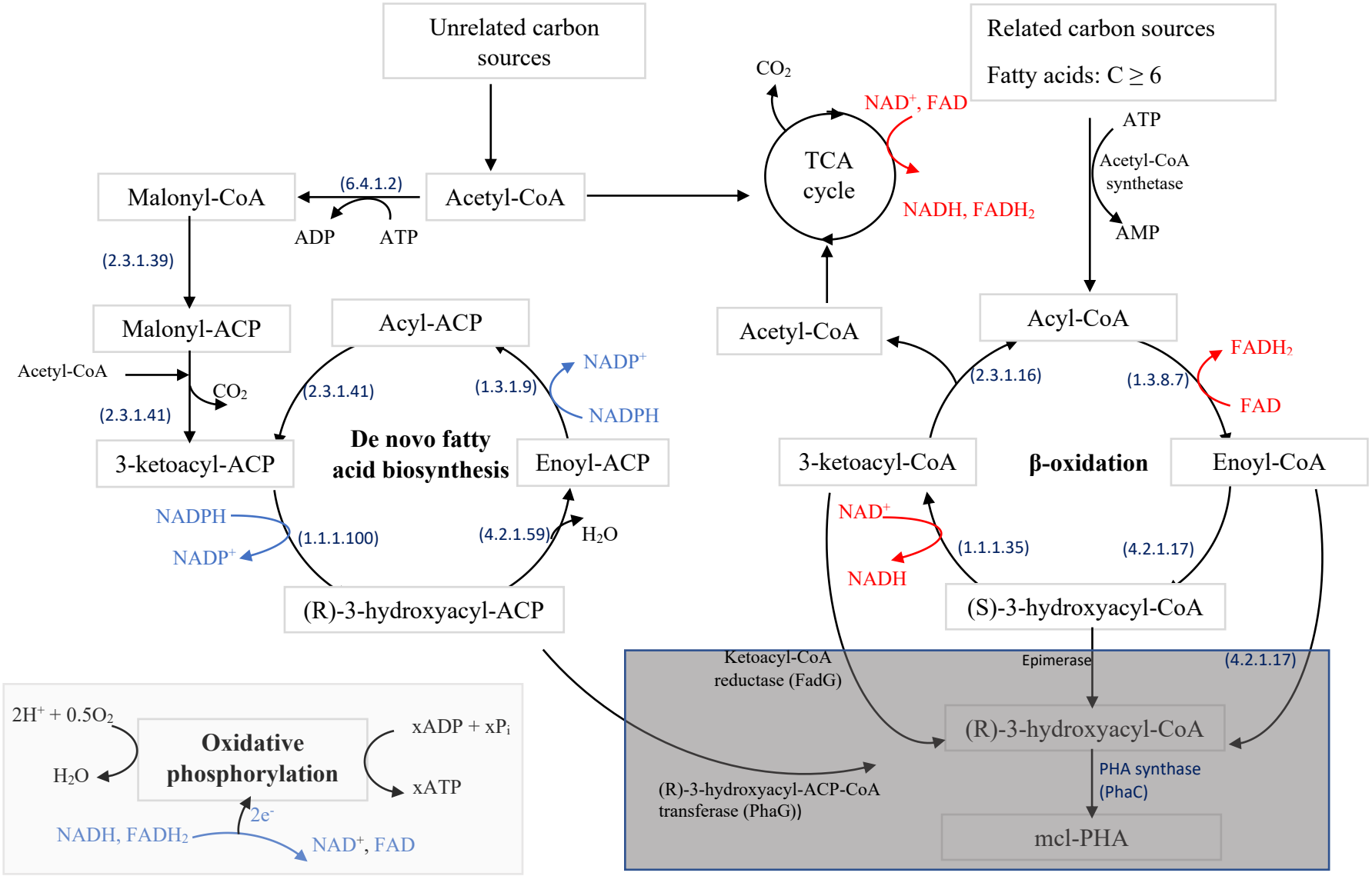

Figure 2.4. Pathways for synthesis of mcl-PHA from de novo synthesis and fatty acid β-oxidation.

Madison & Huisman, 1999). According to Xu and coauthors, there is no significant difference in PHA monomer distribution produced by *Pseudomonas putida* NX-1, regardless of whether protocatechuate or glucose is used as the substrate (Zhaoxian Xu et al., 2021).

2.5.2.2 The fatty acid β-oxidation pathway

The fatty acid β-oxidation pathway is another typical route for mcl-PHAs production, as shown in Figure 2.4. When grown on structurally related carbon sources, such as alkanes, alkenes, fatty acids ($C \geq 6$), and aldehydes, bacteria use this route to produce mcl-PHAs (Preusting et al., 1990) (need more reference). First, a carbon source is transformed into an acyl-CoA molecule, and then the acyl-CoA molecule is oxidized into three intermediate products of the β-oxidation pathway. These products include enoyl-CoA, hydroxyacyl-CoA, and 3-ketoacyl-CoA. All these intermediate molecules have the potential to be transformed into (R)-3-hydroxyacyl-CoA through the actions of three enzymes such as epimerase, trans-enoyl-CoA hydratase (PhaJ), and R-specific ketoacyl-CoA reductase (FadG). Finally, R-3-hydroxyaceyl-CoA polymerized into mcl-PHA using PHA synthase (PhaC). Unlike the fatty acid de novo pathway, the kinds of substrate used in the fermentation medium directly impact the final monomer composition of mcl-PHA that is produced through the β-oxidation route. The length of a PHA monomer is identical to the length of carbon source or decreased by 2, 4, or 6 carbon atoms when the carbon substrate has 6 to 12 carbon atoms (Gao et al., 2016; X. J. Jiang et al., 2013; Madison & Huisman, 1999). Microbes accumulated a higher mcl-PHA content from structurally related carbon sources compared to from unrelated carbon sources. However, structurally related carbon sources are relatively expensive and more toxic compared to structurally unrelated carbon sources like glucose (Rai et al., 2011).

2.6 Challenges in PHA production

Zeneca Group PLC (formerly Imperial Chemical Industries) launched the world's first commercial PHA in the 1980s with the brand name called Biopol, which is a scl-PHA copolymer (PHB-*co*-HV). Later, Yield10 Bioscience Inc. (formerly Metabolix, Inc.) acquired the Biopol business (Rudnik, 2008). Since then, due to their promising properties (biocompatible and biodegradable), PHAs have been produced by several industries, as shown in Table 2.2.

Table 2.2. Example of companies in PHA production

Company	Country	Founded	Source
Yield10 Bioscience Inc	USA	1992	www.yield10bio.com
Danimer Scientific	USA	2004	https://danimerscientific.com
Tianan Biopolymers	China	2000	http://www.tianan-enmat.com
Bio-on	Italy	2007	http://www.bio-on.it.com
Mango materials	USA	2010	https://www.mangomaterials.com
PolyFerm Canada	Canada	1999	http://polyfermcanada.com
Bluepha Co., Ltd	China	2016	https://www.bluepha.com
TerraVerdae Bioworks inc	Canada	2009	https://terraverdae.com
Genecis Bioindustries	Canada	2016	https://www.genecis.com
Bioextrax	Sweden	2014	https://bioextrax.com

Despite the benefits and growing interest in PHA production, one of the challenges is that PHAs are more expensive than plastics made from petroleum, such as polyethylene or polypropylene, because of the cost of carbon sources, extraction, and purification processes. Carbon sources contributed about 50% of the production cost during PHA production (Możejko-Ciesielska &

Kiewisz, 2016). In addition, poor PHA yield and content, in which primary carbon sources channeled to the biosynthesis of non-PHA biomass, maintenance, and respiration of cells, make PHA prices higher than synthetic polymers. Although high scl-PHA yield from carbohydrates have been reported, the bioconversion of unrelated carbon sources to mcl-PHA is ineffective due to a low PHA content per dry cell weight (DCW) (Fu et al., 2014; W. et al., 1990). Few studies have shown that the recombinant strain of *Pseudomonas* putida KT2440 increases PHA content from 34% to 47% and 22% to 67.1% of PHA from glycerol and glucose, respectively (Poblete-Castro et al., 2014a; Poblete-Castro & Andre Luis Rodriguez, 2014). However, most of the mcl-PHA studies have used relatively expensive related carbon sources such as nonanoic acid and decanoic acid to obtain high PHA content ~ 76% CDM (Gao et al., 2016; X. J. Jiang et al., 2013). Considering these findings, nutrient limitation has been studied as a potential strategy for improved PHA production from low-cost carbon sources.

2.6.1 Nutrient limitation for improved PHA production

When bacteria are cultured on structurally unrelated carbon sources, PHA accumulation is highly triggered under conditions providing excess carbon sources with at least one limited essential nutrients (nitrogen, phosphorus, oxygen, sulfur, and potassium) (Anderson & Dawes, 1990; Valappil et al., 2008). Selected studies for the production of PHA under nutrient limited conditions are presented in Table 2.2. Nitrogen limitation is an effective and widely used strategy to trigger scl-PHA synthesis from several carbon sources such as glucose or waste glycerol (Cavalheiro et al., 2012; Mozumder et al., 2014), glucose (Mozumder et al., 2014; Valappil et al., 2008), wood hydrolysates (Dietrich et al., 2019), lignin derivatives or Kraft lignin (Kumar et al., 2017), and volatile fatty acids (Khatami et al., 2022). In addition, few studies have used phosphorus, potassium, and sulfur limitation to synthesize short-chain length PHA (Melnicki et al., 2009; Singh

& Mallick, 2008; Valappil et al., 2008). The presence of high NADPH promoted the formation of PHA as it is enhanced the reduction of the acetoacetyl-CoA to by β-hydroxybutyryl-CoA by acetoacetyl-CoA reductase for PHB synthesis (Senior & Dawes, 1971) (S.-J. Lim et al., 2002).

Under nutrient-excess conditions, acetyl-CoA is diverted primarily to the tricarboxylic acid (TCA) cycle to generate energy and form essential amino acids for cell growth and constituents. During the TCA cycle, high levels of free coenzyme A (CoA-SH) are produced, which inhibits the conversion of acetyl-CoA to acetoacetyl-CoA by **β**-ketothiolase. In addition, NADPH is rapidly oxidized under balanced conditions, which decreases its concentrations (Du et al., 2001; Kessler & Witholt, 2001). In contrast, the generation of high NADPH under unbalanced conditions caused the inactivation of the key enzymes that contribute to the TCA cycle, such as citrate synthase and isocitrate dehydrogenase (Zarei et al., 2013) and enhanced the reduction of the acetoacetyl-CoA to by β-hydroxybutyryl-CoA by acetoacetyl-CoA reductase for PHB synthesis and inhibited the TCA cycle (Senior & Dawes, 1971) (S.-J. Lim et al., 2002). Therefore, nitrogen limitations inhibit the synthesis of microbial protein and cell growth and allow carbon sources to be accumulated in the cell in the form of PHA granules (Wen et al., 2010). Similarly, medium-chain length PHA (mcl-PHA) synthesis from structurally unrelated carbon sources via the *de novo* fatty acid synthesis pathway requires the limitation of essential nutrients such as nitrogen, phosphorus, or oxygen. During the synthesis of medium chain length PHA, β-ketoacyl-acyl carrier protein (ACP) reductase (FabG), NADPH-dependent enzyme, is responsible for the conversion of β-ketoacyl-ACP intermediates to β-hydroxyacyl-ACP intermediates, which is the first reductive stage in the elongation cycle of the de novo fatty acid synthesis (Lai & Cronan, 2004).

Table 2.3. Selected studies for the production of PHA under nutrient limited conditions

Reference	Organism	Substrate	Limitation	%PHA (%CDM)	Types of PHA	Mode of operation
(Mozumder et al., 2014)	*C. necator* DSM 545	glucose, waste glycerol	Nitrogen	62.7 (glycerol), 76 (glucose)	PHB	Fed batch
(Cavalheiro et al., 2012)	*Cupriavidus necator* DSM 545	γ-butyrolactone, Propionic acid, waste glycerol	Nitrogen	36.1	P(3HB-4HB-3HV)	Fed-batch
(Dietrich et al., 2019)	Paraburkholderia sacchari IPT 101	Wood hydrolysates	Nitrogen	71	PHB	Fed-batch
(Khatami et al., 2022)	*Burkholderia cepacian*	VFAs	Nitrogen	54.9	PHB-PHV	Batch bioreactor
(Kumar et al., 2017)	*Pandoraea* sp. ISTKB	Lignin derivatives and krfat lignin	Nitrogen	21-47	P(HB-*co*-HV)	Batch (shake flask
(Khatami et al., 2022)	*Cupriavidus necator*	VFAs	Nitrogen	77.5	PHB-PHV	Batch bioreactor
(Singh & Mallick, 2008)	*Pseudomonas aeruginosa* MTCC 7925	Ethanol, and glucose	Nitrogen	68.7 (ethanol) and 63.9 (glucose)	P(3HB-3HV-3HHD-3HOD)	Batch
(Singh & Mallick, 2008)	*Pseudomonas aeruginosa* MTCC 7925	Ethanol, and glucose	Phosphorus	62 (ethanol) and 58.7 (glucose)	P(3HB-3HV-3HHD-3HOD)	Batch
(Fu et al., 2014)	*Pseudomonas putida LS46*	Waste glycerol	Nitrogen	17.9	mcl-PHA (P3HO-3HD)	Batch (shake flask)
(Zhangyang Xu et al., 2019b)	*Pseudomonas putida* KT2440	Benzoate	Nitrogen	37.3	mcl-PHA	Batch (shake flask)
(Zhangyang Xu et al., 2021)	*Pseudomonas putida* KT2440	Glycerol with benzoate	Nitrogen	45.7	mcl-PHA	Batch (shake flask)
(Ramírez-Morales et al., 2021a)	*Pseudomonas putida* KT2440	Mixed lignin aromatics	Nitrogen	42.2	mcl-PHA	Batch (shake flask)

Table 2.2. Continued

Reference	Organism	Substrate	Limitation	%PHA (%CDM)	Types of PHA	Mode of operation
(Melnicki et al., 2009)	*Rhodospirillum rubrum*	Na-succinate	Sulfur	*	PHB	Batch (flat Roux-type bottles)
(Carlozzi et al., 2019)	*Rhodopseudomonas sp. S16-VOGS3*	Lactate, acetate, and butyrate	Sulfur	21.8 (lactate), 24.6 (acetate) and 27.6 (butyrate)	PHB	Batch (bioreactor)
(Valappil et al., 2008)	Bacillus cereus SPV	Glucose	Potassium	13·4	P(3HB-3HV)	Batch (shake flask)
			Nitrogen	38	PHB	
			Sulfur	13·15	PHB	
			Phosphorus	33·33	PHB	
(S. Y. Lee et al., 2000)	*Pseudomonas putida KT2442*	Oleic acid	Phosphorus?	51.4	mcl-PHA	Fed-batch
(Shang et al., 2008)	*Pseudomonas putida* KT2442	Corn oil hydrolysate	Phosphorus	27[c]	mcl-PHA	Fed-batch
(Diniz et al., 2004)	*Pseudomonas putida IPT 046*	a mixture of glucose and fructose	phosphate	63	mcl-PHA	Fed-batch
(Andin et al., 2017)	*Pseudomonas putida* KT2440	Oleic acid	Nitrogen	54.4	mcl-PHA	Fed-batch
(X. J. Jiang et al., 2013)	*Pseudomonas putida* KT2440	nonanoic acid: glucose: acrylic acid	Carbon	75.5	mcl-PHA	Fed-batch
(Gao et al., 2016)	Pseudomonas putida KT2440	decanoic acid:acetic acid:glucose	Carbon	74	mcl-PHA	Fed-batch

Table 2.2. Continued

Reference	Organism	Substrate	Limitation	%PHA (%CDM)	Types of PHA	Mode of operation
(Blunt et al., 2018)	*Pseudomonas putida* LS46	LCFAs	Oxygen	31	mcl-PHA	Fed-batch
(Poblete-Castro & Andre Luis Rodriguez, 2014)	*Pseudomonas putida* KT2440,	Crude glycerol	Nitrogen	34	mcl-PHA	Batch (bioreactor)
Poblete-Castro & Andre Luis Rodriguez, 2014)	*Mutant Pseudomonas putida* KT2440	Crude glycerol	Nitrogen	47	mcl-PHA	Batch (bioreactor)

- * PHB yield was not reported, but the authors mentioned that sulfur limitation increased the PHB content by 3.5-fold.
- ? The author mentioned that the medium was phosphorus limitation, However, KH_2PO4 was 4 g/L.
- [c] Calculated value from the final cell (103 g/L) and MCL-PHAs concentrations (28 g/l).

