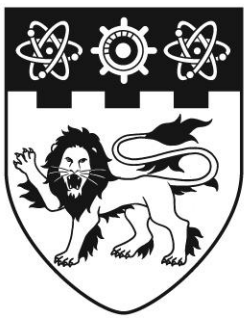


NANYANG TECHNOLOGICAL UNIVERSITY

STYRENE BIOREMEDIATION AND POLYHYDROXYALKANOATE (PHA) PRODUCTION:

ANALYTICAL METHODS DEVELOPMENT AND MICROBIAL STUDY

TAN GIIN YU AMY

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2015

STYRENE BIOREMEDIATION AND POLYHYDROXYALKANOATE (PHA) PRODUCTION:

ANALYTICAL METHODS DEVELOPMENT AND MICROBIAL STUDY

TAN GIIN YU AMY

School of Civil and Environmental Engineering

A thesis submitted to the Nanyang Technological University

in fulfillment of the requirement for the degree of

Doctor of Philosophy

2015

Acknowledgments

I would like to express my gratitude to Associate Professor Wang Jing-Yuan who played more than a supervisory role over the course of my PhD research. His firm belief in my capabilities, and his wisdom, has empowered me with confidence and fresh perspectives on how I could contribute to further environmental engineering research with my biological sciences background. I would like to thank him for his unwavering support and for providing me the opportunity to "evolve academically".

I would also like to thank my mentor Dr Chen Chia-Lung who took time off his busy schedule to teach me laboratory techniques and bounce ideas. He was also generous in dispensing many distilled academic advice, which helped me mature in my academic thinking.

My thanks also go to staff researchers Dr Ge Liya and Li Ling, student researchers Mo Yu (2009 - 2010), Chong Wan Ying (2010 - 2011) and He Xiaoyun (2013 – 2014) for their active involvement in this research project; staff researcher Wang Lin and fellow PhD colleague Pan Chaozhi for their indirect contribution to this research project.

I would like to thank all the staff at Environment Laboratory for their kind assistance in instrument operation. Special thanks go to my reporting laboratory technician Lim-Tay Chew Wang for her assistance and advice in all my research-related administrative matters.

My appreciation also goes to friends and colleagues at Residues and Resource Reclamation Centre (NEWRI-R3C) for their thoughtful inspiration and bringing laughter to my life over the course of my PhD research.

Last but not least, I would like to thank my family, my boyfriend and his family for their unconditional love, patience, and moral support. Special thanks to my boyfriend for being the best and harshest literary critic of this research work.

Table of Contents

Executive Summary

Styrene waste is a principal effluent produced from polystyrene (PS) plastic production and processing industries. The growing demand for PS plastics has imposed significant environmental burden in the form of styrene pollution. The proper monitoring and management of industrial waste styrene is pivotal to public health, ecological and environmental protection. Aerobic biological treatment presents a rapid and green approach to remediate styrene waste but generates high volumes of biomass by-product, resulting in high management and disposal cost. Optimizing the aerobic process for bioconversion of styrene to commercially-valuable medium-chain-length polyhydroxyalkanoate (MCL-PHA) material could potentially alleviate pollution and off-set biomass disposal cost. At the same time, the use of cheap or "free" carbon substrates, such as styrene waste, may lower PHA production cost, bringing it closer to commercialization. Separately, improving analytical methods for styrene waste and PHA analysis is immensely important and beneficial in facilitating the work of both field engineers and researchers. To this end, the objectives of this work were to (1) develop analytical methods to facilitate routine styrene wastewater and PHA analysis, (2) gain an understanding of aqueous styrene-degrading bacterial community, and (3) increase the pool of styrene-degrading *cum* MCL-PHA-producing bacteria.

A new high performance liquid chromatography (HPLC-DAD) method was successfully developed and optimized for the detection of styrene and common co-occuring benzene, toluene, ethylbenzene, xylenes (BTEXS) at high equimolar aqueous concentration of 2.0 mM. Chromatographic separation was achieved within 5.5 min, making it one of the fastest methods to date. A gas chromatography mass spectrometry (GC-MS) method was developed for PHA analysis based on the new finding that there is a highly linear relationship between the carbon number of homologous saturated PHA monomers and their respective retention times and response factors (adjusted $R^2 > 0.98$). High method recovery values (close to 100%) were demonstrated for PHA homo/copolymers. To study the styrene-degrading consortium, next-generation sequencing was performed. The dominant orders Pseudomonadales and Rhizobiales are well-known for styrene degradation and potentially harbor styrene catabolic genes,

respectively, suggesting that they probably form the core styrene-degrading population. Equally prominent were the orders Sphingobacteriales, Clostridiales, and Actinomycetales, which have versatile aromatic catabolic capability and may be responsible for mineralization of styrene metabolites, forming the secondary styrene-degrading population. Bacterial isolation yielded mostly *Pseudomonas* species, which formed MCL-PHA at low levels (< 1% cell dry mass [CDM]), suggesting the consortium's low MCL-PHA production capacity. Instead, a novel *P. putida* NBUS12 was successfully isolated from BTEXS-enriched activated sludge. The bacterium accumulated up to 32.49% CDM of MCL-PHA, making it one of the highest styrene-degrading and MCL-PHA-producing strains presently known.

The newly-developed analytical methods offer benefits over existing methods in terms of protocol simplification and lower analysis price for BTEXS and PHA detection. Additionally, the theoretical concept, behind the GC-MS method, may aid in the future development of methods for better quantitation of more PHA types. New findings, from microbial study, provided valuable information and new bacterial isolates, which could aid in future selection of seeding sludge, bioaugmentation strategies and formulation of defined microbial cocktails. This could reduce reactor start-up time, optimize styrene biodegradation and expand the process for simultaneous MCL-PHA production. More research is needed to investigate the functional roles of consortium members, and determine the PHA-producing sub-population and PHA production capability of aqueous styrene-degrading microbial community. Further research is needed to elucidate the metabolic pathway and enhance the MCL-PHA polymer property of *P. putida* NBUS12.

List of Tables

List of Figures

List of Symbols, Units and Abbreviations

Symbols and units

δ	Chemical shifts
θ	Theta
%	Percentage
°C	Degree Celsius
1/*D*	Inverse Simpson
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
bp	Base pair
cm	Centimeter
COD	Chemical oxygen demand
d	Day
Da	Dalton
eV	Electron volts
g	Centrifugal force
g	Gram
H'	Shannon diversity index
h	Hour
Kb	Kilo base pair
Kg	Kilogram
kV	Kilovolt
L	Liter
M	Molar
m^3	Cubic meter
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
mol	Mole
m/z	Mass-to-charge ratio
M_w	Weight average molar mass
M_n	Number average molar mass
nm	Nanometer
OD_{600}	Optical density at 600 nm
pg	Picogram
ppm	Parts per million
rpm	Revolution per min
s	Second
V	Volt
v/v	Volume/volume
w/w	Weight/weight
w/v	Weight/volume

General abbreviations

ACE	Abundance-based Coverage Estimator
BHT	2,6-di-tert-butyl-4-methylphenol
BLAST	Basic Local Alignment Search Tool
BTEX	Benzene, toluene, ethylbenzene, xylene
BTEXS	Benzene, toluene, ethylbenzene, xylene, styrene
CCA	Canoical correspondence analysis
CDM	Cell dry mass
CI	Confidence interval
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DT	Decomposition temperature
E	Inverse Simpson index
EDTA	Ethylenediaminetetraacetic acid
EH	Epoxide hydrolase
EI	Electron impact
FTIR	Fourier transform infrared spectroscopy
GAC	Granular activated carbon
GC	Gas chromatrography
GPC	Gel permeation chromatography
FID	Flamed ionization detector
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
J'	Shannon diversity index
LC	Liquid chromatography
LCL	Long-chain length
LOD	Limits of Detection
logPOW	Octanol–water partition coefficient
LOQ	Limits of Quantification
MBR	Membrane bioreactor
MCL	Medium-chain length
MS	Mass spectrometer
MSM	Mineral salt medium
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OLR	Organic loading rate
OTU	Operational taxonomic unit
PAD	Phenylacetaldehyde dehydrogenase
PBS	Phosphate buffer saline
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PDI	Polydispersity index
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PhaG	3-hydroxyacyl-acyl carrier protein-CoA transacylase
PID	Photoionization detector

PS	Polystyrene
PLFA	Phospholipid fatty acid
RC	Regenerated cellulose
RF	Response factor
RI	Refractive index
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
RSD	Relative standard deviation
rDNA	Ribosomal deoxyribonucleic acid
RSGP	Reverse sample genome probing
RT	Retention time
SCL	Short-chain length
SDD	Styrene 2,3-dihydrodiol dehydrogenase
SDO	Styrene 2,3-dioxygenase
SDS	Sodium dodecyl sulfate
SIP	Stable-isotope probing
SMO	Styrene monooxygenase
SOI	Styrene oxide isomerase
SPME	Solid phase micro extraction
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TMS	Tetramethylsilane
T_g	Glass to rubber transition temperature
T_m	Crystalline phase melting temperature
TSS	Total suspended solids
UASB	Upflow Anaerobic Sludge Blanket reactor
US EPA	United States Environment Protection Agency
UV	Ultraviolet
UPGMA	Unweighted pair group method with arithmetic mean
VFAs	Volatile fatty acids
VSS	Volatile suspended solids
WHO	World Health Organization
XRD	X-ray diffraction

PHA polymer abbreviations

P3HB	poly(3-hydroxybutyrate), poly(3-hydroxybutyric acid)
P3HV	poly(3-hydroxyvalerate)
P3HB3HV	poly(3-hydroxbutyrate-*co*-3-hydroxyvalerate), poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid)
P3HB4HB	poly(3-hydroxbutyrate-*co*-4-hydroxybutyrate)
P3HBHHx	poly(3-hydroxbutyrate-*co*-3-hydroxyhexanoate)

PHA monomers abbreviations

(*R*)-3-HA-CoA	(*R*)-3-hydroxyacyl-CoA
3HB, C_4	3-hydroxybutyrate, 3-hydroxybutyrtic acid
3HV, C_5	3-hydroxyvalerate, , 3-hydroxyvaleric acid
C_6	3-hydroxyhexanoate, 3-hydroxyhexanoic acid
C_8	3-hydroxyoctanoate, 3-hydroxyoctanoic acid
C_{10}	3-hydroxydecanoate, 3-hydroxydecanoic acid
C_{12}	3-hydroxydodecanoate, 3-hydroxydodecanoic acid
$C_{12:1}$	3-hydroxydodecenoate
C_{14}	3-hydroxytetradecanoate
C_{16}	3-hydroxyhexadecanoate, 3-hydroxyhexadecanoic acid

Publications

Journal papers

Tan, G.-Y.A., Chen, C.-L., Ge, L., Li, L., Tan, S.N., & Wang, J.-Y. (2015). Bioconversion of styrene to poly(hydroxyalkanoate) (PHA) by a new bacterial strain *Pseudomonas putida* NBUS12. *Microbes and Environments*, *30*(1), 76-85.

Tan, G.-Y.A., Chen, C.-L.,Li, L., Ge, L., Wang, L., Razaard, I.M.N., Li, Y., Zhao, L., Mo, Y., & Wang, J.-Y. (2014). Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers*, *6*(3), 706-754.

Tan, G.-Y.A., Chen, C.-L., Ge, L., Li, L., Wang, L., Zhao, L., Mo, Y., Tan, S.N., & Wang, J.-Y. (2014). Enhanced gas chromatography-mass spectrometry method for bacterial polyhydroxyalkanoates analysis. *Journal of Bioscience and Bioengineering*, *117*(3), 379-382.

Tan, G.-Y.A., Chen, C.-L., Zhao, L., Mo, Y., Chang, V.W.C., & Wang, J.-Y. (2012). An HPLC-DAD method for rapid and high resolution analysis of concentrated BTEX and styrene aqueous samples. *Analytical Methods*, *4*(11), 3545-3550.

Related journal paper

Ge, L., **Tan, G.-Y.A.**, Wang, L., Chen, C.-L., Li, L., Tan, S.N., & Wang, J.-Y. (2015). Determination of monomeric composition in polyhydroxyalkanoates by liquid chromatography coupled with online mass spectrometry and offline nuclear magnetic resonance. *Talanta* (*under revision*).

Conference papers and presentations

Tan, G.-Y.A., Chen, C.-L., Wang, J.-Y. (2015). Analyzing bacterial diversity, community structure and polyhydroxyalkanoate (PHA) production potential in styrene-enriched activated sludge. *Second International Conference on Sustainable Urbanization (ICSU 2015)*, 7-9 Jan, Hong Kong, China.

Tan, G.-Y.A., Chen, C.-L., Li, L., Wang, L., Mo, Y., Zhao, L., & Wang, J.-Y. (2013). Hunting for bacteria capable of bioconverting polystyrene waste to polyhydroxyalkanote (PHA). *International Conference on Solid Waste Management 2013 (ICSWHK 2013)*. 5-8 May, Hong Kong, China.

Chen, C.-L., **Tan, G.-Y.A.**, Wang, L., & Wang, J.-Y. (2012). Polyhydroxyalkanote (PHA) production from waste. *Jade Mountain Forum on Sustainable Environment*. 19-22 Apr, Tainan, Taiwan.

Chen, C.-L., **Tan, G.-Y.A.**, & Wang, J.-Y. (2011). Polyhydroxyalkanote (PHA) production from organic solid waste. *Cross Taiwan Strait Conference on Urban Solida Waste Sustainability and Management*. 21-24 Oct, Xiamen, China.

CHAPTER 1

Introduction

Background

Polystyrene (PS) is a petrochemical plastic synthesized from styrene. Since its advent, PS has become an indispensible part of modern life. Owing to its good processing properties, PS is used as a starting material for a wide variety of products ranging from to disposable laboratory consumables, containers, to the ubiquitous arsenal of Styrofoam packaging products. Consequential of its vast application, there is a strong and increasing global demand for PS. Between the period 2010 and 2020, global demand for PS is predicted to grow at a compound annual growth rate of 4.7%, reaching 23.5 million metric tons in 2020 (Plastemart, 2013). The burgeoning demand for PS plastics, however, has imposed significant environmental burden and one of which is styrene pollution.

1.1.1 Sources of styrene waste

Styrene is an industrially-important raw material in plastic production, particularly for PS. Styrene is a mono-aromatic compound, which consists of an unsaturated alkyl group attached to a benzene ring (Figure 1.1), enabling the styrene monomer to undergo polymerization reactions. Repeated polymerization of styrene monomers gives rise to PS plastics. However, sub-optimal polymerization efficiency and the volatile nature of styrene have resulted in unreacted styrene forming a principal component of the waste effluent from PS processing plants (Nemerow & Agardy, 1998; Tossavainen, 1978). It was estimated that up to 11.3 L of styrene-laden wastewater is generated with every Kg of PS (Nemerow & Agardy, 1998). This meant that by 2020, there could be up to 265 million cubic meters of styrene-laden wastewater produced annually. Styrene is also present in off-gas. In the United States alone, about 13.2 kilo metric tons of styrene gas are emitted from styrene-related industries (US EPA, 1994).

Negative health and ecological effects are associated with styrene pollution. It has been found that exposure to styrene, above the maximum concentration level of 0.1 $mg.L^{-1}$, leads to depression of central nervous system, damaged to liver and nerve tissue, potential endocrine disruption, and possibly even cancer (US EPA, 2010; WHO, 2011). Apart from its association with adverse human health effects, styrene

also has negative ecological and environment impact. Styrene exhibits toxicity to various cellular complexities including green algae, zooplankton, earthworm and freshwater fish (Cushman et al., 1997). In the atmosphere, styrene readily reacts with ozone to form photochemical smog (US EPA, 1994). Hence, the proper monitoring and management of industrial waste styrene is pivotal to public health, ecological and environmental protection. On a local perspective, Singapore is a major producer of PS, accounting for about 1.6% of global production (APIC, 2010). The large volumes of styrene waste effluent generated worldwide and in Singapore meant that the monitoring and treatment of waste styrene, from PS production industry, are important issues both locally and internationally.

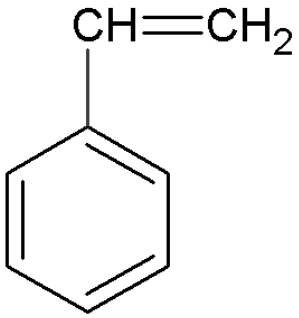


Figure 1.1 Chemical structure of styrene.

1.1.2 Analytical methods for styrene monitoring

Styrene monitoring encompasses the analysis of styrene levels in both off-gas and wastewaters. To do so, styrene analysis methods, based on gas chromatography (GC) and high performance liquid chromatography (HPLC) systems, have been developed. GC-based methods are regarded as well-established and have been adopted as Standard Methods by US EPA (*e.g.*, EPA 5030B/C, EPA 502.2, EPA 8260B, EPA 524.2 and 524.3) (NEMI). While GC-based methods could be employed for the analyses of both gaseous and aqueous styrene, these methods are only well-suited for gaseous styrene analysis and cannot be directly applied to aqueous styrene. This is due to the incompatibility between water and GC gas column stationary phase/GC detector. Additionally, styrene wastewaters have been reported to contain high levels of styrene (up to 2.0 mM) (Aliabadi et al., 2012), which are beyond the styrene detection capability of GC (up to 1.9 μM)

(Demeestere et al., 2007; NEMI). This meant that pre-analysis sample preparation steps (*e.g.*, sample extraction and dilution) are necessary, leading to an increased exposure risk to harmful carcinogens/solvents, higher analysis cost, potential sample loss and measurement inaccuracies. HPLC-based methods, on the other hand, can perform direct analysis of aqueous styrene and has higher styrene detection capability (*i.e.*, 1.73 mM) (Colin et al., 1986), making HPLC-based methods more suitable for styrene wastewater analysis.

1.1.3 Treatment methods for styrene waste

Many treatment methods have been proposed for industrial waste styrene. These treatment methods formed two board categories namely non-biological methods (air-stripping and capture, chemical oxidation, granular activated carbon [GAC] removal, *etc*) and biological methods (biofiltration, activated sludge process, biofilm, membrane bioreactor process [MBR], anaerobic process, *etc*). While non-biological approaches provide a rapid means of styrene removal, these approaches are costly and often produce undesirable secondary pollutants (Farhadian et al., 2008). Conversely, biological methods leverage on microorganisms as biocatalysts for the elimination, attenuation or transformation of organic contaminants into harmless material such as CO_2 and water (Farhadian et al., 2008). This makes them a "greener" approach and the preferred treatment process for styrene styrene waste effluent.

Many studies have demonstrated that styrene biodegradation is favored under aerobic conditions (Durán et al., 2008). It appears that oxygen molecule is an essential signal to induce styrene catabolism (O'Leary et al., 2002), and is also an important reactant for the initiation of styrene biodegradation (Tischler & Kaschabek, 2012). However, high biomass yield is associated with aerobic treatment. In the activated sludge process for an example, sludge management and disposal represents a significant expenditure in the activated sludge process, and was estimated to be as high as 60% of the total wastewater treatment cost (Wei et al., 2003). This begs the question of whether the high operating cost is justifiable for merely styrene removal, and if waste styrene and the final biomass by-product

could be put to better use.

1.1.4 Coupling styrene remediation to production of valuable end-products

To alleviate treatment cost, there has been a surge in interest to utilize bacterial biomass as biofactories for bioconversion of organic waste into valuable chemicals or raw materials (Drepper et al., 2006). Biotransformation of styrene to commercially valuable products namely polyhydroxyalkanoate (PHA), styrene oxide and 3-vinylcatechol have been reported (Ward et al., 2005; Warhurst et al., 1994). Among the aforementioned products, only PHA is formed as an intracellular product whereas styrene oxide and 3-vinylcatechol are secreted into the extracellular cultivation medium (Ward et al., 2005). Hence, PHA is the most suitable target end-product in the up-recycling of organics in wastewater. This is because the intracellular PHA storage enables PHA product to be kept securely within the bacterium and separated from waste effluent matrix, ensuring the recovery of a high-purity product. Furthermore, the resultant biomass by-product becomes a valuable store of PHA, which can potentially create commercial value to the PHA-containing biomass generated from the biological treatment process.

1.1.5 Polyhydroxyalkanoate (PHA) and its analytical methods

There has been considerable amount of commercial interest in PHA polymer due to its biodegradability and biocompatibility. At the molecular level, PHA is a biopolyester comprising of (*R*)-*β*-hydroxy fatty acids monomers, also termed as "PHA monomers". PHA monomers harbor a functional *R* group, which is usually a saturated alkyl group that varies in carbon length from methyl (C_1) to pentadecyl (C_{15}) (Tan et al., 2014b). Depending on the total number of carbon atoms within a PHA monomer, PHA can be classified as either short-chain length PHA (SCL-PHA; 3 to 5 carbon atoms), medium-chain-length PHA (MCL-PHA; 6 to 14 carbon atoms), or long-chain length PHA (LCL-PHA; 15 or more carbon atoms) (Khanna & Srivastava, 2005). Various combinations of PHA monomers constitute to the chemical diversity of PHA, endowing PHA with a wide range of polymer properties (Rai et al., 2011). Having a reliable and accurate way to analyze PHA is key to understanding and predicting its polymer properties. Many methods have been

developed for the detection and analysis of microbial PHA (*e.g.*, colony/cell staining, crotonic acid assay, fourier transform infrared spectroscopy [FTIR], HPLC and GC) (Tan et al., 2014b). Among these methods, GC-based methods are most widely-used as these methods could achieve both qualitative and quantitative analyses of the PHA monomeric composition within a relatively short analysis time.

1.1.6 Microbial MCL-PHA production from styrene

PHA has diverse applications ranging from biodegradable packaging materials to pharmaceutical and biomedical products. In particular, MCL-PHA possesses material properties which are suitable for high-end medical applications including heart valves, cardiac patches, scaffold for tissue engineering, surgical sutures, stents and controlled drug delivery matrices (Rai et al., 2011). MCL-PHA monomers, arising from the depolymerization of MCL-PHA, are also know to exhibit antimicrobial activity and serve as a platform for the production of a plethora of pharmaceutical products including fungicides, flavors, pheromones, vitamins and *etc* (Allen et al., 2012). Presently, while the commercialization of SCL-PHA has been realized, MCL-PHA commercialization continues to be hindered by high production cost. It has been suggested that using cheap or "free" carbon substrates, such as waste, may decrease PHA cost (Choi & Lee, 1999). The production of MCL-PHA, however, appears to be both bacterial and substrate-dependent. Based on pure culture studies, MCL-PHA is typically produced by bacteria from the *Pseudomonas* genus utilizing mono-aromatics, alkene, *n*-alkanes and *n*-alkanoate substrates (Tan et al., 2014b). At the same time, the genus *Pseudomonas* is frequently detected in styrene-degrading microbial communities (Alexandrino et al., 2001; Arnold et al., 1997; Greene & Voordouw, 2004; Portune et al., 2015); styrene-degrading pure culture *Pseudomonas* species have also been reported (Tischler & Kaschabek, 2012). This meant that styrene, as a substrate, and its naturally-occurring bacterial degraders constitute a favorable combination for MCL-PHA production. Indeed, to date, four pure *P. putida* strains (*i.e.*, *P. putida* S12, *P. putida* CA-1, *P. putida* CA-3 [NCIMB 41162] , and *Pseudomonas* sp. TN301) have been found to bioconvert styrene to MCL-PHA (Hartmans et al., 1990; Narancic et al., 2012; O'Connor et al., 1995).

Taken together, the concept of bioconverting of waste styrene to MCL-PHA is advantageous as it provides an environmentally friendly treatment approach to address the issue of waste styrene generated from PS plastic processing industries. More importantly, coupling styrene treatment process to the production of a valuable MCL-PHA biomaterial creates additional value for the biological treatment process and enables MCL-PHA to be cheaply produced from waste carbon substrates. This has the potential benefits of off-setting biological treatment costs and lowering MCL-PHA production costs, bringing MCL-PHA closer to commercialization.

1.2 Problem Statements

PHA production is typically carried out in aqueous environment due to the ease of achieving homogeneity in cultivation conditions required for inducing PHA accumulation (*e.g.*, feast/famine and aerobic/anaerobic cycles) in PHA-producing microbes (Serafim et al., 2008). As such, this PhD study will focus on the microbial production of MCL-PHA from styrene under aqueous environment. While the technical feasibility of bioprocess has been proven in pure bacterial cultures, extending the application to treat styrene waste still faces a number of challenges. There are also limitations to the current analytical methods for styrene and PHA analyses. The limitations and challenges are described below and formed the basis for this PhD study.

(A) Limitations of existing chromatographic methods for aqueous styrene analysis

Styrene is one of the main components in wastewater generated from PS production and processing plants. High aqueous styrene concentrations of up to 2.0 mM have been previously reported (Aliabadi et al., 2012). Nevertheless, monitoring styrene levels may present a challenging task. This is due to the mixing of wastewaters exiting PS and other petrochemical processing plants (Ahmad et al., 2008; Fallah et al., 2010). Mono-aromatic compounds such as benzene, toluene, ethylbenzene and xylenes (BTEX), and polyaromatic compounds such as naphthalene and indene are

known to co-occur with styrene in these wastewater mixtures. Specifically, styrene and BTEX have been reported at similar concentrations (B:T:E:X:styrene ratio [in mM] of 9.3:4.2:1:2.5:6.6) (Ahmad et al., 2008). Structural similarity, especially between mono-aromatic compounds, may lead to a loss in chromatographic resolution on HPLC systems, particularly so if the compounds co-exist at high aqueous concentrations (AlSalka et al., 2010; Campos-Candel et al., 2009). Hence, the presence of BTEX may interfere with styrene detection. Having a simple and fast analysis method, suited for the detection and quantification of aqueous samples, with high styrene and BTEX (BTEXS) concentration, would greatly facilitate the routine environmental monitoring and remediation of contaminated waters. Current HPLC methods are restricted to analyzing styrene and BTEX separately as these methods were developed for application-specific analyses (*e.g.*, BTEX analysis in BTEX-contaminated water samples and occupational environments, and styrene analysis in biological samples and food samples) (AlSalka et al., 2010; Campos-Candel et al., 2009; Colin et al., 1986; Gawell & Larsson, 1980). Hence, there is no chromatography method available for the direct analysis of aqueous samples containing high concentrations of BTEXS. This necessitates the need to develop such a method.

(B) Limitations of existing chromatographic methods for PHA analysis

The analysis of PHA biopolymer is a challenging task due to the myriad of naturally-occurring PHA monomers, which can occur simultaneously within a single biopolymer. For an example, MCL-PHA can comprise up to 9 or more PHA monomers. This translates to a high calibration workload and cost. Furthermore, traditional GC methods, coupled to flamed ionization detector (GC-FID) or mass spectrometer (GC-MS), typically require PHA reference standards for robust identification and quantification. However, many PHA reference standards are either expensive, not readily-available or are commercially-unavailable (Escapa et al., 2011; Furrer et al., 2007). Researchers have sought to overcome this problem by using a combination of GC-MS (for tentative identification of PHA monomers) and GC-FID (for the routine quantification of PHA monomers identified by GC-MS) (Ward et al., 2006) to keep calibration workload manageable and calibration cost

low; chemically or biologically-synthesize PHA analytical standards that are commercially unavailable (Sun et al., 2009); using commercially-available PHA analytical standards to quantify chemically-similar but commercially-unavailable PHA monomers (Oehmen et al., 2005). These measures can lead to errors in measurements. Developing an accurate analytical method for PHA analysis has thus proven to be a difficult, costly, tedious and time-consuming pursuit. This warrants a need to develop a simple GC calibration method which reduces calibration workload and cost while enabling accurate detection and quantification of a wide range of PHA monomers using a limited inventory of PHA reference standards.

(C) Limited information on styrene-degrading microbial community structure in aqueous environment

Industrial biological treatment of styrene is usually conducted under non-sterile environment. To enhance the process for simultaneous styrene remediation and MCL-PHA production under aerobic, non-sterile aqueous environment is a challenging task due to several reasons. First, styrene is a recalcitrant compound and requires specialized microbes for its assimilation (Babaee et al., 2010). However, the providence of aeration, which is essential for rapid styrene metabolism, causes styrene removal through abiotic air stripping instead of the intended biotic action (Fallah et al., 2010). Therefore, styrene does not remain sufficiently long enough in the aqueous environment to enrich for styrene-degrading and PHA-producing microbes. Second, even though some pure *P. putida* cultures (*i.e.*, *P. putida* S12, *P. putida* CA-1, *P. putida* CA-3 [NCIMB 41162], and *Pseudomonas* sp. TN301) could assimilate styrene for MCL-PHA production (Hartmans et al., 1990; Narancic et al., 2012; O'Connor et al., 1995), the prolonged survival and performance of pure cultures may be compromised under non-sterile environments (Coats et al., 2007). Mixed microbial cultures on the other hand, present higher microorganism diversity, have low sterility demand and greater robustness (Cabrol & Malhautier, 2011; Satoh et al., 1998), which makes them more suited for applications involving waste. However, mixed cultures tend to produce lesser PHA compared to pure cultures due to the presence of low-PHA-accumulating and non-PHA-accumulating microorganisms (Lee, 1996; Salehizadeh

& Van Loosdrecht, 2004). Bioaugmentation or formulating defined microbial cocktails, which are representative of the naturally-occurring microflora, using styrene-degrading and/or high PHA-producing bacterial strains, may overcome the aforementioned challenges.

To facilitate the identification of suitable seeding sludge, achieve successful bioaugmentation or the formulation of defined microbial cocktails, aimed at process optimization and extension to concurrent PHA production, it is important to have an understanding of the aqueous styrene-degrading microbial population. Much of the current understanding about styrene-degrading microbial community structure has been confined to biofilter and soil studies (Alexandrino et al., 2001; Arnold et al., 1997; Greene et al., 2000; Portune et al., 2015). However, the microbial population in aqueous environments was found to be distinct from that in non- or semi-aqueous environments (Tresse et al., 2002). Hence, the information, gleaned from biofilter and soil studies, may not provide an accurate picture of the microbial population in aqueous environments. Further studies are needed to characterize styrene-degrading microbial community structure in aqueous environments.

Thus far, the presence of potential PHA-accumulating microbial members within styrene-degrading mixed consortia has yet to be reported. Nevertheless, the microbial community analysis was reported for mixed consortia enriched on other carbon substrates. These carbon substrates include volatile fatty acids (VFAs) (Serafim et al., 2008), alkanoic acids (Lee et al., 2011), and *etc*. A comparison of these studies reveals that different carbon substrates will result in distinct PHA-producing microbial population due to the adaptation of microbial community. Hence, much of the current understanding about PHA-producing bacteria within mixed consortia may not be transferable to styrene-degrading microbial communities. Further studies are needed to identify potential PHA-producing members present within a styrene-degrading microbial consortium in aqueous environments.

(D) Limited availability and diversity of styrene-degrading *cum* MCL-PHA-producing pure microbial cultures

To achieve successful bioaugmentation or formulation of defined microbial cocktails, which is representative of the naturally-occurring microflora, for process optimization and simultaneous MCL-PHA production, it is essential to have a large and diverse pool of pure microbial cultures. Current understanding of microbes capable of both styrene-degradation and MCL-PHA accumulation are limited to only four pure bacterial cultures, from the *Pseudomonas* species (*P. putida* CA-3, *P. putida* CA-1, *P. putida* S12 and *Pseudomonas* sp. TN301) (Hartmans et al., 1990; Narancic et al., 2012; O'Connor et al., 1995). However, most of them bioaccumulate MCL-PHA at very low cellular content (3% to 14% cell dry mass [CDM]). The only exception was *P. putida* CA-3 which could accumulate MCL-PHA up to 33% CDM (Nikodinovic et al., 2008). However, *P. putida* CA-3 is a patented bacterium and is not readily-accessible for further scientific investigation and bioprocess application. Therefore, there is a lack of bacterial strains with the metabolic capacity to bioconvert styrene into MCL-PHA efficiently, which warrants the need to increase the pool of pure microbial cultures.

Furthermore, while *Pseudomonas* species is known to occur as part of a styrene-degrading consortium, the consortium is a diverse one, comprising of many other bacterial genera and species (Alexandrino et al., 2001; Arnold et al., 1997; Greene et al., 2000). Culture-dependent studies have also unraveled many taxonomically-distinct groups of styrene-degrading bacteria (Greene & Voordouw, 2004; Tischler & Kaschabek, 2012) as well as PHA-accumulating bacteria (Koller et al., 2010; Tan et al., 2014b). Cross-referencing between styrene-degrading and PHA-accumulating bacteria reveals some overlaps between the two groups of bacteria. These genera include *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Xanthomonas* and *Azohydromonas* (formerly *Alcaligenes*). These genera have not been reported to produce PHA from styrene but had been proven to do so using other carbon sources (Reddy et al., 2003; Verma et al., 2002). In particular, some species from the genera *Corynebacterium* and *Streptomyces* are known to biosynthesize MCL-PHA (Allen et al., 2012; Shahid et al., 2013). Presently, it is unknown if there may be other

bacteria genera, apart from *Pseudomonas*, which are capable of converting styrene into MCL-PHA, either as a pure culture or as part of a consortium. Hence, further investigation is required.

1.3 Research Objectives

There are two main objectives for this PhD work. The first objective was to develop simple and rapid analytical methods to facilitate the analysis of PHA, aqueous styrene and other common co-occurring mono-aromatic compounds (*i.e.*, BTEX). The second objective was to identify the key bacterial players of the styrene-degrading community, characterize the MCL-PHA production capabilities of culturable bacterial species and increase the pool of pure bacterial cultures capable of bioconverting styrene to MCL-PHA. Specific objectives were:

1. To develop a new and fast method suited for direct analysis of aqueous samples containing high concentrations of BTEXS,
2. To develop a new and simple method for reliable qualitative and quantitative analysis of a wide range of PHA compounds using limited reference standards,
3. To characterize the aqueous bacterial community structure of styrene-assimilating consortium in a laboratory-scale reactor fed with styrene as sole carbon source, and
4. To isolate and characterize styrene-degrading and MCL-PHA-producing bacterial cultures.

1.4 Scope of Work

To achieve the research objectives, the following scope of work was carried out:

Study 1

1. Assess the suitability of a new commercial high performance liquid chromatography (HPLC) column, Acclaim Phenyl-1, for the direct analysis of BTEXS at high aqueous concentrations,

2. Determine the optimal HPLC operation parameters (*i.e.*, mobile phase composition, sample injection volume and flow rate), and
3. Determine the analytical parameters (*i.e.*, Limits of Detection/Limits of Quantification [LOD/LOQ], repeatability, reproducibility and method recovery) for the newly-developed HPLC method.

Study 2

1. Derive mathematical expressions describing the relationship between homologous PHA carbon number and retention time or response factor under GC analysis, and
2. Evaluate the accuracy of the mathematical expressions using PHA monomer and PHA polymer reference standards.

Study 3

1. Test the styrene degradation capability of styrene-enriched activated sludge consortium using HPLC method developed in Study 1,
2. Analyze the changes in the bacterial community structure under long-term styrene enrichment using next-generation sequencing approach,
3. Conduct alpha and beta diversity analyses of styrene-degrading bacterial community, and
4. Identify potential PHA-producing members present within the styrene-degrading bacterial community.

Study 4

1. Isolate and identify pure bacterial cultures capable of bioconverting styrene to MCL-PHA,
2. Determine the MCL-PHA quantity and composition using GC method developed in Study 2,
3. Investigate the influence of temperature and pH on growth of pure styrene-degrading and MCL-PHA-producing bacterial culture,
4. Visual inspection of intracellular MCL-PHA granules in pure bacterial culture, and

5. MCL-PHA polymer extraction and characterization.

1.5 Organization of Thesis

The thesis consists of seven chapters. Chapter 1 provides a general introduction, the problem statements, research aims, research objectives and scope of work. Chapter 2 provides a literature review on the current status of BTEXS and PHA analytical methods as well as styrene waste bioremediation and PHA production with a focus on bacteria species and bacterial metabolic pathway bridging the two biological processes. Chapters 3 to 6 present the research findings of Study 1 to 4. Study 1 (Chapter 3) details the analytical method development for styrene analysis and concurrent analysis of BTEXS mixture. Study 2 (Chapter 4) presents the analytical method development for PHA analysis. Study 3 (Chapter 5) documents the bacterial community structure of a styrene-degrading consortium. Study 4 (Chapter 6) details the isolation and characterization of bacteria capable of bioconverting styrene to MCL-PHA. Chapter 7 provides the overall conclusion and recommendations for future work. The scope of work is summarized in Figure 1.2.

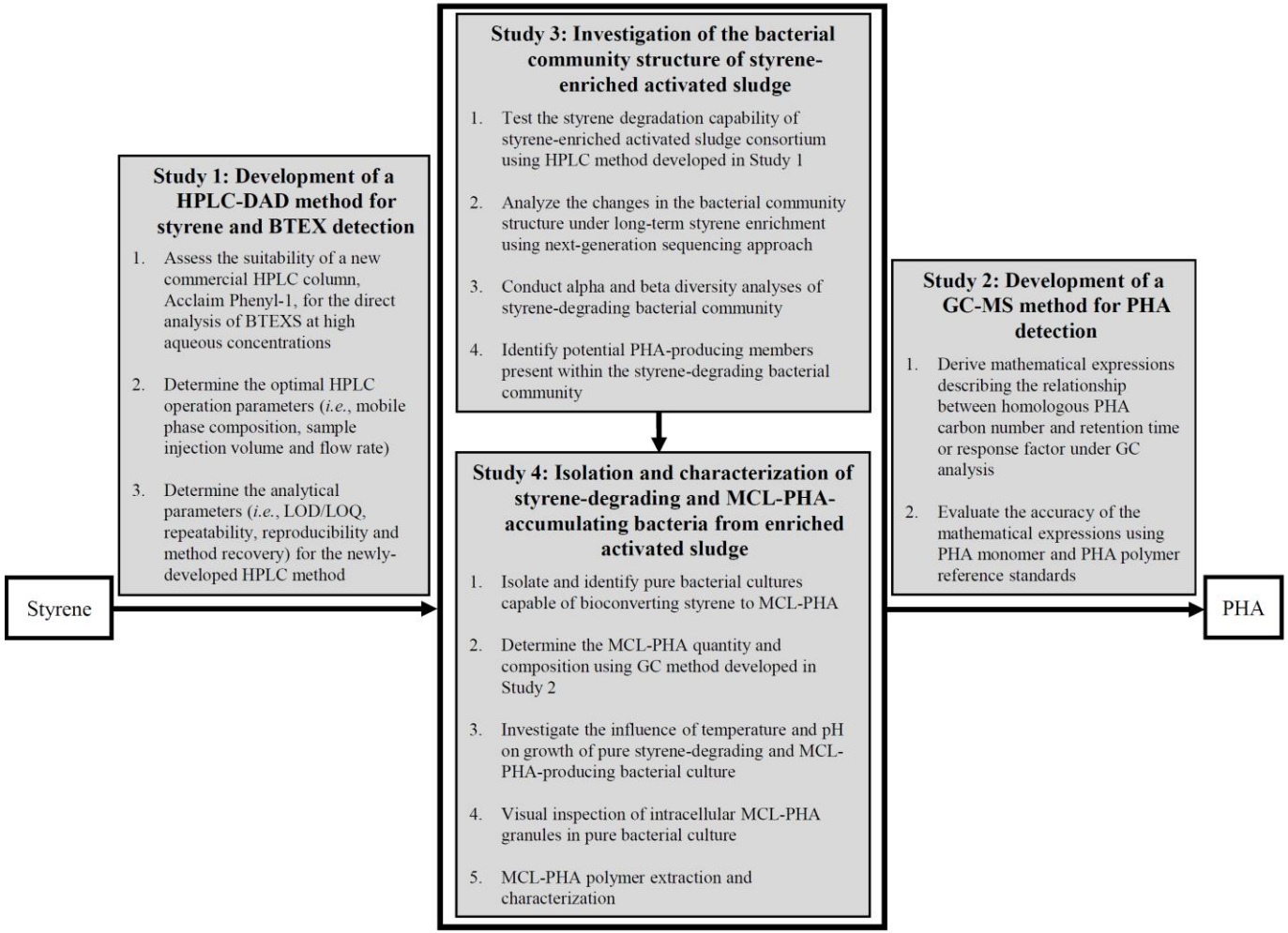


Figure 1.2 Summary of scope of work for this thesis.

CHAPTER 2

Literature Review

2.1 Styrene Waste

Styrene is the main raw material as well as the principal pollutant generated from the industrial synthesis of PS plastics. PS production is a batch process that involves two main steps – bulk polymerization and suspension method (Nemerow & Agardy, 1998). In bulk polymerization, polymerization of pure styrene monomers is performed at 90 °C, in polymerization vessel, to about 30% completion to form a prepolymer (*i.e.*, partially polymerized mass). Following which, the prepolymer is transferred to a suspension-polymerization reactor, containing water, suspending and dispersing agents, where the prepolymer is dispersed into smaller droplets and held in a suspension medium for the completion of polymerization. After complete PS polymerization, the polymer suspension is transported to a blowdown tank where unreacted styrene monomers are air-stripped and emitted as off-gas. The final polymer product is washed and dewatered. The styrene-laden residual suspension medium and washing water, which totaled at 11.3 L per Kg PS, are discharged as wastewaters.

Literatures reported that the concentration range of industrial styrene emission is between 0.01 ppm, and 300 ppm while that of styrene wastewaters is between 2.2 $mg.L^{-1}$ and 300 $mg.L^{-1}$ (Ahmad et al., 2008; Aliabadi et al., 2012; Araya et al., 2000; Durán et al., 2008; Fallah et al., 2010; Tossavainen, 1978). At the aforementioned concentration ranges, the levels of styrene gas and aqueous styrene have exceeded World Health Organization (WHO) guideline values by 140 to 4,000,000 times (air quality: $7e^{-5}$ ppm, 30-min average) and by 100 to 15,000 times (drinking water quality: 0.02 $mg.L^{-1}$), respectively (WHO, 2000, 2011). If left untreated, styrene pollution will bring about to serious health effects and negative ecological repercussions (Cushman et al., 1997). As such, the monitoring and treatment of styrene waste is pivotal to environment and public health protection.

2.1.1 Analytical methods for monitoring gaseous and aqueous styrene samples

Styrene monitoring comprises of styrene analysis in both gas and aqueous samples. Many styrene analysis methods, based on GC and HPLC systems, are currently

available (Table 2.1).

Gaseous styrene analysis

Gaseous styrene samples are typically analyzed on GC systems coupled to various detectors (*e.g.*, FID, PID and MS). Since GC analysis requires analyte to be in gaseous form, gaseous styrene samples can be directly injected into GC systems. GC methods are also adopted as Standard Methods (*e.g.*, EPA 5030B/C, EPA 502.2, EPA 8260B, EPA 524.2 and 524.3) for styrene analysis (NEMI). GC-MS is usually utilized for qualitative analysis to verify the presence of styrene in gaseous samples while GC-FID and GC-PID are used for quantification purposes.

Aqueous styrene analysis

Styrene is a principal component of wastewater generated from PS production and processing plants (Nemerow & Agardy, 1998), However, the sample matrix complexity increases when styrene wastewater is mixed with wastewaters from other petrochemical processing plants (Ahmad et al., 2008; Fallah et al., 2010). Mono-aromatic BTEX compounds reportedly co-occur with styrene in these wastewater mixtures at similar concentrations (Ahmad et al., 2008). Due to the structural similarity of mono-aromatic compounds, achieving good resolution is a challenge for chromatographic separation, especially when the compounds co-exist at high aqueous concentrations (AlSalka et al., 2010; Campos-Candel et al., 2009).

Conventionally, the focus in water sample analysis has been the trace detection of BTEXS in water intended for portable use in accordance to WHO guidelines (NEMI; WHO, 2011). GC-based methods are advantageous in terms of their higher detection sensitivities and chromatographic resolutions. Styrene could be detected at concentrations as low as 2.88×10^{-6} mM (Table 2.1), which is much lower than the stipulated WHO guidelines (1.90×10^{-4} mM) (WHO, 2011). Similarly, the lower detection limits for BTEX, achieved by GC-based methods, are about 10^2 folds to 10^5 folds lower than WHO guidelines (WHO, 2011). Despite superior detection sensitivities and chromatographic resolutions, applying GC for aqueous sample analysis is less straightforward as compared to gaseous sample analysis. This is due

to the inherent incompatibility between water and GC gas column stationary phase/GC detector. This complicates the analysis of volatile mono-aromatics, thus requiring sophisticated sample preparation to extract the compounds from aqueous samples. Sample preparation is either an offline solvent extraction step or online treatments requiring costly accessories such as purge and trap, headspace, and solid phase micro extraction (SPME) (Farhadian et al., 2009). Sample may also require dilution to prevent detector saturation (Farhadian et al., 2008). These preparative steps prior to GC analysis increase human exposure risk to harmful carcinogens/solvents. Additionally, the volatile nature of BTEXS inadvertently constitutes to sample loss during sample preparation, possibly compromising the accuracy of the measurement. While a direct aqueous injection method for GC has been developed to circumvent the problem (Kubinec et al., 2005), the two-step temperature ramp oven programme can be time-consuming. The total analysis time of GC-based methods could take as long as 45 min (Table 2.1), which lowers the overall analysis throughput.

On the other hand, HPLC requires analyte to be in dissolved in aqueous and/or solvent media and is able to accept higher compound concentrations, which makes it well-suited for the analysis of aqueous samples. Hence, unlike GC-based methods, complicated and time-consuming sample preparations are typically not required for HPLC-based methods. Sample preparation usually involves a simple filtration step. Compared to GC-based methods, HPLC-based methods offer several advantages in terms of minimal sample loss, time-savings, higher throughput, and lower capital cost. The total analysis time of HPLC-based methods is usually less than 20 min (Table 2.1), which is shorter than that of GC-based methods. Therefore, HPLC is emerging as a popular alternative and complementary tool to GC for the analysis of BTEXS compounds in water samples. HPLC-UV has been applied for styrene analysis and could detect styrene at concentration between 4.80×10^{-4} and 1.73 mM (Colin et al., 1986). HPLC-UV, HPLC-DAD and HPLC-fluorescence were applied for BTEX analysis, but at lower concentration ranges compared to styrene (Table 2.1). To date, while the feasibility of HPLC for styrene and BTEX analysis has been demonstrated, there is currently no available HPLC method for the concurrent

detection of BTEXS as existing methods were developed for application-specific analyses. For an example, the BTEX analysis methods were specifically developed for analyzing BTEX-contaminated water samples (AlSalka et al., 2010) and BTEX-contaminated occupational environments (Campos-Candel et al., 2009) while styrene analysis methods were specifically developed for analyzing styrene levels in biological samples (Colin et al., 1986) and food samples (Gawell & Larsson, 1980).

HPLC methods based on reversed-phase C8 and C18 columns with methanol- or acetonitrile-water mobile phases were commonly reported (AlSalka et al., 2010; Farhadian et al., 2009; Kelly et al., 1996). Recently, a *β*-cyclodextrin stationary phase column was also reported for BTEX analysis and has an advantage over C8/C18 columns in its ability to achieve complete separation of *m*-xylene and *p*-xylene (Campos-Candel et al., 2009). However, the upper detection limit of these existing HPLC methods remains inadequate for analysis of high analytes concentrations (AlSalka et al., 2010; Campos-Candel et al., 2009). This is due to the deterioration of resolution, especially between ethylbenzene and xylenes, at high analyte concentrations. Taken together, the limited analyte range and upper detection limits of existing HPLC-based methods warranted more efforts in the development of new HPLC method for the direct and concurrent analysis of concentrated BTEXS aqueous samples.

2.1.2 Non-biological styrene treatment methods

Aside from routine monitoring of styrene effluents, treatment of these styrene waste effluents are equally, if not, more important. Non-biological methods achieve styrene remediation through various mechanisms. These mechanisms include mechanical air-stripping which transfers styrene from liquid phase into gaseous phase; physical adsorption of styrene through the use of GAC or oil absorptive resins (US EPA, 1974; Wu & Zhou, 2009); chemical coagulation of styrene using coagulants such as caustic, magnesium chloride, and polyacrylamide (Amosov et al., 1976); oxidation of styrene to form unstable styrene epoxides using oxidants such as hydrogen peroxide (De Paula et al., 2011); membrane-based separation of styrene through microfiltration (Fallah et al., 2010) or pervaporation (Aliabadi et al.,

Table 2.1 Summary of methods for styrene and BTEX in aqueous media.

System	Sample Preparation	Analysis Time (min)	Compound Detection Concentration (mM)					Reference(s)
			Styrene	Benzene	Toluene	Ethylbenzene	Xylenes	
GC-PID/FID	Purge and trap[1,2,3,4], headspace, SPME, solvent extraction	10-20[1], 45[2]	2.88×10^{-6}	2.56×10^{-6}	1.41×10^{-6}	1.88×10^{-8} - 4.71×10^{-8}	9.42×10^{-6}	(AlSalka et al., 2010; NEMI)
GC-MS		40[3], 28-30[4]	For verification purposes	For verification purposes	6.50×10^{-5}	2.83×10^{-7} - 5.65×10^{-7}	9.42×10^{-7}	
HPLC-DAD	Purging from water samples to acetonitrile	32[5]	n.d.	3.84×10^{-6}- 0.02	4.34×10^{-6} -0.02	4.71×10^{-6} - 0.01	1.88×10^{-6} -0.01	(AlSalka et al., 2010)
HPLC-UV	Filtration	4-20[6]	4.80×10^{-4}- 1.73	6.40×10^{-5}- 3.80×10^{-4}	4.34×10^{-5} -3.30×10^{-4}	n.d.	9.42×10^{-5} -4.70×10^{-4}	(Colin et al., 1986; Farhadian et al., 2009; Kelly et al., 1996)
HPLC-fluorescence	Filtration	12	n.d.	0.05-1.15	5.42×10^{-3} - 0.098	7.54×10^{-3}- 0.15	1.32×10^{-3} -0.16	(Campos-Candel et al., 2009)

[1] EPA 5030B and EPA 5030C for aqueous and water miscible liquid samples
[2] EPA 502.2 for source intended for drinking purpose
[3] EPA 8260B for nearly all types of samples
[4] EPA 524.2 and 524.3 for source intended for drinking purpose
[5] Includes sample preparation and analysis time
[6] 4 mins for styrene analysis and 20 mins for analysis of benzene, toluene and xylene mixture
n.d. represents "not determined"

2012; Peng et al., 2003). These non-biological treatment methods typically offer a rapid and efficient solution for styrene removal. For an example, up to 90% reduction in aqueous styrene can be achieved within 20 min via air-stripping (Hsieh, 2000; Thibodeaux, 1974) while treatment efficiencies up to 99.47% can also be achieved for styrene adsorption with GAC or oil absorptive 4-tert-butylstyrene-divinylbenzene-ethylene/propylene/diene resins (US EPA, 1974; Wu & Zhou, 2009).

While non-biological approaches provide a rapid means of styrene removal, these approaches are costly due to the high amount of energy required, particularly in the providence of mechanical agitation for air-stripping, and maintenance of a vacuum or gas purge and low temperature for membrane pervaporation (O'Brien et al., 2000). High treatment cost arising from chemicals supplementation is also associated with treatment methods such styrene adsorption, coagulation and oxidation. Apart from high treatment cost, non-biological methods often produce undesirable secondary pollutants. For an example, air-stripping was often conducted without downstream air emission control, causing air-pollution (Arnold et al., 1997; Farhadian et al., 2008). In adsorption and chemical approaches, hazardous waste such as spent GAC/resins and chemical by-products are formed respectively, requiring additional expenditure in post-treatment processes to ensure that the final disposed waste products are within legislated limits. Taken together, non-biological methods may not present a cost-effective and environmentally-friendly solution for styrene waste treatment.

2.1.3 Biological styrene treatment methods

Unlike non-biological methods, biological methods leverage on microorganisms as biocatalysts for the elimination, attenuation or transformation of organic contaminants into harmless material such as CO_2 and water (Farhadian et al., 2008). Styrene-degrading capabilities has been detected in seeding inoculums from various environments such as soil (Jang et al., 2006; Okamoto et al., 2003), activated sludge (Arnold et al., 1997; Babaee et al., 2010), pond sediments (Bouwer, 1989), and *etc*. This shows that many naturally-occurring microorganisms can assimilate styrene

and form harmless by-products, presenting a "green" approach for the removal of styrene. Compared to non-biological processes, biological treatment strategies are also more economical and effective (Vidali, 2001). Hence, biological treatment methods are widely-applied for the treatment of styrene effluents (Ahmad et al., 2008; US EPA, 1974). Furthermore, microorganisms can be used as whole-cell catalyst for bioconversion of waste substrates into valuable by-products, potentially off-setting treatment cost (Drepper et al., 2006).

Anaerobic process

Both aerobic and anaerobic methods for treatment of styrene waste have been reported. In anaerobic processes, styrene is biodegraded into VFAs and subsequently converted into methane. This was demonstrated by Araya and colleagues (2000) using an Upflow Anaerobic Sludge Blanket (UASB) reactor. However, there have been few reports on anaerobic processes for styrene bioremediation due to the recalcitrant nature of styrene under anaerobic conditions (Field, 2002). The activation of styrene for metabolism occurs via an aromatic side-chain oxygenation. In the absence of oxygen molecules, this proved to be challenging and requires specialized microorganisms capable of catalyzing water-mediated hydroxylation of styrene (Tischler & Kaschabek, 2012). This was evinced in the study by Araya and colleagues (2000) who reported problematic start-up with non-acclimatized sludge. They also observed reactor instability when fed with industrial effluent containing 6 $mg.L^{-1}$ styrene and other impurities at organic loading rates lower than 3 $kg.COD.m^{-3}.d^{-1}$. Styrene was also found to have inhibitory effect on acetoclastic and methanogenic activity between concentrations of 0.45 mM to 1.6 mM, affecting the methane yield. As such, anaerobic processes may not be robust enough to tolerate styrene toxicity. Cultivating specialized anaerobes for metabolizing styrene may require long start-up time and does not necessary generate high methane yield. Anaerobic bioremediation of mono-aromatic compounds typically proceeds at slow degradation kinetics (Corseuil et al., 1998), which meant that anaerobic methods may not present the most cost-efficient method for styrene removal.

Aerobic processes: advantages and associated problems

Many studies have demonstrated that styrene biodegradation is favored under aerobic conditions over anoxic or anaerobic conditions (Durán et al., 2008). The supply of oxygen is key to aerobic styrene biodegradation for two reasons. The first reason is that oxygen, together with styrene, form the chemical signal required for activating the genetic expression of genes encoding for styrene catabolic enzymes (O'Leary et al., 2002). The second reason is that oxygen is an important reactant molecule for the initiation of styrene biodegradation (Tischler & Kaschabek, 2012). Hence, aerobic degradation of styrene exhibits a faster kinetics with greater elimination capacity and is also more robust toward changes in composition and styrene loading (Durán et al., 2008).

Aerobic treatment methods include two-phase aqueous-organic solvent system (Aalam et al., 1993; Han et al., 2006; Osswald et al., 1996), MBR (Fallah et al., 2010), biofilm-based systems (*e.g.*, biofilter, biofilm MBR and submerged immobilized biofilm) (Chang et al., 2000; Gross et al., 2007; Gross et al., 2010; Pérez et al., 2014) and the activated sludge process (US EPA, 1974). Two-phase aqueous-organic solvent system involves the addition of organic solvents (*e.g.*, silicone oil, *n*-hexadecane, bis(2-ethylhexyl)phthalate and dibutylphtalate) into the aqueous cultivation medium to form an organic surface layer for enhancing styrene solubilization for biological degradation while MBR and biofilm-based treatment methods are advantageous due to their ability to achieve high active biomass concentration for fast biotic styrene removal. Despite the advantages associated with two-phase aqueous-organic solvent system, MBR and biofilm-based approaches, these systems have not been widely-applied due to the difficulties in scaling up, high cost and negative environmental impact. For the two-phase aqueous-organic solvent system, the very same organic layer that impeded styrene volatilization also constituted to oxygen mass transfer problem. This severely limits the reactor size, particularly reactor depth. Furthermore, the organic solvents, which typically make up about 20% volume/volume (v/v) of the total working volume, are usually non-biodegradable or even toxic to the environment. Overall, the limitation

in reactor size, the high cost of chemical supplementation and downstream environmental impact makes the two-phase aqueous-organic solvent system an impractical system for large-scale styrene wastewater treatment. MBR and biofilm on the other hand, are prone to biofouling, caused by excessive microbial growth (Fallah et al., 2010; Gross et al., 2010). For biofilm systems, biofouling greatly hinders the mass transfer of oxygen and substrates leading to a deterioration of removal efficiency over time. Similarly in MBR, biofouling increases the transmembrane pressure, reducing the reactor efficiency after prolonged operation. Fallah et al. (2010) reported severe fouling issues when the hydraulic retention time (HRT) was decreased from 24 h to 18 h after 100 days of continuous operation. The same group also posited that the increase in styrene organic loading rate (OLR) may have been responsible for the formation of fouling particles. Biofouling problems necessitate periodic reactor downtime for maintenance or membrane replacement, driving up the overall operation cost. Hence, MBR and biofilm-based approaches are costly in the long run and may not be suitable for continual treatment of large volumes of styrene waste.

On an industrial-scale, the most widely applied aerobic method for the treatment of styrene under aqueous environment is the activated sludge process. Countries using the activated sludge process for styrene treatment include USA, Singapore, Taiwan Republic of China, Brazil, Spain, France, Saudi Arabia, and Iran (The Advent Corporation; Ahmad et al., 2008; Fallah et al., 2010; Hsieh, 2000; Reis et al., 2007; US EPA, 1974). The popularity of activated sludge process is attributed to its low operation cost, low start-up time, high efficiency and ease of operation. Figure 2.1 illustrates a typical activated sludge process where the suspended but settable particles in raw wastewater are first removed in a primary clarifier before entering the aeration tank. It is in the aeration tank where the bulk of the soluble organics fraction are degraded, through the biological action of the activated sludge microbes, and converted into biomass also known as “secondary sludge”. In the secondary clarifier, the biological sludge is allowed to settled and separated from the treated water. Some biological sludge is recycled back to the aeration tanks while excess sludge is treated for sludge disposal.

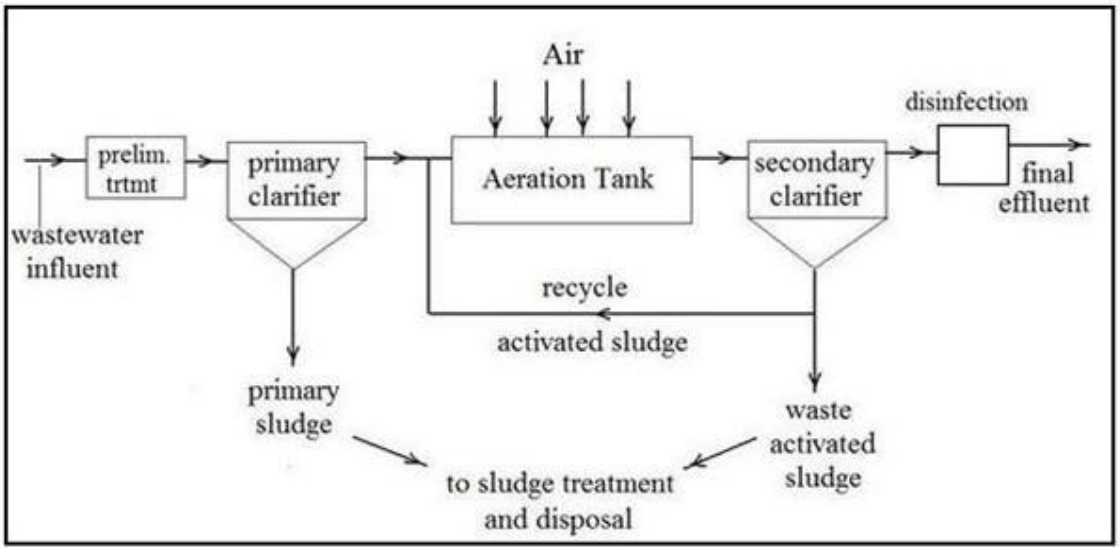


Figure 2.1 Typical flowsheet of the activated sludge process. Adopted from http://www.engineeringexcelspreadsheets.com/?p=147.

