The Hong Kong Polytechnic University

Department of Civil and Structural Engineering

Study of Bio-degradable Plastics (Polyhydroxyalkanoates) Production from Activated Sludge Wastewater Treatment Process

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A thesis submitted in partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

Conventional polymers, which are also known as ‘plastic’, are large contributions to the waste problem. It has for many years been recognized that reducing plastic refuse could go a long way in preventing a landfill crisis. Consequently, many countries are introducing legislation or environmental regulations on reducing plastic usage. However, the remarkable usefulness of polymers probably precludes any serious slowdown in their production. In answer to this dilemma, development and production of biodegradable plastics may be a worthy option. Polyhydroxyalkanoates (PHAs), stored as bacterial reserve materials for carbon and energy, are biodegradable substitutes to petroleum-based plastics that can be produced from renewable raw materials.

Many PHA-polymers have interesting properties, such as biodegradability, and have a wide array of uses ranging from single-use bulk, commodity plastics, to specialized medical applications. PHAs is a kind of polymers synthesized by microorganism. PHAs can be produced under controlled conditions by biotechnological processes. However PHAs is still not used in large-scale in

commercial due to the high production cost of PHAs.

The primary aim of this research is to produce PHAs possibly in large scale and at a cost comparable to synthetic plastics.

After a historical review, degradation of PHAs is shown in detail. This is followed by a discussion of PHAs characterizations, and possibilities for the synthesis of novel PHAs applying different micro-organisms are discussed. In addition, detection, analysis, and extraction methods of PHAs from microbial biomass are presented. Strategies for PHAs production are discussed in detail in addition to the use of a cheap carbon source (activated sludge) in a SBR system from the point of economic view.

Before lab-scale and pilot-scale study of optimization of PHAs production, a Plackett-Burman design was presented to investigate the key factors during PHAs production process. Based on the statistical screening analysis and discussions, C:N ratio was found to be a key variable during the PHAs optimization process. Feast/Famine was suggested 1/3 and SRT was designed as 10 days in study. But oxygen concentration and pH control were not controlled

because they were not critical in enriching the PHAs accumulation.

Study of part one in Stage I study was focus on different C:N ratio, to achieve high PHAs production rate. Results showed that the optimized C:N point was 100:1 under which the maximum PHAs content was obtained per unit nutrient substrate consumed in both Tai Po wastewater treatment sample and refuse transfer station leachate sample.

In part two of Stage I, bacterial identification was applied by MIDI Instant FAMETM Sherlock System and sampling when SBR systems were running under C:N ratio of 100:1 in both two groups. Some bacteria from two sample, *Bacillus sphaericus*, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*, were identified. They had been confirmed by previous research having abilities of storing PHAs.

The last part of Stage I study demonstrated that the monomeric unit composition can be controlled by carbon substrate. When butyric acid (C_4) was used as sole carbon source, PHB homopolymer rather than PHBV was accumulated. On the other hand, when valeric acid (C_5) was used as sole carbon

source, not only PHV homopolymer but PHBV appeared simultaneously. When glucose (C_6) was used as sole carbon source, HB mole fractions was 78% and HV mole fractions was 22%. In a comparison of results in three sets of experiments, PHBV was accumulated when valeric acid or glucose was used. No PHV but PHB solo appeared only when butyric acid was used as solo carbon source. But proportion of HB mole fraction was higher than HV mole fraction no matter what kind of carbon source was utilized.

Based on the empirical data of lab-scale experience, Stage II pilot-scale system was set-up. The results stated that producing PHAs from activated sludge was stable and possibly run in large scale. C:N ratio of 100:1, such a nitrogen-deficiency condition was confirmed to be a significant reference for PHAs industrial application. In addition, *Bacillus cereus*, *Rhodopseudomonas palustris*, *Rhodopseudomonas sphaeroides* and *Salmonella typhimurium* were identified in sample from the pilot-scale SBR system.

Comparison between laboratory-scale and pilot-scale study indicated that the large scale production of PHAs was feasible. As C:N ratio increased, the Y p/x, Y p’/x’ and Y p”/x” were all increasing. Their maximum were 0.386, 0.533 and

0.526 g polymer accumulated / g biomass with C:N ratio from 60:1 to 140:1 among three sets of experiment, respectively. Y p/s , Y p’/s’ and Y p”/s” obtained their maximum point under C:N ratio of 100:1 among three groups system. Cost-benefit comparison was also conducted between this project and traditional pure culture of PHAs. Comparison results addressed that producing PHAs under certain designed condition by mixed culture was strongly competitive to pure culture.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Professor H. Chua, for his valuable guidance, comments and support during the whole period of my research study.

Special thank is given to Mr. W.S. Lam, Mr. C.W. Lau and Dr. Peter H. F. Yu for their greatest and kindness support in laboratory works. I also want to devote my thanks to the current and former members of our Research Group, namely Dr. S.Y. Chan, Dr. Y. F. Tsang and Dr. Y. J. Wang for their encouragement and help in the past years. Appreciate Mr. Y. H. Liu, Miss L. F. Chak and Miss Yuki for their cooperation and coordination in conducting the experiment and field sampling work.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

3HDD	3-hydroxydodecanoate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
ADF	aerobic dynamic feeding
ATP	adenosine triphosphate
AWWA	American Water Works Association
BOD	biochemical oxygen demand
C	Carbon
COD	chemical oxygen demand
E. coli	*Escherichia coli*
GC	Gas Chromatograph
HDPE	high-density polyethylene
HRT	hydraulic retention time
LCL	long-chain-length
LDPE	low-density polyethylene
MCL	medium-chain-length
MLVSS	mixed-liquor volatile suspended solids
N	Nitrogen
OLR	organic loading rate
PHAs	polyhydroxyalkanotes
PHB	polyhydroxybutyrate

PHBV	poly-3-hydroxybutyrate-co-hydroxyvalerate
PHV	poly-3-hydroxyvalerate
RAS	return activated sludge
SCL	short-chain-length
SBR	sequencing batch reactor
sp.	species
STW	sewage treatment works
TKN	total kjeldahl nitrogen
TSA	Trypticase Soy Agar
WEF	Water Environment Federation

CHAPTER 1 INTRODUCTION

1.1 Introduction and overview

In the second half of the twentieth century, a production of certain materials truly skyrocketed, a group of materials never seen before on our planet. They were synthetic polymers (which is often vernacularly referred to as ‘plastics’). Plastics have been widely used because they are flexible, resistant to thermal change, lightweight and are waterproof. Plastic materials are present in daily life in the form of disposable utensils, packaging, furniture, machinery housings and accessories, enhancing life quality and comfort (Khanna and Srivastava, 2009; Castilho et al., 2009). The use of plastics has grown rapidly over the past few decades, with about 150 million tonnes of plastic materials being consumed yearly worldwide (London Metal Exchange, 2009).

Plastics are very heterogenous considering both properties and practical use, and are production of petroleum. But petroleum is unsustainable, currently in light of some industry like plastic production that necessarily accompany it, and over the longer term in light of the finite nature of these resources. It is now generally

recognized that plastic waste are being notably increased by human activity, with significant undegradable polymers waste due to its high resistance to natural degradation process. The fact that the plastics are mostly non-degradable is the primary cause of still increasing amount of solid waste, whose main parts are just synthetic polymers. While the production of polymers involved in supporting human daily needs provides economies that the traditional polymers industry enjoys, but resident in the earth could not enjoy if landfill space is less and become critically deficient. Even they would involve in a serious pollution problem. Petroleum derived plastics not only take decades to get decomposed in nature but also produce toxins at the time of degradation. This was a major reason for the search for new polymers, which can be eliminated from the environment in an eco-friendly fashion (Gross, 2002).

In Hong Kong about 1,941 tones of municipal solid waste produced each day were plastic packaging materials, disposable products and plastic waste generation is forecast to increase by 15% per year over the next few decades (Hong Kong Environmental Protection Department, 2011).

Not only in Hong Kong, this is a large contribution to the environmental problem,

and it has for many years been recognized that reducing plastic refuse could go a long way in preventing a landfill crisis. When discarded in nature, conventional polymers can persist for many decades, at best a mere eyesore, at worst posing a threat to wildlife.

Consequently, many countries are introducing more and more stringent environmental regulations on plastic usage. Proposed or already passed legislation in the USA and Europe aims at reducing the use of polymers (Leaversuch, 1987). On Jun1, 2008 the Chinese government brought out the ban of the free use of plastic bag in supermarket in China Mainland, as London did years ago. Hong Kong is also facing an imminent and serious waste problem. The public widely recognize the indiscriminate use of plastic shopping bags as a major and visible environmental problem in Hong Kong. Based on the statics from the Environmental Protection Department, it is estimated that over eight billion plastic shopping bags are disposed of at our landfills every year. This translates into more than three plastic shopping bags per person per day, which apparently go beyond our needs (Environmental Protection Department, 2009). In order to reduce the use of the plastic bags, the Environmental Protection Department amended the “Environment Levy Scheme on Plastic Shopping Bags”

on July7, 2009 to persuade the people to take their own bags instead of using plastic shopping bags. Although the Environment Levy Scheme on Plastic Shopping Bags has started, there are still many plastic bags used and disposal in the landfills. However, the remarkable usefulness of polymers probably precludes any serious slowdown in their production. In answer to this dilemma, any plastic refuse policy must include solutions other than landfilling. Therefore the biodegradable plastics which have good biodegradability, biocompatibility, and non-toxicity, are considered as the potential substitute for the convenient petroleum based plastics.

In response to problems associated with plastic waste and its effect on the environment, development and production of biodegradable plastics might shine. Polyhydroxyalkanotes (PHAs) currently occupy a special position among biodegradable polyesters. PHAs are polyesters that accumulate as inclusions in a wide variety of bacteria. These bacterial polymers have properties ranging from stiff and brittle plastics to rubber-like materials. Because of their inherent biodegradability, PHAs are regarded as an attractive source of nonpolluting plastics and elastomers that can be used for specialty and commodity products.

However, the economic factor shadows the mass production of bio-plastics. Today price with natural producer like Alcaligenes eutrophus is around US $16 per kg. This is about 18 times more expensive than polypropylene ---- conventional plastics. A new technology to produce PHAs is required to help bio-plastics compete with or even outperform the petrochemically based alternatives on price.

The major drawback in commercialisation of PHAs is the high cost of production. The cost of raw material itself accounts for 40%-50% of the total production cost. Looking to cheaper raw material will have economics reducing the price. For PHAs to be commercially viable price should come to US $3-5 per kg. In addition, most important aspect is its biodegradable nature helps to overcome the problem of environmental hazard. And the value of clean environment will otherwise also outweigh conventional plastics on price factor in favour of more and more use of biodegradable plastics.

1.2 Objectives

1.2.1 General objective

PHAs stand at the forefront of practical use. Most of the published work concerning PHAs production focused on understanding the storage mechanisms but not on the optimization of PHAs production. Therefore, it is expected that the reported PHAs yields could be improved by optimizing process operating parameters and reactor operating conditions.

In addition, the application of PHAs has been rather limited because of their higher cost, inferior thermal and mechanical properties, and worse processability as compared to the conventional oil-based polymers. Much effort has therefore been made to address it.

So, the primary study aim in creating the technology is to make it more cost effective to ‘grow’ PHAs. The possibility of producing PHAs in large scale and at a cost comparable to synthetic plastics will be addressed. An effect to develop more efficient fermentation processes makes a possibility that PHAs could manufacture on a commercial scale.

1.2.2 Specific objectives

The key and specific elements of the project are to:

- Reduce cost of producing PHAs

PHAs are biodegradable polymers produced by microbes to overcome environmental stress. Commercial production of PHAs is limited by the high cost of production compared to conventional plastics. And the cost of raw material itself accounts for 40%-50% of the total production cost. Use ‘waste’—activated sludge as feed, enable it to compete as potential candidate for commercial production of PHAs. Besides, improving the intracellular PHAs content is important for decreasing the extraction and recovery cost of PHAs downstream processing. Research on this field in the past decade focused on areas such as process configuration, reactor operational strategies, metabolic pathway analysis, microbial characterization and polymer characterization. Therefore, enhancing the intracellular PHAs content should be critically considered and studied, which senses another way to reduce cost of producing PHAs.

- Optimal conditions of producing PHAs

A system run to investigate various condition, like effect of C:N ratio, on

enhancing PHAs production yield. Thus, it means to design and set up the experiment system under the well analysis of different operation condition to let it be activated.

- Carbon source effect of PHAs composition

Another hindrance is the brittle nature and low strength of polyhydroxybutyrate (PHB), the most widely studied PHAs. The needs are to produce PHAs, which have better elastomeric properties suitable for commercial applications. The knowledge of the influence of PHAs structure with various polymer properties is important for the practical applications of PHAs. A group of experiment is running to identify whether a relationship exist between carbon source and polymer composition. If exist, this would be a value information for PHAs' commercial applications.

- Feasibility of pilot-scale study

Currently, all studies of PHAs production just stop at lab-scale level, and do not mention pilot-scale process. Thus, another sub-objective is to design and set up the experiment system under the well analysis of the details of the plants, the experiment system could be as practical as possible. Theoretically, the system is

the SBR system. However, the operation parameters are not the same due to the different purposes. The system of this study is for PHAs accumulation but not for wastewater treatment. The system should be automatic, easy-operating, and variable and will not bring any bad effects towards the treatment plant.

1.3 Significances of study

This research explores ways to improve the economics of manufacturing biodegradable plastics by producing useful by-products (PHAs) from potentially environmentally damaging waste discharges. A system was designed for PHAs producing test. The operator could be benefited by the low costs of raw material for producing PHAs and high yield of PHAs under certain conditions. Excess sludge from wastewater or sewage plant, using to produce PHAs in a simple, cost-effective and environment-friendly technology is superior to be transported to landfill.

1.4 Structure of thesis

Chapter 1 explains the background of the study and provides an introduction to the current status and needs of PHAs. This chapter also outlines the objectives and significance of the study. In Chapter 2, relevant studies on PHAs are

reviewed. Background information such as functional group, structure, properties, bio-synthesis pathway and applications of PHAs is also summarized in this chapter. The methods of sampling, chemical analysis, biological analysis and system set-up are described in Chapter 3. A preliminary study before lab-scale and pilot-scale experiment, using Plackett-Burman design to investigate significant factors, is presented in Chapter 4. The results and discussion of this study are presented in two separate chapters. The lab-scale study is shown in Chapter 5. Then the pilot-scale study of PHAs production in an industrial wastewater plant is discussed in Chapter 6. Comparison between lab-scale and pilot-scale study is conducted in Chapter 7. Lastly, overall conclusion and recommendations are incorporated into Chapter 8.

CHAPTER 2 LITERATURE REVIEW

2.1 History of PHAs

Polyhydroxyalkanoates (PHAs) are polymers of hydroxyalkanoic acids that are accumulated intracellularly as granule inclusions by prokaryotic microorganisms (eubacteria and archaea) as carbon and energy reserves or reducing-power storage materials (Steinbüchel and Lütke-Eversloh, 2003; Choi and Lee, 1999a). They are synthesized in the presence of excess carbon, especially when another essential nutrient, such as nitrogen or phosphorus, is limiting (Koller et al., 2008; Anderson and Dawes, 1990).

The first determination of the composition of PHAs was as early as in the year 1926 by the work of Lemoigne. In a soil bacillus resembling Bacillus megaterium, the anaerobic degradation of an unknown material was discovered. Lemoigne identified this material as a homopolymer whose building unit is the 3-hydroxybutyric acid, or polyhydroxybutyrate (PHB), and described it tentatively as a reserve material. It became the first determination of the PHAs.

At the end of the 1950s, the presence of the polyhydroxybutyrate was confirmed as an energy and carbon source and storage in many other bacteria by Macrac and Wilkinson. In 1974, there were isolated, aside from the PHB, also copolymers containing 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) (Wallen and Rohwedder, 1974). 3HV and 3HHx which were detected in chloroform extracts of activated sewage sludge, as the major and minor constituents respectively. Since then, the researcher identified many microorganisms capable of synthesis of various PHAs, and found out about 91 most possible PHAs structures.

2.2 Nature of PHAs

2.2.1 Molecular structure

The PHAs is proved to be the carbon and energy storage material of cells, which is to slow down the process of cell autolysis and death (Ma T. C., 2004). Such kinds of polymers usually contain only one or a few different types of monomer residues, even though a cell may synthesize dozens of different kinds of monomers, most of its polymer are homogeneous (Charlotte W. P., et al., 2004).

As its name implies, PHAs is the polymerization of the hydroxyalkanoates. In other words, the hydroxyalkanoate is the monomer of the PHAs. PHAs are composed together by ester bond (figure 2.1). The ester is composed from the hydroxyl group and carboxyl group under acidic condition, termed the esterification (Figure 2.2). The general expression of the hydroxyalkanoic acid is shown in Figure 2.3. It stands for a group of the organics which contain hydroxyl and carboxyl in the structure itself. Then monomers of hydroxyalkanoic acid could connect together by the ester bond and form PHAs, which is expressed as Figure 2.4. Approximately 150 different hydroxyalkanoic acids are now known to occur as constituents of PHAs (Figure 2.5). PHAs have physical properties that are based on the number of carbon atoms in the individual monomer units as well as on the physical structure of these monomers. Many different monomers in Fig.2.6 can be incorporated into PHAs by supplementing the growth media of the microorganism with feedstock of the related monomer precursor. These related precursors are generally various fatty acid variants that can be processed into PHA monomers.

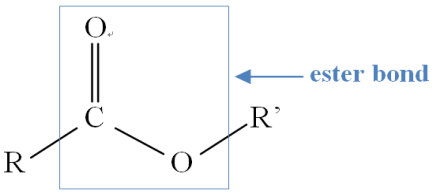


Figure 2.1 The ester bond in hydroxyalkanoic acid

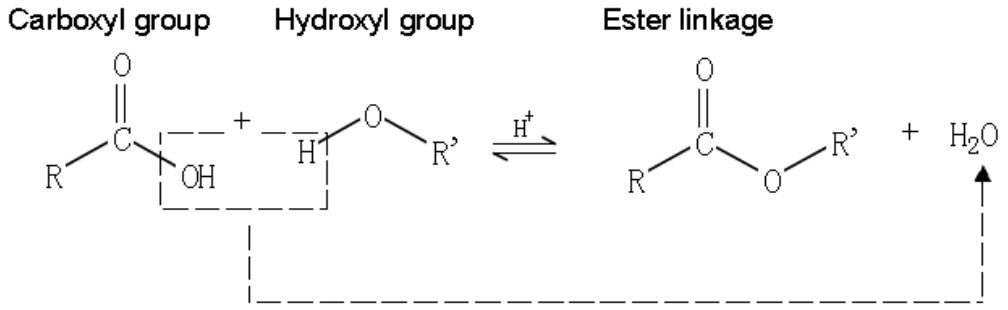


Figure 2.2 The esterification formula

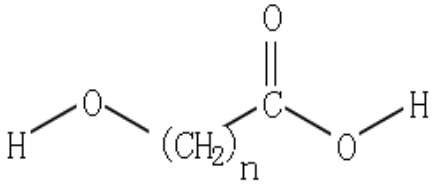
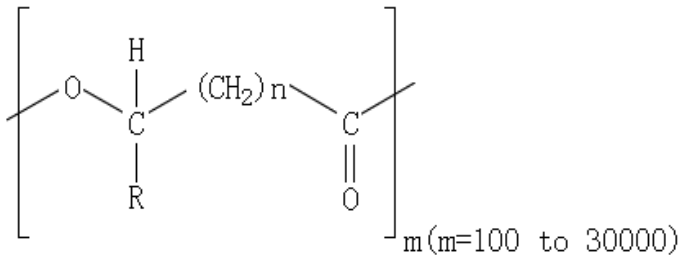