Furthermore, nutrient limitation (such as nitrogen) is required to increase (R)-3-hydroxyacyl-ACP-CoA transferase (PhaG) expression. This enzyme converts (R)-3-hydroxyacyl-ACP into (R)-3-hydroxyacyl-CoA derivative (the last precursor molecule for mcl-PHA polymerization from unrelated carbon sources such as gluconate, citrate, and glycerol) (Fiedler et al., 2000) (Dabrowska et al., 2021; Q. Wang et al., 2012). Ramírez-Morales et al., 2021 reported that nitrogen limitation is required to enhance the content of mcl-PHA (42.2% CDW) from mixed lignin derivatives (p-coumarate, ferulate, and benzoate) (Ramírez-Morales et al., 2021a). Similarly, under nitrogen-limited conditions, PHA content in *Pseudomonas putida* was significantly increased during co-feeding of glycerol and benzoate (Zhangyang Xu et al., 2021) or benzoate (Xu et al., 2019). In another study under the phosphate limitation condition, *Pseudomonas putida* IPT 046, cultivated on the mixture of glucose and fructose, accumulated a high PHA content (63% CDW) (Diniz et al., 2004).

In contrast, some studies reported that limitation of essential nutrients is not required for the synthesis of mcl-PHA from structurally related carbon sources such as nonanoic acid, decanoic acid, and octanoic acid (Gao et al., 2016; Jiang et al., 2013; Sun et al., 2007). Sun et al., reported that no PHA was synthesized in *Pseudomonas putida* KT2440 using glucose as a carbon source without nitrogen or phosphorus limitation, whereas high PHA (75.4% CDW) was produced from nonanoic acid without nitrogen or phosphorus limitation. In contrast, limitation of nonanoic acid during the exponential feeding strategy significantly improved the PHA accumulation in *Pseudomonas putida* KT2440. Few studies reported that mcl-PHA yield from oleic acid has improved when *Pseudomonas putida KT2442 and Pseudomonas putida* KT2440 were cultivated under phosphorus limitation and nitrogen limitation, respectively (Andin et al., 2017; Lee et al., 2000). This indicated that the correlation between mcl-PHA synthesis and nutrient limitation

depends on the types of carbon source, strain, and fermentation conditions (Sun et al., 2007). In contrast, studies showed that carbon limitation is also required during the fed-batch production of mcl-PHA. Under this condition, the co-add of an energy and carbon source (e.g., glucose) and a PHA precursor (e.g., nonanoic acid) is necessary to achieve a high cell density and PHA content, respectively, as negligible amounts of mcl-PHA are produced using de novo fatty acid biosynthesis pathway from unrelated carbon sources under carbon limitation (Jiang et al., 2013; Sun et al., 2007).

2.7 Extraction of PHA

After fermentation, the PHA polymer must be extracted from dried biomass and purified to use for the targeted application. The drying step before extraction can be performed by either freeze drying (lyophilization) or thermal treatment. The extraction and purification steps are another factor that drives up the production costs of PHA (Colombo et al., 2020).

The most widely used strategy for PHA extraction is using chemical solvents to disrupt microbial cells and dissolve PHA polymers. Among these solvents, the most frequent approach for the extraction of PHA involves the use of halogenated solvents (López-Abelairas et al., 2015). Excellent extraction yields and polymer quality (i.e., high molecular weight and low impurities) are achieved using chlorinated solvents such as chloroform, 1,2-dichloroethane, dichloromethane, 1,1,2-trichloroethane, sodium hypochlorite, and 1,1,2,2-tetrachloroethane (Pagliano et al., 2021). After extraction, the PHA polymer is precipitated by adding an anti-solvent such as ethanol, methanol, hexane, acetone, water, and ether. Hexane, ether, and acetone are used only to precipitate scl-PHAs, as the mcl-PHAs are soluble in these solvents (Koller et al., 2013). The purity of the final product may be significantly improved by repeatedly dissolving and precipitating the polymer (Volova et al., 2003). However, the utilization of chlorinated organic solvents can alter

the nature of the polyhydroxyalkanoates (PHA) granules, which limits the use of PHA in some applications. In addition, the high cost of the solvents and the hazards involved with this approach are significant challenges (Jacquel et al., 2008). As a result, due to environmental regulations, their industrial use is limited, and a greater need is emerging for safer, less harmful alternatives (Colombo et al., 2020). Efficient PHA extraction has been done using non-chlorinated solvents such as aromatic ketone (anisole, cyclohexanone and phenetole) (Rosengart et al., 2015), and non-ionic surfactants (Tween® 20) (Colombo et al., 2020). Rosengart et al., 2015 showed that the recovery yield and PHA characteristics using anisole and cyclohexanone extraction are comparable to those of the PHAs obtained using chloroform extraction. Extraction of mcl-PHA from *Pseudomonas putida* was conducted by pre-treatment of biomass followed by acetone extraction. Re-dissolving the PHA in acetone and then reprecipitating it in cold methanol would result in an even higher degree of purity (X. Jiang et al., 2006).

In addition to chemical extraction, biological extraction methods have been applied to recover PHA. Enzyme digestion is one of these methods, which requires mild operating conditions and low energy requirements. In the study conducted by Gutt et al., lysozyme without any additional pre-treatment was applied to digest bacterial cells and recover PHA. However, a very poor purity was obtained due to the contamination of proteins and membrane lipids of the cell (Gutt et al., 2016). A combination of enzyme digestion with other treatments has been used to improve the recovery and purity levels of PHAs. For example, Kathiraser et al., found high purity (93%) and recovery (90%) of PHA extracted from *Pseudomonas putida* using a combined heat treatment with Alcalase (protease enzyme) treatment followed by continuous ultrafiltration (Kathiraser et al., 2007) . The high price of enzymes is a significant barrier to the wide-scale application; however,

this may be partially mitigated by using combinations of commercial enzymes with mechanical or thermal treatments (Gonzalez et al., 2021).

Another biological-based method for the recovery of PHA is using a mealworm, *Tenebrio molitor*. In this approach the mealworm has grown on the freeze-dried *C. necator* cells, and the PHA granules are excreted as feces from the mealworm's digestive tract after it consumes the cells. The PHA granules were nearly 100% pure after additional treatment with water, detergent, and heat. Compared to chloroform extraction, PHA showed no signs of a decrease in molecular weight distribution (Murugan et al., 2016). In recent study, these residual mealworms were used for fish supplements due to the high protein and low lipid contents. According to the finding, a diet containing up to 75% mealworms effectively provided the red hybrid tilapia with the necessary nutrients and energy (Zainab-L et al., 2022) . In another study, PHB-containing cells were fed to *Zophobas morio* Fabricius larvae to extract PHB. The extracted PHB was compared to the PHB extracted using chloroform/sodium hypochlorite. They found the biological approach was more rapid and productive, resulting in excellent purity and thermal stability PHB (de Aguiar et al., 2021). It is concluded that biological extraction process is more environmentally benign.

2.8 Application of Polyhydroxyalkanoates

When considering the application of polyhydroxyalkanoates (PHAs) in various industries, it is important to note that the properties of short-chain length PHA (scl-PHAs) and medium-chain length (mcl-PHAs) differ significantly. Compared to short-chain length PHAs, medium-chain length PHAs are a more elastomeric kind of bio-polymer due to their low glass transition temperature, low crystallinity, high elongation to break, and low tensile strength. Therefore, scl-PHA is more suitable for applications requiring high stiffness and heat resistance, such as packaging materials, fibers, and films, while mcl-PHAs are more suitable for applications

requiring flexibility and biocompatibility. Researchers are working to develop hybrid PHAs that combine the desirable properties of both scl-PHA and mcl-PHAs for various applications. This section discusses the application of polyhydroxyalkanoates in several possible applications.

2.8.1 Medical industry

- **Drug delivery**

In conventional drug delivery, it is difficult to maintain drug concentrations at the target site for the required time. This issue may be avoided by the use of controlled drug release, which allows the molecule to be released at a specified rate for a certain amount of time. Different innovative methods using different biomaterials have been developed for delivering drugs to specific areas (Nair & Laurencin, 2006). Among them, polyhydroxyalkanoates (PHAs) have been considered a promising candidate for preparing the delivery system. Due to their high porosity in the matrices, drugs are released so quickly in short chain length PHAs-based drug delivery system (Rai et al., 2011). Li & Chang, 2005 investigated the in vitro release of gentamicin from composite microspheres made from PHBV and wollastonite. This study showed that a microporous layer was formed on the surface of the PHBV/wollastonite composite microspheres, which is responsible for the regulated release characteristic of gentamicin (Li & Chang, 2005). The mcl-PHA -dendrimer matrix was used for the clinical transdermal drug delivery system using tamsulosin as a model drug. The PHA consists of 3-hydroxyoctanoic acid (92%) and 3-hydroxyhexanoic acid (8%). With the assistance of this PHA matrix, the clinically required dose of tamsulosin was penetrated through the skin model (Wang et al., 2003). Another study used a copolymer of scl-PHA and MCL-PHA, such as poly (3-hydroxybutyrate and 3-hydroxyhexanoate), as a matrix for the controlled release of triamcinolone acetonide was investigated. In the first 24 h, more than 90% of the loaded triamcinolone acetonide was released. Here, The ratio of polymer to drug proved to be

the most significant factor in determining the drug loading capacity and release characteristics (Bayram et al., 2008).

- **Cartilage**

Cartilage tissue is characterized by a lack of blood vessels and cannot regenerate. Therefore, complete joint replacement surgery is often necessary to treat cartilage losses. (Armiento et al., 2018). However, early stages of cartilage degeneration may be successfully treated using PHAs as biomaterials. Recent study has shown that electrospinning may be used to create polymer scaffolds that mimic the collagen fibers that are present in articular cartilage utilizing biodegradable poly(3-hydroxybutyrate) and poly(3-hydroxyoctanoate). In addition, it was found that there was a high cell viability on the scaffolds, which suggested that the scaffolds most closely approximated articular cartilage. Using various mixtures of poly(3-hydroxybutyrate) and poly(3-hydroxyoctanoate) resulted in excellent ultrastructure and mechanical properties of cartilage for clinical usages (Ching et al., 2016). PHAs are the next-generation material for this field because of their mechanical qualities, capacity to induce cell activity, and the creation of collagen (Gregory et al., 2022).

- **Bone tissue engineering**

Bone is a highly vascularized tissue with a great capacity for regeneration during minor fractures. However, surgical repair is necessary to treat significant bone defects and fractures. Because of this, biomaterial-based bone implants have been seen as a valuable tool in bone tissue engineering. (Stevens, 2008). PHAs, due to their biocompatibility, have been the focus of much research for bone tissue engineering applications (Lim et al., 2017). A recent study developed antimicrobial polymer composites made of polyhydroxyalkanoates, such as P(3HB) and P(3HO-co-3HD-co-3HDD), with antimicrobial hydroxyapatite (HA), selenium, and strontium for bone tissue

regeneration applications. The study indicated that the composite samples caused a significant decrease in the number of *E. coli* 8739 and *S. aureus* 6538P cells cultivated on the surface of the composites due to direct contact between the materials and the bacteria and the release of active molecules that inhibited bacterial growth (Marcello et al., 2021). In addition, another research generated polyhydroxyalkanoates scaffolds using pressurized gyration. These scaffolds were effectively employed in bone, cardiovascular, and nerve applications, which can be used to regenerate both defective hard and soft tissues (Basnett et al., 2021).

- **Nerve and skin regeneration**

The skin is an organ most susceptible to various injuries, including burns, and using skin substitutes has improved the potential for healing from burn injuries like a wound. Natural-based biomaterials give a considerable amount of potential to be employed in producing nerve and skin tissue regeneration due to the outstanding biocompatibility and biodegradable properties of these materials. Both in vitro and in vivo studies indicated that non-cytotoxic wound dressings made from PHAs, such as P(3HB- *co* -4HB) with collagen peptides and P(3HB- *co* -3HV) with guar gum, significantly improved wound closure and contraction compared to commercial gauze (Pramanik et al., 2015; Vigneswari et al., 2016). In addition, several PHA materials, including the P(4HB) approved by the U.S. Food and Drug Administration(FDA), have been used as sutures for skin healing (Gregory et al., 2022). In another study, PHAs have been modified with graphene/gold (RGO/Au) for nerve tissue regeneration applications. A copolymer with 3 -hydroxybutyrate) and 3-hydroxyhexanoate, P(3HB-*co*-3HHx), has been used in the study. It is concluded that the PHA-modified scaffold can both repair and regenerate the peripheral nerve (Liu et al., 2020).

- **Heart valves**

According to the World Health Organization (WHO), cardiovascular diseases constitute the leading cause of mortality globally. As a result, there has been much of interests in therapies for cardiac tissue. One approach that has received much attention is the combination of a natural or synthetic biomaterial with suitable cells and growth factors (Giraud et al., 2007). For example, the poly-3-hydroxyoctanoate, P(3HO), is an excellent biomaterial for designing heart tissue since it is biocompatible, biodegradable, and elastomeric. According Bagdadi et al., materials made of P(3HO) displayed mechanical characteristics comparable to those of myocardial muscle that can maintain synchronized heart pumping. In addition, the combination of Arginine-Glycine-Aspartic acid) (RGD) and Vascular Endothelial Growth Factor (VEGF) with P(3HO) suggested that the prepared cardiac patches could duplicate the structure of the cardiac extracellular matrix, which enhanced cell adhesion and cell growth (Bagdadi et al., 2018).

2.8.2 Agriculture industry

- **Mulch Films**

Mulch film is a plastic protective covering spread over agricultural land to prevent the growth of weeds and maintain the property of the soil. Polyethylene was the material commonly used for the production of mulching film. However, after each growing season, the material gradually transformed into macro and microplastics, resulting in the soil's pollution with plastics (Lamont, 2005). Biodegradable plastic mulches have recently been considered a potentially beneficial replacement for polyethylene contamination (Serrano-Ruiz et al., 2021). Although research reports on PHA-related mulch films are few, international patents have been filed. For example, a bioactive mulch film manufactured from poly (3 - hydroxybutyrate) or copolymer poly (3 - hydroxybutyrate - co - valerate) or medium chain length polyhydroxyalkanoates (mcl-PHAs) is

patented by Terraverdae Bioworks inc (U.S. Patent No. 10,433,543B2) and a biodegradable polyester mulch foil containing polyhydroxyalkanoates by BASF SE (U.S. Patent No. 20130029124A1). The high cost of PHAs makes it challenging to produce PHA-based agricultural mulch, pots, and nets that can be used for commercial sale.

- **Controlled release fertilizer**

Coated fertilizers, in which nutrients are delivered in a regulated way, have recently become necessary. Thermoplastic resins, including polyvinylidene chloride, polyolefin, and copolymers, are often used to produce commercially available controlled-release fertilizers with polymer coatings today; however, these materials do not biodegrade efficiently in soil and build up over time (Lawrencia et al., 2021). Therefore, PHA has become the most technically possible because of its biodegradability. In the study conducted by Kontárová and colleagues, poly-3-hydroxybutyrate (P3HB) was coated with struvite and dried biomass to examine the gradual release of nitrogen fertilizer and its impact on the development of maize. It found that coated fertilizers in maize nutrition are an appropriate technique to deliver nutrients to plants per their needs with no adverse effect of poly(3-hydroxybutyrate) (Kontárová et al., 2022). Studies showed that PHA-based controlled fertilizer facilitates the release of fertilizer; however, unlike commercial controlled-release fertilizer, it serves as a source of carbon and energy that improves the soil microbial population and fertility (Boyandin et al., 2013; Murugan et al., 2020). Compared to the traditional source of phosphorus, the phosphorus released using calcium phosphate nanoparticles blended with PHA reduces phosphorus loss by more than 80 percent by providing the same plant capacity (Sigmon et al., 2021).