Despite its popularity, operating the activated sludge process is a challenging task as styrene is not easily biodegradable and requires specialized microbial consortia for its assimilation (Babaee et al., 2010). Without which, the activated sludge process may become a significant emission source of styrene. In a study by Hsieh (2000), simulating the activated sludge treatment process, it was shown that up to 58% of styrene loss was due to air-stripping while only 26% of styrene loss was due to biological action. Apart from causing air pollution, the unintended air-stripping of styrene also meant that styrene does not remain sufficiently long enough in the aqueous environment to enrich for styrene-degrading microbial community. This sets up a cycle where abiotic styrene loss continues to precede over biotic styrene loss, undermining the purpose of an activated sludge process. To overcome this problem, bioaugmentation with selected pure cultures or pre-adapted mixed culture has been suggested as a means to increase the biological rate constant for styrene biodegradation (Hsieh, 2000). Bioaugmentation has been shown to enhance treatment process. In a recent study by Babaee et al. (2010), comparing the styrene biodegradation kinetics of industrial activated sludge (from a wastewater treatment unit of a petrochemical plant) and municipal activated sludge, industrial activated sludge did not show any lag phase in styrene biodegradation and completely mineralized styrene compound within 9 days for styrene concentrations up to 100

mg.L^{-1}. Conversely, municipal activated sludge displayed a lag phase in styrene biodegradation and mineralized less than 50% of styrene after 9 days when incubated with 100 mg.L^{-1} of styrene. These evidence strongly suggested that bioaugmentation with microbial inocula, pre-adapted to styrene, is pivotal to the successful operation of activated sludge process for styrene wastewater treatment.

While bioaugmentation with styrene-degrading inocula is advantageous in enhancing the activated sludge process, the main drawback of this strategy is high sludge production. The yield coefficient of biomass with respect to oxygen demand in activated sludge plants is typically 0.5 (Neyens et al., 2004). The yield coefficients, specific to styrene degradation, can range from 0.2 (for industrial activated sludge) up to 1.8 (for styrene-degrading pure culture) (Babaee et al., 2010; Nikodinovic-Runic et al., 2011). Hence, the management of sludge represents a significant expenditure in the activated sludge process. Treatment and sludge disposal was estimated to be as high as 60% of the total wastewater treatment cost (Wei et al., 2003). To alleviate the cost and value-add to the biomass by-product, there has been a surge in interest to utilize bacterial biomass as biofactories for bioconversion of organic waste into valuable chemicals and materials (Drepper et al., 2006).

2.1.4 Microbes involved in styrene degradation

The microbes, involved in styrene degradation, have been studied at both the pure culture level and at the community level. To date, styrene-degrading pure cultures have been identified in the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Tsukamurella*, *Bacillus*, *Sphingomonas*, *Xanthobacter*, *Enterobacter*, *Pseudomonas*, *Xanthomonas* and *Sphinogobacterium*. These genera are distributed across 4 bacterial phyla (Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes) and 5 bacterial classes (Actinobacteria, Bacilli, Alphaproteobacteria, Gammaproteobacteria and Sphingobacteria) (Table 2.2). While these pure cultures are capable of complete styrene catabolism, bacterial community analysis has revealed that the styrene-degrading consortium is a far more complex community.

The styrene-degrading bacterial communities have been previously examined through culture-dependent isolation method, and culture-independent methods including stable-isotope probing (SIP) *cum* phospholipid fatty acid (PLFA) analysis and next-generation sequencing (Table 2.3). Two independent studies (Arnold et al., 1997; Greene & Voordouw, 2004), using culture-dependent isolation method, found that bacterial isolates from the phylum Proteobacteria and Actinobacteria formed part of the styrene-degrading community. At the genera level, styrene-degrading species from *Azospirillum*, *Pseudomonas*, *Alcaligenes* (Proteobacteria), and *Rhodococcus* (Actinobacteria) were isolated from soil contaminated with 3% weight/weight (w/w) styrene and other aromatic hydrocarbons (Greene & Voordouw, 2004). The genera of styrene-metabolizing bacterial isolates, from styrene-degrading biofilter, were slightly different and included *Pseudomonas*, *Sphingomonas*, *Xanthomonas*, an unidentified genus (Proteobacteria), and *Tsukamurella* (Acintobacteria) (Arnold et al., 1997). While culture-dependent isolation method could provide concrete evidence of the identity and functional role of consortium member, it is inherently bias towards culturable bacteria. From these two existing studies, it appears that Proteobacteria and Acintobacteria bacterial members tend to dominate in isolation studies. Valuable information on less-culturable, fastidious bacteria or unculturable bacteria is lost. Furthermore, isolation studies do not provide any conclusive quantitative information on the abundance of consortium member. Hence, it is not possible to infer the level of importance of bacterial isolates within the styrene-degrading community.

It has been suggested that more than 99% of naturally-occurring microbes are unculturable microbes (Amann et al., 1995), prompting the use of culture-independent techniques for the study of styrene-degrading bacterial community. One of the earliest studies was conducted by Alexandrino et al. (2001) on an experimental and a full-scale styrene-degrading biofilter, using a combination of SIP and PLFA. The group employed deuterated styrene ($^{2}[H]_{8}$ styrene) as a tracer compound and investigated its eventual assimilation as bacterial phospholipid fatty acid. PLFA was conducted to identify both isotope-labeled and unlabeled lipids. In

Table 2.2 Distribution of styrene-degrading capabilities among different classes of prokaryotic microorganisms. Adopted and modified from Tischler et al. (2012).

Bacterial Phylum	Bacterial Class	Genus	References
Actinobacteria	Actinobacteria	*Corynebacterium*	(Itoh et al., 1996)
		Mycobacterium	(Burback & Perry, 1993)
		Nocardia	(Hartmans et al., 1990)
		Rhodococcus	(Hartmans et al., 1990; Jung & Park, 2005; Patrauchan et al., 2008; Tischler et al., 2009; Warhurst et al., 1994; Zilli et al., 2003)
		Streptomyces	(Przybulewska et al., 2006)
		Tsukamurella	(Arnold et al., 1997)
		Gordonia	(Alexandrino et al., 2001)
Firmicutes	Bacilli	*Bacillus*	(Przybulewska et al., 2006)
Proteobacteria	Alphaproteobacteria	*Sphingomonas*	(Arnold et al., 1997)
		Xanthobacter	(Hartmans et al., 1989; Hartmans et al., 1990)
	Gammaproteobacteria	*Enterobacter*	(Grbić-Galić et al., 1990)
		Pseudomonas	(Alexandrino et al., 2001; Beltrametti et al., 1997; Ikura et al., 1997; Kim et al., 2005; Lin et al., 2010; Marconi et al., 1996; O'Connor et al., 1997; Panke et al., 1998; Park et al., 2006; Rustemov et al., 1992; Velasco et al., 1998)
		Xanthomonas	(Arnold et al., 1997)
Bacteroidetes	Sphingobacteria	*Sphinogobacterium*	(Przybulewska et al., 2006)

Table 2.3 Summary of styrene-degrading bacterial community.

Source	Approach	Approach Type	Bacterial Community at Phylum Level[1]	Reference
Contaminated soil	Isolation	Culture-dependent	Proteobacteria and Actinobacteria	(Greene et al., 2000)
Biofilter	Isolation	Culture-dependent	Proteobacteria and Actinobacteria	(Arnold et al., 1997)
Biofilter	Stable-isotope probing (SIP) and phospholipid fatty acid (PLFA) analysis	Culture-independent	Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and some unidentified microbial players	(Alexandrino et al., 2001)
Biofilter pre-inoculated with *P. putida* and develop under non-sterile conditions	Next-generation sequencing (454 pyrosequencing)	Culture-independent	Proteobacteria (40 – 70%) and Acidobacteria (2 – 8%); Chloroflexi and Firmicutes(1 – 8% each); Actinobacteria (2%); Verrucombicrobia (1 – 3%); Planctomycetes (0.5 – 2%); Bacteroidetes and Gemmatimonadetes (up to 2% each); Deinococcus-Thermus, Lentisphaerae and Spirochaetes (up to 1% each); Armatimonadetres, Chlamydiae, Tenericutes and TM7 (up to 0.5% each); Unclassified (10 – 42%)	(Portune et al., 2015)

[1] Percentage relative abundances of bacterial phyla are provided in parentheses

both experimental biofilter and full-scale biofilter, lipids that were characteristic of bacterial members from the phyla Proteobacteria (Pasteurellaceae, Moraxellacea and *Xanthomonas*), Actinobacteria (Microbacteriaceae, Streptomycetaceae and *Gordonia*), Firmicutes (*Bacillus-Staphylococcus* group), and Bacteriodetes (*Bacteroides-Cytophaga*) were detected, indicating the presence of these bacterial members in biofilter consortia. However, isotope-labeled lipids were only detected in some of these bacterial members. For the experimental biofilter, the highest proportion of isotope labeling was found in *Pseudomonas*-like fatty acids (palmitic acid [16:0], 9,10-methylenehexadecanoic acid [17:0 cyclo9-10], and vaccenic acid [18:1 *cis*11]), indicating that styrene was most probably degraded by the genus *Pseudomonas*. Interestingly, for the full-scale biofilter, isotope labeling was also detected in *Pseudomonas*-like fatty acids but not at high levels. Instead, intensive isotope labeling was observed for palmitic acid (16:0) and *cis*-11-hexadecenoic acid (16:1 *cis*11), suggesting the presence of another unknown styrene-degrading taxon, possibly microeukaryotes or bacterial taxa Pasteurellaceae. It was also interesting to note that despite the presence of some known styrene-degraders such as *Xanthomonas* spp. and *Gordonia* spp. in both biofilter samples, isotope incorporation was not detected in their fatty acids. Hence, contrary to findings from culture-dependent isolation studies, combined SIP and PLFA showed that some of these known styrene-degraders do not appear to assimilate styrene directly when they are part of a consortium. While SIP, used in conjunction with PLFA, was able to offer *in-situ* information on active styrene-degraders within the microbial community, some of the limitations of this method include the unequal incorporation of isotope labels in fatty acids and the lack of a phylogenetic database, which makes it less reliable for identifying quantifying and determining the abundance of various consortium members. Additionally, this technique may not be sufficiently sensitive to detect minor consortium members. Hence, gaining a complete and precise picture of the microbial community structure is not possible.

The advent of next-generation sequencing, coupled by its decreasing cost, increased analysis throughput, has made it an attractive option for microbial profiling studies. At present, it is a technique that provides one of the most comprehensive and near

complete information about microbial community. Recently, Portune et al. (2015) applied 454 pyrosequencing to study a styrene-degrading biofilter that was pre-inoculated with a styrene-degrading *P. putida* strain. Despite pre-inoculation, a complex microbial community developed after 80 days of operation. The styrene-degrading microbial community was revealed to be a much more diverse community compared to previous findings (Alexandrino et al., 2001; Arnold et al., 1997; Greene & Voordouw, 2004). The research group observed that the system was consistently dominated by the phyla Proteobacteria (40% to 70%) and a large amount of bacterial sequences which could not be classified at the phylum level (10% to 42%). Other more prominent phyla detected were Acidobacteria (2% to 8%), Chloroflexi and Firmicutes (1% to 8% each), Actinobacteria (2%), Planctomycetes (0.5% to 2%), Bacteroidetes and Gemmatimonadetes (up to 2% each). Minor phyla such as Deinococcus-Thermus, Lentisphaerae, Spirochaetes, Armatimonadetres, Chlamydiae, Tenericutes and TM7 were detected at low levels (less than 1%). At the genera level, up to a total of 261 bacterial genera were detected, and among them, *Azoarcus*, *Pseudomonas* and *Brevundimonas* had the highest relative abundance (1% to 8% each). Canoical correspondence analysis (CCA), revealed that many bacterial genera, including *Pseudomonas*, *Brevundimonas*, *Achromobacter* and *Hydrogenophaga*, were positively associated with conditions of increasing styrene outlet conditions, implying that they may play important roles in styrene degradation under such conditions. Among them, a significant association (p <0.05) were observed for genera *Brevundimonas, Achromobacter* and *Hydrogenophaga*. Interestingly, styrene degradation has not been described in these genera, but genetic evidence suggested that they possessed catabolic potential for styrene degradation. Also noteworthy, is the observation that some known styrene-degrading genera such as *Rhodococcus, Xanthobacter*, and *Sphingomonas* occurred at low abundances (between less than 1% and 5%). Similar to the study by Alexandrino et al. (2001), the study by Portune et al. (2015) provided additional evidence to support the idea that some known styrene-degrading genera may not necessary form the core styrene-assimilating population when they are part of a consortium.

Collectively, these existing findings have yielded some interesting observations and continue to refine the scientific understanding about styrene degraders, non-styrene-degraders and their role in styrene-degrading community. Currently, the activated sludge process is one of the most commonly applied aerobic methods for the treatment of styrene wastewaters (Babaee et al., 2010; US EPA, 1974). Knowledge of the aqueous styrene-degrading microbial community would facilitate seed sludge selection, bioaugmentation and formulation of defined microbial cocktails to enhance biological styrene removal. While studies on biofilter and contaminated soil samples have shed some light on styrene-degrading microbial consortia, these studies were limited to consortia from non-aqueous and semi-aqueous environments. There is currently a lack of understanding on the styrene-degrading microbial consortia present in aqueous environment. Furthermore, it has been reported that styrene-degrading microbial communities would evolve differently to adapt to various environs (Tresse et al., 2002). Hence, the existing information may not accurately reflect the microbial population in aqueous environments. Examination of the aqueous styrene-degrading microbial community is therefore necessary.

2.1.5 Styrene degradation pathways

Styrene could be degraded through either aerobic or anaerobic pathways. The aerobic biodegradation of styrene is known to occur via either the direct ring cleavage pathway or side-chain oxidation pathway (Figure 2.2). In the direct ring cleavage pathway, oxygen atoms are added directly to the aromatic nucleus through a dioxygenation process, catalyzed by styrene 2,3-dioxygenase (SDO), to form a styrene *cis*-glycol. The *cis*-glycol is subsequently re-aromatization to 3-vinylcatechol by styrene 2,3-dihydrodiol dehydrogenase (SDD). 3-vinylcatechol undergoes ring cleavage at either the *meta* or *ortho* position to yield 2-vinyl-cis,cis-muconic acid and 2-hydroxy-6-oxoocta-2,4,7-trienoic acid before entering the central metabolic pathways. In the side-chain oxidation pathway, styrene first undergoes oxygenation, catalyzed by styrene monooxygenase (SMO), to form styrene oxide. Styrene oxide is highly unstable and is rapidly isomerized to phenylacetaldehyde by styrene oxide isomerase (SOI) and further converted to phenylacetic acid by phenylacetaldehyde dehydrogenase (PAD). In an alternative

side-chain oxidation pathway, the styrene oxide is hydrolyzed by epoxide hydrolase (EH) to form phenyl-1,2-diol, followed by subsequent oxidation to mandelic acid and decarboxylation to benzoate.

In comparison to aerobic pathway, the anaerobic pathway for styrene degradation is less well-studied. However, three hypothetical pathways, involving the phenylacetate and 2-ethylphenol intermediates, have been proposed (Grbić-Galić et al., 1990) (Figure 2.3). In the phenylacetate route, oxygen was added to the side chain group of styrene by hydratase-catalyzed addition of water to form 2-phenylethanol. The compound is further oxidized by a series of dehydrogenases to form phenylacetaldehyde, followed by phenylacetate. In the 2-ethylphenol route, a hydroxyl group is added to the aromatic ring of styrene through water-mediated hydroxylation to form 2-ethylphenol. The compound subsequently either undergoes ring cleavage to form 2-ethylhexanol or side-chain oxidation to form 2-hydroxyphenyl-acetic acid.

2.1.6 Up-recycling of styrene to value-added products

The application of bacterial biomass as whole-cell catalyst for bioconverting styrene to valuable products has a relatively young history of 30 years but has gained much momentum since its inception. The production of a commercially valuable product from styrene biotransformation was first demonstrated for styrene oxide in 1970s, followed by 3-vinylcatechol in 1990s, and PHAs in 2005 (Ward et al., 2005; Warhurst & Fewson, 1994).

Styrene oxide and 3-vinylcatechol are metabolic intermediates formed during styrene catabolism through the side-oxidation pathway and direct-ring cleavage pathway respectively (Tischler & Kaschabek, 2012). Styrene oxide is an important starting monomer for the chemical synthesis of pharmaceutical drugs such as nematocide levamisole (Panke et al., 2000). The production of styrene oxide from styrene has been reported in *Mycobacterium* sp. E3 and many Pseudomonads including *P. putida* mt-2, *P. putida* F1, *P. putida* S12, and *Pseudomonas* sp. VLB120 (Mooney et al., 2006; Panke et al., 1998). Similar to styrene oxide,

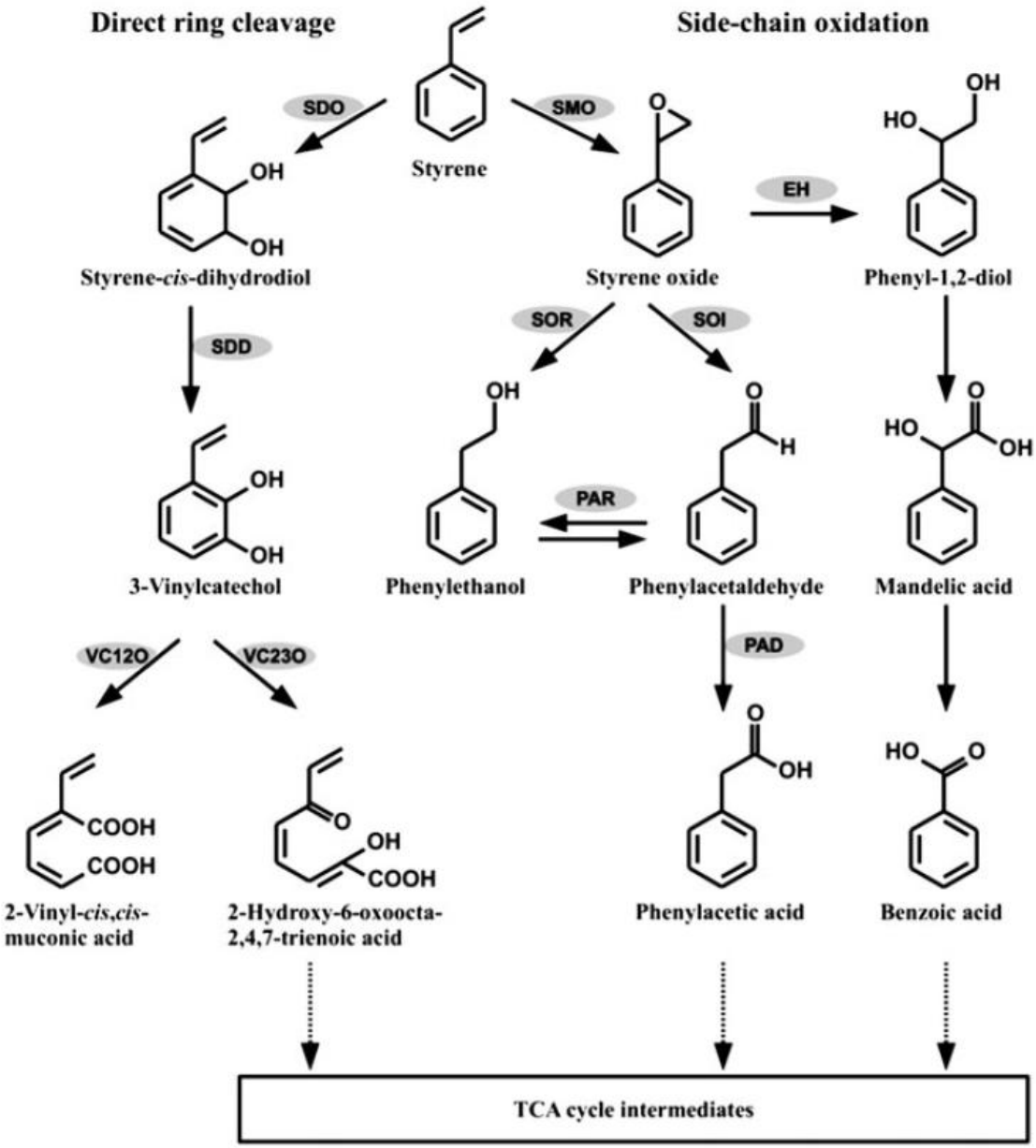


Figure 2.2 Microbial pathways of aerobic styrene degradation. Enzymes are indicated as abbreviations (styrene 2,3-dioxygenase, SDO; styrene 2,3-dihydrodiol dehydrogenase, SDD; vinylcatechol 1,2-dioxygenase, VC120; vinylcatechol 2,3-dioxygenase, VC230; styrene monooxygenase, SMO; styrene oxide reductase, SOR; phenylacetaldehyde reductase, PAR; styrene oxide isomerase, SOI; phenylacetaldehyde dehydrogenase, PAD; epxoide hydrolase, EH). Adopted from Tischler and Kaschabek (2012).

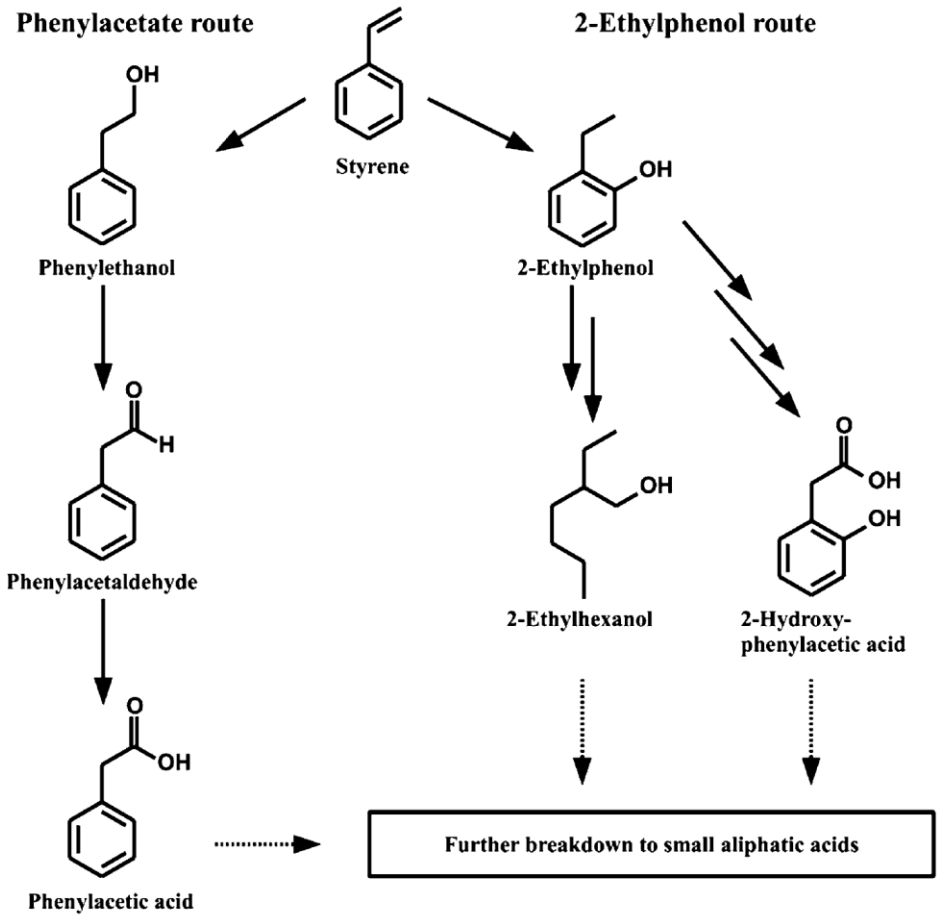


Figure 2.3 Hypothetical microbial pathways of anaerobic styrene degradation. The pathways are based on identified metabolites from pure or mixed cultures. No data about involved enzymes are available so far. Adopted from Tischler and Kaschabek (2012).

3-vinylcatechol is also a starting molecule for the synthesis of pharmaceutical products, polymers and dyes (Mooney et al., 2006). The production of 3-vinylcatechol from styrene was first reported in *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst et al., 1994). Although styrene oxide and 3-vinylcatechol are potential products that can be formed from styrene, the bioconversion process may not be applicable to aqueous waste medium. This is because both products are secreted into the extracellular cultivation medium, usually supplied with a solvent layer for the stable accumulation of the products (Gross et al., 2010; Panke et al., 2000). In a complicated aqueous waste matrix, these catabolic intermediates may be chemically and/or biologically labile due to the presence of impurities and

biological agents, reducing the recovery of these compounds. This makes the production of styrene oxide and 3-vinylcatechol from waste styrene in aqueous medium impractical. As such, the production of styrene oxide and 3-vinylcatechol are largely confined to pure cultures and cultivation in synthetic media.

PHA is a biodegradable and biocompatible polyester with diverse applications. Specifically, MCL-PHA possesses material properties which are suitable for high-end medical uses (Rai et al., 2011) while its depolymerized monomers are active compounds with potential pharmaceutical applications (Allen et al., 2012). To date, four pure *Pseudomonas* strains are reportedly capable of assimilating styrene to form MCL-PHA. These strains are *P. putida* S12, *P. putida* CA-1 and *P. putida* CA-3 (NCIMB 41162) and *Pseudomonas* sp. TN301 (Hartmans et al., 1990; Narancic et al., 2012; O'Connor et al., 1995). Unlike styrene oxide and 3-vinylcatechol that are monomeric in form and accumulate in the cultivation media, PHA is a polymer that is accumulated and stored as lipid granules within the bacterial cell (Galia, 2010; Peters & Rehm, 2005). There are three important implications of this feature. First, in a complex media such as wastewater, the final PHA product is kept securely within the bacterium and separated from wastewater matrix, ensuring the recovery of a high-purity product. Second, the resultant biomass by-product becomes a valuable store of PHA with commercial value for the downstream PHA extraction. Third, the downstream extraction of PHA product is simplified. In addition, the channeling of carbon substrates for PHA production also limits biomass growth. Taken together, PHA production brings about several advantages:

1. Cost savings on reduced volumes of biomass generation,
2. Creates commercial value for the resultant PHA-containing biomass by-product, and
3. Cost savings on additional biomass volume reduction post PHA extraction.

From an economic point of view, these advantages make it particularly attractive for MCL-PHA production from waste substrates in aqueous medium using activated

sludge. MCL-PHA recovery may off-set treatment operation cost and cost associated with post-operation sludge management. Previous studies by Hartmans et al. (1990) and Ward et al. (2005), demonstrating the technical feasibility of bioconverting styrene to MCL-PHA by Pseudomonads pure strains, provide a promising start for MCL-PHA production from styrene waste under aqueous medium. PHA is discussed in greater depth in the following Section 2.2.

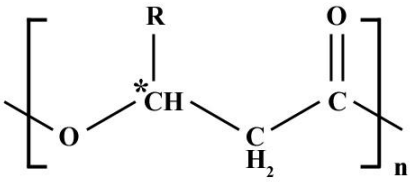
2.2 Polyhydroxyalkanoate (PHA)

PHA is a biopolyester produced by some bacteria strains and was first discovered by Lemogine in 1926 (Anderson & Dawes, 1990). The PHA-producing bacteria, also termed as "PHA accumulators" usually synthesize PHA under nutrients stress conditions where an essential nutrient (*e.g.*, nitrogen, phosphorus, *etc*) is limiting in the presence of excess carbon (Mooney et al., 2006). The excess carbons are bioaccumulated as PHA and stored in the form of intracellular lipid granules. PHA in turn acts as an energy reserve and promotes the long-term survival of PHA accumulators (Kadouri et al., 2005).

At a molecular level, PHA polymer is composed of (*R*)-*β*-hydroxy fatty acids monomer. A PHA molecule is typically made up of 600 to 35,000 monomer units (Khanna & Srivastava, 2005) and has a molecular mass between 50,000 and 1,000,000 Da (Madison & Huisman, 1999). These monomers, also termed as "PHA monomers" harbor a functional *R* group. The *R* group is usually a saturated alkyl group that varies from methyl (C_1) to pentadecyl (C_{15}) (Figure 2.4) (Tan et al., 2014b). SCL-PHA, MCL-PHA and LCL-PHA comprises of PHA monomers between 3 to 5 carbon atoms, 6 to 14 carbon atoms and 15 or more carbon atoms, respectively, (Khanna & Srivastava, 2005). Apart from saturated alkyl groups, functional *R* groups can also take the form of unsaturated alkyl groups, branched alkyl groups, substituted alkyl groups although these forms are less common (Zinn et al., 2001).

2.2.1 Current analytical methods for PHA detection and quantification

Various methods are available for the detection and analysis of intracellular



Poly(3-hydroxyalkanoate)

R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure 2.4 Chemical structure and nomenclature of PHA. Adopted from Tan et al. (2014b).

microbial PHA. These methods include colony/cell staining, crotonic acid assay, FTIR, chromatography-based analysis. Colony/cell staining is often used as a front-line method for high throughput screening and identification of novel microbes with PHA production potential owing to the relative ease of sample preparation and short analysis time. In colony/cell staining method, Nile red or Nile blue A dye is directly added into the solid growth medium (Spiekermann et al., 1999), liquid cell cultures (Melnicki et al., 2009; Wu et al., 2003) or onto heat-fixed smeared cells (Ostle & Holt, 1982). Under UV illumination, Nile red and Nile blue A dye stains PHA to give a pink/red/yellow/orange appearance enabling the PHA-producing microbes to be identified and isolated. Although staining method provide a simple way to screen for PHA-producing microbes efficiently, this methods is also prone to detection errors. False positives may arise from the staining of other non-PHA lipid storage compounds (Spiekermann et al., 1999). Hence, this method could only be employed as a presumptive test of PHA production potential. Another downside of this

method is that it can neither quantify PHA nor provide qualitative information about PHA monomeric composition.

Conversely, crotonic acid assay, FTIR and chromatography-based analysis methods could provide quantitative and qualitative information about PHA, but to varying extents. Crotonic acid assay was one of the earliest PHA quantitation methods developed (Law & Slepecky, 1961; Slepecky & Law, 1960; Ward & Dawes, 1973) but it is limited to SCL-PHA (*i.e.*, P3HB) determination and is prone to overestimation (Karr et al., 1983; Valappil et al., 2007). FTIR, on the other hand, has been applied to detect and distinguish between the different types of PHA (*i.e.*, SCL-PHA, MCL-PHA, and SCL-MCL-PHA), present within intact cells or as purified polymers. Characteristic ester carbonyl band for intracellular SCL-PHA, MCL-PHA, and SCL-MCL-PHA were observed at 1732 cm^{-1}, 1744 cm^{-1} and 1739 cm^{-1}, respectively, whereas the same band for purified polymer SCL-PHA, MCL-PHA, and SCL-MCL-PHA were observed at 1728 cm^{-1}, 1740 cm^{-1} and 1732 cm^{-1}, respectively (Hong et al., 1999). The solvent-less nature of the FTIR technique and short analysis time eliminates risk exposure to hazardous chemicals while providing fast data output. However, FTIR-based methods have lower detection sensitivities, are inapt at describing or detecting changes in PHA monomeric composition, and cannot discriminate between PHA blends and copolymers (Hong et al., 1999). Hence, FTIR-based methods tend to be more suitable for routine monitoring of PHA production for standard bioprocesses with well-characterized PHA products.

LC- and GC-based methods are the most frequently used analytical technique due to automated sample analysis, and the ability to provide accurate PHA quantification and qualitative information about PHA monomeric composition. Compared to FTIR-based methods, chromatography-based methods have higher detection sensitivities ranging from 0.014 to 14 μg for HPLC and 0.05 pg to 15 mg for GC depending on the type of detectors and chemical derivatization methods used (de Rijk et al., 2005). At present, GC remains the preferred method for qualitative and quantitative analysis of PHA monomers owing to its high separation power and detection sensitivity (de Rijk et al., 2005). One of the earliest works on GC

determination of PHA was reported by Braunegg et al. (1978), who developed a method for accurate and reproducible determination of P3HB content in bacterial biomass using GC-FID. They showed that after subjecting P3HB-containing bacterial biomass to methanolysis, P3HB could be completely recovered in the form of its methyl esters derivatives and quantified to levels as low as 10^{-5} $g.L^{-1}$. GC-FID analysis was subsequently expanded to poly(3-hydroxyvalerate) (P3HV) and MCL-PHA (Comeau et al., 1988; Lageveen et al., 1988). The robustness of GC-FID determination however, is dependent on the inclusion of appropriate PHA analytical standards. Conversely, GC-MS ensures more reliable detection, identity confirmation and quantification of PHA monomers, as well as enables tentative identification of novel PHA monomers in the absence of analytical standards (Grubelnik et al., 2008; Lee & Choi, 1995). Nevertheless, GC-MS can only provide a tentative identification of PHA. Further validation using suitable PHA analytical standards is still essential in ensuring the accuracy of the detection method. However, there is currently a lack of PHA analytical standards to represent the wide chemical diversity of PHA monomers. System calibration is costly and also presents significant workload, particularly for PHA polymers such as MCL-PHA and LCL-PHA which may comprise of up to 9 or more PHA monomers. This has made PHA analysis a particularly challenging task (Escapa et al., 2011; Furrer et al., 2007). Some researchers attempted to overcome these problems through using a combination of GC-MS (for tentative identification of PHA monomers) and GC-FID (for the routine quantification of PHA monomers identified by GC-MS) (Ward et al., 2006) to keep calibration workload manageable and calibration cost low; chemically or biologically-synthesize PHA analytical standards that are commercially unavailable (Sun et al., 2009); using commercially-available PHA analytical standards to quantify chemically-similar but commercially-unavailable PHA monomers (Oehmen et al., 2005). However, these measures can lead to errors in measurements, and can be a tedious, time-consuming and expensive process. Hence, there is a need to develop a simple GC-MS calibration method which minimizes calibration workload and cost while enabling the analysis of a wide range of PHA monomers even with a limited inventory of PHA analytical standards.

2.2.2 Properties and applications of PHA

As a polymeric material, PHA is completely biodegradable. PHA's biodegradation has been observed in various environs including anaerboic sewage, sea water (Choi & Lee, 1999), lake (Brandl & Püchner, 1991) and soil (Kim et al., 2000). PHA is also a biocompatible material because it is a product of cell metabolism and its biodegradation by-product, (*R*)-*β*-hydroxy fatty acid, is usually present as a soluble component in blood (Zinn et al., 2001). This was further evinced by i*n vivo* studies where implantation of PHA material was made in rabbits and mice for a 6-month period without eliciting inflammation response (Pouton & Akhtar, 1996; Qu et al., 2006). The biodegradability and compatibility features make PHA a commercially-valuable material. Coupled with the variable functional *R* group, the family of PHAs exhibit a wide variety of physical-mechanical properties, ranging from hard crystalline to elastic. For an example, SCL-PHAs typically have a glass to rubber transition temperature (T_g) of -48 °C to 4 °C and a crystalline phase melting temperature (T_m) of 53 °C to 180 °C (Lu et al., 2009). This meant that SCL-PHAs tend to be more crystalline and exhibit thermoplastic properties. On the other hand, MCL-PHAs generally have lower T_g values between -50 °C and 26 °C, and lower T_m values between 42 and 67 °C (Rai et al., 2011; Zinn, 2010) giving MCL-PHAs their elastomeric properties. The low stiffness and high flexibility of MCL-PHAs makes them suitable for soft tissue regeneration such as heart valves, cardiac patches, scaffold for tissue engineering, surgical sutures, stents and controlled drug delivery matrices (Rai et al., 2011). MCL-PHA monomers, arising from the depolymerization of MCL-PHA, are also know to exhibit antimicrobial activity and serve as a platform for the production of a plethora of pharmaceutical products including fungicides, flavors, pheromones, vitamins and *etc* (Allen et al., 2012). Apart from medical and pharmaceutical applications, MCL-PHAs are also used in plastic coatings and pressure sensitive adhesives (Madison & Huisman, 1999).

To date, although many types of PHA have been identified, only four types of PHA are produced industrially. These PHAs are P3HB, P3HB-3HV, poly(3-hydroxbutyrate-*co*-4-hydroxybutyrate) (P3HB4HB), and poly(3-hydroxbutyrate-*co*-3-hydroxyhexanoate) (P3HBHHx) (Chen, 2010a). With the exception of P3HB-

HHx, which is a copolymer of SCL monomers and MCL monomers, all other commercial PHAs are SCL-PHAs. Despite the potential applications of other non-SCL-PHAs including MCL-PHA, their commercialization is hindered due to high production cost. It has been estimated that at least 30% or more of PHA cost is attributed to carbon and nutrients cost (Choi & Lee, 1999). This has led to an interest to explore different carbon sources for PHA production as a means to economically produce MCL-PHA and other types of non-SCL-PHA.

2.2.3 PHA-producing bacteria and carbon sources for PHA production

The feasibility of a carbon source as a feed substrate for PHA biosynthesis is largely bacterial-dependent. This is so because the successful bioconversion of any carbon source to PHA needs to fulfill two criteria: (1) Whether the bacteria possess the relevant metabolic pathways for assimilation of the targeted carbon source, and (2) whether the bacteria harbor the gene *phaC*, which encodes PHA synthase enzyme, for PHA synthesis. If only the first criterion is met, carbon utilization will occur without PHA accumulation. Conversely, if only the second criterion is met, despite harboring the *phaC* gene, bacteria are unlikely to grow or accumulate PHA.

To date, PHA production has been reported in more than 100 bacterial genera (Table 2.4) (Koller et al., 2010), of which species are commercially available from culture collections (Tan et al., 2014b). Acting as biocatalysts, these PHA-producing microorganisms enable the coupling of a myriad of carbon catabolic pathways together with PHA anabolic pathways, thereby playing a key role in the diversification of PHA production from various carbon sources. These carbon sources include saccharides (*e.g.*, fructose, maltose, lactose, xylose, arabinose, *etc*.), *n*-alkanes (*e.g.*, hexane, octane, dodecane, *etc*.), *n*-alkanoic acids (*e.g.*, acetic acid, propionoic acid, butyric acids, valeric acid, lauric acid, oleic acid, *etc*.), *n*-alcohols (*e.g.*. methanol, ethanol, octanol, glycerol, *etc*.), and gases (*e.g.*, methane, carbon dioxide) (Anderson & Dawes, 1990; Tan et al., 2014b; Verlinden et al., 2007). Wastestreams, which provide a free source of carbons, have also been identified for PHA production (Koller et al., 2010). These include waste frying oil, vinegar waste, waste fats, food waste, agricultural waste, domestic wastewater, plant oil mill

Table 2.4 Polyhydroxyalkanoate (PHA)-accumulating genera of prokaryotic micro-organisms. Adopted from Koller et al. (2010).

Acidovorax	*Erwinia*	*Oscillatoria*[1]
Acinetobacter	*Escherichia* (rec.!)[1]	*Paracoccus*
Actinobacillus	*Ferrobacillus*	*Paucispirillum*
Actinomycetes	*Gamphospheria*	*Pedomicrobium*
Aeromonas	*Gloeocapsa*[1]	*Photobacterium*
Alcaligenes[1,2]	*Gloeothece*[1]	*Protomonas*
Allochromatium	*Haemophilus*	*Pseudomonas*[1,2]
Anabaena[2]	*Halobacterium*[1,3]	*Ralstonia*[1,2]
Aphanothece[1]	*Haloarcula*[1,2,3]	*Rhizobium*[1,2]
Aquaspirillum	*Haloferax*[1,2,3]	*Rhodobacter*
Asticcaulus	*Halomonas*[1]	*Rhodococcus*[2]
Azomonas	*Haloquadratum*[3]	*Rhodopseudomonas*
Azospirillum	*Haloterrigena*[3]	*Rhodospirillum*[2]
Azotobacter[1,2]	*Hydrogenophaga*[1,2]	*Rubrivivax*
Bacillus[1,2]	*Hyphomicrobium*	*Saccharophagus*
Beggiatoa	*Klebsiella* (rec.!)	*Shinorhizobium*
Beijerinckia[2]	*Lamprocystis*	*Sphaerotilus*[1]
Beneckea	*Lampropedia*	*Spirillum*
Brachymonas	*Leptothrix*	*Spirulina*[1]
Bradyrhizobium	*Methanomonas*	*Staphylococcus*
Burkholderia[1]	*Methylobacterium*[2]	*Stella*
Caryophanon	*Methylosinus*	*Streptomyces*
Caulobacter	*Methylocystis*	*Synechococcus*[1]
Chloroflexus	*Methylomonas*	*Syntrophomonas*
Chlorogloea[1]	*Methylovibrio*	*Thiobacillus*
Chromatium	*Micrococcus*	*Thiococcus*
Chromobacterium	*Microcoleus*	*Thiocystis*
Clostridium	*Microcystis*	*Thiodictyon*
Comamonas[1,2]	*Microlunatus*[2]	*Thiopedia*
Corynebacterium[2]	*Moraxella*	*Thiosphaer*[1]
Cupriavidus[1,2]	*Mycoplana*[1]	*Variovorax*[1,2]
Cyanobacterium[2]	*Nitrobacter*	*Vibrio*
Defluviicoccus[2]	*Nitrococcus*	*Wautersia*[1,2] (today *Cupriavidus*)
Derxia[2]	*Nocardia*[1,2]	*Xanthobacter*
Delftia[1,2]	*Nostoc*	*Zoogloea*[1]
Ectothiorhodospira	*Oceanospirillum*	

[1] Detailed knowledge of the growth and production kinetics available
[2] Accumulation of copolyesters known
[3] Archaea

effluents, crude glycerol from biodiesel production, plastic waste, landfill gas and *etc*. Styrene and other aromatic compounds as well as a wide variety of non-aromatic carbon sources have been identified as suitable carbon substrates for MCL-PHA production (Tan et al., 2014b). An overview of the commercially-available MCL-PHA-producing bacterial strains, carbon sources and complex wastestreams for MCL-PHA production is presented in Table 2.5.

2.2.4 PHA bioaccumulation pathways

PHAs play a pivotal role in priming the bacteria for stress survival. PHAs promote the long-term survival of bacteria under nutrients-scarce conditions by acting as energy reserves. Bacteria that harbor PHA also showed enhanced stress tolerance against transient environmental assaults such as UV irritation, heat and osmotic shock (Kadouri et al., 2005). As such, under stressful conditions, PHA-producing bacteria tend to channel resources towards PHA production instead of growth (Steinbüchel & Hein, 2001). For this event to occur, it means that PHA biosynthetic routes need to be intricately linked with central metabolic pathways such that metabolic intermediates can be flexibly routed for energy and growth expenditure or PHA bioaccumulation. This also meant that the crucial coupling between carbon catabolism and PHA anabolism is provided by the bacteria's central metabolic pathways. These central metabolic pathways include glycolysis, Krebs Cycle, *β*-oxidation, *de novo* fatty acids synthesis (Lu et al., 2009; Madison & Huisman, 1999).

There are five main PHA biosynthetic routes in naturally-occurring bacteria. The MCL-PHA synthesis routes are illustrated as Route A and Route B in Figure 2.5. In Route A, sugar substrates are metabolized to form acetyl-CoA, which then enters the *de novo* fatty acids synthesis pathway together with malonyl-CoA. PHA precursors are derived from the *de novo* fatty acids synthesis pathway, which is a chain-lengthening cycling reaction that catalyzes the addition of one molecule of acetyl-CoA to lengthen the malonyl-CoA derivative carbon backbone by two carbons per cycle (Lu et al., 2009). This yields (*R*)-3-hydroxyacyl-ACP intermediates of varying carbon numbers, which can be channeled to form PHA

Table 2.5 Summary of MCL-PHA-producing microbial strains available in culture collections and carbon sources. Adopted from Tan et al. (2014b).

Microorganism	Culture Collection Number[2]	Carbon Source	PHA Monomer or Polymer[3]	PHA Content (% CDM)	Average PHA Productivity ($g.L^{-1}.h^{-1}$)	Reference(s)
Gram negative bacteria						
Pseudomonas aeruginosa PAO1	ATCC 47085	Oil and wax products from polyethylene (PE) pyrolysis	MCL-PHA	25.0	n.g.	(Guzik et al., 2014)
Pseudomonas frederiksbergensis GO23[1]	NCIMB 41539	Terephthalic acid from polyethylene terephthalate (PET) pyrolysis	MCL-PHA	24.0	0.004	(Kenny et al., 2008)
Pseudomonas marginalis	DSM 50276	1,3-Butanediol, octanoate	SCL-MCL-PHA, MCL-PHA	11.9 - 31.4	n.g.	(Lee et al., 1995)
Pseudomonas mendocina	ATCC 25411, DSM 50017	1,3-Butanediol, octanoate	SCL-MCL-PHA	13.5 - 19.3	n.g.	(Lee et al., 1995)
Pseudomonas oleovorans	ATCC 8062, DSM 1045	4-Hydroxyhexanoic acid	SCL-MCL-PHA	18.6	n.g.	(Valentin et al., 1994)

Table 2.5 *Cont.*

Pseudomonas putida CA-3[1]	NCIMB 41162	Styrene	MCL-PHA	31.8	0.063	(Nikodinovic-Runic et al., 2011)
		Styrene from polystyrene (PS) pyrolysis	MCL-PHA	36.4	0.033	(Ward et al., 2006)
Pseudomonas putida GO16[1]	NCIMB 41538	Terephthalic acid from polyethylene terephthalate (PET) pyrolysis	MCL-PHA	27.0	~0.005, 0.008[4]	(Kenny et al., 2008)
Pseudomonas putida GO19[1]	NCIMB 41537	Terephthalic acid from polyethylene terephthalate (PET) pyrolysis	MCL-PHA	23.0	~0.005, 0.008[4]	(Kenny et al., 2008)
Pseudomonas putida GPo1 (formerly *Pseudomonas oleovorans*)	ATCC 29347	1-Alkenes, *n*-alkanes	MCL-PHA	2.0 - 28.0	n.g.	(Lageveen et al., 1988)
		n-alkanoates	SCL-MCL-PHA, MCL-PHA	5.0 - 60.0	n.g.	(Elbahloul & Steinbuchel, 2009; Gross et al., 1989)
Pseudomonas putida KT2440	ATCC 47054	Nonanoic acid	MCL-PHA	26.8 - 75.4	0.250 - 1.110	(Z. Sun et al., 2007)
		4-hydroxyhexanoic acid	MCL-PHA	25.3 - 29.8	n.g.	(Valentin et al., 1994)
		Glucose	MCL-PHA	32.1	0.006	(Davis et al., 2013)

Table 2.5 *Cont.*

Pseudomonas putida F1	ATCC 700007, DSM 6899	Benzene, ethylbenzene, toluene	MCL-PHA	1.0 - 22.0	n.g.	(Nikodinovic et al., 2008)
Pseudomonas putida mt-2	NCIMB 10432	Toluene, *p*-xylene	MCL-PHA	22.0 - 26.0	n.g.	(Nikodinovic et al., 2008)
		Acetic acid, citric acid, glucose, glycerol, octanoic acid, pentanoic acid, succinic acid		4.0 - 77.0	n.g.	(Shahid et al., 2013)
Thermus thermophilus HB8	ATCC 27634, DSM 579	Whey	SCL-MCL-PHA	35.6	0.024	(Pantazaki et al., 2009)
Gram positive bacteria						
Bacillus megaterium	CCM 1464, DSM 509, IFO 12019, NBRC 12109	Citric acid, glucose, glycerol, succinic acid, octanoic acid	P3HB, SCL-MCL-PHA, MCL-PHA	3.0 - 48.0	n.g.	(Shahid et al., 2013)
Various *Bacillus* spp. type strains	Refer to Valappil et al. (2007)	Acetate, *n*-alkanoate, 3-hydroxybutyrate, propionate, sucrose, valerate	C_4, C_5, C_6	2.2 - 47.6	n.g.	(Valappil et al., 2007)

Table 2.5 *Cont.*

Corynebacterium glutamicum	ATCC 15990, DSM 20137, NCIB 10337	Acetic acid, citric acid, glucose, glycerol, succinic acid	P3HB, MCL-PHA	4.0 - 32.0	n.g.	(Shahid et al., 2013)

[1] Refers to patent strain

[2] ATCC, American Type Culture Collection (Manassas, VA, USA); CCM, Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic); DSM, German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); IFO, Institute for Fermentation, Osaka (Yodogawa-ku, Osaka, Japan [*collection transferred to* NBRC]); NBRC, NITE Biological Resource Center (Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan); NCIB, National Collection of Industrial Bacteria (Torry Research Station, Aberdeen, Scotland, UK [*incorporated with* NCIMB]); NCIMB, National Collections of Industrial Food and Marine Bacteria (Aberdeen, UK).

[3] PHA is indicated as monomers unless nuclear magnetic resonance (NMR) verification was performed; SCL-PHA, short-chain length PHA; MCL-PHA, medium-chain length PHA; SCL-MCL-PHA, short-chain length-*co*- medium-chain length PHA; P3HB, poly(3-hydroxybutyrate); C_4, 3-hydroxybutyrate; C_5, 3-hydroxyvalerate; C_6, 3-hydroxyhexanoate.

[4] Refers to the maximal PHA productivity rate recorded

CDM, cell dry mass

n.g., not given

precursors (*R*)-3-hydroxyacyl-CoA ([*R*]-3-HA-CoA) through the action of an enzyme 3-hydroxyacyl-acyl carrier protein-CoA transacylase (PhaG) (Zheng et al., 2005). PHA accumulation via Route B is frequently reported for fatty acids and oils (Costa et al., 2009; Fernández et al., 2005), which derives PHA precursors through *β*-oxidation pathway. *β*-oxidation pathway is a chain-shortening cycling reaction where each complete cycle removes two carbon atoms as acetyl-CoA (Gottschalk, 1986).While Route B generally leads to the production of MCL-PHA, the chain-shortening function of *β*-oxidation pathway also means that PHA precursors of shorter carbon numbers can be produced for SCL-PHA formation (Akiyama et al., 1992; Madison & Huisman, 1999; Steinbüchel & Hein, 2001). SCL-PHA is also synthesized through Route C, D and E, usually from sugars and volatile fatty acids (acetic acid, propionate acid).

2.3 Bioconversion of Styrene to MCL-PHA

The concept of styrene bioconversion to PHA is relatively new and has only been in existence for about 10 years. In the past decade, there has been some scientific progress made on the understanding of microbes, capable of such metabolism, as well as the coupling between styrene catabolic and PHA anabolic pathway.

2.3.1 Microbes involved in bioconversion of styrene to MCL-PHA

The feasibility of the biological conversion of styrene to PHA was first reported in the patent strain *P. putida* CA-3 (NCIMB 41162) (Ward et al., 2005), and later discovered in three other pure *Pseudomonas* spp. namely, *P. putida* S12, *P. putida* CA-1 and *Pseudomonas* sp. TN301 (Hartmans et al., 1990; Narancic et al., 2012; O'Connor et al., 1995). All three strains reportedly synthesize MCL-PHA with similar monomeric composition consisting of 3-hydroxyhexanoate (C_6), 3-hydroxyoctanoate (C_8), 3-hydroxydecanoate (C_{10}), 3-hydroxydodecanoate (C_{12}) and 3-hydroxydodecenoate ($C_{12:1}$) monomers. Among the four strains, *P. putida* CA-3 was the most well-studied. *P. putida* CA-3 was found to be highly efficient at metabolizing styrene and displayed the highest cellular PHA content at 30% CDM. When styrene was supplied at a total concentration of 19.3 $g.L^{-1}$ over a 48 h period, *P. putida* CA-3 metabolized more than 99% of styrene for biomass growth

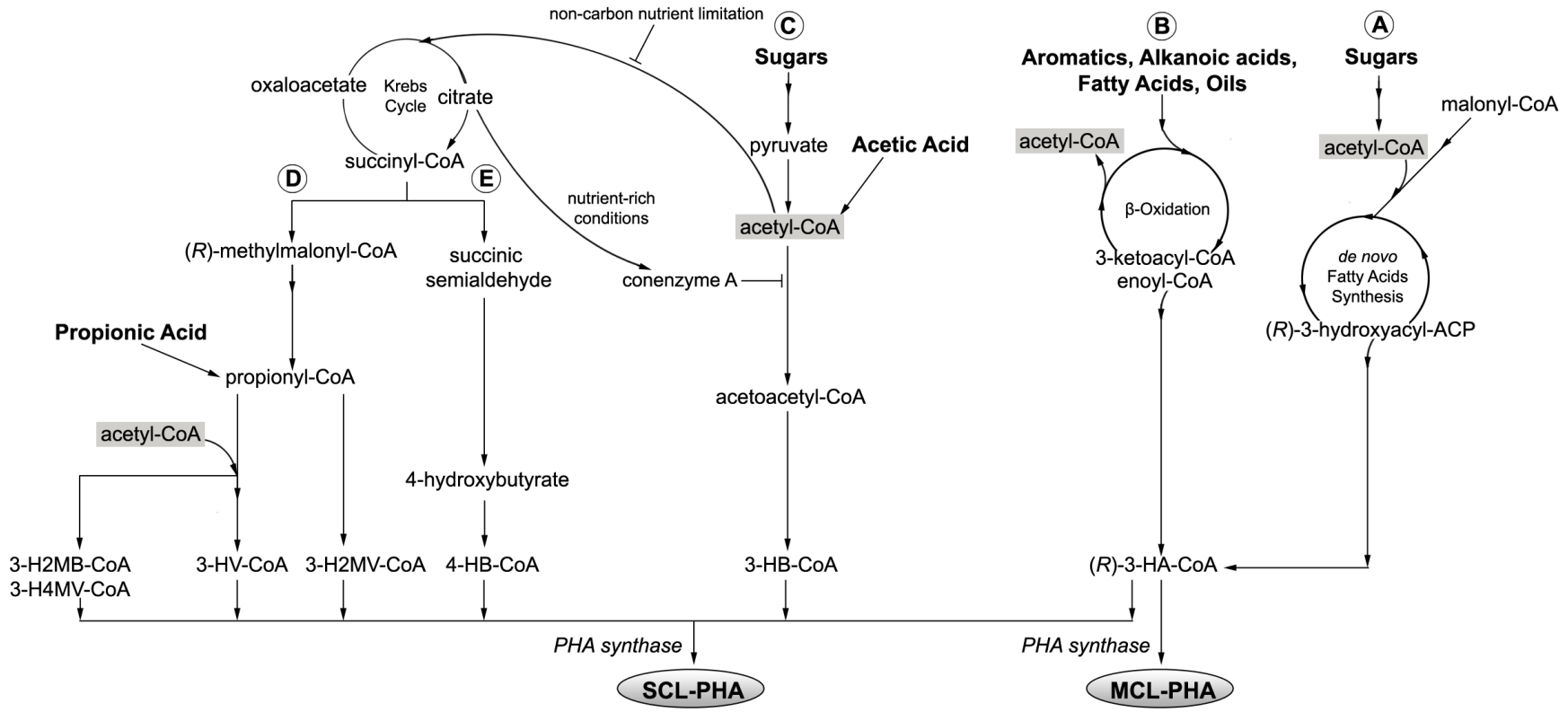


Figure 2.5 Microbial routes of PHA synthesis. The enzyme PHA synthase, which is responsible for the polymerization of PHA precurors to form SCL-PHA or MCL-PHA, is encoded by the gene *phaC* (3-H2MB-CoA, 3-hydroxy-2-methylbutyryl-CoA; 3-H4MV-CoA, 3-hydroxy-4-methylvaleryl-CoA; 3-HV-CoA, 3-hydroxyvaleryl-CoA; 3-H2MV-CoA, 3-hydroxy-2-methylvaleryl-CoA; 4-HB-CoA, 4-hydroxybutyryl-CoA; 3-HB-CoA, 3-hydroxybutyryl-CoA; (*R*)-3-HA-CoA; (*R*)-3-hydroxyacyl-CoA).

(10.56 $g.L^{-1}$) and PHA accumulation (3.36 $g.L^{-1}$) with a PHA productivity of 0.07 $g.L^{-1}.h^{-1}$ (Nikodinovic-Runic et al., 2011). While the study did not report the monomeric constituents of MCL-PHA, based on previous studies, it can be presumed that the MCL-PHA produced by *P. putida* CA-3 was likely to comprise of MCL monomers C_6, C_8, C_{10}, C_{12} and $C_{12:1}$ with a predominance of C_{12} monomers (Nikodinovic et al., 2008; Ward et al., 2006). The MCL-PHA polymer has a decomposition temperature (DT) of 265 °C, T_g of 43 °C, and T_m of 38 °C, which are polymer properties comparable to that of a typical elastomer (van der Walle et al., 2001; Ward et al., 2005). Despite its good performance, *P. putida* CA-3 is a patented bacterium and is not readily-accessible for further scientific investigation and bioprocess application.

Although less well-studied, the bioconversion of styrene to MCL-PHA, with similar monomeric composition, has also been reported in other *Pseudomonas* species *P. putida* S12, *P. putida* CA-1 and *Pseudomonas* sp. TN301. However, these strains accumulate MCL-PHA at lower cellular contents (14% CDM for *P. putida* S12, 8% CDM for *P. putida* CA-1 and 3% CDM for *Pseudomonas* sp. TN301) (Narancic et al., 2012; Tobin & O'Connor, 2005). Therefore, there is currently a lack of bacterial strains with the metabolic capacity to bioconvert styrene into MCL-PHA efficiently, which warrants the need to increase the pool of pure microbial cultures.

Furthermore, to date, only *Pseudomonas* species are found to be capable of performing this bioconversion process. From the literature, styrene degradation capabilities are found to be widespread across many bacterial genera and species (Table 2.2). The same is true for PHA accumulation capabilities (Table 2.4) (Tan et al., 2014b). Cross-referencing between styrene-degrading and PHA-accumulating bacteria reveals some overlaps between the two groups of bacteria. These genera include *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Clostridium*, *Xanthomonas* and *Alcaligenes*. Although PHA accumulation in these genera remained unreported for styrene, PHA accumulation was reported from other carbon sources such as acetate, sucrose, alkanoic acids (Tan et al., 2014b; Valappil et al., 2007; Verlinden et al., 2007). Hence, there may be other bacteria capable of

converting styrene into PHA. To address the limited availability of pure microbial cultures and to investigate if there may be other genera of styrene-degrading *cum* PHA-producing pure microbial cultures, further research is necessary.

2.3.2 Metabolic pathway for bioconversion of styrene to MCL-PHA

As established in Section 2.2.3, the bioconversion of styrene to PHA can only occur if bacteria possess styrene catabolic pathway and PHA synthesis pathway. In addition, there must be coupling between styrene catabolism and PHA anabolism for the channeling of degradation intermediates towards PHA synthesis. To date, two major pathways for aerobic styrene degradation (Section 2.1.5, Figure 2.2), three hypothetical pathways for anaerobic styrene degradation (Section 2.1.5, Figure 2.3), and five main PHA synthetic routes (Section 2.2.4, Figure 2.5) have been identified. Among the aforementioned pathways and route, there is currently only one known coupling between an aerobic styrene degradation pathway and a MCL-PHA synthesis route. This coupling has been elucidated in *P. putida* CA-3. In this bacterium, the side-chain oxidation of styrene to form phenylacetatic acid was reportedly responsible for styrene catabolism and subsequently providing MCL-PHA precursors, varying between six carbons to twelve carbons, for MCL-PHA synthesis through Route A (Figure 2.5) (O'Connor et al., 1995; O'Leary et al., 2005). This mechanism was discovered by Ward et al. (2005), who showed that chemical inhibition of enzyme PhaG, by 2-bromooctanoic acid, inhibited MCL-PHA accumulation in *P. putida* CA-3, providing the first evidence that MCL-PHA synthesis from styrene occurs via Route A. The generation of acetyl-CoA from styrene degradation intermediates was later affirmed by O'Leary et al. (2005) using random mutagenesis approach. They showed that disrupting the *β*-oxidation gene 3-hydroxyacyl-CoA dehydrogenase resulted in the disruption of phenylacetic acid consumption and downstream MCL-PHA production. This led O'Leary and co-workers (2005) to propose that phenylacetic acid, the side-chain oxidation product, undergoes *β*-oxidation to yield acetyl-CoA as the end product of styrene metabolism and acetyl-CoA molecules are in turn channeled to *de novo* fatty acids synthesis pathway (Figure 2.5, Route A) for MCL-PHA biosynthesis.