Figure 2.3 The general expression of hydroxyalkanoic acid



n = 1 R = hydrogen 3-hydroxypropionate

R = methyl 3-hydroxybutyrate

R = ethyl 3-hydroxyvalerate

R = propyl 3-hydroxyhexanoate

R = pentyl 3-hydroxyoctanoate

R = nonyl 3-hydroxydodecanoate

n = 2 R = hydrogen 4-hydroxybutyrate

R = methyl 4-hydroxyvalerate

n = 3 R = hydrogen 5-hydroxyvalerate

R = methyl 5-hydroxyhexanoate

n = 4 R = hexyl 6-hydroxydodecanoate

Figure 2.4 General structures of PHAs

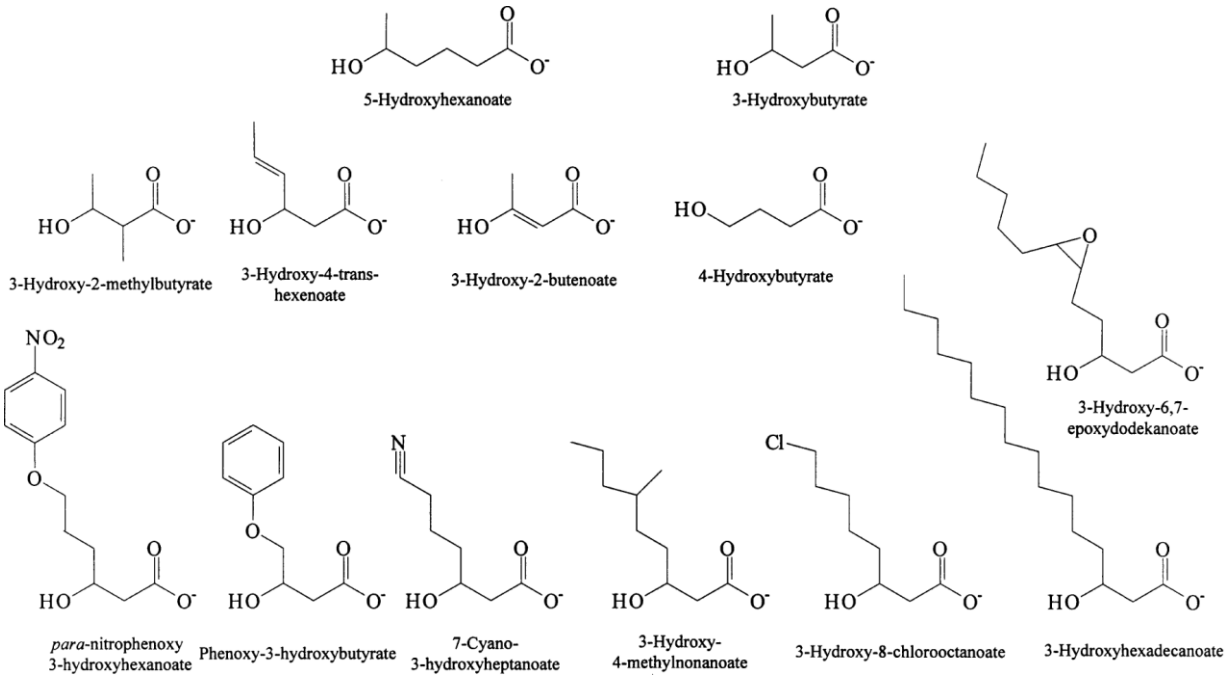


Figure2.5 Representative constituents (hydroxyalkanoic acids) of PHAs

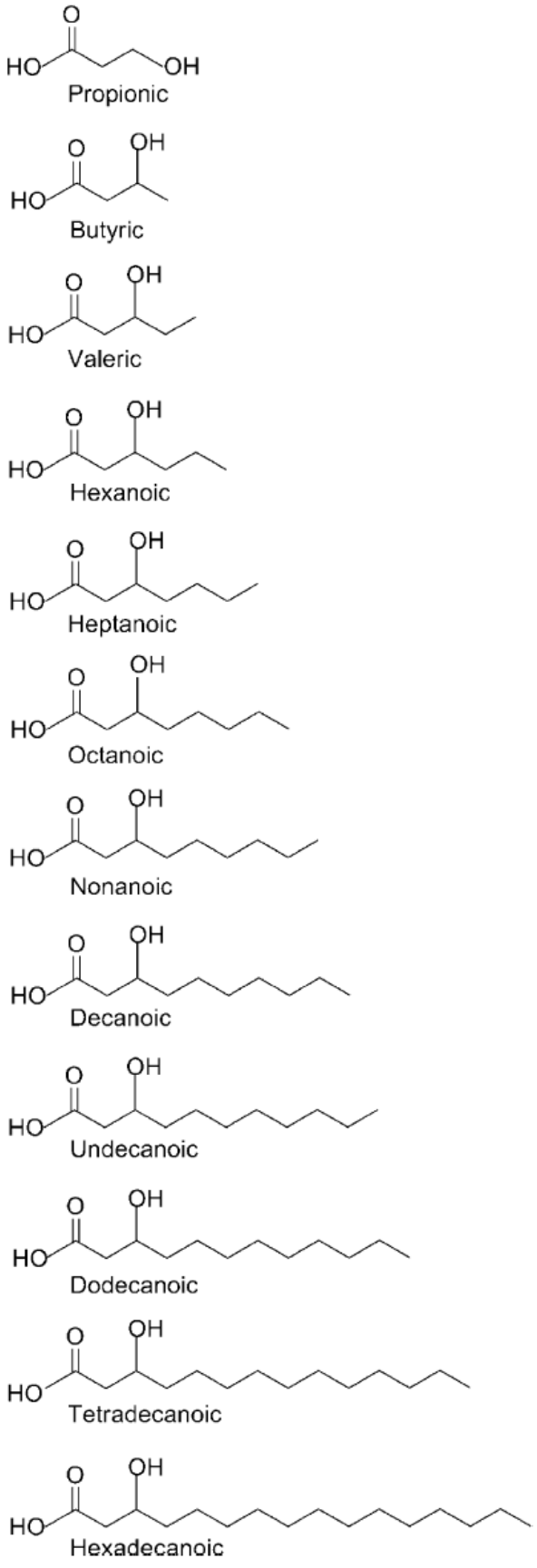


Figure 2.6a Structures of PHAs monomer units: Saturated 3-hydroxyacids

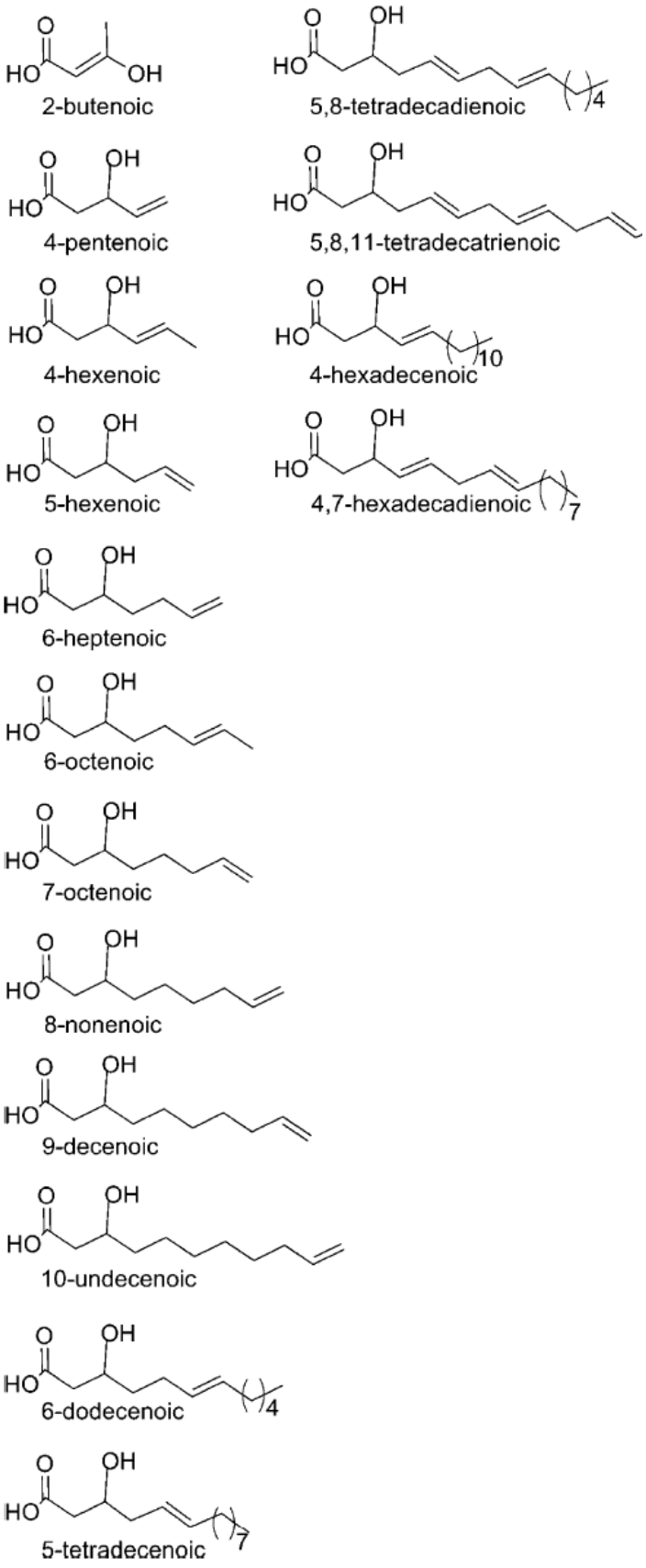


Figure 2.6b Structures of PHAs monomer units: Unsaturated 3-hydroxyacids

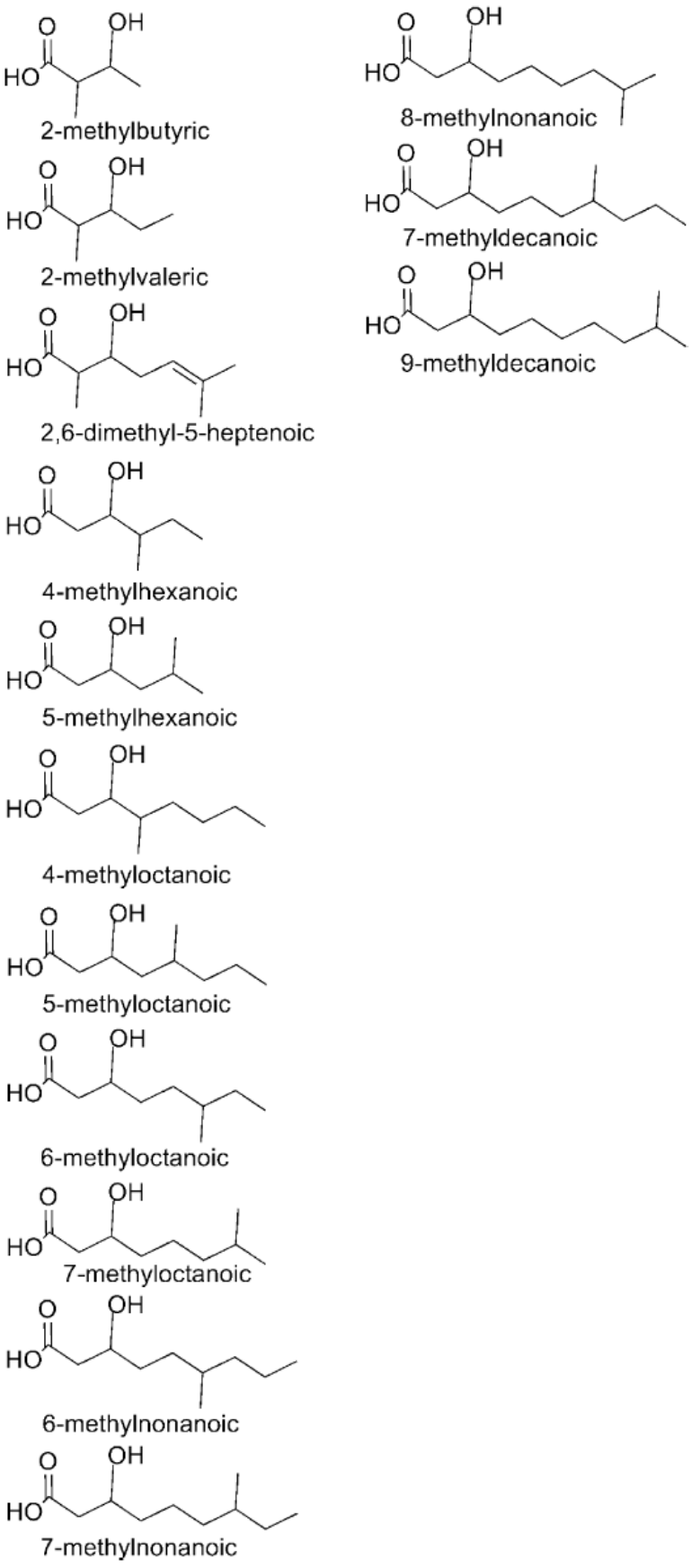


Figure 2.6c Structures of PHAs monomer units: Branched 3-hydroxyacids

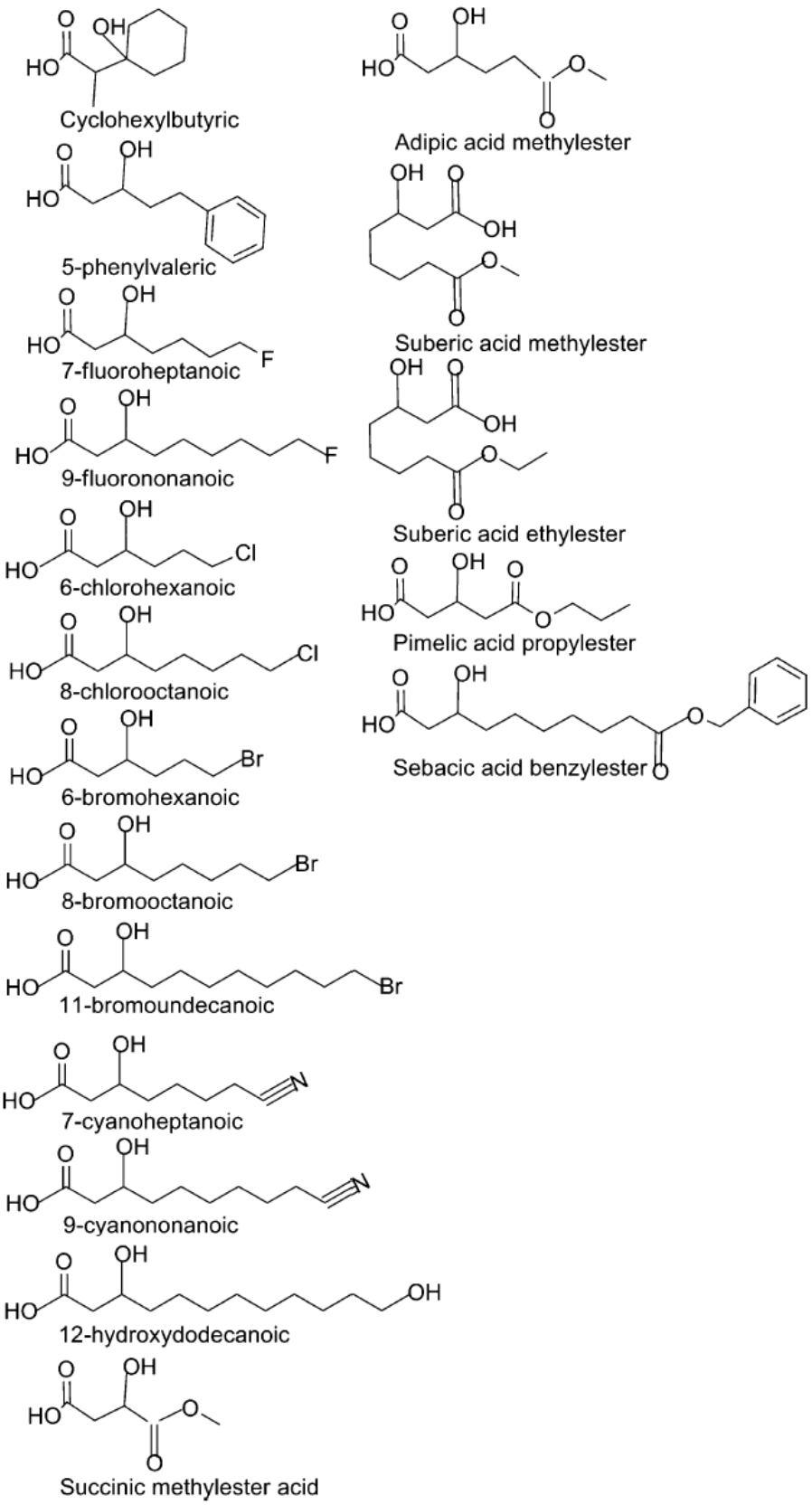


Figure 2.6d Structures of PHAs monomer units: 3-hydroxyacids with substituted side chains

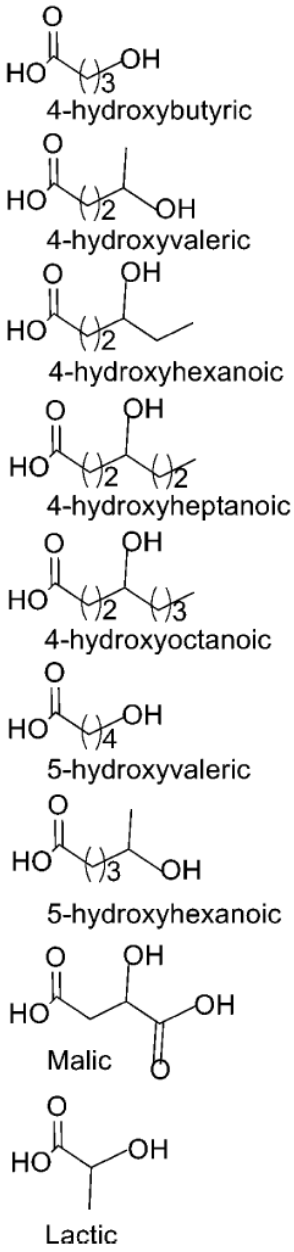


Figure 2.6e Monomers other than 3-hydroxyacids that can be incorporated into PHAs.

2.2.2 Groups of PHAs

PHAs can be divided into three groups depending on the number of carbon atoms in the monomeric units (Lee et al., 1995). One group of PHAs is short-chain-length (SCL) PHAs consisting of 3-5 carbon atoms; the second group is medium-chain-length (MCL) PHAs consisting of 6-14 carbon atoms, the last group is long-chain-length (LCL) PHAs consisting of greater than 14 carbon atoms.

2.2.3 Two major monomers of PHAs

Many documents have focus on two members of the PHAs, which are the PHB and PHV. PHB stands for Polyhydroxybutyrate (Figure.2.7) while the PHV is for Polyhydroxyvalerate (Figure.2.8). They are found in wide range of microorganisms (Shamala T. R., et al., 2003; Anastasia A. P., et al., 2003). Compared with PHB, the PHV is less abundant in the bacterial cells. And their co-polymer PHBV, Poly(3-hydroxybutyrate-co-3-hydryxyvalerate) (Figure.2.9), is found rich in cells and catch most research interest. In most cases, mixtures of various hydroxyalkanoates are obtained unless homopolymers or homologous monomers are available (Gao et al., 2011).

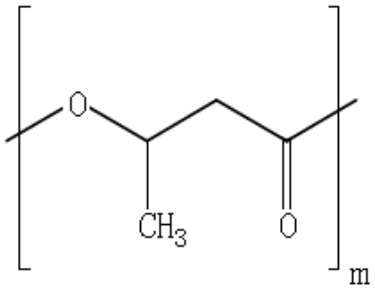


Figure 2.7 Structure of poly-3-hydroxybutyrate, PHB

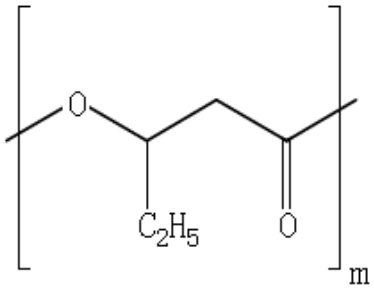


Figure 2.8 Structure of poly-3-hydroxyvalerate, PHV

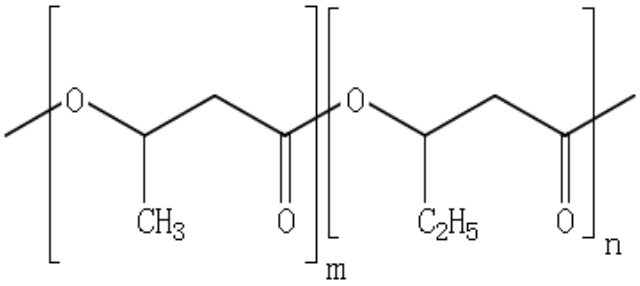


Figure 2.9 Structure of poly-3-hydroxybutyrate-co-hydroxyvalerate, PHBV

The most common short-chain-length monomer in SCL PHAs polymers is 3-hydroxybutyrate (3HB) which is the monomer of PHB. PHB can be produced from a number of different carbon sources.

2.3 Characteristics of PHAs

Interest in PHAs has been growing since the late 1980s. This is a new class of natural polyesters, which are not subject to rapid non-biological hydrolysis and whose properties (molecular weight, crystallinity, mechanical strength, and degradability) very substantially.

2.3.1 Biodegradability

One of the properties that distinguishes PHAs from petroleum-based plastics is their biodegradability. Biodegradability is defined as the capacity of to be broken down, especially into innocuous products, by the action of living things like micro-organisms. Bacteria and fungi are the main participants in the process of biodegradation in the natural world. Some synthetic polymers can be microbially degraded (Steinbüchel, 1992; Pranamuda et al., 1995), but the process is normally slow. Biodegradation of PHAs is dependent upon a number of factors such as the microbial activity of the environment and the exposed surface area. In addition, temperature, pH, molecular weight and crystallinity are important factors. All of PHAs products can be completely degradable to carbon dioxide

and water through natural microbiological mineralization. Consequently, neither their production nor their use or degradation has a negative ecological impact.

2.3.2 A sustainable closed cycle

But it is not only biodegradability that makes PHAs so fascinating; it is also their synthesis from renewable carbon sources, based on agriculture or even on industrial wastes, allowing a sustainable closed cycle process (Figure 2.10) for the production and use of such polyesters instead of the end-of-the-pipe technologies connected to production and use of classical plastics (Figure 2.11).

Biodegradable plastics can be broken down in the environment by micro-organisms in a process called ‘biodegradation’. This process produces carbon dioxide (CO_2) and water (H_2O) under aerobic conditions. Photoautotrophs like plants, algae, and some bacteria fix this inorganic carbon to organic carbon (carbohydrates) using sunlight for energy. Then bacteria can accumulate PHAs under certain fermentation condition. PHAs was one useful source for biodegradable plastic production. In general, the use of waste in biodegradable composites can help to reduce waste, thus contributing to a healthier environment (Satyanarayana et al., 2009). Therefore, a review how carbon based

biodegradable polymeric materials fit into nature's cycle.

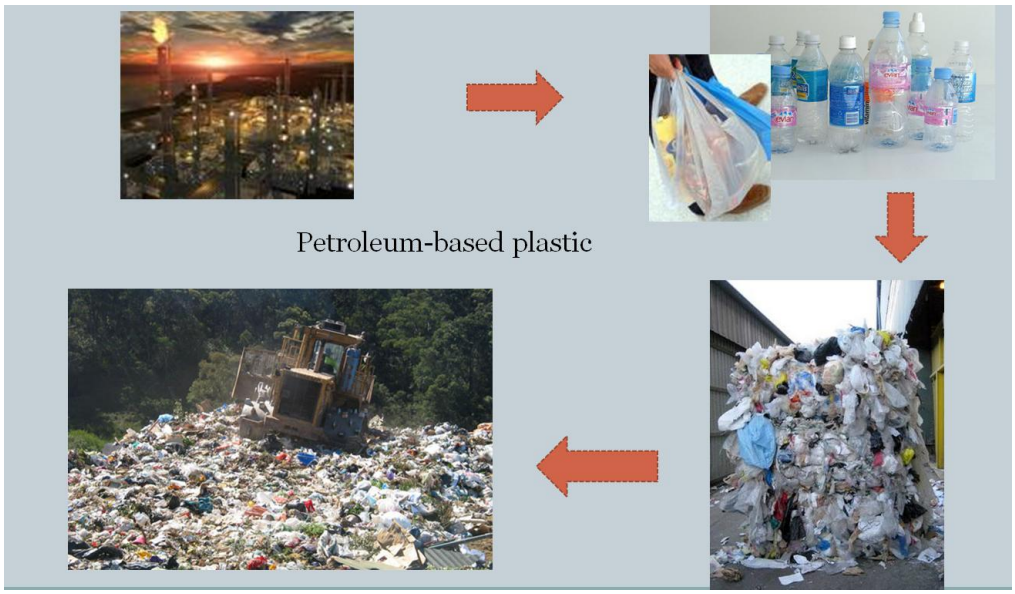


Figure 2.10 Non-circulating of petroleum-based plastic

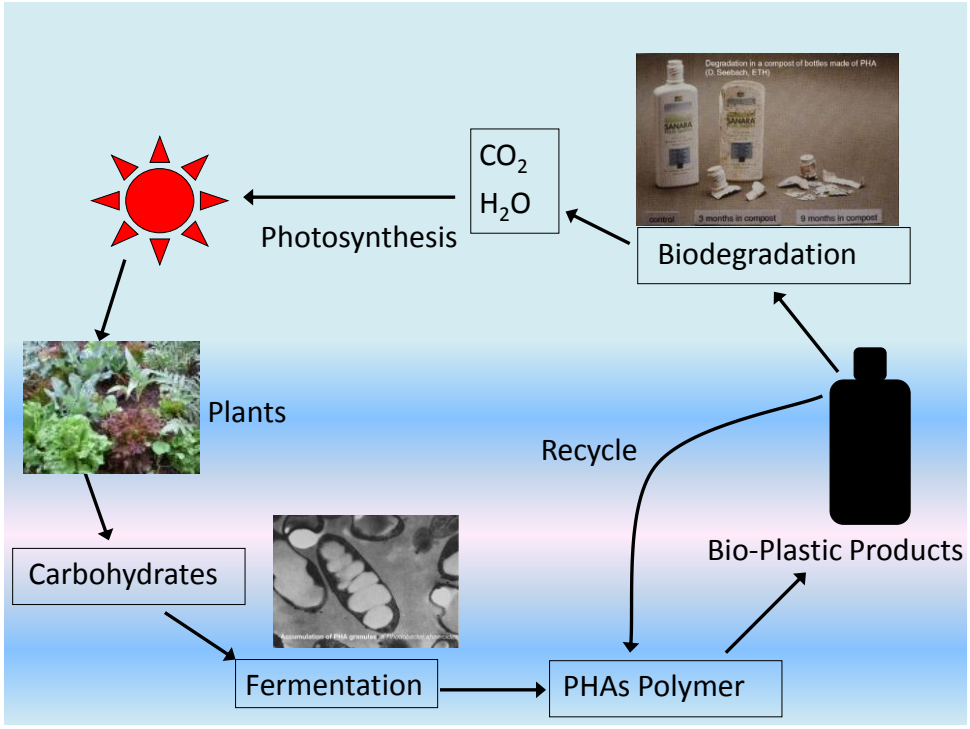


Figure 2.11 A sustainable closed cycle of biodegradable plastics

2.3.3 Mechanical of PHAs

The mechanical of PHAs can also be changed by blending, modifying the surface or combining PHAs with other polymers, enzymes and inorganic materials, making it possible for a wider range of applications.

2.3.3.1 Comparison of properties of different PHAs

As mentioned before, PHAs is divided into three groups: SCL, MCL and LCL PHAs. These monomers can form homopolymers or copolymers with various mechanical properties. Polymers composed solely of SCL monomer units generally have thermoplastic properties, while polymers composed of MCL subunits generally have elastomeric properties. PHAs copolymers with a relatively high mol% of SCL monomers and low mol% of MCL monomers have properties similar to the bulk commodity plastic polypropylene (Abe, Doi, 2002). Ouyang et al. (2007) demonstrated that PHAs copolymers composed of increasing mol% of 3-hydroxydodecanoate (3HDD) monomers had higher crystallinity and tensile strength compared to MCL PHA copolymers with low 3HDD mol% compositions.

PHB and copolymer PHBV are as two most striking PHAs. Table 2.1 shows their physical properties (Lee, 1996; Poirier et al., 1995). The homopolymer PHB is a stiff and relatively brittle thermoplastic. Many studies of the physical properties of bacteria PHAs have been reported with PHB and co-polymer PHBV. PHB is 100% stereospecific with the asymmetric carbon atoms having D(-) configuration which results highly crystalline (Doi and Abe, 1990; Steinbüchel,1991). Its melting point (175℃) is just slightly lower than it degrading temperature (185℃), this makes it processing by injection molding difficult. PHB has several useful properties such as moisture resistance, water insolubility and optical purity, this differentiate PHB from other currently available biodegradable plastics which are either water soluble or moisture sensitive. PHB also shows good oxygen impermeability (Lindsay, 1992; Ojumu et al., 2004; Deepak et al., 2009).

Table 2.1 Properties of PHB and PHBV

Property	PHB	PHBV		
		3 mol %	14 mol %	25 mol %
Melting point (℃)	175	169	150	137
Glass-transition temp (℃)	15	-	-	-1
Crystalline (%)	80	-	-	40
Young's modulus	3.5	2.9	1.5	0.7
Tensile strength (MPa)	40	38	35	30
Elongation to break (%)	6	-	-	-
Impact strength (v/m)	50	60	120	400

Copolymer PHBV has been developed and has much improved mechanical properties. PHBV becomes tougher (increase in impact strength) and more flexible (decreases in young's modulus) as the fraction of 3-hydroxyvalerate unit increases. The elongation to break was also reported to increase as the comonomer fraction increases (Lee, 1996). Furthermore, the melting temperature decreases with increasing comonomer fraction without any change in the degradation temperature. This allows the thermal processing of the copolymer without thermal degradation. Therefore the material properties can be controlled

by adjusting the fraction of HV during the fermentation.

2.3.3.2 Comparison of properties of various PHAs with fossil fuel plastics

PHB generally produces a stiff, thermoplastic material with relatively poor impact strength (Table 2.2). A study by Ouyang et al. demonstrated that PHAs composed of increasing mol% of 3-hydroxydodecanoate (3HDD) monomers had higher crystallinity and tensile strength compared to MCL PHA copolymers with low 3HDD mol% compositions. However, incorporation of other SCL monomers, such as 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB), into PHAs can dramatically improve the physical properties of the polymer. These improvements broaden the number of applications in which SCL PHAs can be used.

Table 2.2 Comparison of properties of various PHAs with fossil fuel plastics (Gracias, Somorja, 1998; Sudesh, K., et al., 2005; Hidalgo-Bastida, et al., 2007)

Polymer	Tm(℃)	Tg(℃)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
P(3HB)	180	4	3.5	40	5
PHBV (20 mol% PHV)	145	-1	0.8	20	50
PHBV (6 mol% PHV)	133	-8	0.2	17	680
P(4HB)	53	-48	250	0.97-1.64	150
Polypropylene	176	-10	1.7	38	400
Low-density polyethylene (LDPE)	88-100	-30	0.2	10-78	150-600
High-density polyethylene (HDPE)	112-132	—	7.5	33-155	12-700

The mechanical properties of PHB including Young's modulus (3.5GPa) and tensile strength (MPa) are similar to those of polypropylene (Table 2.2), but the elongation to break of PHB (5%) is dramatically lower than that of polypropylene (400%).

2.4 Microorganisms accumulating PHAs

Approximately 150 different hydroxyalkanoic acids are at present known, as constituents of bacterial storage polyesters. Table 2.3 concludes the PHAs accumulated by different bacteria in previous studies.

Table 2.3 PHAs accumulated by different bacteria

Bacteria	Source	Substrate	PHAs	Ref
Actinobacillus sp. Strain EL-9	soil	Alcoholic distillery waste	3HB	Son et al, 1996
Aeromonas hydrophilia 4AK4	-	Glucose + lauric acid (P limitation)	3HB-co-3HHx	Chen et al,2001; Liu et al, 2011
Alcaligenes eutrophus	-	Starchy waste	3HB; HB-co-38%HV	Yu,2001
Alcaligenes eutrophus	-	Glucose, Digested Sludge	C_4 monomer	Kim & Chang, 1995; Lee & Yu, 1997

Alcaligenes latus	-	Sucrose	3HB	Yamane et al, 1996; Wang &Lee, 1997
Alcaligenes latus DSM 1122	-	Malt waste	3HB	Wong et al, 2002
Alcaligenes latus DSM 1122	-	Soy waste	3HB	Wong et al, 2002
Alcaligenes latus DSM 1124	-	Malt waste	3HB	Wong et al, 2002
Alcaligenes latus DSM 1124	-	Soy waste	3HB	Wong et al, 2002
Arobacterium sp.	Activated Sludge	Glucose, Pentose, sugar	3HB-co-3HV	Lee et al, 1995
Azotobacter beijerinckii DSM 1041	-	Corn-steep liquor + molasses	HB	Purushothaman et al, 2001
Azotobacter chroococcum	-	Starch	3HB	Kim & Chang,1998
Azotobacter vinelandii	-	Glucose + fish peptone	3HB	Page & Comish, 1993
Azotobacter vinelandii	-	Swine waste	3HB-co-3HV	Cho et al,1997
Bacillus subtilis, Bacillus megaterium, Bacillus firmus, Bacillus sphaericus	Grassland Soil	NB	HB	Aslim et al, 2002
Bukholderia sp.	-	Mineral Media (N,P limitation), Sucrose/ Gluconate,LB	3HB, 3HPE	De Andrade Rodrigues et al, 2000
Chromobacterium violaceum	Soil and water of subtropical regions	Valerate (C source), N limitation	HV	Liebergesell et al, 1994

Hydrogenophaga peseudoflova	-	Luria-bertani medium Modified PHA syntheisi mineral medium	3HB-co-4HB; 4HB	Choi et al, 1999
Klebsiella sp.	Activated Sludge	Malt waste	3HB:7%3 HV	Wong et al, 2002
Klebsiella sp.	Activated Sludge	Soy waste	3HB-co-21%3 HV	Wong et al, 2002
Lactobacilus delbrueckii, Alcaigenes eutrophus	-	Sugar → lactate	HB	Katoh et al, 1999
Legionella pneumophilia	Fresh water inhabitants	ABCD medium	3HB	James et al, 1999
Methylcocytis sp.	GB25 DSMZ 674	Mineral salt Medium	3HB	Wendlandt et al, 2001
Methylobacteriu m organophilem	-	Methanol	3HB	Kim et al,1996
Nocardia coralline N 724	-	NB, Mineral Sait Medium + Glucose + 3HP	3HB, 3HV, 3HP	Valentin & Dennis, 1996
Psedomonas putida mutants GPp120-GPp124	P.putida KT2442	0.1NE2	C6, C8	Ren et al, 1998
Psedomonas corrugata	Alfalfa roots	Trocylglyceros, glucose, oleric acid, soybean oil, coconut oil (C source)	C8-C12	Solaiman et al, 2002
Pseudomonas citronellolis	-	Tallow fee fatty acid	C8, C10	Cromwick et al, 1996

Pseudomonas oleovorans	-	Tallow fee fatty acid	C8, C10	Cromwick et al, 1996
Pseudomonas putida	-	Tallow fee fatty acid	C8, C10	Cromwick et al, 1996
Pseudomonas putida	-	Oleic acid	Poly(3HHx-co-3HO-co-3HD-co-3HDD-co-3HTD	Weusthuis et al, 1997
Pseudomonas resinovorans	-	Tallow fee fatty acid; Unhydrolyzed Tallow	C8, C10	Cromick et al, 1996
Pseudomonas resinovorsan	-	Triglyceride + Medium E	C4- C14	Ashby & Foglia, 1998
Pseudomonas sp. 61-3 (FERMP-13108)	Soil	Mineral salt + sugar + NH_4CL	3HB,3HB-co-3 HA	Kato et al, 1999
Ralstonia eutropha	-	Glucose	3HB	Kim et al, 1994; Ryu et al, 1997
Ralstonia eutropha	-	Tapioca hydrolysate	3HB	Kim &Chang, 1995
Ralstonia eutropha	-	Glucose + Propionic acid	3HB-co-3HV	Kim et al, 1994
Ralstonia eutropha	-	Invert sugar + propionic acid + oleic acid	3HB-co-3HV	Marangoni et al, 2000
Recombinant *E.* Coli	pACE5	M9 + 1% glucose	β -HB-co-β HV	Eshenlauer et al,1996
Recombinant *E.* Coli	pKS, pAED4, PJM9131	Soy waste	HB	Liu et al, 2002
Recombinant *E.* Coli	-	Glucose	3HB	Lee et al, 1994; Lee & Chang, 1994

Recombinant *E.* Coli	-	Glucose + YE + CSL + casein hydrolysate	3HB	Wang &Lee, 1997b
Recombinant *E.* Coli	-	Sucrose	3HB	Lee & Chang, 1993
Recombinant *E.* Coli	-	Whey	3HB	Wong & Lee, 1998
Recombinant Klebsiella aerogenes	-	Molasses	3HB	Zhang et al, 1994
Rhodobacter sphaeroides (IFO12203)	Wastewater	Volatile fatty acid medim	HB-co-HV	Sidikmarsudi & Setiadai, 1997
Rhodococcus ruber NCIMB 40126, *Rhodococcus erythropolis* DSMZ 43060, *Rodococcus opacus* MR22	-	NB, Mineral Sait Medium + Glucose + 3HP	3HB, 3HV, 3HP	Füchtenbusch et al, 1998
Sphaerotilus natans LY2000	Activated sludge	Defined Media	3HB-co-3HV	Liu et al, 2002
Staphylococcus sp.	Sesame Oil Waste	Malt Waste	3HB	Wong et al, 2002
Staphylococcus sp.	Sesame Oil Waste	Soy Waste	3HB	Wong et al, 2002
Various	Activated sludge	Defined media	3HB-co-3HB	Hu et al, 1997; Satoh et al, 1998

2.5 Applications of PHAs

PHAs have been drawing as biocompatible plastics for a wide range of applications. Early investigations of PHA granules by electron microscopy after freeze-etching showed that the polymer in the granule underwent a cold drawing process indicating the plastic nature of polyester and suggesting that it can be processed as a conventional thermoplastic. They can be used as disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles and cups (Auras, 2004).

PHBV received European approval for food contact use in 1996. This opens opportunities in food service and packaging industry. This implied that one of PHAs practical applications is packaging films (for food packages), bags, containers and paper coatings.

In addition to its potential as plastic material PHAs is useful source of stereoregular compounds which can serve as chiral precursors for the chemical synthesis of optically active compounds, particularly in synthesis of some drugs or insect pheromones. These substances are biologically active only in the correct

stereochemical configeration.

PHAs can be easily depolymerised to a rich source of optically pure bifunctional hydroxy acids. PHB, for example can be readily hydrolysed to R-3-hydroxybutyric acid and is used in the synthesis of Merck's anti-glaucoma drug Truspot. Along with R-1,3-butanediol, it is also used to synthesise b-lactams.

Biocompatibility of PHAs coupled with its slow hydrolytic degradation leads to potential in reconstructive surgery. According to Lee (1996) the degradation product of PHB, D(-)-3-hydroxybutyrate has been detected in relatively large amount in human blood plasma. Therefore, it is highly plausible that implanting PHB in mammalian tissues would not be toxic. Recent studies have demonstrated the use of PHAs in the production of stents and in the tissue engineering of heart valves (Dvorin E.L. et al., 2003; Bunger C.M. et al., 2007; Mendelson, K., et al., 2007). PHAs have been confirmed by many researches as safe medical applications. This application includes surgical pins, sutures, staples, swabs, wound dressings, bone replacements/plates and blood vessel replacements. Also, PHAs can be applied as stimulation of bone growth by piezoelectric properties.