2.8.3 Packaging industry

In packaging and coating applications, biodegradable materials, including PHAs, are considered a reasonable alternative for replacing single-use conventional plastics. Despite their biodegradability, their high production cost prevents them from being widely used by the packaging industry. Still, industries are focusing on producing PHAs and registering their patents on application of PHAs in the packaging industry. To mention a few, Danimer Scientific produces PHA with the brand name Nodax® containing pol(hydroxybutyrate-hexanoate) with a wide range of applications in packaging sectors. Solon™ is the PHA produced by RWDC industries that can be used in single-use straws, plates, coffee cups, and fast-food containers. Another company, Mango Materials, produces PHA pellets for packaging film and sheet applications.

However, like other biopolymers, PHAs lack critical packaging-related performances, such as oxygen, aroma, and moisture barrier qualities, that are required to store food items for an extended time. Combining PHAs with other biodegradable polymers for multicomponent packaging materials has resulted in a considerable reduction in oxygen and moisture transfer rate to food items (Trinh et al., 2023). In the study conducted by Urbina et al., polyhydroxyalkanoates (PHA) were coated on the nanopapers, a composite from bacterial cellulose and bacterial cellulose nanocrystal, to prepare packaging films that enhanced their hydrophobicity nature. Furthermore, oxygen permeability was reduced with the addition of natural extract, with a slight decrease in hydrophobicity (Urbina et al., 2019). Polyhydroxyalkanoates (PHAs) can be laminated or metalized with aluminum (Al) or aluminum oxides (AlOx) to increase barrier qualities; however, this process may reduce their biodegradability (Vea et al., 2021)

3 Materials and Methods

3.1 Materials and chemicals

The bacterial strain, *Pseudomonas putida* KT2440 (ATCC 47054), was acquired from the American Type Culture Collection. Upon arrival, stock culture was prepared in 25% pure glycerol and stored at -80 °C. All other reagents used in the study were purchased from Fisher Scientific or Sigma Aldrich and used as received.

3.2 Methods

3.2.1 Inoculum preparation

The inoculum preparation and fermentation conditions were conducted according to Xu et al., 2021 with some modifications. Briefly, stock culture was streaked on fresh Luria-Bertani (LB) agar medium and incubated at 28 °C for 24 h. After that, a single colony was selected and inoculated into LB liquid medium (10 mL) at 28 °C and 180rpm for 14-16 h. Then, the microbial cell pellets were collected by centrifugation (7000 rpm for 10 min) and resuspended in 10 mL corresponding mineral salt media (MSM). This mixture was used as inoculum for shake flask fermentation.

3.2.2 Shake flask fermentation

3.2.2.1 Nitrogen limited conditions

Inoculums (10 mL) were transferred into 500 mL shaking flasks with 100 mL of specified mineral salt medium (MSM) and cultivated in the batch process at 28 °C and 180 rpm for 72 h. Samples were taken every 12 h to measure optical density at 600 nm. All experiments were conducted in triplicates.

The mineral salt media (MSM) prepared according to Pernicova et al., 2020 which consisted the following chemicals (g/L): $Na_2HPO4 \cdot 12\ H_2O$ (9.0), KH_2PO4 (1.5), $MgSO_4 \cdot 7H_2O$ (0.2), NH_4Cl (0.2), $CaCl_2 \cdot 2H_2O$ (0.02), Fe(III)NH_4citrate (0.0012), and 1 mL/L microelements solution. Microelements solution included the following components (g/L): EDTA (50.0); $FeCl_3 \cdot 6H_2O$ (13.8); $ZnCl_2$ (0.84); $CuC_{l2} \cdot 2H_2O$ (0.13); $CoCl_2 \cdot 6H_2O$ (0.1); $MnCl_2 \cdot 6H_2O$ (0.016), and H_3BO_3 (0.1). The final pH value of the mineral salt medium was 7 ± 0.1. Four different mineral salt media (MSM) were prepared by supplying various carbon sources, as shown in Table 3.1. The total carbon concentrations were kept constant (total C 325.8 mM) under all tested conditions. The carbon-to-nitrogen molar ratio (C/N) in all conditions was 87 to 1. Coniferyl alcohol derivatives consisted of 0.312 g/L of vanillin and 0.318 g/L of ferulic acid; sinapyl alcohol derivatives consisted of 0.36 g/L of syringic acid and 0.315 g/L of syringol; p-coumaryl alcohol derivatives consisted of 0.299 g/L of p-coumaric acid and 0.312 g/L of 4-hydroxy methyl benzoate. The indicated concentration of each lignin monomer is equivalent to 16.35 mM of carbon.

Table 3.1. Formulation of mineral salt medium under nitrogen limited conditions.

Carbon sources	**Carbon content (mM)**	
	Glycerol	**Lignin derivatives**
Glycerol (10 g/L)	325.8	-
Glycerol (9 g/L) + *p*-coumaryl alcohol derivatives	293.1	32.7
Glycerol (9 g/L) + sinapyl alcohol derivatives	293.1	32.7
Glycerol (9 g/L) + coniferyl alcohol derivatives	293.1	32.7

3.2.2.2 Sulfur limited conditions

Inoculums were transferred into 500-mL shaking flasks with 100 mL of specified mineral salt medium (MSM) and cultivated in the batch process at 28 °C and 180 rpm for 72 h. Samples were

collected at 0, 12, 24, 48, and 72 h to measure the pH value, optical density (OD_{600nm}) and investigate the substrate consumption. All experiments were conducted in triplicates (Xu et al., 2021).Mineral salt media (MSM) for sulfur limited condition were prepared after the modification the media provided above section (Section 3.2.2.1) and consisted of the following chemicals (g/L): $Na_2HPO4{\cdot}12\ H_2O$ (9.0), KH_2PO4 (1.5), $MgCl_2.6H_2O$ (0.165), NH_4Cl (2.0), $CaCl_2.2H_2O$ (0.02), Fe (III)NH_4citrate (0.0012), and 1 mL/L microelements solution (as prepared in Section 3.2.2.1) (Pernicova et al., 2020). The final pH value of the mineral salt medium was 7 ± 0.1. The mineral salt media (MSM) were supplied with 9 g/L glycerol (total C 293.1 mM) plus coniferyl alcohol derivatives (total C 32.7 mM), which is equimolar of vanillin and ferulic acid (16.35 mM each). $MgCl_2$. $6H_2O$ was used instead of $MgSO_4$. $7H_2O$. 2g/L of ammonium chloride (NH_4Cl) was used to avoid nitrogen limitation. Therefore, to investigate the effects of sulfur concentration on PHA accumulation, Na_2SO_4 was added to the MSM to adjust the carbon-to-sulfur molar ratios to targeted value as shown in Table 3.2.

Table 3.2. Formulation of mineral salt media to investigate effect of carbon to sulfur molar ratios.

C/S (mol/mol)	Added Na_2SO_4 (g/L)	Added sulfur (mM)
400	0.116	0.8145
1000	0.046	0.3258
3000	0.015	0.1086
5000	0.0093	0.0652
7000	0.0066	0.0465
10000	0.0046	0.0326
20000	0.0023	0.0163

3.2.2.3 Effects of lignin derivatives on cell growth and PHA yield

To examine the impact of lignin concentration on PHA accumulation and cell development, we employed a range of lignin derivatives (ferulic acid and vanillin) and glycerol concentrations as shown in Table 3.3. The carbon-to-sulfur molar ratio (C/S) of the medium was 3000 throughout all conditions. The fermentation was conducted at process at 28 °C and 180 rpm for 96 h. Samples were collected at 0, 24, 48, 72, and 96 h to measure the pH value, optical density (OD_{600nm}) and investigate the substrate consumption. All experiments were conducted in triplicates.

Table 3.3. The amount glycerol and lignin monomer added to the medium at different tested conditions.

Lignin monomer content (%mol) *	**Added glycerol (g/L)**	**Added lignin monomer**	
		Vanillin (g/L)	Ferulic acid (g/L)
0	10	-	-
10	9	0.310	0.316
30	7	0.929	0.948
50	5	1.549	1.580
70	3	2.169	2.213
100	-	3.098	3.161

* The overall carbon content at each condition was 325.8 mM, equivalent to 10 g/L of glycerol. Glycerol (10 g/L) was considered as a control in this study.

3.2.3 Cell dry weight analysis

At the end of cultivation, the cell pellets were collected by centrifugation (7000 rpm for 10 min) and the supernatant was used to analyze the carbon source consumption. Then the cell pellets were washed twice with 0.9% NaCl solution. The cell pellets were lyophilized for 2 days, weighed, and stored for further analysis. The cell dry weight (g/L) were calculated as follows.

$$Cell\ dry\ weight\ (g/L) = \frac{Weight\ of\ lyophilized\ cells\ (g)}{Volume\ of\ culture\ (0.1\ L)} \quad (1)$$

3.2.4 Extraction of PHA polymer

The PHA extraction step was performed according to previous publication (Xu et al., 2021). To extract PHA from the microbial cells, approximately 40-50 mg (exact mass was noted) of ground freeze-dried cells and 7 mL of chloroform were placed in a glass vial. Following this, the glass vial was tightly sealed with a Teflon screw cap and incubated in a shaking water bath at 60 °C and 150 rpm for 12 h. After extraction, the mixture was filtered to remove cell debris through a 0.2 µm nylon membrane filter into a separate glass vial. Then, the filtrate was concentrated by purging nitrogen gas at 50 °C to around 1 mL. To precipitate PHA polymer, 10 mL cold methanol was added to the concentrated mixture, vortexed, and stored at 4 °C for 3 days. The precipitated PHA polymer was separated by centrifugation at 3000 rpm for 5 min, dried by purging nitrogen gas, and then weighed.

The PHA content (%CDW) and PHA yield (g/L) were determined using the following formulas:

$$PHA\ content\ (\%\ CDW) = \frac{weight\ of\ extracted\ PHA\ polymer\ (g)}{weight\ of\ dried\ cells\ used\ for\ exraction\ (g)} \times 100\% \quad (2)$$

$$PHA\ yield\ (g/L) = \frac{\text{PHA content}(\%)}{100\%} \times cell\ dry\ weight\ (g/L) \quad (3)$$

3.2.5 Analysis of soluble compounds in fermentation broth

In experiment conducted in section 3.2.2.1, glycerol and lignin derivatives consumptions were analyzed as follows. In order to analyze glycerol, 1 mL of supernatant (collected in Section 3.2.2) was filtered through a 0.2 µm nylon filter into 2 mL high-performance liquid chromatography (HPLC) vial. The analysis was conducted using high-performance liquid chromatography (Agilent technologies 1200 series, CA, USA) coupled with a refractive index detector (RID) and Aminex HPX-87H (Bio-Rad, Richmond, CA, USA) column at 65 °C. The mobile phase was 4 mM sulfuric acid at 0.6 mL/min (Xu et al., 2021).

The lignin monomer consumption was analyzed using Gas chromatograph (GC) coupled with Mass Spectroscopy (MS) and Flame Ionization Detector (FID). For lignin monomer analysis, 1 mL of supernatant was mixed with 1 mL ethyl acetate and vortexed to extract lignin monomers. The ethyl acetate phase at the top of the mixture was transferred into a glass vial, mixed with 125µL internal standard solution, and then dried by purging under nitrogen gas at 40 °C. The internal standard solution contains 4.6 mg/mL of 2-chlorobenzyl alcohol dissolved in ethyl acetate. The dried residue was then derivatized by adding 50 µL N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), and 100 µL pyridine and incubating the mixture at 60 °C for 30 min. Then the derivatized mixture was transferred into a GC vial for GC analysis. The instrument used was an Agilent 6890N GC-FID with Agilent 7683B series autosampler and injector. The GC column was an Agilent DB Petro column (100 m × 0.25 mm × 0.5 µm) purchased from Agilent Technologies (Santa Clara, CA, USA). The relative response factor (RRF) of each aromatic compound was determined. Helium was used as the mobile phase with a 1.27 mL/min flow rate. The inlet was operated in split mode (split ratio 10:1) with an injection temperature of 280 ºC. The GC oven temperature was initially started at 100 ºC, ramped to 200 ºC at 20 ºC/min

held for 1 min, then ramped to 280 ºC at 10 ºC/min and held 2 min, finally ramped to 300 ºC at 5 ºC/min and held for 5 min.

To determine the consumption of glycerol and lignin monomers for experiment conducted in sections 3.2.2.2 and 3.2.2.3, high-performance liquid chromatography (HPLC) was employed using both a refractive index detector (FID) and an ultraviolet (UV) detector. Samples of the fermentation broth were taken and analyze the consumption of carbon sources over the fermentation period. Then, 1 mL of fermentation broth was centrifuged at 12000 × g for 5 min, and the supernatant was stored until further analysis. The analysis was conducted using the Agilent 1200 series high-performance liquid chromatography (HPLC) system (CA, USA) coupled with an Aminex HPX-87H (Bio-Rad, Richmond, CA, USA), a refractive index detector (FID), and a ultraviolet (UV) detector. The supernatant was passed through a 0.2 µm nylon filter to a 2 mL vial for HPLC analysis. The HPLC analysis was conducted using gradient elution of mobile phase using 5 mM sulfuric acid and acetonitrile as an organic modifier. Briefly, an elution gradient was conducted using 5 mM sulfuric acid for 1 min, 15% acetonitrile in 5 mM sulfuric acid for 20 min, and then 5 mM sulfuric acid for 30 min. The flow rate of the mobile phase was 0.6 mL/min with column temperature at 65 °C. In addition, a refractive index detector (RID) was used to analyze glycerol, and whereas an ultraviolet (UV) detector at 254 nm was used to analyze ferulic acid and vanillin of the fermentation broth.

3.2.6 PHA monomer composition analysis

To analyze the PHA monomers, a two-step derivatization process was employed. In the first step, methanolysis, PHA polymers produced from each sample were placed in glass vials. After that, 1 mL of chloroform and 1 mL of methanol with 15% sulfuric acid were added to the vial.

Benzoic acid (1.5-2.3 mg) was added to each vial and closed with a Teflon screw cap. Vials were incubated at 100 °C for 4 h in an oil bath. After that, the mixture was cooled to room temperature, and 1 mL of deionized water was added, vortexed, and left undisturbed until the organic phase separated. The bottom phase containing PHA monomers was transferred into a clean vial, mixed with 1 mL of deionized water, and vortexed again. The bottom phase was transferred into another vial, and sodium sulfate anhydrous was added to remove the remaining water. The mixture was filtered using a 0.2 μm nylon filter and transferred into a GC vial. This methanolized product was analyzed by gas chromatography coupled with mass spectrometry (GC-MS) to identify the PHA monomers. PHA monomers were identified based on the National Institute of Standards and Technology (NIST) library. Some PHA monomers (C8:0, C10:0, C14:0 3-hydroxy acids) were further confirmed with their standards, while the remaining PHA monomers were identified using only the NIST library.

After identifying the monomers, the methanolized products were further derivatized using silylating agents to quantify the monomers. Briefly, the methanolized product of 200 μL was transferred to the GC vial and dried at 50 °C using nitrogen gas. Then it was derivatized after adding 100μL of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) (99:1) as a silylating agent, and 100 μL of pyridine as a solvent to the dried products. The silylation was performed at 70 °C for 45 min. After the silylation, the mixture was cooled to room temperature and transferred to a GC vial (a vial insert was used). The silylated product was analyzed by gas chromatography coupled with a flame ionization detector (GC-FID) to quantify the identified PHA monomers. The relative response factors (RRFs) for PHA monomers (C8:0, C10:0, C14:0 3-hydroxy acids) were determined using analytical standards. The average RRF value of these standards was used for the quantification of all identified PHA monomers (C6:0,

C8:0, C10:0, C12:0, C12:1, C14:0, and C14:1 3-hydroxy acids). The standards were derivatized as described above using benzoic acid as an internal standard.

> **Note: The silylating agent was handled in a dry environment. The fume hood was purged with air for 30 min to minimize the humidity. The micropipette and pipette tips were also dried at 60 °C for around 1 h before handling solvents. The container was quickly opened and closed as soon as possible to minimize exposure to moisture when handling solvents. Also, anhydrous pyridine was used as a solvent.**

The GC-MS/FID was conducted as follows. The PHA monomer composition was performed on an Agilent GC-MS/FID with DB-Petro column (100 m length × 0.25 mm inner diameter × 0.5 μm film) purchased from Agilent Technologies (Santa Clara, CA, USA). Helium was used as the mobile phase with a 1.27 mL/min flow rate. The inlet was operated in split mode (split ratio 10:1) with an injection temperature of 280 ºC. The GC oven temperature was initially started at 40 ºC, ramped to 280 ºC at 10 ºC/min, and held for 15 min.