Elucidating the metabolic activities of *P. putida* CA-3 has provided much insight on the bioconversion of styrene to MCL-PHA. However, the understanding of this process is confined to only this bacterium. Besides Route A, existing literature, based on other styrene-related mono-aromatic compounds, suggests that there may be alternative couplings between styrene cataoblic pathways and PHA anabolic pathways. For an example, it has been found that the reaction steps of direct ring cleavage pathway for styrene are consistent with the peripheral degradation pathways of other related mono-aromatics namely benzene, toluene and ethylbenzene, which yield catechol, 3-methylcatechol, and 3-ethylcatechol, respectively (Tischler & Kaschabek, 2012). *P. putida* F1 could catalyze the degradation of the aforementioned aromatic compounds through these peripheral degradation pathways (Yu et al., 2001). *P. putida* F1 was later observed by Nikodinovic et al. (2008) to accumulate MCL-PHA from benzene, toluene and ethylbenzene, suggesting that the peripheral direct ring cleavage pathways were likely to have provided degradation intermediates for MCL-PHA synthesis. Therefore, it may be possible that other couplings between styrene degradation pathway and PHA biosynthetic route exist and remain to be identified. Hence, increasing the pool and diversity of styrene-degrading *cum* PHA-producing pure microbial cultures may help shed more light in this area in the future.

CHAPTER 3

Study 1:
Development of a HPLC-DAD Method for Styrene and BTEX Detection

3.1 Introduction

Styrene is the main component in wastewaters generated from PS-related industries (Nemerow & Agardy, 1998), and have been detected at aqueous concentrations as high as 2.0 mM (Aliabadi et al., 2012), which is more than 4 orders of magnitude above the guideline value prescribed by WHO (1.9×10^{-4} mM) (WHO, 2011). Improper treatment or disposal of styrene wastewaters results in environmental pollution and brings about serious health and ecological repercussions (Cushman et al., 1997; US EPA, 2010; WHO, 2011). Periodic environmental monitoring and remediation of styrene-contaminated waters is crucial for safeguarding public health and the environment. Therefore, it is imperative to have an analytical method which is suitable for the analysis of concentrated aqueous styrene.

Currently, GC is widely employed for analysis of styrene in aqueous samples (Babaee et al., 2010; Chambers et al., 2006; Fallah et al., 2010). However, GC has limitations in its application to water samples with high styrene concentrations. This is due to its incompatibility with water and the potential detector saturation with upper styrene detection limit capped at 1.9 μM (Demeestere et al., 2007; NEMI). This makes GC-based methods ill-suited for effluent monitoring or bioremediation studies where high styrene concentrations are often encountered (Fallah et al., 2010). The limitations of GC based methods could be circumvented by HPLC. Unlike GC, HPLC is well-suited for the direct analysis of aqueous-based samples and is able to accept higher compound concentrations. The first objective of this study was to develop a fast and simple HPLC method suited for the analysis of aqueous samples containing high styrene concentrations.

Styrene wastewaters may also be mixed with wastewaters exiting from other petrochemical processing plants (Ahmad et al., 2008; Fallah et al., 2010), complicating the sample matrix. Other toxic and carcinogenic mono-aromatic BTEX compounds may co-occur with styrene at high concentrations in these effluents and at similar concentrations ratio (B:T:E:X:Styrene ratio [in mM] of 9.3:4.2:1:2.5:6.6) (Ahmad et al., 2008; Greene & Voordouw, 2004). Structural similarity between mono-aromatic compounds can compromise separation

resolution on HPLC systems and particularly so if the compounds co-exist at high aqueous concentrations (AlSalka et al., 2010; Campos-Candel et al., 2009). Since styrene and BTEX compounds differ only in the functional *R* group attached to the benzene ring, BTEX can potentially interfere with styrene detection under HPLC conditions. While HPLC detection of styrene (Colin et al., 1986; Gawell & Larsson, 1980) and BTEX (AlSalka et al., 2010; Campos-Candel et al., 2009) has been described separately, there is no HPLC method for the concurrent detection of styrene and BTEX, particularly when these compounds co-occur at high concentrations. Hence, the second objective of this study was to expand the HPLC method for the simultaneous detection of BTEXS.

3.2 Materials and Methods

3.2.1 Chemicals and materials

Benzene was purchased from Sigma-Aldrich (St Louis, MO, USA) while all other chemicals used in this study were purchased from Merck (Darmstadt, Germany). All chemicals used were of purities 99.0% or greater while all solvents used in the analysis procedures were of HPLC grade.

3.2.2 Analytical standards preparation

Styrene analytical standards (0.0002, 0.002, 0.02, 0.2 and 2.0 mM) were prepared by dilution in methanol. BTEXS mixture analytical standards were prepared by first mixing the compounds together at equimolar concentrations of 1.2 M followed by dilution in methanol to achieve five standard solutions of equimolar BTEXS mixture (0.0002, 0.002, 0.02, 0.2 and 2.0 mM). The standard solutions were prepared in HPLC amber vials where the vials were completely filled with standard solution in order to eliminate any gas phase. The vials were immediately crimp-sealed using aluminum cap with Teflon-lined septa. After preparation, the standard solutions were immediately kept chilled at 15 °C prior to analysis. Five independent experiments were conducted and the mean values were used to obtain the standard calibration curves for each compound.

3.2.3 HPLC operating conditions

HPLC analysis was performed on UltiMate 3000 HPLC equipped with a DAD detector (Thermo Scientific Dionex, USA). The sample vials were kept chilled on an autosampler tray thermostatted at 15 °C. Sample injection was performed by an autosampler. The sample injection volume was varied between 1 and 20 µL. Chromatographic separation was conducted on an Acclaim Phenyl-1 reversed-phase column (4.6 x 150 mm, 3 µm) with a pre-column heater (Thermo Scientific Thermo Scientific Dionex, USA). The column oven temperature was programmed at 50 °C according to manufacturer's recommendation. The solvent and ultrapure water mobile phase compositions (%/%) were varied from 50%/50% to 70%/30% volume percent at isocratic mode. Flow rates between 0.8 and 2.0 mL.min^{-1} were also examined. A wavelength scan from 190 nm to 300 nm was performed to determine the peak UV absorbance for styrene. An overview of the parameters optimization procedure is illustrated in Figure 3.1. The optimized HPLC parameters were subsequently applied to complex matrices (ultrapure water, tap water and domestic waste water), spiked with styrene or equimolar BTEXS mixture at 0.02 mM and 2.0 mM.

3.2.4 Analytical parameters

Data collection was done by Chromeleon v6.80 software (Thermo Scientific Dionex, USA). The same software was also used for the determination of resolution and LOD/LOQ. Specifically, the resolution factor was calculated using the expression:

$$\text{Resolution} = 2(t_2 - t_1)/(W_1 - W_2) \qquad \text{Eq (3.1)}$$

where t_1 and t_2 are the retention times of two analytes and W_1 and W_2 are the respective widths of each adjacent peak at its base. The LOD and LOQ were expressed as the concentration of analyte that gives a detector signal which is 3 times and 10 times the noise level, respectively. Other analytical parameters obtained using the software included elution time, slope and R^2 coefficient. The upper limit of linearity was determined as the highest concentration tested in this work while the LOQ value was expressed as the lower limit of linearity.

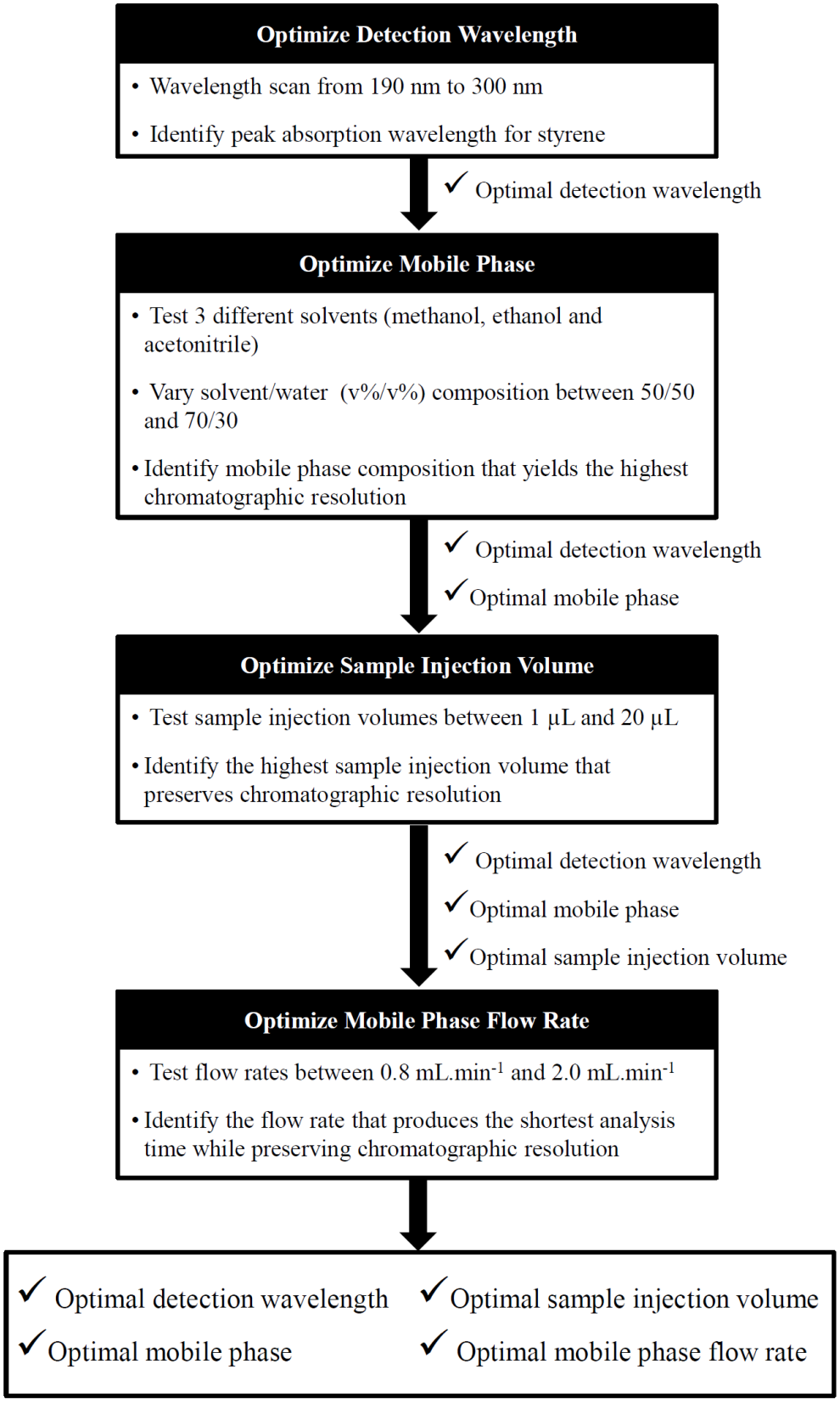


Figure 3.1 Flow diagram of the HPLC-DAD parameters optimization procedure.

Repeatability and reproducibility of the method were tested by performing injections of three independent samples on one day and injections of five independent samples on three days, respectively. To evaluate repeatability and reproducibility, the relative standard deviation (RSD) of sensitivity was used. Sensitivity was calculated using the expression:

$$\text{Sensitivity} = E/C \qquad \text{Eq (3.2)}$$

where E and C are the peak height and analyte concentration respectively.

3.3 Results

3.3.1 HPLC-DAD method development for aqueous styrene analysis

A new commercial HPLC column, Acclaim Phenyl-1 reversed-phase column, was tested for HPLC-DAD analysis of styrene at high concentrations of up to 2.0 mM. Using methanol/water as the mobile phase, styrene could be eluted from the column. The elution time of styrene was 12.5 min under 50% methanol and could be decreased by about 8 min to 4.1 min when the methanol content was increased to 70% (Figure 3.2). The wavelength scan from 190 nm to 300 nm revealed that styrene has strong UV absorption at wavelengths ranging from 190 nm to 220 nm and 230 nm to 260 nm (Figure 3.3). The maximum absorption occurred at about 201 nm, which was chosen as the detection wavelength for subsequent experiments. Under the HPLC operating conditions of 10 µL sample injection volume, detection wavelength of 201 nm, 60% methanol mobile phase, and flow rate of 1.2 $mL.min^{-1}$, the operating pressure varied between 170 and 180 bars. The LOD and LOQ for styrene were 0.0002 mM and 0.0006 mM, respectively. The linear range was 0.0006 mM to 2.0 mM with a slope of 196.40 and R^2 coefficient of 0.9992 ± 0.0538, indicating high linearity (Figure 3.4). The repeatability and reproducibility of the method for two tested styrene concentrations (0.02 mM and 2.0 mM) were within RSD values of ±10.60% (Table 3.1), which complied with US EPA quality control criteria. Aqueous samples of varying matrix complexities, ranging from the less complicated matrices of ultrapure water and tap water to the more complicated matrices of industrial wastewater and domestic wastewater, were spiked with

styrene at 0.02 mM and 2.0 mM. Method recovery values were between 86.4 ± 3.4% and 115.0 ± 3.2% (Table 3.2), which were within the quality control criteria prescribed for United States Environment Protection Agency (US EPA) methods (*i.e.*, between 80% and 120%).

3.3.2 Method optimization for concurrent analysis of aqueous BTEXS

To extend the HPLC-DAD method for simultaneous analysis of BTEXS, operation parameters such as mobile phase, sample injection volume and flow rate were determined and optimized with the aim of achieving rapid detection with high chromatographic resolution. Various solvent mobile phases were first tested for the chromatographic separation of BTEXS mixture at a high equimolar concentration of 2.0 mM. The solvent mobile phases chosen for testing were methanol, ethanol and acetonitrile, which are solvents commonly used in the HPLC detection of aromatic compounds (AlSalka et al., 2010; Chen et al., 2008; Liang et al., 2008; Tønnesen & Karlsen, 1986). The solvent/water compositions were varied between 50%/50% and 70%/30% at isocratic mode. The best separation was achieved using methanol as the mobile phase with 6 resolved peaks ascribed, in sequence of elution, to benzene, toluene, styrene, ethylbenzene, *o*-xylene, and *m*-, *p*-xylene (Figure 3.5).

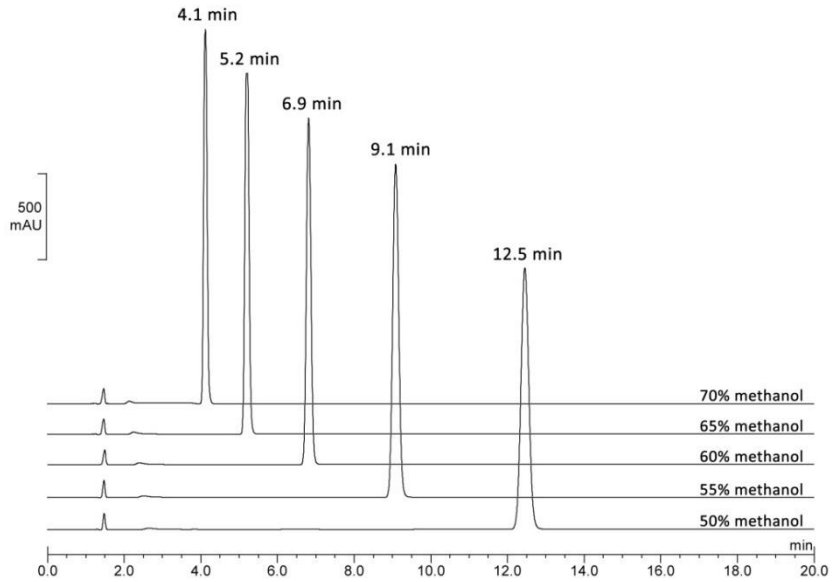


Figure 3.2 Elution profile of a 2.0 mM styrene analytical standard under mobile phases with various methanol contents. Analysis was performed using 10 µL sample injection volume at a flow rate of 1.2 mL.min $^{-1}$.

To determine the range of methanol content that produces fast separation with reasonable chromatographic resolution, methanol/water compositions between 50%/50% and 70%/30% were studied. Increasing methanol content from 50 to 65% did not change the elution order but resulted in reduced elution time for all tested compounds, shortening the overall analysis time from 16 min to 6.5 min (Figure 3.6A). The separation quality was evaluated in terms of resolution between adjacent peaks where a resolution of 1.5 or greater represents well-resolved adjacent peaks (Tijssen et al., 1998).

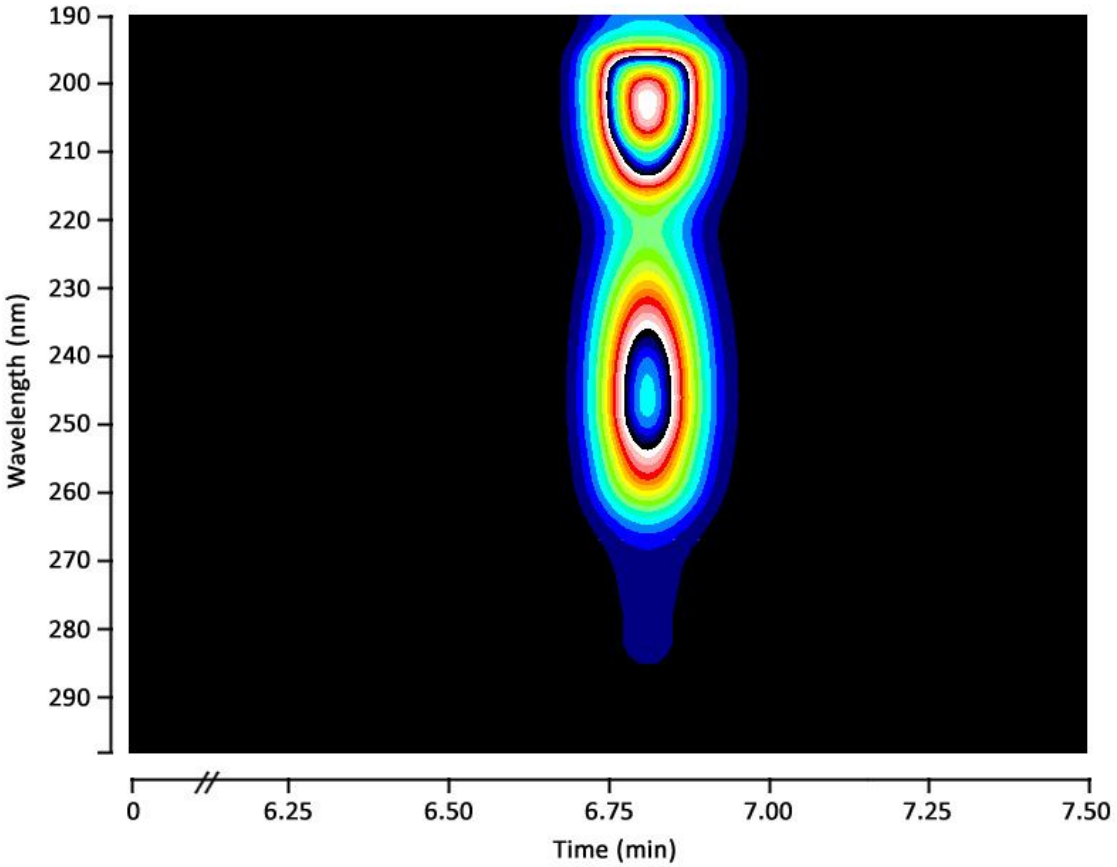


Figure 3.3 DAD profile of a 2.0 mM styrene analytical standard.

Figure 3.6B shows that 50% methanol resulted in high resolution (> 1.5) between all compounds with the exception of ethylbenzene/*o*-xylene (resolution of 1.1). 55 to 60% methanol provided high resolution of close to or greater than 1.5 for all tested compounds. When methanol content was increased beyond 60% methanol, the resolution for between all compounds remained above 1.5 with the exception of

o-xylene/*m*-, *p*-xylene (1.0 at 65% methanol and 0 at 70% methanol), causing the adjacent peaks to overlap partially or completely. Taken together, 55 to 60% methanol was regarded as most optimal for BTEXS separation. An interesting phenomenon was also observed in which the resolution for ethylbenzene/*o*-xylene increased from 1.1 to 1.8 despite a smaller elution time difference of 0.3 min at 65% methanol compared to 0.4 min at 50% methanol (Figure 3.6).

Following the optimization of mobile phase, the optimal range of injection volume was determined by varying this parameter between 1 and 20 µL. High resolutions (> 1.5) between the first four peaks (*i.e.*, benzene/toluene, toluene/styrene and styrene/ethylbenzene) were observed for all tested injection volumes (Figure 3.7). For the last three peaks (*i.e.*, ethylbenzene/*o*-xylene and *o*-xylene/*m*-, *p*-xylene) however, chromatographic resolutions close to or above 1.5 were only observed for injection volumes of up to 10 µL. As such, volume between 1 and 10 µL was considered as the optimal injection volume for concentrated BTEXS samples.

Flow rate is also a parameter known to influence analysis time and chromatographic resolution. Using 60% methanol as the mobile phase and 10 µL sample injection volume, the mobile phase flow rate was varied between 0.8 and 2.0 $mL.min^{-1}$ to determine the optimal flow rate for rapid analysis without compromising resolution. Increasing the flow rate from 0.8 to 2.0 $mL.min^{-1}$, which is the manufacturer's recommended maximum flow rate, halved the analysis time from 12 to 5.5 min (Figure 3.8A) with negligible decrease in resolution (Figure 3.8B). At a flow rate of 2.0 $mL.min^{-1}$, the elution times of benzene, toluene, styrene, ethylbenzene, *o*-xylene, and *m*-, *p*-xylene were 2.5, 3.4, 4.2, 4.6, 4.8, and 5.0 min, respectively, shortening the analysis time (Figure 3.5B).

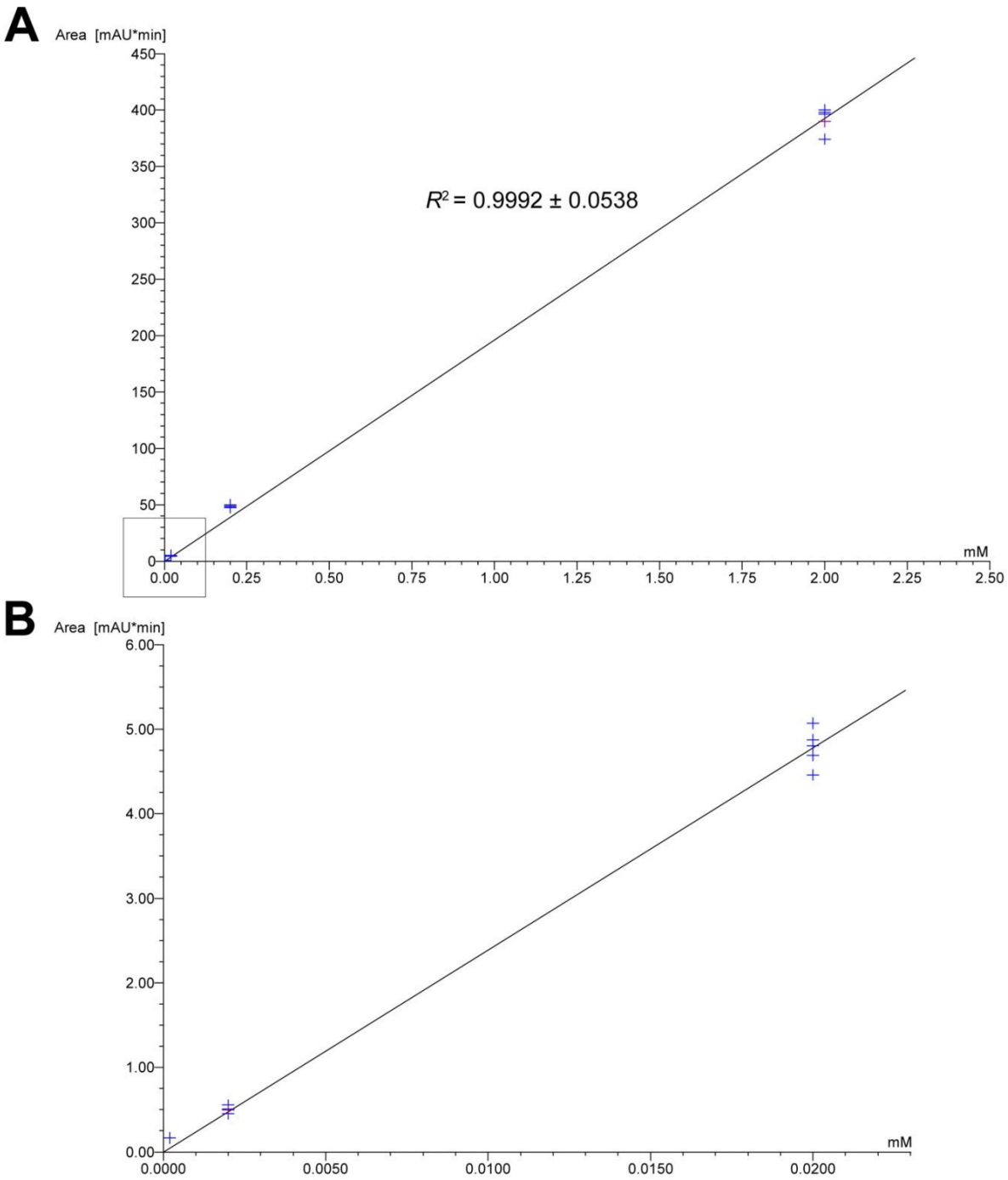


Figure 3.4 Styrene calibration curve. (A) The complete curve constructed based on styrene analytical standards of concentrations 0.0002 mM, 0.002 mM, 0.02 mM, 0.2 mM and 2.0 mM. (B) A magnified portion of the calibration curve, demarcated with a box in (A), with styrene analytical standards of concentrations 0.0002 mM, 0.002 mM and 0.02 mM.

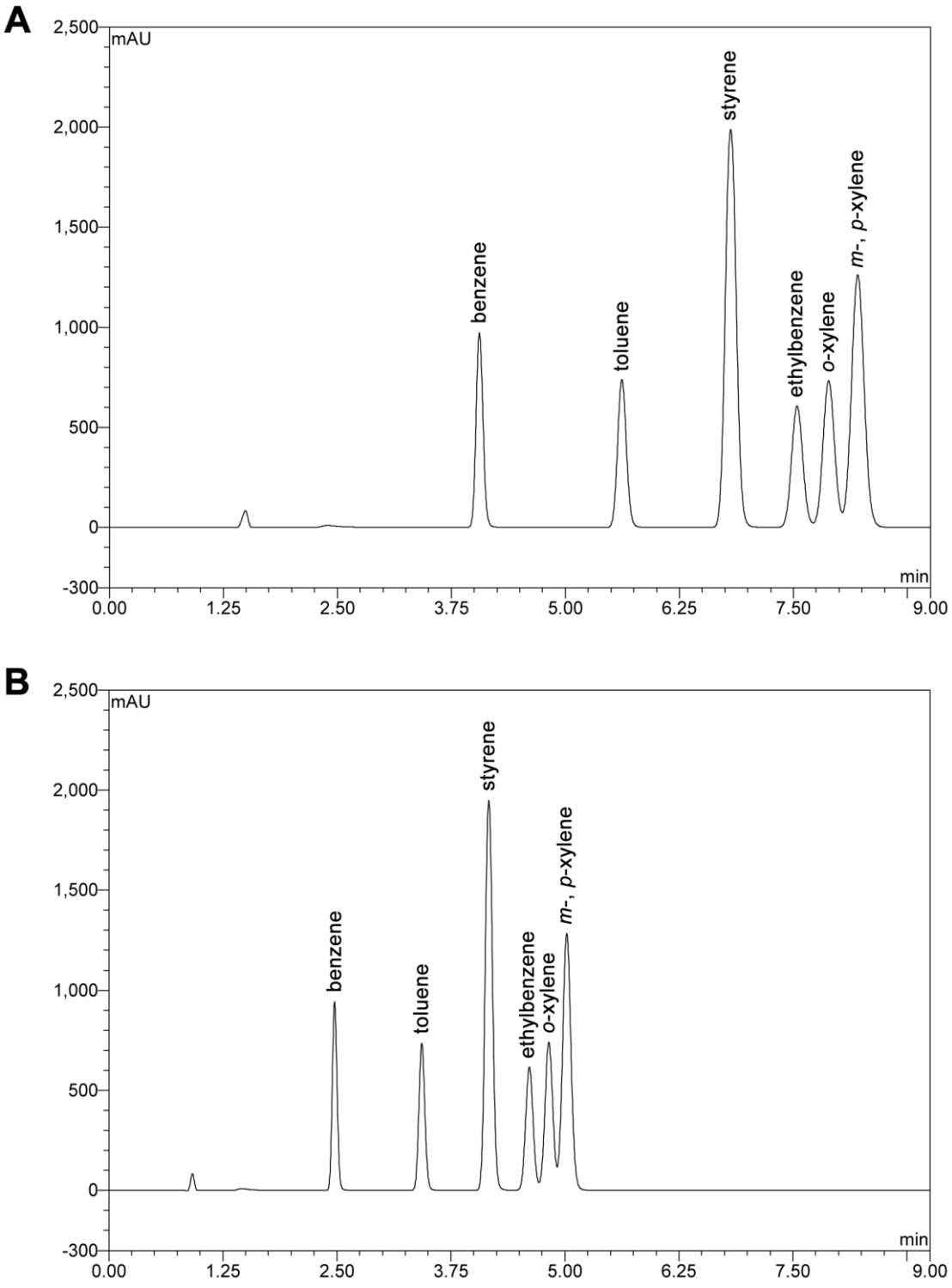


Figure 3.5 Chromatogram of a standard 2.0 mM equimolar mixture of BTEXS. Separation was conducted with 60% methanol mobile phase and 10 µL sample injection volume at a flow rate of (A) 1.2 mL.min $^{-1}$ and (B) 2.0 mL.min $^{-1}$.

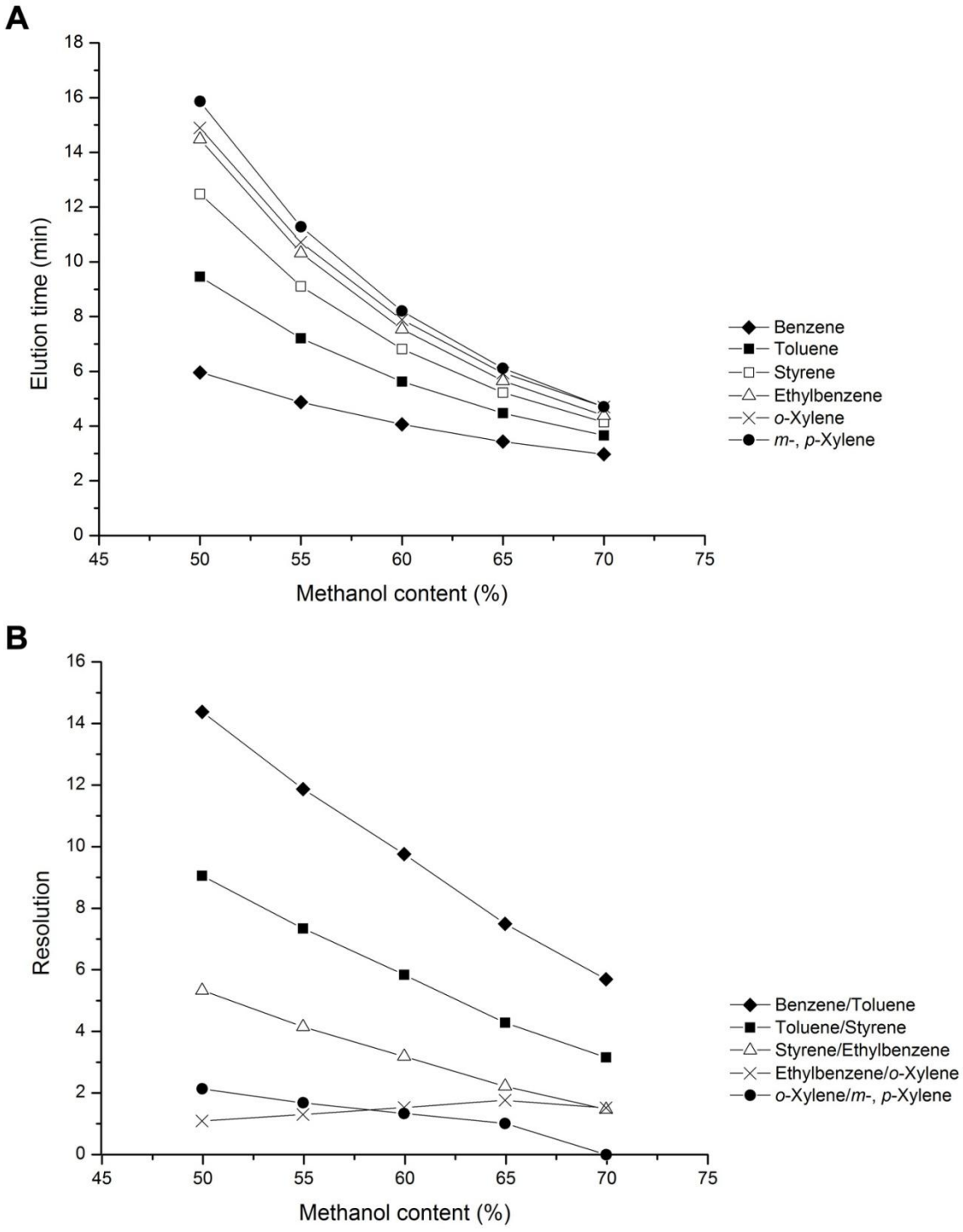


Figure 3.6 Effect of methanol content on the (A) elution time and (B) resolution for a standard 2.0 mM equimolar mixture of BTEXS. Sample injection volume and flow rate were 10 µL and 1.2 mL.min^{-1}, respectively. Results are the average of three independent experiments with standard deviations within ±0.006 and ±0.10 for elution time and resolution, respectively.

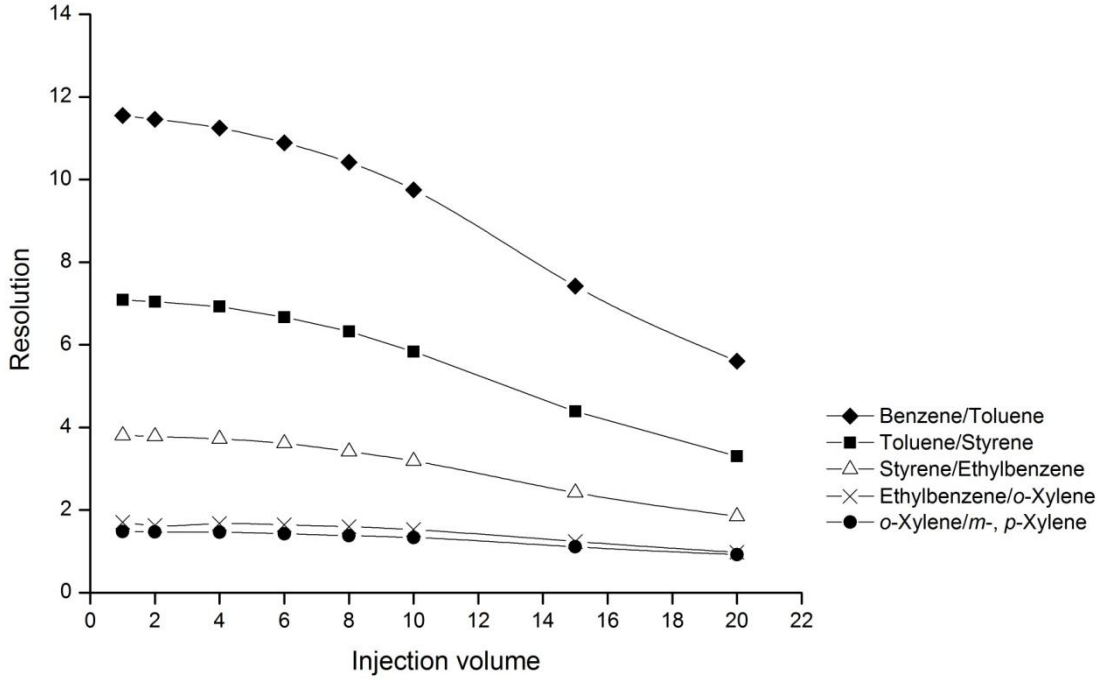


Figure 3.7 Effect of injection volume on the resolution of a standard 2.0 mM equimolar mixture of BTEXS. Separation was conducted with 60% methanol mobile phase at a flow rate of 1.2 mL.min $^{-1}$. Results are the average of three independent experiments with standard deviations within ±0.12.

3.3.3 Determination of method analytical parameters

To determine the analytical parameters for chromatographic separation of BTEXS mixture, the HPLC operating conditions were set at a mobile phase of 60% methanol; sample injection volume of 10 µL; and flow rate of 1.2 mL.min^{-1}. Under these conditions, the elution time of styrene remained unchanged (*i.e.*, 6.9 min). Benzene and toluene eluted ahead of styrene at 4.1 min and 5.7 min, respectively, while ethylbenzene, *o*-xylene, and *m*-, *p*-xylene eluted after styrene at 7.6, 8.0, and 8.3 min, respectively (Figure 3.5A and Table 3.1). In addition to styrene, based on the LOD and LOQ values, this method was also found to be suitable for the detection of trace amounts of toluene, ethylbenzene and xylenes at the lower detection limits prescribed by US EPA (US EPA, 2012). The only exception was benzene where the LOD (0.0003 mM) and LOQ (0.001 mM) values were above US EPA-prescribed limits (0.00006 mM). Apart from styrene, high linearity was also observed for BTEX with R^2 coefficients of 0.9997 ± 0.0280 or greater. The

repeatability and reproducibility of the method for two tested BTEXS equimolar concentrations (0.02 mM and 2.0 mM) was found to be within RSD values of ±13.6%, meeting US EPA quality control criteria. Method recovery values for BTEXS in different aqueous matrices of varying matrix complexities (*i.e.*, ultrapure water, tap water, industrial wastewater and domestic wastewater) were between 86.4 ± 3.4% and 115.9 ± 2.9% (Table 3.2), which fell within the quality control criteria prescribed for US EPA methods.

3.4 Discussion

Existing methods for analysis of BTEXS are focused on trace detections and typically developed on GC platforms (Almeida & Boas, 2004; Chambers et al., 2006). GC-based methods however, cannot be directly applied for aqueous samples containing high BTEXS concentrations, necessitating the need to develop new analytical methods for this type of sample matrix. This study attempted to bridge the research gap by developing a fast and simple HPLC-DAD method suitable for the analysis of aqueous samples containing high styrene concentrations as well as aqueous samples where BTEXS co-occur at high concentrations.

A newly-developed commercial HPLC column Acclaim Phenyl-1 reversed-phase column was chosen for this study's purpose as the column's stationary phase comprised of silanes bearing C_{11} alkyl aromatic moiety with a terminal electron-withdrawing group. The retention mechanism is based on a combination of hydrophobic and pi-pi interactions (Thermo Scientific Dionex, 2010). Conventional HPLC methods for styrene or BTEX analysis employ C8 column (Farhadian et al., 2009) or C18 column (AlSalka et al., 2010; Colin et al., 1986; Kelly et al., 1996; Khaksar & Ghazi-Khansari, 2009), which comprises of octyl or octadecyl carbon chain-bonded silica, respectively, and provide chromatographic selectivity through hydrophobic interactions. Compared to C18 column, the additional chromatographic selectivity of Acclaim Phenyl-1 column based on *pi-pi* interactions was anticipated to provide enhanced chromatographic separation for aromatic compounds such as BTEXS.

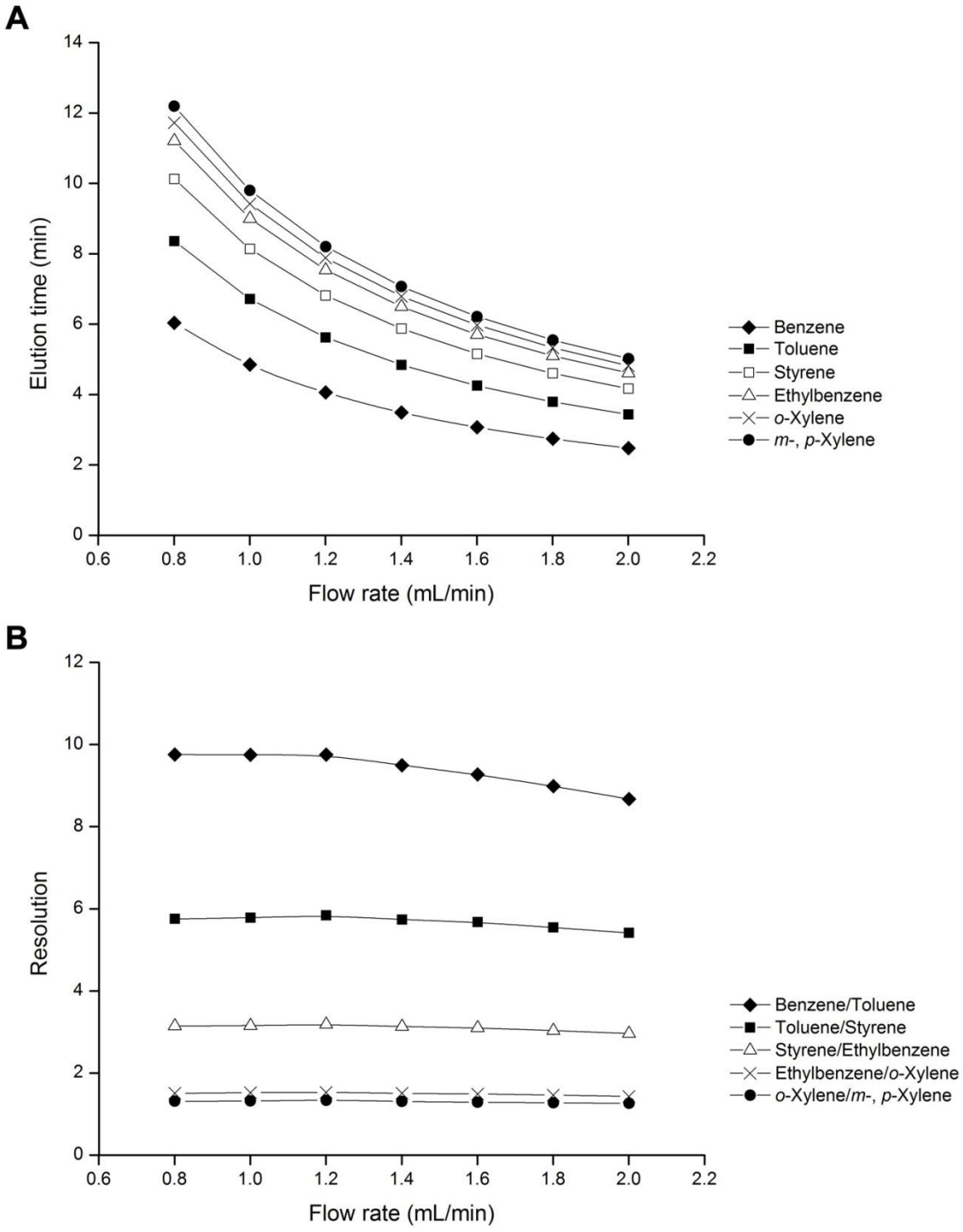


Figure 3.8 Effect of flow rate on the (A) elution time and (B) resolution of a standard 2.0 mM equimolar mixture of BTEXS. Separation was conducted with 60% methanol mobile phase and a sample injection volume of 10 µL. Results are the average of three independent experiments with standard deviations within ±0.006 and ±0.09 for elution time and resolution, respectively.

Table 3.1 Analytical parameters of the developed method for BTEXS determination.

Compound	Elution Time (min)	Linear Range (mM)	Slope	R^2 Coefficient ± RSD[1]	LOD (mM)	LOQ (mM)	Concentration (mM)	Repeatability (RSD%)[2]	Reproducibility (RSD%)[3]
Benzene	4.1	0.001 to 2	63.09	0.9999 ± 0.0143	0.0003	0.001	0.02	7.27	6.30
							2	0.75	1.61
Toluene	5.7	0.001 to 2	67.56	0.9999 ± 0.0071	0.0004	0.001	0.02	6.76	5.10
							2	1.94	2.56
Styrene	6.9	0.0006 to 2	196.40	0.9992 ± 0.0538	0.0002	0.0006	0.02	6.47	4.63
							2	10.60	8.51
Ethylbenzene	7.6	0.002 to 2	65.75	0.9999 ± 0.0060	0.0005	0.002	0.02	6.34	4.62
							2	0.56	2.36
o-Xylene	8.0	0.001 to 2	65.02	0.9999 ± 0.0091	0.0004	0.001	0.02	6.30	13.62
							2	11.27	8.87
m-, *p*-Xylene	8.3	0.0008 to 2	146.58	0.9997 ± 0.0280	0.0002	0.0008	0.02	6.47	4.69
							2	5.62	4.63

[1] Tabulated based on results from five independent samples determination
[2] Tabulated based on response factors from injections of three independent samples performed on 1 day
[3] Tabulated based on response factors from injections of five independent samples performed on 3 days

Table 3.2 Method recovery in different types of aqueous matrices (n = 3).

Compound	Concentration (mM)	Recovery (%) ± s.d.[1]			
		Ultrapure Water	Tap Water	Industrial Wastewater	Domestic Wastewater
Benzene	0.02	111.9 ± 6.2	111.3 ± 3.1	112.4 ± 8.2	114.3 ± 7.9
	2.0	94.3 ± 5.1	95.3 ± 4.5	95.1 ± 3.3	96.3 ± 2.3
Toluene	0.02	109.4 ± 8.5	109.3 ± 4.4	109.7 ± 10.5	108.7 ± 9.2
	2.0	93.7 ± 5.1	94.0 ± 4.6	94.3 ± 3.7	95.1 ± 3.2
Styrene	0.02	112.2 ± 5.5	113.0 ± 6.2	112.5 ± 2.7	115.0 ± 3.2
	2.0	86.7 ± 4.6	86.8 ± 4.0	86.4 ± 3.4	87.4 ± 3.0
Ethylbenzene	0.02	111.3 ± 7.6	110.2 ± 7.6	111.3 ± 7.3	112.3 ± 8.9
	2.0	98.6 ± 4.0	98.7 ± 3.6	99.5 ± 2.7	99.9 ± 2.6
o-Xylene	0.02	115.9 ± 2.9	109.2 ± 10.3	111.9 ± 7.0	111.4 ± 7.7
	2.0	110.6 ± 3.9	110.5 ± 3.6	112.1 ± 2.9	111.6 ± 2.4
m-, *p*-Xylene	0.02	113.5 ± 8.9	109.6 ± 10.1	110.2 ± 8.3	111.2 ± 9.4
	2.0	92.1 ± 4.5	92.2 ± 4.2	92.7 ± 3.5	93.1 ± 2.6

[1] s.d. refers to standard deviation

Styrene could be eluted from the Acclaim Phenyl-1 column using methanol/water as the mobile phase. At a flow rate of 1.2 $mL.min^{-1}$, styrene could be rapidly eluted at 4.1 min when 70% methanol was used as the mobile phase (Figure 3.2), which was comparable to previous reports citing elution times of between 3.2 min and 11.6 min (Colin et al., 1986; Ekins et al., 1996; Flanjak & Sharrad, 1984; Gawell & Larsson, 1980; Khaksar & Ghazi-Khansari, 2009; Marconi et al., 1996; Tawfik & Huyghebaert, 1998). While this finding did not represent the shortest analysis time for styrene, the analysis time is expected to be further shorten by increasing the methanol content in the mobile phase or increasing flow rate although further experiments would be needed for verification. Using a DAD detector, styrene was found to show strong UV absorption at wavelengths ranging from 190 nm to 220 nm and 230 nm to 260 nm (Figure 3.3), which was in good agreement with previous reports of styrene HPLC detection at 210 nm (Ekins et al., 1996), 245 nm (Colin et al., 1986; Flanjak & Sharrad, 1984; Khaksar & Ghazi-Khansari, 2009; Tawfik & Huyghebaert, 1998) and 254 nm (Gawell & Larsson, 1980; Marconi et al., 1996). Within the range of 190 nm to 220 nm, peak UV absorption occurred at about 201 nm and this wavelength was subsequently chosen as the detection wavelength. The Acclaim Phenyl-1 column was found to be suitable for the analysis of styrene at high aqueous concentration of up to 2.0 mM, which was close to the maximum solubility of styrene under ambient temperature. Compared to existing HPLC-based (Colin et al., 1986; Khaksar & Ghazi-Khansari, 2009) and GC-based methods (Chambers et al., 2006; Gilbert-López et al., 2010; NEMI), the upper detection limit of this method was by far one of the highest to date. There was also high linearity (R^2 coefficient of 0.9992 ± 0.0538) over a large concentration range from 0.0006 mM to 2.0 mM (Figure 3.4). This meant that apart from analyzing styrene at high concentrations, the method is also suitable for the detection of trace levels of styrene at the lower detection limits prescribed by US EPA (US EPA, 2012). Repeatability and reproducibility values (RSD of ±10.60%), and method recovery values (between 86.4 ± 3.4% and 115.0 ± 3.2%) in various aqueous matrices (*i.e.*, ultrapure water, tap water, industrial wastewater and domestic wastewater) were all within the quality control criteria prescribed for US EPA methods.

The styrene analytical method was further expanded for the concurrent analysis of BTEX as these mono-aromatic compounds commonly occur alongside styrene at high concentrations, particularly in petrochemical wastewaters (Ahmad et al., 2008; Greene & Voordouw, 2004). Among the various solvents (*i.e.*, methanol, ethanol and acetonitrile) and solvent/water composition tested, methanol/water achieved the best chromatographic separation for BTEXS mixture at a high equimolar concentration of 2.0 mM. Six resolved peaks (*i.e.*, benzene, toluene, styrene, ethylbenzene, *o*-xylene, and *m*-, *p*-xylene) were obtained for 7 compounds when methanol content between 50% and 65% were used (Figure 3.5). Increasing methanol content from 50% to 65% led to a decrease in the overall analysis time from 16 min to 6.5 min (Figure 3.6A). A reasonable explanation could be that higher methanol content created a more hydrophobic mobile phase environment, favoring the dissolution of BTEXS. Since hydrophobic interactions are partially responsible for column retention of BTEXS, a reduction in these interactions would hasten the elution of aromatic analytes. The effect was most prominent for compounds with greater hydrophobic character such as toluene, styrene, ethylbenzene and xylene isomers which has octanol–water partition coefficient values (logPOW) between 2.65 and 3.20 (Farhadian et al., 2009; Tischler & Kaschabek, 2012). The reduction in elution time for the aforementioned compounds was between 6 and 11 min (Figure 3.6A). In contrast, the least hydrophobic benzene (logPOW of 2.13) registered a modest 3 min reduction in elution time. Additionally, higher methanol content in the mobile phase also led to a decrease in resolution between most compounds. The only exception was ethylbenzene/*o*-xylene where the resolution increased from 1.1 (50 % methanol) to 1.8 (65% methanol) and 1.5 (70% methanol) (Figure 3.6B). This outlier was most likely attributed to the changes in *pi-pi* selectivity of the stationary phase aromatic group in response to the dielectric constant of the mobile phase (Carey, 2003; Vitha & Carr, 2006). Since mobile phase containing between 55 and 60% methanol resulted in peak resolutions of close to or greater than 1.5, it was considered as most optimal for BTEXS separation. Similar to most chromatography methods (Almeida & Boas, 2004; AlSalka et al., 2010; Shim & Yang, 1999; Shin & Kwon, 2000), the current method was also unable to resolve positional isomers *m*-xylene and *p*-xylene peaks under

the tested conditions. Nevertheless, to our knowledge, this study provided the first reference to demonstrate the feasibility of chromatographic separation of BTEXS at high concentrations.

Under Acclaim Phenyl-1 column, injection volumes of between 1 µL and 10 µL produced resolutions close to or above 1.5 and were considered as the optimal injection volume for concentrated BTEXS samples (Figure 3.7). Beyond 10 µL, resolutions for ehtylbenzene/*o*-xylene and *o*-xylene/*m*-, *p*-xylene deteriorated to values of 1.0 and 0.9 respectively at 20 µL injection volume, producing notable overlap between peaks (data not shown). High sample injection volumes of 50 µL to 100 µL were frequently reported for BTEX separation on C8/C18 columns (AlSalka et al., 2010; Farhadian et al., 2009; Kelly et al., 1996). However, such high sample injection volumes were found to be detrimental to the Acclaim Phenyl-1 column. Column overloading and appearance of residual peaks were observed at injection volumes greater than 50 µL (data not shown). Although attempts to remove residual peaks by flushing the column with 100% methanol were successful, the column could not be restored to its former resolving ability. Hence, overloading the column, particularly with concentrated BTEXS samples, is not recommended.

Increasing flow rate reduced analysis time with negligible effect on resolution (Figure 3.8). Complete BTEXS analysis could be achieved in as fast as under 5.5 min using a flow rate of 2.0 $mL.min^{-1}$ (Figure 3.5B). Compared to existing HPLC methods, which usually have an analysis time of 10 to 15 min (AlSalka et al., 2010; Campos-Candel et al., 2009; Farhadian et al., 2009; Kelly et al., 1996), the proposed method provided time-savings between 4 and 9.5 min per sample run (*i.e.*, up to 60% decrease in analysis time), leading to higher analysis throughput. This makes the proposed method one of the fastest analytical methods reported to date and is advantageous under circumstances where analysis of large number of samples are required. Furthermore, the amount of solvent required per analysis was about 25% lesser compared to existing methods, generating cost-savings from both solvent usage and disposal.

Under the optimal HPLC operating parameters (mobile phase, 60% methanol; injection volume, 10 µL; flow rate, 1.2 mL.min^{-1}; detection wavelength, 201 nm), a wide linear range with high linearity (R^2 coefficients > 0.9997 ± 0.0280) was also observed for BTEX compounds (Table 3.1). The LOQ values of toluene, ethylbenzene, *o*-xylene, and *m*-/*p*-xylene were 0.001 mM, 0.002 mM, 0.001 mM, and 0.0008 mM, respectively, which made the method suitable for trace detection of these aforementioned compounds at the lower detection limits prescribed by US EPA (US EPA, 2012). However, the method was not sensitive enough for trace analysis of benzene and GC-based methods would be required. Repeatability and reproducibility values were within RSD values of ±13.6% while method recovery values ranged from 86.4 ± 3.4% to 115.9 ± 2.9% (Table 3.2), which met the quality control criteria prescribed for US EPA methods. A summary of the optimal protocol for HPLC-DAD analysis of styrene and BTEXS mixture is provided in Table 3.3. Taken together, this method is well-suited for direct aqueous sample analysis without the need for dilution, and can be employed as an alternative method or complement existing GC/HPLC methods for the routine monitoring of highly-polluted styrene and BTEXS-contaminated industrial effluents.

Table 3.3 Optimized protocol for HPLC-DAD analysis of styrene and BTEXS.

Parameter	Styrene	BTEXS
Column	Acclaim Phenyl-1 reversed-phase column (4.6 x 150 mm, 3 µm)	
Column oven temperature	50 °C	
Autosampler temperature	15 °C	
Detector	DAD	
Detection wavelength	201 nm	
Mobile phase solvent	Methanol	
Solvent/water (%/%) mobile phase composition	70/30	55/45 to 60/40
Sample injection volume	Up to 20 µL	Up to 10 µL
Flow rate	Up to 2.0 mL.min^{-1}	
Analysis time	4.5 min	5.5 min

3.5 Summary

A simple, rapid and reliable HPLC-DAD detection method for the direct analysis of aqueous samples containing high styrene concentration or high BTEXS concentrations was developed. The analysis of a single styrene compound could be completed in under 4.5 min using a 70%/30% methanol/water mobile phase and flow rate of 1.2 $mL.min^{-1}$. For the analysis of BTEXS mixture, the optimal HPLC operation parameters, which provided high resolution of close to or greater than 1.5 for adjacent analyte peaks, were determined to be 55 to 60% methanol for mobile phase, a maximum of 10 µL for sample injection volume, up to 2.0 $mL.min^{-1}$ for flow rate, and a detection wavelength of 201 nm. Under the optimal conditions, chromatographic separation of concentrated BTEXS samples was completed within 5.5 min. To our knowledge, the method is also the first to demonstrate the feasibility of chromatographic separation of styrene together with BTEX, expanding the range of aromatic compounds detected in a single run. The method is versatile in its applicability for trace analysis of styrene, toluene, ethylbenzene and xylenes as well as direct analysis of BTEXS at high aqueous concentrations. Several advantages of the proposed method include protocol simplification, high analysis throughput, minimal sample loss, less solvent waste generation and lower price of analysis. These key developments are immensely important and beneficial for both field engineers and researchers alike, who have to perform routine monitoring or handle a large number of samples.

CHAPTER 4

Study 2:
Development of a GC-MS Method for PHA Detection

4.1 Introduction

PHA is a family of biopolyesters consisting of (*R*)-3-hydroxyalkanoic acids repeat units of varying carbon lengths (Verlinden et al., 2007). Depending on the functional *R* group, PHA may be classified as SCL-PHA (3 to 5 carbon atoms), MCL-PHA (6 to 14 carbon atoms), LCL-PHA (15 or more carbon atoms) (Khanna & Srivastava, 2005). The chemical heterogeneity of PHA monomers has made the task of detecting and quantifying PHA a laborious and difficult one.

GC-FID is one of the most commonly-used methods (Furrer et al., 2007; Wang et al., 2006). However, the robustness of GC-FID to identify and quantify PHA is greatly dependent on the selection and inclusion of appropriate PHA analytical standards. On the other hand, GC-MS enables putative PHAs to be identified through the comparison of mass spectra pattern against the National Institute of Standards and Technology (NIST) Standard Reference Library, which makes it more robust in the detection of new putative PHAs (Lee & Choi, 1995; Lütke-Eversloh et al., 2001). Nevertheless, GC-MS can only provide a tentative identification of PHA. Further validation using suitable PHA analytical standards is pivotal in ensuring the accuracy of the detection method.

Currently, the lack of readily- or commercially-available PHA analytical standards to represent the chemical diversity of PHA monomers has made its analysis particularly challenging (Escapa et al., 2011; Furrer et al., 2007). Existing literary descriptions of GC-MS method are mostly confined to commercially-available PHA standards (Oehmen et al., 2005; Werker et al., 2008). Some of the ways to circumvent this problem include the chemical synthesis of PHA analytical standards or deriving analytical standards through the biosynthesis of PHA by well-characterized PHA accumulators (Sun et al., 2009); using commercially-available PHA analytical standards to quantify chemically-similar but commercially-unavailable PHA monomers (Oehmen et al., 2005). Furthermore, for each analyte, system calibration typically requires at least 3 calibration points with 3 to 5 independent calibration standard samples for each calibration point. This represents significant calibration workload and calibration cost. A combination of GC-MS (for

tentative identification of PHA monomers) and GC-FID (for the routine quantification of PHA monomers identified by GC-MS) (Ward et al., 2006) can keep calibration workload manageable and calibration cost low. However, these aforementioned approaches may lead to errors in measurements, and can be a tedious, time-consuming and expensive process. This warrants a need to develop a GC-MS calibration method which minimizes calibration workload and cost while enabling the analysis of a wide range of PHA monomers with a limited inventory of PHA analytical standards.

GC-MS calibration for hydrocarbon compounds such as PHA is typically performed through calculating a response factor (RF) for each PHA analytical standard while the identification of PHA is done by comparing the retention times (RTs) of the putative PHAs against the RTs of analytical standards (Lee & Choi, 1995; Werker et al., 2008). Previous studies have reported correlations between the carbon number (*i.e.,* molecular weight) of homologous hydrocarbon series and RF (Jorgensen et al., 1990; Tong et al., 1985), as well as between carbon number and retention indices (Castello, 1999). These correlations can help to estimate the RF and RT of other hydrocarbon homologues for which analytical standards are unavailable. This study postulated that such correlations may also exist between the carbon number and RF/RT for homologous PHAs. Based on this postulation, the objective of this study was to develop a simple GC-MS calibration method that enables reliable qualitative and quantitative analysis of PHAs in the absence of reference standards using homologous saturated PHA monomers 3-hydroxyalkanoic acids between carbon number 4 and 16.