Besides reconstructive surgical application, PHAs can be used as biodegradable carriers for long term dosage of drugs, medicines, insecticides, herbicides, insecticides or fertilizers.

CHAPTER 3 METHDOLOGY

In this study, two systems for accumulating PHAs were set up in laboratory-scale and pilot-scale respectively. The data from the lab-scale experiments were used to be a reference for establish and operation of pilot-scale system. Sequencing batch reactor (SBR) systems were both used for the development of a system and operating procedures for the high production and internal storage of PHAs in laboratory-scale and pilot-scale study. The flow chart in Fig. 3.1 shows the study approach of this study.

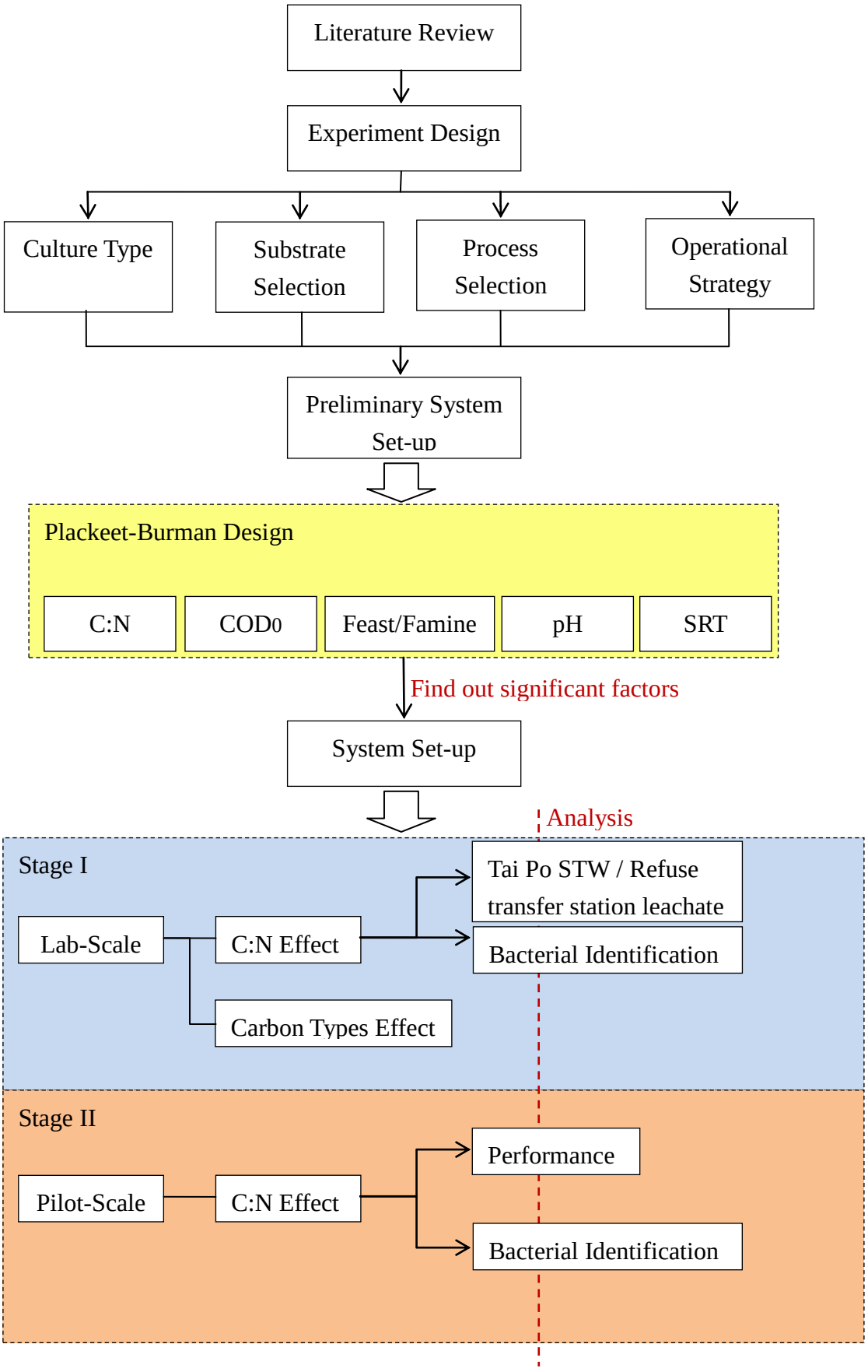


Figure 3.1 Scheme of the study

The lab-scale experiments were based on the comprehensive literature reviews and preliminary experiments results. Both laboratory-scale and pilot-scale reactors were set up with similar system configurations. The operation of the pilot-scale experiment was targeted to scale up that of the lab-scale experiment. The different operation conditions were tested for PHAs optimal value. Comparison of lab-scale and pilot-scale system performance took the real case fluctuations into considerations. This would also offer value for further application in PHAs commercial production. The findings generated from the pilot-scale experiment were used to predict cost of producing PHAs.

3.1 Design concepts of experiments

3.1.1 Selection of culture type – Using mixed culture

The PHAs cell content in activated sludge (70% of cell dry weight) is lower than the maximum value reported for pure cultures (Lee, 1997; Punrattanasim, 2001; Reddy et al.,2003). However, using mixed culture rather than pure culture to producing PHAs is feasible and efficient. Because mixed culture does not require additional and expensive step of environmental disinfection and longer selection time while pure culture does. It can also save cost of technology. Thus, there is no need for sterilization and sterile fermentation systems, which contributes to

the reduction of the final PHAs price. In addition, the molecular weights and melting point temperatures of PHAs produced by the mixed culture of activated sludge biomass were comparable to those obtained from pure cultures and have the potential to be used for commercial applications (Punrattanasim, 2001). In further stage II experiment, if using pure culture, the production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate, low polymer production, and the cost of maintaining a single strain environment.

3.1.2 Substrate selection – Activated sludge from secondary settlement tank

The next question and consideration for us was what material appropriate be a substrate to accumulate PHAs in this study. One of objectives in our study is to reduce cost of producing PHAs. Due to the large impact of the carbon source price on production costs, one of the most important approaches to reduce costs is to use wastes as raw material for the fermentation process. Activated sludge is a good substance as a cheap substrate to reduce production cost. The microbial communities in activated sludge can adapt well to the complex substrates present in the agroindustrial wastes (Salehizadeh and Van Loosdrecht, 2004). There is scientific evidence of a stable ecological function with diverse microbial populations in activated sludge (Kaewpipat and Grady Jr., 2001; Stamper et al.,

2003).

Seeding sludge obtained from return activated sludge (RAS) from secondary settlement tank was more suitable than other parts of wastewater treatment plant system. Because bacterial populations from RAS were under nutrient limitation conditions, they would get used to designed environment with nutrient deficiency soon.

3.1.3 Selection of process – Sequencing batch reactor (SBR) systems

SBR method is through the time of the arrangement, completed within a pond of water, reaction, sedimentation and drainage and a series of processes, which constitutes a cycle. This process volatile combination, coupled with a shorter application time, had not been summed up a complete set of design, control methods. Specifically, SBR has following advantages: (1) A flexible operation mode, due to reactions within the same reactor, working under different conditions; (2) Sludge inside SBR with high activity, the settlement good performance; (3) Processing equipment with less simple structure, easy operation and maintenance management. Therefore, SBR system is a suitable process choice for our project.

3.1.4 Operational strategy

The most well-studied strategy for enrichment of mixed cultures with PHAs production capability is based on alternating availability and unavailability of carbon substrate under aerobic conditions, so called “feast and famine” or “aerobic dynamic feeding” (ADF) conditions (Serafim et al., 2004; Dias et al., 2005; Dionisi et al., 2006).

The feast is the phase when microorganisms experience substrate excess, whereas the famine is the phase when no external substrate is present in the liquid medium. The feast and famine phases were easily recognized on the basis of dissolved oxygen profile: dissolved oxygen concentration was low during the feast phase, as a result of high bacterial metabolic activity, whereas it was high during the famine phase, as a resultof reduced metabolic activity (Dionisi et al., 2006).

Why choose conditions of “feast and famine” not “feast” only to stimulate PHAs growth? When exposed to an excess of external substrate, bacterium in activated sludge cannot grow at the maximal rate because of the internal limitation. But

bacterium can remove the substrate by storing it inside the cell. They store the substrate during the initial feast phase, and then use the stored polymer as energy and an internal carbon source in the famine phase. In this manner these bacterium can balance their growth rate and internal storage rate in dynamic processes (Van Loosdrecht, et al., 1997). Therefore, when they are cultured under conditions of alternating excess and lack of external substrates, storage is becoming more evident. Under these conditions, activated sludge is able to accumulate PHAs up to 50% of cell dry weight (Dionisi, 1995).

3.2 Preliminary study – Plackett-Burman Design

After discussion and analysis in Session 3.1., it concluded that producing PHAs by activated sludge in SBR under “feast/famine” condition was a cost-effective way. However, numbers of independent variables (factors) affect PHAs production. It might appear to vary all possible components and study separately each effect in turn. Such a procedure, however, was wasteful either of labour or accuracy, while to carry out a complete factorial experiment. So, if a preliminary test helped to find out some key factors, it would save a lot of time and avoid heavy work load. Plackett-Burman Design was a way to minimize the variance of the estimates of dependencies using a limited number of

experiments. In this part of study, two-level factorial design based on Plackett–Burman folded method was used to screen the most important parameters affecting the production of PHAs. Then the key parameters affecting producing PHAs were identified and used as impact factors in lab-scale study to seek an optimal values leading to the best storage response.

3.2.1 Introduction of Plackett-Burman Design

Plackett–Burman Designs are experimental designs presented in 1946 by R. L. Plackett and J. P. Burman. It was an economical and efficient design and commonly used in industrial designed experiments. This method did not require to carrying out a complete factorial experiment, and also obtained reliable results with limited number of experiments. Interactions between the factors were considered negligible.

Plackett-Burman Design based on Hadamard matrices and its process represented in matrix form. In a Plackett-Burman Design, main effects are, in general, heavily confounded with two-factor interactions. The Plackett-Burman design is a screening experiment that can run for up to (N-1) factors in an N-run design. If one is trying to estimate less than N parameters, then one simply uses a

subset of the columns of the matrix. In addition, Plackett-Burman designs are used when N is a multiple of 4 (i.e. N = 4, 8, 12, 16, 20, 24…). Thus, Plackett-Burman designs are for up to (4m-1) factors in 4m runs. The Plackett-Burman design in 12 runs, for example, may be used for an experiment containing up to 11 factors. In addition, Plackett-Burman design generating orthogonal matrices whose elements are all either 1 or -1, which present high level and low level for each factor. The appropriate high level and low level are usually determined by relevant reference.

A matrix in Plackett-Burman designs is organized by some rules. The first row of the basic cyclic design is given opposite N, the number of experiments. For any particular value of N, the appropriate row is selected and written down as the first row. A catalog of first row for several of the smaller designs is listed below (Table 3.1).

Table 3.1 A catalog of first row in Plackett Burman design for N = 8,12,16,20,24

N=8	+ + + - + - -
N=12	+ + - + + + - - - + -
N=16	+ + + + - + - + + - - + - - -
N=20	+ + - - + + + + - + - + - - - - + + -
N=24	+ + + + + - + - + + - - + + - - + - + - - - -

The remainder of the matrix is generated by shifting this row cyclically one place (N-2) times and then adding a final row of minus signs. The result will be a matrix which contains N rows and (N-1) columns. The Plackett-Burman design in 12 runs was taken as an example again to demonstrate the interaction (Table 3.2).

Table 3.2 Illustration of a Plackett–Burman design for 12 runs

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	+	+	−	+	+	+	−	−	−	+	−
2	−	+	+	−	+	+	+	−	−	−	+
3	+	−	+	+	−	+	+	+	−	−	−
4	−	+	−	+	+	−	+	+	+	−	−
5	−	−	+	−	+	+	−	+	+	+	−
6	−	−	−	+	−	+	+	−	+	+	+
7	+	−	−	−	+	−	+	+	−	+	+
8	+	+	−	−	−	+	−	+	+	−	+
9	+	+	+	−	−	−	+	−	+	+	−
10	−	+	+	+	−	−	−	+	−	+	+
11	+	−	+	+	+	−	−	−	+	−	+
12	−	−	−	−	−	−	−	−	−	−	−

3.2.2 Procedure

In the first step, a list of factors was enumerated and ordered in any way which makes sense. Then each parameter was selected an upper and lower value. These values should be the endpoints of a plausible range for the parameter. The ranges do not need to be identically sized, but their selection should be reasoned. So the

range could be reference from the culture conditions routinely used for activated sludge process. The analysis will indicate the effects of varying each parameter by the amount of the chosen range. In addition, index to measure factors was based on the main objective that was producing PHAs in a cost-effective way. Secondly, an appropriate pattern of Plackett-Burman Design needed a discussion. This number of runs of experiment was usually depended on how many variables to be investigated. If the number of variables is less than N, a so-called 'Dummy' variable which does not influence the outcome of the experiment will be introduced. After running the prescribed scenarios based on the matrix, results would be analysed by Minitab.

Flask culture was more easy in operation with different variables and would reduce cost and time of experiment. Therefore, a small scale lab, flask culture was selected in Plackett-Burman Design study. All Erlenmeyer flasks were pre-sterilized before used in culturation.

3.3 Laboratory-scale study

After completion of Plackett–Burman design test, factors to acclimatize activated sludge with high PHAs storage capacity were determined. The most significant parameters were C:N ratio. So C:N ratio was set to be a parameter of inoculating conditions in Stage I experiments to find out the PHAs accumulation in different C:N molar ratio. It is useful for regulation during PHAs production.

After finding the optimal C:N ratio for producing PHAs, bacterial identification would be taken for the best growth sample under such specific condition. Bacterial identification test helps to understand more about accumulating PHAs. Also, another bacterial identification test would be completed at the optimal condition for PHAs growth at pilot-scale. Comparative bacterial analysis between lab-scale and pilot-scale would be discussed at Chapter 7.

Another part of lab-scale study was about different carbon types resulting different PHAs composition. The monomeric composition of PHAs polymers can be influenced by several factors, including the organism producing the PHAs polymer, the carbon source on which cells are grown, how that carbon source is

metabolized in the cells, the types of monomer-supplying enzymes used, and the type of PHAs synthase used to synthesize the polymer (Sparks & Scholz, 2008; Lu et al., 2009). But they did not give details of which a carbon source or various carbon proportions will lead to which kind of compositions of PHAs. So details and growing process in cell are not for our study any more. We just focus on ‘input’ and ‘output’, which means different proportions of carbon materials considered as ‘input’, while resulting PHAs composition as ‘output’. Thus the aim of this part was to find out a relation between carbon source and PHAs composition.

3.3.1 Varing C:N ratio to optimize PHAs production in lab-scale

Plackett-Burman design test results suggest an important effect of different values of carbon-nitrogen (C:N) ratio on the development of the PHAs generation. To study this effect in more detail, we proposed to set-up a system.

This system was designed to enrich for microorganisms capable of producing PHAs. The activated sludge was first thickened by settling for at least 2 hours. Then 100 ml of the thickened activated sludge was transferred and cultivated in the lab-scale reactor. The reactor was operated in a mode of C:N ratio.

3.3.1.1 Raw material collection area and characteristics

The activated sludge used as a PHAs accumulator while wastewater samples as carbon source and nutrient substrate in this set of experiments were collected from two different organizations. They were Tai Po sewage treatment plant and Transfer Station leachage treatment plant. They were both municipal wastewater treatment plant.

The background of these two sewage treatment plants is described below:

(1) Background of Tai Po Sewage Treatment Works

Tai Po Sewage Treatment Works (Tai Po STW) is a major secondary sewage treatment works and located within the Tai Po Industrial Estate in Tai Po. It occupies 13 hectares of land and serves a population of 250,000 in Tai Po District, which produces 95,000 m^3 of sewage per day. It currently comprises four Stages: I, II, IVA, and IVB works. The Stage I/II Works and the Stage IV Works are operated independently for some treatment processes and otherwise for the others.

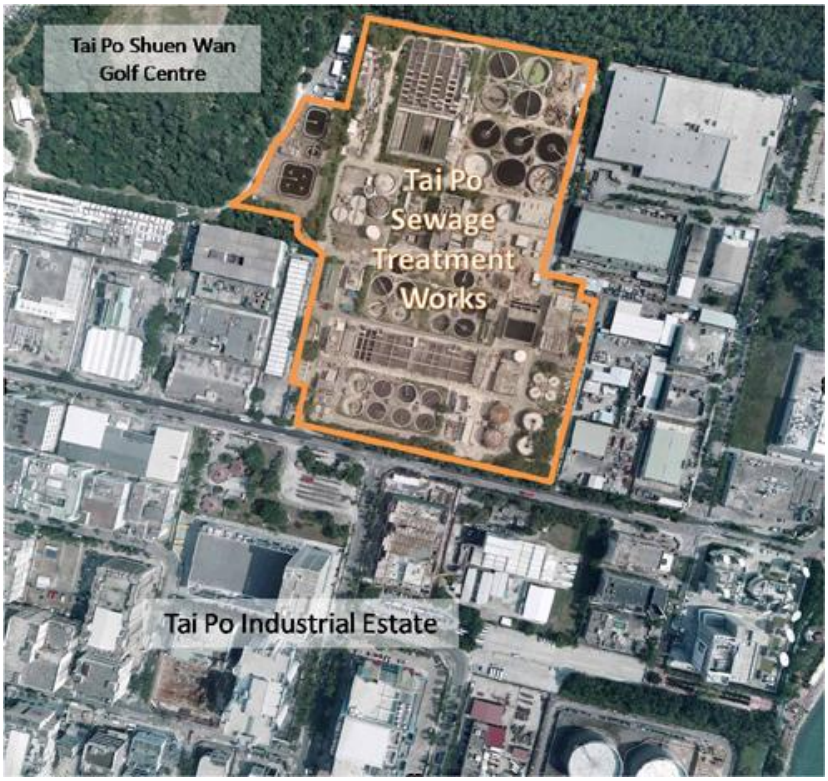


Figure 3.2 Location of Tai Po Sewage Treatment Works

To cope with the rapid development in the District and more stringent effluent discharge standards, The TPSTW - Stage V is upgrading the existing STW to provide additional sewage treatment capacity from the present daily average dry weather flow of 100,000 m^3/day to 120,000 m^3/day. This project will be completed in September 2013 to meet the demands of both the existing and future developments, as well as to meet the revised discharge license requirements. (Drainage Services Department, 2009). The characteristics of Tai Po STW were stated as following (Table 3.3).

Table 3.3 Tai Po Sewage Treatment Works characteristics

Parameter	**Raw Sewage Influent**	**Treat Effluent**
Biochemical Oxygen Demand (BOD) (mg/L)	196	5
Total Suspended Solids (TSS) (mg/L)	383	8
Nitrogen (mg/L)	49	8
***E. Coli* (counts/100ml)**	10^7	53,000

(2) Background of refuse transfer station leachate treatment plant

It has become difficult to establish refuse treatment facilities and disposal sites near urban areas in Hong Kong, resulting in calls for disposal at remote locations and long transport of refuse. The refuse transfer system in Hong Kong is illustrated in the figure (Figure 3.3). This refuse transfer system can establish a high efficiency in refuse collection and transport as shown in the flowchart (Figure 3.4). Refuse transfer station usually associated with leachate problem.

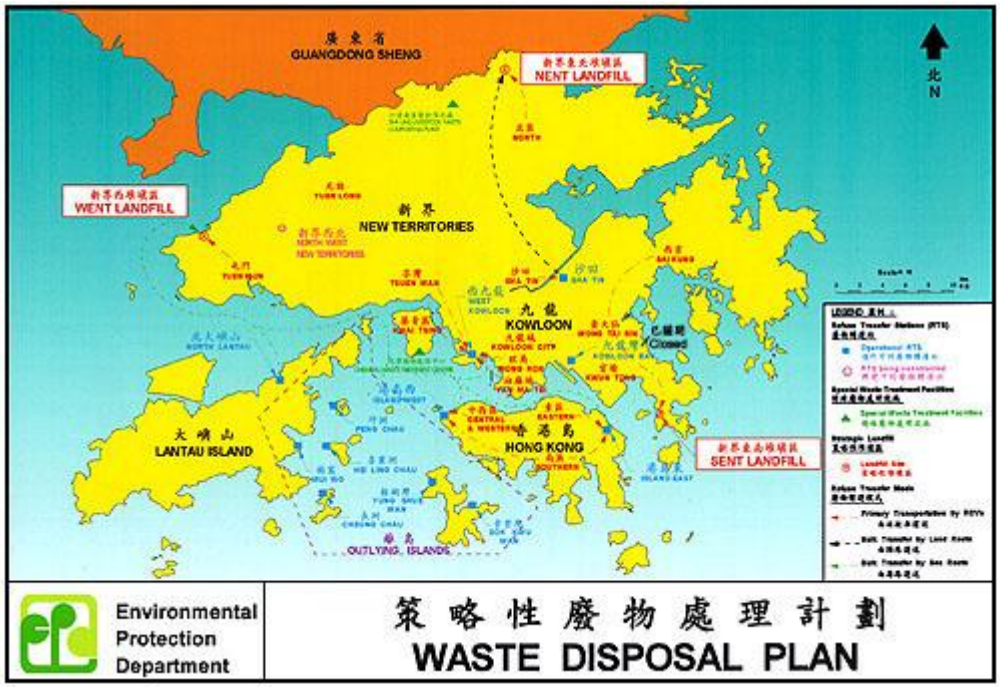


Figure 3.3 Hong Kong's refuse transfer system

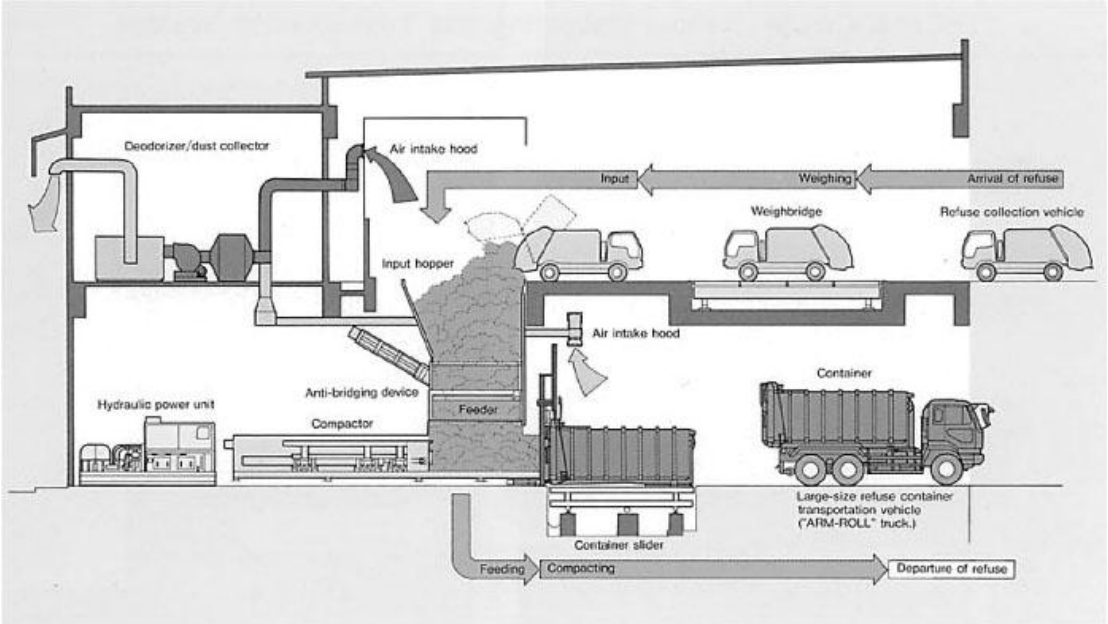


Figure 3.4 The refuse handling flowchart

In order to better understand the performances of two samples, a comparison of

their background information was conducted first. The properties of these two sewage treatment plants are shown in Table 3.4.

Table 3.4 Properties of two sewage treatment plants

Location	**Chemical Oxygen Demand (COD) (mg/L)**	**Total Kjeldahl Nitrogen (TKN) (mg/L)**
Tai Po water treatment plant	386	99
Refuse Transfer Station leachate treatment plant	13868	132

3.3.1.2 Reactor system set-up under different C:N ratio

Activated sludge from a laboratory-scale reactor was inoculated into an SBR reactor with 1000 ml of working volume. Two reactors with activated sludge and wastewater from Tai Po STW and leachate were operated under the same or similar conditions: with the same hydraulic retention times and the same sludge retention times. Activated sludge was cultured under aerobic condition for 5 days and consumed all original substrate before test started. Then each SBR was operated with aerobic dynamic substrate feeding in which a short period of substrate feeding alternates with a long period of absence of substrate. Program was designed as follow. Each SBR cycle consisted of a fixed ‘‘famine’’ phase of 3.75 h (3 hr 45 min), a ‘‘feast’’ phase 0.75 h, 1 h of settling (agitation and air

bubbling switched off), and 15min withdrawing half of the volume, which was replaced with fresh medium (wastewater sample) during the first 15 min at the beginning of the next cycle. The total cycle duration was 6 h, the reaction time was 4. 5 h (4h 30 min) and the hydraulic retention time (HRT) was 1 day. At the end of the aerobic period, before settling, a defined volume of biomass was removed to keep a sludge retention time (SRT) of 10 days. Because the organic loading rate (OLR) equal to or less than 20 gCOD/L/day the selected biomass showed a transient response that was mainly due to storage, whereas at OLR equal to or higher than 25 gCOD/L/day biomass response was mainly due to growth, with little or no contribution of storage (Dionisi et al., 2006). This means that biomass was unstable at the intermediate OLR from 20 to 25 gCOD/L/day, with a shift from storage to growth response. So the value of OLR was fixed at 20 gCOD/L/day in this study. Oxygen was supplied by an air compressor through a ceramic membrane disperser inside the reactor. During “feast and famine” time, SBR was maintained under air bubbling oxygen concentrations in the range 2-3 mg/L. Timers controlled the stirring, the air pump, and the pumps for medium feed and removal. The reactor was operated without pH control. the temperature was kept at 22℃.

The C:N ratios are blank, 20:1, 40:1, 60:1, 80:1, 100:1, 120:1, 140:1, 160:1, 180 and 200:1 for each sample (Tai Po Sewage Treatment and Refuse Transfer Station Leachate Treatment Plant).

Glucose was used as a carbon source and ammonium chloride as a nitrogen source to adjust C:N ratio for investigation of optimal operation condition. The characteristic of the glucose and ammonium chloride was shown in the following table (Table 3.5).

Table 3.5 Characteristics of substrates

	Glucose	Ammonium Chloride
Other Names	Glc, CHO	-
Molecular Formula	$C_6H_{12}O_6$	NH_4Cl
Molar Mass (g/mol)	180.16	53.49
Appearance	White deliquescent powder	White powder

3.3.2 Identification of dominating strains in microbial communities

Bacterial identification test gives information on the microbial strains dominating in a complex microbial consortium. These tests were performed in order to better

investigate the production of PHAs. In this part of study, we isolated and identified bacteria from mix liquid sample. There are several ways to test bacterium. The MIDI Instant FAME^{TM} Sherlock Microbial Identification System was used. This technique identifies microorganisms based on the unique fatty acid pattern of each strain. The fatty acid profile is compared against extensive databases which currently contains over 2000 species, including aerobic bacteria, anaerobic bacteria and yeasts.

3.3.2.1 Isolation the bacterial colonies

Since activated sludge sample was a complex microbial communities of mixed populations, isolation of individual colonies works were required first. The streak plate technique was used to isolate pure bacterial colonies from a mixed population of bacteria.

According to the standard procedure, all bacterial culture should be growth on Trypticase Soy Agar (TSA) for 24 hours before protein extraction process. TSA plates were Tryptic Soy Broth mixed with 15% agar, heated to sterilize media and melt agar then poured into a Petri dish. Aseptically transfer the contents of the applicator to a small section of the plate (approx. 10% of total plate surface

area) with a sterile loop of wire (Figure 3.5), then turn the plate 90 degrees and sample some bacteria from the initial streak and drag the loop across the surface of TSA in a zig-zag fashion. Then turn the plate another 90 degrees and sample some bacteria from the second streak and isolate as above again. Finally turn the plate another 90 degrees and sample some of the bacteria from the third streak. TSA plates were incubate upside down for 24 hours at 30℃.

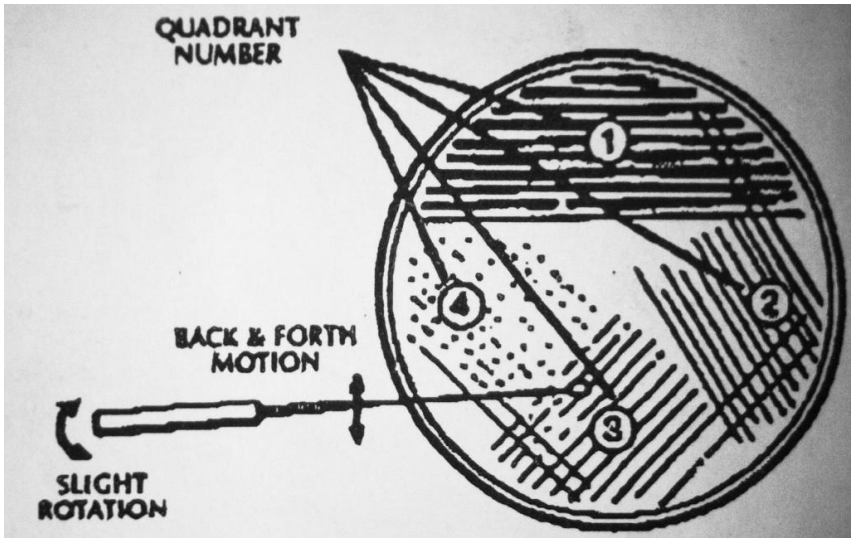


Figure 3.5 Quadrant streak plate techniques to isolate individual bacterial colonies

To ensure bacteria isolated successfully, TSA plate with more than 30 individual colonies would be repeated to do streaking till with less than 30 individual colonies.