3.2.7 Statistical analysis

All experiments were carried out in three separate replicates, unless mentioned otherwise. The data were presented as the mean value together with the standard deviation. In addition, for the purpose of statistical analysis, a one-way analysis of variance (ANOVA) with the Tukey test was carried out; a value of $p < 0.05$ was considered to show the presence of significant differences.

4 Results and discussion

4.1 Microbial conversion of lignin and glycerol to polyhydroxyalkanoates by *Pseudomonas putida* KT2440 under nitrogen-limited conditions

4.1.1 Growth curve and cell dry weight of *P. putida* KT2440

In this study, three different lignin monomer combinations were used to identify the most promising carbon source for PHA production using *P. putida* KT2440. The bacterium was cultivated on mineral salt medium (MSM) containing different carbon sources (total carbon 325.8 mM), including glycerol (control) or glycerol with lignin monomers, supplemented with 0.2 g/L (3.7 mM) of ammonium chloride (NH_4Cl), This resulted in a final carbon to nitrogen (C/N) molar ratio of 87 to 1. The lignin monomers used in this investigation included coniferyl alcohol derivatives (vanillin and ferulic acid; total C 32.7 mM), sinapyl alcohol derivatives (syringic acid and syringol; total C 32.7 mM), and p-coumaryl alcohol derivatives (*p*-coumaric acid and 4-hydroxy methyl benzoate; total C 32.7 mM). These monomers were added to the fermentation media by replacing 10% of total carbon content in glycerol (10 g/L) (control). Figure 4.1. shows the optical density of the fermentation broth measured at 600 nm for a period of up to 72 h, to evaluate the cell growth. Among all the tested conditions, including the control (10 g/L of glycerol), cell growth was dramatically enhanced when 10% of glycerol was replaced by coniferyl alcohol derivatives (i.e., vanillin and ferulic acid). After 48 hours, the OD reached a high of around 5, which was maintained nearly constant until the fermentation process ended.

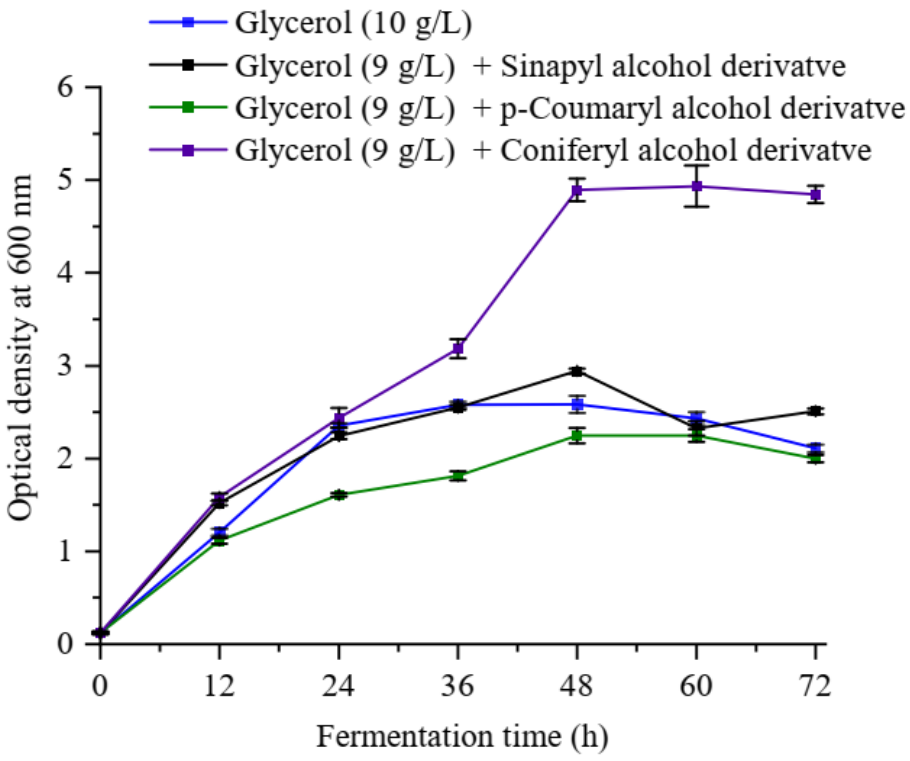

Figure 4.1. Growth curve of *Pseudomonas putida* KT2440 when grown in different carbon sources under nitrogen limited conditions. Data in the curve are presented as the means ± standard deviation (error bars) of triplicate experiments.

To investigate the cell growth and account for potential effects of the fermentation media on the optical density, the cell dry weight (g/L) was determined at the end of the fermentation. When cultivating *P. putida* KT2440 in a medium containing coniferyl alcohol derivatives and glycerol, the cell biomass produced was found to be at its highest (1.14 ± 0.03 g/L), which was significantly higher than the results obtained under any other tested conditions (Figure 4.2). This finding supported the observations made in the growth curve (Figure 4.1). Interestingly, the addition of sinapyl alcohol derivatives, such as syringic acid and syringol, to the fermentation medium did not significantly affect cell dry weight compared to glycerol (10 g/L). However, the cell dry weight significantly decreased when *p*-coumaryl alcohol derivatives (such as *p*-coumaric acid and 4-hydroxy methyl benzoate) were combined with glycerol.

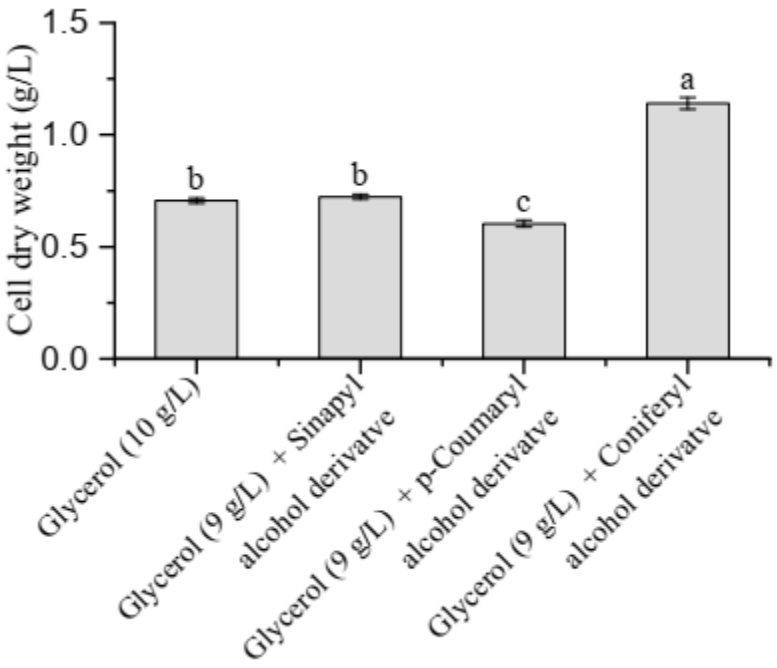

Figure 4.2. Cell dry weight (g/L) of *Pseudomonas putida* KT2440 at 72 h under nitrogen limited conditions. Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different ($P<0.05$).

In line with our findings, Xu et al. (2021) observed improved cell growth of *P. putida* KT2440 when single aromatic compound (such as vanillin, vanillic acid, and sodium benzoate) was co-fed with glycerol as carbon sources, compared to glycerol alone, even though authors did not keep the total carbon content constant (Xu et al., 2021). Our study extends these findings by maintaining a constant total carbon level and focusing on the effect of monomer mixtures derived from different lignin monomer units. We found that mixtures of aromatic compounds derived from coniferyl alcohol monomeric units increased cell growth, while aromatics derived from *p*-coumaryl alcohol monomeric units negatively affected cell growth. In contrast, no cell growth changes were observed when aromatics derived from sinapyl alcohol were used compared to glycerol alone at the same carbon concentrations. Furthermore, our results support the observations by Kukurugya et al. (2019) who reported a 37% increase in the growth rate of *Pseudomonas putida* KT2440 in the medium containing a mixture of glucose and benzoate (a total of 100 mM carbon), as compared

to glucose alone at the same total carbon concentration (100 mM). The increase in growth rate was attributed to changes in protein abundance in microbial cells grown on the mixture of benzoate and glucose. The impact of lignin mixture addition on cell growth could depend on the types of lignin monomers.

4.1.2 PHA content and PHA yield by *Pseudomonas putida* KT2440

The influence of co-feeding lignin derivatives on PHA content and PHA yield was investigated to assess their potential as carbon sources in comparison to glycerol. The results showed that PHA content increased when lignin derivatives with glycerol were utilized compared to glycerol alone ($3 \pm 1\%$ CDW), as shown in Figure 4.3. Maximum PHA content ($40.0 \pm 0.8\%$ CDW) was obtained when coniferyl alcohol derivatives were used as a carbon source, followed by $23 \pm 1\%$ CDW and $15 \pm 4\%$ CDW, when sinapyl alcohol and p-coumaryl derivatives were used, respectively. The addition of lignin derivatives under nitrogen-limited conditions resulted in a significant increase in the PHA content (%CDW). Coniferyl alcohol units demonstrated the highest level of PHA content when compared to the two other types of lignin units, such assinapyl alcohol derivatives and p-coumaryl alcohol derivatives. In terms of PHA yield (g/L), co-feeding of glycerol and lignin monomers also led to a significant improvement, reaching a maximum value of 0.456 ± 0.002 g/L on glycerol with coniferyl alcohol derivatives such as ferulic acid and vanillin (Figure 4.4). Although, the yield was lower when sinapyl alcohol and p-coumaryl derivatives were used compared to coniferyl alcohol derivatives, but it was still significantly higher than when glycerol alone was used as a carbon source. In conclusion, the co-feeding of lignin derivatives and glycerol enhanced both PHA content and yield, with coniferyl alcohol derivatives demonstrating the greatest potential as an alternative carbon source for PHA production.

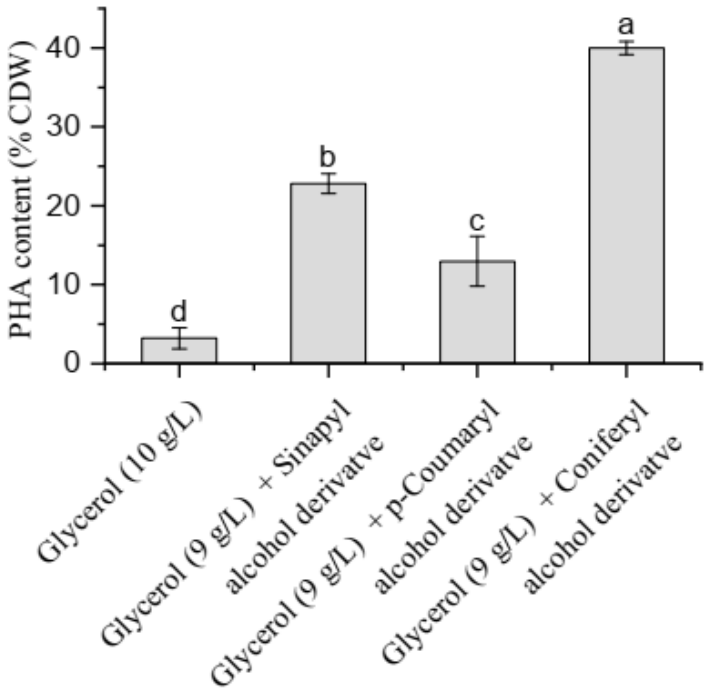

Figure 4.3. PHA content (% CDW) by *Pseudomonas putida* KT2440 at 72 h under nitrogen limited conditions. Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different (P<0.05).

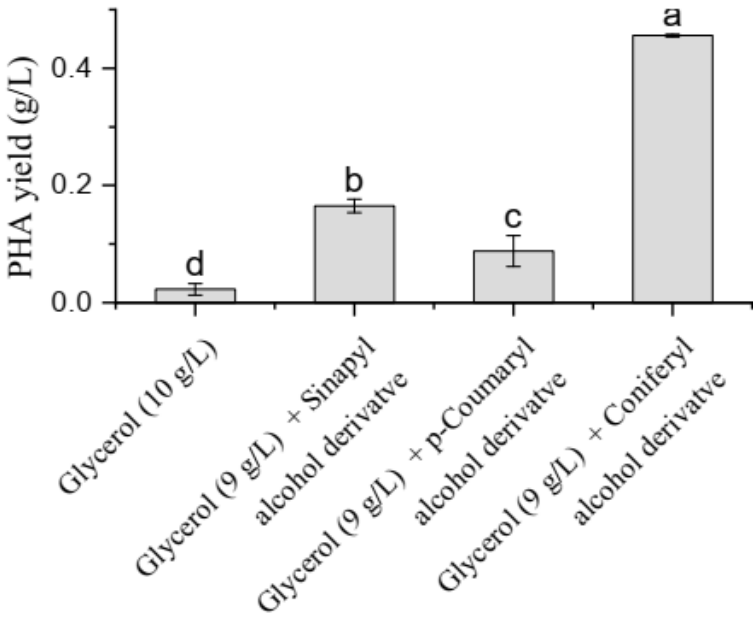

Figure 4.4. PHA yield (g/L) by *Pseudomonas putida* KT2440 at 72 h under nitrogen limited conditions. Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different (P<0.05).

The effect of lignin-derived compounds on PHA synthesis and cell growth may be related to their structural heterogenicity. For example, in the presence of 4-hydroxy benzoic acid and protocatechuic acid, the growth of *P. putida* Gpo1, *P. putida* JCM 13063, and *P. aeruginosa* was higher than the growth of these *Pseudomonas* strains on 15 different lignin-derived compounds including p-coumaric acid, caffeic acid, ferulic acid, and syringic acid, and others. Under all conditions, however, PHA accumulation was less than 1% CDW. The microbial conversion of lignin derivatives to PHA is also strongly dependent on microbial strain type (Tomizawa et al., 2014). Co-feeding lignin monomers with glycerol enables microbes to access various biosynthetic pathways that sustain redox balance and satisfy the requirements for higher PHA yield (Kukurugya et al., 2019; Xu et al., 2021). NADPH is crucial for biosynthetic processes, including PHA production. Our findings support the conclusions drawn by the previous study by Xu et al. (2021), which explored the influence of lignin-derived compounds (such as vanillin, vanillic acid, and sodium benzoate) on PHA synthesis. They demonstrated that co-feeding lignin derivatives with glycerol enhances PHA production in *Pseudomonas putida* KT2440 by maintaining an optimal redox balance and increasing NADPH availability. As a result, the addition of lignin derivatives was found to yield higher PHA production compared to using glycerol alone as a carbon source (Xu et al., 2021).

However, our study introduces a novel approach by using a mixture of lignin monomers with glycerol instead of single monomers with glycerol, which is more representative of the natural composition of lignin. This strategy allows for better understanding of how different lignin monomeric units contribute to PHA synthesis. Furthermore, we maintained a constant total carbon content during all experimental conditions to ensure that the observed increase in PHA yield was not due to increasing carbon content. This approach allows for a more controlled comparison

between the various conditions and highlights the specific impact of lignin monomer mixtures on PHA production. By incorporating these novel elements, our study not only confirms the findings of previous literature but also extends our understanding of the role of lignin monomer mixtures in enhancing PHA content in *Pseudomonas* strains.

4.1.3 Substrate consumption by *Pseudomonas putida* KT2440

When *P. putida* KT2440 was cultivated on glycerol alone (10 g/L), glycerol consumption was observed to be 55 ± 2 %. The addition of aromatic compounds derived from sinapyl alcohol, coniferyl alcohol, and *p*-coumaryl alcohol lignin monomer units to the fermentation medium reduced glycerol consumption to 41 ± 2%, 37 ± 1%, and 33 ± 1%, respectively. In terms of lignin monomers consumption, 99.3 ± 0.6 % of coniferyl alcohol lignin derivatives added to the fermentation media were consumed by *P. putida* KT2440 at the end of the fermentation. However, only 35 ± 18% of sinapyl alcohol derivatives and 34 ± 30% of *p*-coumaryl alcohol derivatives were consumed at 72 h. This could potentially explain why a medium containing glycerol and coniferyl alcohol derivatives exhibited a significant increase in cell growth and PHA yield.

Table 4.1. Glycerol and lignin monomers consumption under nitrogen limitations at 72 h based on their initial concentrations.