4.2 Materials and Methods

4.2.1 PHA monomers, PHA polymers and chemicals

All PHA monomer analytical standards and PHA polymers were procured from Sigma-Aldrich (St Louis, MO, USA). The PHA monomer standards included 3-hydroxybutyric acid (C_4; Cat. no. 54920), 3-hydroxyoctanoic acid (C_8; Cat. no. H3898), 3- hydroxydecanoic acid (C_{10}; Cat. no. H3648), 3-hydroxydodecanoic acid (C_{12}; Cat. no. H3398) and 3-hydroxyhexadecanoic acid (C_{16}; Cat. no. H4398). The

PHA polymers included poly(3-hydroxybutyric acid) (P3HB; Cat. no. 363502), poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) containing between 11 and 13 mol% of C_5 monomeric unit (P3HB3HV; Cat. no. 403121), and a MCL-PHA polymer with composition verified by proton nuclear magnetic resonance (H^1-NMR) and carbon-13 nuclear magnetic resonance (C^{13}-NMR) as C_{10} monomeric repeats (Figure A1). The internal analytical standard methyl benzoate was procured from Merck (Darmstadt, Germany). Chemicals used for the methanolysis reaction included sulphuric acid (Merck, Darmstadt, Germany), LiChrosolv® grade methanol (Merck, Darmstadt, Germany) GC grade chloroform (Sigma-Aldrich, St Louis, MO, USA), and anhydrous sodium sulfate (Alfa Aesar, Ward Hill, MA).

4.2.2 PHA analytical standard preparation

PHA monomer analytical standards were subjected to acidic methanolysis to yield their constituent 3-hydroxyalkanoic acid methyl esters according to the procedure adapted from Oehmen et al. (2005) and Wang et al. (2010). Briefly, 5 mg (1250 $mg.L^{-1}$) of PHA monomer standard was dissolved in the methanolysis solution comprising of 2 mL of chloroform and 2 mL of acidified methanol (15% [v/v] H_2SO_4). Stock solution of methyl benzoate was prepared by dilution in methanol and added to the methanolysis mixture at a final concentration of 5 $mg.L^{-1}$ as an internal standard. The methanolysis reaction was performed in 9 mL pyrex test tubes tightly-capped with Teflon-lined screw caps and incubated at 100 °C for 3 h on a dry-block heater (Grant Instruments, Cambridge, UK). The reaction mixture was subsequently cooled to room temperature. Ultrapure water (1 mL) was added and the mixture was vortex vigorously for 1 to 2 min to remove particulate debris from the chloroform phase. The mixture was left to stand for 1 h to allow phase separation. Using a glass pipette, the top layer (water) was removed. The bottom layer (chloroform) was dried with 0.5 to 1 g of anhydrous sodium sulphate before transferring into amber GC vials and crimp-sealed with aluminum cap with Teflon-lined septa.

4.2.3 GC-MS analysis

Methanolysed sample (1 μL) was injected at a split ratio of 50:1 into a HP6890 GC

Series equipped with the 5975I MS detector (Agilent Technologies, Palo Alto, Calif). Chromatographic analysis was achieved on a HP-5MS capillary column (30 m by 0.25 mm, 0.25-µm film thickness; Agilent Technologies, Palo Alto, Calif). The temperatures of the injection port, interface, quadrupole and ion source was set at 250, 280, 120 and 250 °C, respectively. Oven temperature was programmed at an initial temperature of 40 °C and subsequently raised with a rate of 10 °C.min^{-1} to 280 °C and held for 5 min. Helium was used as a carrier gas at a flow rate of 1.2 mL.min^{-1}. Solvent delay was set at 2.5 min. The mass spectrometer setup was operated in electron impact (EI) mode at 70 eV, full scans (mass range of *m/z* 40 to 600 with 0.1 mass accuracy).

4.2.4 Data collection and analysis

Data collection was performed by HPChem Station software (Hewlett-Packard, Palo Alto, Calif). The observed response factor for each PHA monomer analytical standard was calculated using the expression:

$$\text{Response factor, RF} = (A_A \times C_i)/(A_i \times C_A) \qquad \text{Eq (4.1)}$$

where is A_A the sum of peak areas of the four main fragment ions of the PHA analytical standard (*i.e.*, *m/z* 43, 71, 74 and 103 ions), A_i is the peak area of the characteristic *m/z* 105 ion of the methyl benzoate internal standard, and C_A and C_i are the concentrations of the PHA analytical standard and methyl benzoate internal standard, respectively.

The relationships between the carbon number of PHA analytical standards and their respective RTs as well as their respective RFs were analyzed using OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA, USA).

4.2.5 Method recovery

The method recovery for PHA quantification was determined by subjecting known amounts of PHA monomers and PHA polymers to acidic methanolysis followed by GC-MS analysis. The concentration of PHA monomers (C_4/C_8/C_{10}/C_{12}/C_{16}) used

was 5 mg (*i.e.*, 1250 mg.L^{-1}) while the concentration of PHA polymers used was 5.1 mg (*i.e.*,1275 mg.L^{-1}) for PHB, between 27.7 mg and 31.1 mg (*i.e.*,6925 and 7775 mg.L^{-1}) for PHBHV pellets, and between 5 mg and 7.7 mg (*i.e.*,1250 and 1925 mg.L^{-1}) for MCL-PHA. Methyl benzoate (5 mg.L^{-1}) was included as an internal standard. Recovery was calculated as a percentage of deviation of the measured values from the actual values using the following expression:

$$\text{Method recovery (\%)} = [(W_m - W_A)/(W_A)] \ 100\% \qquad \text{Eq (4.2)}$$

where W_m and W_A are the calculated weight and actual weight of the PHAs, respectively.

4.3 Results

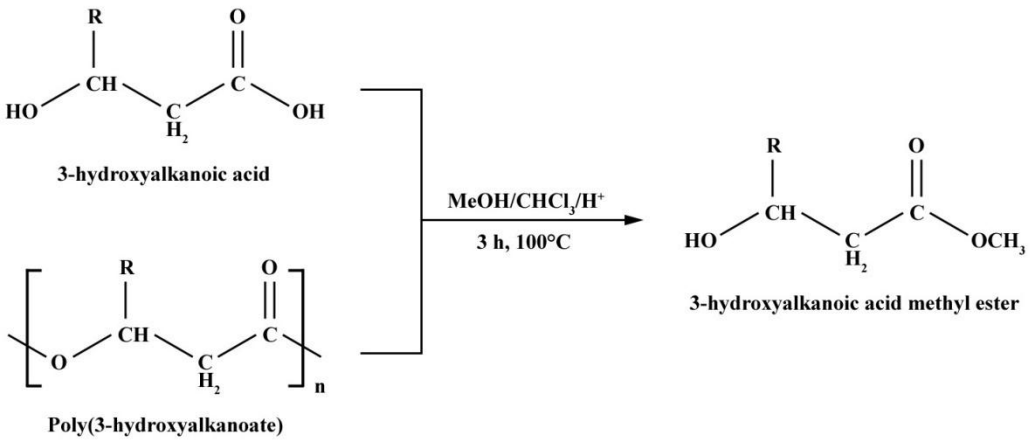
4.3.1 GC-MS detection of PHA monomers

Homologous saturated PHA monomers with alkyl *R* groups, 3-hydroxyalkanoic acids, were chosen for this study due to the commercial-availability of these analytical standards, providing a good starting point for testing this study's postulation. 3-hydroxyalkanoic acids of varying carbon number ranging from SCL-PHA monomer (C_4), to MCL-PHA monomers (C_8, C_{10}, C_{12}) and LCL-PHA monomer (C_{16}) were subjected to methanolysis and GC-MS analysis. The methanolysis reaction chemically converted 3-hydroxyalkanoic acids to their respective 3-hydroxyalkanoic acid methyl esters (Figure 4.1). Under the GC-MS conditions, the observed RTs of the different 3-hydroxyalkanoic acid methyl esters were as follow: C_4, 4.69 min; C_8, 10.88 min; C_{10}, 13.67 min; C_{12}, 16.09 min; and C_{16}, 20.28 min (Figure 4.2 and Table 4.1). An increase in the carbon number of PHA monomer was correlated to an increase in RT. Internal standard methyl benzoate was detected at 8.53 min. The mass spectra of the 3-hydroxyalkanoic acid methyl esters were characteristic of fragmentation patterns previously reported (de Rijk et al., 2005), with a fragment ion at *m*/*z* 103, formed by an α cleaveage to the hydroxyl functional group; at *m*/*z* 74, arising from McLafferty rearrangement; at *m*/*z* 71, possibly from the expulsion of methanol from *m*/*z* 103; and at *m*/*z* 43, attributed to either the saturated alkanoic portion or methyl ester moiety of the

molecule (Figure 4.3). The observed RFs for the various 3-hydroxyalkanoic acid methyl esters were as follow: C_4, 0.302 ± 0.023; C_8, 1.176 ± 0.375; C_{10}, 1.716 ± 0.322; C_{12}, 2.254 ± 0.323; and C_{16}, 3.078 ± 0.805 (Table 4.1).

4.3.2 Relationship between PHA carbon number and retention time (RT) or response factor (RF)

PHA carbon number was observed to correlate positively with both observed RTs and observed RFs (Figure 4.4). A linear relationship was found between the carbon number of PHA monomer standards and observed RTs (Eq 4.3) with an adjusted coefficient of determination (R^2) of 0.987 (Figure 4.4A). Similarly, a linear relationship was observed between PHA carbon number and observed RFs (Eq 4.4)



R group	Carbon no.	PHA monomer	PHA polymer
methyl	C_4	3-hydroxybutyric acid	Poly(3-hydroxybutyrate)
ethyl	C_5	3-hydroxyvaleric acid	Poly(3-hydroxyvalerate)
propyl	C_6	3-hydroxyhexanoic acid	Poly(3-hydroxyhexanoate)
butyl	C_7	3-hydroxyheptanoic acid	Poly(3-hydroxyheptanoate)
pentyl	C_8	3-hydroxyoctanoic acid	Poly(3-hydroxyoctanoate)
hexyl	C_9	3-hydroxynonanoic acid	Poly(3-hydroxynonanoate)
heptyl	C_{10}	3-hydroxydecanoic acid	Poly(3-hydroxydecanoate)
octyl	C_{11}	3-hydroxyundecanoic acid	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	3-hydroxydodecanoic acid	Poly(3-hydroxydodecanoate)
undecyl	C_{14}	3-hydroxytetradecanoic acid	Poly(3-hydroxytetradecanoate)
tridecyl	C_{16}	3-hydroxyhexadecanoic acid	Poly(3-hydroxyhexadecanoate)

Figure 4.1 Methanolysis reaction for PHA monomer 3-hydroxyalkanoic acid and PHA polymer poly(3-hydroxyalkanoate). The nomenclature and carbon number for the homologous series of saturated PHA monomers and polymers is determined by the functional alkyl *R* group.

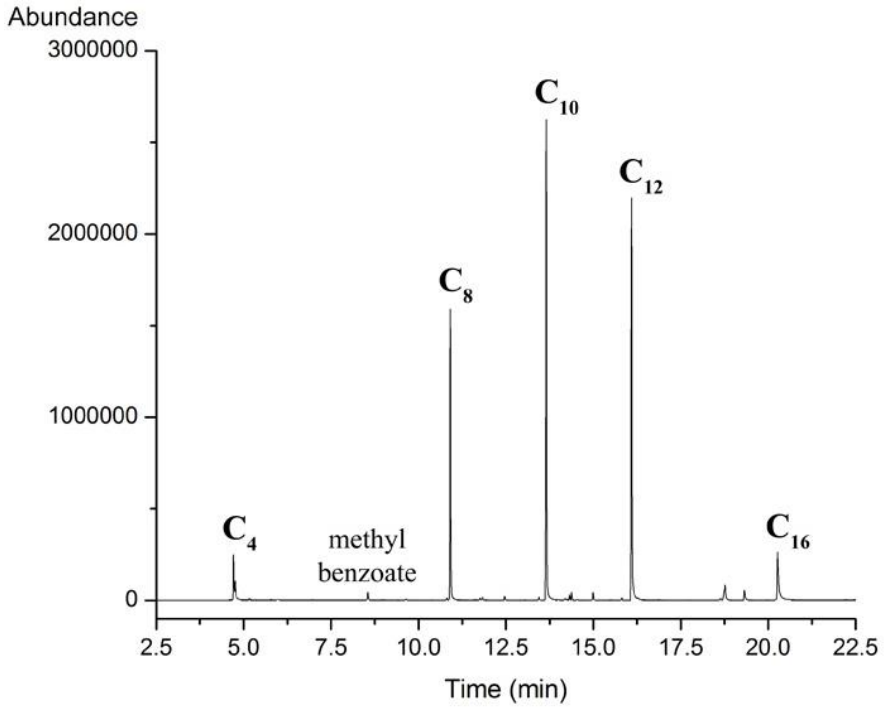


Figure 4.2 GC-MS total ion chromatograms of 3-hydroxyalkanoic acid methyl esters derived from PHA monomer analytical standards and methyl benzoate internal standard. (C_4, C_8, C_{10}, C_{12}, C_{16} represent methyl esters of 3-hydroxybutyric acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxyhexadecanoic acid, respectively).

with an adjusted R^2 of 0.997 (Figure 4.4B). These results indicated strong linear relationships with more than 98% of the total variability among the RT and RF values explained by the linear regression of RT/RF on carbon number of PHA.

$$RT = 1.299 \times (\text{carbon no.}) + 0.129 \qquad \text{Eq (4.3)}$$

$$RF = 0.235 \times (\text{carbon no.}) - 0.646 \qquad \text{Eq (4.4)}$$

The predicted RT and RF values for the PHA monomers were calculated using Eq (4.3) and Eq (4.4), respectively, and compared against experimentally-observed values. With the exception of C_4 monomer where the observed RT and predicted RT differed by about 13.6%, the difference for the rest of the PHA monomers was smaller and found to be within 4% (Table 4.1). The percentage difference between observed RFs and predicted RFs were between 0.7% (C_{10}) and 4.9% (C_8).

Table 4.1 Analytical parameters of the developed method for PHA determination.

PHA	Observed Retention Time ± s.d. (min)[1]	Predicted Retention Time (min)[2]	Observed Response Factor ± s.d.[1]	Predicted Response Factor[3]	Actual Concentration ($mg.L^{-1}$)	Observed Recovery ± s.d. (%)[1, 4]	Estimated Recovery ± s.d. (%)[1, 5]
Monomer							
C_4	4.69 ± 0.01	5.33	0.302 ± 0.023	0.294	1250	102.3 ± 7.2	105.1 ± 7.4
C_8	10.88 ± 0.03	10.52	1.176 ± 0.375	1.234	1250	100.0 ± 31.9	95.3 ± 30.4
C_{10}	13.67 ± 0.03	13.12	1.716 ± 0.322	1.704	1250	100.0 ± 18.8	100.7 ± 18.9
C_{12}	16.09 ± 0.03	15.72	2.254 ± 0.323	2.174	1250	95.6 ± 14.3	99.1 ± 14.9
C_{16}	20.28 ± 0.01	20.91	3.078 ± 0.805	3.114	1250	124.0 ± 26.1	122.6 ± 28.8
Polymer							
C_4 (P3HB)	4.69 ± 0.00	5.33	-	0.294	1275	-	113.8 ± 14.1
C_4 (P3HB3HV)	4.66 ± 0.01	5.33	-	0.294	6925 - 7775	-	114.3 ± 4.2
C_5 (P3HB3HV)	6.22 ± 0.00	6.62	-	0.529			
C_{10} (MCL-PHA)	13.58 ± 0.01	13.12	-	1.704	1250 - 1925	-	100.5 ± 10.4

[1] Tabulated based on results from at least three independent sample determinations ($n \geq 3$)
[2] Tabulated using Eq (5.3)
[3] Tabulated using Eq (5.4)
[4] Tabulated using Observed Response Factor values
[5] Tabulated using Predicted Response Factor values

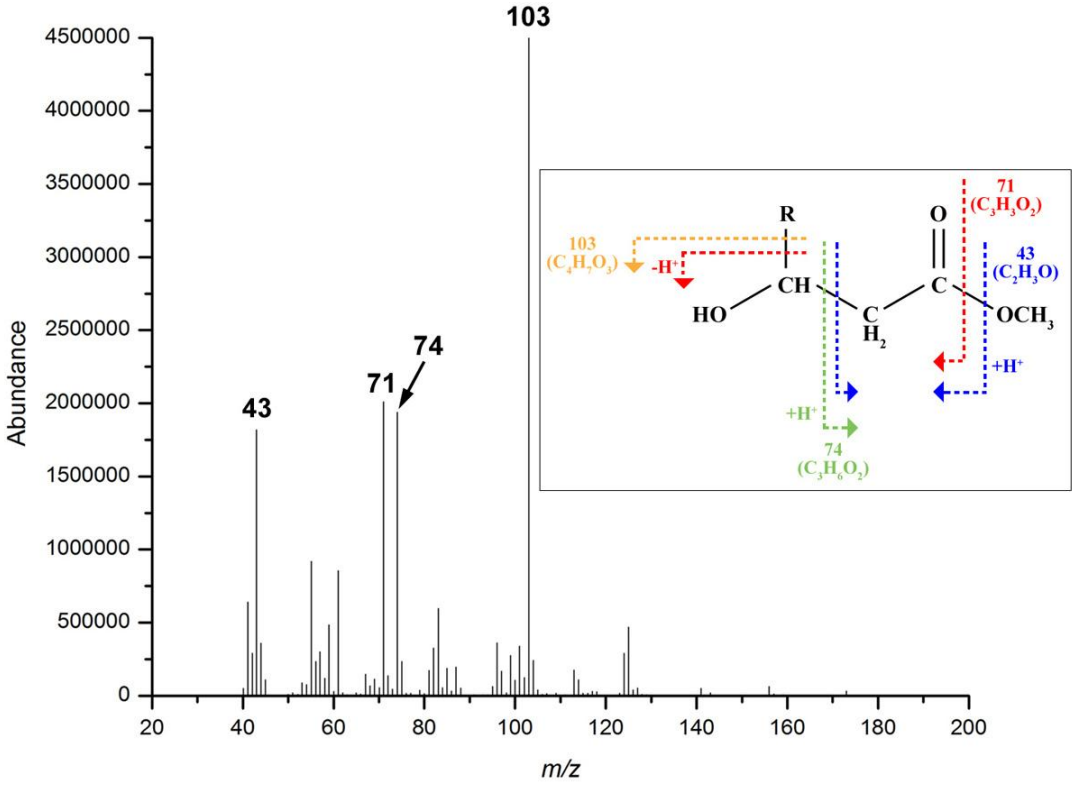


Figure 4.3 Mass fragmentation spectrum of PHA. Inset shows the fragment ions generated, with their respective chemical formula provided within the parentheses, under positive-ion mode EI-MS analysis.

4.3.3 Method evaluation and recovery

The predictive value of the linear relationship between carbon number and RT, as described by Eq (4.3), was evaluated using SCL-PHA polymer standards P3HB and P3HB3HV, where P3HB3HV contained between 11 to 13 mol% of a C_5 monomeric unit that was not included as an analytical standard in the present study. A MCL-PHA polymer, whose composition was verified by H^1-NMR and C^{13}-NMR as C_{10} monomeric repeats, was also included as a standard (Figure A1). After methanolysis, P3HB was chemically converted to form C_4 methyl esters while P3H3BHV was chemically reacted to form C_4 and C_5 methyl esters, and MCL-PHA was chemically reacted to C_{10} methyl esters (Figure 4.5). The observed RT of C_4 methyl esters from P3HB was 4.69 min; C_4 and C_5 methyl esters from P3HB3HV were 4.66 min and 6.22 min; and C_{10} methyl esters from MCL-PHA was 13.58 min (Table 4.1). The observed RTs of C_4 methyl esters derived from both P3HB and

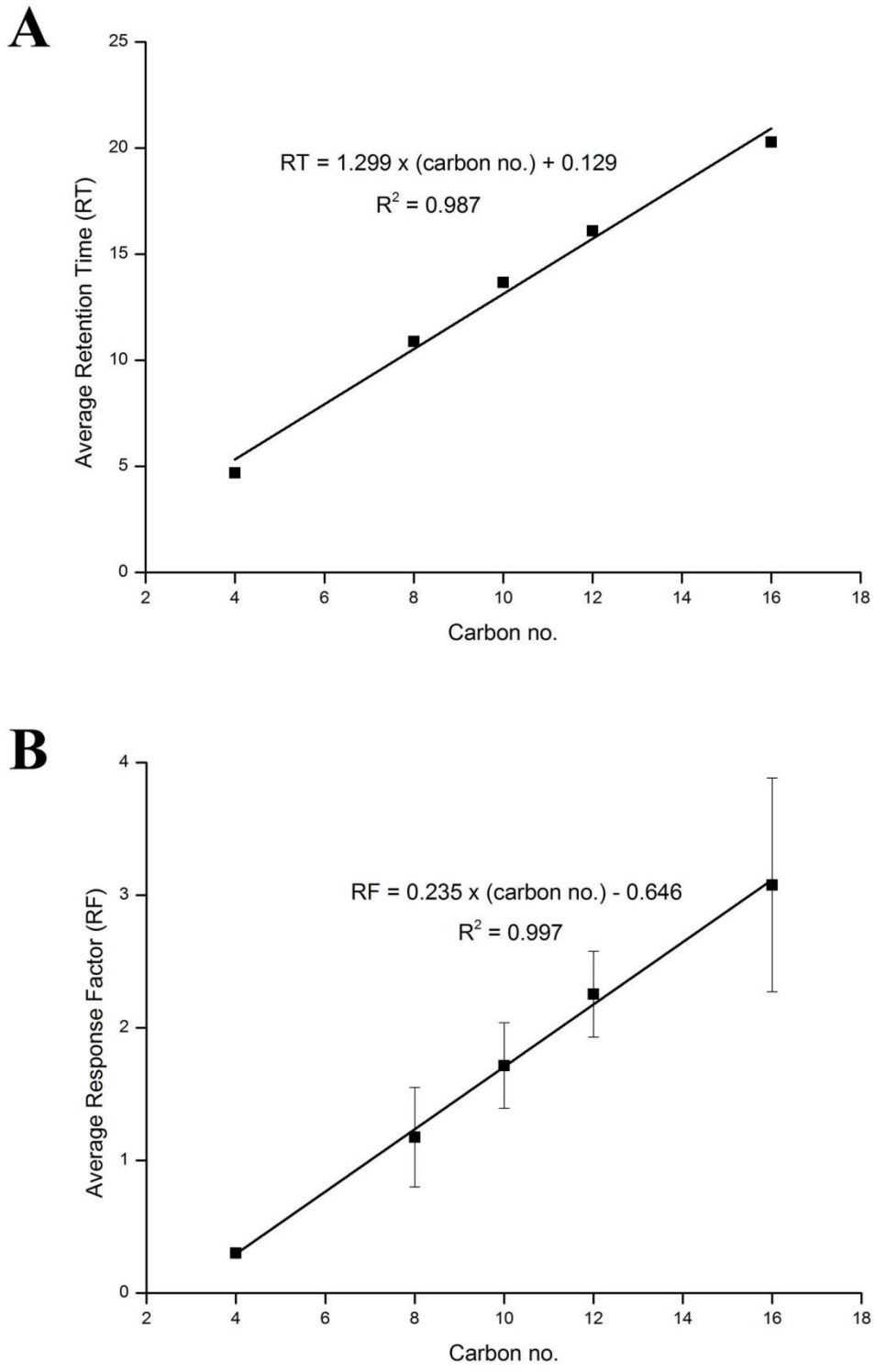


Figure 4.4 Graphs depicting the linear relationship between PHA carbon number and (A) average retention time (RT), and (B) average response factor (RF). The mean values represent values from at least three independent experiments ($n \geq 3$) and the error bars represent standard deviations.

P3HB3HV were more similar to that derived from C_4 PHA monomer standard (4.69 min) as compared to the RT value predicted by Eq (4.3) (5.33 min). However, the observed RTs of C_5 methyl esters from PHBHV and C_{10} methyl esters from MCL-PHA were similar to the predicted RT values (C_5, 6.62 min; C_{10}, 13.12 min) with a percentage difference of about 6.4% and 3.4%, respectively. Although there was a greater discrepancy between the predicted and observed RT value for C_4 methyl esters, it was also observed that the mass fragmentation information provided by GC-MS can help to cross-validate the predicted RT value, minimizing errors in PHA detection.

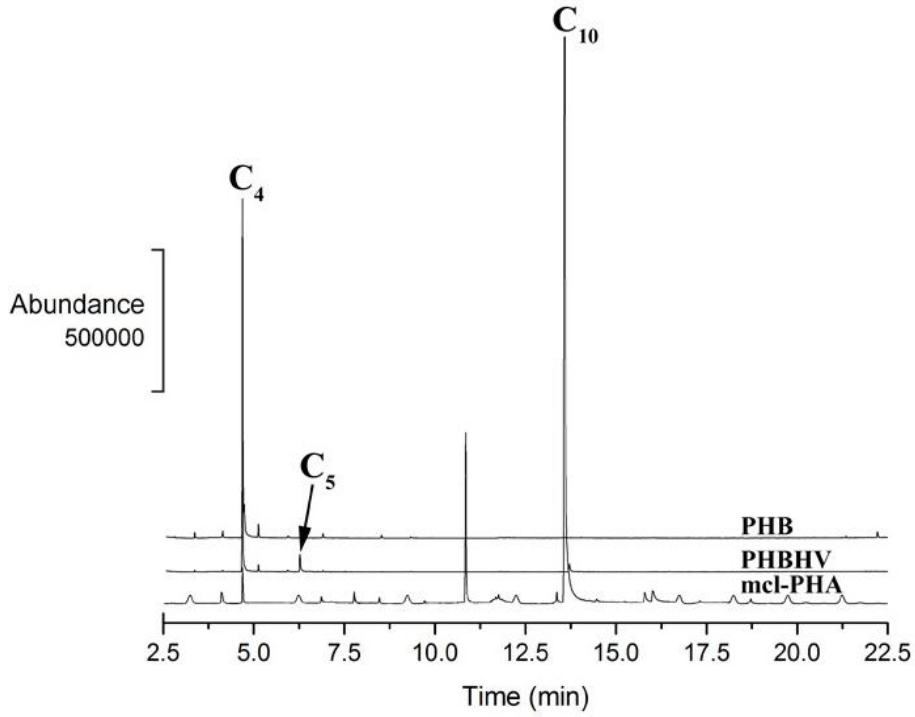


Figure 4.5 GC-MS total ion chromatograms of 3-hydroxyalkanoic acid methyl esters derived from PHA polymers P3HB, P3HB3HV and MCL-PHA. (C_4, C_5, C_{10} represent methyl esters of 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 3-hydroxydecanoic acid, respectively).

The predictive value of the linear relationship between carbon number and RF, as described by Eq (4.4), was evaluated in terms of method recovery using known amounts of PHA monomers and PHA polymers. Method recovery values obtained based on the observed RFs (ranging from 0.302 ± 0.023 to 3.078 ± 0.805) and

predicted RFs (ranging from 0.294 to 3.114) were compared. Based on predicted RF values, the estimated recovery for all the tested PHA monomers (*i.e.*, C_4, C_8, C_{10}, C_{12}, and C_{16}) were between 95.3 ± 30.4% and 122.6 ± 28.8%, which were similar to the recovery values obtained using observed RF values (between 95.6 ± 14.3% and 124.0 ± 26.1%) (Table 4.1). For PHA polymers, the estimated recoveries for P3HB and MCL-PHA were 113.8 ± 14.1% and 100.5 ± 10.4%, respectively. To quantify the estimated recovery for P3HB3HV, the predicted RF values for both C_4 and C_5 methyl esters had to be considered. Based on Eq (4.4), the RF value for C_4 and C_5 methyl esters was predicted to be 0.294 and 0.529, respectively (Table 4.1). According to the predicted RF values, the estimated recovery was 114.3 ± 4.2%. The estimated recovery values for all tested PHA polymers were within the range of 80 to 120%, which is the acceptable range according to the quality control criteria prescribed for most US EPA methods. In addition, the monomeric composition of P3HB3HV was found to be 90.6 ± 1.5 mol% of C_4 monomer and 9.4 ± 1.5 mol% of C_5 monomer, which was a reasonable estimation of the actual values (*i.e.*,87 to 89 mol% of C_4 monomer and 11 to 13 mol% of C_5 monomer).

4.4 Discussion

PHA biopolymers usually comprised of several kinds of monomeric units, some of which may not have readily-available analytical standards. This study attempted to overcome this issue by developing a GC-MS calibration method based on the relationship between the carbon number of homologous saturated PHA monomers and their respective RT and RF. The carbon number of PHA monomers was found to correlate with RT and RF in a linear fashion, which can be described by Eq (4.3) and Eq (4.4), respectively (Figure 4.4). The adjusted R^2 values were more than 0.98 for both equations, indicating the high strength of the linear relationships. This also suggested that the equations may enable the RT and RF of saturated PHA monomeric homologues to be estimated with high accuracy. Such correlations have been previously demonstrated for homologous series of *n*-alkanes (Tong et al., 1985), ketones, secondary alcohols, nitrogen heterocycles, and *etc* (Jorgensen et al., 1990). To our best knowledge, the present study presents the first reference demonstrating these linear correlations for PHA monomers under GC-MS analysis.

The linear equation Eq (4.3) was used to predict the RTs for various PHA monomers and polymers. The predicted RT values were generally found to be a reasonable estimate of the observed values. No more than 6.4% difference was found between the predicted and observed RT values for PHA polymers and most of the PHA monomers tested (Table 4.1). Most notably, in the absence of a C_5 analytical standard, the RT of C_5 methyl esters from P3HB3HV was predicted as 6.62 min, which was similar to the observed value of 6.27 min. The only exception was C_4 methyl esters derived from C_4 PHA monomer, P3HB and P3HB3HV where a 13.6% difference was observed. It appears that Eq (4.3) may provide a more reliable estimation of RT for methyl esters of PHA with C_5 or higher carbon number, and was less reliable in estimating the RT for C_4 methyl esters. Nevertheless, the discrepancy between predicted and observed RT values can be easily overcome by cross-validating with mass fragmentation information. As such, Eq (4.3) proved to be useful in providing a reliable estimation for the RTs of homologous PHA, especially when coupled with mass spectra information.

Likewise, RF values predicted by Eq (4.4) (*i.e.,* 0.294 to 3.114) proved to be a close estimate of the observed RF values (*i.e.,* 0.302 ± 0.023 to 3.078 ± 0.805) (Table 4.1). This was supported by further evaluation in terms of method recovery using known amounts of PHA monomers and polymers. For PHA monomers, the estimated recovery values calculated based on predicted RFs (between 95.3 ± 30.4% and 122.6 ± 28.8%) were similar to the observed recovery values obtained using observed RF values (*i.e.,* between 95.6 ± 14.3% and 124.0 ± 26.1%). For PHA polymers P3HB and MCL-PHA, the estimated recovery values were 113.8 ± 14.1% and 100.5 ± 10.4%, respectively. Importantly, Eq (4.4) could reliably predict the RF value for C_5 monomer (0.529), which was omitted from method development in this present study, and together with the predicted RF values for C_4 monomer (0.294), yielded an estimated recovery for P3HB3HV of 114.3 ± 4.2%. As such, the estimated recovery values for all tested PHA polymers fell within the range of 80 to 120%, which is the acceptable range according to the quality control criteria prescribed for most US EPA methods. Furthermore, the predicted RF values

estimated the monomeric composition of P3HB3HV as 90.6 ± 1.5 mol% of C_4 monomer and 9.4 ± 1.5 mol% of C_5 monomer, which was similar to the actual values (*i.e.*,87 to 89 mol% of C_4 monomer and 11 to 13 mol% of C_5 monomer). These findings highlighted that Eq (4.4) could predict RF values for homologous saturated PHA monomers with high accuracy and the predicted RFs could be applied to the quantification of both PHA homopolymers as well as PHA copolymers even in the absence of some analytical standards.

Collectively, the results suggested that the proposed method can provide a reliable identification and quantification of saturated PHA monomer homologues between the carbon number of C_4 and C_{16}. The proposed method may be particularly advantageous in facilitating the analysis of some saturated PHA monomers with odd-number carbon length for which analytical standards are less readily available or expensive to procure (Mittendorf et al., 1998). This also eliminates the need to include a full spectrum of PHA standards. The proposed method resulted in calibration protocol simplification, significantly reducing the number of calibration points and calibration standard samples. This leads to an 80% decreased in calibration workload and cost.

It should be noted that the present study dealt with only saturated PHAs with alkyl functional *R* groups, which are by no means exhaustive of all the PHAs currently known to science. There are many types of PHA that currently exist without readily-available commercial standards. These PHAs include most unsaturated PHAs (Furrer et al., 2007) and branched PHAs (Lu et al., 2009). The chemical and physical modifications of naturally-occurring PHAs (Olivera et al., 2010; Zinn & Hany, 2005) and the creation of genetically-modified organisms (GMOs) to produce PHAs with specialized functional groups (Escapa et al., 2011) has led to the rapid development of new types of PHAs. While GC-MS is able to provide a tentative identification of these unconventional PHAs, the analysis currently needs to be further supported by NMR methods for compound validation and quantification (Escapa et al., 2011). Nevertheless, it is anticipated that as more PHA analytical standards become available, the present study may serve as a

methodological reference upon which GC-MS calibration methods for other types of PHA could be similarly modeled after.

4.5 Summary

A GC-MS calibration method which could be applied to reliably detect and quantify PHA polymers and a series of PHA monomer using a limited number of analytical standards was successfully developed. Through this study, the carbon number of a homologous saturated PHA monomer series (C_4/C_8/C_{10}/C_{12}/C_{16}) was observed as having a strong linear relationship with its respective RT (adjusted R^2 of 0.987) and RF (adjusted R^2 of 0.997). The linear equations describing PHA carbon number and RT (RT = 1.299 x [carbon no.] + 0.129), and PHA carbon number and RF (RF = 0.235 x [carbon no.] - 0.646) could predict RT and RF values within 4.9% accuracy of experimentally-observed values for most of the PHA monomers tested (*i.e.*, C_8/C_{10}/C_{12}/C_{16} monomers). The only exception was the predicted RT value for C_4 monomer which differed from the observed RT value by 13.6%. Nevertheless, cross-validation with mass fragmentation information was found to minimize error in the detection of C_4 monomer. Method recovery values for known amounts of PHA monomers were estimated at between 95.3 ± 30.4% and 122.6 ± 15.11%, which were similar to the experimentally-observed values (between 95.6 ± 14.3% and 124.0 ± 26.1%). The calibration method was successfully applied to the quantification of known amounts of PHA homopolymers P3HB and C_{10}-containing MCL-PHA with estimated recovery values of 113.8 ± 14.1% and 114.3 ± 4.2%, respectively. In the absence of C_5 PHA standard, the method was demonstrated to facilitate the detection and quantification of P3HB3HV copolymer, predicting a RT value of 6.62 min for C_5 monomer which was similar to the observed value (6.22 min) and an estimated recovery value of 100.5 ± 10.4%. This method is advantageous in its simplification of calibration work, and in facilitating the detection and quantification of PHA polymer, particularly when PHA polymer comprises of several different PHA monomers and where analytical standards are expensive or less readily available.

CHAPTER 5

Study 3:
Investigation of the Bacterial Community Structure of Styrene-Enriched Activated Sludge

5.1 Introduction

The industrial production of PS plastics is primarily responsible for styrene-laden wastewater and off-gas generation (Abduli et al., 2006; Tossavainen, 1978). Mixed microbial cultures, such as activated sludge, are frequently applied for the bioremediation of styrene-laden effluent and contaminated soil (Dehghanzadeh et al., 2005; Fallah et al., 2010; Greene et al., 2000). Bioremediation also offers the possibility for bioconverting waste into valuable PHA products, with potential benefits to lower PHA production cost and off-set biological treatment cost through PHA recovery. The feasibility of this concept was demonstrated for waste carbon substrates from various organic wastestreams (*i.e.*, biodiesel wastewater, oily waste and VFAs); depending on the microbial community structure as well as operating conditions, mixed cultures could accumulate between 1% CDM and 67% CDM cellular PHA content (Dobroth et al., 2011; Salehizadeh & Van Loosdrecht, 2004; Serafim et al., 2008). However, the production of MCL-PHA from styrene using mixed cultures currently remains an unproven concept. Hence, an examination of the styrene-degrading microbial consortium could serve as a knowledge base for identification of suitable seeding sludge, process optimization, bioaugmentation and formulation of defined microbial cocktails, aimed at enhancing styrene elimination and expanding the biological process for simultaneous MCL-PHA production.

The biofilter and soil styrene-degrading microbial consortium were previously investigated using both culture-dependent and culture-independent approaches. Culture-dependent method, such as isolation, and a combination of culture-dependent method and culture-independent method, such as reverse sample genome probing (RSGP), has identified the presence of biofilter and soil styrene-degrading bacterial species from the genera *Pseudomonas*, *Azospirillum*, *Rhodococcus*, *Alcaligenes*, *Tsukamurella*, *Sphingomonas*, and *Xanthomonas*, which belonged to the phyla Proteobacteria and Actinobacteria (Arnold et al., 1997; Greene et al., 2000). Through the use of culture-independent methods alone, the styrene-degrading microbial community was discovered to be a far more diverse community. PLFA analysis, using isotope-labeled styrene, indicated the presence of biofilter bacterial members from the phyla Proteobacteria, Actinobacteria,

Firmicutes, as well as some unidentified microbial players (Alexandrino et al., 2001). Pyrosequencing provided an even more detailed insight into the biofilter microbial community, revealing sixteen bacterial phyla and an unclassified bacterial phylum (Portune et al., 2015). While much information on the styrene-degrading microbial consortia could be gleaned from non- and semi-aqueous environments such as biofilters and contaminated soil studies, these styrene-degrading microbial communities reportedly evolve differently to adapt to various environs (Tresse et al., 2002), which meant that the existing information may not accurately describe the microbial population in aqueous environments. The objective of this study is to bridge this research gap by investigating the aqueous microbial community composition, under prolonged styrene enrichment, with the use of next-generation 16S rDNA sequencing technique.

5.2 Materials and Methods

5.2.1 Operation of styrene-degrading bioreactor and sample collection

The styrene-degrading bioreactor was a 2 L glass reactor with 1.4 L working volume. Enriched domestic activated sludge, obtained from a BTEXS-degrading reactor following 15 months of enrichment on BTEXS, were inoculated to 1X mineral salt medium (MSM) at total suspended solids (TSS) and volatile suspended solids (VSS) values of 3.33 $g.L^{-1}$ and 3.07 $g.L^{-1}$, respectively. The cultivation medium comprised of 3.70 $g.L^{-1}$ KH_2PO_4, 5.80 $g.L^{-1}$ K_2HPO_4, 0.2 $g.L^{-1}$ $MgSO_4.7H_2O$, and 1.0 $mL.L^{-1}$ microelements solution (2.78 $g.L^{-1}$ $FeSO_4.7H_2O$, 1.98 $g.L^{-1}$ $MnCl_2.4H_2O$, and 2.81 $g.L^{-1}$ $CoSO_4.7H_2O$, 1.67 $g.L^{-1}$ $CaCl_2.2H_2O$, 0.17 $g.L^{-1}$ $CuCl_2.2H_2O$ and 0.29 $g.L^{-1}$ $ZnSO_4.7H_2O$ in 0.1 M HCl). The pH of MSM was adjusted to 7.2 using 3 M NaOH. Nitrogen source, in the form of $(NH_4)_2SO_4$, was added at 16 mM. Liquid styrene was added weekly at 1.8 mM as the sole carbon source. The cultivation medium was replaced with fresh medium weekly. Due to the volatile nature and therefore, short liquid retention time of styrene, the substrate was also continuously delivered in a gaseous form as part of the aeration source in order to ensure constant exposure of sludge microogranisms to styrene. This was achieved by placing a styrene reservoir in between the bioreactor and an aeration pump. The aeration pump delivered atmospheric air into the styrene

reservoir inducing the volatilization of styrene. The gaseous mixture, consisting of atmospheric air and styrene was delivered to the bioreactor via an air sparger at a flow rate of 440 ± 7.5 $mL.min^{-1}$, which delivered gaseous styrene to the reactor at an average rate of 0.55 ± 0.03 $g.h^{-1}$. The bioreactor was operated for a total of 19.5 months. De-sludging was performed after 16 months of operation where 50% sludge volume was removed, reducing TSS and VSS values to 13.08 $g.L^{-1}$ and 9.77 $g.L^{-1}$, respectively. Sludge samples (2 mL) were collected at various time points during the operation period (0, 3, 11, 17 and 19.5 months). The samples were centrifuged at 9,425 g, 15 °C for 5 min and the supernatant was discarded, leaving behind the sludge pellet. The sludge pellets were stored at -20 °C prior to total genomic DNA extraction.

5.2.2 Styrene biodegradation batch experiment

The styrene degradation test was carried out in a 60 mL amber glass serum bottle with a total working volume of 6.7 mL and 53.3 mL of headspace. The bottle was first filled with 1X MSM containing 16 mM of $(NH_4)_2SO_4$. Liquid styrene was added to the cultivation medium at 2.3 mM, which also corresponded to a total amount of 1.65 mg. After styrene addition, the serum bottle was immediately crimp-sealed with Telfon-lined silicone septum and aluminum crimp cap, and incubated overnight at 30 °C to allow for liquid-gas partitioning of styrene. Sludge, taken from the styrene-degrading bioreactor after 16 months of enrichment, was added into the serum bottle at a concentration of 300 VSS $mg.L^{-1}$. Sludge addition was performed via injection using a plastic syringe with needle attached. Negative control containing 300 VSS $mg.L^{-1}$ of double-autoclaved sludge (*i.e.*, sludge which has been autoclaved twice), and empty control containing only the cultivation medium without any sludge addition were similarly set up. The serum bottles were incubated at 30 °C with shaking at 120 rpm for 48 h. Styrene determinations were performed at 0, 2, 4, 6, 8 and 10 h. One bottle of setup, negative control and empty control were sacrificed for each analysis. For aqueous styrene determination, 2 mL of liquid culture was drawn from the serum bottle using a glass syringe with a needle attached. Liquid culture was filtered through a 0.45 µm regenerated cellulose (RC) filter membrane (Satorius Stedim, Germany). The filtrate was collected in and

completely filled the HPLC amber vials to eliminate any gas phase. The vial was immediately crimp-sealed using aluminum cap with Teflon-lined septum, and kept chilled at 15 °C prior HPLC-DAD analysis. For headspace gaseous styrene determination, 10 µL of headspace gas was sampled by a gas-tight syringe and manually injected into a GC-FID. The styrene analytical methods are described in the following section. Triplicate experiments were performed.

5.2.3 Styrene chromatographic determination methods

Aqueous styrene was determined by HPLC-DAD (Thermo Scientific Dionex, USA) using a mobile phase of 60% methanol with a flow rate of 1.2 $mL.min^{-1}$, a sample injection volume of 10 µL and a detection wavelength of 201 nm. All other operation parameters were kept the same, as previously described by Tan et al. (2012) (Chapter 3). Headspace gaseous styrene was determined by manual injection into a GC-FID (Agilent Technologies, Palo Alto, Calif) at a split ratio of 1:10. Analysis was achieved on a ZB-5HT column (30 m by 0.25 mm, 0.25-µm film thickness; Phenomenex, Torrance, CA, USA). The temperatures of the injection port and detector heater were 250 and 300 °C, respectively. Oven temperature was programmed at an initial temperature of 40 °C and subsequently raised with a rate of 5 $°C.min^{-1}$ to 65 °C. Helium was used as a carrier gas at a flow rate of 4.0 $mL.min^{-1}$. A calibration curve was used to determine the styrene amount.

5.2.4 Total genomic DNA extraction

Total genomic DNA of sludge samples were extracted using a chemical-lysis method described by Liu et al. (1997). Briefly, the cell pellets were lysed in an extraction buffer (100 mM Tris-HCl, 100 mM Ethylenediaminetetraacetic acid (EDTA), 0.75 M sucrose), Lysozyme (10 $mg.mL^{-1}$), Achromopeptidease (1 $mg.mL^{-1}$), ProteaseK (20 $mg.mL^{-1}$) and 10% sodium dodecyl sulfate (SDS) at 37 °C. Phenol/Chlorofinsorm/IAA (25:24:1) and Chlorofinsorm/IAA (24:1) were used at least twice on the supernatant to purify the DNA solutions. Cold isopropanol was used to precipitate the DNA. DNA pellet was subsequently washed by 70% ethanol twice and dissolved in 100 µL water.

5.2.5 16S rDNA next-generation sequencing, data processing and analysis

Extracted DNA of sludge samples were sent to the Research and Testing Laboratory (RTL; Lubbock, TX, USA) for next-generation 16S rDNA sequencing. Sequencing was performed on the Illumina MiSeq platform (Illumina Inc, San Diego, CA, USA) with at least an average of 15,000 reads per sample. The primer set used was 515F/909R, developed by RTL, which spans the V4 to V5 region of the bacterial 16S rRNA gene. Raw sequences were processed and analyzed using the software Mothur (version 1.33.0) (Schloss et al., 2009) according to the protocol described by Kozich et al. (2013), which is a standard operating procedure developed to process 16S rDNA sequences generated from Illumina MiSeq platform. Briefly, sequences that were shorter than 350 bp, longer than 400 bp or contained ambiguous bases were discarded. The remaining sequences were sorted and grouped based on sequence identity into groups of unique sequence. Sequences were aligned to the SILVA-based bacterial reference alignment (Schloss, 2009), followed by pre-clustering based on a pseudo-single linkage algorithm (Huse et al., 2010). Using Mothur's in-built UCHIME algorithm (Edgar et al., 2011), the data was checked for chimeras, and sequences, deemed as chimeric, were removed. Using Mothur's classify.seq command, its in-built Bayesian classifier and Mothur-formatted version of the Ribosomal Database Project (RDP-II) training set (v.9) (Wang et al., 2007), taxonomy alignment was performed for the data at a pseudobootstrap cutoff value of 80%. The data was further checked for sequences arising from archaea, chloroplasts, mitochondria and eukaryotes, and these sequences were also removed to produce the final processed sequences using Mothur's remove.lineage command. The sequences were clustered into operational taxonomic units (OTUs) using Mothur's cluster.split command to split at the order level (*i.e.*, taxlevel = 4) and clustered to a 0.15 cutoff. To obtain information on the number of sequences in each OTU for each sludge DNA sample, a shared filed was created using Mothur's make.shared command. Using Mothur's classify.otu command with a default confidence threshold of 51%, consensus taxonomy information for each OTU was obtained. The shared file was used for OTU-based analyses. Rarefaction curves were generated using Mothur's rarefaction.single command. Using Mothur's summary.single command, values for diversity indices

(Inverse Simpson [1/*D*] and Shannon diversity index [*H'*]), richness abundance estimators (Chao1 and Abundance-based Coverage Estimator [ACE]), observed OTUs, and Good's coverage were obtained for each sludge DNA sample. Alpha diversity analysis including diversity indices, richness abundance estimators, observed OTUs and Good's coverage were computed based on the number of normalized sequences for all 5 sampling points (*i.e.*, 84,942) at the 95% confidence interval (CI) from 1,000 iterations of the subsamples. Evenness indices, based on Inverse Simpson index (*E*) and Shannon diversity index (*J'),* were also obtained with Mothur's summary.single command. A phylip-formatted distance matrix, based on Bray-Curtis dissimilarity, was calculated using Mothur's dist.shared command through 1,000 iterations of the subsamples (subsample size of 84,942). Using the tree.shared command, the distance matrix was used in hierarchical clustering by the unweighted pair group method with arithmetic mean (UPGMA) algorithm to generate a dendrogram describing the similarities between the samples.

5.3 Results

5.3.1 Styrene biodegradation test

To ascertain the styrene degradation capability of the enriched sludge, the biodegradation test was performed using 16-months styrene-enriched sludge. Prior to sludge inoculation, it was observed that 1.65 mg of styrene partitioned such that 1.24 mg existed as a gas form in the serum bottle headspace and 0.41 mg existed as an aqueous form in the cultivation medium (Figure 5.1). Within 4 h to 6 h of inoculation with sludge, between 65.6% and 75.5% of styrene were degraded. Aqueous styrene decreased to low levels between 0.015 and 0.075 mg while headspace styrene gradually decreased to 0.55 mg (4 h) and subsequently to 0.33 mg (6 h) (Figure 5.1A). By 8 h, aqueous styrene was undetectable while headspace styrene dropped to 0.04 mg (98.0% styrene removal). Headspace styrene decreased slightly to 0.03 mg by 10 h, achieving a total styrene removal of 98.4%. No further reduction of styrene was observed up to 48 h (data not shown). In the negative control and blank control setups inoculated with double-autoclaved sludge and without sludge addition, respectively, up to a 40% styrene reduction was observed within 4 h. In both setups, headspace styrene decreased by about 0.44 mg to

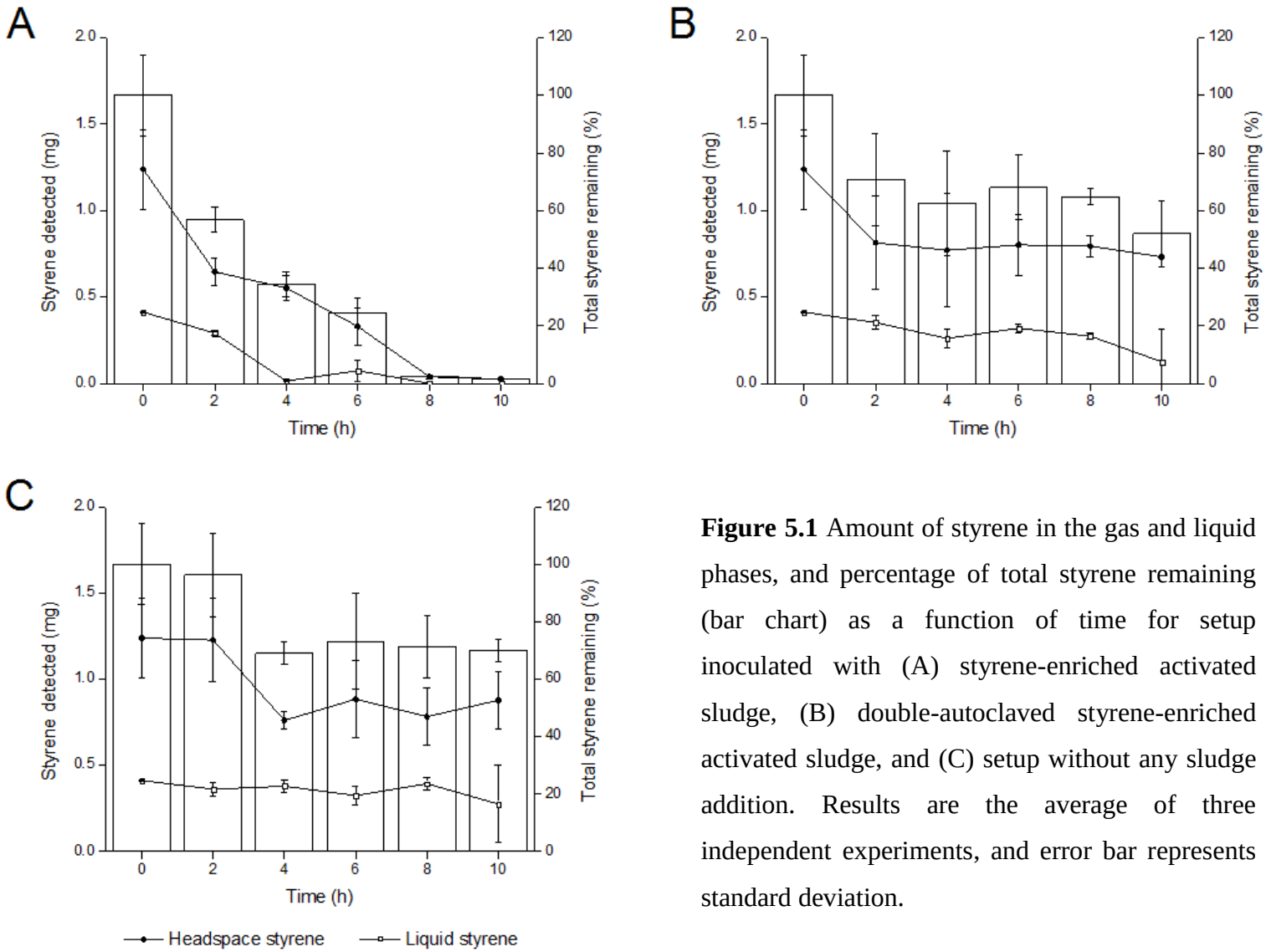


Figure 5.1 Amount of styrene in the gas and liquid phases, and percentage of total styrene remaining (bar chart) as a function of time for setup inoculated with (A) styrene-enriched activated sludge, (B) double-autoclaved styrene-enriched activated sludge, and (C) setup without any sludge addition. Results are the average of three independent experiments, and error bar represents standard deviation.

0.80 mg while aqueous styrene decreased slightly by about 0.11 mg to 0.3 mg (Figures 5.1B and 5.1C). Beyond 4 h however, styrene levels remained at levels between 50% and 70% without further decrease (data not shown).

5.3.2 Alpha and beta diversity analyses of bacterial community

The sludge was sampled at five different time points during the styrene enrichment period, specifically, at 0, 3, 11, 17 and 19.5 months. The Illumina MiSeq platform (Illumina Inc, San Diego, CA, USA) yielded between 129,861 and 282,514 raw reads (Table 5.1). During data processing, about 27 to 34% of the raw reads were detected as either chimeric sequences, of non-bacterial origin, or did not meet the quality control criteria; these sequences were removed, resulting in a total of 84,942 to 195,383 post-processing sequences. Using the normalized post-processing sequence size (*i.e.*, 84,942), between 306 and 1,033 observed OTUs were detected. The number of OTUs in sludge inoculum (*i.e.*, 0 months) was 478 and this number decreased to 306 during the initial stages of styrene enrichment (*i.e.*, 3 months). With further enrichment, the OTUs increased from 306 to 590 at 11 months, and increased further to 1,033 at 17 months after desludging was performed, before decreasing slightly to 887 at 19.5 months. This trend in observed OTUs was supported by a similar trend in Chao1 and ACE richness abundance estimator values (Table 5.1). Rarefaction curve showed that the number of observed OTUs did not reached asymptotic level for all the samples, suggesting that the sequencing depths of up to 195,383 sequences were insufficient in providing the complete bacterial diversity (Figure. 5.2). The rarefaction curve data also was supported by richness estimator values, which were found to be higher than the observed OTUs at all sampling time points. Nevertheless, more than 99.4% coverage were detected for all the samples at the 95% CI level (Table 5.1), which meant that majority of the OTU information were captured by next-generation sequencing and that more than 167 extra sequences would be required before a new OTU could be detected.

The inoculum sludge had diversity indices values of 6.39 ($1/D$) and 2.34 (H'), richness abundance estimator values of 1,143 (Chao1) and 1,658 (ACE), and evenness indices values of 0.013 (E) and 0.380 (J') (Table 5.1). A drop in diversity

Table 5.1 Summary of raw, processed sequences and results from alpha diversity analysis.

	Samples[2]				
	0 month	3 months	11 months	17 months	19.5 months
Raw reads	129,861	192,519	203,910	282,514	130,878
Sequences post-processing	84,942	139,868	145,220	195,383	89,973
Sequences removed during processing (%)	34.6	27.3	28.8	30.8	31.3
Normalized sequences	84,942	84,942	84,942	84,942	84,942
OTUs[1]	478	306	590	1,033	887
Inverse Simpson (1/*D*)	6.39 (6.35 – 6.44)	2.95 (2.93 - 2.96)	3.33 (3.31 – 3.34)	5.27 (5.24 – 5.31)	10.33 (10.23 – 10.42)
Shannon diversity (*H'*)	2.34 (2.33 – 2.35)	1.47 (1.47 – 1.48)	1.81 (1.80 – 1.82)	2.39 (2.38 – 2.39)	2.96 (2.95 – 2.97)
Chao 1 richness	1,143 (942 – 1,431)	651 (517 – 870)	1,480 (1,231 – 1,825)	2,288 (2,029 – 2,614)	2,322 (2,004 – 2,731)
ACE richness	1,658 (1,485 – 1,862)	795 (701 – 911)	2,439 (2,212 – 2,698)	4,178 (3,888 – 4,497)	4,632 (4,263 – 5,042)
Good's coverage (%)	99.7	99.9	99.8	99.7	99.4
Inverse Simpson evenness (*E*)	0.013	0.010	0.006	0.005	0.012
Shannon evenness (*J'*)	0.380	0.258	0.284	0.344	0.436

[1] Operational taxonomic units observed after normalization
[2] Values within parentheses are the 95% confidence intervals of respective estimators

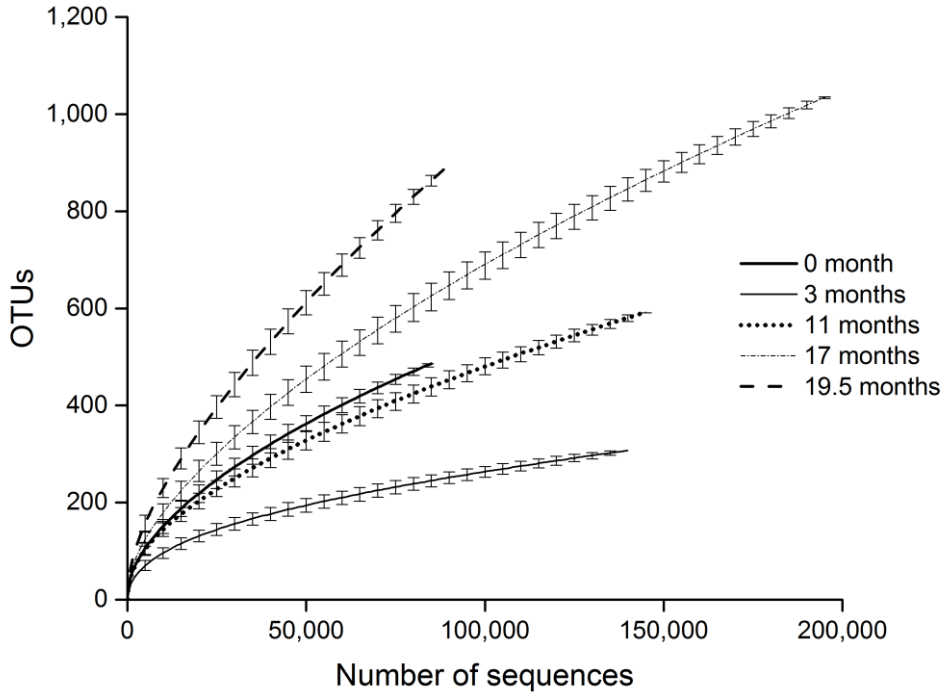


Figure 5.2 Rarefaction curves at 3% cutoff for styrene enrichment time points at 0, 3, 11, 17 and 19.5 months. Error bar represents 95% confidence interval.

was observed in the beginning of styrene enrichment (3 months; 1/*D* = 2.95, *H'* = 1.47). The drop in diversity was attributed to a decrease in both richness (Chao1 = 651, ACE = 795) and evenness (*E* = 0.010, *J'* = 0.258). Under prolonged enrichment, diversity increased to peak at 19.5 months with values of 10.33 (1/*D*) and 2.96 (*H'*). It is worth noting that the evenness indices values did not vary much for sampling periods between 3 months and 19.5 months, ranging from 0.005 to 0.012 (*E*), and 0.258 to 0.436 (*J'*); while richness indices increased from 651 to 2,322 (Chao1), and from 795 to 4,632 (ACE). Hence, the increase in diversity under long-term enrichment were attributed to the increase in richness (*i.e.*, more species present in a sample). At the 95% CI level, only the CIs of Chao1 and ACE for 17 months and 19.5 months overlapped, which meant that there was no statistical difference ($p > 0.05$) in the richness between these two samples. There was however, a statistical difference ($p < 0.05$) in the richness amongst the first four samples (*i.e.*, 0, 3, 11 and 17 months), and between the first three samples (*i.e.*, 0, 3 and 11 months) and the final sample (*i.e.*, 19.5 months).

Dendrogram, generated based on Bray-Curtis dissimilarity, showed that the four samples taken during styrene enrichment (*i.e.*, 3, 11, 17 and 19.5 months) were clustered closely together and separately from the sludge inoculum (*i.e.*, 0 month) (Figure 5.3). Among the styrene-enriched samples, samples from 3 months and 11 months shared the highest similarity, and were clustered together. Between samples from 17 months and 19.5 months, the 17-month sample was spatially closer to, and therefore, sharing greater similarity with 3-month and 11-month samples.