3.3.2.2 MIDI test

This robust methodology is an excellent technique for comparison and identification of a wide range of isolates, both Gram positive and Gram negative. Compared to other microbial identification systems, MIDI technique has more superiors and benefits. Its sample preparation and analysis time is rapid. The identification process takes less than 15 minutes from pure culture to identification, while other phenotypic identification systems require 4-24 hours. Its procedure used less than 3 mg of cells, compared to 40 mg of cells needed with other identification systems like biochemical test system. In addition, MIDI system has a large database of environmental organisms and can identify over 2,000 bacterial and yeast species.

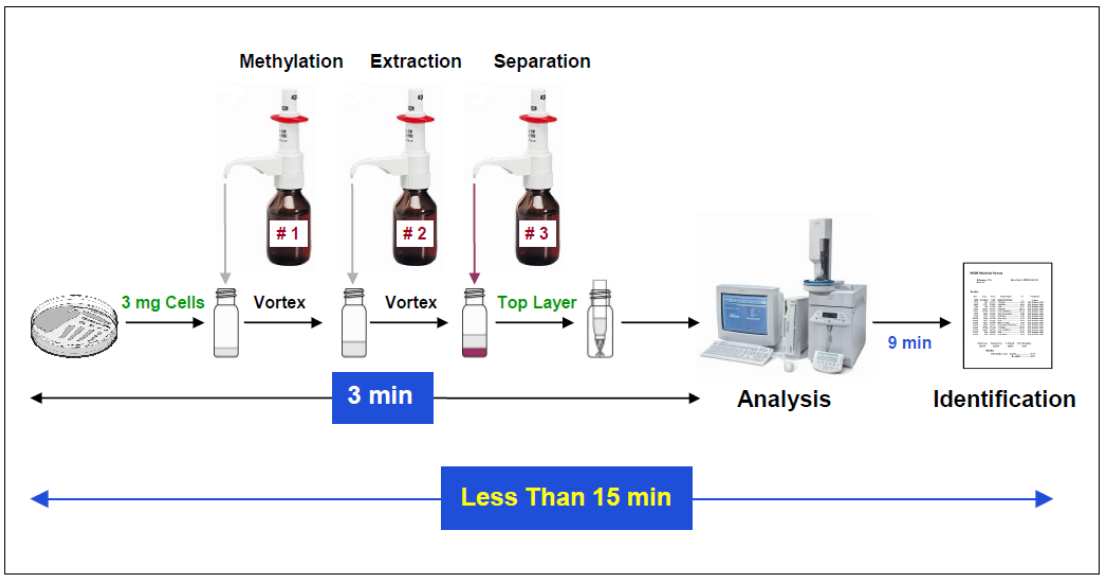


Figure 3.6 The procedure of MIDI test

3.3.3 Batch experiment to investigate relationship between carbon source and PHAs composition

Another batch experiment was to figure out how different carbon source affect PHAs composition. What is the reason to do this part research? Cause different combinations of PHAs monomers yield different polymer properties, such as the mechanical, physical and thermal properties. This result will allow for better processing on commercial applications.

Common precursors to PHAs synthesis include simple sugars such as glucose and fructose, and organic acids such as acetic and propionic acid. This group of experiment aimed to discuss whether type and proportion of carbon substrate regulate the polymeric structure of the PHAs.

In order to ensure a single carbon source in one batch experiment, synthetic wastewater was feed in the third part of Stage I study. The relative amounts of acetic, propionic acids and glucose were calculated on a COD basis. SBR operating condition referred to an optimal condition to produce PHAs in Stage I

part one. Thus C:N ratio was adjusted to the value which results maximum PHAs production and kept the same for all running in this part of study. In each batch, 10 g polymer-free activated sludge was washed with distilled water to remove any residual nitrogenous matters before inoculated in SBR. The SBR feed also contained the mineral medium with the following composition: 4000 mg/L $(NH_4)_2SO_4$, 500 mg/L K_2HPO_4, 385 mg/L KH_2PO_4, 50 mg/L $CaCl_2 \cdot 2H_2O$, 100 mg/L $MgSO_4 \cdot 7H_2O$, 2 mg/L $FeCl_3 \cdot 6H_2O$, 4 mg/L $Na_2SiO_3 \cdot 6H_2O$, 0.1 mg/L $ZnSO_4 \cdot 7H_2O$, 0.03 mg/L $MnCl_2 \cdot 4H_2O$, 0.3 mg/L H_3BO_3, 0.2 mg/L $CoCl_2 \cdot 6H_2O$, 0.02 mg/L $NiCl_2 \cdot 6H_2O$, 0.01 mg/L $CuCl_2 \cdot 2H_2O$, 0.03 mg/L $NaMoO_4 \cdot 2H_2O$. 20 mg/L thiourea was also added to inhibit nitrification.

Three sets of SBR were proceeding in different carbon weight ratio in the medium for this part study. In the first set, butyric acid (C_4) to valeric acid (C_5) as carbon sources were respectively adjusted to 100:0, 75:25, 50:50, 25:75 and 0:100 (g/g). In the second set, butyric acid (C_4) to glucose (C_6) weight ratios were from 100:0, 75:25, 50:50, 25:75 to 0:100 (g/g) decreasing progressively. In the last set of experiment, glucose and fructose were used as carbon source. They have the same molecular formula ($C_6H_{12}O_6$) but they differ structurally. Their weight ratios were varied as 100:0, 75:25, 50:50, 25:75 and 0:100 (g/g).

3.3.4 Sampling and analytical methods in Stage I

3.3.4.1 Sampling

During the lab-scale SBR running, the mixed liquor of each C:N ratio in the batch reactor was sampled at regular intervals for analytical determinations. The measurements included residual COD, residual TKN, DO, the overall biomass and polymer accumulation.

In the second part of Stage I, accumulator having the maximum yield of PHAs under certain C:N ratio condition was picked up for MIDI test. On account of a long time and huge work to isolate pure colonies from activated sludge, as well as high cost of MIDI test, only sludge obtaining the maximum yield of PHAs was selected for bacterial identification.

The culture broth in three sets of experiment in Stage I part three were periodically sampled for analysis.

3.3.4.2 Analytical techniques

Analytical techniques used in Stage I study were all according to American Public Health Association (APHA), American Water Works Association (AWWA) and the Water Environment Federation (WEF) Standard Method for the Examination of Water and Wastewater 2005.

The dry weight of the overall biomass was measured as mixed-liquor volatile suspended solids (MLVSS) technique (Standard code: 2540D). The testing of COD was according to APHA (2005) stand method (Standard code: 5220C) for water analysis. BOD and COD ratio is around 0.5 ($\pm$0.05) in this project. Hence, the COD is used to represent the BOD in this experiment. Sample from Tai Po STW and Refuse transfer station leachate treatment plant were examined total kjeldahl nitrogen (TKN) (Standard code: 4500-Norg). The TKN was determined by Tecator Autoanalyzer (KJELTEC AUTO 1030 ANALYZER). Sample was centrifuge first, and then only the supernatant was taken for TKN test. In addition, dissolved oxygen (DO) and pH were tested (Standard code: 2540D).

For PHAs determination, 50 ml of sludge were centrifuged under 3000 rpm for

20 min after sampling. The supernatant was frozen over night to make sure no liquor exists. The frozen obtained was lyophilized in free dryer system (Figure 3.7). Afterwards 20mg lyophilized sample was resuspended in 2 ml acidic methanol (20% H_2SO_4) with 0.65 mg/ml of methyl benzoate (as internal standard). To this mixture 2 ml of chloroform was added and the solution was kept in a thermoblock at 100℃ for 3.5 h. After cooling, 0.5 ml of distilled water was used for extraction. The chloroform phase was collected in another Pyrex tube (volume, 10ml) and molecular sieves (0.3 nm) were added for water adsorption. 1 μl of the chloroform phase obtained was injected on column into a Gas Chromatograph (GC), equipped with a programmable auto-sampler VARIAN CP-8400 (Figure 3.8) and a 30 m × 0.25 mm Factor Four™ capillary column. Nitrogen was used as the carrier gas. The temperature of injection port was set to 260℃, and the flame ionization detector port was set to 220℃. The temperature program used was: 1 min at 40℃, rise of 30℃/min to 50℃; after 2 min of program start the oven rise was 8℃/min to 160℃, and kept for 1 min at 160℃. The injection port temperature follows the oven temperature program. A calibration curve was obtained by injecting standards of HB previously submitted to the procedure described for reactor samples. DL-3-Hydroxybutyric acid sodium salt and a polymer P(HB-co-HV) of known HV content were dissolved in

acidified methanol as the standards.

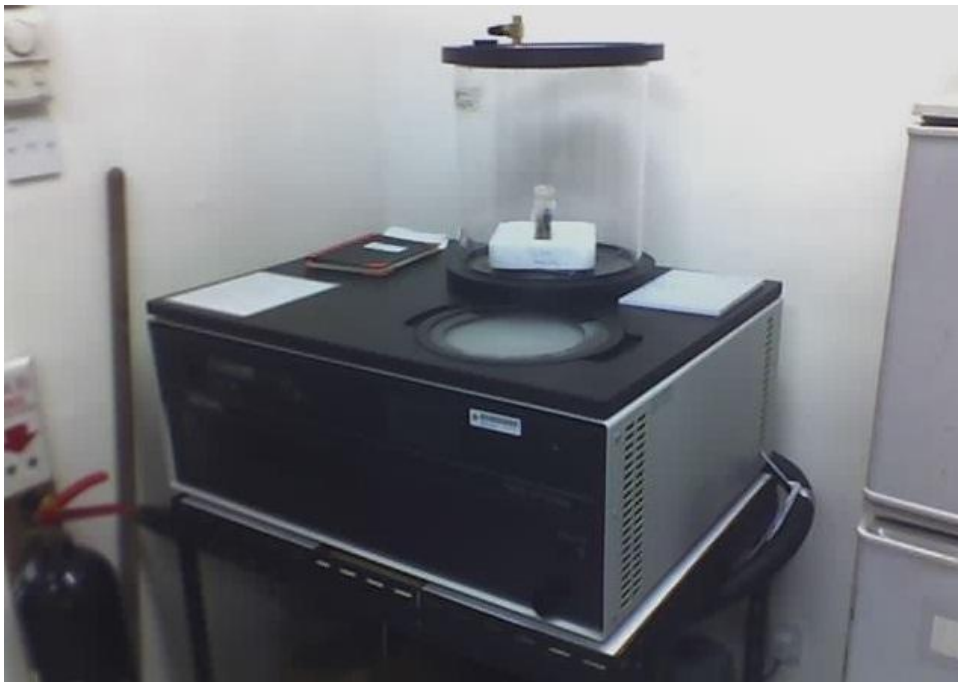


Figure 3.7 The free dryer system

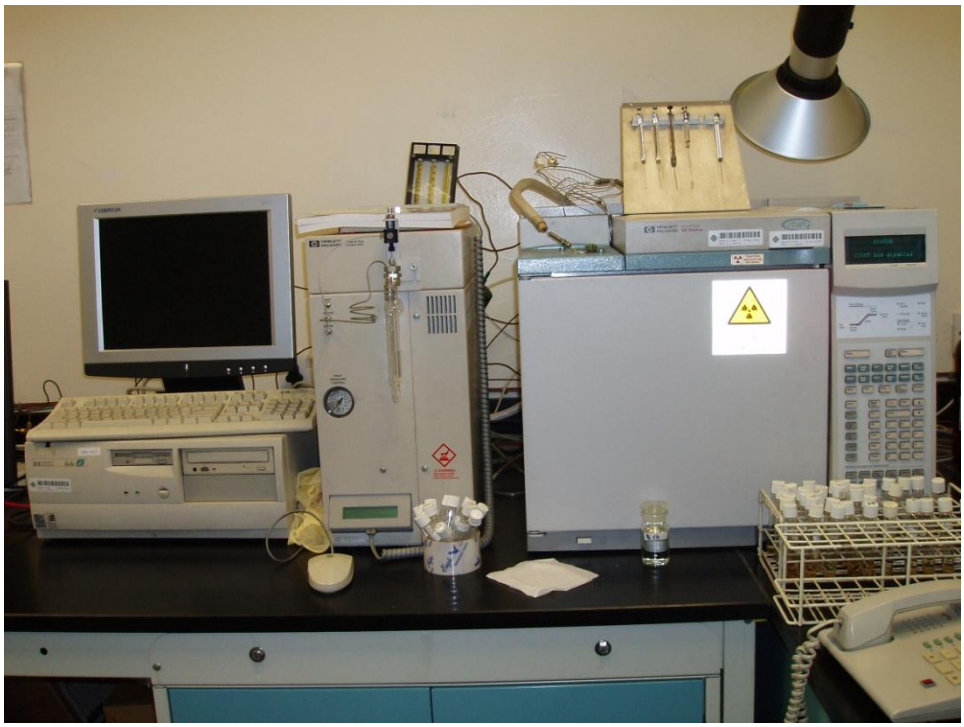


Figure 3.8 Gas Chromatograph equipped with a programmable VARIAN

CP-8400

3.4 Pilot-scale study

3.4.1 Background information of pilot-scale study wastewater treatment plant

The pilot-scale study was conducted in a dyeing wastewater treatment plant located in Xintang town, Zhengcheng, Guangdong Province, Mainland China. Xintang town is the main manufactory of denim and jeans in the world. This dyeing wastewater treatment plant is situated in one of Industrial Gardens of Environmental Protection in Xingtang.

The process used in this dyeing treatment plant is CEAO (China Ever Anaerobic Oxidation) technology, which is developed by ChinaEver Inc. (Du L. et al, 2007). This process, which consists of physical-chemical tank and A/O process, is suitable for treating medium concentration dyeing water. The A/O is the major treating unit, which is low in power consumption (Zhang J., 2007). The whole of the process is presented in the Figure 3.9 and the overall print of the plant is shown in Figure 3.10.

Figure 3.9a The process diagram of the Purification Plant of Water Quality in Xinzhou Industrial Garden of Environmental Protection

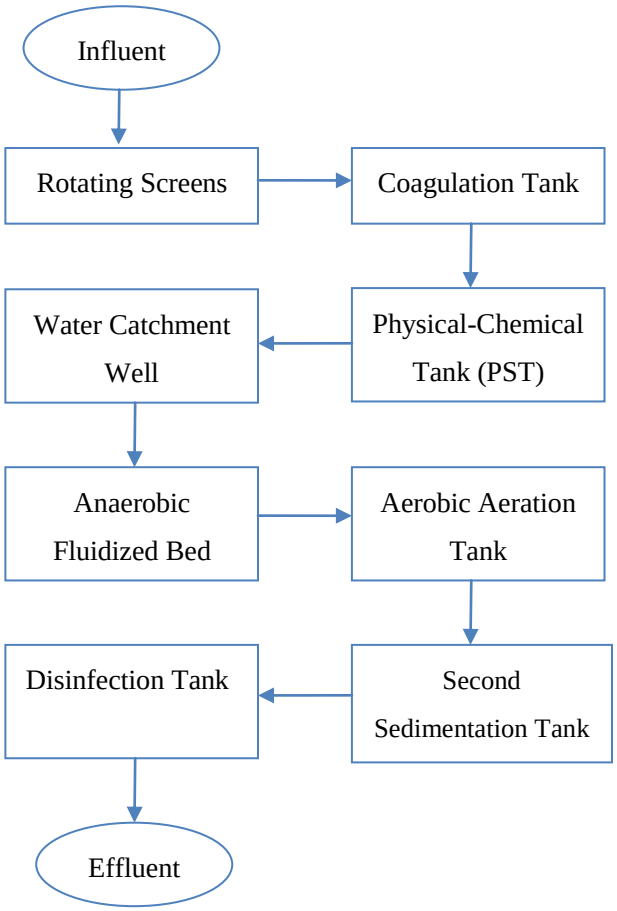


Figure 3.9b The process diagram of the Purification Plant of Water Quality in Xinzhou Industrial Garden of Environmental Protection

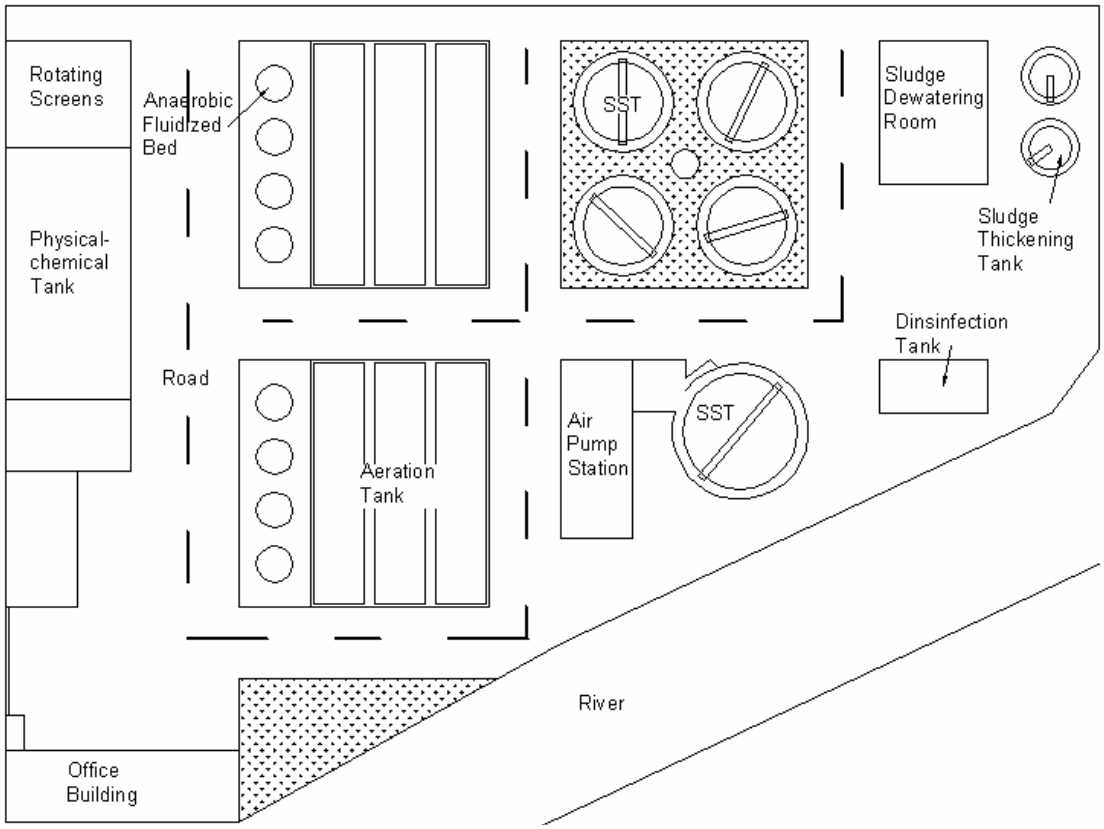


Figure 3.10 The overall print of the Purification Plant of Water Quality in Xinzhou Industrial Garden of Environmental Protection

The plant treats the wastewater from jeans, tatting, knitting and yarn dyeing factories with the total capacity of 50 kilo tons per day. The influent and effluent properties are shown in Table 3.6.

Table 3.6 Characteristics of influent and effluent in Purification Plant of Water Quality in Xinzhou Industrial Garden of Environmental Protection

	COD (mg/L)	SS (mg/L)	BOD (mg/L)	pH	Chroma (Hazen)
Influent	600-1200	580-880	177-400	9.5-11	355-530
Effluent	⩽80	⩽70	⩽20	6-9	⩽40

3.4.2 Design of pilot-scale PHAs production bioreactor

In Stage II study, the return activated sludge (RAS) was used to start up to the SBR system. And the dyeing wastewater will be feed to the SBR system as the substance. Why not used raw wastewater directly as the substance? Because the raw wastewater contained lots of unbiodegradable materials like the residue fibre of textile and the BOD/COD ratio was low. Therefore, the wastewater fed from the effluent of the anaerobic fluidized bed was more suitable to the SBR system operation.

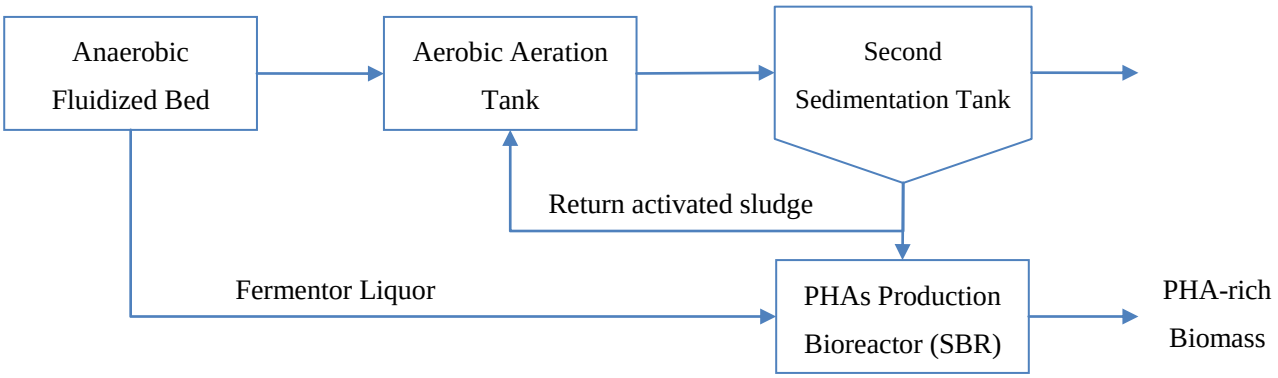


Figure 3.11 The diagram of feeding for SBR system

With these considerations, the PHAs production SBR was decided to be constructed near the 5# SST (Second Sedimentation Tank). The place had a suitable area to set up the system. Simultaneously, the location was not so far away from the anaerobic fluidized bed effluent (Figure 3.12).

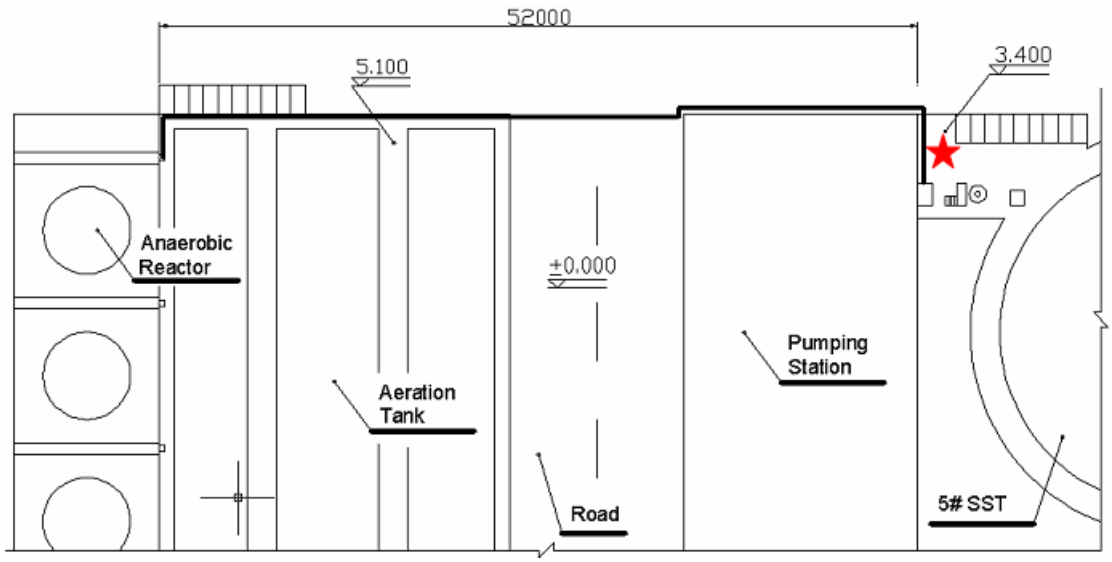


Figure 3.12 Location of pilot-scale SBR system in Purification Plant of Water Quality in Xinzhou Industrial Garden of Environmental Protection

The PHAs production SBR system consists of six sub-systems:

(1) The suction and equalization system

This part was to transfer the wastewater from the anaerobic reactors to the storage tank. This system was controlled by a floatable water level switch (FWLS) in the storage tank (Figure 3.13). When the water level of the storage tank was below certain level, the submersible sewage pump in the top of the anaerobic fluidized bed would turn on to supply wastewater for the storage tank.

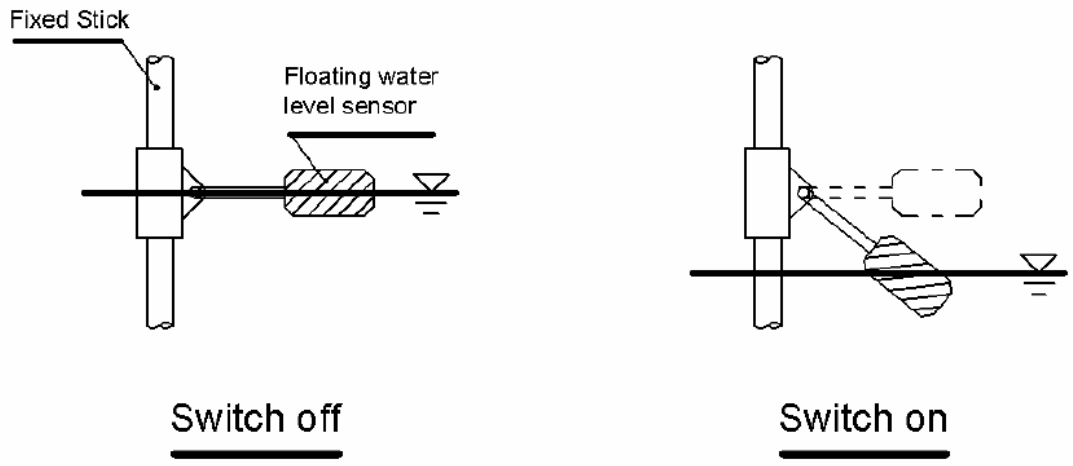


Figure 3.13 A floatable water level in the SBR system

(2) The filling system

This function was the input of raw wastewater as carbon source feeding the reaction tank. This system was controlled by WLS (Figure 3.14) in the reaction tank and the time switch (TS).

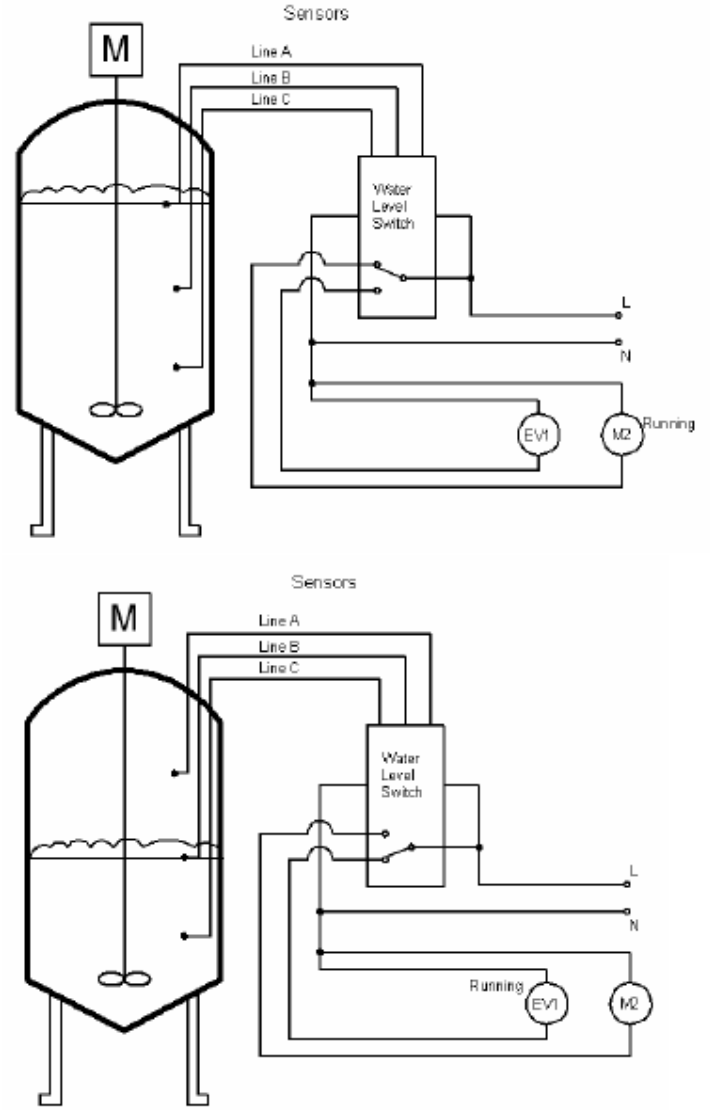


Figure 3.14 The schematic diagram of SBR system

(3) The reaction and stirring system

This part was responsible for supply the air for the reaction under ADF condition. And it also guaranteed the reaction under complete mixing conditions was. This system was controlled by the TS.

(4) The decanting system

This part was in charge of carefully pouring the supernatant after the settling procedure. The system was controlled by WLS (water level switch) in the bioreactor and the TS.