Carbon source	**Glycerol consumption (%)**	**Lignin monomer consumption (%)**
Glycerol (10 g/L)	55 ± 2^{A}	-
Glycerol (9 g/L) + Sinapyl alcohol derivatives	41 ± 2^{B}	35 ± 18^{B}
Glycerol (9 g/L) + p-Coumaryl alcohol derivatives	33 ± 1^{D}	34 ± 30^{B}
Glycerol (9 g/L) + Coniferyl alcohol derivatives	37 ± 1^{C}	99.3 ± 0.6^{A}

4.1.4 PHA monomer analysis

The gas chromatography-mass spectrometry and flame ionization detector (GC-MS/FID) analysis were used to investigate the monomeric composition of PHA. Its monomeric composition significantly influences the final properties of PHA. For instance, the thermal and mechanical characteristics of mcl-PHA are strongly dependent on the type of monomer that constitutes the majority of the polymer (Jiang et al., 2013). As depicted Figure 4.5, the GC-chromatograph indicated that the polymer has seven mcl-PHA monomers, including 3-hydroxyhexanoate (C6:0), 3-hydroxyoctanoate (C8:0), 3-hydroxy decanoate (C10:0), 3-hydroxydodecanoate (C12:1 and C12:0), and 3-hydroxy tetradecanoate (C14:1 and C14:0) under all tested conditions. This indicated that the addition of lignin derivatives did not alter the monomer distribution of PHA. As shown in Table 4.2, among the seven identified medium chain-length PHA monomers, the two dominant monomers were the 3-hydroxydecanoate (55.2 ± 0.4 to 63.6 ± 0.3 mol%) and 3-hydroxyoctanoate (C8:0) (20 ± 11 to 28.0 ± 0.4 mol%), and the molar fraction of each of the remaining monomers was below 10%, which was consistent with the previous report (Xu et al., 2021).

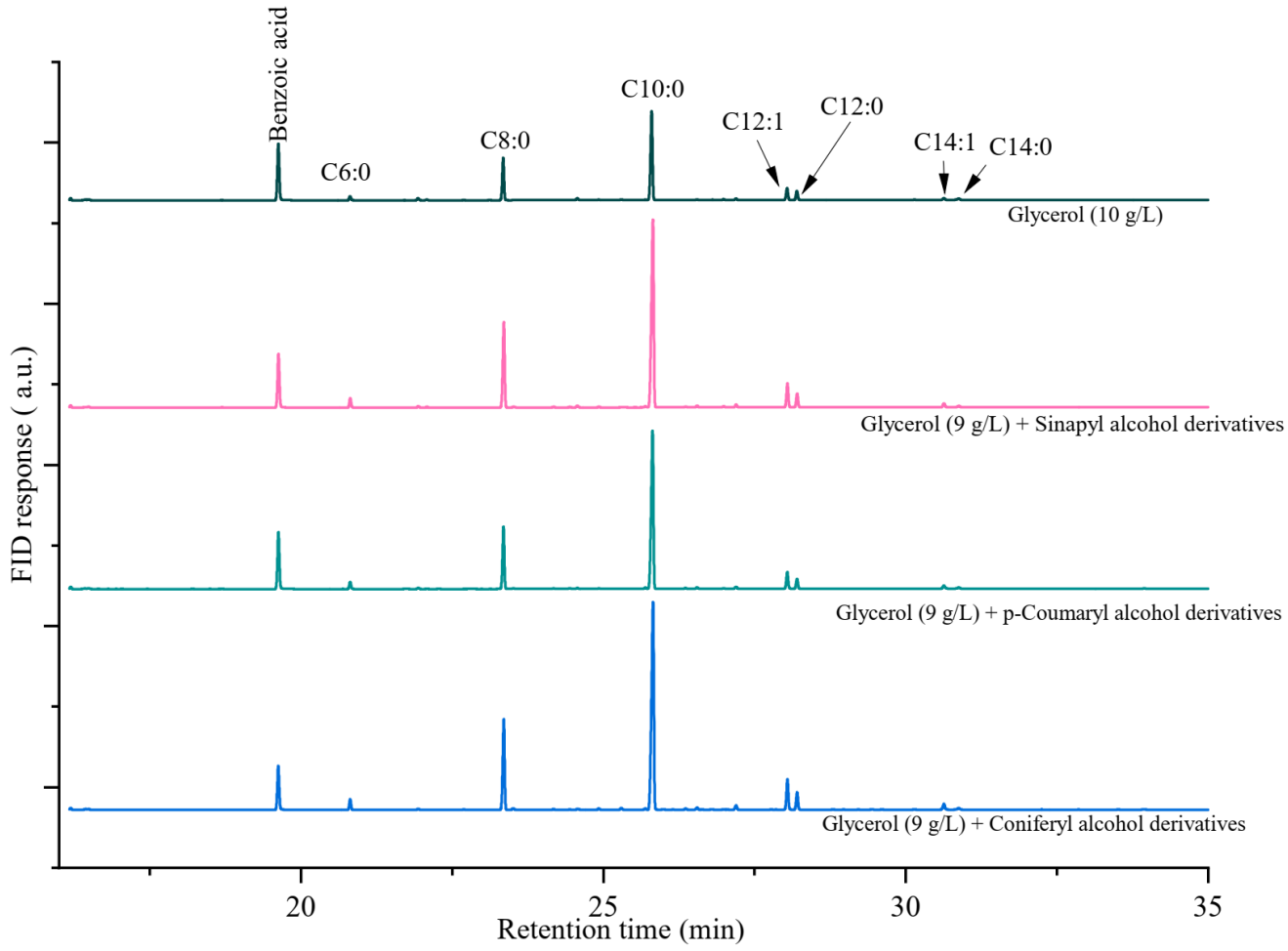

Figure 4.5. The GC-FID chromatogram of mcl-PHA monomers produced by *Pseudomonas putida* KT2440 under nitrogen limitation using glycerol and various lignin derivatives as carbon sources.

Table 4.2. Monomer composition of mcl-PHA produced by *Pseudomonas putida* KT2440 under nitrogen limited conditions. 3-hydroxyhexanoate (C6:0), 3-hydroxyoctanoate (C8:0), 3-hydroxydecanoate (C10:0), 3-hydroxydodecenoate (C12:1), 3-hydroxydodecanoate (C12:1), 3-hydroxytetradecanoate (C14:0), 3-hydroxytetradecenoate (C14:1)

Carbon sources	**Mole fraction (%) of PHA monomers**						
	C6:0	**C8:0**	**C10:0**	**C12:1**	**C12:0**	**C14:1**	**C14:0**
Glycerol (10 g/L)	3.56 ± 0.09^{a}	28.0 ± 0.4^{a}	55.2 ± 0.4^{c}	6.8 ± 0.6^{a}	4.50 ± 0.0^{a}	1.2 ± 0.2^{a}	0.8 ± 0.4^{a}
Glycerol (9 g/L) + Sinapyl alcohol derivatives	3.4 ± 0.4^{a}	20 ± 11^{a}	61 ± 1^{b}	8 ± 5^{a}	5.5 ± 3^{a}	1 ± 1^{a}	0.6 ± 0.4^{a}
Glycerol (9 g/L) + *p*-Coumaryl alcohol derivatives	3.2 ± 0.3^{a}	23.9 ± 0.2^{a}	63.6 ± 0.3^{a}	4.9 ± 0.1^{a}	2.81 ± 0.03^{a}	1.04 ± 0.03^{a}	0.50 ± 0.02^{a}
Glycerol (9 g/L) + Coniferyl alcohol derivatives	3.20 ± 0.06^{a}	24.4 ± 0.5^{a}	61.5 ± 0.5^{b}	6.01 ± 0.03^{a}	3.4 ± 0.1^{a}	1.092 ± 0.004^{a}	$0.0.39 \pm 0.01^{a}$

4.1.5 Conclusion

This study showed that PHA yield and PHA content were enhanced when 10% glycerol was replaced with coniferyl alcohol derivatives, sinapyl alcohol derivatives, or *p*-coumaryl alcohol derivatives while the total carbon was kept constant (325.8 mM). Furthermore, both PHA content and cell growth were increased during the co-feeding of glycerol and coniferyl alcohol derivatives (vanillin and ferulic acid). Therefore, lignin with high coniferyl monomer units, such as softwood lignin, could be considered promising carbon sources for microbial production of polyhydroxyalkanoates (PHA).

4.2 Microbial conversion of lignin and glycerol to polyhydroxyalkanoates by *Pseudomonas putida* KT2440 under Sulfate-limited conditions

4.2.1 Effect of carbon to sulfur molar ratio on cell growth

The effect of carbon-to-sulfur molar ratios (C/S) on the cell growth of *Pseudomonas putida* KT2440 was investigated by varying sulfate concentration, as shown in Figure 4.6. Different conditions of carbon-to-sulfur molar ratios (C/S: 400, 1000, 3000, 5000, 7000, 10000, and 20000) were used, keeping the total carbon content (325.8 mM)constant in all conditions using glycerol with 10% coniferyl alcohol derivatives (ferulic acid and vanillin), with various Na_2SO_4 concentrations (i.e., 0.116 g/L to 0.0023 g/L) to achieve the corresponding carbon-to-sulfur ratios. The optical density (OD_{600nm}) of the fermentation broth was used to study cell growth. The optical density of the cells continued to rise and reached a stationary phase at 48 h under all conditions except C/S 7000, where the OD kept rising until the end of the fermentation. At the end of fermentation, the maximum OD value was 10.7 ± 0.4 at a C/S molar ratio of 1000, and the minimum OD value was 3.91 ± 0.01 at C/S 20000. *Pseudomonas putida* KT2440 did not exhibit a diauxic growth pattern when cultivated in a medium containing glycerol, ferulic acid, and

vanillin under all conditions, indicating that there was simultaneous consumption of carbon sources (Narang & Pilyugin, 2007).

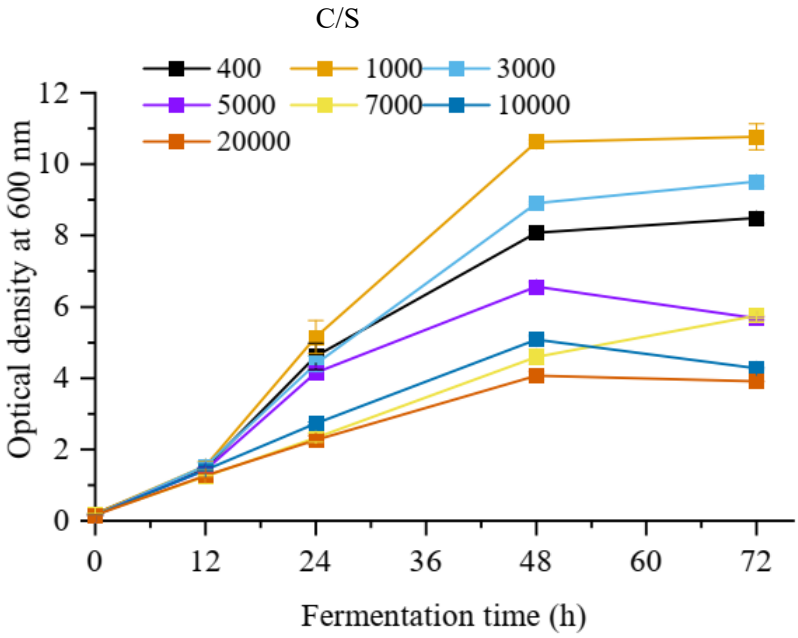

Figure 4.6. Growth curve of *Pseudomonas putida* KT2440 at different carbon to sulfur molar ratios. OD was measured at 600 nm.

In addition to optical density (OD) values, the cell dry weight of *Pseudomonas putida* was determined at the end of the fermentation as shown in Figure 4.7. The maximum dry cell weight of *P. putida* KT2440 was 3.4 ± 0.1 g/L at C/S 1000, which was significantly higher than the results obtained under other tested conditions. However, cell dry weight was 2.62 ± 0.04 g/L at the C/S 400 and 2.45 ± 0.04 g/L at the C/S 3000, indicating no significant difference was observed between these two conditions. When sulfur concentrations in the fermentation media were further decreased, the cell dry weight of *Pseudomonas putida* also decreased, reaching a minimum cell dry weight (~1 g/L) at C/S 10000 and C/S 20000, indicating no statistical difference between them. Both cell dry weight and growth curve results indicated that the cell growth of *Pseudomonas putida* KT2440 was significantly affected by the carbon-to-sulfur molar ratio, with a decrease in

growth observed, particularly at high C/S molar ratios or when sulfur availability was deficient in the fermentation medium. In the literature, it has been shown that sulfur limitation can inhibit the synthesis of sulfur-containing proteins and cofactors, which has resulted in low microbial cell growth of *Rhodospirillum rubrum* (Melnicki et al., 2009) and *Rhodosporidium toruloides* (Wu et al., 2011); similar results in cell growth were observed in this study specifically at lower sulfur concentrations. These findings highlight the importance of optimizing C/S molar ratios in the medium for efficient microbial growth.

The initial pH value was around 7 for all conditions and the pH value of fermentation broth was measured at different fermentation time. As shown in Figure 4.8, the pH value at C/S 400 decreased with time and reached a pH value of 4.5 after 48 h of fermentation, a minimum pH value at which *Pseudomonas putida* KT2440 can not grow (Reva et al., 2006). After 48 h, the pH level at C/S 1000 decreased to ~6 and remained relatively constant until 72 h. However, there were no significant change of pH values with time for other conditions compared to initial value. These results suggested that the pH value was generally more stable during fermentation under lower sulfur conditions. When sulfur is limited, the growth rate of the organism decreased, the metabolic pathways that produce acidic by-products or carbon dioxide (CO_2) might be affected, leading to a slower decrease in pH. However, further studies would be needed to elucidate the specific metabolic changes occurring in response to varying sulfur concentrations and their impact on pH. These findings have the potential to be significantly valuable in large-scale fermentation by eliminating the need for the addition of base to maintain a constant pH value.

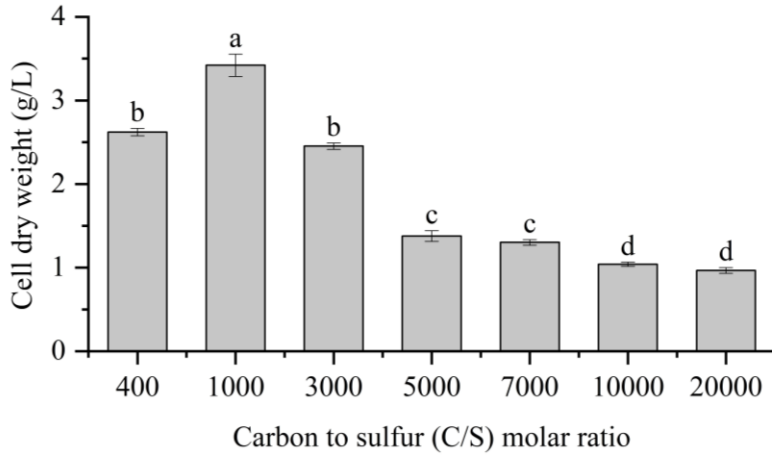

Figure 4.7. Cell dry weight of *Pseudomonas putida* KT2440 at 72 h under different carbon to sulfur molar ratios. Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different (P<0.05).

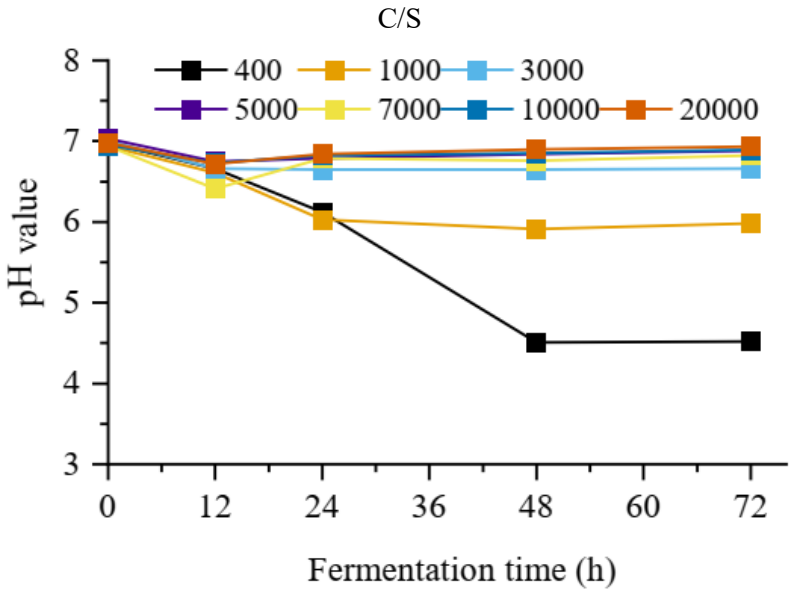

Figure 4.8. pH value of fermentation broth with time at different carbon to sulfur molar ratio conditions.