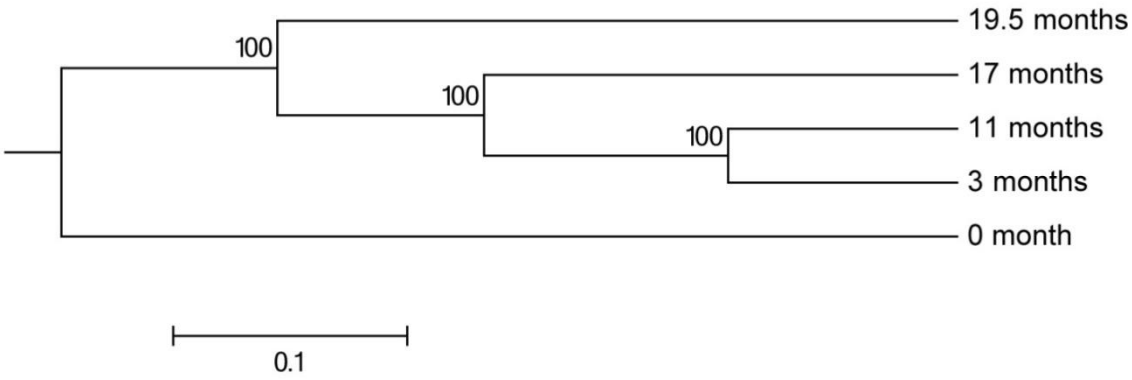


Figure 5.3 Dendrogram based on Bray-Curtis dissimilarity for styrene enrichment time points at 0, 3, 11, 17 and 19.5 months. The numbers beside the nodes indicated bootstrap values based on 1,000 iterations. The scale bar represents the difference in Bray-Curtis indices between samples.

5.3.3 Bacterial community structure analysis at phyla level

A total of 12 bacterial phyla and 1 unclassified group were represented in the sludge over the five sampling time points (Figure 5.4). The top five phyla were identified as Proteobacteria, Bacteriodetes, Firmicutes, unclassified phylum, and Actinobacteria (Figure 5.4). Collectively, these phyla make up more than 97% of the entire bacterial community at all sampling time points. Proteobacteria was the dominant phylum in styrene-enriched sludge, forming more than half of the community throughout the enrichment period (51.6% to 59.4%). Aside from Proteobacteria, an overall increase was also observed for Bacteriodetes and the unclassified phylum. Bacteriodetes rose from 11.5% (0 months) to levels between 18.3% and 27.9%; while bacteria, which are unclassified at the phylum level, increased from 4.2% (0 months) to levels between 6.3% and 6.9%. Conversely, an

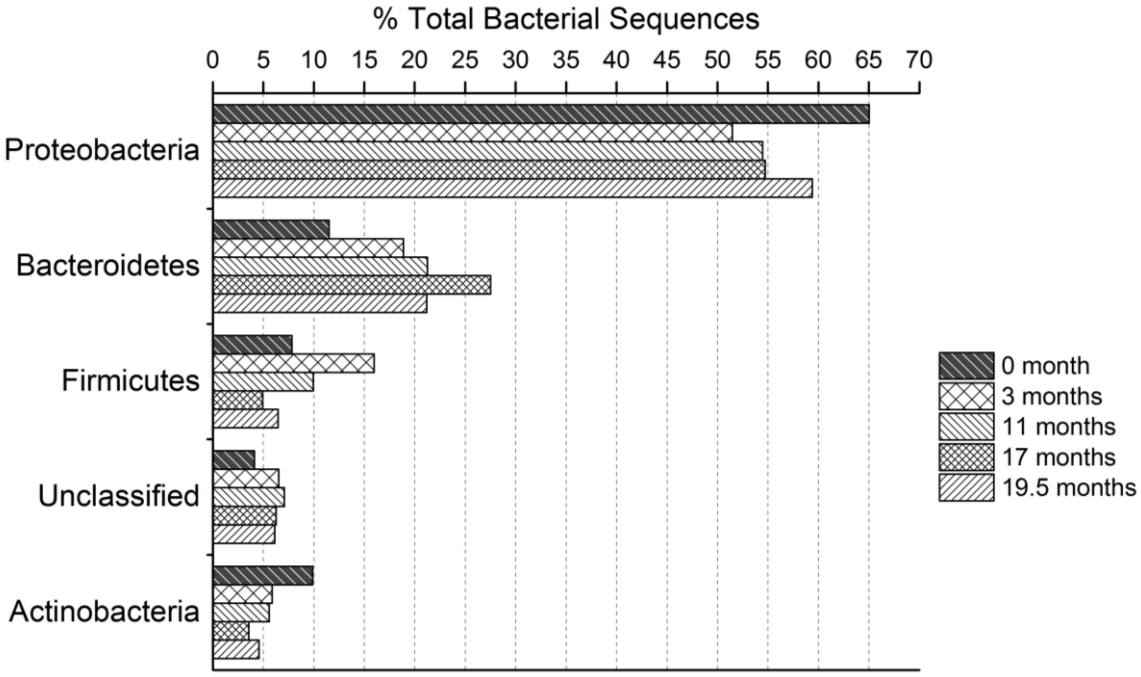


Figure 5.4 Percentage of total bacterial sequences at various styrene enrichment time points (*i.e.*, 0, 3, 11, 17 and 19.5 months) that are assigned to major bacterial phyla. The Unclassified phylum consisted of bacterial sequences that could not be classified into any known phyla.

overall decrease was observed for Actinobacteria and Firmicutes. Actinobacteria decreased from 9.8% (0 months) to levels between 3.5% and 5.9% while Firmicutes decreased from 8.4% (0 months) to levels between 4.9% (19.5 months).

Minor phyla Plantomycetes, Deinococcus-Thermus, Verrucomicrobia, Chloroflexi, Acidobacteria, Aquificae, Chlamydiae, and Gemmatimonadetes each represented less than 1% of the bacterial community (Figure 5.5). Collectively, they formed about 3% of the community. Planctomycetes and Deinococcus-Thermus increased from 0.2% up to 0.8% and 0.6%, respectively. Verrucomicrobia and Acidobacteria were originally undetectable in the sludge inoculum and emerged during the enrichment process at 3 months and 17 months, respectively. After emergence, both phyla persisted at about 0.3% (Verrucomicrobia) and 0.1% (Acidobacteria). Chloroflexi, on the other hand, decreased from 0.6% to 0.2%.

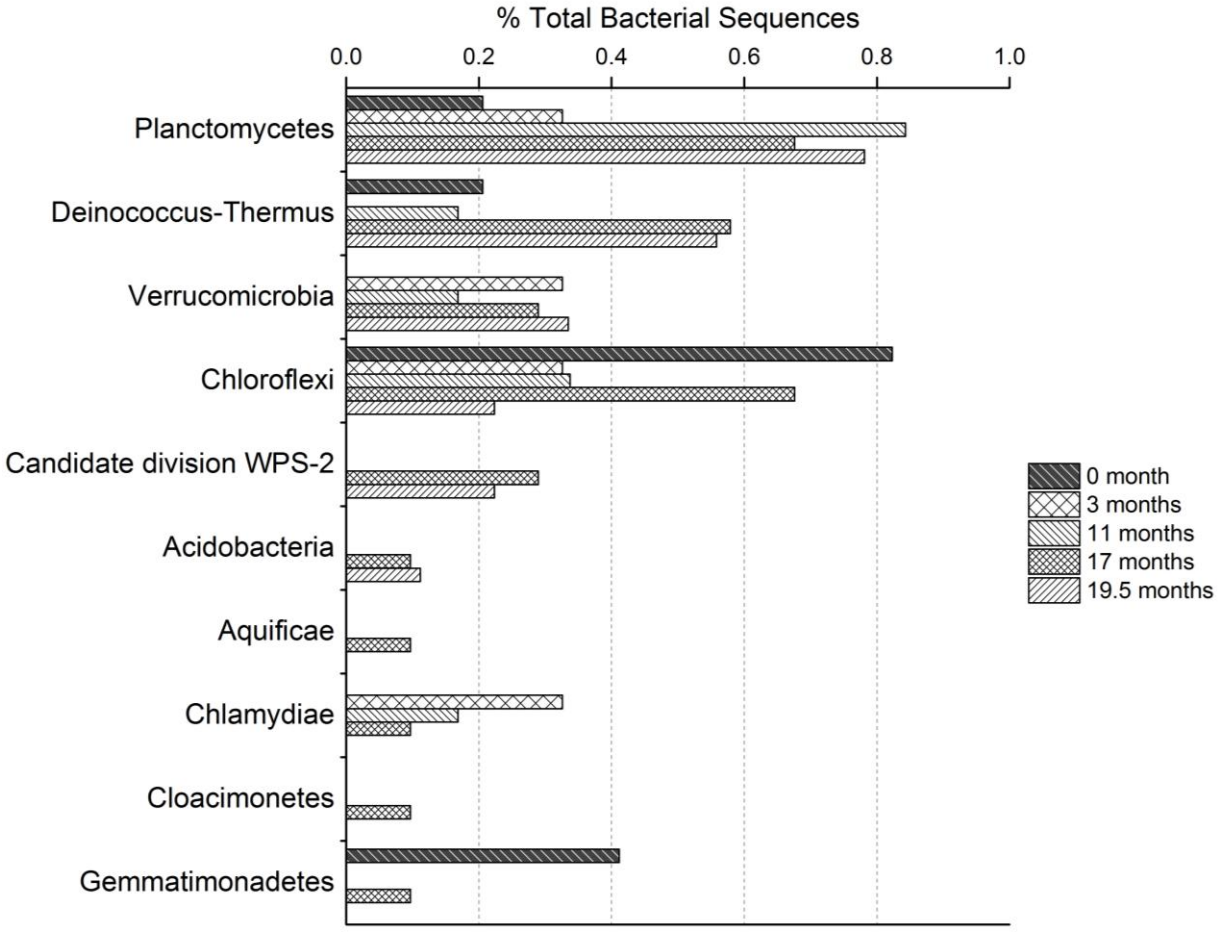


Figure 5.5 Percentage of total bacterial sequences at various styrene enrichment time points (*i.e.*, 0, 3, 11, 17 and 19.5 months) that are assigned to minor bacterial phyla.

Perturbations such as switching of carbon substrates from BTEXS to styrene and desludging were associated with a transient spike/decrease and emergence/disappearance of some phyla. During the initial enrichment period, exposure of BTEXS-enriched sludge inoculum to styrene as the sole carbon source caused a drop in Proteobacteria (64.9% to 51.6%), surge in Firmicutes (8.4% to 16.3%), emergence of Chlamydiae (0.3%), and disappearance of Deinococcus-Thermus (0.2% to 0%). Desludging at 16 months resulted in a spike in Bacteroidetes (21.7% to 27.7%) and Chloroflexi (0.2% to 0.7%), emergence of Aquificae (0.1%) and Gemmatimonadetes (0.1%), and a slight decrease in Actinobacteria (5.6% to 3.5%) and Planctomycetes (0.8% to 0.7%).

5.3.4 Bacterial community structure analysis at class and order level

Major phyla that increased with styrene enrichment: Proteobacteria and Bacteroidetes

Under the phylum Proteobacteria, bacterial classes Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria and Gammaproteobacteria were detected. While Proteobacteria consistently formed more than half of the sludge bacterial community throughout the enrichment period (Figure 5.4), there was a shift in the dominance of bacterial classes under this phylum. In the BTEXS-enriched sludge inoculum, Alphaproteobacteria and Betaproteobacteria were the two dominant bacterial classes (60.2%) while Gammaproteobacteria was detected at low level of 2.3% (Table 5.2). During styrene enrichment however, Gammaproteobacteria increased by 10-fold to 22.2% and Betaproteobacteria decreased by 3-fold from 28.7% to 8.9% while Alphaproteobacteria decreased slightly but continued to persist at high levels (12.7% to 30.4%), resulting in the dominance of Alphaproteobacteria and Gammaproteobacteria (19.5 months, 43.7%). The increase in Gammaproteobacteria was largely ascribed to the increase in bacterial order Pseudomonadales (0.6% to 16.7%), followed by an unclassified order (0% to 3.4%) and Xanthomonadales (1.3% to 2.1%) (Table 5.2). The decrease in Betaproteobacteria was attributed to the decrease in the order Burkholderiales (24.5% to 6.8%). Alphaproteobacteria was represented by the order Rhizobiales (between 6.9% and 20.7%), Sphingomonadales (between 0.9% and 2.8%), and an unclassified order (between 1.0% and 6.4%). Under styrene enrichment, there was also a slight increment in Deltaproteobacteria (0.6% to about 2.0%) with the order Myxococcales emerging and forming 1.6% of the total bacterial population at 19.5 months. Epsilonproteobacteria and its corresponding order Campylobacterales were initially undetectable in the sludge inoculum, but emerged and persisted at about 0.1%. For Bacteroidetes, classes Bacteroidia, Sphingobacteria, Flavobacteria and an unclassified order were detected. Among these classes, the increase in Sphingobacteria and its order Sphingobacteriales from 4% to levels between 8.5% and 23.7% (Table 5.2) were observed to be most reflective of the increase in Bacteroidetes.

Table 5.2 Bacterial sequences assigned to classes and orders under phyla that increased with styrene enrichment.

Phylum	Class	Order(s)[1]	Total Bacterial Sequences in Sample (%)				
			0 month	3 months	11 months	17 months	19.5 months
Proteobacteria	Alphaproteobacteria		31.6	12.7[4]	19.3	30.4[4]	21.5
		Rhizobiales	24.3	6.9[4]	13.1	20.7[4]	17.0
		Unclassified[2]	3.8	1.0[4]	3.2	6.4[4]	3.2
		Sphingomonadales	1.9	2.6[4]	1.7	2.8[4]	0.9
	Betaproteobacteria		28.7	16.0[4]	15.3	9.0	8.9
		Burkholderiales	24.5	15.0[4]	11.5	7.5	6.8
	Gammaproteobacteria		2.3	17.3[4]	10.8	9.0[4]	22.2
		Pseudomonadales	0.6	14.1[4]	7.3	5.7[4]	16.7
		Xanthomonadales	1.3	2.3	1.4	1.0[3]	2.1
		Unclassified[1]	0.0	0.7	1.7	2.1	3.4
	Deltaproteobacteria		0.6	2.0	2.0	1.2[3]	2.3
		Myxococcales	0.0	0.7	0.8	0.3[3]	1.6
	Epsilonproteobacteria		0.0	0.0	0.2	0.1	0.1
		Campylobacterales	0.0	0.0	0.2	0.1	0.1
Bacteroidetes	Sphingobacteria		4.0	8.5	14.9	23.7[4]	17.5
		Sphingobacteriales	4.0	8.5	14.9	23.7[4]	17.5
	Bacteroidia		2.9	5.9	2.5	1.2	1.2
		Bacteriodales	2.9	5.9	2.5	1.2	1.2
	Flavobacteria		2.7	1.3	1.5	0.5[4]	1.2
		Flavobacteriales	2.7	1.3	1.5	0.5[4]	1.2
	Unclassified[3]		1.9	2.6	2.7	2.3	1.5

Table 5.2 *Cont.*

Planctomycetes	Planctomycetacia		0.2	0.3	0.8	0.7[4]	0.8
		Planctomycetales	0.2	0.3	0.8	0.7[4]	0.8
Deinococcus-Thermus	Deinococci		0.2	0.0[4]	0.2	0.6	0.6
		Deinococcales	0.2	0.0[4]	0.2	0.6	0.6
Verrucomicrobia	Opitutae		0.0	0.3	0.2	0.2	0.1
		Opitutales	0.0	0.3	0.2	0.2	0.1
Acidobacteria	Acidobacteria Gp3		0.0	0.0	0.0	0.1	0.1
		incertae sedis	0.0	0.0	0.0	0.1	0.1

[1] Consisted of dominant bacterial order(s) detected for each given bacterial class

[2] Consisted of bacterial sequences that could be classified into a bacterial class but could not be further classified into any known orders

[3] Consisted of bacterial sequences that could be classified into a bacterial phlyum but could not be further classified into any known classes

[4] Transient changes in percentage of total bacterial sequences (*i.e.*, increase, decrease and disappearance) deemed as associated with perturbations due to switching of carbon substrates from BTEXS to styrene at 0 month and desludging at 16 months

Minor phyla that increased with styrene enrichment: Planctomycetes, Deinococcus-Thermus, Verrucomicrobia and Acidobacteria

The phyla Planctomycetes, Deinococcus-Thermus and Acidobacteria were each represented by a single bacterial class and its corresponding bacterial order. Planctomycetes was represented by the class Planctomycetacia and type order Planctomycetales; Deinococcus-Thermus was represented by the class Deinococci and order Deinococcales; and Acidobacteria was represented by the class Acidobacteria Gp3 and order incertae sedis. Under the phylum Verrucomicrobia, the main bacterial class and order detected was Opitutae and Opitutales, respectively, which emerged at 3 months and persisted at levels of around 0.1% to 0.2% (Table 5.2).

Major phyla that decreased with styrene enrichment: Firmicutes and Actinobacteria

Clostridia and Bacilli were the two main bacterial classes identified under phylum Firmicutes. Clostridia and its representative order Clostridiales decreased slightly from 6.1% to 4.8% and from 5.9% to 4.7%, respectively (Table 5.3). Conversely, Bacilli and its representative order Bacillales remained at constant levels ranging from 0.7% to 2% and 0.6% to 1.5%, respectively. For the Actinobacteria phylum, most bacteria belonged to the order Actinomycetales, which exhibited a gradual decrease from 9.8% to 3.8% under styrene enrichment (Table 5.3).

Minor phylum that decreased with styrene enrichment: Chloroflexi

The bacterial class Thermomicrobia and an unclassified class were detected under Chloroflexi phylum. A slight decrease from 0.2% to 0.1% was observed for Thermomicrobia and its order Sphaerobacterales while the unclassified bacteria decreased from 0.4% to 0.1% (Table 5.3).

5.4 Discussion

Mixed cultures have been long and widely-applied for the bioremediation of styrene wastewaters (Ahmad et al., 2008; Fallah et al., 2010; US EPA, 1974). However, to date, studies on styrene-degrading microbial consortia were largely based on

Table 5.3 Bacterial sequences assigned to classes and orders under phyla that decreased with styrene enrichment.

Phylum	Class	Order(s)[1]	Total Bacterial Sequences in Sample (%)				
			0 month	3 months	11 months	17 months	19.5 months
Firmicutes	Clostridia		6.1	12.7[3]	6.8	3.9[3]	4.8
		Clostridiales	5.9	12.4[3]	6.6	3.6[3]	4.7
	Bacilli		1.3	1.0[3]	2.0	0.7[3]	0.8
		Bacillales	0.8	1.0	1.5	0.6[3]	0.8
Actinobacteria	Actinobacteria		9.8	5.9	5.6	3.5[3]	4.5
		Actinomycetales	9.8	5.9	5.3	3.1[3]	3.8
Chloroflexi	Thermomicrobia		0.2	0.3[3]	0.2	0.4[3]	0.1
		Sphaerobacterales	0.2	0.3[3]	0.2	0.3[3]	0.1
	Unclassified[2]		0.4	0.0[3]	0.0	0.2[3]	0.1

[1] Consisted of dominant bacterial order(s) detected for each given bacterial class

[2] Consisted of bacterial sequences that could be classified into a bacterial phlyum but could not be further classified into any known classes

[3] Transient changes in percentage of total bacterial sequences (*i.e.*, increase, decrease, disappearance and emergence) deemed as associated with perturbations due to switching of carbon substrates from BTEXS to styrene at 0 month and desludging at 16 months

non-aqueous and semi-aqueous environments such as soil, biofilter and biotrickling filters (Alexandrino et al., 2001; Arnold et al., 1997; Greene et al., 2000; Portune et al., 2015). Little is known about the composition of styrene-degrading microbial community as well as the potential PHA-producing members in aqueous environments. This study was conducted to address the knowledge gap through investigating the changes in the microbial community structure of activated sludge under long-term styrene enrichment.

Validation of styrene degradation capability in enriched sludge

Styrene is considered a relatively recalcitrant compound, requiring specialized microbial consortia for its biodegradation. Compared to enriched mixed cultures, non-enriched cultures tend to exhibit a lag phase in styrene degradation and greater sensitivity to the toxic effects of styrene due to the lack of microbial species possessing styrene metabolism capabilities (Babaee et al., 2010). This could be circumvented through long-term exposure of mixed cultures to styrene, providing a favorable selection pressure for styrene-degrading microbial species. For this study, municipal activated sludge, previously enriched on BTEXS for 15 months, was used as an inoculum source for styrene enrichment. After 16 months of enrichment, 300 VSS $mg.L^{-1}$ of styrene-enriched sludge was capable of removing 1.62 mg of styrene within 10 h, achieving 98.4% of total styrene removal (Figure 5.1A). The observed styrene degradation rate was higher when compared to a similar study, which reported 6 days for the degradation of 1.7 mg styrene using 650 $mg.L^{-1}$ of petrochemical activated sludge (Babaee et al., 2010); but was comparable to another study which reported the degradation of 1.07 mg to 2.16 mg of styrene within 4 to 8 h using 2.5 g of biomass from an active styrene filter bed (Jorio et al., 2005). Hence, the mixed culture, cultivated through the course of this study, could be considered as an active styrene-degrading consortium.

Negative control setup, containing double-autoclaved sludge, and blank control setup, without any sludge addition, had up to a 50% decrease in styrene amount (Figures 5.1B and 5.1C). However, unlike the test setup containing enriched sludge, there was no further decrease in styrene amounts for control setups. Similar

phenomenon has also been observed previously (Jorio et al., 2005), suggesting that styrene could be lost through abiotic means, which may include adsorption onto cellular material, glass wall interface of serum bottle and reaction with Telfon-lined spetum.

Overview of bacterial community

The initial bacterial community structure of the BTEXS-enriched sludge inoculum and its evolution over the course of styrene enrichment was investigated through samples taken at 0, 3, 11, 17 and 19.5 months into the enrichment. The application of next-generation sequencing on the Illumina MiSeq platform (Illumina Inc, San Diego, CA, USA) yielded sequencing depths ranging from 84,942 to 195,383 sequences, and detected between 306 and 1,033 OTUs (Table 5.1). While rarefaction curve, richness abundance estimators and Good's coverage values indicated that the bacterial diversity were not completely captured in this study, this is a common observation across many studies (Claesson et al., 2010; Jiang et al., 2013; Portune et al., 2015; Schloss et al., 2009). Nevertheless, the high throughput nature of next-generation sequencing achieved between 99.4% and 99.9% of sample coverage, providing a near-complete OTU information that should sufficiently reflect the bacterial consortia.

At the superficial level, there were no major changes to the types of phyla detected between the sludge inoculum (*i.e.*, 0 month) and styrene-enriched samples (*i.e.*, 3, 11, 17 and 19.5 months). It was observed that 97% of the bacterial community was consistently dominated by the major phyla Proteobacteria, Bacteriodetes, Firmicutes, Actinobacteria, and an unclassified phylum (Figure 5.4). The remaining 3% of the bacterial community comprised of minor phyla Plantomycetes, Deinococcus-Thermus, Verrucomicrobia, Chloroflexi, Acidobacteria, Aquificae, Chlamydiae, and Gemmatimonadetes (Figure 5.5). Emergence and disappearance of phyla were detected, but these instances were only observed in minor phyla (*i.e.*, Deinococcus-Thermus, Verrucomicrobia, Acidobacteria, Aquificae, Chlamydiae, and Gemmatimonadetes). Despite the seemingly constant phyla composition, beta analysis, based on Bray-Curtis dissimilarity, revealed that the initial BTEXS-

enriched bacterial community had evolved into a different community within 3 months of styrene enrichment, and continued evolving under prolonged enrichment (Figure 5.3). In line with this observation, was an overall increase in species richness (Chao 1: 1,143 to 2,322; ACE: 1,658 to 4,632) and therefore, an increase in bacterial diversity (Inverse Simpson: 6.39 to 10.33; Shannon diversity: 2.34 to 2.96). One of the contributing factors may have been the rise in the abundance of bacterium that could not be assigned to a phylum (unclassified phylum: 4.2% to 7%) (Figure 5.4) or an order (unclassified [Gammaproteobacteria]: 0% to 3.4%) (Table 5.2). The increase in unassigned bacteria sequences was also observed in styrene-degrading biofilter, and could form up to 41% of the bacterial community (Portune et al., 2015). The findings, from this study and the recent study Portune et al. (2015), indicated that there is currently much unknown bacterial information associated with styrene-degrading consortia.

Phyla that increase under styrene enrichment

Shifts in the relative abundance of classifiable phyla were also observed and changes in the bacterial community structure became more apparent at lower taxonomic levels such as class and order. Specifically, the major phyla Proteobacteria and Bacteroidetes, and minor phyla Planctomycetes, Deinococcus-Thermus, Verrucomicrobia and Acidobacteria were observed to increase with styrene enrichment, suggesting that these phyla probably play an important role in styrene degradation and/or flourish under the given condition. Throughout the enrichment period, more than half of the bacteria consortium was dominated by the phylum Proteobacteria. Nonetheless, a sharp decrease from 64.8% (0 months) to 51.6% (3 months) during the initial start up period was observed before the phylum steadily increased to 59.4% (19.5 months) (Figure 5.4). This was linked to bacterial succession at the class level. Most notably, Betaproteobaceria decreased by nearly 20% and became less dominant while Gammaproteobacteria increased by roughly the same amount to form a more significant population (Table 5.2). Since the sludge inoculum was derived from municipal activated sludge after 15 months of enrichment on BTEXS, these findings suggested that Betaproteobaceria plays a bigger role in BTEXS degradation while Gammaproteobacteria is more important

for styrene degradation. The notion that Betaproteobaceria is associated with BTEX degradation was further supported by previous studies on bacterial consortia in BTEX-contaminated aquifers, which detected Betaproteobaceria at dominant levels between 27% and 75% (Alfreider & Vogt, 2007; Lee et al., 2010). At the order level, Betaproteobacteria was represented by Burkholderiales (Table 5.2). This bacterial order reportedly has widespread aromatic catabolic potential and the highest heterogeneity in the distribution of aromatic catabolic genes among the bacterial orders of Proteobacteria (Pérez-Pantoja et al., 2012; Pérez-Pantoja et al., 2010). Members of Burkholderiales are known to possess enzymes for the direct BTEX metabolism, and their degradation capabilities have been demonstrated in pure cultures (Kanehisa & Goto, 2000; Kanehisa et al., 2014; Pruden & Suidan, 2004). Burkholderiales also harbors genes for metabolism of styrene degradation intermediates, including benzoic acid, catechol, phenylacetyl-CoA and *etc* (Kanehisa & Goto, 2000; Kanehisa et al., 2014; Pérez-Pantoja et al., 2012; Pruden & Suidan, 2004). The broad aromatic catabolic capability of Burkholderiales could be the main reason why it flourished under BTEXS mixture feed and became a more prominent member in the sludge inoculum (24.5%) (Table 5.2). To date, however, there were neither reports of styrene-degrading species (Tischler & Kaschabek, 2012) nor genetic evidence for direct styrene metabolism among Burkholderiales and other Betaproteobacteria members (Kanehisa & Goto, 2000; Kanehisa et al., 2014), which could explain their reduced dominance when styrene became the sole carbon source (Betaproteobacteria, 8.9%; Burkholderiales, 6.8%).

Gammaproteobacteria was largely represented by Pseudomonadales, which showed the greatest increase (16.1%) among all the bacterial orders detected in the consortium, suggesting its importance in styrene degradation (Table 5.2). Indeed, members of Pseudomonadales, particularly from the genus *Pseudomonas*, are often linked to the styrene removal ability of mixed cultures. In styrene-degrading biofilter, the abundance of *Pseudomonas* was positively-correlated to styrene inlet concentrations, achieving removal efficiencies of 83% to 97% (Portune et al., 2015). SIP labeling, using deuterated styrene, further demonstrated styrene assimilation by a primary styrene-degrading biofilter population with *Pseudomonas*-like fatty acid

profile (Alexandrino et al., 2001). Numerous studies have also reported the isolation of styrene-degrading *Pseudomonas* strains from active biofilter (Arnold et al., 1997), bioreactors (O'Connor et al., 1995; Tobin & O'Connor, 2005) and contaminated environment samples (Greene et al., 2000; Narancic et al., 2012; Tobin & O'Connor, 2005). The styrene degradation pathways were elucidated for *Pseudomonas*, and were found to involved either direct ring cleavage with the generation of 3-vinylcatechol intermediate or side-chain oxidation with the generation of phenylacetaldehyde intermediate (Tischler & Kaschabek, 2012). Collectively, this information provides strong evidence that Pseudomonadales members form the core styrene-metabolizing bacterial population in this study's enriched activated sludge.

In contrast to Betaproteobacteria and Gammaproteobacteria, Alphaproteobacteria persisted at high levels under both BTEXS feed (31.6%) and styrene feed (12.7% to 30.4%) (Table 5.2). This result is in good agreement with existing findings. Independent studies on consortia, exposed to BTEX and styrene, have similarly reported the abundance of Alphaproteobacteria at levels between 20% and 30% (Lee et al., 2010; Portune et al., 2015). Alphaproteobacteria was most prominently-represented by the order Rhizobiales (between 17.0% and 24.3%). Aside from Rhizobiales, the order Sphingomonadales was also detected, albeit at much lower abundance between 0.9% and 2.8%. Genome analysis have unraveled high aromatic catabolism versatility among Rhizobiales and Sphingomonadales members, particularly in families Bradyrhizobiaceae, Xanthobacteraceae, Hyophomonadaceae, Phyllobacteriaceae, and Sphingomonadaceae (Pérez-Pantoja et al., 2010), which were also the bacterial families detected in this study (data not shown). Catabolic genes include those involved in the direct and indirect metabolism of BTEXS (Kanehisa & Goto, 2000; Kanehisa et al., 2014; Pérez-Pantoja et al., 2010), which most likely enhanced Rhizobiales' and Sphingomonadales' adaptability to BTEXS mixture feed. When the substrate feed was switched to styrene, Sphingomonadales decreased slightly from 1.9% to 0.9% while Rhizobiales remained a prominent population (6.9% to 20.7%) (Table 5.2). Styrene-degrading pure cultures, from both bacterial orders, was previously reported, but were limited to the genera *Xanthobacter* (Rhizobiales) and *Sphingomonas* (Sphingomonadales) (Tischler &

Kaschabek, 2012). Both genera were not detected at high abundances throughout the enrichment period (between 0% and 0.3%, data not shown). Instead, the bulk of the Alphaproteobacteria members detected, particularly from Rhizobiales, are currently not known to assimilate styrene directly. While Rhizobiales is most well-known for its nitrogen-fixing property and is typically associated with plant as a root-nodule endosymbiont (Masson-Boivin et al., 2009), there are emerging genetic evidence hinting at its involvement in direct styrene degradation. Intriguingly, 51% polypeptide identity was found between NodW, a root nodulation response regulator, and StyR, a response regulator responsible for up-regulating styrene degradation genes (O'Leary et al., 2002). Another study identified a novel functional *smoA* gene, from loam soil metagenome, which showed 95% genetic similarity to uncharacterized homologs from Rhizobales; the gene encodes styrene monooxygenase, which is an enzyme that catalyzes the epoxidation of styrene, making the compound more liable to degradation (van Hellemond et al., 2007). This genetic evidence, coupled with Rhizobiales' dominant presence in styrene-enriched sludge, strongly suggests that Rhizobiales could be part of a core styrene-degrading population alongside Pseudomonadales. However, further studies, focusing on the function of Rhizobiales, would be required to confirm this theory.

Bacteroidetes formed the second largest bacterial population after Proteobacteria and was detected at 21.4% following 19.5 months of enrichment (Figure 5.4). Under Bacteroidetes, the class Sphingobacteria and its order Sphingobacteriales was initially detected at 4%, but increased by more than 13%, and became as equally dominant as Rhizobiales and Pseudomonadales (*i.e.*, 17.5%) (Table 5.2). Styrene-degrading capability has been reported for the genus *Sphingobacterium* (Sphingobacteriales) (Przybulewska et al., 2006), but *Sphingobacterium* existed at non-dominant levels (0.1% to 0.3%, data not shown), suggesting that it is not the main styrene-degrading population in this study. Strikingly, the abundance of Bacteroidetes and Sphingobacteria in styrene-enriched sludge was much higher than what was previously reported in styrene-degrading biofilter (Bacteroidetes, about 2%; Sphingobacteria, less than 1%) (Portune et al., 2015). Sphingobacteriales have also been identified in BTEX-contaminated groundwater (22% to 23%) (Lee et al.,

2010), phenol-degrading reactors (Basile & Erijman, 2010), soil contaminated with cyclic explosive compound hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (about 15%) (Jayamani et al., 2013), and sites polluted by polychlorinated biphenyls (PCBs) (Luo et al., 2008). Genes involved in the catabolism of styrene metabolites homogentisate and fumarylacetoacetate (*i.e.*, homogentisate 1,2-dioxygenase and fumarylacetoacetate hydrolyase), phenolic compounds (*i.e.*, oxidoreductases, 2-keto-4-pentenoate hydradatase and 2-oxohepta-3-ene-1,7-dioic acid hydratase) and benozate (*i.e.*, protocatechuate 3,4-dioxygenase) were identified, while oxidioreductases-encoding genes involved in the catabolism of biphenyl, toluene and xylene were predicted from Sphingobacteriales genomes (Kanehisa & Goto, 2000; Kanehisa et al., 2014; Ntougias et al., 2014). These findings imply that Sphingobacteriales is genetically-primed to adapt and survive on various aromatic and cyclic hydrocarbon compounds. Nevertheless, it is unclear why there is a huge discrepancy of about 20% between the abundance of Bacteroidetes/Sphingobacteria detected in this study and peat-inoculated styrene-degrading biofilter (Portune et al., 2015). Inoculum abundance and richness did not appear to be a contributing factor. Based on existing reports, the abundance of Bacteroidetes and Sphingobacteria in peat (Bacteroidetes, 0% to 3.6%; Sphingobacteria, 0% to 3.3%) and air samples (Bacteroidetes, 5% to 20%; Sphingobacteria, 0% to 6%) (Bowers et al., 2011; Sun et al., 2014) were similar to this study's sludge inoculum (Bacteroidetes, 11.5%; Sphingobacteria, 4.9%) (Figure 5.4 and Table 5.2). Higher richness was also observed in peat (Chao1: 5,291 to 17282; ACE: 9,388 to 32,330) (Sun et al., 2014) compared to this study's sludge inoculum (Chao1: 1,143; ACE: 1,658) (Table 5.1). Hence, it could be that Bacteroidetes/Sphingobacteria has a higher proliferation rate in aqueous environment or the group benefited from a favorable synergism within aqueous consortium. More studies would be needed to address these speculations.

Proteobacteria members such as Xanthomonadales (Gammaproteobacteria; 1.3% to 2.1%), Myxococcales (Deltaproteobacteria; 0% to 1.6%) and Campylobacterales (Epsilonproteobacteria; 0% to 0.1%) were detected at low levels but found to emerge/increase under styrene enrichment. Similar trend was also observed for some minor bacterial phyla and their corresponding orders. This includes the

phyla/orders Planctomycetes/Planctomycetales (0.2% to 0.8%), Deinococcus-Thermus/Deinococcales (0.2% to 0.6%), Verrucomicrobia/Opitutales (0% to 0.1%) and Acidobacteria/incertae sedis (0% to 0.1%) (Table 5.2). Xanthomonadales members, particularly from the genus *Stenotrophomonas*, are known to degrade styrene and BTEX (Dao et al., 2014; Lee et al., 2002). However, *Stenotrophomonas* formed only about 0.2% of the consortium (data not shown). While existing studies show a positive relationship between *Stenotrophomonas* abundance and styrene inlet concentrations, *Stenotrophomonas* remained at low levels of 2% or less (Juneson et al., 2001; Portune et al., 2015). The low abundance of *Stenotrophomonas* suggests that it is not part of the main styrene-degrading population. Furthermore, majority of the Xanthomonadales members detected belonged to the genus *Dokdonella* (1.2%, data not shown), which are not known to be styrene-degrading. Bacterial orders from Deltaproteobacteria (*i.e*, Myxococcales) and minor phyla (*i.e*, Planctomycetales, Deinococcales, and Acidobacteria Gp3 incertae sedis) are also not known to assimilate styrene, but their presence have been noted in styrene-degrading and phenol-degrading mixed cultures, and BTEX-contaminated sites (Feris et al., 2004; Lee et al., 2010; Portune et al., 2015; Silva et al., 2012). Species from some of these orders reportedly harbor aromatic catabolism pathways. *Arcobacter* sp. (Campylobacterales) possesses the enzymes catechol 2,3-dioxygenase and 2-hydroxymuconate-semialdehyde hydrolase, which are involved in the breakdown of styrene metabolite 3-vinylcatechol (Kanehisa & Goto, 2000; Kanehisa et al., 2014). A recent analysis on the core metabolic pathway of eleven type strains, from the order Planctomycetales, revealed genetic potential for the metabolism of diverse aromatic compounds including ethylbenzene, toluene, benzoate, and *etc*, as well as dispensable pathways for styrene degradation (Guo et al., 2014). Since the catabolism pathways for many aromatic compounds are known to share common metabolites and many ring-cleavage enzymes have broad substrate-specificity (Pérez-Pantoja et al., 2010), it is plausible that the emergence and/or increase of these bacterial orders was due to their ability to utilize styrene degradation intermediates, generated as a result of styrene metabolism by the core styrene-degrading population, such as Pseudomonadales, within the consortium. These styrene metabolites include phenylacetate and homogentisate generated from

the side chain oxidation pathway; 3-vinylcatechol, generated from the direct ring cleavage pathway; and more downstream metabolites such as benzoate, phenylalanine, Kreb Cycle intermediates, which could support non-styrene-assimilating sub-populations within the consortium (Kanehisa & Goto, 2000; Kanehisa et al., 2014; Tischler & Kaschabek, 2012).

Phyla that decrease under styrene enrichment

Major phyla Firmicutes and Actinobacteria, and minor phylum Chloroflexi initially occur in BTEXS-enriched inoculum at 8.4%, 9.8% and 0.6%, respectively, but decreased by about 0.4% to 5% with styrene enrichment, suggesting that these phyla may play a less important role in styrene degradation (Figure 5.4). Within Firmicutes, the classes Clostridia and Bacilli were detected. It was interesting to note that most of the Firmicutes members belonged to Clostridia (4.8%), which is a class of obligate anaerobes (Table 5.3). Clostridia was also reported at levels of 1% to 2% in styrene-degrading filter (Portune et al., 2015). Its presence indicates that despite aeration, the dissolved oxygen content in styrene-degrading systems may be low. Dissolved oxygen contents, associated with active styrene-degrading system or aromatic-contaminated sites, were detected at about 50% to 90% lower than non-active system and non-polluted sites (Lee et al., 2010; Nikodinovic-Runic et al., 2011). This is probably because the aerobic degradation of styrene and other aromatic compounds is typically initiated by the addition of oxygen atoms to the aromatic ring or side chains (Pérez-Pantoja et al., 2010; Tischler & Kaschabek, 2012), resulting in rapid oxygen consumption and therefore, lower dissolved oxygen contents. In this study, the lack of stirring could have also given rise to pockets of anaerobic zones within the system, favoring Clostridia's survival. Clostridia was represented by the order Clostridiales. (Grbić-Galić et al., 1990) reported the isolation of non-styrene-assimilating *Clostridium* species (Clostridiales) from styrene-degrading anaerobic consortia as well as the detection of styrene degradation compounds phenylacetic acid, benzyl alcohol, benzaladehyde, benozate and phenol in the cultivation medium. Members of Clostridiales are known to harbor xenobiotic compound-degrading enzymes that could assimilate the aforementioned compounds. This include benzoyl-CoA reductase and

3-hydroxyacyl-CoA dehydrogenase, which participate in the metabolism of toluene, and styrene metabolites benzoate and phenylalanine (Carmona et al., 2009; Kanehisa & Goto, 2000; Kanehisa et al., 2014; Shah et al., 2013). Hence, this could account for Clostridiales' adaptability and presence in styrene-degrading microbial community.

The classes Bacilli (Firmicutes) and Acintobacteria (Actinobacteria) were detected at levels ranging from 0.7% to 2% and 3.5% to 5.9%, respectively (Table 5.3). Both classes were also identified in styrene-degrading biofilters and at levels similar to this study (Alexandrino et al., 2001; Portune et al., 2015). Aromatic-degrading ability was reported for pure cultures from both classes. Specifically, Bacilli members could assimilate benzene, toluene and xylene (Jacques et al., 2007). Actinobacteria members could metabolize a wide range of aromatics including BTEX, catechol, PAH, biphenyl, and napthalene (Hori et al., 2009b; Jacques et al., 2007; Kämpfer, 2010), which could explain their higher abundance in BTEXS sludge inoculum (9.8%) (Table 5.3). Styrene degradation has also been reported in the genera *Rhodococcus*, *Mycobacterium*, *Tsukamurella* (Actinobacteria) and *Bacillus* (Bacilli) (Arnold et al., 1997; Burback & Perry, 1993; Jung & Park, 2005; Warhurst et al., 1994). However, *Bacillus* was not detected in this study whereas all known styrene-degrading species from Actinobacteria were detected at low levels (0% to 0.3%) (data not shown). Therefore, known styrene degraders from Actinobacteria and Bacilli did not appear to be a core styrene-degrading population. Similar findings were also previously reported by Alexandrino et al. (2001). The researchers used SIP method to investigate a styrene-degrading biofilter, and failed to observe styrene assimilation by Bacilli and Acintobacteria despite being presence in the biofilter. Taken together, the findings from this work and previous work indicated that Bacilli and Actinobacteria does not appear to be the primary degraders in styrene-degrading consortium.

The minor phylum Chloroflexi is not known to degrade styrene but have been noted in styrene-degrading biofilter (1% to 7%) (Portune et al., 2015), phenol-degrading sludge (Silva et al., 2012), anaerobic benzene-degrading environment (Herrmann et

al., 2008), PAH-contaminated groundwater site (23%) (Winderl et al., 2008). Genes mapped to styrene, toluene and benzoate degradation have also been identified in Chloroflexi (Kanehisa & Goto, 2000; Kanehisa et al., 2014).

Collectively, Firmicutes, Actinobacteria, and Chloroflexi members, detected in this study, are frequently associated with environs exposed to aromatic compounds. Despite their apparent limited potential for direct styrene assimilation, they possess versatile catabolic potential for the metabolism of many other mono-aromatic compounds. This suggests that they could be secondary degraders, who are responsible for the mineralization of styrene degradation by-products. More studies will be needed to clarify their functional roles.

Potential PHA-producing members

The PHA bioaccumulation trait is widespread within the bacterial domains and occurs in species from more than 100 bacterial genera (Koller et al., 2010). In this study, many of these bacterial genera were detected in the styrene-enriched sludge. These genera include *Pseuodmonas* and *Acinetobacter* (Pseudomonadales, Gammaproteobacteria), *Hyphomicrobium*, *Methylobacterium*, *Nitrobacter*, *Rhizobium*, *Rhodopseudomonas* and *Xanthobacter* (Rhizobiales, Alphaproteobacteria), *Paracoccus* (Rhodobacterales, Alphaproteobacteria), *Azospirillum* (Rhodospirillales, Alphaproteobacteria), *Comamonas*, *Cupriavidus* and *Thiobacillus* (Burkholderiales, Betaproteobacteria), *Rhodococcus* (Actinomycetales, Actinobacteria), and *Clostridium* and *Syntrophomonas* (Clostridales, Clostridia). While many of these genera belonged to orders, which dominated the system and formed about 41% to 56.5% of the styrene-degrading consortium (data not shown), the total abundance of these genera were only 2.8% to 5.9% (data not shown). Nonetheless, it was also observed that there were a total of 13.5% to 24.5% bacterial sequences, which belonged to the same family as the aforementioned genera but could not be classified at the genus level (data not shown). These unclassified sequences could have resulted in an underestimation of these genera. In a study by Portune et al. (2015), the authors found that the unclassified sequences within the family *Pseudomonadaceae* shared high sequence identity to genus *Pseudomonas*. Hence, it may be possible that the genera, with

PHA-producing potential, are higher than the levels detected. It should be noted, however, that there are variation in PHA biosynthesis abilities among species from the same genus, which could in turn be influenced by predisposition of PHA synthesis genes, carbon source and nutrients conditions (Choi & Lee, 1999). As such, this study is unable to provide conclusive identification of the PHA producers. Future studies would be needed to assess the PHA accumulation capability and to identify the PHA producers of styrene-enriched sludge.

5.5 Summary

This study investigated the bacterial community structure of a styrene-degrading activated sludge. BTEXS-enriched sludge served as the sludge inoculum for styrene enrichment, and changes in bacterial composition over a 19.5-month period was documented using next-generation 16S rDNA sequencing technique. Styrene exposure increased species richness (Chao 1: 1,143 to 2,322; ACE: 1,658 to 4,632), bacterial diversity (Inverse Simpson: 6.39 to 10.33; Shannon diversity: 2.34 to 2.96) and altered the bacterial profile. The most abundant phylum was Proteobacteria, which consistently made up more than half of the consortium (51.6% to 64.9%). However, most strikingly, a shift in the dominance of Proteobacteria classes from Alphaproteobacteria and Betaproteobacteria (0 month: 60.2%) to Alphaproteobacteria and Gammaproteobacteria (19.5 months: 43.7%) was observed under prolonged styrene enrichment. The shift was due to the decreased abundance of Betaproteobaceria (28.7% to 8.9%) and increased abundance of Gammaproteobacteria (2.3% to 22.2%), specifically, the well-known styrene-degrading bacterial order Pseudomonadales (0.6% to 16.7%). Alphaproteobacteria was represented by the order Rhizobiales (6.9% to 20.7%), which harbors putative styrene catabolic *smoA* gene. Hence, Pseudomonadales and Rhizobiales probably form the primary styrene-degrading bacterial population in activated sludge.

Aside from Proteobacteria, 11 other bacterial phyla and 1 unclassified phylum were also detected. Amongst them, the presence of Aquificae, Chlamydiae, and Gemmatimonadetes was transient and low (0% to 0.3%). Their temporary existence in the consortium appeared to be associated with perturbations which include the

switch in carbon substrates from BTEXS to styrene (between 0 and 3 months) and desludging (16 months). Hence, these phyla are not considered to be part of the stable bacterial population in this study. An overall increase in abundance was observed for Bacteriodetes (11.5% to 21.4%), Planctomycetes (0.2% to 0.8%), Deinococcus-Thermus (0.2% to 0.6%), Verrucomicrobia (0% to 0.3%), Acidobacteria (0% to 0.1%) and the unclassified phylum (4.2% to 6.3%), which suggested that this study's styrene-degrading environ was favorable to these phyla. In contrast, an overall decrease in abundance was noted for Firmicutes (8.4% to 6.3%), Actinobacteria (9.8% to 4.5%) and Chloroflexi (0.6% to 0.2%), suggesting that the same environ was less favorable to these phyla. Some genera with known styrene-degrading species were identified in this study including the Bacteroidetes genus *Sphingobacterium* and Actinobacteria genera *Rhodococcus*, *Mycobacterium* and *Tsukamurella*. However, these genera were detected at very low levels between 0% and 0.3%, which meant that they are not the core styrene-degraders in this system. Aside from these species, there is limited evidence on direct styrene metabolism for most members of the aforementioned phyla. Nevertheless, many literatures have reported the presence of these members in aromatic-degrading consortia and aromatic-contaminated environs, as well as their diverse aromatic catabolic capability and potential. Hence, it is likely that these members assimilate the styrene metabolites, and through synergistic degradation, work alongside the styrene-degraders to achieve complete mineralization of styrene.

This study presents the first reference of the styrene-degrading bacterial community in aqueous environment, providing a knowledge base to facilitate the selection of seeding sludge for similar bioprocesses. Specifically, bacterial orders Pseudomonadales and Rhizobiales are postulated as key to the styrene biodegradation capability of the microbial community, and should be considered as important biomarkers. Sphingobacteriales does not appear to form the primary styrene-degrading population but this order of bacteria occurs at much higher abundance in the aqueous consortium as compared to biofilter consortium, suggesting that it could serve as an additional biomarker in aqueous seeding sludge. Other biomarkers include Clostridiales, and Actinomycetales. This study also

identified potential PHA-producing members, notably, bacterial genera from the the dominant bacterial orders Pseudomonadales and Rhizobiales (*Pseuodmonas*, *Acinetobacter*, *Hyphomicrobium*, *Methylobacterium*, *Nitrobacter*, *Rhizobium*, *Rhodopseudomonas* and *Xanthobacter*) and other less dominant or minor bacterial orders Rhodobacterales, Rhodospirillales, Burkholderiales, Actinomycetales, Clostridales (*Paracoccus*, *Azospirillum*, *Comamonas*, *Cupriavidus*, *Thiobacillus*, *Rhodococcus*, *Clostridium* and *Syntrophomonas*). This suggests that PHA-producing members could be native to aqueous styrene-degrading consortium, providing a logical basis for future process optimization, bioaugmentation and formulation of defined microbial cocktails to enhance the biological process for simultaneous styrene elimination and PHA production.

However, this study has several limitations. First, the bacterial community was largely classified at the order level. Beyond the order level and particularly at the genus level, there were a high percentage of unclassified sequences. The same outcome was observed with different taxonomy databases (*i.e.*, SILVA and Ribosomal Database Project [RDP] databases) and at two different commonly-used pseudobootstrap cutoff values (*i.e.*, 60% and 80%) (Gaidos et al., 2011; Mizrahi-Man et al., 2013; Schloss, 2009). Hence, it was not possible to affirm the specific identity for most bacterial community members. As this study is restricted to genomic 16S rDNA analysis, this study could also neither confirm the identity of PHA-producers nor provide conclusive evidence on the functional role of bacterial members. Bacterial isolation study could help provide confirmative information on the identity and role of culturable microbes within styrene-degrading consortium. Furthermore, strategies such as bioaugmentation and creating defined microbial cocktails are greatly dependent on the availability of pure bacterial isolates. Hence, bacterial isolation study could also yield bacterial cultures that could be applied as bioaugmentation strains or microbial cocktail members.

CHAPTER 6

Study 4:
Isolation and Characterization of Styrene-Degrading and MCL-PHA-Accumulating Bacteria from Enriched Activated Sludge

6.1 Introduction

The bioconversion of styrene to PHA has been described in four bacterial strains, all of which belonged to *Pseudomonas*, under the class of Gammaproteobacteria (Tobin & O'Connor, 2005). These bacterial strains are *Pseudomonas* sp. TN301, *P. putida* CA-3 (NCIMB 41162), *P. putida* CA-1, and *P. putida* S12 (Narancic et al., 2012; Tobin & O'Connor, 2005; Ward et al., 2005). These bacterial strains assimilate styrene to form saturated PHA precursors (*R*)-3-hydroxyalkanoates of varying carbon chain length between C_6 to C_{12} and unsaturated $C_{12:1}$ precursors. The polymerization of the aforementioned PHA precursors gave rise to MCL-PHA, through the *de novo* fatty acids synthesis pathway (Hume et al., 2009).

Most of these *Pseudomonas* species accumulate MCL-PHA at low cellular contents of between 3 and 14% CDM. The only exception is *P. putida* CA-3, which can store MCL-PHA up to 33% CDM and 31.8% CDM under shake-flask and fermentor conditions, respectively (Nikodinovic-Runic et al., 2011; Nikodinovic et al., 2008). However, *P. putida* CA-3 is a patented bacterium and is not readily-accessible for further scientific investigation and bioprocess application. Therefore, there is a lack of bacterial strains with the metabolic capacity to bioconvert styrene into MCL-PHA efficiently, necessitating the need to increase the pool of pure microbial cultures.

Chapter 5 has unraveled an aqueous styrene-degrading consortium with diverse bacterial composition. Aside from *Pseudomonas*, potential PHA-producing members were noted. Presently, it is unknown if there may be other bacteria genera, which are capable of converting styrene into MCL-PHA. Hence, further investigation is required. Additionally, this study could provide confirmative information on the identity and role of culturable microbes within mono-aromatic-degrading consortia such as the styrene-degrading consortium.

6.2 Materials and Methods

6.2.1 Isolation of styrene-degrading bacteria

Pure bacterial cultures were isolated from an aerobic bioreactor, seeded with

domestic activated sludge from a local water reclamation plant, at two time points of the enrichment process. The first time point was after 11 weeks of enrichment on 1X MSM supplemented with weekly additions of BTEXS at equimolar concentration of 1.8 mM. The second time point was post 15 months enrichment on BTEXS, followed by an additional 19.5 months enrichment on styrene. Styrene enrichment was performed on 1X MSM supplemented with gaseous styrene at a rate of 0.55 ± 0.03 g.h^{-1} and weekly addition of liquid styrene at 1.8 mM (Chapter 5, Section 5.2.1). The enriched sludge samples were serially diluted (10^{-1} to 10^{-4}) in 1X phosphate buffer saline (PBS) and 100 µL of each dilution was evenly-spread on solid cultivation media. The plates were incubated at 30 °C for 2 to 5 days. Colonies appearing on solid cultivation media were transferred onto fresh media until pure bacterial isolates, as determined by colony morphology and color, were obtained. Bacterial isolation, at the first time point, was performed on 1X MSM containing 1 g.L^{-1} sodium benzoate as carbon source, 16 mM of $(NH_4)_2SO_4$ as nitrogen source, and 1.5% weight/volume (w/v) agar noble as solidifying agent; these bacterial isolates were denoted with the prefix "NBUS". Bacterial isolation, at the second time point, was performed on 1X MSM containing 40 µL styrene as carbon source, supplied by placing a pipette tip on the agar, 16 mM of $(NH_4)_2SO_4$, and either 1.5% (w/v) agar noble or 0.8% (w/v) Gelzan as solidifying agent. Bacterial isolates, obtained from agar noble and Gelzan plates, were denoted with the prefixes "ANSUS" and "GSUS", respectively. Pure bacterial cultures were also isolated from an aerobic bioreactor, seeded with industrial activated sludge from a local petrochemical wastewater treatment facility, after 11 weeks of enrichment on 1X MSM supplemented with BTEXS at equimolar concentration of 1.8 mM. Isolation was performed on 1X MSM containing 1 g.L^{-1} sodium benzoate as carbon source, 16 mM of $(NH_4)_2SO_4$ as nitrogen source, and 1.5% (w/v) agar noble as solidifying agent; these bacterial isolates were denoted with the prefix "NBIWW". To test the styrene-utilization capability of the pure bacterial cultures, isolated on sodium bezonate (*i.e.*, NBUS and NBIWW isolates), a bacterial starter culture for each isolate was cultivated with 1X MSM containing 1 g.L^{-1} of sodium benzoate at 30 °C with shaking at 100 rpm for 2 days. Bacterial culture (10 µL) was pipetted onto 1X MSM agar containing 40 µL styrene supplied by placing a pipette tip on

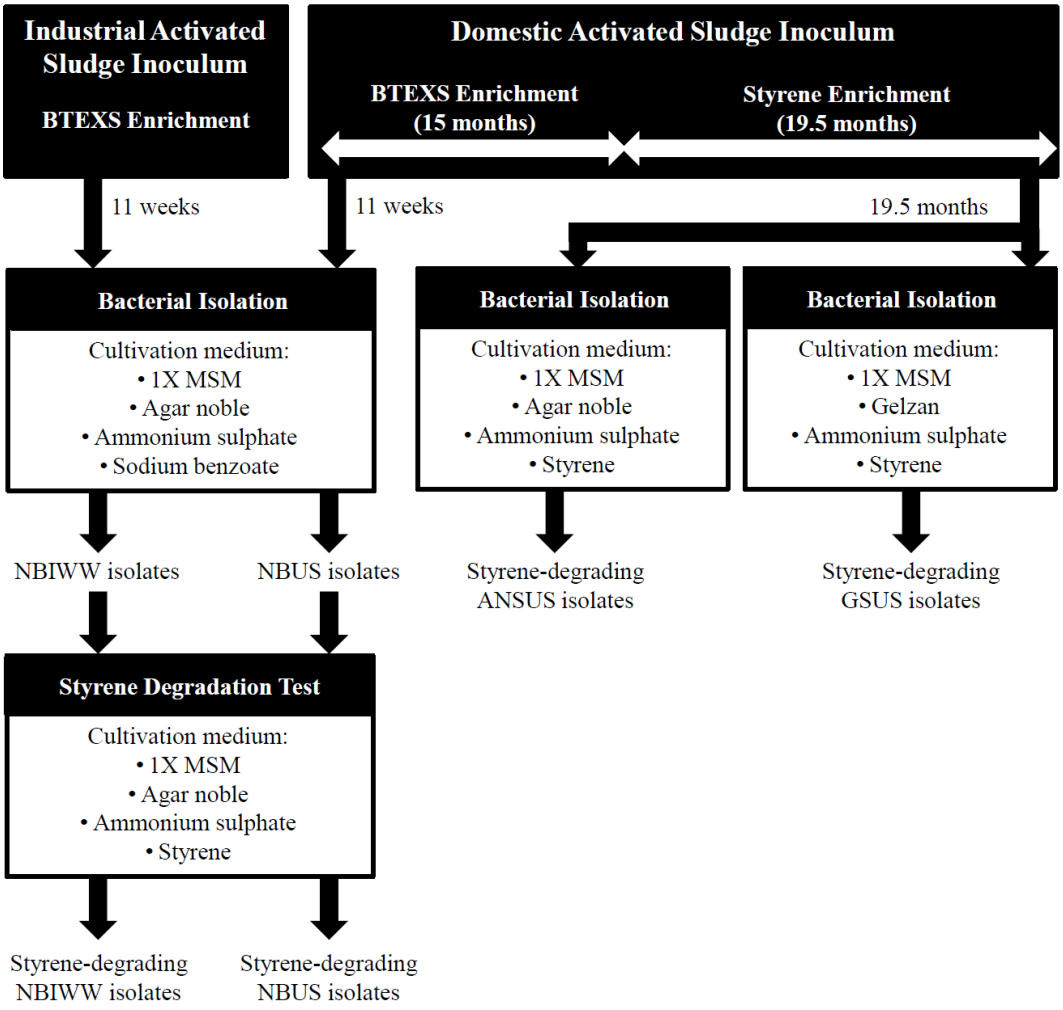


Figure 6.1 Flow diagram of the styrene-degrading bacterial isolation procedure.

the agar. The petri dish was tightly-sealed with parafilm and placed in an air-tight jar at 30 °C for up to 5 days. A negative control setup, without carbon substrate, was included. A summary of the isolation procedure is provided in Figure 6.1.

6.2.2 Total genomic DNA extraction, 16S rDNA and *phaC* genetic characterization of bacterial isolates

Total DNA of styrene-degrading bacterial isolates were extracted according to a chemical-lysis method (Liu et al., 1997) described in Section 5.2.4. Extracted DNA material served as template for PCR amplification of bacterial 16S rDNA using primer set 8F/1490R (Weisburg et al., 1991) and PHA synthase *phaC* gene using general primer set G-D/G-1R, which amplifies a section of the genes encoding

Class I PHA synthase (*phaC*) and Class II PHA synthases (*phaC1* and *phaC2*) (Romo et al., 2007). PCR reaction mixture consisted of 1X GoTaq Colorless Mastermix (Promega, Madison, WI), with 0.2 µM of each primer, 3.0 mM $MgCl_2$, 1 µL of DNA template, and nuclease-free water to complete the 50 µL reaction mixture. Amplification was performed on a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories Inc., Richmond, California) according to temperature programs previously described (Romo et al., 2007; Weisburg et al., 1991). Gel electrophoresis was used for detection of PCR products. Detection of 16S rDNA PCR products were performed using gel electrophoresis at 100V for 30 min, on 1% (w/v) agarose containing 0.2X Gel Red (Biotium, Hayward, CA, USA), and in 1X tris-acetate-EDTA (TAE) buffer. The size of 16s rDNA PCR products was estimated using a 1 kb molecular mass marker (Promega, USA). Detection of *phaC* PCR products were performed under gel electrophoresis conditions of 70V for 75 min, on 1.5% (w/v) agarose containing 1X Gel Red (Biotium, Hayward, CA, USA). The size of *phaC* PCR products was estimated using a 100 bp DNA molecular mass marker (Promega, USA). PCR products were visualized under UV light and recorded with a gel documentation system (UVItec, UK). Sequencing of the 16S rDNA PCR product was performed by a local sequencing service company (Axil Scientific Pte Ltd, Singapore). Partial 16S rDNA sequences of bacterial isolates (> 1,400 bp) were compared to available sequences in GenBank using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program. MEGA5.2 software (Tamura et al., 2011) was used to align the 16S rDNA sequences (with provided ClustalW function) and construct a neighbor-joining tree with Juke-Cantor correction. Bootstrapping for 1,000 replicates was used to estimate the confidence of the tree's topology.