(5) The EAS discharging system

Drainage of the excessive sludge was performed after settling. This system was controlled by WLS in the reaction tank and the TS.

(6) The dosing system

Feed the reactor with necessary dosage under purpose. This system was controlled by the TS. The dosing system consisted of a plastic dosing tank with 150L volume a mid-storage tank and a dosing pump. The plastic dosing tank was filled with glucose at certain concentration. The solution would be fed to the midway storage tank. At the pre-set dosing time, the dosing pump transfered the fixed volume of the solution in the mid-storage tank to the reactor. The concentration in the dosing tank was calculated by considering the residue COD concentration in the reactor and COD concentration in the influent. The overall print of the SBR system was shown as following.

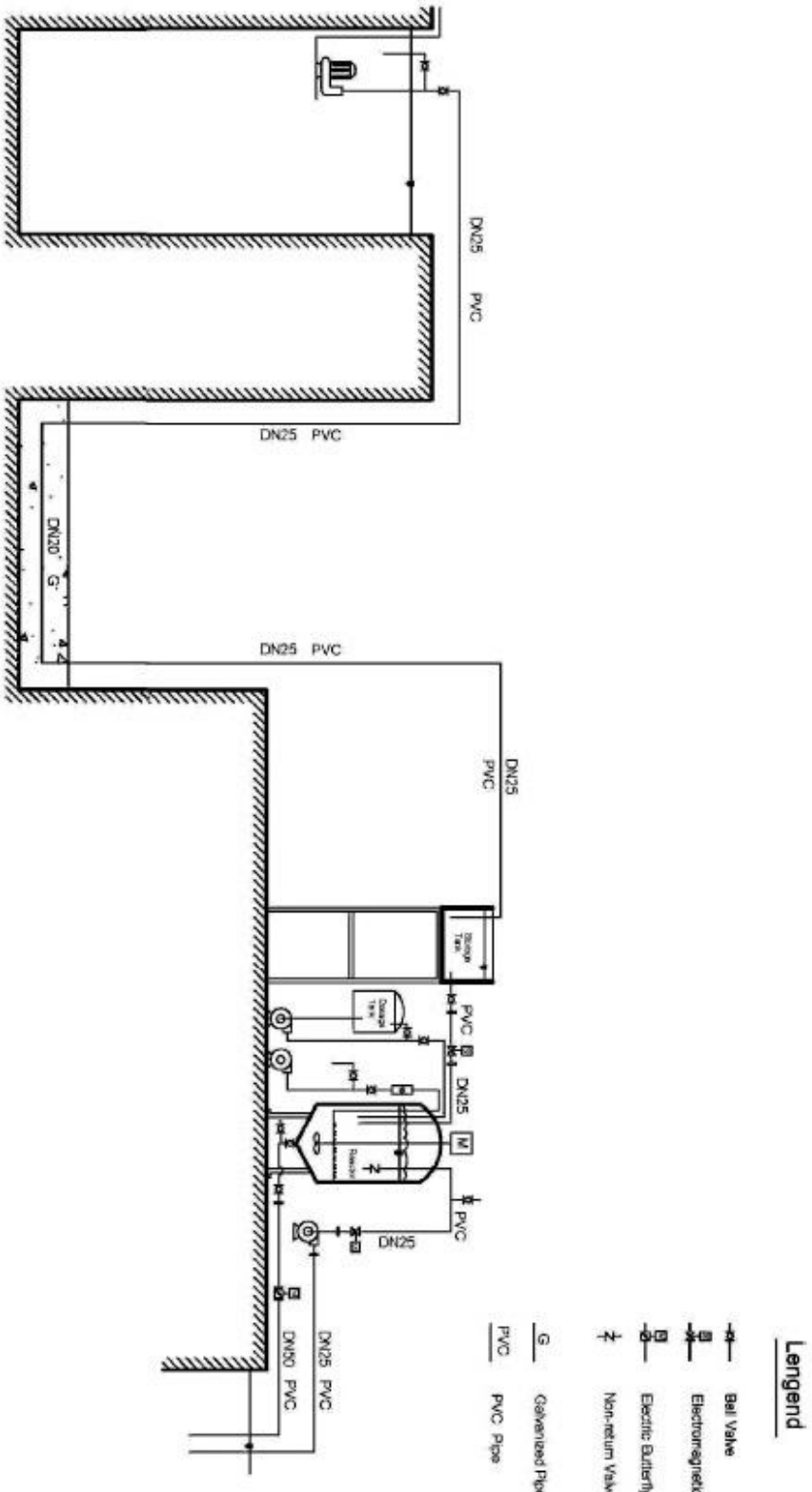


Figure 3.15 The overall print of the SBR system

3.4.3 Sampling and analytical methods in Stage II

The sludge in the pilot-scale SBR was sampled at regular intervals for analytical determinations of the substrates and storage compounds. The measurements included the overall biomass (at the end of cycle) and polymer concentration (at the end of cycle and at substrate depletion). Sampling method of pilot-scale system was the same as previous lab-scale run. DO was determined on site. COD, TKN, overall biomass weight and PHAs content examination were carried out in lab on campus. It usually spent about 3 hr from the site to the lab.

3.4.4 Bacterial identification

In order to identify potential members of microbial communities in pilot-scale SBR, MIDI test was conducted.

3.4.5 Statistical analysis

To better interpret the obtained data, statistical analyses were conducted. Two-sample t-test statistics was adopted to assess the variation among different sets of experiment. The association between each two metal elements was assessed by calculating the Pearson correlation coefficient. In this study, Minitab 15 was utilized to perform all the statistical analyses.

CHAPTER 4 PRELIMINARY RESULTS USING PLACKETT-BURMAN DESIGN METHOD

Though activated sludge is a good substance as a cheap substrate to produce PHAs, there is a long list of factors that may affect its producing process and low yield rate of PHAs is gained without acclimatization. In order to distinguish the key variables for SBR performance to increase producing PHAs by activated sludge, Plackett-Burman Design experiments were conducted to screen parameters before laboratory-scale and pilot-scale study. The technique, Plackett-Burman Design, is adequate to provide information about the factor that really affect the process when an experimenter is faced with a modest number of factors.

4.1 Variables and index

4.1.1 Variables

To accumulate PHAs in a cost-effect way, bacteria in activated sludge was used

as a candidated. The growth of bacteria is a complex process. In order to find out the most important variables that result in the production of PHAs by activated sludge wastewater treatment process, any proposed factor affecting the accumulative procedure was listed first. Biosynthesis of PHAs is stimulated in cases of imbalanced substrate supplies. Under macronutrient limitation, overflow metabolism is induced, which is thought to be part of a survival strategy (Babel, 1992). Most bacteria accumulate PHAs when their growth is limited by an essential nutrient, such as N, P, Mg, K, O, or S in the presence of an excessive carbon supply. In particular, nitrogen limitation is considered a factor that affects PHAs accumulation, cell dry weight, and polymer content during PHAs production (Chua, 1999). So C:N ratio was proposed in the investigation. In wastewater treatment process, one usually use of C:N:P ratio is between 100:20:1 and 100:5:1 for ideal biological growth (Russell, 2006). It reflects the minimal N and P requirements for healthy heterotrophic growth. Therefore, in the test, C:N ratio was set as 50:1 in high level while 20:1 in low level.

Second, providing a supply of oxygen was important during the wastewater treatment process. The oxygen supports bacterial growth which breaks down the organic. However, excess oxygen condition does not benefit the PHAs

accumulation in activated sludge. At the lower dissolved oxygen (DO) supply rates, a higher proportion of the substrate was preserved as PHAs than at higher DO supply rates. At low DO concentrations, the limited availability of adenosine triphosphate (ATP) prevented significant biomass growth and most ATP was used for acetate transport into the cell. In contrast, high DO supply rates provided surplus ATP and hence higher growth rates, resulting in decreased PHAs yields (Third et al., 2003). Therefore, oxygen management is crucial to conserving reducing power, as excessive aeration rates decrease the PHAs yield and allow higher biomass growth. So oxygen concentration, such a traditional parameter in wastewater treatment process, should be considered in PHAs accumulation. The preferred oxygen concentration in wastewater treatment reactor system was around 7-8 mg/L that would be input as high level in Plackett-Burman Design. While lower DO concentration was adjusted in 2-3 mg/L due to lower DO concentrations (0.5–2.0 mg/L) produced sludge with poorer settling properties. Aeration was automatically controlled by DO controller DOCN601 to keep the DO value in the desired range.

Third, a operational strategy, “feast and famine” which enriched PHAs production, is selected in our study. However, feast/famine (FE/FA) ratio may

affect the PHA storage performance. This suggested to take a part in Plackett-Burman Design to confirm such opinion and find out its suitable phase ratio of FE/FA in producing PHAs. A small feast to famine ratio (1/5) was designed as low level and a larger FE/FA ratio (1/3) was designed as high level during the screening.

In wastewater treatment process, the range of pH value is usually between 6.0 and 8.5. According to this, in order to gain more insights of pH effect, pH 6.5-7.0 and pH 7.5-8.0 were desinged as low level and high level, respectively.

In SBR system, sludge retantion time (SRT) is a significant factor affecting the mixed liquor suspended solids and microbial characteristics. The SRT might have affected the PHA accumulation capability of activated sludge. To investigate PHAs production behaviors, the operating SRT was varied notable with 4 days and 10 days. SRT with 4 days was considered as low level while 10 days was considered as high levels.

A total of five components [variables, k = 5] including air bubbling oxygen concentration, C:N ratio, Feast:Famine ratio, pH and SRT, were selected for the

study with each variable represented at two levels, high concentration (+) and low concentration (–) . Table 4.1 illustrates the variables and their corresponding levels used in the experimental design.

Table 4.1 Experimental field definition for the Plackett-Burman Design

Term	Variables	High level (+1)	Low level (–1)
X_1	air bubbling oxygen concentrations (mg/L)	7-8	2-3
X_2	C:N ratio	50:1	20:1
X_3	Feast/Famine (h/h)	1/3	1/5
X_4	pH	7.5-8.0	6.5-7.0
X_5	SRT (d)	10	4

4.1.2 Index

In order to estimate the efficiency of PHAs production, which explained the higher performance in terms of PHAs storage, two key indexs needed to be introduced. One was net growth of PHAs, when in high value it impled the greater yield of PHAs. The other one index was content of substrate consumed, because carbon source cost contributed an important part of bio-plastic cost.

Economic and efficient PHAs production due to more PHAs accumulation by using less substrate. So low value of the second index suggested that the PHAs storage behavior was strong, which was in contrast with the first index. Then in the analysis process, the significance (p-value) of the effect of individual variable on these two index were estimated by t-test.

4.2 Appropriate pattern chosen in this Plackett-Burman design

Plackett-Burman design helps to find out which variables exert a significant influence on the output and which ones are inconsequential. It contains patterns for all designs up to 100, except for 92 (Baumert et al., 1962). So an appropriate pattern should be decided first. There were five variables stated previously. With an N-Run Plackett-Burman design, we can look at up to (N-1) factors. The 8 was the most closed and the next larger multiple of four over five, so N=8 patten was chosen (Table 4.2), which requires running the fewest sensitivity analysis of various scenarios. In fact, only five factors to vary, how to complete such patten of a Plackett-Burman design? The solution was to introduce a so-called 'Dummy' variable which does not influence the outcome of the experiment. The main effect of a dummy variable should be zero. The main effect of a dummy variable is a measure for the error in estimating main effects. Table 4.3 illustrates the full

Plackett-Burman design with 5 variabls and 2 dummy factors. Each row represents a trial run and each column represents an independent (assigned) or dummy (unassigned) variable.

Table 4.2 Patten of 8-run in Plackett-Burman Design

N=8	+ + + – + – –

Table 4.3 Experimental field definition for the 8-run matrix in Plackett-Burman Design

Term	Variables	High level (+1)	Low level (–1)
X_1	air bubbling oxygen concentrations (mg/L)	7-8	2-3
X_2	C:N ratio	50:1	20:1
X_3	Feast/Famine (h/h)	1/3	1/5
X_4	pH	7.5-8.0	6.5-7.0
X_5	SRT (d)	10	4
X_6	D_1	–	–
X_7	D_2	–	–

* Dummy 1 and Dummy 2 was expressed as D_1 and D_2.

In conclusion, a 8-run Plackett-Burman design was applied to evaluate five factors (including two dummy variables). Each variable was examined at two levels: –1 for the low level and +1 for the high level.

4.3 Screening results

During this part of study, flask culture was used for determination of the optimum conditions for the fermentation process. All Erlenmeyer flasks were pre-sterilized then used in cell culture in this preliminary study.

Table 4.4 represented the Plackett-Burman experimental design for 8 trials with two levels of concentrations for each variable.Each row of the Plackett-Burman matrix specified a distinct scenario of the model. Each column was associated with a particular factor/component. Column 1 was assigned to the first item in the established parameter list, Column 2 the second, etc.

Table 4.4 Illustration of a Plackett–Burman design for 8 runs

Run	X_1	X_2	X_3	X_4	X_5	D_1	D_2
1	+	+	+	−	+	−	−
2	−	+	+	+	−	+	−
3	−	−	+	+	+	−	+
4	+	−	−	+	+	+	−
5	−	+	−	−	+	+	+
6	+	−	+	−	−	+	+
7	+	+	−	+	−	−	+
8	−	−	−	−	−	−	−

Each scenario (an assemblage of upper and lower values for the parameters) will produce output (target quantities of index). Minitab Version 15.0 was used for regression and graphical analysis of the experimental data obtained. Analysis of variance (ANOVA) was conducted to determine the significance of regression coefficients. The significance of regression coefficients was tested by t-test. The t-test for any individual effect showed an evaluation of the probability of finding the observed effect. A p-value was a measure of how much evidence one has against the null hypothesis. A p-value less than 0.05 indicate that model terms are

significant, while, less than 0.01 indicate that models terms are highly significant.

Table 4.5 and Table 4.6 represented the results of Plackett-Burman experiment with respect to content of PHAs production (Y_1), consumption of COD (Y_2), the effect, standard error, *t*-value (xi), *p*-value (xi) and confidence level of each component.

Table 4.5 Analysis of PHAs production (Y_1) in Plackett-Burman design test

Variables	**Standard error**	**t-value**	**p-value**	**Confidence (%)**
air bubbling oxygen conc. (mg/L)	0.1762	−1.74	0.394	60.59
C:N ratio	0.1762	8.78	0.004	99.59
Feast/Famine (h/h)	0.1762	0.72	0.127	87.29
pH	0.1762	1.13	0.664	33.59
SRT (d)	0.1762	5.50	0.049	95.09

Table 4.6 Analysis of COD consumption (Y_2) in Plackett-Burman design test

Variables	Standard error	t-value	p-value	Confidence (%)
air bubbling oxygen conc. (mg/L)	0.1411	4.01	0.412	58.80
C:N ratio	0.1411	−17.54	0.001	99.99
Feast/Famine (h/h)	0.1411	−2.23	0.047	95.29
pH	0.1411	3.42	0.092	90.79
SRT (d)	0.1411	5.11	0.241	75.89

The result data described a wide variation in PHAs production content, from 0.034 to 0.184 g/L, in the 8 trials. Analysis of the regression coefficients and the *t*-values of 5 factors (Table 4.5) showed that X_2, X_3, X_4 and X_5 had positive effects on PHAs production. The effect of these three variables were positive, which meant that an increase in the C:N ratio, the Feast/Famine ratio, pH and the SRT value increased the efficiency of PHAs accumulating process. But X_1 had a negative effect on PHAs production, which meant that lower air bubbling oxygen concentration more helped an increase of Y_1.

If the *p*-value is less than 0.05 and confidence level of each component were at or above 95% , the variables were considered to be significant. Moreover, a p-value

less than 1% indicate that terms are highly significant. The *p*-value of variables X_1, X_3 and X_4 were all larger than 0.01 in Y_1 analysis, so that they were considered insignificant. The *p*-value of X_5 was 0.049, which was just less than the critical value 0.05. It was acceptable to be accounted as a significant variable in PHAs storage. Only C:N ratio with p-value was less than 0.01 and at 99.59% confidence level. It was a highly significant term. The t-value of X_2 was positive. Therefore, results indicated that X_2 enhanced the PHAs growth as it increased.

In content of COD consumed, the result displayed a variation from 3.25 to 4.33 g/L, in the Plackett-Burman design test. Analysis of the regression coefficients and the t-values of 5 factors (Table 4.6) showed that X_1 X_4 and X_5 had positive effects on PHAs production. The effect of these variables were positive, which meant that an increase in the air bubbling oxygen concentration, the pH value or SRT increased the consumption of COD. Using smaller amount of COD benefited the efficiency of PHAs production in application. This implied that variables having negative effects of Y_2 in high level offer better PHAs productive conditions. And variables having positive effects on Y_2 in lower level stimulated the efficiency of PHAs production.

The *p*-value was a measure whether variable was a key influence in process. In Y_2 statistic analysis, only *p*-value of X_2 (C:N ratio) was less than 0.01. Although the *p*-value of X_3 (Feast/Famine) was larger than 0.01, it was less than 0.05. Effect of Feast/Famine should not be ingnored. Due to t-value was negative, X_3 negatively affected Y_2. The smaller amount of Y_2 would benefit PHAs optimization. Therefore, higher level of Feast/Famine ratio (Feast/Famiine = 1/3) contributed optimization of PHAs production. Effects of others variables can be ignored due to their p-value were all larger than 0.05. A research concluded that pH had a remarkable effect on the enrichment of PHAs in a SBR (Villano et al, 2010). But our results disagreed on this.

Combined Y_1 and Y_2 analysis, it was no doubt that X_2 (C:N ratio) was a key variable in influence of PHAs optimization process. Use of C:N ratio adjustment during the fermentation and scale up for laboratory and pilot scale fermentors may give further ideas on potential of its use for PHAs industrial production. So the optimal concentrations of this key component, C:N ratio needed to be further studied in the following lab-scale study.

Assessment of X_3 (Feast/Famine ratio) in both Y_1 and Y_2 analysis, its effect were

positive and negative respectively. To obtain more PHAs accumilation and less COD consumption, it should take high level in both test. No conflict that Feast/Famine ratio was decided as 1/3 in nest step study to optimize PHAs yield.

X_5 (SRT) was another variable with *p*-value less than the critical value 0.05. It was compared in both Y_1 and Y_2 analysis. It had postive effects in both analysed results. To obtain more PHAs accumilation, X_5 shold be chosen in high level. In the other hand, to consume COD as little as possible, X_5 should be chosen in lower level. There was a disagreement in both analysis, but X_5 's *p*-value was less than 0.05 in Y_1 analysis not Y_2 analysed result. So X_5 was prefered as 10 days SRT finally.

4.4 Conclusion of Plackett-Burman design experiment

Using Plackett-Burman design was significant during the preliminary study procedure. One of reasons was the sheer elegance of these experiments. They maximize the number of factors for a given size experiment. The construction of the design was relatively straightforward. Also, the interpretation of the results from experiments tended to be clearcut.

Based on the statistical screening analysis and disscussion above, C:N ratio was a key variable during the PHAs optimization process. It should be studied in detail in further study. Feast/Famine ratio and SRT were found significant. Feast/Famine was taken as 1/3 and SRT was designed as 10 days in lab-scale and pilot-scale study. But according to Dionisi's research, while the length of the feast phase was not higher than 20% of the overall length of one cycle, the running tended to polymer storage response rather than a growth response (Dionisi et al., 2007). Our results was disagreed with such statement.

From this part of results, it can be concluded that oxygen concentration and pH control was not critical in enriching the PHAs accumulation.

CHAPTER 5 RESULTS IN LABORATORY-SCALE SYSTEM

After address the key concerns in preliminary test, a design of lab-scale study was confirmed. From the Plackett-Burman design experiment, it was clear that C:N ratio was the most significant variable in optimization of PHAs production. Therefore, C:N ratio was the main controller in the lab-scale study in order to find the specific point that maximized the ability of producing PHAs.

5.1 Investigation of different C:N ratio affect PHAs production

Several batch experiments with different degrees of nutrient deficiency (different C:N ratio) were carried out in order to evaluate its impact on PHAs production. After the reactor was operated to attain a stable condition under the C:N ratio of 20:1, the carbon concentration in the wastewater was increase to result in C:N ratio of 40:1, 60:1, 80:1, 100:1, 120:1, 140:1, 160:1, 180 and 200:1. In each run, the SBR was operated for at least 2 months, in order to describe the performance of the experiment under “steady state conditions”.

5.1.1 Groups of Tai Po STW sample performance

Determination showed the wastewater sample from Tai Po STW with 0.386 g/L COD and 0.099 g/L TKN. To reach and keep a certain the value of OLR at about 20 g COD/L/day as designed, glucose and ammonium chloride was added to increase C:N ratio to 20:1. The SBR was operating as designed before. When stable operation was attained in the SBR under C:N ratio of 20:1, samples of the mixed liquor were periodically 45 min collected for analysis during the 4 h 30 min reaction time in a randomly selected operation cycle. Analysis of the parameters included residual organic and nutrient concentrations, biomass growth and polymer accumulation. The values of these parameters were shown in Table 5.1. The concentration of carbon, measured as residual COD, in the mixed liquor of the SBR reduced with time from 3.244 g/L to 0.279 g/L, with 91.4% COD removal. Simultaneously the residual TKN decreased from 0.161 g/L to 0.027 g/L. However, as the residual COD and TKN decreased, the overall biomass increased from 2.080 g to 2.633 g, obtaining 0.553 g net biomass growth. As the biomass increased, COD, TKN, and DO decreased but polymer accumulation increased. This indicated that the biomass has the ability to convert organic pollution, at least in part, into bacterial reserve material, PHAs. During the same period, the polymer content increased from 0.114 g to 0.176 g,

accumulating 0.062 g net intracellular polymers.

Table 5.1 SBR performance under C:N ratio of 20:1 (Tai Po STW sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.244	0.161	2.080	0.114
0.75	2.660	0.129	2.434	0.117
1.5	2.069	0.103	2.563	0.126
2.25	1.630	0.092	2.600	0.130
3	0.751	0.068	2.624	0.152
3.75	0.394	0.048	2.629	0.163
4.5	0.279	0.027	2.633	0.176

After the experiment under C:N ratio of 20:1 was completed, ammonium chloride concentration was reduced to result in C:N ratio of 40:1. The SBR was operated about two weeks until stable condition was achieved. It was found that when C:N ratio was increased to 40:1, similar observations were obtained as performance in the case under C:N ratio of 20:1. The residual COD and TKN decrease from time while the overall biomass and polymer accumulation increase. The residual COD reduced over time from 3.238 g/L to 0.268 g/L, with 91.7% COD removal. At the same running time, the residual TKN decreased from 0.079 g/L to 0.014 g/L. However, as the residual COD and TKN decreased, the overall biomass increased from 2.073 g to 2.619 g, obtaining 0.546 g net biomass growth. Also, the polymer content increased from 0.120 g to 0.211 g, accumulating 0.091

g net intracellular polymers. The performance of each parameter was shown in Table 5.2.

Table 5.2 SBR performance under C:N ratio of 40:1 (Tai Po STW sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.238	0.079	2.073	0.120
0.75	2.687	0.067	2.260	0.144
1.5	1.943	0.056	2.342	0.160
2.25	1.495	0.039	2.425	0.179
3	0.885	0.028	2.529	0.191
3.75	0.557	0.021	2.571	0.194
4.5	0.268	0.014	2.619	0.211

After consummation of former groups of experiment, ammonium chloride content was further reduced to create a C:N ratio of 60:1. For this running, nine days was required for the reactor to a steady state. It was found that the residual COD reduced over time from 3.262 g/L to 0.199 g/L, with 93.9% COD removal. On the other hand, the residual TKN decreased from 0.054 g/L to 0.006 g/L. A recurrence of similar development appeared. As the residual COD and TKN decreased, the overall biomass increased from 2.115 g to 2.642 g, obtaining 0.527 g net biomass growth. The polymer content increased from 0.126 g to 0.223 g, accumulating 0.097 g net intracellular polymers (Table 5.3).

Table 5.3 SBR performance under C:N ratio of 60:1 (Tai Po STW sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.262	0.054	2.115	0.126
0.75	2.381	0.045	2.178	0.167
1.5	1.859	0.038	2.263	0.174
2.25	1.207	0.029	2.369	0.190
3	0.750	0.021	2.432	0.196
3.75	0.326	0.011	2.517	0.202
4.5	0.199	0.006	2.642	0.223

Under C:N ratio of 80:1, the residual COD reduced over time from 3.246 g/L to 0.231 g/L during the reaction time, with 92.9% COD removal. Simultaneously the residual TKN decreased from 0.041 g/L to 0.006 g/L. On the contrary, the overall biomass increased from 2.106 g to 2.625 g, resulting 0.519 g net biomass growth. The polymer content increased from 0.139 g to 0.281 g, accumulating 0.142 g net intracellular polymers (Table 5.4).

Table 5.4 SBR performance under C:N ratio of 80:1 (Tai Po STW sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.246	0.041	2.106	0.139
0.75	2.241	0.032	2.148	0.123
1.5	1.850	0.027	2.274	0.137
2.25	1.298	0.017	2.317	0.158
3	1.006	0.012	2.401	0.196
3.75	0.617	0.008	2.493	0.202
4.5	0.231	0.006	2.625	0.281

Under C:N ratio of 100:1, the residual COD reduced over time from 3.252 g/L to 0.272 g/L during the reaction time, with 91.6% COD removal. Simultaneously the residual TKN decreased from 0.032 g/L to 0.006 g/L. On the contrary, the overall biomass increased from 2.049 g to 2.532 g, with 0.483 g net biomass growth. The polymer content increased from 0.158 g to 0.342 g, accumulating 0.184 g net intracellular polymers (Table 5.5).

Table 5.5 SBR performance under C:N ratio of 100:1 (Tai Po STW sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.252	0.032	2.049	0.158
0.75	2.504	0.026	2.140	0.165
1.5	2.179	0.020	2.181	0.169
2.25	1.756	0.015	2.243	0.213
3	1.041	0.011	2.264	0.275
3.75	0.403	0.007	2.311	0.315
4.5	0.272	0.006	2.532	0.342

The SBR was operated around a week to a steady state after adjusted C:N ratio to 120:1. Under C:N ratio of 120:1, the residual COD reduced over time from 3.271 g/L to 0.258 g/L during the reaction time, with 92.1% COD removal. Simultaneously the residual TKN decreased from 0.026 g/L to 0.004 g/L. But the overall biomass and polymer accumulation were on the increase. The overall

biomass increased from 2.029 g to 2.506 g, with 0.477 g net biomass growth. The polymer content increased from 0.134 g to 0.316 g, accumulating 0.182 g net intracellular polymers (Table 5.6).

Table 5.6 SBR performance under C:N ratio of 120:1 (Tai Po STW sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.271	0.026	2.029	0.134
0.75	2.518	0.021	2.110	0.159
1.5	2.002	0.017	2.212	0.169
2.25	1.030	0.012	2.232	0.187
3	0.766	0.008	2.272	0.261
3.75	0.501	0.005	2.354	0.294
4.5	0.258	0.004	2.506	0.316

Under C:N ratio of 140:1, the residual COD reduced over time from 3.236 g/L to 0.289 g/L during the reaction time, with 91.1% COD removal. Simultaneously the residual TKN decreased from 0.023 g/L to 0.002 g/L. The overall biomass increased from 2.113 g to 2.575 g, with 0.462 g net biomass growth. The polymer content increased from 0.145 g to 0.323 g, receiving 0.178 g net intracellular polymers (Table 5.7).

Table 5.7 SBR performance under C:N ratio of 140:1 (Tai Po STW sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.236	0.023	2.113	0.145
0.75	2.651	0.019	2.143	0.173
1.5	2.283	0.016	2.179	0.182
2.25	1.521	0.011	2.221	0.208
3	1.197	0.007	2.258	0.270
3.75	0.777	0.003	2.427	0.318
4.5	0.289	0.002	2.575	0.323

Under C:N ratio of 160:1, the residual COD reduced over time from 3.224 g/L to 0.266 g/L during the reaction time, with 91.7% COD removal. Simultaneously the residual TKN decreased from 0.020 g/L to 0.002 g/L. On the other hand, when the C:N ratio was further increased to 160:1, the overall biomass increased from 2.225 g to 2.671 g, with 0.446 g net biomass growth. The polymer content increased from 0.136 g to 0.313 g, receiving 0.177 g net intracellular polymers (Table 5.8).

Table 5.8 SBR performance under C:N ratio of 160:1 (Tai Po STW sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.224	0.020	2.225	0.136
0.75	2.249	0.015	2.270	0.179
1.5	1.844	0.012	2.307	0.193
2.25	1.353	0.008	2.345	0.235
3	0.735	0.005	2.374	0.245
3.75	0.569	0.004	2.484	0.263
4.5	0.266	0.002	2.671	0.313

Under C:N ratio of 180:1, the residual COD reduced over time from 3.236 g/L to 0.247 g/L during the reaction time, with 92.4% COD removal. Simultaneously the residual TKN decreased from 0.018 g/L to 0.001 g/L. However, the overall biomass increased from 2.226 g to 2.645 g, with 0.419 g net biomass growth. The polymer content increased from 0.156 g to 0.326 g, receiving 0.169 g net intracellular polymers (Table 5.9).

Table 5.9 SBR performance under C:N ratio of 180:1 (Tai Po STW sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.236	0.018	2.226	0.156
0.75	2.598	0.015	2.268	0.203
1.5	2.156	0.013	2.284	0.225
2.25	1.653	0.009	2.331	0.246
3	1.097	0.006	2.397	0.269
3.75	0.654	0.004	2.534	0.328

4.5	0.247	0.001	2.645	0.326

Under C:N ratio of 200:1, the residual COD reduced over time from 3.235 g/L to 0.199 g/L during the reaction time, with 93.8% COD removal. Simultaneously the residual TKN decreased from 0.016 g/L to 0.001 g/L. However, the overall biomass increased from 2.219 g to 2.607 g, with 0.388 g net biomass growth. The polymer content increased from 0.137 g to 0.304 g, receiving 0.167 g net intracellular polymers (Table 5.10).