4.2.2 PHA content and PHA yield by *P. putida* KT2440

PHA content (%CDW) and PHA yield (g/L) at the end of the fermentation were considered to assess the effect of sulfur limitation on PHA production by *Pseudomonas putida* KT2440. Our

results showed that reduced sulfur concentration (Na_2SO_4) enhanced the PHA accumulation of *Pseudomonas putida* KT2440 as PHA content significantly increased with an increased carbon-to-sulfur (C/S) molar ratio (Figure 4.9). When *Pseudomonas putida* KT2440 was cultivated at C/S molar ratios of 1000, 3000, 5000, 7000, 10000, and 20000, the PHA content was 11 ± 2%, 30 ± 1%, 24.0 ± 0.4%, 24 ± 2%, 21 ± 2%, and 18.1 ± 0.6%, respectively, which was significantly higher than 2.7 ± 0.8% at C/S 400. Maximum PHA content was achieved at a carbon-to-sulfur molar ratio of 3000, which was significantly higher than the PHA content found in all other conditions. There was no significant difference in PHA content (%) at C/S 5000, C/S 7000, and C/S 10000, but a further increase of carbon to sulfur molar ratio to C/S 20000 showed a significant decrease.

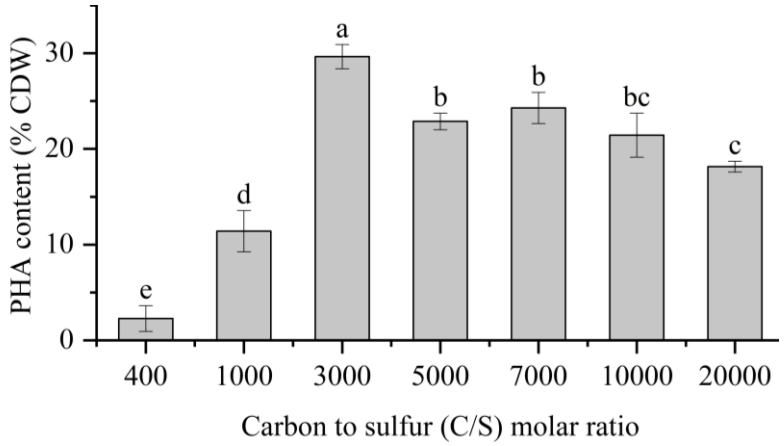

Figure 4.9. PHA content (% CDW) under different carbon to sulfur molar ratio conditions. Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different ($P<0.05$).

Similarly, the PHA yield (g/L) increased significantly once the sulfur content of the medium was decreased (Figure 4.9). PHA yield dramatically rose from 0.06 ± 0.03 to 0.73 ± 0.04 g/L when the carbon-to-sulfur molar ratio was raised from 400 to 3000. Similar to PHA content, there was no significant difference in PHA yield in the C/S 5000–10000 range; however, further raising the C/S ratio resulted in a lower PHA yield. The results of this study revealed that when *Pseudomonas putida* KT2440 was grown on a medium under sulfur-limited conditions, PHA yield and PHA

content were improved. These indicated that carbon sources could be channeled more preferentially to the PHA synthesis pathways. Similarly in previous studies, the sulfur limitation has also been used to enhance the microbial production of hydrogen and PHA by photosynthetic bacteria, *Rhodospirillum rubrum* (Melnicki et al., 2009). Valappil and other coauthors have compared the effects of nitrogen, phosphate, potassium, and sulfur limitations on PHA synthesis by *B. cereus* SPV using glucose. They found that the PHA content in sulfur limitation was 13.5 % CDW after 48 h and 1.29 % CDW after 72 h, which was the lowest compared to other situations (Valappil et al., 2008). Here, it is important to mention that the impact of nutrient limitation on PHA production varies with the type of microbial strain, carbon source, and limiting nutrient (Montiel-Corona & Buitrón, 2022). However, the effect of sulfur limitation on PHA accumulation in *Pseudomona*s species had been unknown. Therefore, this is the first report that uses a sulfur limitation strategy to improve the PHA production by *Pseudomonas putida* KT2440 using glycerol and lignin-based aromatic as carbon sources.

Nitrogen limitation is known to promote PHA synthesis in *P. putida* KT2440 when the bacteria are cultured on structurally unrelated carbon sources (Możejko-Ciesielska & Serafim, 2019; Xu et al., 2019). Consequently, we used nitrogen limitation in the first part of our studies. As demonstrated in Sections 4.1.1 and 4.1.2, nitrogen limitation resulted in a PHA content of 40 ± 0.8% and a PHA yield of 0.456 ± 0.002 g/L. In contrast, sulfur limitation approach achieved a PHA content of 30 ± 1% and PHA yield of 0.73 ± 0.04 g/L at a carbon-to-sulfur molar ratio of 3000. Although the PHA content under sulfur limitation was lower than under nitrogen limitation, the PHA yield was significantly higher under sulfur limitation. Higher PHA content per cell dry weight plays an essential role in reducing cost associated with purification and recovery of PHA from microbial cells during industrial PHA production, as it reduces the cost of extraction solvent

and energy, and waste disposal (Salehizadeh & Van Loosdrecht, 2004; Yang et al., 2011), which is the advantages of nitrogen limitation. On the other hand, sulfur limitation has advantages over nitrogen limitation, as higher PHA yield is essential for the overall productivity and economic viability of the process. A higher PHA yield means that more PHA is produced per unit volume of fermentation broth, which can result in lower production costs. Both PHA content of the cell and PHA yield are important in industrial production, but their significance depends on the specific context and objectives of the process.

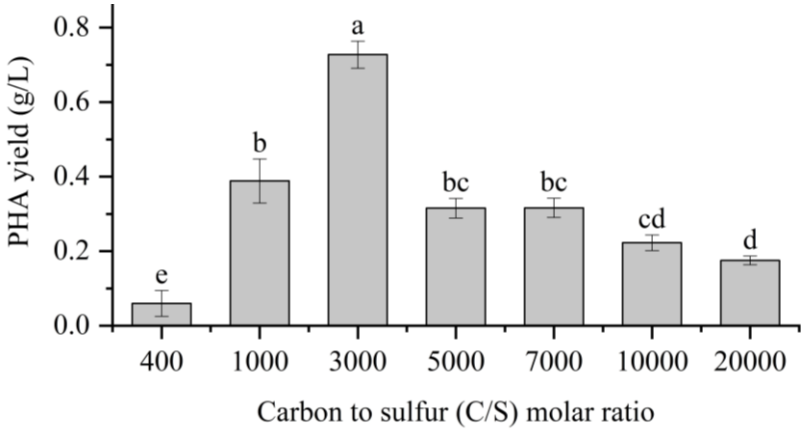

Figure 4.10. PHA yield (g/L) under different carbon to sulfur molar ratio conditions. Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters is significantly different ($P<0.05$).

4.2.3 Utilization of carbon sources by *Pseudomonas putida* KT2440

The contents of glycerol, ferulic acid, and vanillin were measured during the fermentation process to understand better how *Pseudomonas putida* used various carbon sources under sulfur-limited conditions (Figure 4.11). Glycerol was completely consumed at 48h when *Pseudomonas putida* KT2440 was cultivated on a medium with a C/S molar ratio of 1000. This is consistent with the growth curve where maximum OD was achieved at this condition. However, at C/S 400, glycerol residual was 2.16 ± 0.05 g/L at 48 h and remained at the same level until 72h. The possible reason

might be that the pH of the medium was dropped to 4.5, which inhibited cell growth and glycerol consumption (Reva et al., 2006). In other conditions, glycerol was continuously consumed until the fermentation ended and was never exhausted. At the end of the fermentation, there was a significant difference in glycerol residual in all conditions, as shown in Figure, indicating that the glycerol consumption was decreased when the carbon to sulfur (C/S) molar ratio of the growth medium increased, which subsequently decreased cell biomass. On the other hand, vanillin was consumed within 24 h of inoculation, while ferulic acid was consumed within 12 h in all conditions except C/S 7000. The result showed that both glycerol and lignin-derived compounds were consumed simultaneously by *Pseudomonas putida* KT2440. This is consistent with the growth curve of *Pseudomonas putida* KT2440.

It is important to note that a significant quantity of lignin is created as a by-product during the process of producing lignocellulosic biofuel (Zhou et al., 2022). During the lignin degradation pathway, ferulic acid and vanillin are dominant intermediate metabolites, which bacteria can further convert into polyhydroxyalkanoates (PHAs) (Zhou et al., 2020). Additionally, glycerol is another by-product that is formed during the process of producing biodiesel from animal fats and plant oils (Monteiro et al., 2018). Therefore, effective exploitation of these substrates is required to lower not only the cost of PHA production but also the cost associated with biofuel and biodiesel, ultimately improving the sustainability of the emerging biorefinery industries.

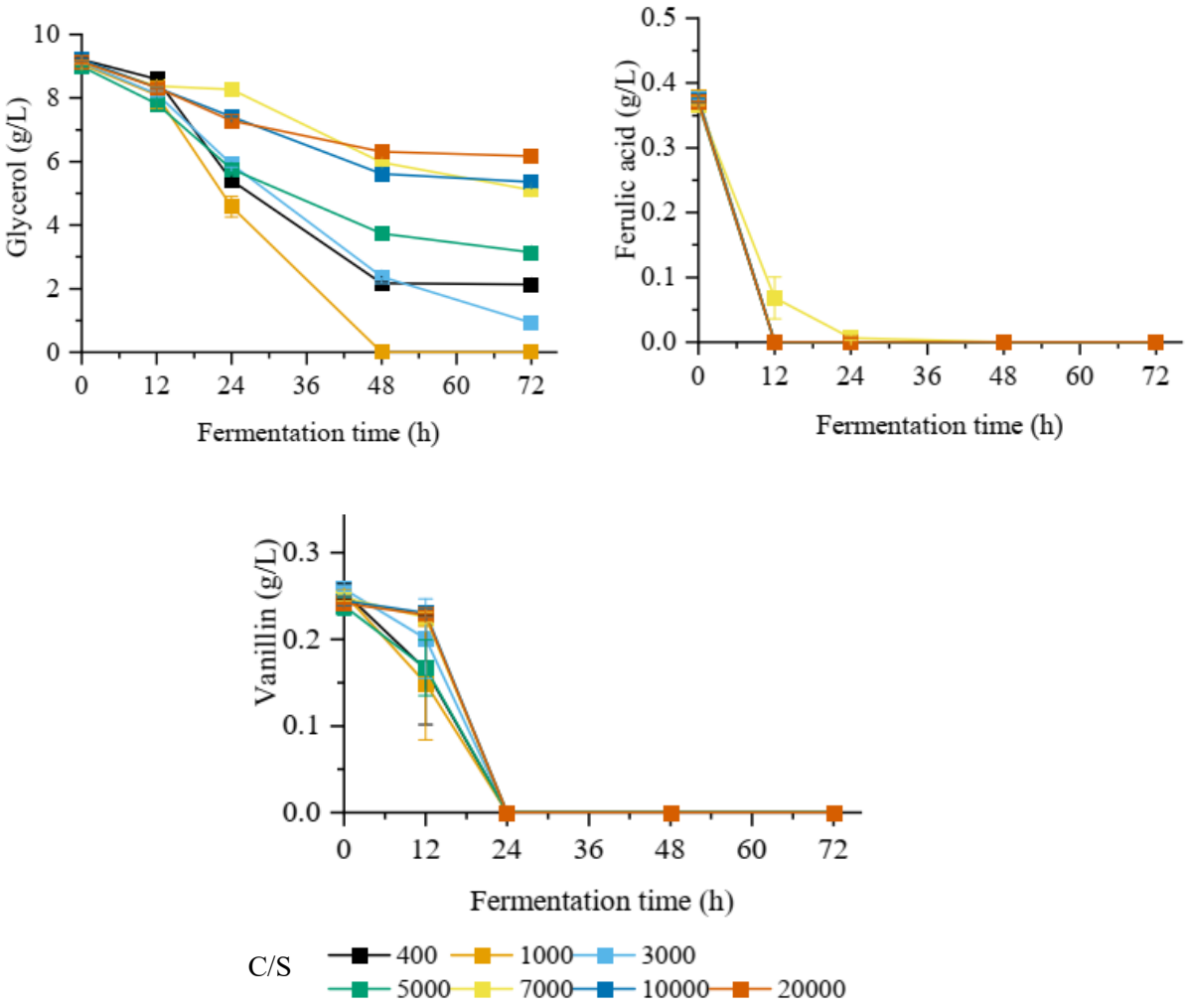

Figure 4.11. Carbon sources (glycerol, vanillin, and ferulic acid) utilization by *Pseudomonas putida* KT2440 different carbon to sulfur molar ratios. Data are presented as the means ± standard deviation (error bars) of triplicate experiments.

4.2.4 PHA monomer composition Analysis

The thermal and mechanical properties of PHA depend on the type of PHA monomers and their monomeric compositions (Jiang et al., 2013). PHA samples were analyzed by gas chromatography-mass spectrometry and flame ionization detector (GC-MS/FID) to determine the monomers compositions. The results showed the existence of mcl-PHA monomers at all conditions (Figure 4.12). Four PHA monomers, including 3-hydroxyoctanoate (C8:0), 3-hydroxy decanoate

(C10:0), 3-hydroxydodecanoate (C12:1 and C12:0), were found at a C/S molar ratio of 400. In other conditions, seven mcl-PHA monomers, including 3-hydroxyhexanoate (C6:0), 3-hydroxyoctanoate (C8:0), 3-hydroxy decanoate (C10:0), 3-hydroxydodecanoate (C12:1 and C12:0), and 3-hydroxy tetradecanoate (C14:1 and C14:0) were identified.

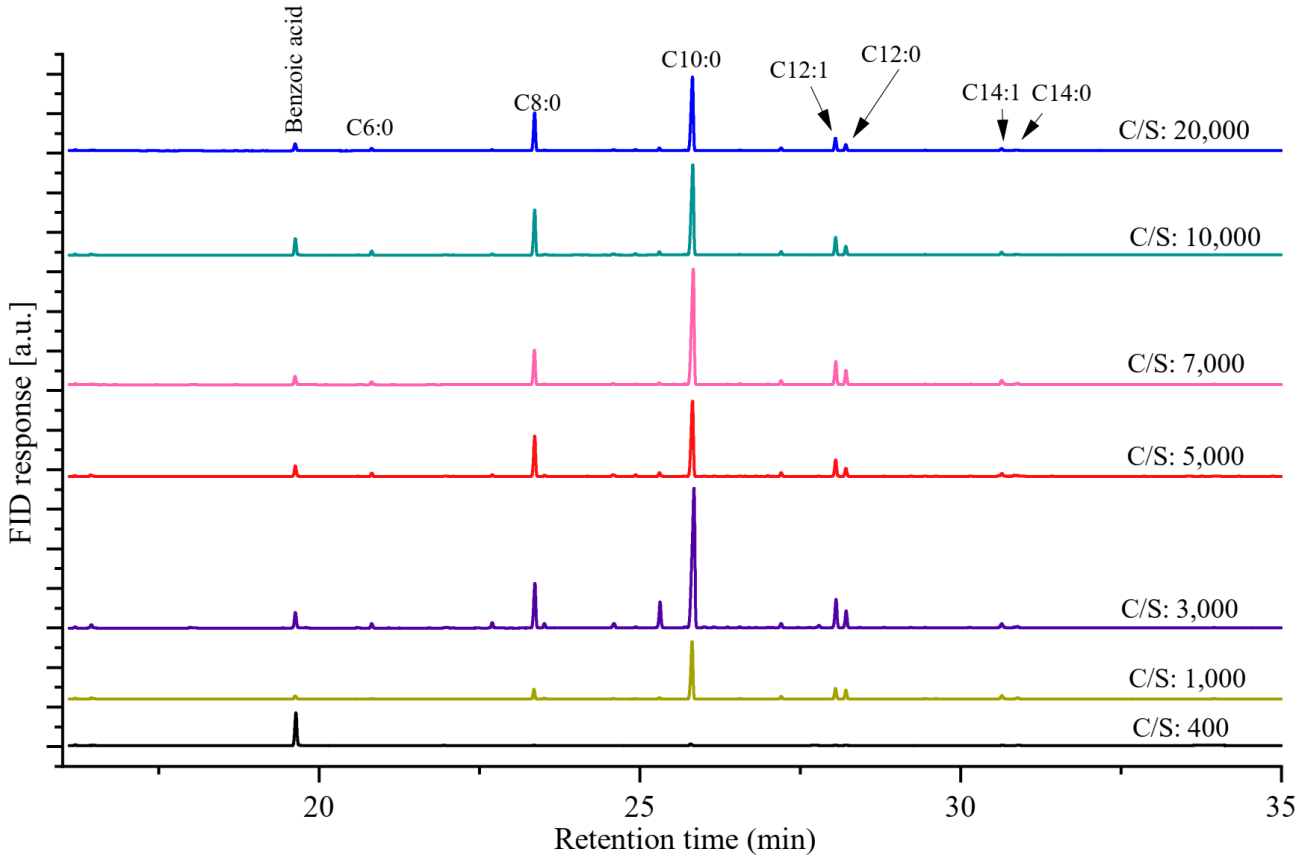

Figure 4.12. The GC-FID chromatogram of mcl-PHA monomers produced by *Pseudomonas putida* KT2440 various carbon to sulfur molar ratios.