6.2.3 Screening for MCL- PHA accumulation in styrene-degrading bacterial isolates

Bacterial starter cultures were transferred to a 250 mL conical flask, containing 50 mL of 1X MSM and 0.25 $g.L^{-1}$ NH_4Cl (67 $mg.L^{-1}$ nitrogen; nitrogen-limited), at a final optical density at 600 nm (OD_{600}) of 0.01. Styrene (350 µL) was added to a central fused column, which enabled styrene to partition into the headspace and

liquid medium. The conical flasks were tightly-sealed and incubated at 30 °C at 200 rpm. After 48 h, the bacterial cultures were centrifuged (9840 *g*, 15 °C, 10 min) to harvest the cell pellets. Cell pellets were washed twice with 1X PBS buffer and freeze-dried. The CDM of freeze-dried biomass was determined. MCL-PHA content was quantified by subjecting dried biomass (5 to 10 mg) to methanolysis followed by GC-MS analysis according to the procedures described by Tan et al. (2014a) (Chapter 4). Since the amount of styrene added is not completely converted into biomass cells and MCL-PHA, to obtain a more accurate estimate of MCL-PHA yield from styrene, PHA productivity and non-PHA biomass productivity values were used to calculate the amount of styrene biotically consumed based on the following stoichiometric equations:

Non-PHA biomass synthesis: $C_8H_8 + NH_4 + 5.25\ O_2 \rightarrow C_5H_7O_2N + 3\ CO_2 + 2.5\ H_2O$

C_{10} PHA monomer synthesis: $10\ C_8H_8 + 46\ O_2 \rightarrow 4\ C_{10}H_{18}O_2 + 4\ H_2O + 40\ CO_2$

PHA yield was computed based on the amount of MCL-PHA produced for a given amount of styrene biotically consumed.

6.2.4 Biochemical assay, temperature and pH shake-flask studies

Gram staining was performed with a three-step Gram stain procedure kit (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Biochemical assay was performed using Biolog GEN III MicroPlate™ and analyzed using Biolog MicroLog 3 v5.2.01 software (Biolog, Inc., Hayward CA, USA). For temperature and pH study, bacterial starter culture was inoculated to 10 mL of 1X MSM, containing 1 $g.L^{-1}$ of sodium benzoate, at a final OD_{600} of 0.05. To determine the optimum growth temperature of the selected bacterial isolate, the bacterial culture was incubated at four different temperatures (*i.e.*, 25, 30, 35 and 40 °C). The optimum pH for growth medium was investigated at 30 °C in the pH range of 3 to 11 (*i.e.*, 3, 4, 5.5, 6, 7, 8, 10 and 11) with pH adjustment by the addition of either HCl or NaOH. In both studies, bacterial growth over the course of 72 h was monitored by changes in OD_{600} readings with a Cary UV-Vis spectrophotometer

(Agilent Technologies, Palo Alto, California) at 12 h intervals and 24 h intervals for temperature and pH studies, respectively. Results from the temperature and pH studies were plotted using B-Spline and Spline curve-fitting functions, respectively, with OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA, USA).

6.2.4 PCR amplification of *phaZ* gene and phylogenetic tree construction

For amplification of PHA depolymerase *phaZ* gene, the primers DEV15R and DEV15L (Solaiman & Ashby, 2005), were reverse-complemented and modified to DEV15R-RC (5'-GCA TCG GCG CCA ACC TGG-3') and DEV15L-RC (5'-GRA ACT TCA TGA TGA TCG GGG-3'). Amplification was performed with temperature program: 1 cycle at 95 ºC for 5 min, followed by 25 cycles at 94 ºC for 30 sec, 63.4 ºC for 30 sec and 72 ºC for 45 sec, and a final cycle at 72 ºC for 5 min. DNA template from known PHA-accumulator *P. putida* mt-2 (NCIMB 10432) served as a positive control while DNA template from non-PHA accumulator *Escherichia coli* CN13 (ATCC 700609) served as a negative control. A PCR reaction without DNA template was also included to serve as an empty control. Sequencing of the *phaZ* PCR product was performed by Axil Scientific Pte Ltd (Singapore). Partial *phaZ* sequences (> 600 bp) were compared to available sequences in GenBank using the NCBI BLAST program and to other existing PHA-producing Pseudomonads described by Solaiman and Ashby (2005). Phylogenetic tree construction, based on partial *phaZ* sequences, was performed using MEGA5.2 software (Tamura et al., 2011) according to the procedures previously-described. Phylogenetic tree of PhaZ protein sequences was constructed using the Neighbor-joining (*p*-distance) algorithm with bootstrapping for 1,000 replicates to estimate the confidence of the tree's topology.

6.2.6 Transmission electron microscopy (TEM)

Bacterial cells were recovered by centrifugation (9425 *g*, 10 min). Cell pellet was resuspended in fixation buffer, containing 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde, for 2 h. Following fixation, the immobilized cells were washed thrice with 0.5X PBS and post-fixed with 1% (v/v) osmium tetraoxide for 2 h. The cells were washed thrice with 0.5X PBS and dehydrated through a graded

acetone series. The dehydrated cells were embedded with propylene oxide and Spurr resin (1:1 ratio [v/v]) and polymerized. Ultrathin sections, between 70 and 100 nm in thickness, were cut with a Leica UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL, USA). Ultrathin sections were stained with uranyl acetate followed by lead citrate before viewing with a JEOL JEM 2010F TEM (JEOL, Tokyo, Japan), equipped with a field-emission gun, with an accelerating voltage of 200 kV. Imagining was done at magnifications of 25,000 to 40,000X. Image capture was performed with a GATAN charge-coupled device camera and analyzed using commercial GATAN software (Gatan Inc., Pleasanton, CA, USA).

6.2.7 Bacterial cultivation for MCL-PHA polymer extraction

Overnight bacterial starter culture was inoculated to a 1 L cultivation reactor containing 500 mL of 1X MSM at an initial OD_{600} of 0.1. Temperature and agitation were controlled at 30 °C and 100 rpm, respectively. The initial pH of the cultivation media was adjusted to 7.19 ± 0.15. A styrene reservoir was set up using a serum bottle and placed in between the air pump and cultivation reactor. The air pump was connected to the inlet of the reservoir to induce the volatilization of styrene. Gaseous styrene exited the reservoir via a reservoir outlet and was supplied to the culture via an air sparger. The flow rate of gas mixture was set to 500 $mL.min^{-1}$ using an airflow regulator, delivering gaseous styrene to the reactor at an average rate of 3.63 ± 0.54 $g.h^{-1}$. Nitrogen source NH_4Cl was added at an initial concentration of 191 $mg.L^{-1}$ (50 $mg.L^{-1}$ nitrogen) and further additions of 95.5 $mg.L^{-1}$ (25 $mg.L^{-1}$ nitrogen) at 24 and 48 h to maintain nitrogen at a limiting level for MCL-PHA accumulation. After 72 to 120 h of cultivation, cell pellets were harvested from bacterial cultures by centrifugation (9840 *g*, 15 °C, 10 min) and washed twice with 1x PBS prior to freeze-drying. MCL-PHA polymer from 4.58 g freeze-dried cells was extracted with dichloromethane (10% [w/v]) at 55 °C for 8 h using a Soxhlet apparatus. The extract was filtered through Whatman No.4 filter paper to remove cellular debris. Ice-cold methanol was added to the filtrate at 1:1 (v/v) ratio slowly under vigorous stirring to precipitate MCL-PHA. MCL-PHA was subsequently separated from methanol-dichloromethane mixture by centrifugation

at 9425 g (-1 °C, 5 min), casted on a glass surface and air-dried overnight. MCL-PHA yield was estimated based on the procedures described previously in Setion 6.2.3.

6.2.8 MCL-PHA polymer analysis

For GC-MS analysis, MCL-PHA polymer (5 to 10 mg) was derivatized according to the procedures described by Tan et al. (2014a) (Chapter 4). For ^{1}H-NMR and ^{13}C-NMR analyses, 30 mg of PHA polymer was dissolved in 0.7 mL deuterated chloroform containing 0.03% (v/v) tetramethylsilane (TMS). The solution was transferred to a NMR tube (5 mm O.D., 7 inch length; Sigma-Aldrich, St Louis, MO, USA). NMR spectra were recorded on a Bruker DRX-400 spectrometer at 400 MHz (Bruker, Switzerland) by using deuterated chloroform as a solvent with TMS as an internal standard. Chemical shifts were given in ppm (δ) relative to the chemical shift of solvent residual signals for deuterated chloroform at 7.26 and 77.00 ppm, respectively. The weight average molar mass (M_w), number average molar mass (M_n), and polydispersity index (PDI) were determined by gel permeation chromatography (GPC). An Agilent 1100 series GPC system equipped with a LC pump, PLgel MIXED-C column (5 μm, 7.5 x 300 mm), and refractive index (RI) detector were used (Agilent Technologies, Palo Alto, California). The column was calibrated with polystyrene standards (EasiVial PS-H; Agilent Technologies, Palo Alto, California). Tetrahydrofuran (THF), containing 250 ppm of 2,6-di-tert-butyl-4-methylphenol (BHT) as inhibitor, was used as the mobile phase at a flow rate of 1 mL.min^{-1}. T_g and T_m of MCL-PHA polymer were determined using differential scanning calorimetry (DSC) on Mettler-Toledo DSC1 (Mettler-Toledo, Switzerland) at a heating rate of 10 °C.min^{-1} from -70 to 80 ºC with a nitrogen purge at 80 mL.min^{-1}. The mid-point of heat capacity change was determined as T_g while the maximum endothermic point was taken as T_m. DT was determined as the onset temperature of the derivative thermogravimetry curve using thermogravimetric analysis (TGA) on Netzsch STA 499 F3 (Netzsch, Germany) with a heating rate of 10 °C.min^{-1} from 35 °C to 600 ºC under a nitrogen flow rate of 50 mL.min^{-1}. Crystallinity of PHA polymer was determined by powder X-ray diffraction (XRD) (Bruker AXS, D8 Advance, Karlsruhe, Germany) using Cu-K

radiation (K=1.5418 Å) over a 2 range of 10 to 50 °, 0.03 °.min^{-1}, 1 s.step^{-1}.

6.3 Results

6.3.1 Bacterial isolation, genotypic and phenotypic characterization

A total of 24 bacterial isolates were isolated. Of these, 17 bacterial isolates were obtained on agar medium containing sodium benzoate; 3 bacterial isolates were obtained on agar medium containing styrene; and 4 bacterial isolates were obtained on Gelzan medium containing styrene. Bacterial cultures, isolated on sodium benzoate medium, were further tested for their ability to grow using styrene as the sole carbon source. Out of 17 benzoate-degrading bacterial isolates, only 4 isolates (*i.e.*, NBUS2, NBUS3, NBUS6 and NBIWW2) were unable to metabolize styrene (data not shown).

The bacterial isolates were tested for *phaC* gene using a general primer set, designed on the basis of conserved nuleic acid sequences in the regions of the Class I and Class II PHA synthase genes (Romo et al., 2007). Class I PHA synthase gene is reported in various genera such as *Rhododoccus* (Pieper & Steinbüchel, 1992), *Stenotrophomonas* (Romo et al., 2014), *Cupriavidus*, *Alcaligenes* and *etc* (Romo et al., 2007) while Class II PHA synthase genes are highly-conserved among PHA-producing *Pseudomonas* species (Solaiman & Ashby, 2005). A PCR product with an expected size of about 500 bp was obtained for most bacterial isolates (Figure 6.2). This indicated the presence of *phaC* gene and thereby, the genetic potential of these bacterial isolates to synthesize PHA. Only two isolates, ANSUS1 and GSUS1, both with close evolutionary relationship to *Pseudomonas* sp. (data not shown), did not show any positive result for PCR detection of *phaC* gene.

Phylogentic clustering, based on 16S rDNA sequences, were performed for bacterial isolates that tested positive for *phaC* gene. The bacterial isolates belonged to either the phyla Proteobacteria or Actinobacteria (Figure 6.3). Under the phylum Proteobacteria, one bacterial isolate (*i.e.*, GSUS9) was identified as the genus *Stenotrophomonas* while the rest were identified as the genus *Pseudomonas.* Under the phylum Actinobacteria, both isolates NBUS4 and GSUS3 were identified as the

genus *Rhodococcus*. Based on genetic analysis and colony morphology, 10 distinct groups were identified. The 4 bacterial isolates (*i.e.*, NBUS2, NBUS3, NBUS6 and NBIWW2) that could degrade benzoate but not styrene were clustered together as Group 1. The styrene-degrading bacterial isolates were clustered as Group 2 (NBUS9, NBIWW1 and NBIWW3), Group 3 (NBUS5, NBUS12 and NBIWW6), Group 4 (NBUS1 and NBUS8), Group 5 (ANSUS2 and GSUS2), Group 6 (ANSUS3, ANSUS4 and GSUS4), Group 7 (NBUS7, NBUS11, NBIWW4 and NBIWW7), Group 8 (GSUS9), Group 9 (NBUS4), and Group 10 (GSUS3). Table 6.1 provides a summary of the phenotypic and genotypic characterization for these 10 groups.

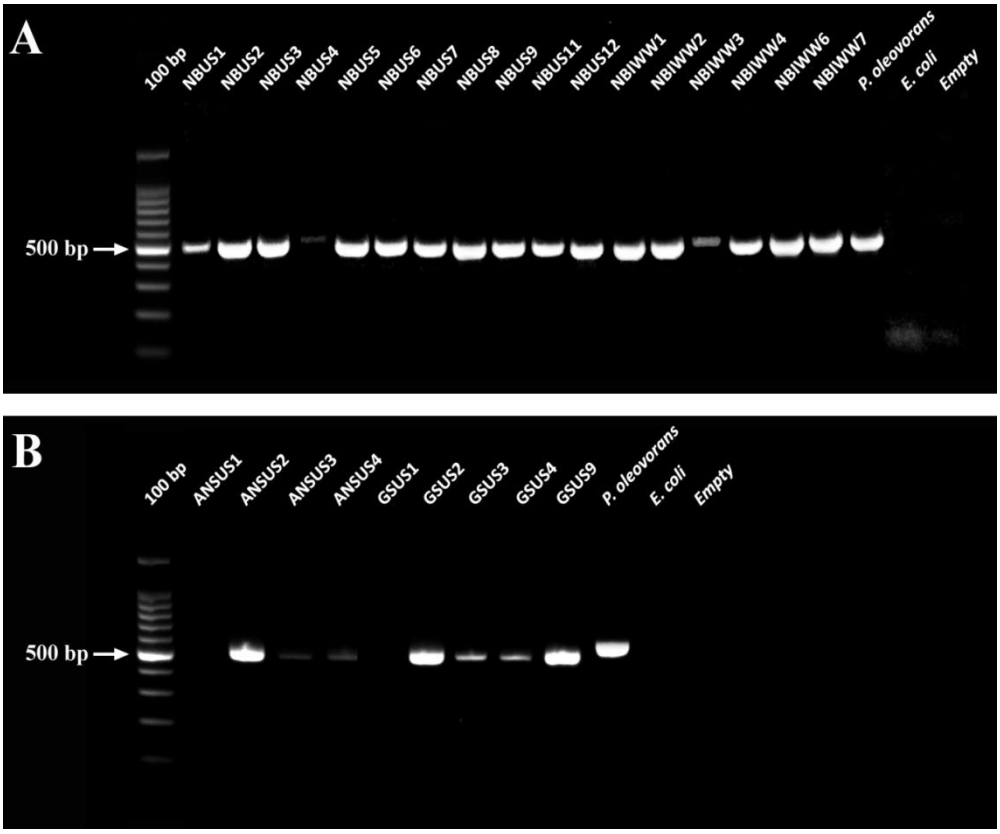


Figure 6.2 PCR detection of *phaC* gene in bacteria isolated on (A) sodium benzoate and (B) styrene. A 100 bp DNA molecular mass marker was included. The presence of a *phaC* gene is indicated by a PCR band of size around 500 bp. PHA accumulator *P. oleovorans* served as a positive control while non-PHA accumulator *E. coli* served as a negative control. Empty is an empty control with no DNA template added.

Phylogenetic analysis revealed that Group 1 and 2 bacterial isolates were closely-related to styrene-degraders *Pseudomonas* sp. LQ26 and *Pseudomonas* sp. VLB120 (Lin et al., 2010; Panke et al., 1998) while bacterial isolates from Group 3, 6, 7, 8 and 10 were closely-related to species known to degrade various mono-aromatic, poly-aromatic and crude oil compounds (Table 6.1). These species however, are not known to accumulate PHA. On the other hand, Group 4, 5 and 9 bacterial isolates were clustered together with bacterial strains known to accumulate PHA from mono-aromatics (Figure 6.3). Group 4 was closely-related to *P. putida* F1, which could biosynthesize PHA from benzene, toluene and ethylbenzene (Nikodinovic et al., 2008); and with *Pseudomonas* sp. TN301, which could assimilate styrene and a wide range of aromatic compounds for PHA production (Narancic et al., 2012). Group 5 shared close evolutionary relationship with *P. putida* mt-2 and *P. fulva* TY16, which reportedly produce PHA from BTEX (Ni et al., 2010; Nikodinovic et al., 2008); and with *P. putida* KT2440, a bacterium capable of bioconverting many non-aromatic substrates into PHA (Tan et al., 2014b; Wang & Nomura, 2010). Group 9 was clustered together with *Rhodococcus* sp. JDC-11 and *R aetherivorans* IAR1, which are bacterium strains capable of degrading phthalic acid esters and metabolizing toluene for PHA biosynthesis, respectively (Hori et al., 2009b; Liang et al., 2010).

Genetic comparisons between known styrene-degrading *cum* PHA-producing bacteria and the newly-isolated bacteria were made. Within the *Pseudomonas* genus, with the exception of *Pseudomonas* sp. TN301, the 16S rDNA gene sequences of all existing styrene-degrading and PHA-producing *Pseudomonas* strains (*i.e.*, *P. putida* CA-3, *P. putida* CA-1 and *P. putida* S12) were unavailable. Hence, genetic comparison could only be made between styrene-utilizing Pseudomonads isolates and *Pseudomonas* sp. TN301. Group 2, Group 3 and Group 4 bacterial isolates were evolutionarily closer to *Pseudomonas* sp. TN301 (99% genetic identity, 97% coverage), followed by Group 5 and 6 (99% identity, 100% coverage), and Group 7 (96% identity, 96% coverage). To date, no bacterium from the genera *Stenotrophomonas* and *Rhodococcus* has been reported to have the metabolic capability of bioconverting styrene to PHA. As such, similar genetic comparisons

Table 6.1 Phenotypic and genotypic characterization of *phaC*+ bacterial isolates.

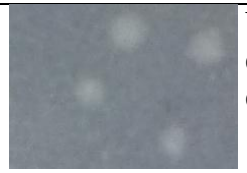
Group	Isolate(s)	Colony Morphology		Closest Identified Relative Species Name	Aromatic Degradation	Genetic Identity (%)	Coverage (%)	Reference
1	NBUS2, NBUS3, NBUS6, NBIWW2		Yellowish, opaque, of circular shape	*Pseudomonas* sp. VLB120 and *Pseudomonas* sp. LQ26	Styrene	100	100	(Lin et al., 2010; Panke et al., 1998)
2	NBUS9, NBIWW1, NBIWW3		Yellowish, opaque, of circular shape with undulated edge			100	99	
3	NBUS5, NBUS12, NBIWW6		Greenish-yellow, opaque, of irregular shape	*P. putida* BCNU 106	BTEX	100	99	(Park et al., 2007)
4	NBUS1, NBUS8		Yellowish, opaque, of circular shape	*P. putida* F1[1]	Benzene, toluene, ethylbenzene	100	100	(Nikodinovic et al., 2008)
				Pseudomonas sp. TN301[1]	Styrene, various mono-aromatics and poly-aromatics compounds	99	97	(Narancic et al., 2012)

Table 6.1 Cont.

5	ANSUS2, GSUS2		Pale brown, opaque, of circular shape with slight undulated edge	*P. putida* mt-2[1]	Toluene, p-xylene	99	96	(Nikodinovic et al., 2008)
				P. fulva TY16[1]	Benzene, toluene, ethylbenzene	99	100	(Ni et al., 2010)
				P. putida KT2440[1]	n.a.	100	100	-
6	ANSUS3, ANSUS4, GSUS4		Off-white, opaque, of circular shape	*P. putida* AS90	Nonylphenol polyethoxylates	100	100	(Liu et al., 2006)
7	NBUS7, NBUS11, NBIWW4, NBIWW7		Yellowish and filamentous	*P. aeruginosa* L-4	Catechol	99	99 to 100	(NCBI, 2014a)
				Pseudomonas sp. ADP	Atrazine	99	100	(Mandelbaum et al., 1995)
				Pseudomonas sp. wust-c	n.a.	99	100	-
8	GSUS9		White, cloudy, of circular shape with undulated edge	*S. maltophilia* JV3	n.a.	100	100	-
				S. maltophilia VUN 10010	Pyrene, fluorene, phenanthrene	99	100	(Boonchan et al., 1998)

Table 6.1 *Cont.*

9	NBUS4		White, circular with undulated edge	*Rhodococcus* sp. JDC-11	Phthalic acid esters	99	99	(Liang et al., 2010)
				R. aetherivorans IAR1[1]	Toluene	96	98	(Hori et al., 2009b)
10	GSUS3		White, circular with fading edges	*R. qingshengii* BLH-Y4	Crude oil[2]	100	100	(NCBI, 2014b)

[1] Bacterial strain is also a PHA-producer
[2] Information on the crude oil components, which the bacterium is capable of degrading, is unavailable
Information that is unavailable is indicated as "n.a."

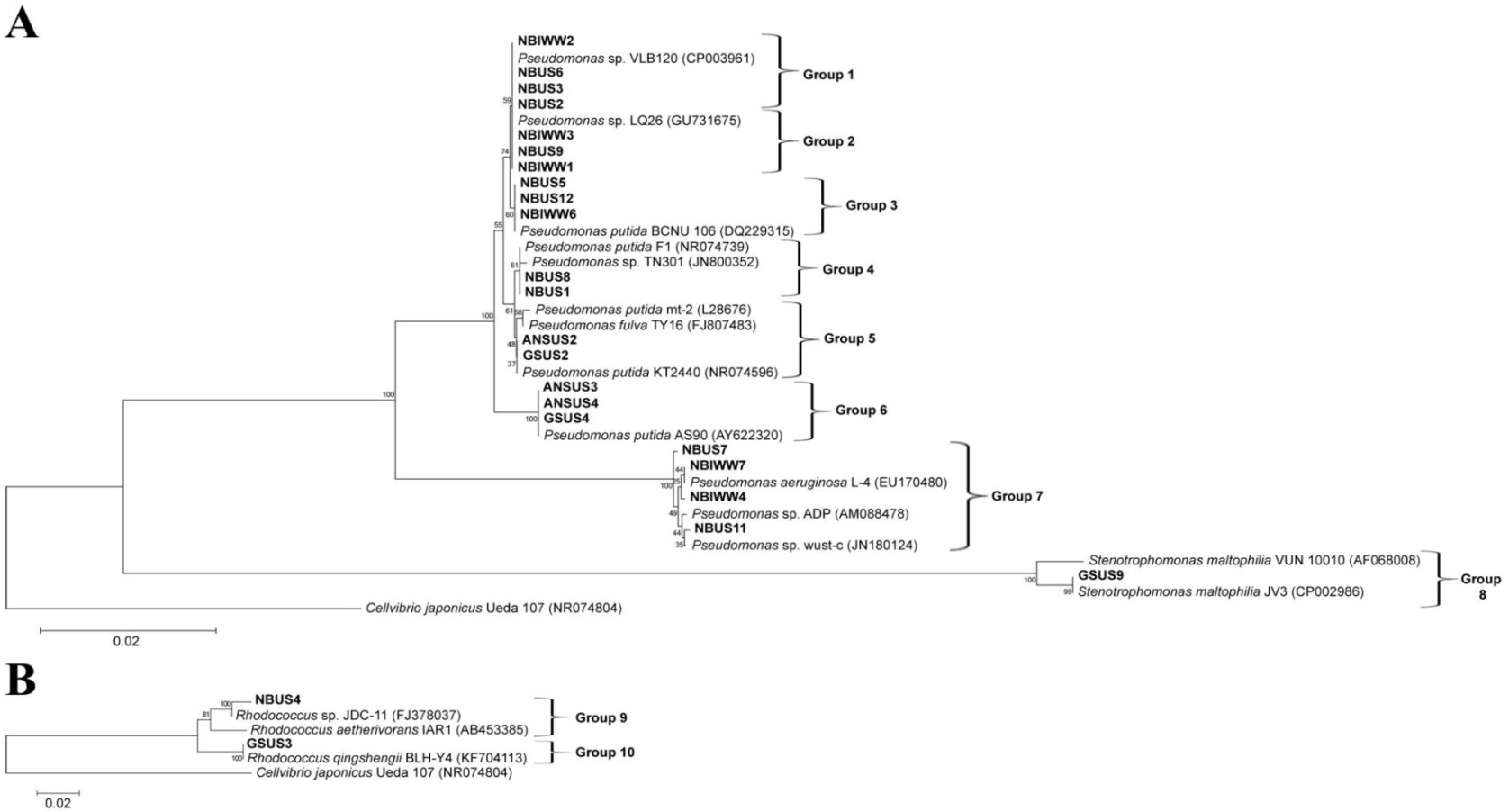


Figure 6.3 Neighbor-joining phylogenetic tree based on partial 16S rDNA sequences of bacterial isolates (designated in bold) and closely-related bacteria for the phyla (A) Proteobacteria and (B) Actinobacteria. *Cellvibrio japonicas* Ueda107 (NR074804) was included as the outgroup. The scale bar represents the estimated number of nucleotide changes per sequence position.

Table 6.2 Growth and MCL-PHA accumulation by bacterial isolates using styrene as sole carbon source.

Genus	Group	Bacterial Isolates	CDM ± s.d. ($g.L^{-1}$)	MCL-PHA Content ± s.d. (% CDM)	PHA Monomeric Composition C_6:C_8:C_{10}:C_{12} (wt %)	PHA Yield (g PHA.g styrene^{-1})
Pseudomonas	2	NBUS9 NBIWW1 NBIWW3	0.35 ± 0.07	0.05 ± 0.00	0:0:82:18	0.0005
	3	NBUS5 NBUS12 NBIWW6	0.63 ± 0.14 (0.80 ± 0.03)	13.67 ± 5.22 (23.10 ± 3.25)	1:20:76:3 (1:15:82:2)	0.1424 (0.2296)
	4	NBUS1 NBUS8	0.37 ± 0.19	6.23 ± 1.46	4:30:64:2	0.0670
	5	ANSUS2 GSUS2	0.38 ± 0.09	0.59 ± 0.27	0:36:64:0	0.0064
	6	ANSUS3 ANSUS4 GSUS4	0.33 ± 0.09	0.08 ± 0.04	0:0:100:0	0.0009
	7	NBUS7 NBUS11 NBIWW4 NBIWW7	0.26 ± 0.04	0.08 ± 0.01	0:0:52:48	0.0009
Stenotrophomonas	8	GSUS9	0.42 ± 0.20	-	-	-
Rhodococcus	9	NBUS4	0.37 ± 0.13	-	-	-
	10	GSUS3	0.41 ± 0.15	-	-	-

Values for *Pseudomonas* isolate NBUS12 are provided within parentheses. Cell yield was obtained by taking the average CDM for each group; Average MCL-PHA content was calculated by dividing the PHA yield ($g.L^{-1}$) by cell yield ($g.L^{-1}$). Results were the mean values of at least three independent experiments ($n \geq 3$) and s.d. refers to standard deviation.

were not conducted for Group 8, 9 and 10.

6.3.2 Bioconversion of styrene to MCL-PHA by bacterial isolates

PHA production is reportedly enhanced under nitrogen-limiting conditions for bacterial species from the genera *Pseudomonas*, *Stenotrophomonas* and *Rhodococcus* (Hori et al., 2009a; Singh & Parmar, 2013; Ward et al., 2005). Hence, styrene-degrading bacterial isolates from Group 2 to 10 were tested for their ability to bioconvert styrene into PHA under nitrogen-limiting condition in shake-flask culture. Bacterial isolates, belonging to the genera *Stenotrophomonas* and *Rhodococcus* (*i.e.*, Group 8, 9 and 10), grew to final CDM between 0.37 ± 0.13 $g.L^{-1}$ and 0.42 ± 0.20 $g.L^{-1}$, but no PHA was detected in these bacteria (Table 6.2). Pseudomonad isolates in Group 2, 4, 5, 6 and 7 attained similar final CDM between 0.26 ± 0.04 $g.L^{-1}$ and 0.38 ± 0.09 $g.L^{-1}$. MCL-PHA, comprising of C_6, C_8, C_{10} and C_{12} monomers, were detected. However, the PHA contents and yields in these isolates were generally below 0.6% CDM and 0.0064 g PHA.g $styrene^{-1}$. The only exception was Group 4 which had higher MCL-PHA content of 6.23 ± 1.46% CDM and PHA yield of 0.0670 g PHA.g $styrene^{-1}$. MCL-PHA produced by Group 4 bacterial isolates comprised of C_6, C_8, C_{10} and C_{12} monomers in C_6:C_8:C_{10}:C_{12} ratio of 4:30:64:2 with C_{10} and C_8 monomers predominating the polymer (94 weight % [wt %]).

The highest growth, PHA formation and PHA yield were observed in Group 3 bacterial isolates (0.63 ± 0.14 $g.L^{-1}$; 13.67 ± 5.22% CDM; 0.1424 g PHA.g $styrene^{-1}$) (Table 6.2). Similar to Group 4 bacterial isolates, the monomeric composition of MCL-PHA was mostly comprised of C_8 and C_{10} monomers (96 wt %) with a C_6:C_8:C_{10}:C_{12} ratio of 1:20:76:3. Within Group 3, the bacterial isolate NBUS12 showed the highest growth on styrene and PHA yield, and was selected for further investigation.

6.3.3 Biochemical and genetic characterization of bacterial isolate NBUS12

Pseudomonas isolate NBUS12 is an aerobic Gram-negative rod-shaped bacterium, about 1.5 µm in size (Figure 6.4), and was identified as the species "*putida*" by

Microlog database. Biolog assay also revealed that the bacterium had high reducing power and could tolerate growth conditions up to 4% NaCl. Table 6.3 provides a summary of various carbon sources on Biolog GEN III MicroPlate^{TM} that *P. putida* NBUS12 was able to assimilate, as well as the antibiotic sensitivities of *P. putida* NBUS12. Under shake-flask conditions and with styrene as the sole carbon substrate, *P. putida* NBUS12 grew to a final biomass of 0.80 ± 0.03 $g.L^{-1}$, comprising of PHA at 23.10 ± 3.25% CDM, and with PHA yield of 0.2296 g PHA.g $styrene^{-1}$ (Table 6.2). Bioaccumulated MCL-PHA was visible as intracellular PHA granules within *P. putida* NBUS12 (Figure 6.4). The C_6:C_8:C_{10}:C_{12} monomeric composition was 1:15:82:2 with C_8 and C_{10} monomers accounting for the bulk of the polymer at 97 wt % (Table 6.2).

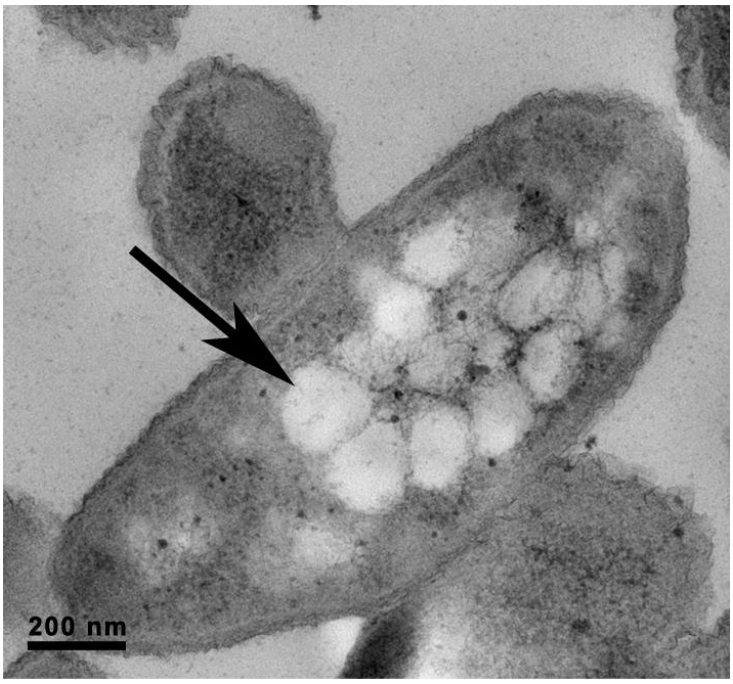


Figure 6.4 TEM micrograph of a *P. putida* NBUS12 bacterium with intracellular MCL-PHA granules. Arrow indicates MCL-PHA granule.

The optimum range of temperature and pH for *P. putida* NBUS12's growth was investigated. The bacterium displayed a lag phase in the first 12 h of incubation at all the temperatures investigated (*i.e.*, 25, 30, 35 and 40 °C) (Figure 6.5). After 12 h, exponential growth was observed at temperatures between 25 and 35 °C. At 25 °C, the bacterium's growth started to plateau from 36 h, achieving a maximum OD_{600}

Table 6.3 Antibiotic sensitivity and carbon assimilation on Biolog GEN III MicroPlate™ by *P. putida* NBUS12.

Chemical Guild	Substrate	Chemical Guild	Substrate
Sugars	*α-D*-Glucose	Hexose acids	*D*-Galacturonic acid
	D-Mannose		*D*-Gluconic acid
	D-Fructose		*D*-Glucuronic acid
	D-Galactose		Glucuronamide
	D-Fucose		Mucic acid
	L-Fucose		Quinic acid
	L-Rhamnose		*D*-Saccharic acid
Hexose-phosphate	*D*- Fructose-6-phosphate	Carboxylic acids, esters and fatty acids	*L*-Lactic acid
			Critic acid
Amino acids	*L*-Alanine		*α*-Ketoglutaric acid
	L-Arginine		*L*-Malic acid
	L-Aspartic acid		*γ*-Aminobutyric acid
	L-Glutamic acid		*β*-Hydroxy-*D,L*-butyric acid
	L-Histidine		Propionic acid
	L-Pyroglutamic acid		Acetic acid
	L-Serine		
	D-Serine	Alcohol	Glycerol
		Antibiotic	Minocycline

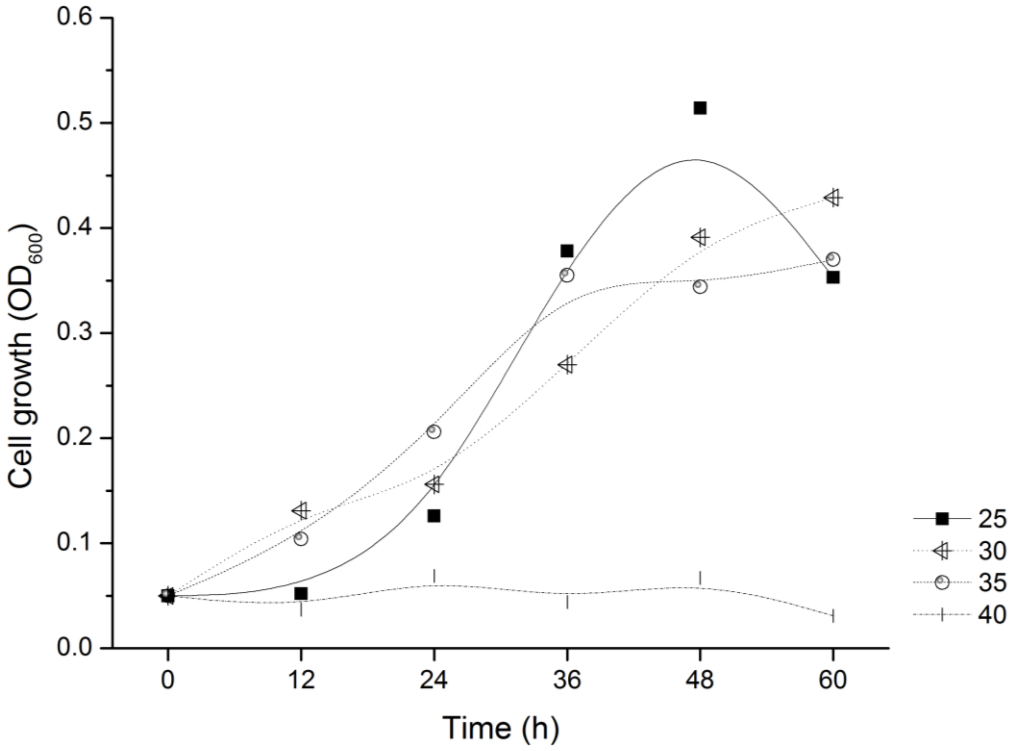


Figure 6.5 The growth of *P. putida* NBUS12 under different temperatures.

value of about 0.2. At 30 °C and 35 °C however, exponential growth was observed throughout the 72 h incubation period, achieving a maximum OD_{600} value of about 0.6. Negligible growth was observed at 40 °C with OD_{600} values remaining low at around 0.1. This could be possibly due to the deactivation of enzymes, involved in growth metabolism, at 40 °C. Figure 6.6 shows the growth curve of the bacterium at pH 3, 4, 5.5, 6, 7, 8, 10 and 11. No growth was observed at acidic pH values of 3 and 4 while a lag phase of 24 h was observed at pH 5.5. Between pH 6 and 11, there was no lag phase in growth and the bacterium grew optimally in this range, reaching OD_{600} values of up to to 1.1. Collectively, the results suggest that the optimal growth temperature was between 25 and 35 °C while the optimal growth pH was between 6 and 11.

Genetic comparisons were made between *P. putida* NBUS12 and *Pseudomonas* sp. TN301 using 16S rDNA sequences, and between *P. putida* NBUS12, *P. putida* CA-3 and other known PHA-producing Pseudomonads (Solaiman & Ashby, 2005) using

phaZ sequences. *phaZ* encodes for the PHA depolymerase enzyme, which is responsible for PHA degradation. *phaZ* is chosen because unlike *phaC*, which is present in two copies, *phaZ* gene is present in a single copy in PHA-producing Pseudomonads(Solaiman & Ashby, 2005). *phaZ* gene is reported as having highly conserved regions among PHA-producing Pseudomonads and the gene locus is also structurally conserved within the Class II PHA operon of Pseudomonads, flanked by the gene loci of *phaC1* and *phaC2* (Solaiman & Ashby, 2005). These features make *phaZ* a suitable genetic signature for PHA-producing Pseudomonads.

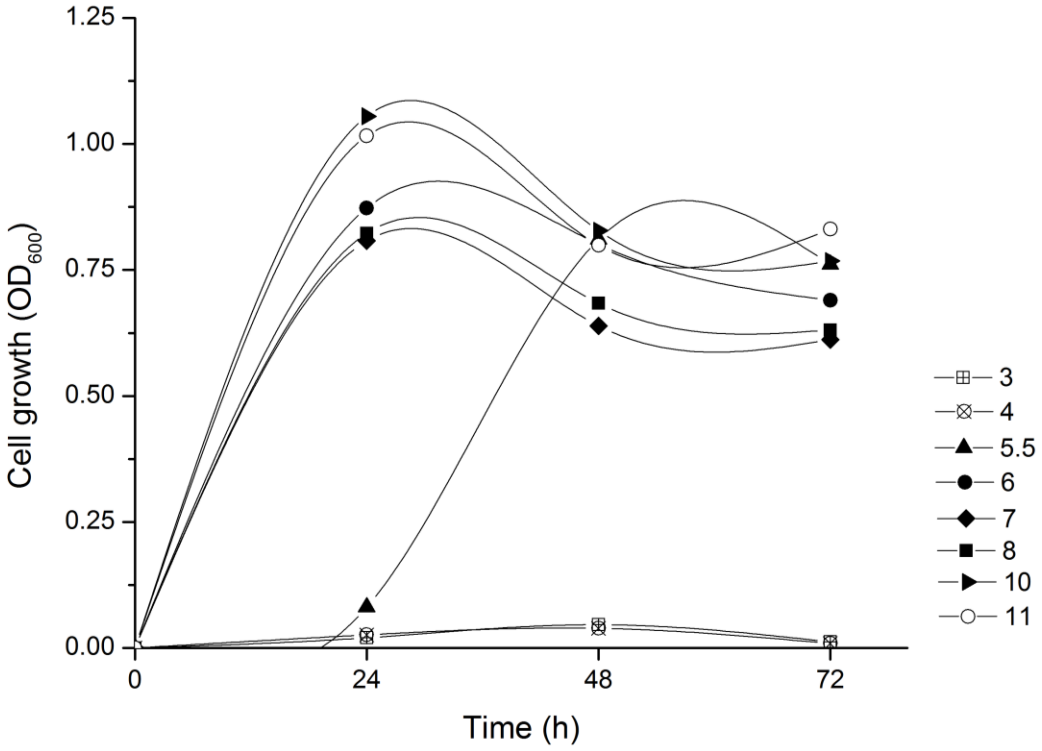


Figure 6.6 The growth of *P. putida* NBUS12 under different pH values.

The 16S rDNA sequence of *P. putida* NBUS12 shared 99% identity with 97% coverage with *Pseudomonas* sp. TN301 but showed higher genetic similarity to *P. putida* BCNU 106 (100% identity, 99% coverage), which is a bacterium strain not known to accumulate PHA (Figure 6.3 and Table 6.1). The *phaZ* gene of *P. putida* NBUS12 also

did not show particularly high genetic similarity to *P. putida* CA-3 (88% identity with 100% coverage) and other known PHA-producing Pseudomonads (80 to 88% identities with 99 to 100% coverage). Instead, BLAST analysis revealed *Pseudomonas* sp. VLB120 as *P. putida* NBUS12's closest relative (100% identity with 100% coverage). ClustalW analysis of *phaZ* sequences indicated that *P. putida* NBUS12 was evolutionarily-closer to PHA-producing *P. putida* as compared to other PHA-producing *Pseudomonas* species (*i.e.*, *P. resinovorans*, *P. stutzeri*, *P. nitroreducens*, and *P. mendocina*) but nonetheless formed a distinct branch together with *Pseudomonas* sp. VLB120, which is a styrene-degrader but not known to produce PHA (Figure 6.7A). Further analysis made with *phaZ* translated amino acid sequences showed that *P. putida* NBUS12 had 100% coverage with 98% and 100% identities to *P. putida* CA-3 and *Pseudomonas* sp. VLB120, respectively. Phylogenetic clustering of PhaZ sequences showed a trend similar to that for *phaZ* sequences where *P. putida* NBUS12 was grouped together with *Pseudomonas* sp. VLB120 as an evolutionarily-distinct branch (Figure 6.7B).

6.3.4 Characterization of MCL-PHA polymer produced from styrene by *P. putida* NBUS12

To obtain sufficient biomass for MCL-PHA polymer extraction, the cultivation volume of *P. putida* NBUS12 was scaled up to 500 mL. Each cultivation run produced an average of 1.28 ± 0.10 $g.L^{-1}$ biomass with 32.49 ± 2.40% CDM of PHA content, corresponding to a total PHA production of 0.42 ± 0.04 $g.L^{-1}$ and PHA mass fraction of 324.90 ± 23.98 $mg.g.CDM^{-1}$. The PHA yield was 0.2959 g PHA.g $styrene^{-1}$. The extracted polymer film was odorless, semi-transparent and of light yellow color (Figure 6.8). GC-MS analysis of the methanolyzed polymer revealed the presence of medium-chain-length C_6, C_8, C_{10}, C_{12} monomers, and an additional 3-hydroxytetradecanoate (C_{14}) monomer with the retention times of 7.82, 10.89, 13.64, 16.07 and 18.26 min, respectively (Figure 6.9). The retention time profile corroborated with results reported in Chapter 4 and existing literature (Tan et al., 2014a). The monomers were present in the C_6:C_8:C_{10}:C_{12}:C_{14} ratio of 2:42:1257:17:1 with C_{10} monomer accounting for 95 wt %

of the polymer.

The NMR analytical results of the MCL-PHA polymer are shown in Figure 6.10. Collectively, GC-MS and NMR analyses suggested that *m* can have a value of 1, 3, 5, 7, and 9 corresponding to C_6, C_8, C_{10}, C_{12} and C_{14} monomeric units, respectively. NMR chemical shifts of different PHA monomeric units are compiled in Table 6.4, which were in good agreement with reported literature and displayed typical profiles of MCL-PHA copolymers (Gross et al., 1989; Huijberts et al., 1994; Muhr et al., 2013). The additional signals at 2.02, 2.35, 5.29 and 5.53 ppm in the ^{1}H-NMR spectrum and signals between 123.3 ppm and 134.2 ppm in the ^{13}C-NMR spectrum indicated the presence of olefinic groups along the alkyl side chain. Therefore, besides saturated monomers, low levels of unsaturated monomers were also present in the polymer. The percentage of unsaturated units in PHA was estimated from the ratio of the integration peak at 2.02 ppm to peak at 2.52 ppm in ^{1}H-NMR spectrum. The MCL-PHA, produced by new bacterial isolate *P. putida* NBUS12, contained about 7.4% abundance of unsaturated units. However, due to the lack of analytical standards, the chemical structures of these unsaturated monomers remained to be elucidated and further verified.

The M_w and M_n of the MCL-PHA polymer were 101,500 Da and 49,300 Da, respectively, while PDI index was 2.06. The T_g was -46.05 °C while T_m was 50.67 °C (Figure A2), giving rise to the amorphous property of the polymer at room temperature. The polymer was also partially crystalline (13.57%) where crystalline peaks were detected at 2θ values of 19.05 and 21.68 under XRD analysis (Figure A3). Thermal degradation of the polymer occurred at 277 °C (Figure A4). These values fell within the range of expected values for MCL-PHA (Table 6.5). Currently, polymer property data for MCL-PHA, produced from styrene, was only available for *P. putida* CA-3 (Ward et al., 2005). Hence, a comparison between this study's MCL-PHA polymer and the MCL-PHA polymer from *P. putida* CA-3 were made (Table 6.5). This study's MCL-PHA was found to have higher M_w and M_n values, and lower PDI value.

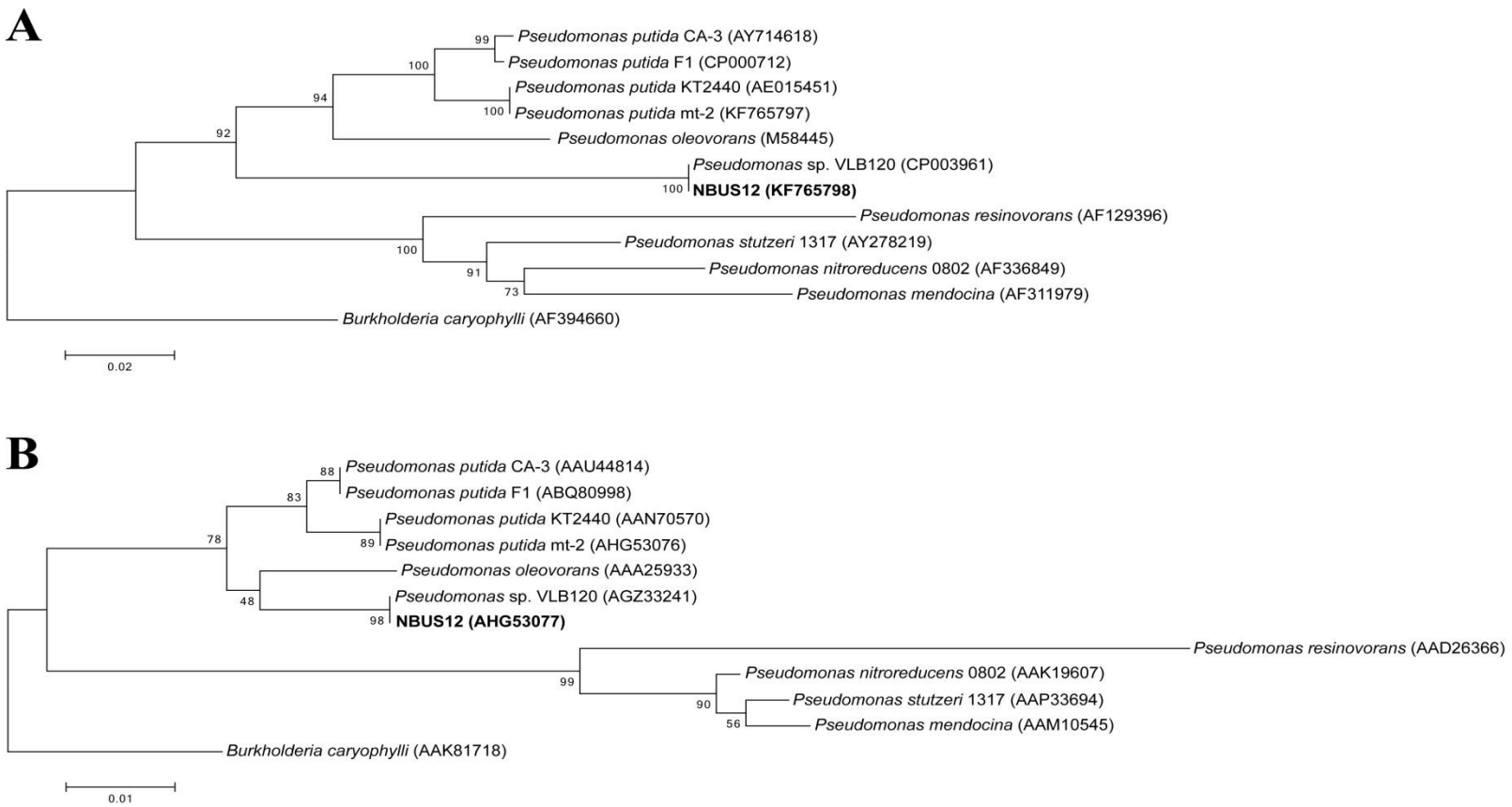


Figure 6.7 Neighbor-joining phylogenetic phylogenetic tree based on (A) partial *phaZ* gene sequence and (B) PhaZ protein sequence of *P. putida* NBUS12 (designated in bold), *Pseudomonas* sp. VLB120 and known PHA-producing Pseudomonad strains described by Solaiman and Ashby (2005) with *Burkholderia caryophylli* as the outgroup. Genbank accession numbers are provided within parentheses. The numbers beside the nodes indicated bootstrap values based on 1,000 replications. The scale bar represents the estimated number of nucleotide changes per sequence position.

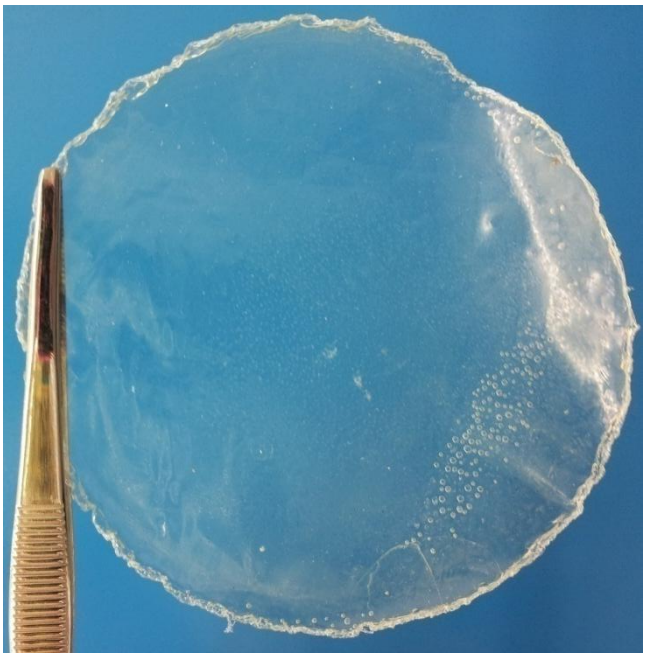


Figure 6.8 MCL-PHA polymer extracted from *P. putida* NBUS12.

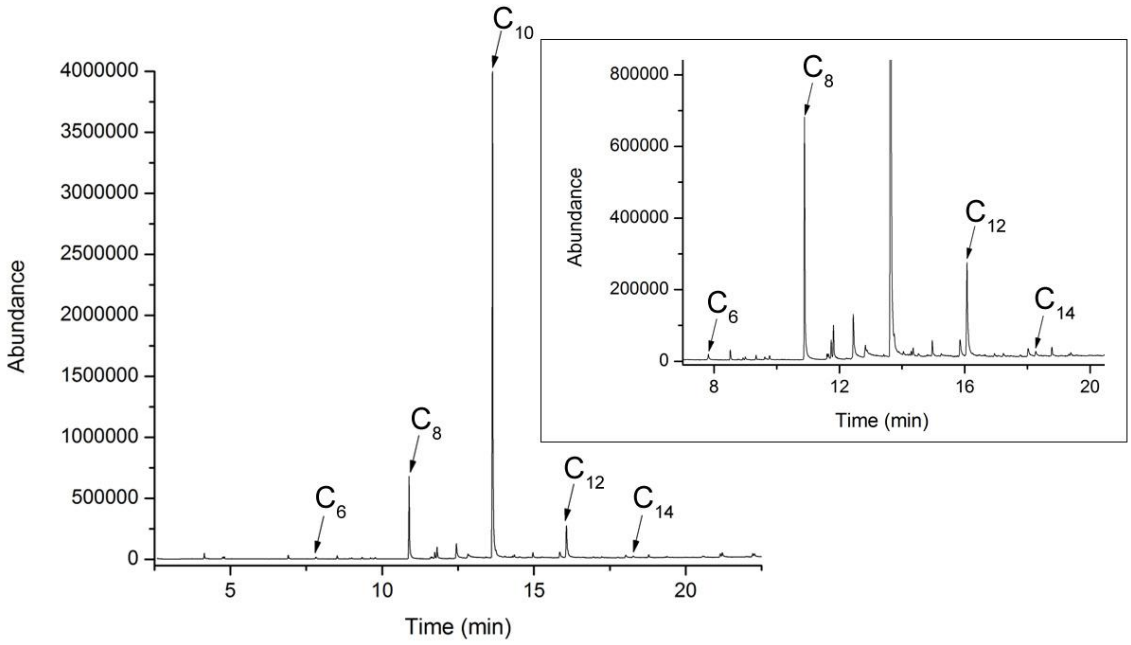


Figure 6.9 GC-MS chromatogram of MCL-PHA monomers detected in the polymer extracted from *P. putida* NBUS12. The insert shows a magnified version of the chromatogram (C_6, 3-hydroxyhexanoate; C_8, 3-hydroxyoctanoate; C_{10}, 3-hydroxydecanoate; C_{12}, 3-hydroxydodecanoate; C_{14}, 3-hydroxytetradecanoate).

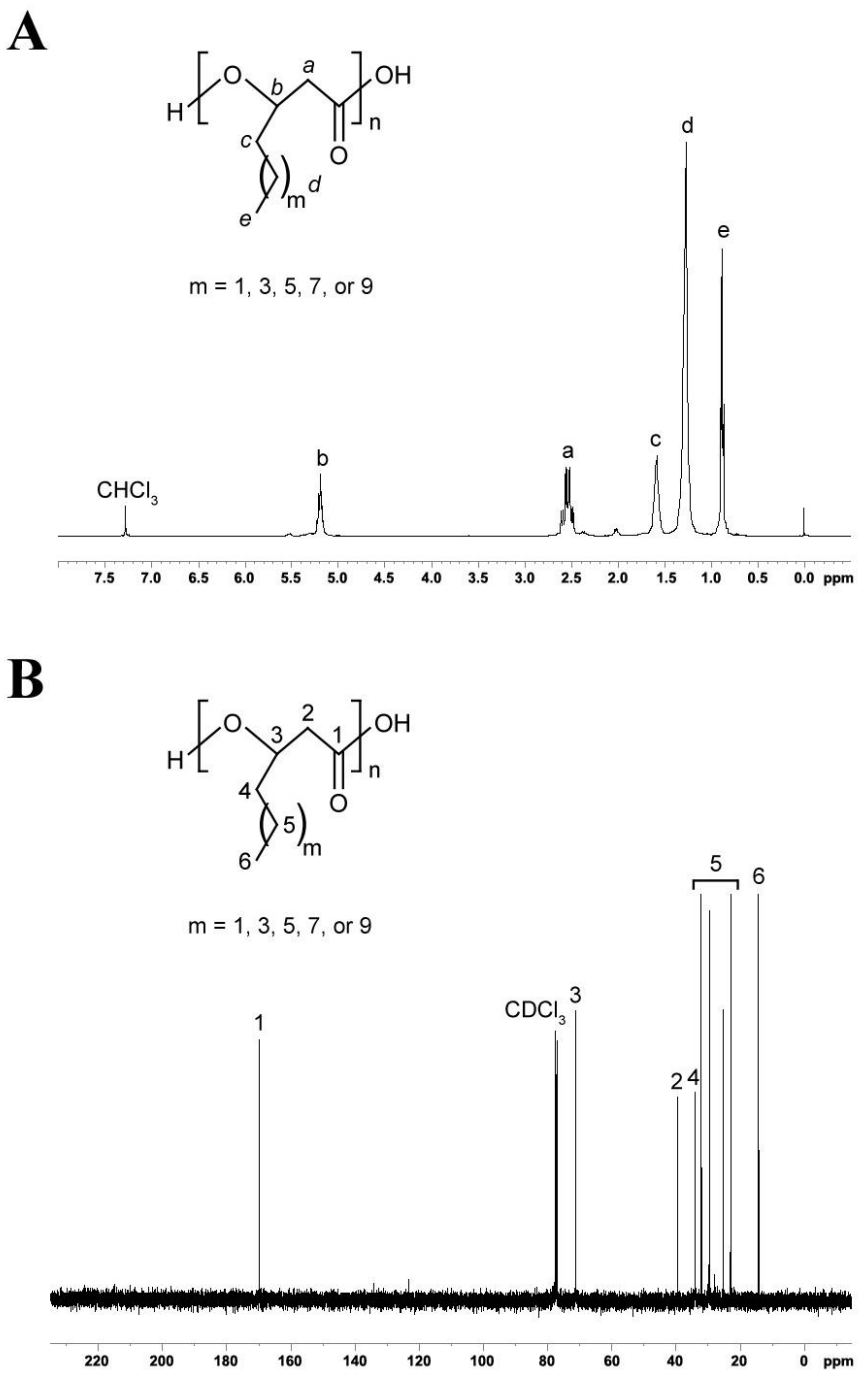


Figure 6.10 (A) ^{1}H-NMR and (B) ^{13}C-NMR spectra of MCL-PHA polymer from *P. putida* NBUS12 using styrene as the sole carbon substrate. The signals corresponding to hydrogen atoms in ^{1}H-NMR spectrum are denoted by alphabet letters while the signals corresponding to carbon atoms in ^{13}C-NMR spectrum are denoted by numericals.

Table 6.4 NMR chemical shift assignments of PHA monomers in MCL-PHA produced from styrene by *P. putida* NBUS12.

	Position[1]	C_6	C_8	C_{10}	C_{12}	C_{14}
^{1}H-NMR	a	2.52	2.52	2.52	2.52	2.52
	b	5.17	5.17	5.17	5.17	5.17
	c	1.58	1.58	1.58	1.58	1.58
	d-1	1.25	1.25	1.25	1.25	1.25
	d-2		1.25	1.25	1.25	1.25
	d-3		1.25	1.25	1.25	1.25
	d-4			1.25	1.25	1.25
	d-5			1.25	1.25	1.25
	d-6				1.25	1.25
	d-7				1.25	1.25
	d-8					1.25
	d-9					1.25
	e	0.87	0.87	0.87	0.87	0.87
^{13}C-NMR	1	169.73	169.73	169.73	169.73	169.73
	2	39.44	39.44	39.44	39.44	39.44
	3	71.19	71.37	71.37	71.37	71.37
	4	36.22	34.15	34.15	34.15	34.15
	5-1	18.65	25.05	25.41	25.41	25.41
	5-2		31.86	29.52	29.52	29.52
	5-3		22.69	32.12	29.69	29.69
	5-4			23.03	29.52	29.69
	5-5			25.41	29.33	29.69
	5-6				32.12	29.52
	5-7				23.03	29.33
	5-8					32.12
	5-9					23.03
	6	14.11	14.27	14.27	14.27	14.27

[1] Refer to Figure 6.10 for atom numbers

Table 6.5 A comparison of MCL-PHA polymer, produced from styrene by *P. putida* NBUS12, with existing reports.