Table 5.10 SBR performance under C:N ratio of 200:1 (Tai Po STW sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.235	0.016	2.219	0.137
0.75	2.522	0.013	2.259	0.164
1.5	2.059	0.010	2.264	0.178
2.25	1.408	0.007	2.329	0.216
3	0.975	0.005	2.417	0.242
3.75	0.327	0.002	2.543	0.272
4.5	0.199	0.001	2.607	0.304

After finish of this part of study by using sample from Tai Po STW, a similar developing trend was observed with C:N ratio from 20:1 to 200:1. As COD and TKN decreased but the overall biomass and polymer accumulation increased under various C:N ratio. These results confirmed that the biomass had the ability

to convert organic pollution into bacterial storage material, PHAs.

Though these appearances seemed in common, further analysis and comparisons were conducted, it was found that the growth rate of the overall biomass and polymer accumulation were diverse among different C:N ratio condition. The net biomass growth was determined as del X. The net polymer accumulation was defined as del P. And the net consumption of COD during the reaction time was defined as del S. So in mathematical operation, specific growth yield, Y x/s, equal to del X divided by del S. Specific polymer yield, Y p/x, can be calculate as del P divided by del X. As for the overall polymer production yield, Y p/s, which was a product of Y x/s and Y p/x, equals to Y x/s multiplied by Y p/x.

Table 5.11 SBR performance of each parameters under different C:N ratio (Tai Po STW sample)

C:N ratio	del X (g)	del P (g)	del S (g)	Y x/s (g/g)	Y p/x (g/g)	Y p/s (g/g)
20:1	0.553	0.062	2.965	0.187	0.112	0.021
40:1	0.546	0.091	2.969	0.184	0.167	0.031
60:1	0.527	0.098	3.063	0.172	0.185	0.032
80:1	0.519	0.143	3.015	0.172	0.275	0.047
100:1	0.483	0.184	2.980	0.162	0.381	0.062
120:1	0.477	0.182	3.012	0.158	0.382	0.061
140:1	0.462	0.178	2.947	0.157	0.386	0.061
160:1	0.446	0.177	2.957	0.151	0.398	0.060

180:1	0.419	0.169	2.989	0.140	0.405	0.057
200:1	0.388	0.167	3.036	0.128	0.432	0.055

Table 5.11 summarized the growth rate of biomass and polymer, and COD consumption situation. It was found that net biomass growth, del X, decreased from 0.553 g to 0.388 g as C:N ratio increased. Accompany to this, Y x/s declined from 0.187 to 0.128 g biomass/ g COD consumed. On the contrary, the net polymer accumulation, del P, increased from 0.062 g to 0.167 g. Similarly, Y p/x increased from 0.112 to 0.432 g polymer accumulated/ g biomass. This change implied that the storage capacity of polymers enhanced with the increase of C:N ratio. It indicated that bacterial communities in SBR showed more contribution of a storage response than growth response under higher C:N ratio (Figure 5.1).

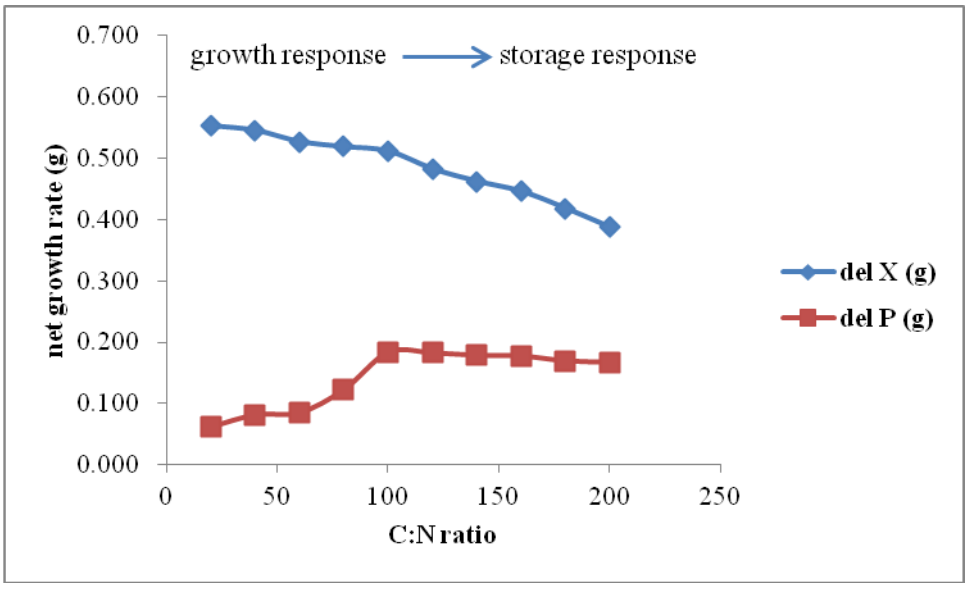


Figure 5.1 Corresponding between growth and storage response in SBR with an increase of C:N ratio (Tai Po STW sample)

In addition to a visual and intuitive analysis of figure, statistical software, Minitab 15, was also applied to confirm whether del X and del P had a relationship. Thus in order to verify decrease in growth of biomass associated with PHAs storage. After analyzed by Minitab, the Pearson correlation coefficient was -0.7744. In statistics, the Pearson correlation coefficient (typically denoted by Greek letter ρ) is a measure of the correlation (linear dependence) between two variables A and B, giving a value between +1 and −1 inclusive. The coefficient ρ of -0.7744 was in reasonable agreement with the hypothesis of conversion between biomass growth response and PHAs storage response, indicating del X and del P having a correlation (Table 5.12).

Table 5.12 Guidelines for the interpretation of a correlation coefficient

Correlation	**Negative**	**Positive**
None	-0.09 to 0.0	0.0 to 0.09
Small	-0.3 to -0.1	0.1 to 0.3
Medium	-0.5 to -0.3	0.3 to 0.5

Large	-1.0 to -0.5	0.5 to 1.0

This performance under high C:N ratio demonstrated that biomass growth became unfavorable under nitrogen deficiency condition. But at the same time, this nitrogen deficiency environment induced sludge microorganisms to storage intracellular carbon reserves in the form of PHAs.

One important aim in the whole study was to reduce cost of producing PHAs. An optimal operating point, under which less carbon source was consumed while more polymers accumulated, would be considered as a significant indicator. The overall polymer production yield, Y p/s, means the PHAs accumulation efficiency of bacteria under various C:N ratio. A larger Y p/s indicated that more PHAs accumulated in the cell and less carbon/raw material used. This parameter Y p/s met the above mentioned requests so that it can be a significant and effective indicator.

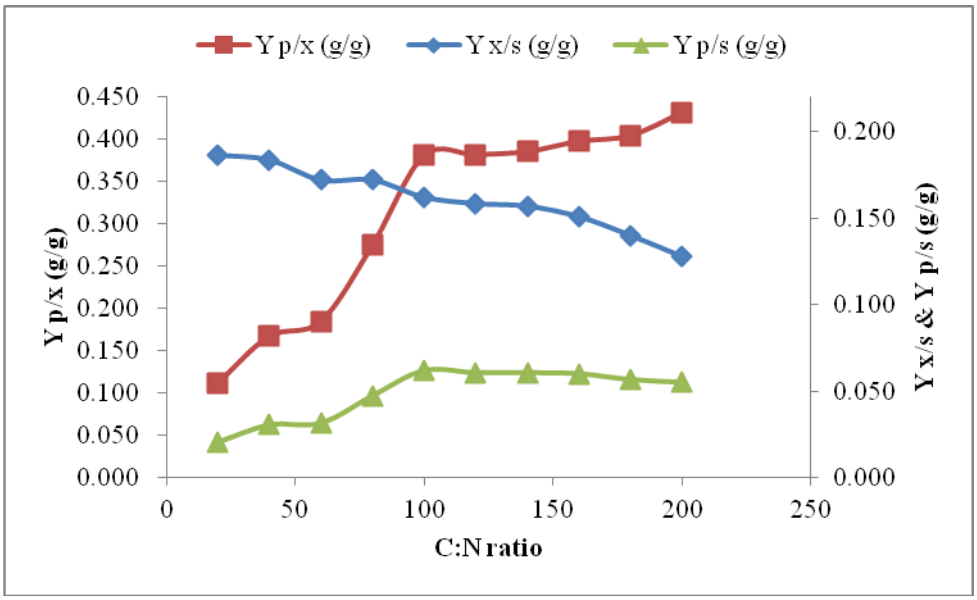


Figure 5.2 The effect of C:N ratio on biomass and polymer growth (Tai Po STW)

Figure 5.2 shows that Y p/s reached a maximum value of 0.062 g polymer/ g COD consumed under C:N ratio of 100:1. When C:N ratio continuously increased from 120:1 to 200:1, Y p/s decreased slightly instead. Y p/s indicated the capacity of PHAs yield per COD consumed. Concept in economics, saving the cost to increase profits, for SBR running with Tai Po STW sample, C:N ratio of 100:1 was its optimal operating condition.

5.1.2 Groups of refuse transfer station leachate treatment plant performance

The background determination showed the sample from refuse transfer station leachate treatment plant with 13.868 g/L COD, 0.132 g/L TKN. The concentrations of COD and TKN were much higher than Tai Po STW ones. It was easy to understand because some waste collected in the refuse transfer station like food wastes or household wastes were organic material. To reach and keep a certain the value of OLR at about 20 g COD/L/day as designed, sample was diluted first, then glucose and ammonium chloride was dosed to increase C:N ratio to 20:1.

When the operation of SBR reached a steady state under C:N ratio of 20:1, samples of the mixed liquor were periodically 45 min collected for analysis in a randomly selected operation cycle like sampling for the Tai Po STW group. Analysis of the parameters included residual organic and nutrient concentrations, biomass growth and polymer accumulation. The performance was shown in Table 5.13. Residual COD reduced with time from 3.198 g/L to 0.289 g/L, with 90.9% COD removal. Simultaneously the residual TKN decreased from 0.159 g/L to 0.039 g/L. However, as the residual COD and TKN decreased, the overall

biomass increased from 2.080 g to 2.712 g, obtaining 0.632 g net biomass growth. During the same period, the polymer content increased from 0.133 g to 0.207 g, accumulating 0.074 g net intracellular polymers.

Table 5.13 SBR performance under C:N ratio of 20:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.198	0.159	2.080	0.133
0.75	2.632	0.127	2.434	0.137
1.5	2.175	0.102	2.563	0.149
2.25	1.733	0.091	2.600	0.153
3	1.279	0.068	2.627	0.180
3.75	0.768	0.048	2.642	0.188
4.5	0.289	0.039	2.712	0.207

After experiment under C:N ratio of 20:1, an influent adjusted to 40 C:N ratio was fed into the SBR. The results displayed that the residual COD reduced over time from 3.256 g/L to 0.293 g/L during the reaction time, with 91.0% COD removal. Simultaneously the residual TKN decreased from 0.081 g/L to 0.015 g/L. On the contrary, the overall biomass increased from 2.073 g to 2.663 g, resulting 0.059 g net biomass growth. The polymer content increased from 0.142 g to 0.239 g, accumulating 0.097 g net intracellular polymers (Table 5.14).

Table 5.14 SBR performance under C:N ratio of 40:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.256	0.081	2.073	0.142
0.75	2.702	0.069	2.260	0.170
1.5	1.954	0.051	2.342	0.189
2.25	1.514	0.038	2.425	0.222
3	1.055	0.029	2.529	0.234
3.75	0.716	0.021	2.571	0.241
4.5	0.293	0.015	2.663	0.239

After the experiment under C:N ratio of 40:1 was completed, ammonium chloride concentration was reduced to result in C:N ratio of 60:1. Similar observation was obtained as performance in the case under C:N ratio of 20:1 and 40:1. The residual COD and TKN decrease from time while the overall biomass

and polymer accumulation increase. The performance of each parameter was shown in Table XX. The residual COD reduced over time from 3.245 g/L to 0.208 g/L, with 93.6% COD removal. At the same running time, the residual TKN decreased from 0.053 g/L to 0.005 g/L. However, as the residual COD and TKN decreased, the overall biomass increased from 2.115 g to 2.663 g, obtaining 0.548 g net biomass growth. Also, the polymer content increased from 0.158 g to 0.292 g, accumulating 0.134 g net intracellular polymers (Table 5.15).

Table 5.15 SBR performance under C:N ratio of 60:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.245	0.053	2.115	0.158
0.75	2.369	0.044	2.178	0.191
1.5	1.856	0.037	2.263	0.218
2.25	1.201	0.028	2.369	0.224
3	0.805	0.020	2.432	0.248
3.75	0.337	0.011	2.517	0.273
4.5	0.208	0.005	2.663	0.292

Under C:N ratio of 80:1, the residual COD reduced over time from 3.228 g/L to 0.266 g/L during the reaction time, with 91.8% COD removal. Simultaneously the residual TKN decreased from 0.039 g/L to 0.005 g/L. When the C:N ratio was further increased to 80:1, the overall biomass increased from 2.106 g to

2.581 g, with 0.475 g net biomass growth. The polymer content increased from 0.174 g to 0.343 g, receiving 0.169 g net intracellular polymers (Table 5.16).

Table 5.16 SBR performance under C:N ratio of 80:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.228	0.039	2.106	0.174
0.75	2.227	0.031	2.148	0.211
1.5	1.848	0.026	2.274	0.237
2.25	1.291	0.018	2.317	0.244
3	1.059	0.012	2.401	0.282
3.75	0.620	0.008	2.432	0.296
4.5	0.266	0.005	2.581	0.343

Under C:N ratio of 100:1, the residual COD reduced over time from 3.243 g/L to 0.292 g/L during the reaction time, with 91.0% COD removal. Simultaneously the residual TKN decreased from 0.032 g/L to 0.004 g/L. However, the overall biomass increased from 2.043 g to 2.465 g, with 0.422 g net biomass growth. The polymer content increased from 0.251 g to 0.457 g, receiving 0.206 g net intracellular polymers (Table 5.17).

Table 5.17 SBR performance under C:N ratio of 100:1 (leachate treatment plant sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.243	0.032	2.043	0.251
0.75	2.504	0.024	2.125	0.316
1.5	2.173	0.020	2.165	0.344
2.25	1.767	0.015	2.227	0.362
3	1.038	0.011	2.247	0.407
3.75	0.778	0.007	2.294	0.439
4.5	0.292	0.004	2.465	0.457

Under C:N ratio of 120:1, the residual COD reduced from 3.208 g/L to 0.190 g/L during the reaction time, with 94.1% COD removal. Simultaneously the residual TKN decreased from 0.026 g/L to 0.004 g/L. On the contrary, the overall biomass increased from 2.049 g to 2.460 g, with 0.411 g net biomass growth. The polymer content increased from 0.256 g to 0.462 g, having 0.206 g net intracellular polymers (Table 5.18).

Table 5.18 SBR performance under C:N ratio of 120:1 (leachate treatment plant sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.208	0.026	2.049	0.256
0.75	2.470	0.021	2.131	0.305
1.5	1.968	0.016	2.233	0.323
2.25	1.572	0.011	2.254	0.358
3	1.091	0.008	2.295	0.387
3.75	0.642	0.005	2.377	0.435
4.5	0.190	0.004	2.460	0.462

Further increase C:N ratio to 140:1, determination showed that the residual COD reduced from 3.253 g/L to 0.238 g/L reaching 92.7% COD removal. At the same period the residual TKN decreased from 0.023 g/L to 0.002 g/L. But the overall biomass increased from 2.112 g to 2.498 g, with 0.386 g net biomass growth. The polymer content increased from 0.261 g to 0.467 g, having 0.206 g net intracellular polymers (Table 5.19).

Table 5.19 SBR performance under C:N ratio of 140:1 (leachate treatment plant sample)

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.253	0.023	2.112	0.261
0.75	2.681	0.020	2.142	0.312

1.5	2.306	0.016	2.158	0.329
2.25	1.529	0.011	2.196	0.353
3	1.204	0.007	3.274	0.412
3.75	0.781	0.003	2.266	0.442
4.5	0.238	0.002	2.498	0.467

After consummation of former experiment, ammonium chloride content was further reduced to create a C:N ratio of 160:1. It was found that the residual COD reduced over time from 3.357 g/L to 0.269 g/L, with 92.0% COD removal. On the other hand, the residual TKN decreased from 0.021 g/L to 0.002 g/L. A similar development appeared again. As the residual COD and TKN decreased, the overall biomass increased from 2.232 g to 2.608 g, obtaining 0.376 g net biomass growth. The polymer content increased from 0.264 g to 0.473 g, accumulating 0.209 g net intracellular polymers (Table 5.20).

Table 5.20 SBR performance under C:N ratio of 160:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.357	0.021	2.232	0.264
0.75	2.349	0.015	2.277	0.349
1.5	1.927	0.012	2.315	0.376
2.25	1.413	0.009	2.353	0.458
3	0.768	0.005	2.346	0.476
3.75	0.590	0.004	2.397	0.513
4.5	0.269	0.002	2.608	0.473

Under C:N ratio of 180:1, the residual COD reduced from 3.267 g/L to 0.245 g/L during the reaction time, having 92.5% COD removal. Simultaneously the residual TKN decreased from 0.018 g/L to 0.001 g/L. However, the overall biomass increased from 2.226 g to 2.587 g, with 0.361 g net biomass growth. The polymer content increased from 0.270 g to 0.477 g, having 0.207 g net intracellular polymers (Table 5.21).

Table 5.21 SBR performance under C:N ratio of 180:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.267	0.018	2.226	0.270
0.75	2.712	0.015	2.268	0.351
1.5	2.351	0.014	2.284	0.389
2.25	1.641	0.009	2.295	0.425
3	1.094	0.006	2.330	0.465
3.75	0.736	0.004	2.427	0.519
4.5	0.245	0.001	2.587	0.477

As C:N ratio increase, Table 5.22 shows the revolution of PHAs storage performance during C:N of 200:1. It illustrated that the residual COD reduced over time from 3.296 g/L to 0.203 g/L during the reaction time, with 93.8% COD removal. At the same time, the residual TKN decreased from 0.016 g/L to 0.001

g/L. However, the overall biomass increased from 2.219 g to 2.569 g, with 0.350 g net biomass growth. The polymer content increased from 0.271 g to 0.480 g, receiving 0.209 g net intracellular polymers.

Table 5.22 SBR performance under C:N ratio of 200:1 (leachate treatment plant sample)

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.296	0.016	2.219	0.271
0.75	2.525	0.013	2.259	0.325
1.5	2.039	0.010	2.264	0.353
2.25	1.703	0.009	2.288	0.427
3	1.132	0.006	2.317	0.479
3.75	0.326	0.002	2.343	0.529
4.5	0.203	0.001	2.569	0.480

In summary, a similar developing trend was observed with C:N ratio from 20:1 to 200:1 resembling the situation in SBR operated by Tai Po STW sample. When the residual COD and TKN declined, the overall biomass and polymer accumulation ascended gradually under different C:N ratio. These results stated that the microorganisms in reactor had the ability to convert carbon source into PHAs when using material from leachate treatment plant as substrate.

Using the same method as Tai Po sample to calculate the net growth of biomass

and polymer, and the net COD consumption under different C:N ratio condition. To assess the performance, specific biomass yield as Y x/s, specific polymer yield as Y x/s and the overall polymer production yield Y p/s were calculated also. The results are depicted in the following table (Table 5.23).

Table 5.23 SBR performance of each parameters under different C:N ratio (leachate treatment plant sample)

C:N ratio	del X (g)	del P (g)	del S (g)	Y x/s (g/g)	Y p/x (g/g)	Y p/s (g/g)
20:1	0.632	0.073	2.909	0.217	0.116	0.025
40:1	0.590	0.097	2.963	0.199	0.164	0.033
60:1	0.548	0.134	3.037	0.180	0.244	0.044
80:1	0.475	0.169	2.962	0.160	0.355	0.057
100:1	0.422	0.205	2.951	0.143	0.487	0.070
120:1	0.411	0.206	3.018	0.136	0.500	0.068
140:1	0.386	0.206	3.015	0.128	0.533	0.068
160:1	0.376	0.209	3.088	0.122	0.556	0.068
180:1	0.361	0.207	3.023	0.119	0.574	0.069
200:1	0.350	0.209	3.093	0.113	0.596	0.067

The growth rate of biomass and polymer, and COD consumption situation were demonstrated. It was found that net biomass growth, del X, decreased from 0.632 g to 0.350 g as C:N ratio increased. Also, Y x/s declined from 0.217 to 0.113 g biomass/ g COD consumed. Whereas the net polymer accumulation, del P,

increased from 0.073 g to 0.209 g. At the same time, Y p/x increased from 0.116 to 0.596 g polymer accumulated / g biomass. These results implied that the storage capacity of polymers enhanced with the increase of C:N ratio. The bacterial communities' growth balance moved from biomass growth response to internal storage response in the reactor (Figure 5.3).

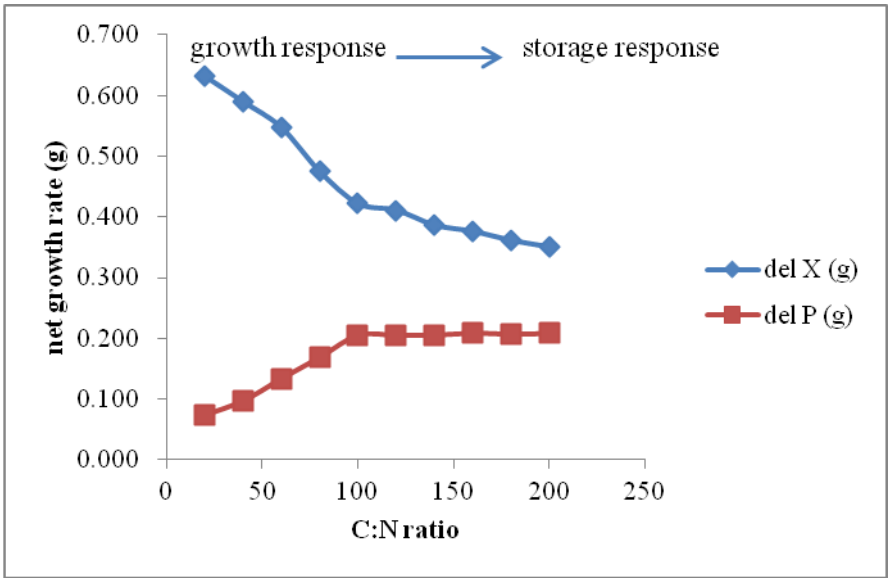


Figure 5.3 Corresponding between growth and storage response in SBR with an increase of C:N ratio (leachate treatment plant sample)

This trend demonstrated that unfavorable biomass growth conditions due to nitrogen deficiency. However, nitrogen deficiency condition benefited the storage rate of intracellular PHAs. The overall trends were no difference with the previous experiment of Tai Po STW sample. In Minitab analysis, the Pearson

correlation coefficient was -0.8106. The coefficient ρ of -0.8106 was in reasonable agreement with the hypothesis as results of leachate treatment plant, indicating a correlation presented between del X and del P again.

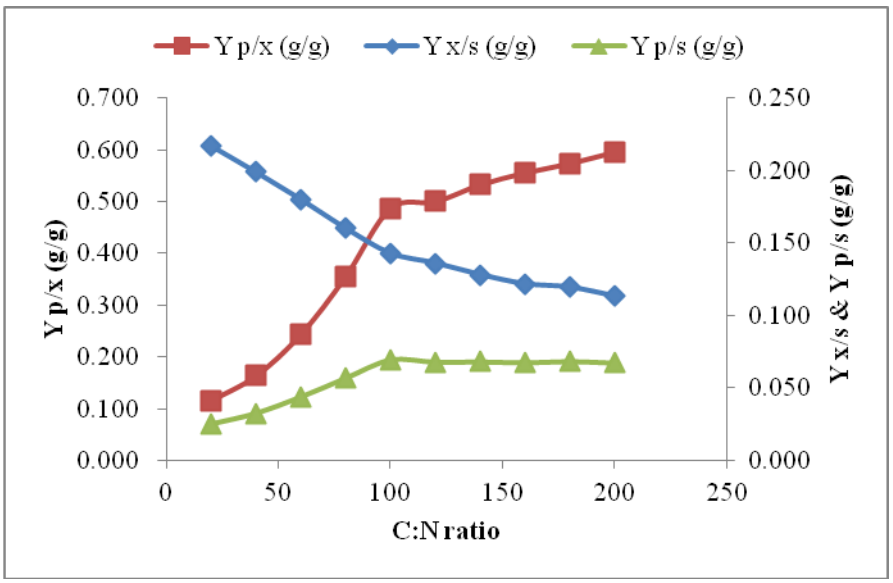


Figure 5.4 The effect of C:N ratio on biomass and polymer growth (leachate treatment plant sample)

In order to letting the biomass store PHAs at the maximal rates, the important indicator, the largest value of Y p/s, needed to be identified again. Table 5.4 shows that Y p/s reached a maximum value of 0.070 g polymer/ g COD consumed under C:N ratio of 100:1. As C:N ratio was continuous increasing from 120:1 to 200:1, Y p/s stopped increasing and decrease slightly from 0.070 to 0.067 g polymer/ g COD consumed. It was clear that C:N ratio of 100:1 was the

SBR optimal operating condition in this part of study (Figure 5.4).

5.1.3 Comparison C:N ratio effect between two groups of lab-scale study

In generally, the evolution of biomass growth and polymer accumulation was similar in two groups of lab-scale study above. The nitrogen-deficient condition restricted the normal growth of biomass in the activated sludge but stimulated the accumulation of PHAs. For a more detailed understanding, data from Tai Po STW and leachate treatment plant sample were put together for an analysis. To distinguish these two, the representation for Tai Po STW experiment was unchanged, while the symbol for leachate treatment plant sample were marked as del X', del P' , $Y_{x'/s'}$, $Y_{p'/x'}$ and $Y_{p'/s'}$.

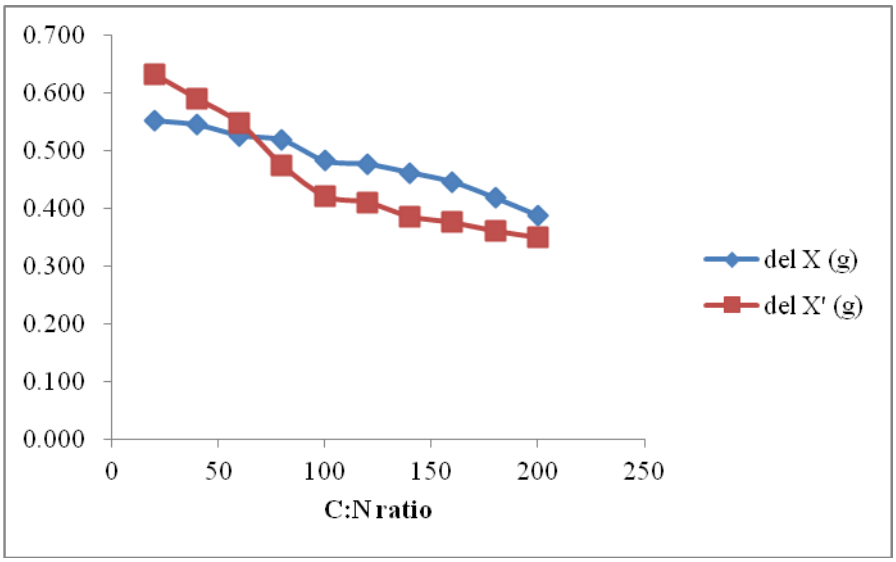


Figure 5.5 Comparison of net biomass growth under different C:N ratio

(lab-scale)

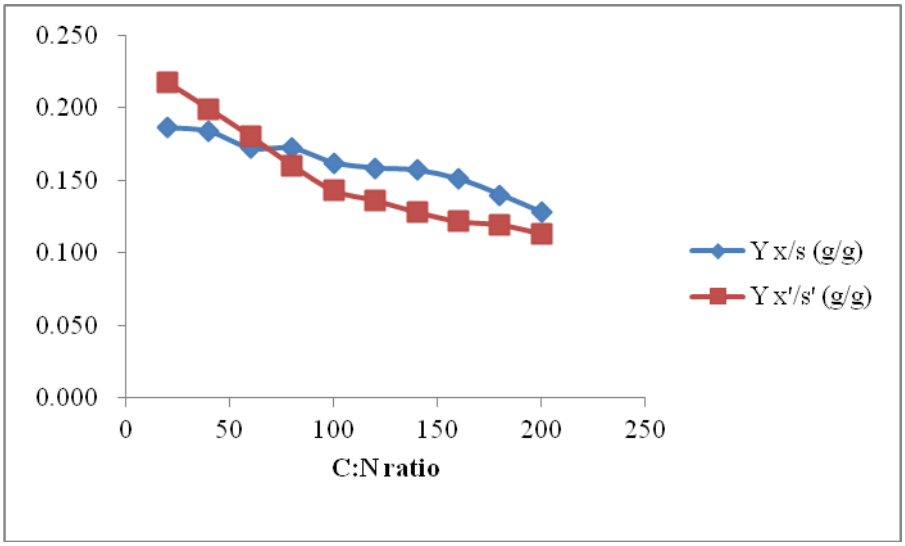


Figure 5.6 Comparison of specific biomass growth under different C:N ratio (lab-scale)

Though both net biomass decreased as C:N ratio increased, the reducing degree was not the same always in leachate treatment plant sample and Tai Po STW sample under different C:N ratio.In Figure 5.5, del X' value was higher than del X at the beginning, which indicated bacteria in leachate treatment plant sample had a stronger capacity of biomass growing under minor nitrogen deficiency condition. But after C:N ratio around 60:1, del X' lessened greater than del X. It seemd capacity of biomass growth weakened in second group of experiment as C:N ratio increased. But this suspicion doubted by more carbon substrates may be enrolled in the leachate treatment plant group at the beginning. Due to

unstable carbon substrate enrolled, del $X^{'}$ dropped greater than del X then. Further, to address such doubt, specific biomass growth per unit substrate consumed requested to have a comparison. Then compared Y x/s and Y $x^{'}/s^{'}$, the shapes of them resembled in Figure 5.6. Y $x^{'}/s^{'}$ value was higher than Y x/s after about C:N = 60:1. So it further demonstrated capacity of biomass growth weakened greater in leachate treatment plant group of experiment as C:N ratio increased, nevertheless its biomass growth capacity was stronger at lower C:N ratio.