Similarly, Xu et al. reported that mcl-PHA produced in nitrogen-surplus conditions consisted of only two monomers, while six monomers were found in nitrogen-limited situations, which indicated that nutrient limitation highly affected the PHA monomer distribution (Xu et al., 2019). As shown in Table 4.3, Under all tested conditions in our study, the most dominant monomer was the 3-hydroxydecanoate (C10:0) (57.1 ± 0.1 to 66.8 ± 0.5 mol%), followed by the 3-

hydroxyoctanoate (C8:0) (15 ± 3 to 29.1 ± 0.3 mol%), which was consistent with previous reports (Beckers et al., 2016; Poblete-Castro et al., 2014; Xu et al., 2021).

Table 4.3. Monomer composition of mcl-PHA produced by *Pseudomonas putida* KT2440 at different carbon to sulfur molar ratios. Data are presented as the means ± standard deviation of triplicate experiments. Data points denoted with different letters in the column are significantly different (P<0.05). 3-Hydroxyhexanoate (C6:0), 3-hydroxyoctanoate (C8:0), 3-hydroxydecanoate (C10:0), 3-hydroxydodecenoate (C12:1), 3 hydroxydodecanoate (C12:1), 3-hydroxytetradecanoate (C14:0), 3-hydroxytetradecenoate (C14:1)

	Mole fraction (%) of PHA monomers						
C/S (mol/mol)	**C6:0**	**C8:0**	**C10:0**	**C12:1**	**C12:0**	**C14:1**	**C14:0**
400	-	15 ± 3 [a]	66 ± 2 [a]	5 ± 5 [a]	14 ± 2 [a]	-	-
1000	1 ± 1 [a]	16 ± 10 [a]	63 ± 5 [a]	9 ± 2 [a]	6 ± 3 [b]	3 ± 1 [a]	1 ± 1 [a]
3000	2 ± 1 [a]	20 ± 7 [a]	63 ± 7 [a]	8.9 ± 0.3 [a]	4.4 ± 0.4 [b]	1.5 ± 0.2 [a]	0.4 ± 0.1 [a]
5000	1.9 ± 0.1 [a]	23 ± 5 [a]	59 ± 5 [a]	8.8 ± 0.3 [a]	4.4 ± 0.2 [b]	1.7 ± 0.3 [a]	0.5 ± 0.1 [a]
7000	2 ± 1 [a]	16.5 ± 0.3 [a]	66.8 ± 0.5 [a]	8.3 ± 0.3 [a]	4.8 ± 0.1 [b]	1.6 ± 0.2 [a]	0.47 ± 0.04 [a]
10000	2 ± 1 [a]	21 ± 10 [a]	61 ± 6 [a]	8 ± 1 [a]	5 ± 3 [b]	2 ± 1 [a]	0.7 ± 0.6 [a]
20000	2.3 ± 0.2[a]	29.1 ± 0.3[a]	57.1 ± 0.1 [a]	6.83 ± 0.02 [a]	3.20 ± 0.03 [b]	1.15 ± 0.02 [a]	0.3 ± 0.2 [a]

4.2.5 Conclusion.

The role of sulfur limitation in PHA production by *Pseudomonas putida* KT2440 had not been investigated previously. This study demonstrated, for the first time, that sulfur-limited conditions greatly stimulated the production of mcl-PHA from glycerol and lignin monomers. We found that *Pseudomonas putida* KT2440 produced significant amount of PHAs under sulfur-limited conditions compared to non-limited condition. The highest PHA content of the cell under sulfur limited condition reached 30 ± 1% at C/S 3000, which was significantly higher than the 2.7 ± 0.8% under non-limited condition. This finding suggests that manipulating sulfur levels can be a useful strategy for optimizing PHA production, in addition to the known strategy of nitrogen limitation.

4.3 Investigation of lignin concentration on PHA production by *Pseudomonas putida* KT2440

4.3.1 Effects of lignin monomers on cell growth

In this study, the effects of lignin ratios on cell growth of *Pseudomonas putida* KT2440 under sulfur-limited conditions (C/S: 30000) was investigated. To explore the effects, six different conditions were used by varying the lignin percent from 0 mol% (glycerol alone) to 100 mol% (lignin derivatives alone) of the total carbon of the media. Under all conditions, the total carbon content of the media was kept constant at 325.8 mM to avoid the effects of carbon supply fluctuation. The carbon source was glycerol, lignin-derived compounds (ferulic acid and vanillin), or a mixture of glycerol and lignin derivatives. The cell growth was assessed by measuring the OD_{600nm} of the fermentation broth at 0, 24, 48, 72, and 96 h and determining cell dry weight at the end of the fermentation (96 h).

As shown in Figure 4.13, the growth curve was generated by plotting the optical density (OD) values at 600 nm (OD_{600nm}) of the fermentation broth against fermentation time. Upon analyzing

the growth curve, it was observed that the optical density generally increased until 48 h for 0 mol% and 10 mol% lignin derivatives. But, after 72 h, the optical density was decreased at 10 mol% lignin derivatives, possibly due the bacteria degrading the accumulated PHA for energy or cellular maintenance purposes, resulting in a reduction in OD. However, when lignin concentrations were increased (30 %, 50%, and 70% mol), the optical density continued to increase until 72 h and maintained nearly constant until the end of the fermentation. In the case of only lignin derivatives (100% mol), a lag phase was observed until 24 h, and the final OD value was significantly lower than the OD value in other tested conditions.

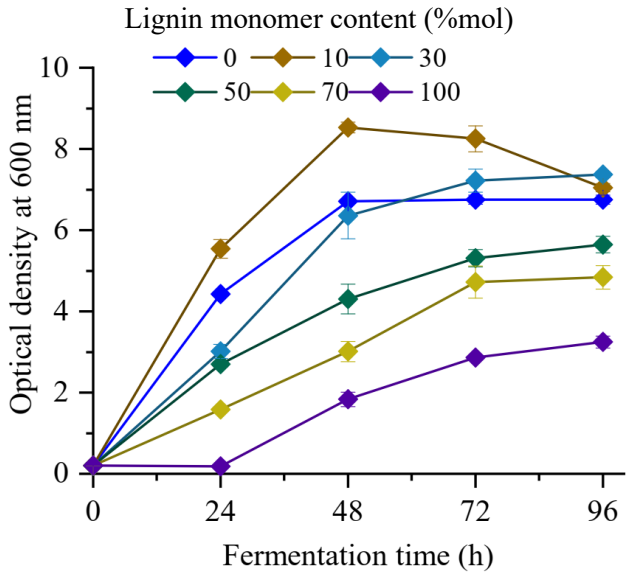

Figure 4.13. Growth curve of *Pseudomonas putida* KT2440 when cells were grown at different lignin concentrations under sulfur limited conditions (C/S: 3000). Data are presented as the means ± standard deviation (error bars) of triplicate experiments.

Cell dry weight was analyzed to provide a more comprehensive understanding of effect of lignin level on the cell growth (Figure 4.14). The maximum dry cell weight of *P. putida* KT2440 was 2.009 ± 0.007 and 2.02 ± 0.03 g/L at 10 % and 30% mol of lignin, respectively, which were significantly higher than the results obtained under other tested conditions. However, *Pseudomonas putida* KT2440 cell dry weight decreased when the lignin monomer

concentration of the medium was increased. The cell dry weight at lignin derivatives alone (100 % mol) was 0.94 ± 0.08 g/L, significantly lower than all conditions. This dry cell weight result mostly supported the observations made in the growth curve.

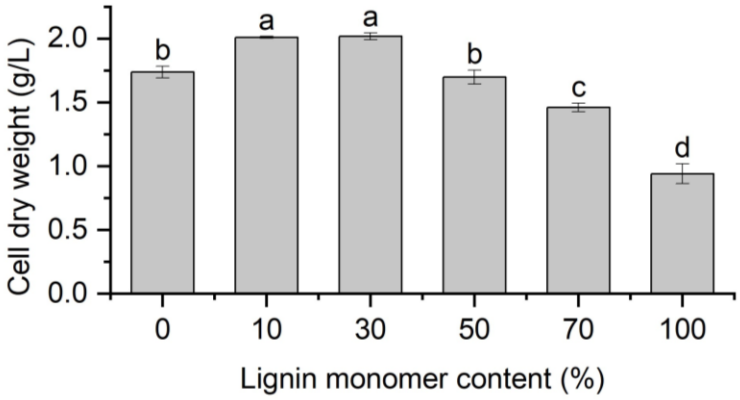

Figure 4.14. Cell dry weight of *Pseudomonas putida* KT2440 at 96 h when cells were grown on different lignin concentrations under sulfur limited conditions (C/S: 3000). Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different (P<0.05).

In this study, we observed that cell growth was significantly enhanced by adding up to 30 % mol of lignin monomer mixture (vanillin and ferulic acid) as a carbon source compared to utilizing glycerol alone under sulfur-limited conditions. Similar results were reported by Xu et al., 2021, under nitrogen-limited conditions during the growth of *Pseudomonas putida* KT2440 on media containing glycerol and benzoate. They found that co-feeding of benzoate (1 g/L) with glycerol (9 g/L) showed cell growth improvement compared to glycerol (10 g/L). The authors suggested that the possible reason for increased cell growth during co-feeding might be the higher overall carbon content, as 1 g/L of benzoate has a higher carbon content (57.3 mM) than 1 g/L of glycerol (32.7 mM). One might expect that cell growth would continue to increase with more lignin monomer additions. Similarly, in our study, despite maintaining a constant total carbon content, cell growth improved during co-feeding, suggesting that the lignin monomers or their mixture might have

contributed to the enhanced growth through other mechanisms. One possible explanation might be that the oxidative stress induced by aromatic compounds can be detrimental to cells at high concentrations; however, at low levels, it can trigger a hormetic response. Hormesis is a phenomenon in microorganisms where a low dose of a stressor can result in beneficial effects, such as enhanced cell growth (Tang et al., 2022). It is possible that the addition of aromatic compounds at low concentrations may lead to a hormetic response by upregulating of ATP synthesis, which could explain the observed growth improvement. (Alhasawi et al., 2015). However, further study is necessary to conclusively determine whether the observed growth improvements are due to hormetic responses.

4.3.2 Effect of lignin concentration on PHA content and yield

The impact of lignin monomer concentration on PHA production by *Pseudomonas putida* KT2440 was evaluated by measuring the PHA content (%CDW) and PHA yield (g/L) after fermentation. As shown in Figure 4.15, the maximum PHA content (33.6 ± 0.7% CDW) was obtained when 10% of total carbon was from lignin monomers, which was significantly higher than the PHA content found in all other tested conditions. Interestingly, no significant difference was observed in PHA content between glycerol alone (0 %mol lignin monomers) and 30 %mol of monomers, 27.5 ± 0.6 and 27 ± 1 % CDW, respectively. However, when the lignin monomer concentration was further increased to 50% or higher, a significant decrease in PHA content was observed, indicating that higher lignin monomer contents have a detrimental effect on PHA content. As shown in Figure 4.16, a similar pattern was observed in PHA yield, with a maximum yield of 0.68 ± 0.02 g/L at 10 %mol of lignin monomer content. However, the cultivation of *Pseudomonas putida* on lignin monomers without adding glycerol (100 %mol lignin) gave a PHA yield of 0.10 ± 0.02 g/L, which was significantly lower than the PHA yield of 0.478 ± 0.009 g/L obtained from a carbon source

containing glycerol alone. These findings indicate that small addition of vanillin and ferulic acid triggered the accumulation of PHA, while higher monomer concentrations were not beneficial for PHA production by *Pseudomonas putida* KT2440.

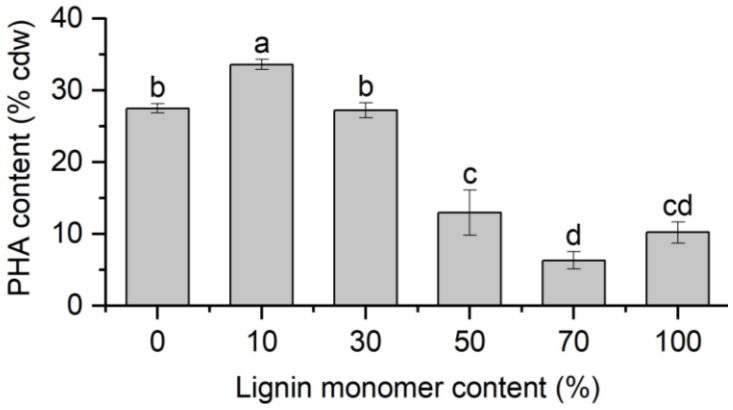

Figure 4.15. Effect of lignin monomers concentration on the PHA content when *Pseudomonas putida* KT2440 was cultivated under sulfur limited conditions (C/S: 3000). Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different (P<0.05).

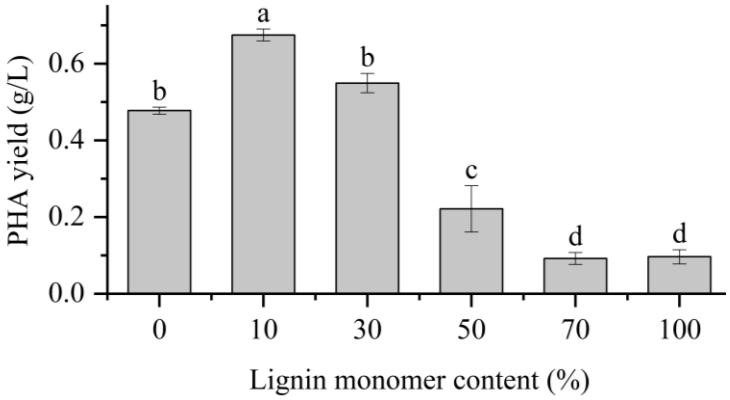

Figure 4.16. Effect of lignin monomers concentration on the PHA yield when *Pseudomonas putida* KT2440 was cultivated under sulfur limited conditions (C/S: 3000). Data are presented as the means ± standard deviation (error bars) of triplicate experiments. Bars denoted with different letters are significantly different (P<0.05).

In the literature, it has been demonstrated that oxidative stress led to an increase NADPH level, which in turn promotes PHA synthesis (Montano-Herrera et al., 2017; Alhasawi et al., 2015). This suggests that the cellular response to oxidative stress may contribute to the regulation of PHA production. Building on this knowledge, Xu et al. (2021) investigated the effect of an aromatic

compound, benzoate, as an inducer of oxidative stress in *Pseudomonas putida* KT2440 They found that that co-feeding small amounts of benzoate with glycerol increased the ratio of NADPH/NADP+, leading to an increase in PHA accumulation in *Pseudomonas putid*a KT2440. However, they also observed that the ratio of NADPH/NADP+ decreased when the benzoate amount in the media increased (Xu et al., 2021). In our study, the addition of 10% mol of lignin-derived compounds (ferulic acid and vanillin) may have similarly induced oxidative stress, resulting in increased NADPH level and ultimately enhancing PHA production.