Properties	Expected Range of Values for MCL-PHA[1]	MCL-PHA Produced by *P. putida* CA-3[2]	This Study's Results
M_w	76,500 to 339,000	76,500	101,500
M_n	25,200 to 182,000	25,200	49,300
PDI	1.25 to 4.4	3.03	2.06
T_g (°C)	-52.0 to -25.8	-41.7	-46.05
T_m (°C)	38.1 to 66.8	38.14	50.67
DT (°C)	227 to 290.5	265	277
Crystallinity (%)	0 to 40	Partially crystalline[3]	13.57

[1] Values obtained from (Chen (2010b); Marchessault et al. (2011); Rai et al. (2011); Zinn (2010))
[2] Values obtained from Ward et al. (2005)
[3] Percentage of crystallinity was not provided

Additionally, this study's MCL-PHA displayed a wider temperature range of between T_g and T_m (96.72 °C), and higher DT value.

6.4 Discussion

Environmental pollution with styrene is pervasive in land, water and air, particularly in wastewaters and off-gases from PS-related industries (Aliabadi et al., 2012; Tischler & Kaschabek, 2012). Styrene waste presents a large volume of inexpensive carbon source that could be bioconverted and upcycled to high-value MCL-PHA biopolymer, potentially lowering the price of PHA and off-setting waste treatment cost. While much work on styrene bioremediation has been accomplished, the bioconversion of styrene into a new resource, such as MCL-PHA, has received limited attention. This study was aimed at addressing this research gap through the isolation and characterization of bacteria that could bioconvert styrene into MCL-PHA.

Aromatic-degrading and PHA-forming bacteria are usually isolated from petrochemical-contaminated sites or bioreactors treating petrochemical industrial effluents (Kenny et al., 2008; Narancic et al., 2012; Ni et al., 2010) as these pre-enriched sources are more likely to contain microorganisms that could break down petrochemical aromatics. In this study, domestic activated sludge from a water reclamation plant treating municipal wastewater and industrial activated sludge from a petrochemical wastewater treatment facility were tested as inoculum sources. In the first round of isolation, sodium benzoate was used as the aromatic carbon source due to its solubility, non-volatility and non-toxic nature. Past research has also shown that benzoate-utilizing bacterial strains were capable of degrading styrene (Lindsay et al., 2008). A total of 17 benzoate-degrading bacterial isolates were isolated from both inoculum sources following 11 weeks of enrichment on BTEXS mixture. The partial *phaC*, which is a gene encoding PHA synthase responsible for PHA biosynthesis, were detected in all bacterial isolates (Figure 6.2A). Among these benzoate-degrading bacterial isolates, 12 were also found to be styrene-degrading while 4 isolates (*i.e.*, NBUS2, NBUS3, NBUS6 and NBIWW2) could not grow on styrene. Benzoate is one of the by-products of styrene

degradation (Tischler & Kaschabek, 2012) and the isolation of a minority of bacteria, which were able to consume benzoate but not styrene, suggested the emergence of a dominant styrene-degrading microbial population supporting a sub-microbial population which thrives on styrene degradation intermediates during the early stages of enrichment. Furthermore, the detection of *phaC* gene in bacterial isolates, from these two microbial populations, may hint at the possibility of PHA biosynthesis from styrene and its degradation intermediates in a mixed culture system. BLAST analysis revealed that all but one bacterial isolate (*i.e.*, NBUS4) belonged to the genus *Pseudomonas* (Table 6.1). Isolate NBUS4 was identified as the genus *Rhodococcus*. The bacterial isolates were phylogenetically clustered into 6 distinct groups: Group 1 (NBUS2, NBUS3, NBUS6 and NBIWW2), Group 2 (NBUS9, NBIWW1 and NBIWW3), Group 3 (NBUS5, NBUS12 and NBIWW6), Group 4 (NBUS1 and NBUS8), Group 7 (NBUS7, NBUS11, NBIWW4 and NBIWW7) and Group 9 (NBUS4) (Figure 6.3, Table 6.1). With the exception of Group 4 and 9, which comprised of bacterial isolates from domestic activated sludge, all other groups consisted of bacterial isolates from both domestic and industrial activated sludge. Hence, it appears that the pre-enriched nature of industrial activated sludge did not result in any notable difference to the type of bacterial isolates obtained.

Due to the slightly higher diversity in bacterial isolates obtained from domestic activated sludge, the sludge was further enriched on BTEXS before switching to styrene, and a second round of bacterial isolation was performed after 19.5 months of enrichment on styrene. Nine styrene-degrading bacterial isolates were obtained, and of which, *phaC* gene was detected in all bacterial isolates with the exception of two *Pseudomonas* species ANSUS1 and GSUS1 (Figure 6.2B). This indicates the presence of both styrene-degrading *cum* non-PHA-forming bacteria as well as styrene-degrading *cum* PHA-producing bacteria within a mixed culture system. The seven *phaC*+ bacterial isolates were clustered into 4 different groups: Group 5 (ANSUS2 and GSUS2), Group 6 (ANSUS3, ANSUS4 and GSUS4), Group 8 (GSUS9) and Group 10 (GSUS3) (Figure 6.3 and Table 6.1). All *phaC*+ bacteria, isolated on agar medium (*i.e.*, ANSUS2, ANSUS3 and ANSUS4), were determined

as *Pseudomonas*. On Gelzan medium, a higher diversity of *phaC*+ bacterial isolates were obtained, including species from *Stenotrophomonas* (GSUS9), *Rhodococcus* (GSUS3) and *Pseudomonas* (GSUS2 and GSUS4). The use of Gelzan as solidifying agent is known to circumvent some of the toxic effects that agar has on some groups of microorganisms, and facilitate the isolation of a wider range of microorganisms (Janssen et al., 2002). It was also noted that the bacterial isolates obtained corroborated well with the findings from the previous study but were not sufficiently representative of the diversity found in styrene-degrading consortium (Chapter 5).

On both isolation attempts, bacteria from the *Pseudomonas* genus formed between 80% and 95% of bacterial isolates obtained, which was much higher than the abundance level detected by next-generation sequencing (0.21% to 1.6%, data not shown). A possible explanation could be that Pseudomonad is an easily-culturable bacterium, which may have resulted in an over-representation of Pseudomonads in isolation studies (Tischler & Kaschabek, 2012; Warmink et al., 2009; Watanabe et al., 2002). Another possible explanation could be the high percentage of bacterial sequences, which could not be classified within the next-generation sequencing and bioinformatics analysis parameters used in this study, resulting in an underestimation of this genus. While Pseudomonads were obtained from both rounds of isolation, it was observed that the Pseudomonad cultures from domestic activated sludge (*i.e.*, bacterial isolates denoted by prefix "NBUS") and styrene-enriched sludge (*i.e.*, bacterial isolates denoted by prefixes "ANSUS" and "GSUS") were phylogenetically-distinct from each other (Figure 6.3). This observation further supported previous findings on the evolution of bacterial population under prolonged enrichment (Chapter 5, Figure 5.3). The bacterial genera *Stenotrophomonas*, *Pseudomonas* and *Rhodococcus* are known for their mono-aromatic biodegradation properties, including styrene metabolism (Jimenez et al., 2004; Kuyukina & Ivshina, 2010; Seo et al., 2009). Bacterial strains from *Pseudomonas* and *Rhodococcus* are also widely-reported to bioconvert aromatic compounds into PHA (Hori et al., 2009b; Tan et al., 2014b). Indeed, the 10 groups of bacterial isolates were found to have close evolutionary relationship with known

aromatic-degrading bacteria (Table 6.1) and/or MCL-PHA producing bacteria including *P. putida* F1, *Pseudomonas* sp. TN301, *P. putida* mt-2, *P. fulva* TY16, *P. putida* KT2440, and *R. aetherivorans* IAR1 (Hori et al., 2009b; Narancic et al., 2012; Ni et al., 2010; Nikodinovic et al., 2008; Wang & Nomura, 2010).

The bacterial isolates were tested for their ability to assimilate styrene for MCL-PHA formation. Despite positive growth on styrene and having the genetic potential for PHA biosynthesis, no PHA was detected in *Stenotrophomonas* and *Rhodococcus* bacterial isolates (Table 6.2). The *Stenotrophomonas* genus was previously described by Palleroni and Bradbury (1993) as non-PHA-forming. This was further supported by Romo et al. (2007), who failed to detect *phaC* gene in *S. maltophilia*. It was therefore interesting to note that *Stenotrophomonas* sp. GSUS9 tested positive for *phaC* gene based on the detection method developed by Romo et al. (2007) (Figure 6.2B). This result could be attributed to pseudogene or unspecific primer amplification, although further verification will be needed. On the other hand, PHA-producing species from the *Rhodococcus* genus have been reported (Tan et al., 2014b), albeit none of them could produce PHA using styrene. PHA-accumulating bacteria, including *Rhodococcus*, are known to exhibit selectivity over carbon substrates for PHA production (Haywood et al., 1991; Lee et al., 2001; Narancic et al., 2012). One of the possible underlying reasons is the inhibition of enzyme PHA synthase by metabolite intermediates. Although this has not been reported in *Rhodococcus*, it has been observed in *P. fluorescens* BM07. The bacterium accumulates PHA from 7-carbon hepatanoic acid to 16-carbon hexadecanoic acid but stores 18-carbon octadecanoic acid as intracelluar free fatty acid instead, possibly because PHA synthase was inhibited by octadecanoic acid (Lee et al., 2001). Another likely explanation is the presence of a competing anabolic pathway for carbon degradation intermediates. This was evinced in *R. aetherivorans* IAR1 where the triacylglycerol anabolic pathway competed with the PHA anabolic pathway for degradation by-products from toluene (Hori et al., 2009a).

All the *phaC*+ Pseudomonad isolates could bioconvert styrene into MCL-PHA

(Table 6.2). Hence, within the context of this study, it appears that a short enrichment period (*i.e.*, 11 weeks) for non-contaminated inoculum source, such as domestic activated sludge, was sufficient to yield styrene-degrading bacteria with MCL-PHA accumulation property, providing a relatively simple and fast enrichment strategy. The MCL-PHA detected were C_6, C_8, C_{10} and C_{12} monomers, which was consistent with previous studies (Narancic et al., 2012; Nikodinovic et al., 2008). Existing literature suggested that the bioconversion process may begin with the side-chain oxidation of styrene and *β*-oxidation to form acetyl-CoA through phenylacetaldehyde before acetyl-CoA enters a downstream *de novo* fatty acid synthesis pathway which generates MCL-PHA precursors of varying chain length for MCL-PHA polymerization (O'Leary et al., 2005). Aside from the aforementioned styrene catabolism pathway, aerobic styrene degradation is also known to occur via side-chain oxidation to form benzoate intermediate and via direct ring cleavage (Tischler & Kaschabek, 2012). While there are currently no evidence of coupling between these two styrene catabolism pathways and MCL-PHA anabolism pathway, the observation that some of this study's bacterial isolates (*i.e.*, Group 2, 3, 4 and 7) were also benzoate-degrading may hint at the possibility of an alternative bioconversion pathway. Further investigation would be required to elucidate the mechanism underlying the isolates' bioconversion property.

Differences in growth and cellular MCL-PHA accumulation were observed among the various groups of Pseudomonad isolates (Table 6.2). Low MCL-PHA contents (between 0.05 ± 0.00% CDM and 0.08 ± 0.01% CDM) and low PHA yields (between 0.0005 g PHA.g styrene^{-1} and 0.0009 g PHA.g styrene^{-1}) were observed for Group 2, 6 and 7. It could be noted that these groups clustered closely with styrene-degraders *Pseudomonas* sp. LQ26 and *Pseudomonas* sp. VLB120, nonylphenol polyethoxylates-degrading *P. putida* AS90, catechol-degrader *P. aeruginosa* L-4 and atrazine-degrader *Pseudomonas* sp. ADP (Lin et al., 2010; Liu et al., 2006; Mandelbaum et al., 1995; NCBI, 2014a; Panke et al., 1998) (Figure 6.3A and Table 6.1). However, bioaccumulation of PHA has not been previously described in these Pseudomonads. Although the results provided the first evidence of MCL-PHA production in these Pseudomonads, the PHA contents were too low

to be considered attractive for application purposes.

Group 4 and 5 both shared close evolutionary relationship to existing MCL-PHA producing *Pseudomonas* strains (Figure 6.3A). Group 4 was closely-related to *P. putida* F1, which reportedly produce MCL-PHA from benzene, toluene and ethylbenzene (Nikodinovic et al., 2008), and with *Pseudomonas* sp. TN301 (99% identity with 97% coverage), which could produce MCL-PHA from styrene (Narancic et al., 2012). Compared to *Pseudomonas* sp. TN301 however, the growth (0.37 ± 0.19 g.L^{-1}) and PHA content (6.23 ± 1.46% CDM) of Group 4 were about 2-folds higher, implying that Group 4 isolates were more efficient at utilizing styrene for PHA formation. Group 5 was closely-related to *P. putida* mt-2 which utilizes toluene, *p*-xylene and various non-aromatic carbons for MCL-PHA production (Nikodinovic et al., 2008); to *P. fulva* TY16, which reportedly produce MCL-PHA from benzene, toluene and ethylbenzene (Ni et al., 2010); and to *P. putida* KT2440 which produces MCL-PHA from glucose and alkanoic acids (Tan et al., 2014b). However, the cellular PHA content and PHA yield for Group 5 was low (0.59 ± 0.27% CDM; 0.0064 g PHA.g styrene^{-1}). Typically, MCL-PHA contents could reach up to between 20 and 30% CDM with aromatic carbon substrates (Tan et al., 2014b), which meant that PHA content of both Group 4 and 5 is low in comparison. Nevertheless, Group 4 and Group 5's close evolutionary neighbors reportedly utilized various aromatic compounds for PHA synthesis and accumulated high amounts of MCL-PHA content (*Pseudomonas* sp. TN301, 23% CDM [naphthalene]; *P. putida* F1, 22% CDM [toluene]; *P. putida* mt-2, 26% CDM [*p*-xylene]; *P. fulva* TY16, 58.9 %CDM [toluene]) (Narancic et al., 2012; Ni et al., 2010; Nikodinovic et al., 2008). This offers the possibility that Group 4 and 5 bacterial isolates may be able to metabolize other toxic aromatic pollutants and accumulate MCL-PHA in higher amounts. Additional studies will be necessary for further verification.

Among all the groups of isolates, Group 3 showed the highest growth, MCL-PHA content and PHA yield (0.63 ± 0.14 g.L^{-1}; 13.67 ± 5.22% CDM; 0.1424 g PHA.g styrene^{-1}) (Table 6.1). An isolate, subsequently identified and designated as *P. putida* NBUS12 (Figure 6.4), displayed the highest growth (0.80 ± 0.03 g.L^{-1}),

MCL-PHA accumulation (23.10 ± 3.25% CDM) and PHA yield (0.2296 g PHA.g styrene^{-1}) on styrene. The cellular MCL-PHA content of *P. putida* NBUS12 was similar to *P. putida* CA-3 (Ward et al., 2005) and about 9 to 20% CDM higher than other styrene-degrading *Pseudomonas* strains (*i.e.*, *P. putida* CA-1, *P. putida* S12, and *Pseudomonas* sp. TN301) (Narancic et al., 2012; Tobin & O'Connor, 2005), making it one of the highest PHA-producing strain among existing styrene-degrading bacteria and warranted its selection for further investigation.

Genetic analyses revealed *P. putida* NBUS12 as an evolutionary-distinct strain from known styrene-degrading and MCL-PHA-producing *Pseudomonas* strains. The 16S rDNA and *phaZ* genes of *P. putida* NBUS12 did not share particularly high similarity to that of *Pseudomonas* sp. TN301 (16S rDNA, 99% identity with 97% coverage) and *P. putida* CA-3 (*phaZ*, 88% identity with 100% coverage) (Figures 6.3A and 6.7A). Instead, higher genetic similarity was observed with non-PHA-producing *Pseudomonas* strains *P. putida* BCNU 106 (16S rDNA, 100% identity with 99% coverage) and *Pseudomonas* sp. VLB120 (*phaZ*, 100% identity with 100% coverage). While most of the genetic variations observed between *phaZ* sequences from *P. putida* NBUS12 and *P. putida* CA-3 were attributed to codon degeneracy (PhaZ, 98% identity with 100% coverage) (Figure 6.7B), some genetic variations resulted in amino acid substitutions (data not shown). Additionally, ClustalW analyses of *phaZ* sequences and PhaZ sequences showed *P. putida* NBUS12 as an evolutionarily-distinct branch from *P. putida* CA-3 and other known PHA-producing Pseudomonads (Solaiman & Ashby, 2005). This suggests that despite its phenotypic-similarity to existing bacterial strains, *P. putida* NBUS12 could be considered as a new styrene-degrading and MCL-PHA-producing bacterial strain.

A 500 mL volume of cultivation using a higher inoculum amount of *P. putida* NBUS12 led to an improvement in final biomass production (1.28 ± 0.10 g.L^{-1}), PHA production (0.42 ± 0.04 g.L^{-1}), which represented a 1.6-fold and 2.2-fold increase, respectively, compared to shake-flask culture (Table 6.2). There was an increase in PHA yield (0.2959 g PHA.g styrene^{-1}) compared to shake-flask cultivation (0.2295 g PHA.g styrene^{-1}). Higher MCL-PHA content (32.49 ± 2.40%

CDM) was also observed and found to be comparable to values obtained by *P. putida* CA-3 (Nikodinovic-Runic et al., 2011). The extracted MCL-PHA polymer mainly comprised of C_6, C_8, C_{10}, C_{12}, C_{14} in the ratio of 2:42:1257:17:1, showing an increased dominance of C_{10} monomer and an additional C_{14} monomer (Figures 6.9 and 6.10), which were previously not observed under shake-flask cultivation (Table 6.2). Variations in monomer content for the same bacterium are known to occur (Rai et al., 2011), and may be ascribed to cultivation conditions which in turn influence the activity of enzymes involved in the *de novo* fatty acid synthesis pathway, leading to the increased synthesis of C_{10} monomer and synthesis of C_{14} monomers (Witholt & Kessler, 1999). PHA monomers such as C_6, C_8, C_{10}, C_{12}, and $C_{12:1}$ are known to arise from styrene metabolism (Narancic et al., 2012; Nikodinovic et al., 2008; Tobin & O'Connor, 2005; Ward et al., 2005). While C_{14} monomer has been previously reported as a minor monomer in MCL-PHA formed from glucose, long-chain alkanoic acids and tallow (Rai et al., 2011), this monomer is not known to derive from styrene. This study presents the first report of C_{14} monomer synthesis from styrene.

The properties of PHA polymer produced by *P. putida* NBUS12 were found to be in good agreement with those expected for MCL-PHA and the polymer was likely to resemble thermoplastic elastomers (Table 6.5) (Muhr et al., 2013; Rai et al., 2011). Unlike SCL-PHA, which typically has much higher T_m (up to 180 °C) and crystallinity (up to 78%) (Peña et al., 2014), the combination of reduced T_m (50.67 °C) and crystallinity (13.57%) made MCL-PHA more malleable and biodegradable (Rai et al., 2011). Greater biocompatibility over SCL-PHA was also reported for MCL-PHA due to a reduced cytotoxicity observed for PHA monomers with longer carbon number (J. Sun et al., 2007). These attributes collectively meant that MCL-PHA, produced from *P. putida* NBUS12, may have potential high-value medical applications such as soft tissue regeneration, controlled drug delivery matrices, or as plastic coatings and pressure sensitive adhesives (Madison & Huisman, 1999; Rai et al., 2011). Compared to MCL-PHA polymer produced from *P. putida* CA-3 using styrene (Ward et al., 2005), this study's polymer displayed higher M_w (101,500 Da) and M_n values (49,300 Da), and lower PDI value (2.06).

Higher M_w values are usually associated with greater PHA polymer tensile strength (Quagliano Javier & Miyazaki Silvia, 2002; Volova, 2004) suggesting that the MCL-PHA obtained in this study may be more suitable for applications requiring higher tensile strength. The wider temperature range of between T_g (-46.05 °C) and T_m (50.67 °C), and higher DT value (277 °C) also meant that this polymer has a wider processing and application temperature, which may expand the applicability of the polymer. Nevertheless, the M_w of this study's polymer was still relatively low compared to MCL-PHA produced from other carbon sources (*e.g.*, sugars, alkanes, alkenes and long-chain fatty acids) where M_w values ranged from 124,000 Da to 339,000 Da (Rai et al., 2011). Factors such as the state of inoculums, cultivation media composition, fermentation conditions and PHA extraction process have been asserted to influence PHA's M_w (Rai et al., 2011). The investigation of the aforementioned factors on M_w of MCL-PHA from *P. putida* NBUS12 could be the subject of future studies.

It is anticipated that *P. putida* NBUS12 could serve as a good candidate for the bioaugmentation of styrene-degrading systems to shorten the start-up time of the system and to enhance PHA production from styrene. However, there has been reports of limited success with bioaugmentation due to poor survival and performance of the exogenous species (Thompson et al., 2005). Given that there were phylogenetic differences in Pseudomonad cultures obtained from the same sludge inoculum but at different time points of the enrichment (Figure 6.3), it is unknown if *P. putida* NBUS12, which was isolated from BTEXS-enriched sludge, could be successfully established as a permanent member of a styrene-degrading consortium. To verify, additional long-term bioaugmentation studies will be needed. *P. putida* NBUS12 could also be utilized for the formulation of microbial cocktail that may achieve higher PHA production as compared to mixed cultures. *P. putida* NBUS12, together with the other pure cultures obtained in this study, belonged to bacterial classes Gammaprotebacteria and Actinobacteria. While these two bacterial classes were dominant in styrene-degrading community, there were also other dominant classes detected, specifically, Alphaproteobacteria, Sphingobacteria and Clostridia (Chapter 5, Tables 5.2 and 5.3). Hence, the diversity of culturable isolates

is currently limited and could not adequately reflect the bacterial community structure of styrene-enriched consortium. Additionally, aside from Pseudomonads, it is not known if there may be other culturable PHA-producing species present within the consortium. Future isolation work, using varied cultivation strategies, would be necessary to increase the diversity of pure isolates for the formulation of microbial cocktails. The long-term robustness and performance of microbial cocktails for bioconversion of styrene to PHA would also need to be assessed.

6.5 Summary

This study demonstrated a simple enrichment strategy for the isolation of bacteria, capable of bioconverting organic pollutant styrene into MCL-PHA, from both petrochemical-contaminated and non-contaminated inoculum sources. Seventeen styrene-degrading and MCL-PHA-producing Pseudomonad isolates were obtained. The best-performing isolate was a novel strain, *P. putida* NBUS12, which could achieve a growth of 1.28 ± 0.10 $g.L^{-1}$, MCL-PHA content of 32.49 ± 2.40% CDM and PHA yield of 0.2295 g PHA.g $styrene^{-1}$, making it one of the highest PHA-producing strain among known styrene-degrading bacteria. MCL-PHA polymer from *P. putida* NBUS12 showed monomeric components distinctive from existing phenotypically-similar bacterial strains and provided the first evidence of C_{14} monomer synthesis from styrene. Besides saturated monomers (*i.e.*, C_6, C_8, C_{10}, C_{12}, C_{14}), the PHA also contains unsaturated monomers, which hold the possibility for further chemical modifications. The chemical modifications can greatly impact PHA properties, and will expand its usage in the medical and environmental areas. The higher degree of monomer heterogeneity may influence and result in different polymer properties, expanding the possible applications of MCL-PHA polymer derived from styrene-based source. Collectively, this study successfully increased the limited pool of unique bacterial cultures which could aid in the development of biotechnologies for simultaneous styrene effluent treatment and MCL-PHA recovery. Future work will be focused on improving *P. putida* NBUS12's PHA production and polymer properties to enhance the economic viability of this bioprocess. The suitability of *P. putida* NBUS12 as a bioaugmentation strain should be assessed as well.

CHAPTER 7

Conclusions and Recommendations

7.1 Conclusions

7.1.1 HPLC-DAD method for analysis of BTEXS at high aqueous concentrations

A HPLC-DAD method, based on a new commercial reversed-phase column Acclaim Phenyl-1, was successfully developed for the concurrent detection of aqueous BTEXS. Optimal operation parameters were determined as detection wavelength of 201 nm, column oven temperature of 50 °C, water/methanol mobile phase compositions of 55%/45% to 60%/40%, flow rate sample injection volumes of up to 10 µL and flow rates of up to 2.0 $mL.min^{-1}$. Under the optimal conditions, chromatographic separation of BTEXS, co-occurring at high equimolar aqueous concentration of up to 2.0 mM, could be achieved in 5.5 min. The method was also suitable for the detection of styrene, toluene, ethylbenzene and xylenes at the lower detection limits prescribed by US EPA. Repeatability, reproducibility and method recovery values in various aqueous matrices (*i.e.*, ultrapure water, tap water, industrial wastewater and domestic wastewater) were all within the quality control criteria prescribed for US EPA methods. The method offered several advantages over existing methods: (1) protocol simplification, minimal sample loss and minimized exposure to harmful compounds owing to direct injection and concurrent analysis of aqueous BTEXS mixture, (2) decreased analysis time by up to 60% which provides high analysis throughput, and (3) lowered analysis price due to savings in chemical usage (by about 25%) and lower solvent waste generation.

The HPLC-DAD method was utilized concurrently with a GC-FID method for the detection and quantification of aqueous styrene and gaseous styrene, respectively, in a biodegradation test (Study 3, Chapter 5). It was observed that the biological elimination of styrene from liquid phase occurred at a faster rate compared to the elimination of styrene from gas phase. This observation further reinforced the idea that styrene biodegradation occurred mostly in the liquid phase. Consequently, the elimination of aqueous styrene provides a force that drives the partition of headspace styrene to the cultivation medium, resulting in the observed elimination of gaseous styrene. There are previous studies which performed the analysis of either liquid styrene or headspace styrene and estimated the overall styrene amount

using the compound's gas-liquid partition coefficient (Babaee et al., 2010; Jung & Park, 2005). This finding proved that this method is unlikely to yield an accurate picture of the true styrene biodegradation kinetics. Therefore, the measurements of styrene in both liquid and gaseous state are necessary for a more reliable quantification of overall styrene elimination in a biological system.

7.1.2 GC-MS method development for analysis of PHA biopolymer

A key novel finding is the existence of a highly linear relationship between the carbon number of homologous saturated PHA monomers and their respective RT and RF. Based on this key discovery, the RTs and RFs of most saturated PHA monomeric homologues were estimated with high accuracy, which enabled the development of a GC-MS calibration method for various PHA monomers, ranging from C_4 to C_{16}, using a limited inventory of reference standards. The only exception was the RT of C_4 monomer where a large difference between observed RT and predicted RT was found. However, this discrepancy was circumvented through cross-validation with mass fragmentation information. Hence, RT prediction should be considered together with mass spectra information to minimize errors resulting from the mathematical prediction. The calibration method was tested using known amounts of various SCL-PHA and MCL-PHA co/homopolymers including PHBHV, which contained C_5 monomer that was excluded from method development. For all the PHA polymers, the method showed high recovery values of close to 100%, demonstrating its reliability and applicability to actual PHA samples even in the absence of some analytical standards. Therefore, this method is the first of its kind to prove the linear correlations between the carbon number of PHA monomer reference standards and RT/RF under GC-MS analysis, and leverage on these relationships to interpolate the RT/RF of other PHA monomers, for which reference standards are unavailable, resulting in successful quantification of PHA polymers. The GC-MS method was also used for the analysis of cellular MCL-PHA in styrene-degrading bacterium isolates and MCL-PHA polymer produced by selected isolate *P. putida* NBUS12. Among other monomers (*i.e.*, C_8, C_{10} and C_{12}), the method also successfully detected and quantified C_6 and C_{14} monomers based on predicted RT and RF values, respectively (Study 4, Chapter 6). It is noteworthy that

the GC-MS results for the MCL-PHA polymer were in good agreement with NMR results, further attesting the accuracy and reliability of the method. The method offered several advantages over existing methods: (1) higher accuracy for PHA analysis even with incomplete analytical standard inventory, (2) calibration protocol simplification and decreased calibration workload (by up to 80%), and (3) decreased calibration cost (by up to 80%). Furthermore, the calibration method may serve as a methodological reference for the future development of similar calibration method for other types of PHA homologous series. This is crucial in circumventing the lack of PHA analytical standards for many PHA monomer types (*e.g.*, unsaturated PHAs, branched PHAs, and PHA with specialized functional *R* groups). Hence, this study has made a significant and positive impact on PHA analysis.

7.1.3 Microbial profile analysis of styrene-degrading bacterial consortium under prolonged enrichment

A styrene-degrading consortium was successfully enriched from a BTEXS-enriched activated seed sludge. The bacterial community was mostly dominated by Proteobacteria, followed by Bacteriodetes, Firmicutes, Actinobacteria and unclassified phylum. The phyla Plantomycetes, Deinococcus-Thermus, Verrucomicrobia, Chloroflexi, Acidobacteria, Aquificae, Chlamydiae, and Gemmatimonadetes were also detected, but at non-dominant levels. At the order level, the most striking increase was observed for Pseudomonadales (Proteobacteria), strongly suggesting that this order is important for styrene elimination. This observation was further supported by numerous existing reports of styrene degradation capability for many pure isolates from Pseudomonadales Arnold et al., 1997; Lin et al., 2010; Narancic et al., 2012; O'Connor et al., 1995; Panke et al., 1998; Tobin & O'Connor, 2005). Rhizobiales (Proteobacteria) was also a prominent order, and may harbor styrene catabolic genes (O'Leary et al., 2002; van Hellemond et al., 2007). Taken together, these findings, coupled with current literature data, suggest that Pseudomonadales and Rhizobiales may form the core styrene-degrading bacterial population in this study's system.

Equally prominent in the system were the orders Sphingobacteriales (Bacteroidetes), Clostridiales (Firmicutes), and Actinomycetales (Actinobacteria). Some genera members of these orders are reportedly styrene-degraders. These include *Sphingobacterium* (Sphingobacteriales) (Przybulewska et al., 2006), *Rhodococcus*, *Mycobacterium*, *Tsukamurella* (Actinomycetales) (Arnold et al., 1997; Burback & Perry, 1993; Jung & Park, 2005; Warhurst et al., 1994). However, these genera members were detected at low levels, indicating that they are not the main styrene-degrading population. Nevertheless, these orders are also known for their versatile aromatic catabolic capability including styrene degradation intermediates (*i.e.*, phenylacetic acid, catechol, homogentisate, fumarylacetoacetate, benzoate, *etc*). This suggests that these orders could form the secondary styrene-degrading bacterial population in this study's system, which was responsible for mineralization of styrene degradation by-products.

Potential PHA-producing bacterial genera, from dominant phyla, were also detected. These genera include *Pseuodmonas*, *Acinetobacter*, *Hyphomicrobium*, *Methylobacterium*, *Nitrobacter*, *Rhizobium*, *Rhodopseudomonas*, *Xanthobacter*, *Paracoccus*, *Azospirillum*, *Comamonas*, *Cupriavidus* and *Thiobacillus* (Proteobacteria), *Rhodococcus* (Actinobacteria), and *Clostridium* and *Syntrophomonas* (Firmicutes). This hints at the possibility that PHA-producing members may be native to aqueous styrene-degrading consortium and the mixed culture has the potential for PHA production.

Collectively, the findings from this study presented the first reference of an aqueous styrene-degrading bacterial community. A key contribution of the study is the identification of biomarkers (*i.e.*, Pseudomonadales, Rhizobiales, Sphingobacteriales, Clostridiales and Actinomycetales), which provided a knowledge scaffold guiding future process enhancement strategies such as selection of seeding sludge, bioaugmentation to reduce reactor start-up time and optimize the biological process. Additionally, the knowledge could be applied to guide the formulation of defined microbial cocktails for simultaneous styrene elimination and MCL-PHA production.

7.1.4 MCL-PHA-producing and/or styrene-degrading bacterial isolates

A total of 20 styrene-degrading bacterial pure cultures, which also tested positive for partial *phaC* gene detection, were isolated through two isolation attempts. Of these, through the first isolation attempt, 5 *Pseudomonas* species were obtained from industrial activated sludge while 7 *Pseudomonas* species and 1 *Rhodococcus* species were obtained from domestic activated sludge following 11 weeks of enrichment on BTEXS. Based on phylogenetic clustering, there was little difference observed in the type of *Pseudomonas* species isolated from both inoculum sources (Figure 6.3A and Table 6.1). The domestic activated sludge was further enriched for a total of 15 months on BTEXS, followed by another 19.5 months on styrene, before another round of isolation was conducted. The second isolation attempt yielded 5 *Pseudomonas* species, 1 *Rhodococcus* species and 1 *Stenotrophomonas* species. Phylogenetic clustering analysis revealed that the type of *Pseudomonas* and *Rhodococus* species, obtained through the second isolation attempt, were distinct from those obtained through the first isolation attempt (Figure 6.3A and Table 6.1). This indicated an evolution in bacterial species within the domestic activated sludge under prolonged BTEXS and styrene enrichment, which supported the observed bacterial community evolution reported in Study 3 (Figure 5.3). The genera of styrene-degrading bacterial isolates (*i.e.*, *Pseudomonas*, *Rhodococus* and *Stenotrophomonas*), from the second isolation attempt, corroborated with existing literature (Jimenez et al., 2004; Kuyukina & Ivshina, 2010; Seo et al., 2009) and were also in good agreement with those detected through bacterial community profile analysis (Study 3, Chapter 5), affirming the presence and styrene-assimilating function of these bacterial genera in styrene-degrading consortium.

Despite testing positive for partial *phaC* gene detection, PHA formation from styrene was not observed in *Rhodococus* and *Stenotrophomonas* isolates. Only *Pseudomonas* isolates were capable of bioconverting styrene to MCL-PHA bioconversion (Table 6.2). Variation in MCL-PHA bioaccumulation abilities were observed for different *Pseudomonas* species. Most *Pseudomonas* isolates, including those obtained from the second isolation attempt, bioaccumulated MCL-PHA at low amounts of less than 1.0% CDM, resulting in low PHA yields (Table 6.2). Hence,

while styrene-degrading consortium may comprise of MCL-PHA-producing *Pseudomonas* species, these species did not appeared to be high PHA producers. The highest MCL-PHA bioaccumulation (up to 32.49 ± 2.40% CDM) and PHA yield (up to 0.2295 g PHA.g styrene^{-1}) was observed in a novel strain designated *P. putida* NBUS12, which was isolated from domestic activated sludge in the first isolation attempt. *P. putida* NBUS12 assimilated styrene for the biosynthesis of MCL-PHA, which was comprised of C_6, C_8, $C_{10,}$ C_{12} and C_{14} monomers. The biopolymer properties were well-aligned with previous reports (Muhr et al., 2013; Rai et al., 2011) and resembled those of thermoplastic elastomers (Table 6.5). Coupled with biodegradable and biocompatible attributes, the MCL-PHA biopolymer could have potential high-value medical and pharmaceutical applications (Madison & Huisman, 1999; Rai et al., 2011). This study has provided alternative bacteria strains for further study and application, which may well facilitate the development of a biotechnology that mines valuable MCL-PHA from styrene waste. The newly-isolated MCL-PHA-producing and/or styrene-degrading bacterial isolates, particularly *P. putida* NBUS12, may be applied as bioaugmentation strains to shorten the start-up time of styrene-degrading reactors or formulated as part of a defined microbial cocktail for enhanced MCL-PHA production from styrene.

7.2 Recommendations

Several research outcomes have been achieved through this work: (1) a new analytical method for BTEXS detection at high aqueous concentrations, (2) a new analytical method for PHA detection, (3) new insights on the bacterial community profile of an aqueous styrene-degrading microbial consortium, and (4) a novel styrene-degrading *cum* MCL-PHA-producing bacterial culture. Nevertheless, the findings from this work have also led to new research questions that need to be addressed in the future.

7.2.1 Developing analytical methods for more PHA monomer types

An accurate and reliable analytical calibration method is integral to the detection and quantification of PHA. Qualitative and quantitative information about PHA

may further impact the selection of biological process strategies and operation parameters. While this work has developed a GC-MS calibration method for saturated PHA monomers with alkyl functional *R* groups, there is currently a lack in analytical methods for other types of PHA monomers such as unsaturated PHAs, branched PHAs, and PHA with specialized functional *R* groups. The analysis of these aforementioned PHA monomer types continues to pose difficulties to researchers due to the lack of readily-available commercial standards. This work may provide a methodological reference upon which GC-MS calibration methods for other types of PHA could be similarly modeled after. Since the chemical diversity of PHA monomers is largely dependent on its functional *R* group, it is anticipated that a carefully-curated but limited inventory of homologous PHA analytical standards, containing the desired functional *R* group, may be sufficient for the development of a GC-MS calibration method that is capable of analyzing a wide range of PHA monomers within the same homologue series. Hence, future research efforts could build upon this work and expand the theoretical concept, behind this work, for the development of analytical methods for other types of PHA monomer.

7.2.2 Widen styrene-degrading microbial community analysis to non-bacterial microorganisms and linking microbial profile to performance

This work presented a description of the bacterial community structure of a styrene-degrading activated sludge, its changes under prolonged styrene exposure, and identified potential PHA-accumulating members. Aside from bacteria, fungi are also present in activated sludge, albeit at abundances ranging from two to three orders below that of bacteria (Cooke & Pipes, 1970). Nevertheless, several fungi species are also known to metabolize styrene (Tischler & Kaschabek, 2012), and have been found to co-exist with bacteria in styrene-degrading consortia (Cox et al., 1993; Hartmans et al., 1990). However, this study, as well as recent studies (Alexandrino et al., 2001; Arnold et al., 1997; Portune et al., 2015), were centered on bacteria population with little to no focus on fungi members in styrene-degrading consortia. Future investigations should examine the non-bacterial population of a styrene-degrading community. It is anticipated that, with the aid of modern

biomolecular techniques such as high-throughput next-generation sequencing, it would now be possible to obtain a more comprehensive picture and enhance the current understanding of the styrene-degrading microbial consortium. While elucidating the microbial players is undoubtedly an important step towards gaining a qualitative understanding on the signature 'microbial markers' of styrene-degrading activated sludge, the function of these consortium members also remains an open question, which could only be inferred from existing literature and bioinformatics databases. Future studies, involving a combination of several biomolecular techniques, such as isolation, cloning, proteomics, transcriptome and metabologenomics analyses, would be crucial in providing greater insights into the functional roles of consortium members.

7.2.3 Clarification of PHA-producing sub-population and PHA production capability of styrene-degrading microbial community

Based on this study and existing literature (Narancic et al., 2012; Tobin & O'Connor, 2005; Ward et al., 2005), only *Pseudomonas* isolates were capable of bioconverting styrene to PHA. However, many bacterial genera, with reported PHA production capabilities, have been detected in the aqueous styrene-degrading bacterial community. It remains unknown if there may be other genera of styrene-degrading *cum* MCL-PHA-producing bacterial genera. The abundance of these bacteria as well as their MCL-PHA production capacity would have practical implications on the application of styrene-enriched mixed cultures or the formulation of microbial cocktails for PHA production from styrene wastewaters. Therefore, there is a need to thoroughly investigate the PHA-producing sub-population and MCL-PHA production capability of styrene-degrading microbial community. This may be achieved through expanding culture-dependent isolation strategies to capture a higher diversity of culturable bacteria. Culture-independent methods such as transcriptomics and protemics, with a focus on PHA biosynthesis genes and proteins expression analyses, could also be utilized to capture information from unculturable bacteria.

7.2.4 Increasing bacterial isolate diversity for the formulation of styrene-degrading *cum* MCL-PHA-producing defined microbial cocktails

The direct application of pure culture for MCL-PHA production from styrene wastewaters may thwarted by poor prolonged survival and performance due to the culture's high sterility demand and sensitivity to impurities. Mixed cultures may generally display greater robustness in complex cultivation matrices (Cabrol & Malhautier, 2011) but tend to produce lesser PHA compared to pure microbial cultures due to the presence of low-PHA-accumulating and non-PHA-accumulating microorganisms (Lee, 1996; Salehizadeh & Van Loosdrecht, 2004). On the other hand, defined microbial cocktail, which is a microbial culture comprising of two or more pure microbial isolates, may circumvent the disadvantages associated with the conventional pure microbial cultures and mixed microbial cultures by displaying greater robustness to cultivation matrix impurities (Bader et al., 2010) while enabling a greater control over the microbial community structure such that high-PHA-producing bacteria can be included as cocktail members while low- and non-PHA-producing bacteria can be excluded.

To formulate a styrene-degrading *cum* PHA-producing defined microbial cocktail that is robust enough to persist under the complex matrix of styrene wastewater, sufficiently diverse bacterial isolates are required. The bacterial community profile analysis has revealed an intricate styrene-degrading consortium, comprising of many bacterial members (Tables 5.2 and 5.3). Despite the presence of many culturable bacteria, only 3 genera of bacteria (*i.e., Pseudomonas, Stenotrophomonas* and *Rhodococcus*), which belonged to 2 bacterial classes (*i.e.*, Gammaproteobacteria and Actinobacteria), were successfully isolated. Of which, only *P. putida* NBUS12 and its closely-clustered bacterial isolates (*i.e.*, NBUS5, NBIWW5) could metabolize styrene and bioaccumulate MCL-PHA at high amounts (Table 6.2). Interestingly, *Stenotrophomonas* and *Rhodococcus* bacterial isolates tested positive for partial *phaC* gene detection, but were not observed to bioconvert styrene to PHA. PHA production has been previously-described in the genus *Rhodococcus* but not the genus *Stenotrophomonas*. It is not known if partial *phaC* gene detected were false positives attributed to pseudogene, unspecific primer

amplification or these two isolates are indeed capable of PHA production but were unable to do so under the tested conditions. There were also many *Pseudomonas* isolates that could accumulate MCL-PHA from styrene, but do so at low levels. Based on phylogentic clustering, some *Pseudomonas* isolates have close evolutionary relationship to existing *Pseudomonas* species that could produce MCL-PHA at high amounts from other aromatic substrates (Figure 6.3 and Table 6.1). It may be possible that these *Pseudomonas* isolates could achieve higher MCL-PHA production with other aromatic carbon substrates such as styrene degradation intermediate compounds. As such, there is a need to address the discrepancy observed in *Stenotrophomonas* and *Rhodococcus* bacterial isolates, to clarify the PHA production of "low PHA-producing" *Pseudomonas* isolates using styrene metabolites, and to increase the diversity of bacterial isolate that is capable utilizing styrene or styrene metabolites for PHA production. To do so, cultivation and isolation strategies could be further expanded to include more carbon substrates, cultivation media, solidifying media, incubation parameters, and *etc*. Increasing bacterial isolate diversity also enables the study of the functional role of different consortium bacterial members, which could help to link microbial profile to reactor performance.

7.2.5 Investigation on *P. putida* NBUS12's metabolic pathway and MCL-PHA polymer property

Some interesting observations were noted for the new styrene-degrading and high MCL-PHA-producing isolate *P. putida* NBUS12. In particular, the bacterium was able to metabolize both styrene and benzoate. Benzoate is typically a degradation by-product from side-chain oxidation of styrene by fungi or from anaerobic styrene catabolism by bacteria (Grbić-Galić et al., 1990; Tischler & Kaschabek, 2012). Currently, the known pathway for styrene to MCL-PHA bioconversion came from the studies of *P. putida* CA-3, and involved the side-chain oxidation styrene to form phenylacetaldehyde intermediate for subsequent MCL-PHA formation (O'Leary et al., 2005). There is currently no evidence of coupling between styrene catabolic pathway and MCL-PHA anabolic pathway through benzoate intermediate. Hence, it is uncertain at this point if the bacterium could utilize such an alternative

bioconversion pathway. Further clarification of the bacterium's bioconversion metabolic pathways would be necessary.

Additionally, *P. putida* NBUS12 was observed to produce MCL-PHA containing C_{14} monomer. This finding presents the first reference of C_{14} monomer, which is by far the monomer with the longest functional *R* group known to arise from styrene metabolism. The incorporation of PHA monomers with large *R* groups reportedly disrupts the crystalline packing structure within MCL-PHA, lowering the polymer's cystallinity (Rai et al., 2011). The biopolymer from *P. putida* NBUS12 was found to display slightly different properties from that of *P. putida* CA-3, which makes the polymer more suitable for applications requiring wider processing/application temperature and higher tensile strength. However, the M_w of the polymer was still relatively low compared to MCL-PHA produced from other carbon sources (Rai et al., 2011). Higher M_w is known to enhance the mechanical properties, increasing tensile strength (Quagliano Javier & Miyazaki Silvia, 2002; Volova, 2004) and thereby expanding the potential application niches of MCL-PHA. Hence, more research is needed to increase the M_w of MCL-PHA from *P. putida* NBUS12 and examine the impact of C_{14} monomer incorporation on polymer property.

References

Aalam, S., Pauss, A., & Lebeault, J.-M. (1993). High efficiency styrene biodegradation in a biphasic organic/water continuous reactor. *Applied Microbiology and Biotechnology, 39*(6), 696-699.

Abduli, M. A., Abbasi, M., Nasrabadi, T., Hoveidi, H., & Razmkhah, N. (2006). Solid waste management in Tabriz Petrochemical Complex. *Iranian Journal of Environmental Health, Science and Engineering, 3*(3), 185-192.

The Advent Corporation. Company Profile and Statement of Qualifications

Ahmad, M., Bajahlan, A., & Hammad, W. (2008). Industrial effluent quality, pollution monitoring and environmental management. *Environmental Monitoring and Assessment, 147*(1), 297-306.

Akiyama, M., Taima, Y., & Doi, Y. (1992). Production of poly(3-hydroxyalkanoates) by a bacterium of the genus *Alcaligenes* utilizing long-chain fatty acids. *Applied Microbiology and Biotechnology, 37*(6), 698-701.

Alexandrino, M., Knief, C., & Lipski, A. (2001). Stable-isotope-based labeling of styrene-degrading microorganisms in biofilters. *Applied and Environmental Microbiology, 67*(10), 4796-4804.

Alfreider, A., & Vogt, C. (2007). Bacterial diversity and aerobic biodegradation potential in a BTEX-contaminated aquifer. *Water, Air, and Soil Pollution, 183*(1-4), 415-426.

Aliabadi, M., Aroujalian, A., & Raisi, A. (2012). Removal of styrene from petrochemical wastewater using pervaporation process. *Desalination, 284*, 116-121.

Allen, A., Daley, P., Ayorinde, F., Gugssa, A., Anderson, W., & Eribo, B. E. (2012). Characterization of medium chain length (*R*)-3-hydroxycarboxylic acids produced by *Streptomyces* sp. JM3 and the evaluation of their antimicrobial properties. *World Journal of Microbiology and Biotechnology, 28*(9), 2791-2800.

Almeida, C. M. M., & Boas, L. V. (2004). Analysis of BTEX and other substituted benzenes in water using headspace SPME-GC-FID: method validation. *Journal of Environmental Monitoring, 6*(1), 80-88.

AlSalka, Y., Karabet, F., & Hashem, S. (2010). Development and optimisation of quantitative analytical method to determine BTEX in environmental water samples using HPLC-DAD. *Analytical Methods, 2*(8), 1026-1035.

Amann, R. I., Ludwig, W., & Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews, 59*(1), 143-169.

Amosov, V. V., Zilberman, A. G., & Kucheryavykh, E. I. (1976). Experience in local treatment of wastewater from petrochemical production. *Chemistry and Technology of Fuels and Oils, 12*(11-12), 850-852.

Anderson, A. J., & Dawes, E. A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews, 54*(4), 450-472.

APIC. (2010). Market Data: Singapore. Retrieved Apr 18, 2012, from http://www.apic-online.org/countrydata_singapore.htm

Araya, P., Chamy, R., Mota, M., & Alves, M. (2000). Biodegradability and toxicity of styrene in the anaerobic digestion process. *Biotechnology Letters, 22*(18), 1477-1481.

Arnold, M., Reittu, A., von Wright, A., Martikainen, P. J., & Suihko, M. L. (1997). Bacterial degradation of styrene in waste gases using a peat filter. *Applied Microbiology and Biotechnology, 48*(6), 738-744.

Babaee, R., Bonakdarpour, B., Nasernejad, B., & Fallah, N. (2010). Kinetics of styrene biodegradation in synthetic wastewaters using an industrial activated sludge. *Journal of Hazardous Materials, 184*(1–3), 111-117.

Bader, J., Mast-Gerlach, E., Popović, M. K., Bajpai, R., & Stahl, U. (2010). Relevance of microbial coculture fermentations in biotechnology. *Journal of Applied Microbiology, 109*(2), 371-387.

Basile, L. A., & Erijman, L. (2010). Maintenance of phenol hydroxylase genotypes at high diversity in bioreactors exposed to step increases in phenol loading. *FEMS Microbiology Ecology, 73*(2), 336-348.

Beltrametti, F., Marconi, A. M., Bestetti, G., Colombo, C., Galli, E., Ruzzi, M., et al. (1997). Sequencing and functional analysis of styrene catabolism genes

from *Pseudomonas fluorescens* ST. *Applied and Environmental Microbiology, 63*(6), 2232-2239.

Boonchan, S., Britz, M. L., & Stanley, G. A. (1998). Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. *Biotechnology and Bioengineering, 59*(4), 482-494.

Bouwer, E. J. (1989). Biotransformation of aromatics in strip-pit pond. *Journal of Environmental Engineering, 115*(4), 741-755.

Bowers, R. M., Sullivan, A. P., Costello, E. K., Collett, J. L., Knight, R., & Fierer, N. (2011). Sources of bacteria in outdoor air across cities in the midwestern United States. *Applied and Environmental Microbiology*, *77*(18), 6350-6356.

Brandl, H., & Püchner, P. (1991). Biodegradation of plastic bottles made from 'Biopol' in an aquatic ecosystem under *in situ* conditions. *Biodegradation, 2*(4), 237-243.

Braunegg, G., Sonnleitner, B., & Lafferty, R. (1978). A rapid gas chromatographic method for the determination of poly-*β*-hydroxybutyric acid in microbial biomass. *European Journal of Applied Microbiology and Biotechnology, 6*(1), 29-37.

Burback, B. L., & Perry, J. J. (1993). Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. *Applied and Environmental Microbiology, 59*(4), 1025-1029.

Cabrol, L., & Malhautier, L. (2011). Integrating microbial ecology in bioprocess understanding: the case of gas biofiltration. *Applied Microbiology and Biotechnology, 90*(3), 837-849.

Campos-Candel, A., Llobat-Estellés, M., & Mauri-Aucejo, A. (2009). Comparative evaluation of liquid chromatography versus gas chromatography using a *β*-cyclodextrin stationary phase for the determination of BTEX in occupational environments. *Talanta, 78*(4-5), 1286-1292.

Carey, F. A. (2003). *Organic chemistry* (Fifth ed.). New York: McGraw-Hill.

Carmona, M., Zamarro, M. T., Blázquez, B., Durante-Rodríguez, G., Juárez, J. F., Valderrama, J. A., et al. (2009). Anaerobic catabolism of aromatic

compounds: a genetic and genomic view. *Microbiology and Molecular Biology Reviews, 73*(1), 71-133.

Castello, G. (1999). Retention index systems: alternatives to the *n*-alkanes as calibration standards. *Journal of Chromatography A, 842*(1–2), 51-64.

Chambers, D. M., McElprang, D. O., Waterhouse, M. G., & Blount, B. C. (2006). An improved approach for accurate quantitation of benzene, toluene, ethylbenzene, xylene, and styrene in blood. *Analytical Chemistry, 78*(15), 5375-5383.

Chang, C. N., Chen, H. R., Huang, C. H., & Chao, A. (2000). Using sequencing batch biofilm reactor (SBBR) to treat ABS wastewater. *Water Science & Technology, 41*(4-5), 433-440.

Chen, C.-L., Wu, J.-H., & Liu, W.-T. (2008). Identification of important microbial populations in the mesophilic and thermophilic phenol-degrading methanogenic consortia. *Water Research, 42*(8–9), 1963-1976.

Chen, G.-Q. (2010a). Industrial production of PHA. In G.-Q. Chen (Ed.), *Plastics from bacteria: Natural Functions and Applications* (Vol. 14, pp. 121-132). Berlin Heidelberg, Germany: Springer.

Chen, G.-Q. (2010b). Introduction of bacterial plastics PHA, PLA, PBS, PE, PTT, and PPP. In G.-Q. Chen (Ed.), *Plastics from Bacteria: Natural Functions and Applications* (Vol. 14, pp. 1-16). Berlin Heidelberg, Germany: Springer.

Choi, J., & Lee, S. Y. (1999). Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Applied Microbiology and Biotechnology, 51*(1), 13-21.

Claesson, M. J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, R. P., et al. (2010). Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Research*.

Coats, E. R., Loge, F. J., Smith, W. A., Thompson, D. N., & Wolcott, M. P. (2007). Functional stability of a mixed microbial consortium producing PHA from waste carbon sources. Applied Biochemistry and Biotechnology. In J. R. Mielenz, K. T. Klasson, W. S. Adney & J. D. McMillan (Eds.), (pp. 909-925): Humana Press.

Colin, P., Sirois, G., & Chakrabarti, S. (1986). Determination of styrene in biological samples by reversed-phase liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications, 375*, 431-437.

Comeau, Y., Hall, K. J., & Oldham, W. K. (1988). Determination of poly-*β*-hydroxybutyrate and poly-*β*-hydroxyvalerate in activated sludge by gas-liquid chromatography. *Applied and Environmental Microbiology, 54*(9), 2325-2327.

Cooke, W. B., & Pipes, W. O. (1970). The occurrence of fungi in activated sludge. *Mycopathologia et mycologia applicata, 40*(3-4), 249-270.

Corseuil, H. X., Hunt, C. S., Ferreira dos Santos, R. C., & Alvarez, P. J. J. (1998). The influence of the gasoline oxygenate ethanol on aerobic and anaerobic BTX biodegradation. *Water Research, 32*(7), 2065-2072.

Costa, S., Lépine, F., Milot, S., Déziel, E., Nitschke, M., & Contiero, J. (2009). Cassava wastewater as a substrate for the simultaneous production of rhamnolipids and polyhydroxyalkanoates by *Pseudomonas aeruginosa*. *Journal of Industrial Microbiology & Biotechnology, 36*(8), 1063-1072.

Cox, H. H., Houtman, J. H., Doddema, H. J., & Harder, W. (1993). Enrichment of fungi and degradation of styrene in biofilters. *Biotechnology Letters, 15*(7), 737-742.

Cushman, J. R., Rausina, G. A., Cruzan, G., Gilbert, J., Williams, E., Harrass, M. C., et al. (1997). Ecotoxicity hazard assessment of styrene. *Ecotoxicology and Environmental Safety, 37*(2), 173-180.

Dao, L., Grigoryeva, T., Laikov, A., Devjatijarov, R., & Ilinskaya, O. (2014). Full-scale bioreactor pretreatment of highly toxic wastewater from styrene and propylene oxide production. *Ecotoxicology and Environmental Safety, 108*, 195-202.

Davis, R., Kataria, R., Cerrone, F., Woods, T., Kenny, S., O'Donovan, A., et al. (2013). Conversion of grass biomass into fermentable sugars and its utilization for medium chain length polyhydroxyalkanoate (mcl-PHA) production by *Pseudomonas* strains. *Bioresource Technology, 150*(0), 202-209.

De Paula, R., Simões, M. M. Q., Neves, M. G. P. M. S., & Cavaleiro, J. A. S. (2011). Oxidation of styrene and of some derivatives with H_2O_2 catalyzed by novel imidazolium-containing manganese porphyrins: a mechanistic and thermodynamic interpretation. *Journal of Molecular Catalysis A: Chemical, 345*(1–2), 1-11.

de Rijk, T. C., van de Meer, P., Eggink, G., & Weusthuis, R. A. (2005). Methods for analysis of poly(3-hydroxyalkanoate) (PHA) composition. In Y. Doi & A. Steinbüchel (Eds.), *Biopolymers Online* (Vol. 3b, pp. 1-12). Weinheim, Germany: Wiley-VCH.

Dehghanzadeh, R., Torkian, A., Bina, B., Poormoghaddas, H., & Kalantary, A. (2005). Biodegradation of styrene laden waste gas stream using a compost-based biofilter. *Chemosphere, 60*(3), 434-439.

Demeestere, K., Dewulf, J., De Witte, B., & Van Langenhove, H. (2007). Sample preparation for the analysis of volatile organic compounds in air and water matrices. *Journal of Chromatography A, 1153*(1–2), 130-144.

Dobroth, Z. T., Hu, S., Coats, E. R., & McDonald, A. G. (2011). Polyhydroxybutyrate synthesis on biodiesel wastewater using mixed microbial consortia. *Bioresource Technology, 102*(3), 3352-3359.

Drepper, T., Eggert, T., Hummel, W., Leggewie, C., Pohl, M., Rosenau, F., et al. (2006). Novel biocatalysts for white biotechnology. *Biotechnology Journal, 1*(7-8), 777-786.

Durán, U., Monroy, O., Gómez, J., & Ramírez, F. (2008) Biological wastewater treatment for removal of polymeric resins in UASB reactor: Influence of oxygen. *Vol. 57* (pp. 1047-1052).

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics, 27*(16), 2194-2200.

Ekins, S., Williams, J. A., Murray, G. I., Burke, M. D., Marchant, N. C., Engeset, J., et al. (1996). Xenobiotic metabolism in rat, dog, and human precision-cut liver slices, freshly isolated hepatocytes, and vitrified precision-cut liver slices. *Drug metabolism and disposition, 24*(9), 990-995.

Elbahloul, Y., & Steinbuchel, A. (2009). Large-scale production of poly(3-hydroxyoctanoic acid) by *Pseudomonas putida* GPo1 and a simplified downstream process. *Applied and Environmental Microbiology, 75*(3), 643-651.

Escapa, I. F., Morales, V., Martino, V. P., Pollet, E., Avérous, L., García, J. L., et al. (2011). Disruption of *β*-oxidation pathway in *Pseudomonas putida* KT2442 to produce new functionalized PHAs with thioester groups. *Applied Microbiology and Biotechnology, 89*(5), 1583-1598.

Fallah, N., Bonakdarpour, B., Nasernejad, B., & Alavi Moghadam, M. R. (2010). Long-term operation of submerged membrane bioreactor (MBR) for the treatment of synthetic wastewater containing styrene as volatile organic compound (VOC): Effect of hydraulic retention time (HRT). *Journal of Hazardous Materials, 178*(1-3), 718-724.

Farhadian, M., Duchez, D., Vachelard, C., & Larroche, C. (2008). Monoaromatics removal from polluted water through bioreactors - a review. *Water Research, 42*(6-7), 1325-1341.

Farhadian, M., Duchez, D., Vachelard, C., & Larroche, C. (2009). Accurate quantitative determination of monoaromatic compounds for the monitoring of bioremediation processes. *Bioresource Technology, 100*(1), 173-178.

Feris, K., Hristova, K., Gebreyesus, B., Mackay, D., & Scow, K. (2004). A shallow BTEX and MTBE contaminated aquifer supports a diverse microbial community. *Microbial Ecology, 48*(4), 589-600.

Fernández, D., Rodríguez, E., Bassas, M., Viñas, M., Solanas, A. M., Llorens, J., et al. (2005). Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: effect of culture conditions. *Biochemical Engineering Journal, 26*(2-3), 159-167.

Field, J. A. (2002). Limits of anaerobic biodegradation. *Water Science and Technology, 45*(10), 9-18.

Flanjak, J., & Sharrad, J. (1984). Quantitative analysis of styrene monomer in foods. A limited East Australian survey. *Journal of the Science of Food and Agriculture, 35*(4), 457-462.

Furrer, P., Hany, R., Rentsch, D., Grubelnik, A., Ruth, K., Panke, S., et al. (2007). Quantitative analysis of bacterial medium-chain-length poly([*R*]-3-hydroxyalkanoates) by gas chromatography. *Journal of Chromatography A, 1143*(1), 199-206.

Gaidos, E., Rusch, A., & Ilardo, M. (2011). Ribosomal tag pyrosequencing of DNA and RNA from benthic coral reef microbiota: community spatial structure, rare members and nitrogen-cycling guilds. *Environmental Microbiology, 13*(5), 1138-1152.