Two figures above confirmed capacity of biomass growth declined, but capacity of polymers synthesised intracellularly as a carbon or energy reserve would be enhanced, which can be verified by Minitab 15 statistic analysis before. It was convinced that a transfer from biomass growth response to intracellular polymers storage response happened in both two groups of experiments. In addition, growth trend of microorganism in leachate treatment plant sample SBR was earlier going to storage response from growth response. To further confirm such conclusions, one comparison between del P and del $P^{'}$ and another comparison between del Y p/x and del Y $p^{'}/x^{'}$ were conducted. Figure 5.7 shows that net polymer accumulations in leachate treatment plant sample SBR were always

higher than in Tai Po STW sample SBR. It also showed in Figure 5.8 that differences between Y p/x and Y p'/x' were not distinct when C:N ratio was smaller than 60:1. But difference between Y p/x and Y p'/x' became larger as C:N ratio increased. These comparisons demonstrated that polymer storage capacity of microorganisms in the former SBR was stronger than the latter. The reason possibly was refuse containing many and variety organic material. It usually included domestics waste and commercial waste (food waste, old furniture etc.). Due to bacteria in sludge which had been used to high carbon circumstance, these bacteria adapted quickly to lab-scale SBR operation, and gained a good biomass growing and accumulating performance. So PHAs producing performance was better in leachate treatment plant sample SBR in high C:N ratio. In addition, difference between del P and del $P^{'}$ was small under C:N ratio of 20:1 and 40:1. Their differences were 0.011 and 0.016 g, respectively. After C:N ratio was more than 60:1, their difference was obvious and higher than 0.02 g always, evenly up to 0.049g. Refer to Figure 5.5 and Figure 5.6, values of del $X^{'}$ and del Y $x^{'}/s^{'}$ were smaller than del X and del Y x/s respectively after C:N ratio of 60:1. These comparisons illustrated that bacterial growth response moved to intracellular energy storage response as polymer accumulation under C:N ratio more than 60:1. From Figure 5.7 and Figures 5.8,

it was obvious met such conclusion also.

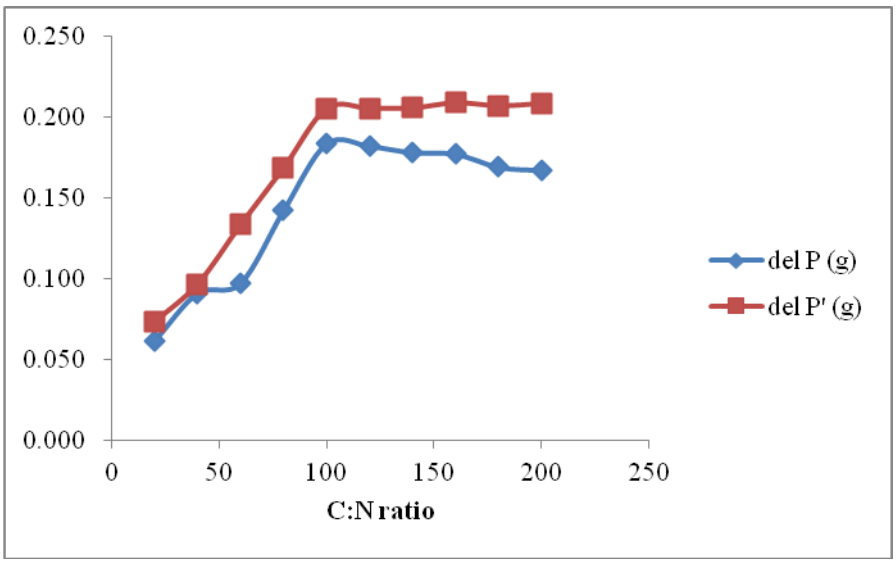


Figure 5.7 Comparison of net polymer growth under different C:N ratio (lab-scale)

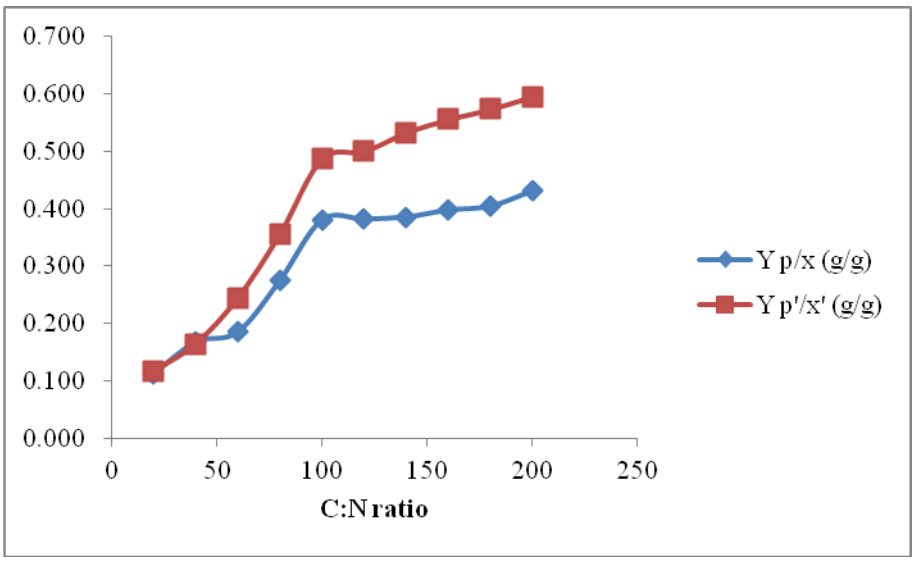


Figure 5.8 Comparison of specific polymer growth under different C:N ratio (lab-scale)

As mentioned before, Y p/s was an important indicator during optimization of producing PHAs. Figure 5.9 compares the polymer accumulation capacity over substrate consumption. Y p/s reached a maximum value after C:N ratio increased to 100:1, then kept decreasing gradually as C:N ratio increased. On the other hand, Y $p^{'}/s^{'}$ reached a maximum value under C:N ratio of 100:1, then fluctuated and declined slightly from 100 to 180 C:N ratio. It further declined after 180 C:N ratio. These two figures implied that nitrogen-deficient condition favor polymers accumulation, but extreme nitrogen deficient condition did not stimulate or maintain a good capacity of polymers storage in both groups of experiment. Even it may negatively affect polymers storage. In addition, a larger Y p/s indicated that more PHAs accumulation and less carbon source was used. Therefore, basis on this, the optimum production of PHAs was conducted as running under 100 C:N ratio. During C:N ratio of 100:1, the maximum PHAs content production from Tai Po wastewater treatment sample and refuse transfer station leachate sample were 14.78% and 18.47%, respectively.

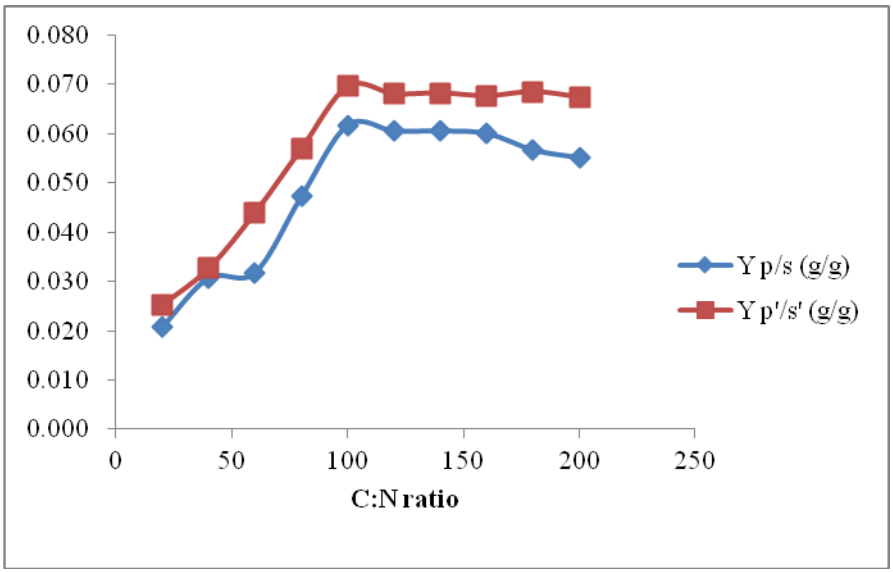


Figure 5.9 Comparison of overall polymer production yield under different C:N ratio (lab-scale)

In conclusion, the nitrogen-deficient condition affected the normal growth of biomass and polymers in the activated sludge. The nitrogen-deficient condition constrained the biomass growing but simulated the intracellular polymer accumulating no matter in sewage treatment plant sample SBR or refuse transfer station leachate treatment plant sample SBR. The optimum operating condition of PHAs production was 100 C:N ratio.

5.2 Analysis of bacterial identification in lab-scale study

When C:N ratio less than 100:1, PHAs accumulations were not in a great amount in mixed microbial process. These circumstances may have a negative impact in

the process that the bacterial population was possibly heterogeneous in terms of the storage capacity. Microorganisms presenting low storage capacities will contribute for the reduction of the average PHAs cell content and increasing the PHAs extraction costs. It is worth noting that the operation of the SBR system should be optimized to obtain a homogeneous population with high and stable storage capacity. Under C:N ratio of 100, the system met such challenges, so that an analysis of microbial communities could provide more useful and significant reference.

In order to identify potential members of the microbial communities in sludge, samples of the mixed cultures that developed during C:N ratio of 100:1 in groups of Tai Po STW and leachate treatment plant SBR were collected and analyzed by MIDI Instant FAMETM Sherlock System. When the integration of peak was performed, an electronic signal from detector was passed to the computer and compared to a stored database. Consequently, bacteria in sample were identified.

Arthrobacter viscosus, *Bacillus sphaericus*, *Brevibacillus choshinensis*, *Micrococcus luteus* and *Micrococcus lylae* were identified in sample from Tai Po STW SBR. One of these bacteria, *Bacillus sphaericus*, was a bacteria which had

a capacity to accumulate PHAs proven by Bui's research (Figure 5.10) (Bui et al., 1997).

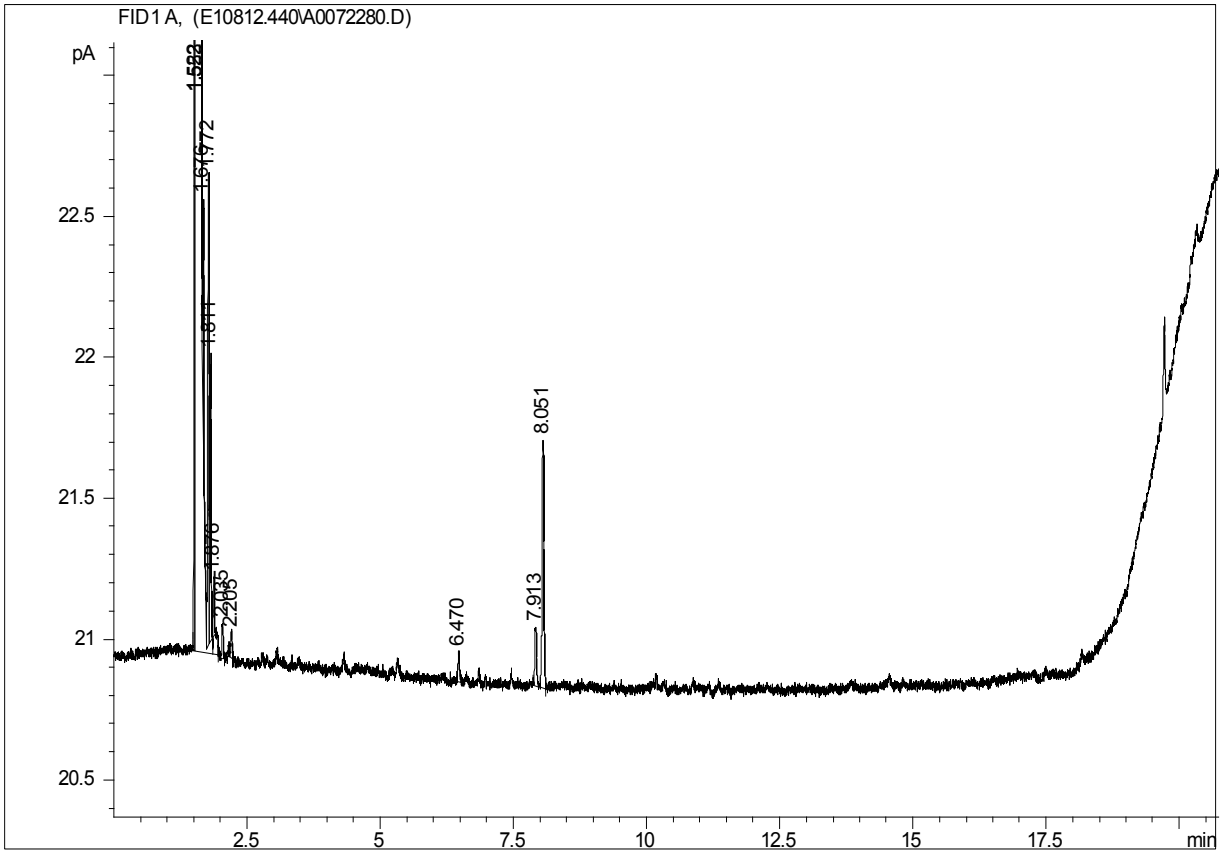


Figure 5.10 MIDI test for activated sludge operated under C:N ratio of 100:1 (Tai Po STW sample)

In sample of leachate treatment plant SBR, *Bacillus megaterium*, *Micrococcus luteus*, *Micrococcus lylae*, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* were indentified (Figure 5.11 & Figure 5.12). The previous research has confirmed that *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* had capacities of synthesizing PHAs (Fuchtenbusch et al., 1998; Pham et al., 2004).

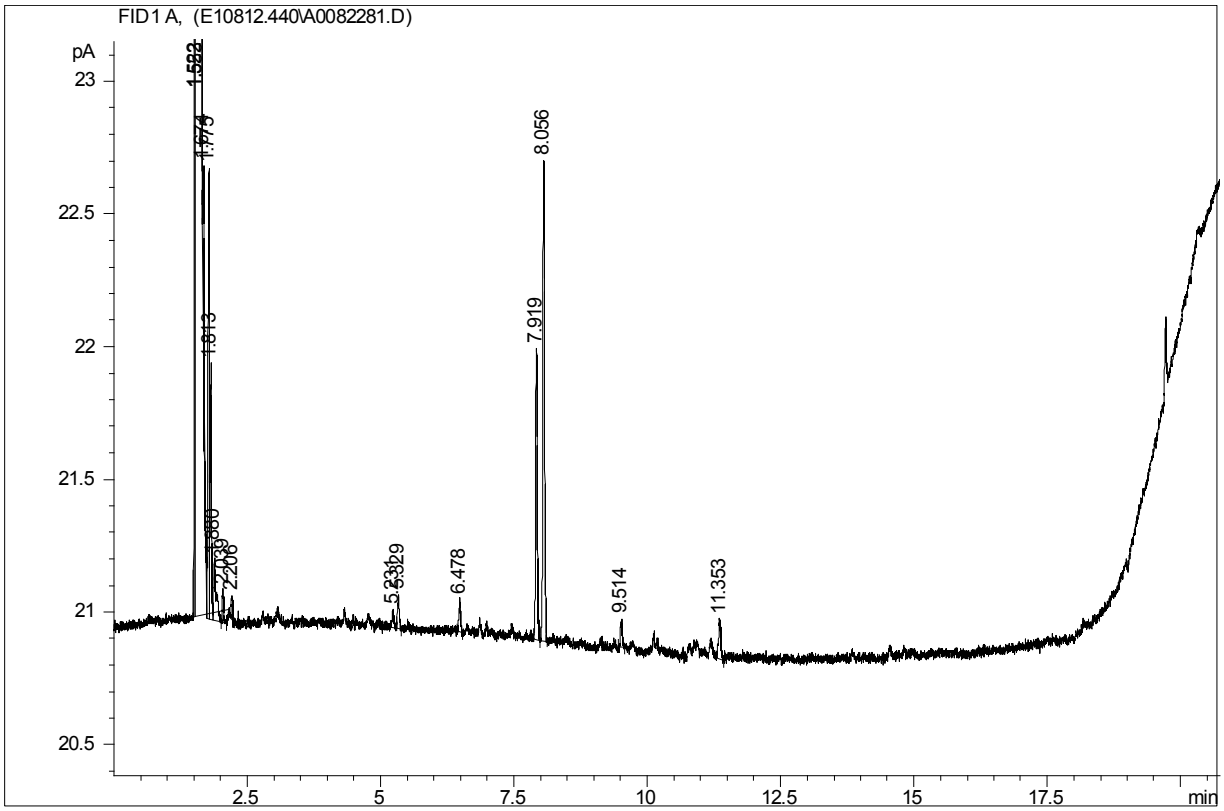


Figure 5.11 MIDI test for activated sludge operated under C:N ratio of 100:1 (leachate treatment plant sample)

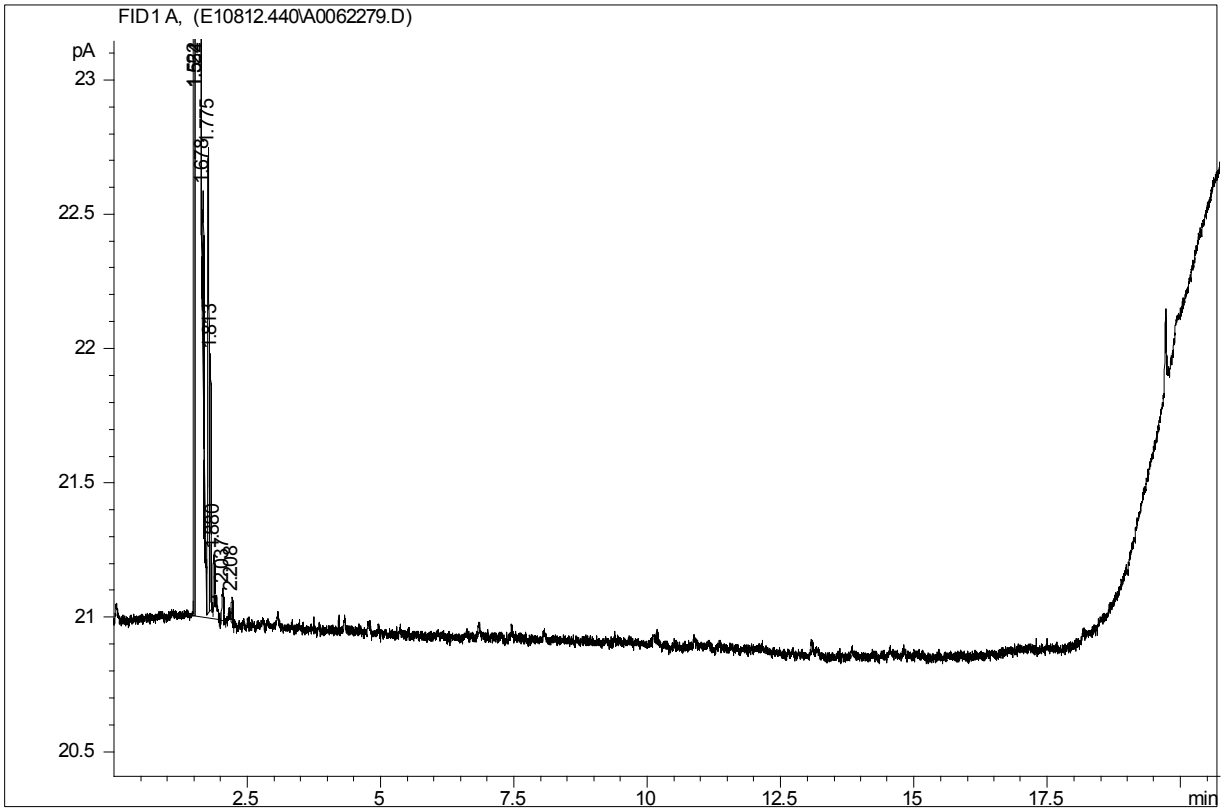


Figure 5.12 MIDI test for activated sludge operated under C:N ratio of 100:1 (leachate treatment plant sample)

The confirmation of some bacteria like *Bacillus sphaericus*, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* demonstrated these activated sludge had ability of accumulating PHAs. Comparison between these two groups of results was found that *Micrococcus luteus* and *Micrococcus lylae* were identified in both two sets of sample. This coincidence conjectured that *Micrococcus luteus* and *Micrococcus lylae* may have capacities of accumulating PHAs. This conjecture was needed more evidence and detection to prove it. Outside the scope of this project, this idea can be added in future study.

5.3 Effect of carbon sources on monomer compositions of PHAs

As mention in Chapter 2 before, HB and HV are the two major monomers in PHAs group. So PHB and PHV were main objects in this part of study that how carbon sources (C_4, C_5 and C_6) affect PHAs compositions. PHAs can have physical properties that range from brittle and thermally unstable to soft and tough, depending upon their PHV/PHB ratios. It was reported that PHBV was more flexible and tougher than PHB for application purposes. And the PHBV copolymer has a lower melting point, which reduces thermal degradation (Byrom, 1992). Therefore, if it would succeed to identify what kind of PHAs compositions were received by using different carbon sources with different proportions, this result would be useful and valuable information and reference for bio-degradable plastic industrial application. From brittle, flexible to elastic, physical properties can be fully controllable.

Results were concluded in the following three figures (Figure 5.13, 5.14 and 5.15). Columns were divided into two parts in each figure. The bottom part of columns stacked from 0% to 100% (1) shows the different ratio weight of C_4, C_5 and C_6 run in the experiments. On the top of columns also stacked from 0% to 100% displays the ratio of HB and HV produced.

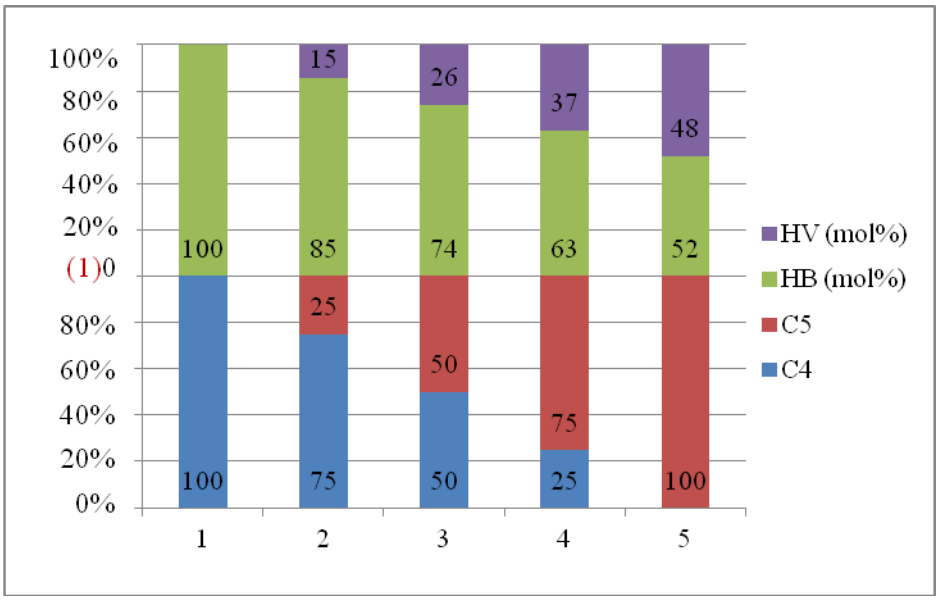


Figure 5.13 Compositions of PHAs under different ratio of C_4 and C_5

Figure 5.13 shows the effects of changes in C_4 to C_5 provided percentages on compositions of PHAs, which was presented on HB and HV mole fraction of PHBV. When butyric acid (C_4) was used as sole carbon source, only PHB homopolymer rather than PHBV was accumulated. On the other hand, when valeric acid (C_5) was used as sole carbon source, not only PHV homopolymer but PHBV appeared simultaneously. However, the highest HV mole fraction appeared when C_5 was used as sole carbon source.

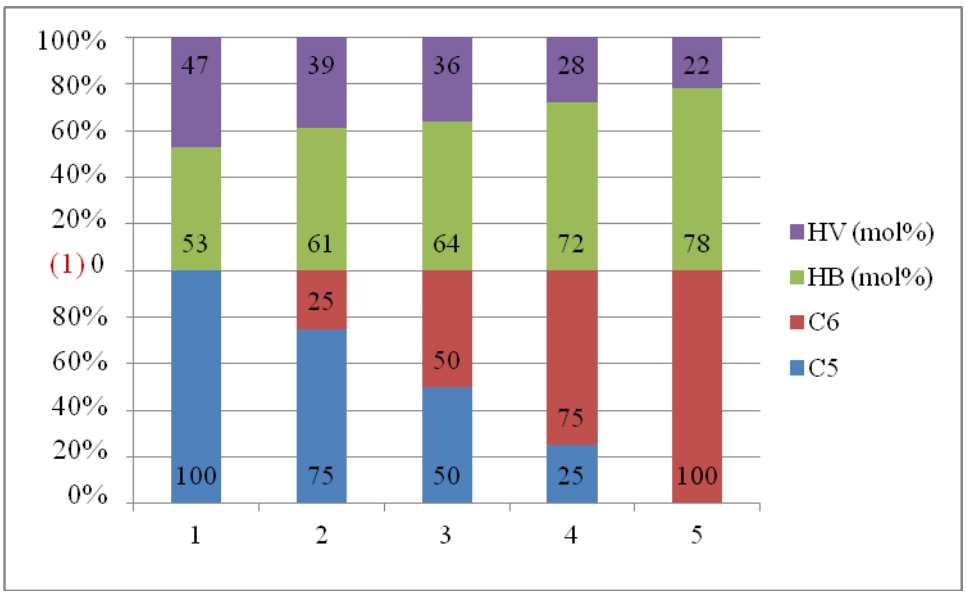


Figure 5.14 Compositions of PHAs under different ratio of C_5 and C_6

In figure 5.14, when glucose (C_6) was used as sole carbon source, HB mole fractions was 78% and HV mole fractions was 22%. When valeric acid (C_5) used as sole carbon source, HB and HV mole fractions were 53% and 47%, respectively. This illustrated that more content of glucose (C_6) utilized, proportion of HB mole fraction was higher.

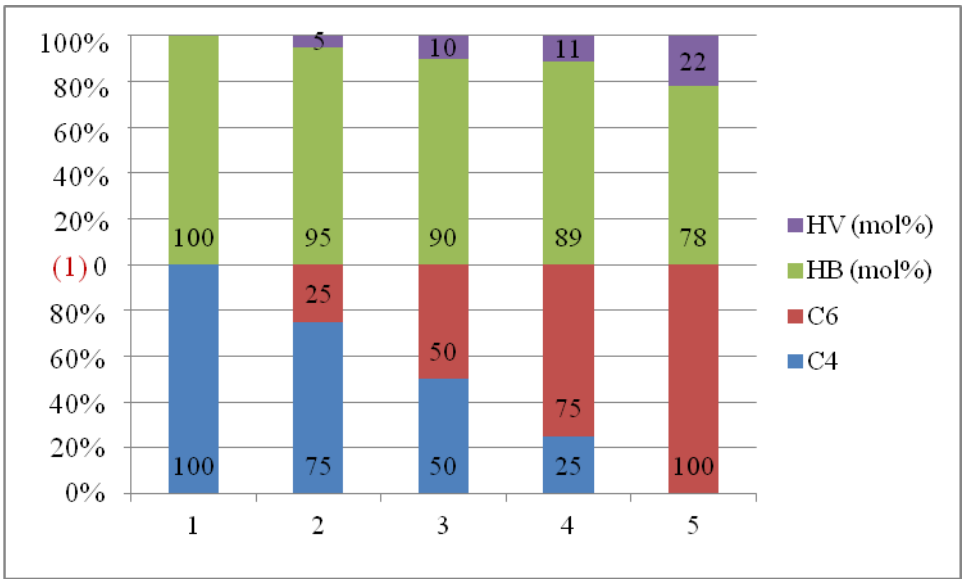


Figure 5.15 Compositions of PHAs under different ratio of C_4 and C_6

In the third group of experiment in this part, the PHV content of the copolymer increased as the glucose concentration in the medium increased. When glucose was used as solo carbon source, HV mole fraction reached 22% and HB was 78% (Figure 5.15).

Compared three sets of experiments, PHBV was accumulated when valeric acid or glucose was used. No PHV but PHB solo appeared only when butyric acid was used as solo carbon source. But proportion of HB mole fraction was higher than HV mole fraction no matter what kind of carbon source was utilized. The minimum difference between HB and HV occurred at C_5 used as sole carbon source. More proportion of C_4 or C_6 used as carbon source, content of HB mole

fractions was resulted in a higher percentage.