4.3.3 Consumption of carbon sources

The contents of glycerol, ferulic acid, and vanillin were measured during the fermentation process to understand better how *Pseudomonas putida* KT2440 used various carbon sources at sulfur-limited condition. As shown in Figure 4.17, when glycerol was co-fed with lignin monomers, glycerol was completely consumed after 72 h of the fermentation period. However, glycerol was continuously consumed until 96 h when glycerol alone was used as a carbon source. During co-feeding of glycerol and 10 %mol of lignin, the glycerol consumption rate between 0 h 24 h was considerably higher than the glycerol consumption rate in other co-feeding conditions. It indicated that adding a small amount of lignin monomers (ferulic acid and vanillin) enhanced glycerol consumption and increased the cell growth of *Pseudomonas putida* KT2440 in a sulfur-limited environment. Similar to our results, Xu et al. (2021) showed that the complete consumption of glycerol during the co-feeding of glycerol (9 g/L) with benzoate (1 g/L) was faster than feeding glycerol (10 g/L). However, when benzoate concentration was increased beyond 1 g/L, the resulting glycerol consumption rate was lower than glycerol alone (10 g/L).

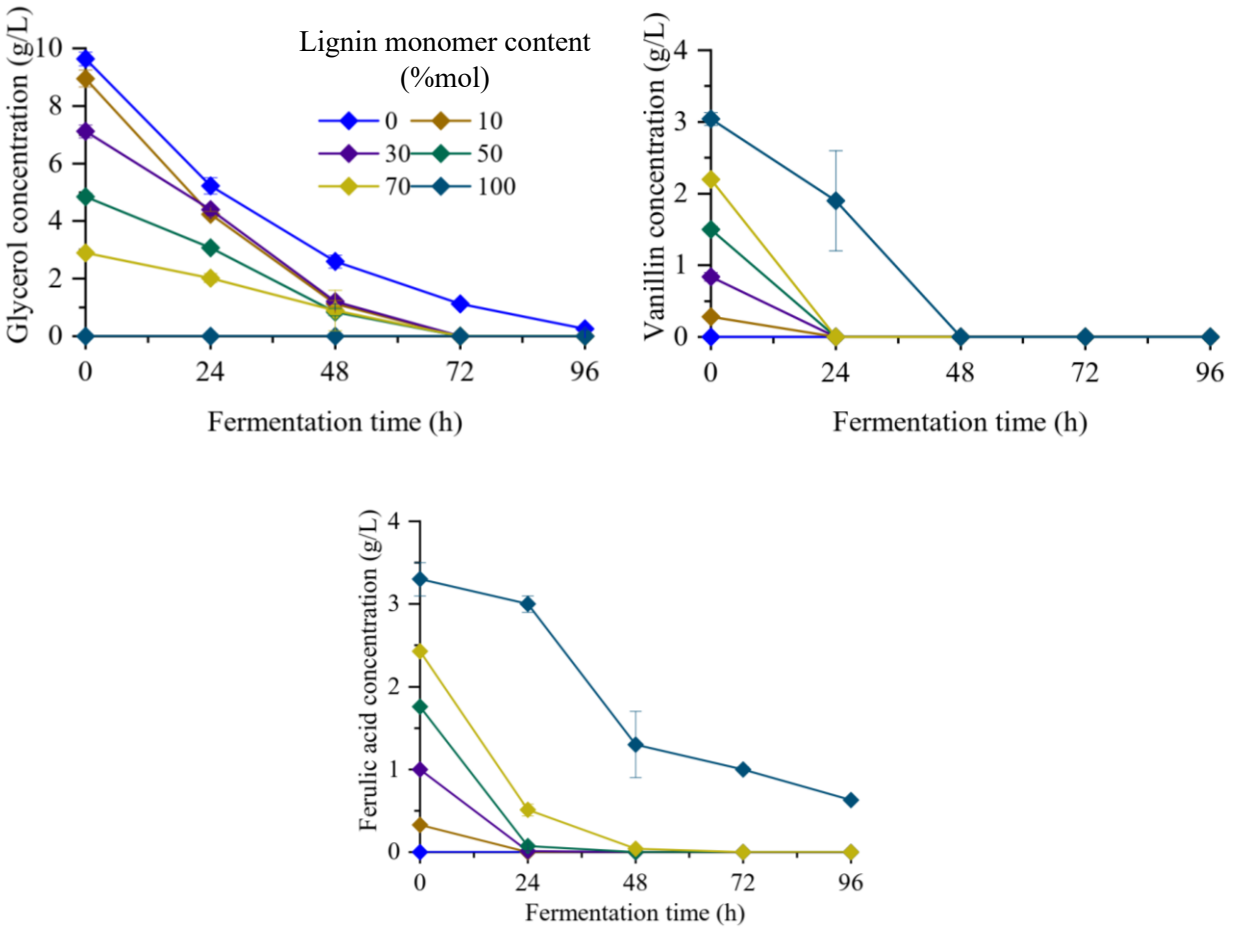

Figure 4.17. Carbon source consumption by *Pseudomonas putida* KT2440 at different lignin monomer concentrations. Data are presented as the means ± standard deviation (error bars) of triplicate experiments.

In terms of aromatics consumption, vanillin was completely consumed within 24 h, while ferulic acid was completely consumed within 48 h when lignin monomers in the media increased up to 70% mol. At high lignin monomer concentration (100% mol) a complete consumption of vanillin was observed after 48 h. In contrast, ferulic acid was not completely consumed until 96 h at higher lignin monomer content (100 %mol), where residual ferulic acid was 0.63 ± 0.02 g/L, indicating that *Pseudomonas putida* KT2440 utilized only around 80% of initial ferulic acid. Although the fermentation started with an equal amount of vanillin and ferulic acid, the vanillin consumption rate was higher than that of ferulic acid. Similarly, Ravi et al., 2017 reported that vanillate was more rapidly consumed than ferulate when *Pseudomonas putida* KT2440 and new isolates were

cultivated on a mixture of lignin model monomers (Ravi et al., 2017). Vanillin is an intermediate metabolite of the ferulic acid funneling pathway in *Pseudomonas* species (Jiménez et al., 2002). Therefore, a high initial vanillin amount in the media might affect the metabolism of ferulic acid as slow consumption of ferulic acid was observed within 24 h (Figure 4.17).

4.3.4 PHA monomer analysis

The monomeric composition of PHA has a significant impact on its final properties. In this study, the monomeric composition of PHA was analyzed by gas chromatography-mass spectrometry and flame ionization detector (GC-MS/FID). As shown in Table 4.4, the result revealed the presence of monomer units with 6 to 14 carbon atoms, signifying that the polymer is medium chain length PHA (mcl-PHA). Seven mcl-PHA monomers were identified regardless of the addition of lignin monomers. The most dominant monomer in all tested conditions was the 3-hydroxydecanoate (C10:0). The amount of this monomer was significantly increased as lignin content increased, whereas co-feeding of lignin derivatives with glycerol resulted in a low content of unsaturated mcl-PHA monomer (3-hydroxy dodecanoate (C12:1), compared to feeding glycerol alone. The thermal and mechanical properties of mcl-PHA highly depend on the nature of the dominant monomers (Jiang et al., 2013). In addition, the unsaturated side chains in these PHAs offer opportunities for further modification through various chemical reactions such as cross-linking, epoxide formation by oxidation or double bond hydration (Lee & Choi, 1995). It concluded that the composition of mcl-PHAs can be altered by selecting different feedstocks and their combinations, ultimately would affecting the final properties of the PHA material.

Table 4.4. Monomer composition of mcl-PHA produced by *Pseudomonas putida* KT2440 at different lignin monomer concentrations. 3-hydroxyhexanoate (C6:0), 3-hydroxyoctanoate (C8:0), 3-hydroxydecanoate (C10:0), 3-hydroxydodecenoate (C12:1), 3-hydroxydodecanoate (C12:1), 3-hydroxytetradecanoate (C14:0), 3-hydroxytetradecenoate (C14:1). Values in the table represent the average ± standard deviation of triplicate of experiments, except 10% mol, which is duplicate experiments. The values that do not share the same letter in the column indicate that they are significantly different ($p<0.05$).

Lignin monomer (%mol)	**Mole fraction (%) of PHA monomers**						
	C6:0	**C8:0**	**C10:0**	**C12:1**	**C12:0**	**C14:1**	**C14:0**
0	1.6 ± 0.7^{a}	15 ± 2^{a}	57.2 ± 0.6^{d}	14.3 ± 0.9^{a}	7.5 ± 0.4^{a}	3 ± 1^{a}	1.2 ± 0.6^{a}
10	1.7 ± 0.4^{a}	19.0 ± 0.5^{a}	61 ± 1^{c}	10.288 ± 0.003^{b}	5.507 ± 0.003^{bc}	1 ± 1^{a}	1.5 ± 0.9^{a}
30	1.9 ± 0.1^{a}	17.7 ± 0.3^{a}	65.3 ± 0.2^{b}	8.64 ± 0.06^{cd}	4.67 ± 0.05^{d}	1.52 ± 0.09^{a}	0.37 ± 0.05^{a}
50	0.6 ± 0.2^{a}	11.71 ± 0.04^{b}	70.0 ± 0.4^{a}	9.0 ± 0.1^{c}	6.1 ± 0.1^{b}	2.0 ± 0.1^{a}	0.59 ± 0.03^{a}
70	1.4 ± 0.7^{a}	16 ± 2^{a}	67 ± 2^{b}	7.9 ± 0.4^{cd}	5.1 ± 0.3^{cd}	1.8 ± 0.2^{a}	0.65 ± 0.05^{a}
100	1.5 ± 0.2^{a}	15.1 ± 0.4^{a}	67.6 ± 0.9^{ab}	7.7 ± 0.5^{d}	5.09 ± 0.06^{cd}	2.19 ± 0.07^{a}	0.83 ± 0.05^{a}

4.3.5 Conclusion

This study investigated the effect of lignin derivatives on PHA production and cell growth under sulfur limited conditions using shaking flask experiments. These finding demonstrated that co-feeding of small amount of lignin derivatives such as ferulic acid and vanillin with glycerol significantly increased both cell growth and PHA production. However, further addition above 30 mol% of lignin derivatives resulted in decrease in both the cell growth, and PHA content and yield. This finding is significant, as it suggests that incorporating small lignin derivatives as a carbon source can boost microbial growth and PHA synthesis, regardless of whether nitrogen or sulfur limitation is applied. The possibilities for future research in this area include exploring the optimal lignin monomer ratios to maximize PHA production, investigating the mechanisms behind the observed effects, and finding ways to overcome the negative impacts of higher lignin concentrations.

5 General conclusions and recommendations

5.1 General conclusions

The global plastic industry has been facing growing environmental concerns due to the unsustainable nature of traditional petroleum-based plastics, which contribute significantly to plastic pollution and have detrimental effects on ecosystems. To address this pressing issue, the development of biodegradable bioplastics from industrial by-products, such as lignin and glycerol, presents a promising solution that not only mitigates plastic pollution but also adds value to by-products. By utilizing these underexploited resources, we could create eco-friendly alternatives to conventional plastics, supporting a circular economy and promoting sustainable development. This study aims to increase the yield of biodegradable bioplastics through the implementation of various nutrient limitation strategies.

In the first study, it was concluded that co-feeding of lignin-based aromatic mixtures and glycerol, significantly improved the yield and content of biodegradable bioplastics (PHA) in *Pseudomonas putida* KT2440 under nitrogen limitations (Section 4.1). Among the three lignin monomer derivatives tested (coniferyl alcohol derivatives, *p*-coumaryl alcohol derivatives, and sinapyl alcohol derivatives), coniferyl alcohol derivatives, which is primarily monomers softwood lignin, demonstrated the most promising results when used in combination with glycerol as a co-substrate. As a result, it is possible that utilizing coniferyl alcohol derivatives in conjunction with glycerol can lead to a more efficient bioplastic production process. Softwood species are abundant in Canada, particularly in the Boreal Forest region, making this approach sustainable (Maltman et al., 2002).

Although nitrogen limitation has been widely studied to improve bioplastic production, it may not always be the most effective strategy for all types of microorganisms or feedstocks. This variability necessitates the exploration of other nutrient limitations to identify the alternative approach for maximizing PHA yield. Considering these factors, the second study focused on developing alternative fermentation strategies for improving PHA yield (Section 4.2). Specifically, sulfur limitation was examined for its potential to enhance PHA production from glycerol and coniferyl alcohol derivatives (vanillin and ferulic acid) in *Pseudomonas putida* KT2440. These findings suggest that sulfur limitation can be an effective strategy for improving PHA production in *Pseudomonas* species, potentially providing better results than nitrogen limitation particularly in PHA yield. In this section, the study highlighted the potential of novel fermentation strategies, such as sulfur limitation, for enhancing PHA production and may help optimize the production of biodegradable plastics from renewable resources, considering the varying effectiveness of different nutrient limitations depending on the specific conditions.

In the last study, it was concluded that incorporating lignin derivatives such as ferulic acid and vanillin (up to 10% mol) with glycerol in sulfur limited conditions significantly enhanced PHA yield compared to glycerol alone (Section 4.3). The importance of using high lignin concentrations lies in its potential to provide a sustainable, relatively low-cost alternative to traditional carbon sources (sugar), which could have significant environmental and economic benefits. However, high lignin concentrations (above 30% mol) were negatively affected cell growth and PHA accumulation. This suggests that high concentrations of lignin monomers may be toxic to the microbial cells or interfere with the PHA synthesis mechanism.

The analysis of PHA monomer distribution revealed a consistent presence of mcl-PHA monomers across various fermentation conditions (nitrogen and sulfur limitation) and lignin concentrations.

The most dominant monomers were found to be 3-hydroxydecanoate and 3-hydroxyoctanoate, despite variations in their molar compositions. Mcl-PHAs exhibit better thermal stability and mechanical properties compared to their short-chain-length counterparts (scl-PHAs), though the dominant monomers may influence the thermal and chemical properties of the resulting PHA products.

In general, this thesis has successfully demonstrated the effectiveness of the nutrient limitation strategy for producing bioplastics by *Pseudomonas putida* KT2440 using glycerol and lignin monomers as feedstocks. The use of industrial by-products as feedstock for bioplastic production is particularly noteworthy for several reasons, including better resource efficiency, production cost reduction, and better valorization of materials.

5.2 Recommendations and future directions

- **Proteomic and metabolic analysis**

Proteomic and metabolic analysis can provide a more comprehensive understanding of the metabolic pathways and regulatory mechanisms involved in PHA production under sulfur-limited conditions. Proteomic analysis can identify changes in protein expression and post-translational modifications that occur in response to sulfur limitation, and this information can be used to identify critical proteins involved in PHA biosynthesis.

Furthermore, as NADPH is a crucial cofactor in PHA biosynthesis, it would be helpful to investigate the formation of NADPH in *Pseudomonas putida* KT2440 in response to sulfur limitation. This would provide insights into how the cells regulate NADPH production under these conditions to support PHA biosynthesis.

- **Fed batch fermentation**

Higher concentrations of lignin monomers significantly reduced both cell growth and PHA accumulation. Therefore, employing a fed-batch fermentation strategy would be helpful to avoid the toxicity of lignin monomers at higher concentrations. In future directions, the fed-batch PHA fermentation process would be started with the initial fermentation medium containing glycerol and a low concentration of lignin monomers. The rate and timing of lignin monomers addition should be carefully controlled to avoid substrate toxicity. This approach can increase the yield and quality of PHA and improve the fermentation process's efficiency.

- **Further optimization of fermentation parameters**

Oxygen availability is critical for lignin degradation and PHA production by *Pseudomonas putida* KT2440 (Ramírez-Morales et al., 2021). While shaking flask experiments can provide valuable preliminary data, bioreactors are a more suitable platform for studying microbial growth and metabolism under controlled conditions that can be monitored and adjusted in real-time. Therefore, the study on the effect of oxygen availability on PHA yield under sulfur limitation in a bioreactor can provide more valuable insights into the relationship between oxygen availability and PHA production by *Pseudomonas putida* KT2440. Furthermore, in a bioreactor, it is possible to monitor and control several key variables, including pH and temperature, which are critical for successful fermentation. Since pH can vary during fermentation and potentially fall outside the optimal range for the microbes involved (observed in some of our results, Section 4.2.1), it is essential to maintain pH within the ideal ranges. These parameters are essential for successfully transferring laboratory-scale findings to commercial applications.

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