Galia, M. B. (2010). Isolation and analysis of storage compounds. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 3725-3741). Berlin Heidelberg, Germany: Springer.

Gawell, B.-M., & Larsson, B. (1980). Determination of styrene in foods by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 198*(2), 198-202.

Gilbert-López, B., Robles-Molina, J., García-Reyes, J. F., & Molina-Díaz, A. (2010). Rapid determination of BTEXS in olives and olive oil by headspace-gas chromatography/mass spectrometry (HS-GC–MS). *Talanta, 83*(2), 391-399.

Gottschalk, G. (1986). *Bacterial metabolism*. Gottingen, Germany: Springer-Verlag Berlin Heidelberg GmbH.

Grbić-Galić, D., Churchman-Eisel, N., & Mraković, I. (1990). Microbial transformation of styrene by anaerobic consortia. *Journal of Applied Microbiology, 69*(2), 247-260.

Greene, E. A., Kay, J. G., Jaber, K., Stehmeier, L. G., & Voordouw, G. (2000). Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. *Applied and Environmental Microbiology, 66*(12), 5282-5289.

Greene, E. A., & Voordouw, G. (2004). Biodegradation of C5+ hydrocarbons by a mixed bacterial consortium from a C5+-contaminated site. *Environmental Technology, 25*(3), 355-363.

Gross, R., Hauer, B., Otto, K., & Schmid, A. (2007). Microbial biofilms: new catalysts for maximizing productivity of long-term biotransformations. *Biotechnology and Bioengineering*, *98*(6), 1123-1134.

Gross, R., Lang, K., Bühler, K., & Schmid, A. (2010). Characterization of a biofilm membrane reactor and its prospects for fine chemical synthesis. *Biotechnology and Bioengineering*, *105*(4), 705-717.

Gross, R. A., DeMello, C., Lenz, R. W., Brandl, H., & Fuller, R. C. (1989). The biosynthesis and characterization of poly(*β*-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. *Macromolecules*, *22*(3), 1106-1115.

Grubelnik, A., Wiesli, L., Furrer, P., Rentsch, D., Hany, R., & Meyer, V. R. (2008). A simple HPLC-MS method for the quantitative determination of the composition of bacterial medium chain-length polyhydroxyalkanoates. *Journal of Separation Science, 31*(10), 1739-1744.

Guo, M., Zhou, Q., Zhou, Y., Yang, L., Liu, T., Yang, J., et al. (2014). Genomic Evolution of 11 Type Strains within Family Planctomycetaceae. *PloS One*, *9*(1), e86752.

Guzik, M. W., Kenny, S. T., Duane, G. F., Casey, E., Woods, T., Babu, R. P., et al. (2014). Conversion of post consumer polyethylene to the biodegradable polymer polyhydroxyalkanoate. *Applied Microbiology and Biotechnology*, 1-10.

Han, J. H., Park, M. S., Bae, J. W., Lee, E. Y., Yoon, Y. J., Lee, S. G., et al. (2006). Production of (*S*)-styrene oxide using styrene oxide isomerase negative mutant of *Pseudomonas putida* SN1. *Enzyme and Microbial Technology*, *39*(6), 1264-1269.

Hartmans, S., Smits, J., Van der Werf, M., Volkering, F., & De Bont, J. (1989). Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Applied and Environmental Microbiology*, *55*(11), 2850-2855.

Hartmans, S., Van der Werf, M. J., & De Bont, J. A. M. (1990). Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Applied and Environmental Microbiology*, *56*(5), 1347-1351.

Haywood, G. W., Anderson, A. J., Roger Williams, D., Dawes, E. A., & Ewing, D. F. (1991). Accumulation of a poly(hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NCIMB 40126. *International Journal of Biological Macromolecules, 13*(2), 83-88.

Herrmann, S., Kleinsteuber, S., Neu, T. R., Richnow, H. H., & Vogt, C. (2008). Enrichment of anaerobic benzene-degrading microorganisms by in situ microcosms. *FEMS Microbiology Ecology, 63*(1), 94-106.

Hong, K., Sun, S., Tian, W., Chen, G.-Q., & Huang, W. (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by fourier transform infrared spectroscopy. *Applied Microbiology and Biotechnology, 51*(4), 523-526.

Hori, K., Abe, M., & Unno, H. (2009a). Production of triacylglycerol and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by the toluene-degrading bacterium *Rhodococcus aetherivorans* IAR1. *Journal of Bioscience and Bioengineering, 108*(4), 319-324.

Hori, K., Kobayashi, A., Ikeda, H., & Unno, H. (2009b). *Rhodococcus aetherivorans* IAR1, a new bacterial strain synthesizing poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from toluene. *Journal of Bioscience and Bioengineering, 107*(2), 145-150.

Hsieh, C.-C. (2000). Removal mechanisms of VOCs in an activated sludge process. *Journal of Hazardous Materials, 79*(1–2), 173-187.

Huijberts, G. N. M., De Rijk, T. C., De Waard, P., & Eggink, G. (1994). ^{13}C nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. *Journal of Bacteriology, 176*(6), 1661-1666.

Hume, A. R., Nikodinovic-Runic, J., & O'Connor, K. E. (2009). FadD from *Pseudomonas putida* CA-3 is a true long-chain fatty acyl coenzyme A synthetase that activates phenylalkanoic and alkanoic acids. *Journal of Bacteriology, 191*(24), 7554-7565.

Huse, S. M., Welch, D. M., Morrison, H. G., & Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environmental Microbiology, 12*(7), 1889-1898.

Ikura, Y., Yoshida, Y., & Kudo, T. (1997). Physiological properties of two *Pseudomonas mendocina* strains which assimilate styrene in a two-phase (solvent-aqueous) system under static culture conditions. *Journal of Fermentation and Bioengineering, 83*(6), 604-607.

Itoh, N., Yoshida, K., & Okada, K. (1996). Isolation and identification of styrene-degrading *Corynebacterium* strains, and their styrene metabolism. *Bioscience, Biotechnology, and Biochemistry, 60*(11), 1826-1830.

Jacques, R. J., Okeke, B. C., Bento, F. M., Peralba, M. C., & Camargo, F. A. (2007). Characterization of a polycyclic aromatic hydrocarbon–degrading microbial consortium from a petrochemical sludge landfarming site. *Bioremediation Journal, 11*(1), 1-11.

Jang, J. H., Hirai, M., & Shoda, M. (2006). Effect of shutdown on styrene removal in a biofilter inoculated with *Pseudomonas* sp. SR-5. *Journal of Hazardous Materials, 129*(1-3), 223-227.

Janssen, P. H., Yates, P. S., Grinton, B. E., Taylor, P. M., & Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied and Environmental Microbiology, 68*(5), 2391-2396.

Jayamani, I., Manzella, M. P., & Cupples, A. M. (2013). RDX degradation potential in soils previously unexposed to RDX and the identification of RDX-degrading species in one agricultural soil using stable isotope probing. *Water, Air, & Soil Pollution, 224*(10), 1-15.

Jiang, X.-T., Peng, X., Deng, G.-H., Sheng, H.-F., Wang, Y., Zhou, H.-W., et al. (2013). Illumina sequencing of 16S rRNA tag revealed spatial variations of bacterial communities in a mangrove wetland. *Microbial Ecology, 66*(1), 96-104.

Jimenez, J. I., Miñambres, B., Garcia, J. L., & Diaz, E. (2004). Genomic insights in the metabolism of aromatic compounds in *Pseudomonas*. In J.-L. Ramos

(Ed.), *Pseudomonas* (pp. 425-462). London: Kluwer Academic / Plenum Publishers.

Jorgensen, A. D., Picel, K. C., & Stamoudis, V. C. (1990). Prediction of gas chromatography flame ionization detector response factors from molecular structures. *Analytical Chemistry, 62*(7), 683-689.

Jorio, H., Brzezinski, R., & Heitz, M. (2005). A novel procedure for the measurement of the kinetics of styrene biodegradation in a biofilter. *Journal of Chemical Technology & Biotechnology, 80*(7), 796-804.

Juneson, C., Ward, O., & Singh, A. (2001). Microbial treatment of a styrene-contaminated air stream in a biofilter with high elimination capacities. *Journal of Industrial Microbiology and Biotechnology, 26*(4), 196-202.

Jung, I.-G., & Park, C.-H. (2005). Characteristics of styrene degradation by *Rhodococcus pyridinovorans* isolated from a biofilter. *Chemosphere, 61*(4), 451-456.

Kadouri, D., Jurkevitch, E., Okon, Y., & Castro-Sowinski, S. (2005). Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Critical Reviews in Microbiology, 31*(2), 55-67.

Kämpfer, P. (2010). Actinobacteria. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 1819-1838). Germany Springer.

Kanehisa, M., & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research, 28*(1), 27-30.

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2014). Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Research, 42*(D1), D199-D205.

Karr, D. B., Waters, J. K., & Emerich, D. W. (1983). Analysis of poly-*β*-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. *Applied and Environmental Microbiology, 46*(6), 1339-1344.

Kelly, W. R., Hornberger, G. M., Herman, J. S., & Mills, A. L. (1996). Kinetics of BTX biodegradation and mineralization in batch and column systems. *Journal of Contaminant Hydrology, 23*(1-2), 113-132.

Kenny, S. T., Nikodinovic-Runic, J., Kaminsky, W., Woods, T., Babu, R. P., Keely, C. M., et al. (2008). Up-cycling of PET (polyethylene terephthalate) to the biodegradable plastic PHA (polyhydroxyalkanoate). *Environmental Science & Technology, 42*(20), 7696-7701.

Khaksar, M.-R., & Ghazi-Khansari, M. (2009). Determination of migration monomer styrene from GPPS (general purpose polystyrene) and HIPS (high impact polystyrene) cups to hot drinks. *Toxicology Mechanisms and Methods, 19*(3), 257-261.

Khanna, S., & Srivastava, A. K. (2005). Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry, 40*(2), 607-619.

Kim, J., Ryu, H. W., Dong, J. J., Tae, H. L., & Cho, K.-S. (2005). Styrene degradation in a polyurethane biofilter inoculated with *Pseudomonas* sp. IS-3. *Journal of Microbiology and Biotechnology, 15*(6), 1207-1213.

Kim, M. N., Lee, A. R., Yoon, J. S., & Chin, I. J. (2000). Biodegradation of poly(3-hydroxybutyrate), Sky-Green® and Mater-Bi® by fungi isolated from soils. *European Polymer Journal, 36*(8), 1677-1685.

Koller, M., Atlić, A., Dias, M., Reiterer, A., & Braunegg, G. (2010). Microbial PHA production from waste raw materials. In G.-Q. Chen (Ed.), *Plastics from Bacteria: Natural Functions and Applications* (Vol. 14, pp. 85-119). Berlin Heidelberg, Germany: Springer.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology, 79*(17), 5112-5120.

Kubinec, R., Adamuščin, J., Jurdáková, H., Foltin, M., Ostrovský, I., Kraus, A., et al. (2005). Gas chromatographic determination of benzene, toluene, ethylbenzene and xylenes using flame ionization detector in water samples with direct aqueous injection up to 250 μl. *Journal of Chromatography A, 1084*(1-2), 90-94.

Kuyukina, M., & Ivshina, I. (2010). Application of *Rhodococcus* in Bioremediation of Contaminated Environments. Biology of *Rhodococcus*. In H. M. Alvarez (Ed.), (Vol. 16, pp. 231-262): Springer Berlin / Heidelberg.

Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G., & Witholt, B. (1988). Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. *Applied and Environmental Microbiology, 54*(12), 2924-2932.

Law, J. H., & Slepecky, R. A. (1961). Assay of poly-*β*-hydroxybutyric acid. *Journal of Bacteriology, 82*(1), 33-36.

Lee, E.-H., Kim, J., Kim, J.-Y., Koo, S.-Y., Lee, S.-D., Ko, K.-S., et al. (2010). Comparison of microbial communities in petroleum-contaminated groundwater using genetic and metabolic profiles at Kyonggi-Do, South Korea. *Environmental Earth Sciences, 60*(2), 371-382.

Lee, E., Jendrossek, D., Schirmer, A., Choi, C., & Steinbüchel, A. (1995). Biosynthesis of copolyesters consisting of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids from 1,3-butanediol or from 3-hydroxybutyrate by *Pseudomonas* sp. A33. *Applied Microbiology and Biotechnology, 42*(6), 901-909.

Lee, E. Y., & Choi, C. Y. (1995). Gas chromatography-mass spectrometric analysis and its application to a screening procedure for novel bacterial polyhydroxyalkanoic acids containing long chain saturated and unsaturated monomers. *Journal of Fermentation and Bioengineering, 80*(4), 408-414.

Lee, E. Y., Jun, Y. S., Cho, K.-S., & Ryu, H. W. (2002). Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. *Journal of the Air & Waste Management Association, 52*(4), 400-406.

Lee, H. O. J., Choi, M. H., Kim, T. U. N., & Yoon, S. C. (2001). Accumulation of polyhydroxyalkanoic acid containing large amounts of unsaturated monomers in *Pseudomonas fluorescens* BM07 utilizing saccharides and its inhibition by 2-bromooctanoic acid. *Applied and Environmental Microbiology, 67*(3-12), 4963-4974.

Lee, S. H., Kim, J. H., Mishra, D., Ni, Y. Y., & Rhee, Y. H. (2011). Production of medium-chain-length polyhydroxyalkanoates by activated sludge enriched under periodic feeding with nonanoic acid. *Bioresource Technology, 102*(10), 6159-6166.

Lee, S. Y. (1996). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends in Biotechnology, 14*(11), 431-438.

Liang, C., Huang, C.-F., & Chen, Y.-J. (2008). Potential for activated persulfate degradation of BTEX contamination. *Water Research, 42*(15), 4091-4100.

Liang, R., Wu, X., Dai, Q., Jin, D., & Wang, Y. (2010). Genetic diversity of phthalic acid esters-degrading bacteria isolated from different geographical regions of China. *Antonie van Leeuwenhoek, 97*(1), 79-89.

Lin, H., Qiao, J., Liu, Y., & Wu, Z.-L. (2010). Styrene monooxygenase from *Pseudomonas* sp. LQ26 catalyzes the asymmetric epoxidation of both conjugated and unconjugated alkenes. *Journal of Molecular Catalysis B: Enzymatic, 67*(3–4), 236-241.

Lindsay, D., Ntoampe, M., & Gray, V. M. (2008). Biodegradation of sodium benzoate by a Gram-negative consortium in a laboratory-scale fluidized bed bioreactor. *Bioresource Technology, 99*(11), 5115-5119.

Liu, W. T., Marsh, T. L., Cheng, H., & Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology, 63*(11), 4516-4522.

Liu, X., Tani, A., Kimbara, K., & Kawai, F. (2006). Metabolic pathway of xenoestrogenic short ethoxy chain-nonylphenol to nonylphenol by aerobic bacteria, *Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90. *Applied Microbiology and Biotechnology, 72*(3), 552-559.

Lu, J., Tappel, R. C., & Nomura, C. T. (2009). Mini-review: biosynthesis of poly(hydroxyalkanoates). *Polymer Reviews, 49*(3), 226-248.

Luo, W., D'Angelo, E. M., & Coyne, M. S. (2008). Organic carbon effects on aerobic polychlorinated biphenyl removal and bacterial community composition in soils and sediments. *Chemosphere, 70*(3), 364-373.

Lütke-Eversloh, T., Bergander, K., Luftmann, H., & Steinbüchel, A. (2001). Identification of a new class of biopolymer: bacterial synthesis of a sulfur-containing polymer with thioester linkages. *Microbiology, 147*(1), 11-19.

Madison, L. L., & Huisman, G. W. (1999). Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiology and Molecular Biology Reviews, 63*(1), 21-53.

Mandelbaum, R. T., Allan, D. L., & Wackett, L. P. (1995). Isolation and characterization of a *Pseudomonas* sp. that mineralizes the *s*-triazine herbicide atrazine. *Applied and Environmental Microbiology, 61*(4), 1451-1457.

Marchessault, R. H., Dou, H., & Ramsay, J. (2011). Microbial medium chainlength poly [(*R*)-3-hydroxyalkanoate] shows liquid crystal behaviour. *International Journal of Biological Macromolecules, 48*(2), 271-275.

Marconi, A. M., Beltrametti, F., Bestetti, G., Solinas, F., Ruzzi, M., Galli, E., et al. (1996). Cloning and characterization of styrene catabolism genes from *Pseudomonas fluorescens* ST. *Applied and Environmental Microbiology, 62*(1), 121-127.

Masson-Boivin, C., Giraud, E., Perret, X., & Batut, J. (2009). Establishing nitrogen-fixing symbiosis with legumes: how many *Rhizobium* recipes? *Trends in Microbiology, 17*(10), 458-466.

Melnicki, M. R., Eroglu, E., & Melis, A. (2009). Changes in hydrogen production and polymer accumulation upon sulfur-deprivation in purple photosynthetic bacteria. *International Journal of Hydrogen Energy, 34*(15), 6157-6170.

Mittendorf, V., Robertson, E. J., Leech, R. M., Krüger, N., Steinbüchel, A., & Poirier, Y. (1998). Synthesis of medium-chain-length polyhydroxyalkanoates in *Arabidopsis thaliana* using intermediates of peroxisomal fatty acid *β*-oxidation. *Proceedings of the National Academy of Sciences, 95*(23), 13397-13402.

Mizrahi-Man, O., Davenport, E. R., & Gilad, Y. (2013). Taxonomic classification of bacterial 16S rRNA genes using short sequencing reads: evaluation of effective study designs. *PloS One, 8*(1), e53608.

Mooney, A., Ward, P. G., & O'Connor, K. E. (2006). Microbial degradation of styrene: biochemistry, molecular genetics, and perspectives for biotechnological applications. *Applied Microbiology and Biotechnology, 72*(1), 1-10.

Muhr, A., Rechberger, E. M., Salerno, A., Reiterer, A., Schiller, M., Kwiecień, M., et al. (2013). Biodegradable latexes from animal-derived waste: biosynthesis and characterization of mcl-PHA accumulated by *Ps. citronellolis*. *Reactive and Functional Polymers, 73*(10), 1391–1398.

Narancic, T., Kenny, S., Djokic, L., Vasiljevic, B., O'Connor, K., & Nikodinovic-Runic, J. (2012). Medium-chain-length polyhydroxyalkanoate production by newly isolated *Pseudomonas* sp. TN301 from a wide range of polyaromatic and monoaromatic hydrocarbons. *Journal of Applied Microbiology, 113*(3), 508-520.

NCBI. (2014a). *Pseudomonas aeruginosa* strain L-4 16S ribosomal RNA gene, partial sequence. Retrieved 13 Oct, 2014

NCBI. (2014b). *Rhodococcus qingshengii* strain BLH-Y4 16S ribosomal RNA gene, partial sequence. Retrieved 13 Oct, 2014

Nemerow, N. L., & Agardy, F. J. (1998). *Strategies of industrial and hazardous waste management*: Van Nostrand Reinhold.

NEMI. National Environmental Methods Index. Retrieved 25 Mar, 2012, from http://www.nemi.gov

Neyens, E., Baeyens, J., Dewil, R., & De heyder, B. (2004). Advanced sludge treatment affects extracellular polymeric substances to improve activated sludge dewatering. *Journal of Hazardous Materials, 106*(2–3), 83-92.

Ni, Y. Y., Kim, D. Y., Chung, M. G., Lee, S. H., Park, H. Y., & Rhee, Y. H. (2010). Biosynthesis of medium-chain-length poly(3-hydroxyalkanoates) by volatile aromatic hydrocarbons-degrading *Pseudomonas fulva* TY16. *Bioresource Technology, 101*(21), 8485-8488.

Nikodinovic-Runic, J., Casey, E., Duane, G. F., Mitic, D., Hume, A. R., Kenny, S. T., et al. (2011). Process analysis of the conversion of styrene to biomass and medium chain length polyhydroxyalkanoate in a two-phase bioreactor. *Biotechnology and Bioengineering, 108*(10), 2447-2455.

Nikodinovic, J., Kenny, S. T., Babu, R. P., Woods, T., Blau, W., & O'Connor, K. E. (2008). The conversion of BTEX compounds by single and defined mixed cultures to medium-chain-length polyhydroxyalkanoate. *Applied Microbiology and Biotechnology, 80*(4), 665-673.

Ntougias, S., Lapidus, A., Lapidus, A., Han, J., Han, J., Mavromatis, K., et al. (2014). High quality draft genome sequence of *Olivibacter sitiensis* type strain (AW-6T), a diphenol degrader with genes involved in the catechol pathway. *Standards in Genomic Sciences, 9*(3).

O'Connor, K. E., Buckley, C. M., Hartmans, S., & Dobson, A. D. W. (1995). Possible regulatory role for nonaromatic carbon sources in styrene degradation by *Pseudomonas putida* CA-3. *Applied and Environmental Microbiology, 61*(2), 544-548.

O'Connor, K. E., Dobson, A., & Hartmans, S. (1997). Indigo formation by microorganisms expressing styrene monooxygenase activity. *Applied and Environmental Microbiology, 63*(11), 4287-4291.

O'Leary, N. D., O'Connor, K. E., & Dobson, A. D. W. (2002). Biochemistry, genetics and physiology of microbial styrene degradation. *FEMS Microbiology Reviews, 26*(4), 403-417.

O'Leary, N. D., O'Connor, K. E., Ward, P. G., Goff, M., & Dobson, A. D. W. (2005). Genetic characterization of accumulation of polyhydroxyalkanoate from styrene in *Pseudomonas putida* CA-3. *Applied and Environmental Microbiology, 71*(8), 4380-4387.

O'Brien, D. J., Roth, L. H., & McAloon, A. J. (2000). Ethanol production by continuous fermentation–pervaporation: a preliminary economic analysis. *Journal of Membrane Science, 166*(1), 105-111.

Oehmen, A., Keller-Lehmann, B., Zeng, R. J., Yuan, Z., & Keller, J. (2005). Optimisation of poly-*β*-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. *Journal of Chromatography A, 1070*(1–2), 131-136.

Okamoto, K., Izawa, M., & Yanase, H. (2003). Isolation and application of a styrene-degrading strain of *Pseudomonas putida* to biofiltration. *Journal of Bioscience and Bioengineering, 95*(6), 633-636.

Olivera, E. R., Arcos, M., Naharro, G., & Luengo, J. M. (2010). Unusual PHA biosynthesis. In G.-Q. Chen (Ed.), *Plastics from Bacteria: Natural Functions and Applications* (Vol. 14, pp. 133-186). Berlin Heidelberg, Germany: Springer.

Osswald, P., Baveye, P., & Block, J. C. (1996). Bacterial influence on partitioning rate during the biodegradation of styrene in a biphasic aqueous-organic system. *Biodegradation, 7*(4), 297-302.

Ostle, A. G., & Holt, J. (1982). Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. *Applied and Environmental Microbiology, 44*(1), 238-241.

Palleroni, N. J., & Bradbury, J. F. (1993). *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al. 1983. *International Journal of Systematic Bacteriology, 43*(3), 606-609.

Panke, S., Witholt, B., Schmid, A., & Wubbolts, M. G. (1998). Towards a biocatalyst for (*S*)-styrene oxide production: characterization of the styrene degradation pathway of *Pseudomonas* sp. strain VLB120. *Applied and Environmental Microbiology, 64*(6), 2032-2043.

Panke, S., Wubbolts, M. G., Schmid, A., & Witholt, B. (2000). Production of enantiopure styrene oxide by recombinant *Escherichia coli* synthesizing a two-component styrene monooxygenase. *Biotechnology and Bioengineering, 69*(1), 91-100.

Pantazaki, A. A., Papaneophytou, C. P., Pritsa, A. G., Liakopoulou-Kyriakides, M., & Kyriakidis, D. A. (2009). Production of polyhydroxyalkanoates from whey by *Thermus thermophilus* HB8. *Process Biochemistry, 44*(8), 847-853.

Park, H., Bae, Y., Cho, S., Kim, S., Moon, J., Ha, K., et al. (2007). Toluene-induced accumulation of trehalose by *Pseudomonas* sp. BCNU 106 through the expression of *otsA* and *otsB* homologues. *Letters in Applied Microbiology, 44*(1), 50-55.

Park, J. B., Bühler, B., Habicher, T., Hauer, B., Panke, S., Witholt, B., et al. (2006). The efficiency of recombinant *Escherichia coli* as biocatalyst for stereospecific epoxidation. *Biotechnology and Bioengineering, 95*(3), 501-512.

Patrauchan, M. A., Florizone, C., Eapen, S., Gómez-Gil, L., Sethuraman, B., Fukuda, M., et al. (2008). Roles of ring-hydroxylating dioxygenases in styrene and benzene catabolism in *Rhodococcus jostii* RHA1. *Journal of Bacteriology, 190*(1), 37-47.

Peña, C., Castillo, T., García, A., Millán, M., & Segura, D. (2014). Biotechnological strategies to improve production of microbial poly-(3-hydroxybutyrate): a review of recent research work. *Microbial Biotechnology, 7*(4), 278-293.

Peng, M., Vane, L. M., & Liu, S. X. (2003). Recent advances in VOCs removal from water by pervaporation. *Journal of Hazardous Materials, 98*(1–3), 69-90.

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D. H., et al. (2012). Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. *Environmental Microbiology, 14*(5), 1091-1117.

Pérez-Pantoja, D., Donoso, R., Junca, H., González, B., & Pieper, D. H. (2010). Phylogenomics of aerobic bacterial degradation of aromatics. In K. N. Timmis (Ed.), *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 1355-1397). Germany: Springer Berlin Heidelberg.

Pérez-Pantoja, D., González, B., & Pieper, D. (2010). Aerobic degradation of aromatic hydrocarbons. In K. N. Timmis (Ed.), *Handbook of hydrocarbon and lipid microbiology* (pp. 799-837). Germany: Springer.

Pérez, M. C., Álvarez-Hornos, F. J., Portune, K., & Gabaldón, C. (2014). Abatement of styrene waste gas emission by biofilter and biotrickling filter: comparison of packing materials and inoculation procedures. *Applied Microbiology and Biotechnology*, 1-14.

Peters, V., & Rehm, B. H. A. (2005). *In vivo* monitoring of PHA granule formation using GFP-labeled PHA synthases. *FEMS Microbiology Letters, 248*(1), 93-100.

Pieper, U., & Steinbüchel, A. (1992). Identification, cloning and sequence analysis of the poly (3-hydroxyalkanoic acid) synthase gene of the Gram-positive bacterium *Rhodococcus ruber*. *FEMS microbiology letters, 96*(1), 73-79.

Plastemart. (2013). Rise in global demand for polystyrene and expandable polystyrene is led by demand from Asia Pacific. Retrieved Jun 30, 2015, from http://www.plastemart.com/Plastic-Technical-Article.asp?LiteratureID=2032&Paper=rise-global-demand-polystyrene-expandable%20polystyrene-EPS-led-by-asia-pacific

Portune, K. J., Pérez, M. C., Álvarez-Hornos, F. J., & Gabaldón, C. (2015). Investigating bacterial populations in styrene-degrading biofilters by 16S rDNA tag pyrosequencing. *Applied Microbiology and Biotechnology*, 1-16.

Pouton, C. W., & Akhtar, S. (1996). Biosynthetic polyhydroxyalkanoates and their potential in drug delivery. *Advanced Drug Delivery Reviews, 18*(2), 133-162.

Pruden, A., & Suidan, M. (2004). Effect of benzene, toluene, ethylbenzene, and *p*-xylene (BTEX) mixture on biodegradation of methyl tert-butyl ether (MTBE) and tert-butyl alcohol (TBA) by pure culture UC1. *Biodegradation, 15*(4), 213-227.

Przybulewska, K., Wieczorek, A., & Nowak, A. (2006). Isolation of microorganisms capable of styrene degradation. *Polish Journal of Environmental Studies, 15*(5), 777.

Qu, X.-H., Wu, Q., Zhang, K.-Y., & Chen, G.-Q. (2006). *In vivo* studies of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) based polymers: biodegradation and tissue reactions. *Biomaterials, 27*(19), 3540-3548.

Quagliano Javier, C., & Miyazaki Silvia, S. (2002). Microbial production of the biodegradable polyester poly-3-hydroxybutyrate (PHB) from *Azotobacter Chroococcum* 6B: relation between PHB molecular weight, thermal stability and tensile strength. In A. Durieux & J. Simon (Eds.), *Applied Microbiology* (Vol. 2, pp. 135-140). Netherlands: Springer.

Rai, R., Keshavarz, T., Roether, J. A., Boccaccini, A. R., & Roy, I. (2011). Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. *Materials Science and Engineering: R: Reports, 72*(3), 29-47.

Reddy, C. S. K., Ghai, R., Rashmi, & Kalia, V. C. (2003). Polyhydroxyalkanoates: an overview. *Bioresource Technology, 87*(2), 137-146.

Reis, J. L. R., Dezotti, M., & Sant'Anna Jr, G. L. (2007). Toxicity evaluation of the process effluent streams of a petrochemical industry. *Environmental Technology, 28*(2), 147-155.

Romo, D. M. R., Grosso, M. V., Solano, N. C. M., & Castaño, D. M. (2007). A most effective method for selecting a broad range of short and medium-chain-length polyhidroxyalcanoate producing microorganisms. *Electronic Journal of Biotechnology, 10*(3), 348-357.

Romo, D. M. R., Obando, M. C., Perdomo, M. I. G., & Izquierdo, P. F. (2014). PHA synthase genes in oil-degrading marine bacteria from the Colombian Pacific Coast. *International Journal of Advanced Biotechnology and Research, 5*(3), 404-414.

Rustemov, S., Golovleva, L., Alieva, R., & Baskunov, B. (1992). New pathway of styrene oxidation by a *Pseudomonas putida* culture. *Microbiology, 61*(1), 1-5.

Salehizadeh, H., & Van Loosdrecht, M. C. M. (2004). Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnology Advances, 22*(3), 261-279.

Satoh, H., Iwamoto, Y., Mino, T., & Matsuo, T. (1998). Activated sludge as a possible source of biodegradable plastic. *Water Science and Technology, 38*(2), 103-109.

Schloss, P. D. (2009). A high-throughput DNA sequence aligner for microbial ecology studies. *PloS One, 4*(12), e8230.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology, 75*(23), 7537-7541.

Seo, J.-S., Keum, Y.-S., & Li, Q. X. (2009). Bacterial degradation of aromatic compounds. *International Journal of Environmental Research and Public Health, 6*(1), 278-309.

Serafim, L. S., Lemos, P. C., Albuquerque, M. G. E., & Reis, M. A. M. (2008). Strategies for PHA production by mixed cultures and renewable waste materials. *Applied Microbiology and Biotechnology, 81*(4), 615-628.

Shah, V., Zakrzewski, M., Wibberg, D., Eikmeyer, F., Schlüter, A., & Madamwar, D. (2013). Taxonomic profiling and metagenome analysis of a microbial community from a habitat contaminated with industrial discharges. *Microbial Ecology, 66*(3), 533-550.

Shahid, S., Mosrati, R., Ledauphin, J., Amiel, C., Fontaine, P., Gaillard, J.-L., et al. (2013). Impact of carbon source and variable nitrogen conditions on bacterial biosynthesis of polyhydroxyalkanoates: evidence of an atypical metabolism in *Bacillus megaterium* DSM 509. *Journal of Bioscience and Bioengineering, 116*(3), 302-308.

Shim, H., & Yang, S.-T. (1999). Biodegradation of benzene, toluene, ethylbenzene, and *o*-xylene by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* immobilized in a fibrous-bed bioreactor. *Journal of Biotechnology, 67*(2–3), 99-112.

Shin, H.-S., & Kwon, O.-S. (2000). The simultaneous analysis of benzene, toluene, ethylbenzene, *o*, *m*, *p*-xylenes and total petroleum hydrocarbons in soil by GC-FID after ultra-sonication. *Bulletin-Korean Chemical Society, 21*(11), 1101-1105.

Silva, C. C., Hayden, H., Sawbridge, T., Mele, P., Kruger, R. H., Rodrigues, M. V., et al. (2012). Phylogenetic and functional diversity of metagenomic libraries of phenol degrading sludge from petroleum refinery wastewater treatment system. *AMB Express, 2*(1), 1-13.

Singh, P., & Parmar, N. (2013). Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria. *African Journal of Biotechnology, 10*(24), 4907-4919.

Slepecky, R. A., & Law, J. H. (1960). A rapid spectrophotometric assay of alpha, beta-unsaturated acids and beta-hydroxy acids. *Analytical Chemistry, 32*(12), 1697-1699.

Solaiman, D. K., & Ashby, R. D. (2005). Rapid genetic characterization of poly(hydroxyalkanoate) synthase and its applications. *Biomacromolecules, 6*(2), 532-537.

Spiekermann, P., Rehm, B. H., Kalscheuer, R., Baumeister, D., & Steinbüchel, A. (1999). A sensitive, viable-colony staining method using Nile red for direct

screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Archives of Microbiology, 171*(2), 73-80.

Steinbüchel, A., & Hein, S. (2001). Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. In W. Babel & A. Steinbüchel (Eds.), *Biopolyesters* (Vol. 71, pp. 81-123). Berlin Heidelberg, Germany: Springer

Sun, H., Terhonen, E., Koskinen, K., Paulin, L., Kasanen, R., & Asiegbu, F. O. (2014). Bacterial diversity and community structure along different peat soils in boreal forest. *Applied Soil Ecology, 74*(0), 37-45.

Sun, J., Dai, Z., Zhao, Y., & Chen, G.-Q. (2007). *In vitro* effect of oligo-hydroxyalkanoates on the growth of mouse fibroblast cell line L929. *Biomaterials, 28*(27), 3896-3903.

Sun, Z., Ramsay, J. A., Guay, M., & Ramsay, B. A. (2007). Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440. *Applied microbiology and biotechnology, 74*(1), 69-77.

Sun, Z., Ramsay, J. A., Guay, M., & Ramsay, B. A. (2009). Fed-batch production of unsaturated medium-chain-length polyhydroxyalkanoates with controlled composition by *Pseudomonas putida* KT2440. *Applied Microbiology and Biotechnology, 82*(4), 657-662.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*(10), 2731-2739.

Tan, G.-Y. A., Chen, C.-L., Ge, L., Li, L., Wang, L., Zhao, L., et al. (2014a). Enhanced gas chromatography-mass spectrometry method for bacterial polyhydroxyalkanoates analysis. *Journal of Bioscience and Bioengineering, 117*(3), 379–382.

Tan, G.-Y. A., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I. M. N., et al. (2014b). Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers, 6*(3), 706-754.

Tan, G.-Y. A., Chen, C.-L., Zhao, L., Mo, Y., Chang, V. W. C., & Wang, J.-Y. (2012). An HPLC-DAD method for rapid and high resolution analysis of concentrated BTEX and styrene aqueous samples. *Analytical Methods, 4*(11), 3545-3550.

Tawfik, M., & Huyghebaert, A. (1998). Polystyrene cups and containers: styrene migration. *Food Additives & Contaminants, 15*(5), 592-599.

Thermo Scientific Dionex, Acclaim Phenyl-1: a unique reversed-phase column with high aromatic selectivity. (2010).

Thibodeaux, L. J. (1974). *A test method for volatile component stripping of waste water* (Vol. 1). Washington, D.C.: U.S. Environmental Protection Agency.

Thompson, I. P., Van Der Gast, C. J., Ciric, L., & Singer, A. C. (2005). Bioaugmentation for bioremediation: the challenge of strain selection. *Environmental Microbiology, 7*(7), 909-915.

Tijssen, R., Katz, E., Eksteen, R., & Schoenmakers, P. (1998). The mechanisms and importance of zone-spreading. In Danilo Corradini, Elena Katz, Roy Eksteen, Peter Schoenmakers & N. Miller (Eds.), *Handbook of HPLC* (Vol. 78, pp. 55-142). New York, USA: CRC Press.

Tischler, D., Eulberg, D., Lakner, S., Kaschabek, S. R., van Berkel, W. J., & Schlömann, M. (2009). Identification of a novel self-sufficient styrene monooxygenase from *Rhodococcus opacus* 1CP. *Journal of Bacteriology, 191*(15), 4996-5009.

Tischler, D., & Kaschabek, S. R. (2012). Microbial styrene degradation: from basics to biotechnology. In S. N. Singh (Ed.), *Microbial Degradation of Xenobiotics* (pp. 67-99). Berlin, Heidelberg, Germany: Springer.

Tobin, K. M., & O'Connor, K. E. (2005). Polyhydroxyalkanoate accumulating diversity of *Pseudomonas* species utilising aromatic hydrocarbons. *FEMS Microbiology Letters, 253*(1), 111-118.

Tong, H. Y., Thompson, T. S., & Karasek, F. W. (1985). Quantitative responses of normal alkanes in gas chromatographic-mass spectrometric analysis. *Journal of Chromatography A, 350*(0), 27-35.

Tønnesen, H. H., & Karlsen, J. (1986). Studies on curcumin and curcuminoids VII. Chromatographic separation and quantitative analysis of curcumin and

related compounds. *Zeitschrift für Lebensmitteluntersuchung und - Forschung A, 182*(3), 215-218.

Tossavainen, A. (1978). Styrene use and occupational exposure in the plastics industry. *Scandinavian Journal of Work, Environment & Health*, 7-13.

Tresse, O. T., Lorrain, M. J. L., & Rho, D. R. (2002). Population dynamics of free-floating and attached bacteria in a styrene-degrading biotrickling filter analyzed by denaturing gradient gel electrophoresis. *Applied Microbiology and Biotechnology, 59*(4), 585-590.

US EPA Drinking Water Contaminants. (2012). Retrieved 25 Mar, 2012, from http://water.epa.gov/drink/contaminants/

US EPA. (1974). *Development document for effluent limitations guidelines and new source performance standards for the synthetic resins - segment of the plastics and synthetic materials manufacturing*: U.S. Environmental Protection Agency.

US EPA. (1994). *Pollution Prevention and Toxics, Styrene Fact Sheet: Support Document , EPA 749-F-95-019a, Case No. 100-42-5*.

US EPA. (2010). *Technical Factsheet on: Styrene*: U.S. Environmental Protection Agency.

Valappil, S. P., Boccaccini, A. R., Bucke, C., & Roy, I. (2007). Polyhydroxyalkanoates in Gram-positive bacteria: Insights from the genera *Bacillus* and *Streptomyces*. *Antonie van Leeuwenhoek, 91*(1), 1-17.

Valentin, H. E., Lee, E. Y., Choi, C. Y., & Steinbüchel, A. (1994). Identification of 4-hydroxyhexanoic acid as a new constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. *Applied Microbiology and Biotechnology, 40*(5), 710-716.

van der Walle, G., de Koning, G., Weusthuis, R., & Eggink, G. (2001). Properties, Modifications and Applications of Biopolyesters

Biopolyesters. In W. Babel & A. Steinbüchel (Eds.), (Vol. 71, pp. 263-291): Springer Berlin / Heidelberg.

van Hellemond, E. W., Janssen, D. B., & Fraaije, M. W. (2007). Discovery of a novel styrene monooxygenase originating from the metagenome. *Applied and Environmental Microbiology, 73*(18), 5832-5839.

Velasco, A., Alonso, S., García, J. L., Perera, J., & Díaz, E. (1998). Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. *Journal of Bacteriology, 180*(5), 1063-1071.

Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., & Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology, 102*(6), 1437-1449.

Verma, S., Bhatia, Y., Valappil, S., & Roy, I. (2002). A possible role of poly-3-hydroxybuiyric acid in antibiotic production in *Streptomyces*. *Archives of Microbiology, 179*(1), 66-69.

Vidali, M. (2001). Bioremediation. An overview. *Pure and Applied Chemistry, 73*(7), 1163-1172.

Vitha, M., & Carr, P. W. (2006). The chemical interpretation and practice of linear solvation energy relationships in chromatography. *Journal of Chromatography A, 1126*(1–2), 143-194.

Volova, T. G. (2004). *Polyhydroxyalkanoates- Plastic Materials of the 21st Century: production, properties, applications*. New York, USA: Nova Science Publishers.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology, 73*(16), 5261-5267.

Wang, Q., & Nomura, C. T. (2010). Monitoring differences in gene expression levels and polyhydroxyalkanoate (PHA) production in *Pseudomonas putida* KT2440 grown on different carbon sources. *Journal of Bioscience and Bioengineering, 110*(6), 653-659.

Wang, Q., Zhang, H., Chen, Q., Chen, X., Zhang, Y., & Qi, Q. (2010). A marine bacterium accumulates polyhydroxyalkanoate consisting of mainly 3-hydroxydodecanoate and 3-hydroxydecanoate. *World Journal of Microbiology and Biotechnology, 26*(6), 1149-1153.

Wang, Y., Ruan, L., Chua, H., & Yu, P. H. (2006). Cloning and expression of the PHA synthase genes *phaC1* and *phaC1AB* into *Bacillus subtilis*. *World Journal of Microbiology and Biotechnology, 22*(6), 559-563.

Ward, A. C., & Dawes, E. A. (1973). A disk assay for poly-*β*-hydroxybutyrate. *Analytical Biochemistry, 52*(2), 607-613.

Ward, P. G., De Roo, G., & O'Connor, K. E. (2005). Accumulation of polyhydroxyalkanoate from styrene and phenylacetic acid by *Pseudomonas putida* CA-3. *Applied and Environmental Microbiology, 71*(4), 2046-2052.

Ward, P. G., Goff, M., Donner, M., Kaminsky, W., & O'Connor, K. E. (2006). A two step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. *Environmental Science & Technology, 40*(7), 2433-2437.

Warhurst, A. M., Clarke, K. F., Hill, R. A., Holt, R. A., & Fewson, C. A. (1994). Metabolism of styrene by *Rhodococcus rhodochrous* NCIMB 13259. *Applied and Rnvironmental Microbiology, 60*(4), 1137-1145.

Warhurst, A. M., & Fewson, C. A. (1994). Microbial metabolism and biotransformations of styrene. *Journal of Applied Microbiology, 77*(6), 597-606.

Warmink, J. A., Nazir, R., & Van Elsas, J. D. (2009). Universal and species-specific bacterial 'fungiphiles' in the mycospheres of different basidiomycetous fungi. *Environmental Microbiology, 11*(2), 300-312.

Watanabe, K., Futamata, H., & Harayama, S. (2002). Understanding the diversity in catabolic potential of microorganisms for the development of bioremediation strategies. *Antonie Van Leeuwenhoek, 81*(1-4), 655-663.

Wei, Y., Van Houten, R. T., Borger, A. R., Eikelboom, D. H., & Fan, Y. (2003). Minimization of excess sludge production for biological wastewater treatment. *Water Research, 37*(18), 4453-4467.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology, 173*(2), 697-703.

Werker, A., Lind, P., Bengtsson, S., & Nordström, F. (2008). Chlorinated-solvent-free gas chromatographic analysis of biomass containing polyhydroxyalkanoates. *Water Research, 42*(10–11), 2517-2526.

WHO. (2000). *WHO air quality guidelines for Europe* (2nd ed.). Copenhagen, Denmark: WHO Press, World Health Organization.

WHO. (2011). *Guidelines for drinking-water quality* (4th ed.). Geneva: WHO Press, World Health Organization.

Winderl, C., Anneser, B., Griebler, C., Meckenstock, R. U., & Lueders, T. (2008). Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. *Applied and Environmental Microbiology, 74*(3), 792-801.

Witholt, B., & Kessler, B. (1999). Perspectives of medium chain length poly (hydroxyalkanoates), a versatile set of bacterial bioplastics. *Current Opinion in Biotechnology, 10*(3), 279-285.

Wu, B., & Zhou, M. (2009). Sorption of styrene from aqueous solutions with oil absorptive resin. *Journal of Environmental Management, 90*(1), 217-221.

Wu, H. A., Sheu, D. S., & Lee, C. Y. (2003). Rapid differentiation between short-chain-length and medium-chain-length polyhydroxyalkanoate-accumulating bacteria with spectrofluorometry. *Journal of Microbiological Methods, 53*(1), 131-135.

Yu, H., Kim, B., & Rittmann, B. (2001). The roles of intermediates in biodegradation of benzene, toluene, and *p*-xylene by *Pseudomonas putida* F1. *Biodegradation, 12*(6), 455-463.

Zheng, L. Z., Li, Z., Tian, H.-L., Li, M., & Chen, G.-Q. (2005). Molecular cloning and functional analysis of (*R*)-3-hydroxyacyl-acyl carrier protein:coenzyme A transacylase from *Pseudomonas mendocina* LZ. *FEMS Microbiology Letters, 252*(2), 299-307.

Zilli, M., Converti, A., & Di Felice, R. (2003). Macrokinetic and quantitative microbial investigation on a bench-scale biofilter treating styrene-polluted gaseous streams. *Biotechnology and Bioengineering, 83*(1), 29-38.

Zinn, M. (2010). Biosynthesis of medium-chain-length poly[(*R*)-3-hydroxyalkanoates]. In G. G.-Q. Chen (Ed.), *Plastics from Bacteria* (Vol. 14, pp. 213-236). Berlin, Heidelberg, Germany: Springer.

Zinn, M., & Hany, R. (2005). Tailored material properties of polyhydroxyalkanoates through biosynthesis and chemical modification. *Advanced Engineering Materials, 7*(5), 408-411.

Zinn, M., Witholt, B., & Egli, T. (2001). Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Advanced Drug Delivery Reviews, 53*(1), 5-21.

Appendix Figures

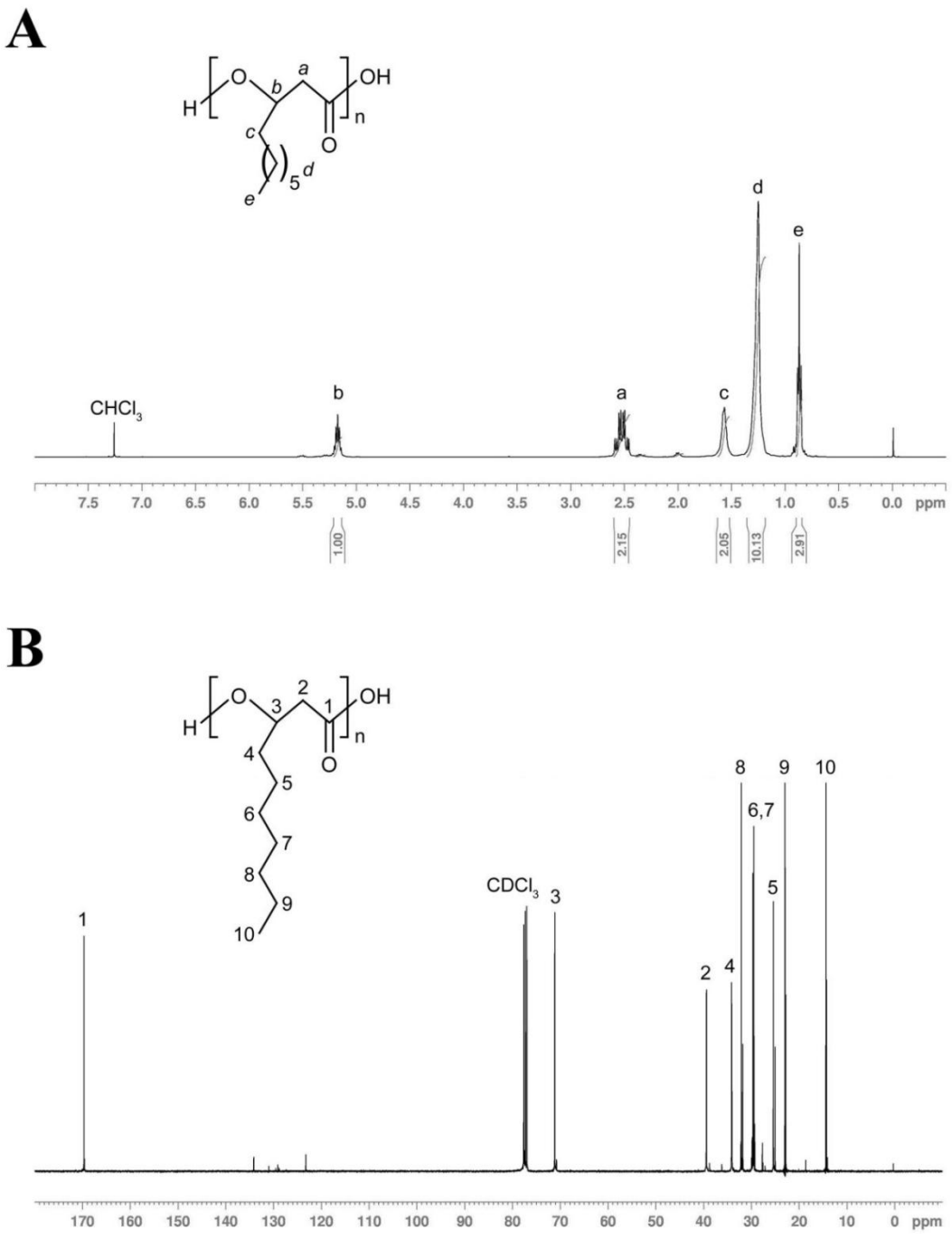


Figure A1 (A) ^{1}H-NMR and (B) ^{13}C-NMR spectra of MCL-PHA polymer. The signals corresponding to hydrogen atoms in ^{1}H-NMR spectrum are denoted by alphabet letters while the signals corresponding to carbon atoms in ^{13}C-NMR spectrum are denoted by numericals.

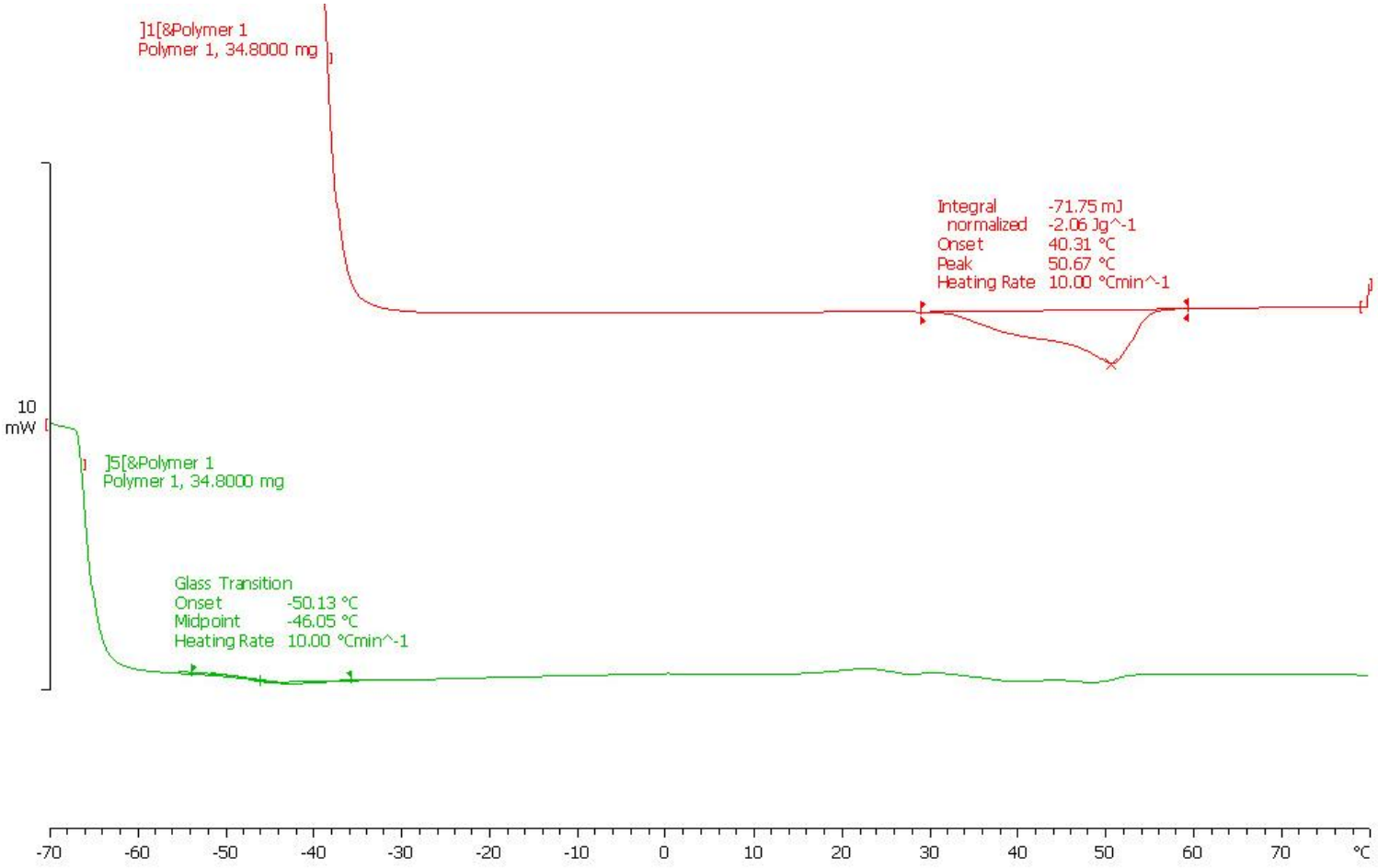


Figure A2 DSC thermogram of MCL-PHA polymer from *P. putida* NBUS12. The mid-point of heat capacity change (-46.05 °C) was determined as T_g while the maximum endothermic point (50.67 °C) was taken as T_m

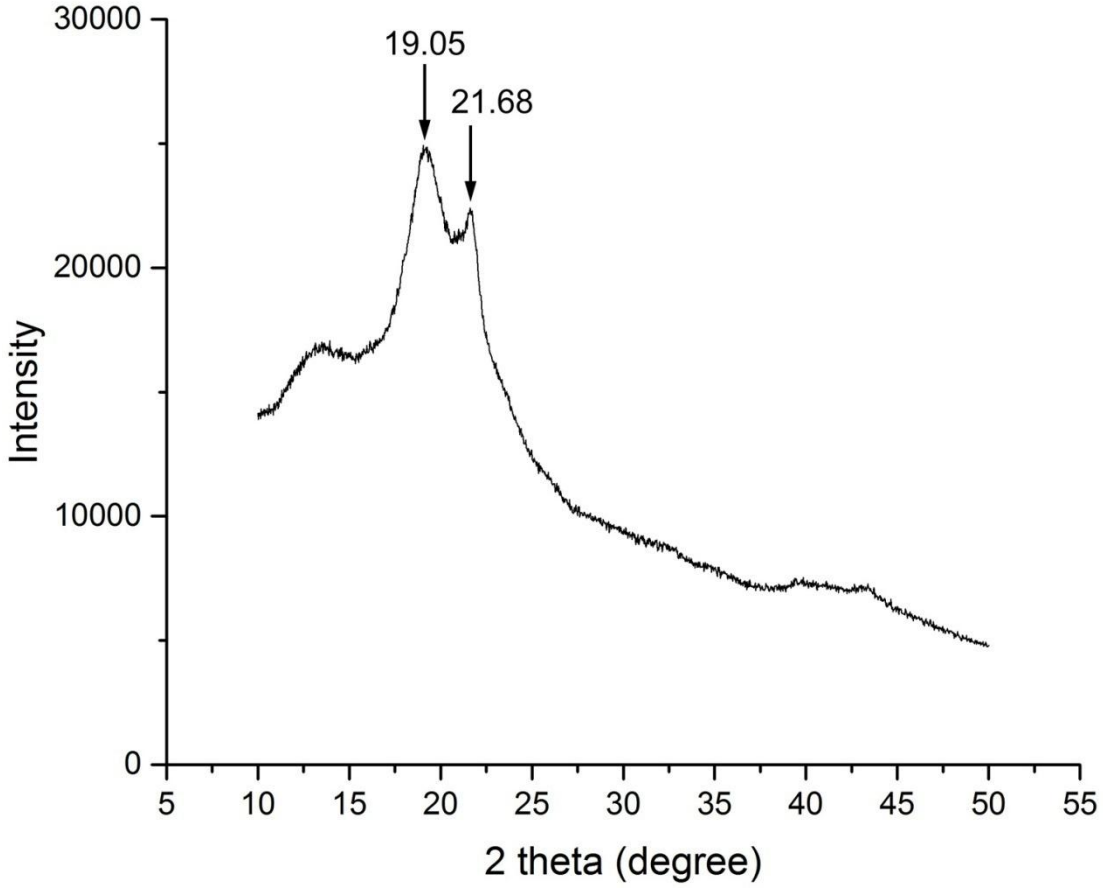


Figure A3 XRD diagram of MCL-PHA polymer from *P. putida* NBUS12. Crystalline peaks were detected at 2 theta values of 19.05 ° and 21.68 °.

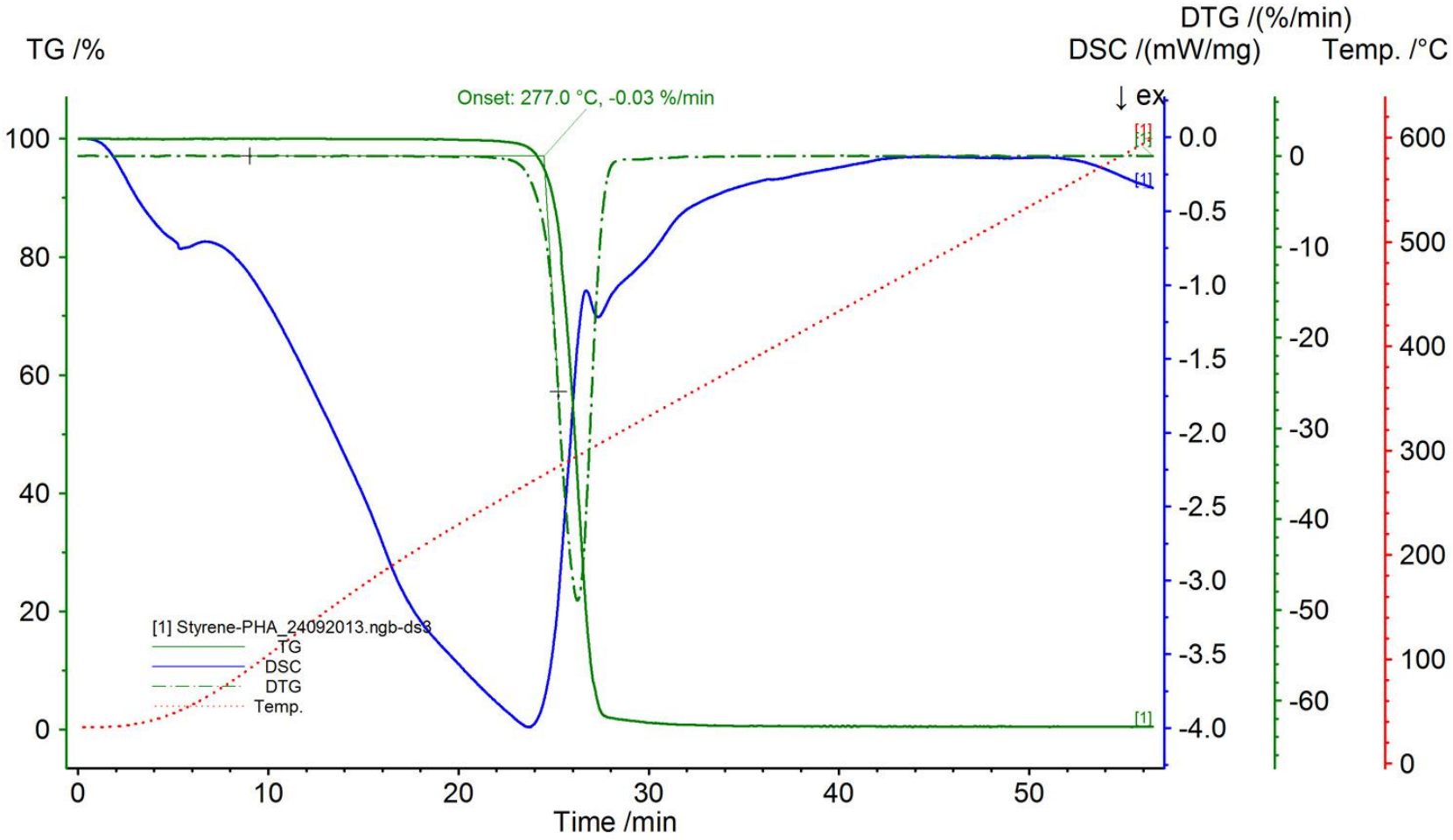


Figure A4 TGA thermogram of MCL-PHA polymer from *P. putida* NBUS12. Decomposition temperature (DT) was determined as the onset temperature of the derivative thermogravimetry curve (277 °C).