5.4 Discussion of Stage I study

In this stage of research, running of lab-scale SBR for production of PHAs could reach a steady state, which implied that design of system was reliable. Functional stability was maintained despite differing and contrasting wastewater sample and microbial populations during PHAs accumulation procession.

Study of part one in Stage I study was focus in the key and significant variables, C:N ratio, to investigate its effect during PHAs production. From an economic point of view, the optimized point under which the maximum PHAs content was obtained per unit nutrient substrate consumed was C:N ratio of 100:1 in both Tai Po wastewater treatment sample and refuse transfer station leachate sample. The nitrogen-deficient condition constrained the biomass growing but simulated the intracellular polymer accumulating no matter in sewage treatment plant sample SBR or refuse transfer station leachate treatment plant sample SBR. However, extreme high nitrogen-deficient condition may negatively affect PHAs storage.

Bacterial identification was applied by MIDI Instant FAMETM Sherlock System

and sampling when SBR systems were running under C:N ratio of 100:1 in both two groups. Some bacteria from two sample, *Bacillus sphaericus*, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*, were identified. They had been confirmed by previous research having abilities of storing PHAs. This indicated these activated sludge had ability of accumulating PHAs.

The last part of Stage I study demonstrated that the monomeric unit composition can be controlled by carbon substrate. The study showed that polymer composition in the final batch stage can readily be controlled independently from the feed composition in the SBR.

CHAPTER 6 RESULTS IN PILOT-SCALE SYSTEM

The pilot-scale system was operated under similar condition as lab-scale. Refer to the previous lab-scale experiment results as well as to reducing cost, investigation of C:N ratio range was narrowed. Five different C:N ratios were studied: 60:1, 80:1, 100:1, 120:1 and 140:1.

The return activated sludge (RAS) of the plant was taken to the reactor and was cultured to store PHAs by applying feast and famine conditions. The effluent of the SBR system was analyzed to examine PHAs concentration. In the start-up period, detection of COD and the SS of the effluent were conducted to determine whether the system was under stable status. The C:N ratio was adjusted by external dosing. The external carbon source (71±5%, glucose solution) was feed in dosing system for adjustment. The COD is greatly increased when adding the external carbon source.

6.1 Investigation of performance in pilot-scale SBR system

The SBR was operating as designed for lab-scale experiments. When stable

operation was attained in the SBR under C:N ratio of 60:1, samples of the mixed liquor were periodically 45 min collected for analysis during the 4 h 30 min reaction time in a randomly selected operation cycle. Analysis of the parameters included residual COD and TKN concentrations, overall biomass growth and polymer (PHAs) accumulation. The values of these parameters were shown in Table 6.1.

Table 6.1 Pilot-scale SBR performance under C:N ratio of 60:1

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.362	0.054	2.115	0.090
0.75	2.454	0.044	2.178	0.109
1.5	1.916	0.038	2.263	0.125
2.25	1.244	0.029	2.369	0.128
3	0.773	0.021	2.432	0.142
3.75	0.336	0.011	2.517	0.156
4.5	0.195	0.006	2.540	0.171

The concentration of carbon, measured as residual COD, in the mixed liquor of the SBR reduced with time from 3.362 g/L to 0.195 g/L, with 94.2% COD removal. Simultaneously the residual TKN decreased from 0.054 g/L to 0.006 g/L. However, as the residual COD and TKN decreased, the overall biomass increased from 2.115 g to 2.540 g, obtaining 0.425 g net biomass growth. As the biomass increased, COD, TKN, and DO decreased but polymer accumulation

increased. This indicated that the biomass has the ability to convert organic pollution, at least in part, into bacterial reserve material, PHAs. During the same period, the polymer content increased from 0.090 g to 0.171 g, accumulating 0.081 g net intracellular polymers.

After the experiment under C:N ratio of 60:1 was completed, ammonium chloride concentration was reduced to result in C:N ratio of 80:1. Similar observation was obtained as performance in the case under C:N ratio of 60:1 and 80:1. The residual COD and TKN decrease from time while the overall biomass and polymer accumulation increase. The performance of each parameter was shown in Table 6.2.

Table 6.2 Pilot-scale SBR performance under C:N ratio of 80:1

React time (h)	**Residual COD (g/L)**	**Residual TKN (g/L)**	**Overall biomass (g)**	**Polymer accumulation (g)**
0	3.357	0.041	2.106	0.109
0.75	2.316	0.032	2.148	0.132
1.5	1.913	0.027	2.274	0.149
2.25	1.343	0.017	2.317	0.153
3	1.041	0.012	2.401	0.177
3.75	0.638	0.008	2.432	0.186
4.5	0.266	0.006	2.468	0.214

The residual COD reduced over time from 3.357 g/L to 0.266 g/L, with 92.1%

COD removal. At the same running time, the residual TKN decreased from 0.041 g/L to 0.006 g/L. However, as the residual COD and TKN decreased, the overall biomass increased from 2.106 g to 2.468 g, obtaining 0.362 g net biomass growth. Also, the polymer content increased from 0.109 g to 0.214 g, accumulating 0.105 g net intracellular polymers.

Table 6.3 Pilot-scale SBR performance under C:N ratio of 100:1

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.375	0.033	2.058	0.142
0.75	2.599	0.027	2.140	0.179
1.5	2.261	0.021	2.181	0.195
2.25	1.823	0.016	2.243	0.205
3	1.080	0.011	2.264	0.230
3.75	0.810	0.008	2.311	0.242
4.5	0.285	0.006	2.367	0.269

Under C:N ratio of 100:1, the residual COD reduced over time from 3.375 g/L to 0.285 g/L during the reaction time, with 91.6% COD removal. Simultaneously the residual TKN decreased from 0.033 g/L to 0.006 g/L. However, the overall biomass increased from 2.058 g to 2.367 g, with 0.309 g net biomass growth. The polymer content increased from 0.142 g to 0.269 g, receiving 0.127 g net intracellular polymers (Table 6.3).

Table 6.4 Pilot-scale SBR performance under C:N ratio of 120:1

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.314	0.027	2.029	0.126
0.75	2.552	0.022	2.110	0.150
1.5	2.051	0.017	2.212	0.159
2.25	1.624	0.012	2.232	0.177
3	1.127	0.009	2.272	0.191
3.75	0.663	0.005	2.354	0.215
4.5	0.252	0.004	2.292	0.245

Under C:N ratio of 120:1, the residual COD reduced over time from 3.314 g/L to 0.252 g/L during the reaction time, with 92.4% COD removal. Simultaneously the residual TKN decreased from 0.027 g/L to 0.004 g/L. When the C:N ratio was further increased to 80:1, the overall biomass increased from 2.029 g to 2.292 g, with 0.263 g net biomass growth. The polymer content increased from 0.126 g to 0.245 g, receiving 0.119 g net intracellular polymers (Table 6.4).

Table 6.5 Pilot-scale SBR performance under C:N ratio of 140:1

React time (h)	Residual COD (g/L)	Residual TKN (g/L)	Overall biomass (g)	Polymer accumulation (g)
0	3.321	0.024	2.113	0.125
0.75	2.740	0.020	2.143	0.150
1.5	2.352	0.017	2.159	0.158
2.25	1.561	0.012	2.198	0.169
3	1.020	0.007	3.275	0.197
3.75	0.711	0.003	2.267	0.212
4.5	0.226	0.002	2.337	0.243

As C:N ratio increase, Table 6.5 displays the revolution of PHAs storage performance under C:N of 140:1. It illustrated that the residual COD reduced over time from 3.321 g/L to 0.226 g/L during the reaction time, with 93.2% COD removal. At the same time, the residual TKN decreased from 0.024 g/L to 0.002 g/L. However, the overall biomass increased from 2.113 g to 2.337 g, with 0.224 g net biomass growth. The polymer content increased from 0.125 g to 0.243 g, receiving 0.118 g net intracellular polymers.

Observed from results under different C:N ratio, it was found that when the residual COD and TKN declined, the overall biomass and polymer accumulation ascended gradually. This showed a similar trend with observation in lab-scale results. These results stated that the microorganisms in reactor had the ability to convert carbon source into PHAs when using activated sludge from this pilot-scale reactor.

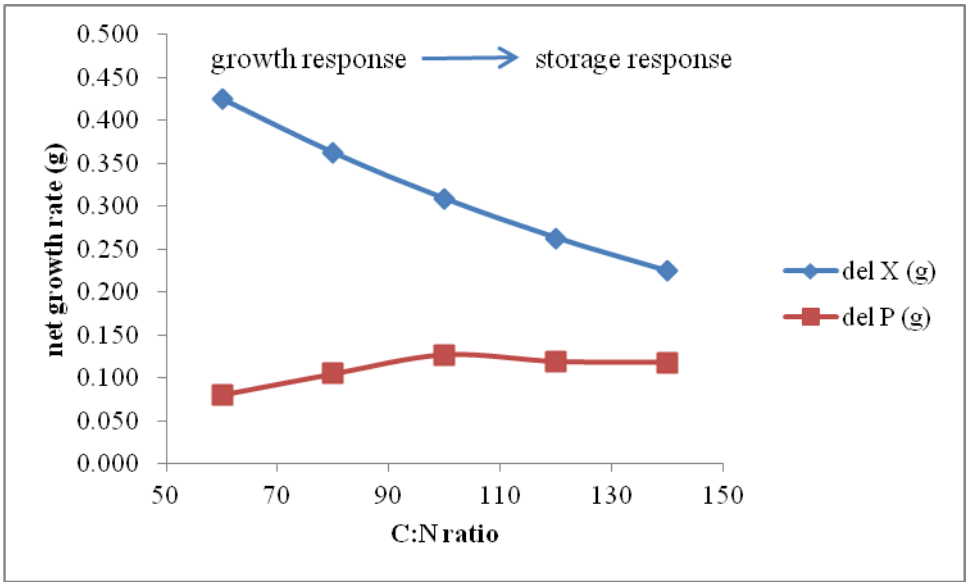


Figure 6.1 Corresponding between growth and storage response in pilot-scale SBR with an increase of C:N ratio

Further analysis and comparisons were conducted, and it was found that the net PHAs accumulation, del P, increased from 0.081 g to 0.118 g. Whereas net biomass growth, del X, decreased from 0.425 g to 0.224 g as C:N ratio increased. These results implied that the storage capacity of polymers enhanced with the increase of C:N ratio once again. The bacterial communities' growth balance moved from biomass growth response to internal storage response in the pilot-scale reactor with the occurrence of nitrogen limitation.

Another important indicator, the overall polymer production yield (Y p/s), was needed to be evaluated again. It had the economic significance of PHAs

commercial production.

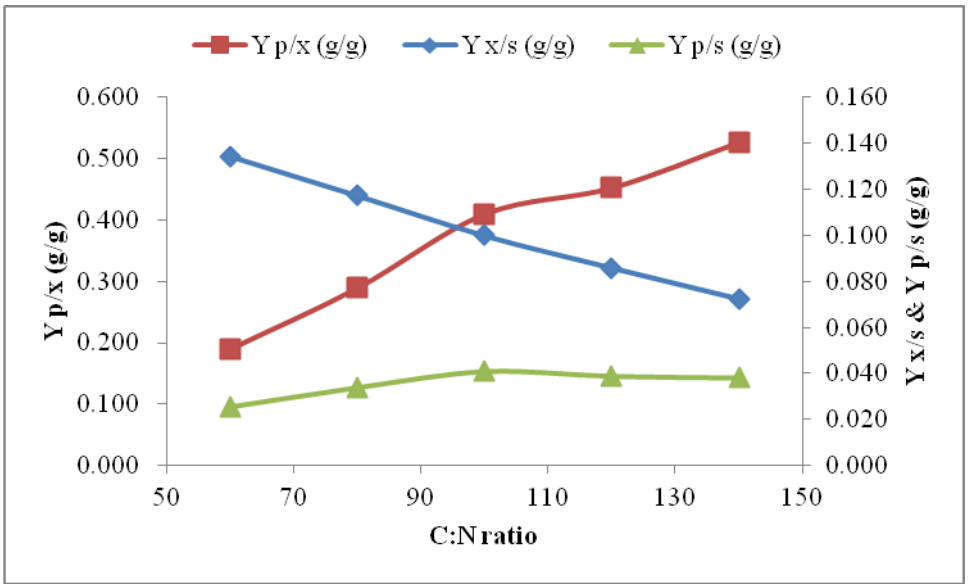


Figure 6.2 The effect of C:N ratio on biomass and polymer growth in pilot-scale SBR

After work of mathematical calculation, it shows that Y x/s declined from 0.134 to 0.072 g biomass/ g COD consumed, which implied growth of biomass was not promoted under aggravated nitrogen limitation environment. But Y p/x was increasing from 0.190 to 0.526 g polymer accumulated / g biomass as C:N ratio increased, which meant capacity of PHAs storage was increasing. The most important indicator for measure manufacturing cost, Y p/s, reached the highest value at C:N ratio of 100:1. This results confirmed C:N ratio of 100:1 was an optimal condition for PHAs production in this SBR system.

Figure 6.2 had a similar trend with Figure 5.2 and Figure 5.4 (Chapter 5). Y p/s was increasing while Y x/s was decreasing as nitrogen deficiency intensified. Y p/s reached a maximum under C:N ratio of 100:1. Such similarity drew an interest to compare these three sets of experiments. Further discuss would be in Chapter Seven.

6.2 Analysis of bacterial identification in pilot-scale study

Sample was collected and analyzed by MIDI Instant FAMETM Sherlock System. When the integration of peak was performed, an electronic signal from detector was passed to the computer and compared to a stored database. Consequently, bacteria in sample were identified. Refer to results of 6.1., PHAs content by unit substrate used was recorded a maximum under C:N ratio of 100:1. Therefore, samples of the mixed cultures during C:N ratio of 100:1were randomly collected and analyzed for bacterial identification.

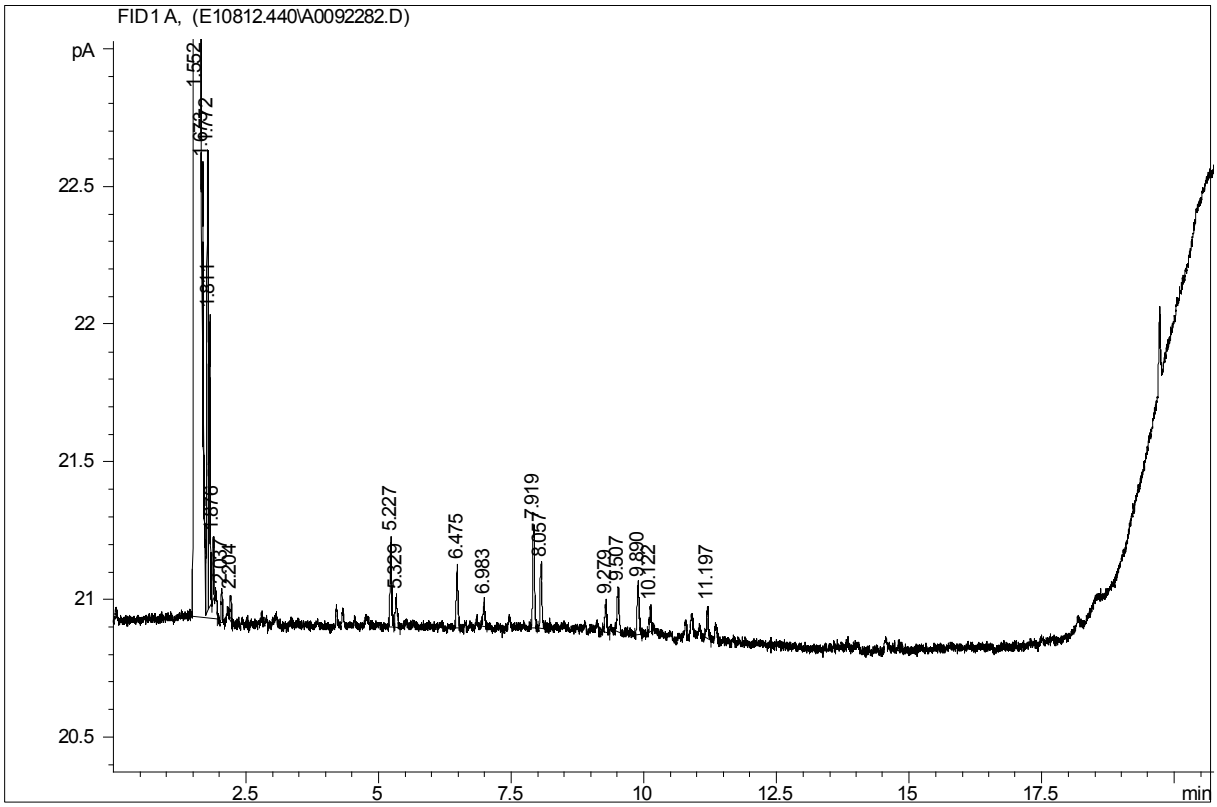


Figure 6.3 MIDI test for activated sludge operated under C:N ratio of 100:1 in pilot-scale SBR

Bacillus cereus, *Rhodopseudomonas palustris*, *Rhodopseudomonas sphaeroides* and *Salmonella typhimurium* were identified in sample from the pilot-scale SBR system. *Bacillus cereus* was one of bacteria having capacities of PHAs accumulation (Caballero et al., 1995). Also *Rhodopseudomonas* sp., was confirmed to accumulate PHAs under microaerobic and photoheterotrophic conditions (Wasimul et al., 1996). It had been reported that under nitrogen-limiting conditions a PHAs content of up to 60 to 70% of the cellular dry weight was detected in *Rhodopseudomonas sphaeroides* over two decades

(Brandl et al., 1991).

6.3 Discussion of Stage II study

Based on the previous lab-scale experience, pilot-scale system was set-up. The results stated that producing PHAs from activated sludge was easy and stable to operate, and possibly run in large scale.

The important indicator, Y p/s, reached a maximum under C:N ratio of 100:1. In order to achieve maximum economic benefits, such nitrogen-deficiency condition was a significant reference for PHAs industrial application.

Bacteria was identified under the best operational condition (C:N = 100:1). *Bacillus cereus*, *Rhodopseudomonas palustris*, *Rhodopseudomonas sphaeroides* and *Salmonella typhimurium* were identified in sample. Some of them were reported having abilities of accumulating biopolymers in past studies.

CHAPTER 7 COMPARISON BETWEEN LAB-SCALE AND PILOT-SCALE STUDY

In this chapter, compariosn between laboratory-scale and pilot-scale study was discussed in order to measure the feasibility of large scale production of PHAs rather than stay at the stage of lab-scale study. Systems performances and producing polymers capacities are criteria to justify the choice of producing PHAs by activated sludge in SBR system. The comparison was based on the same operational strage of three sets of studies. In addition, cost of producing PHAs was mostly concerned. It was related to fix capital cost, operational cost and maintenance cost. Therefore, cost-benefit analysis would be in progress in this chapter.

7.1 Comparison of performance between lab-scale and pilot-scale system

Figure 7.1, Figure 7.2 and Figure 7.3 allowed the comparison between the performances of lab-scale system and pilot-scale system. The specific biomass yield, Y x/s, was found to be in reduction in three groups of experiment with the

increase of C:N ratio. Values of Y x/s and Y x'/s' were always higher than Y x"/s" in the process. When C:N ratio was 60:1, Y x'/s' sampled in leachate treatment plant was the largest than other groups, which indicated their bacteria had the strongest capacity of biomass growth among three sets of experiment. While over C:N ratio of 80:1, Y x/s turned to be the highest one, which meant it had better ability of biomass growth in higher nitrogen-deficiency environment than other two groups. No matter what C:N level was, Y x"/s" was the smallest one, around 1/3 to 1/2 of amount in lab-scale.

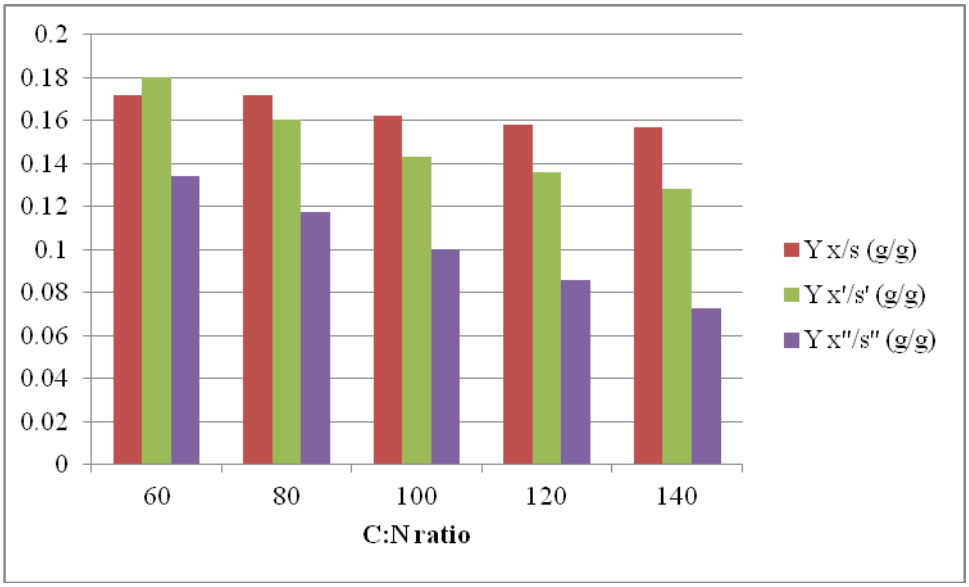


Figure 7.1 The specific biomass grwoth in lab-scale and pilot-scale system.

The specific polymer yield showed the intracelluar accumulating PHAs rate of microorganism in mixed activated sludge. As C:N ratio increased, the Y p/x, Y

p’/x’ and Y p”/x” were all increasing. Their maximum were 0.386, 0.533 and 0.526 g polymer accumulated / g biomass with C:N ratio from 60:1 to 140:1 among three sets of experiment, respectively (Figure 7.2). Such results indicated that a supplement with more carbon substrate would result more storage of PHAs generally.

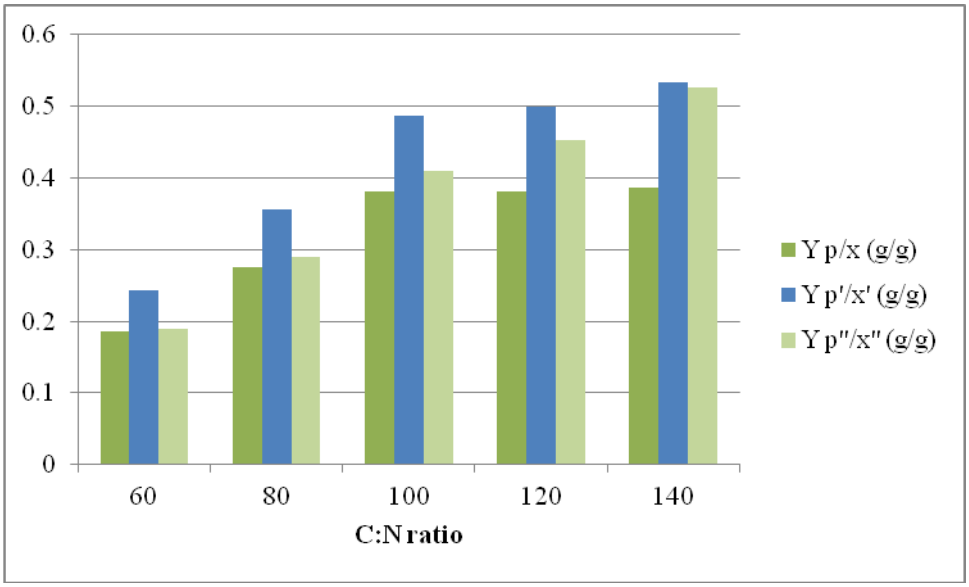


Figure 7.2 The specific polymer growth in lab-scale and pilot-scale system.

Many previous studies focused on the development of high yield in net polymers. The role of overall polymer yield per substrate consumed was ingored. However, high value of net polymers yield by an exchange of a large amount of carbon nutrition was of little significance in munufactoring. The Y p/s was an essential indicator of cost in producing PHAs for economic reasons. In order to produce PHAs in a cost-effective and environmentally friendly way, Y p/s was engaged in

scientific pursuit. When Y p/s was larger, the profit of production was higher. During our research, Y p/s , Y p'/s' and Y p"/s" obtained their maximum point under C:N ratio of 100:1 among three groups system.

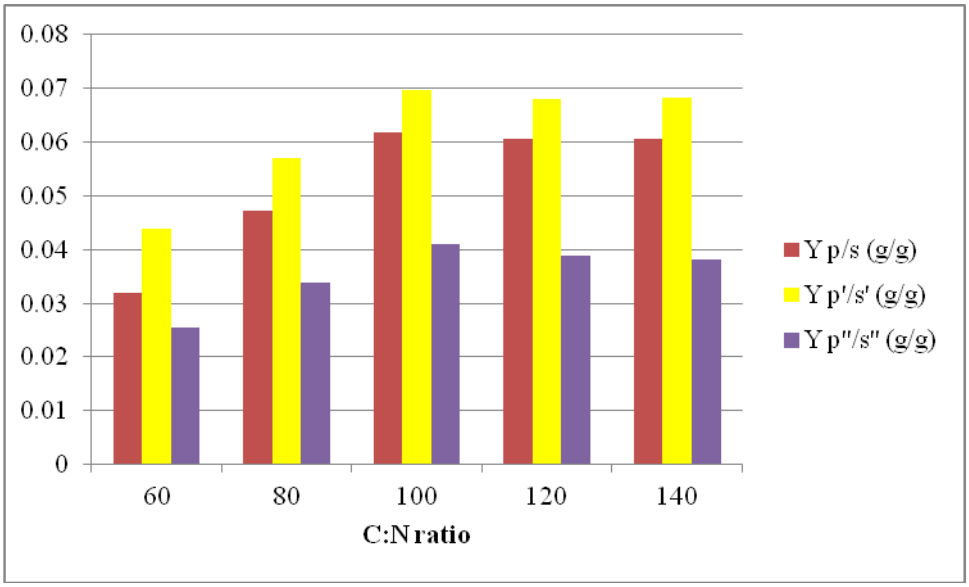


Figure 7.3 The overall polymer grwoth in lab-scale and pilot-scale system.

The maximum of Y p/s and Y p'/s' were 0.062 and 0.070 g polymer accumulated / g COD consumed in Tai Po Sewage WWT and leachate treatment plant, respectively. The Y p"/s" was 0.041 g polymer accumulated / g COD consumed and less than Y p/s and Y p'/s' under similar operational condition. One possible explation of better performance of lab-scale system to produce PHAs was daily wastewater influent quality was not stable in pilot-scale system which would result lower Y p"/s". But 0.041 g polymer accumulated / g COD consumed of Y

p”/s” may still lie in a acceptable range and pratical in application. The overall running of pilot-scale SBR system was well and stable for its designed application. It will be compared with Y p/s in pure culture to disccuss whether such yield worths using in process.

7.2 Cost-benefit comparison between this project and traditional pure culture of PHAs

7.2.1 Y p/s comparison between this project and pure culture of PHAs

As mentioned in Chapter 2, a major obstacle to the introduction of PHAs as a replacement of petroleum-based plastics was the high cost associated with PHAs production. But carbon substrate contributed 40% towards the total production cost (Reis et al. 2003; Salehizadeh and Van Loosdrecht 2004), in order to reduce the capital cost of producing, cheaper carbon substrate (wastewater) and accumulator (bacteria in activated sludge) were employed.

The PHAs accounted 40-50% of dry cell mass in mixed culture with different carbon resource under C:N ratio of 100:1 (Figure 7.2). In the purpose of verifing the optimized strategy in our study worked well and competitive, the

performance of activated sludge in the best grwoth condition (C:N ratio of 100:1) was compared with performances of several common bacteria which can accumulate PHAs in pure culture. *Azotobacter vinelandii* UWD, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas sp.*, *Ralstonia eutropha* and Recombinant *E. coli* were involed in these comparison. Table 2.3 demonstrated various bacteria having different capacities of accumulating PHAs.

Azotobacter vinelandii UWD, a mutant strain that produces PHAs during growth on a variety of unrefined sugar sources including beet molasses, cane molasses, and corn syrup. It accumulated PHAs up to 79% in in glucose medium. However, the Y p/s was 0.003 (Page et al., 1992).

Pseudomonas aeruginosa was another one common PHAs accumlator and grown on different n-alkanoic acids for PHAs accumulation. The PHAs yield measured as % cellular dry weight of the biomass. *Pseudomonas aeruginosa* varied from 0.27% to 13.4%, depending on the type of fatty acid used and the limitation of Mg in the medium. The highest PHAs yield was observed in accumulating 13.4% PHAs in biomass with tridecanoic acid in magnesium (Mg) deprived. And the Y p/s was about 0.091 (Barbuzzi et al., 2004).

In cultures of *Pseudomonas putida*, with glucose and fructose as carbon source, limitation of either nitrogen or phosphorus, and minimum oxygen supply, PHAs yields were reported to be 21% and 63% PHAs per cell dry mass, with the PHAs yield factors per substrate consumed (Y p/s) being 0.15 and 0.019 g/g, respectively (Diniz et al., 2004).

One *Pseudomonas sp.* showed 99% similarity to three recognized strains of the genus Pseudomonas, that was, *Pseudomonas putida* ATCC 17453, *Pseudomonas putida* 17514 and *Pseudomonas sp.* strain ONBA. The PHAs accumulative capacity of this *Pseudomonas sp.*, especially under conditions of both nitrogen and oxygen limitation, PHAs % was 11.2% and Y p/s was as low as 0.033 g polymer accumulated / g substrate consumed (Kourmentza et al., 2009).

Ralstonia eutropha can intrinsically synthesize PHAs from various renewable inexpensive carbon sources, like sugars and plant oils, its recombinant PHAs production is relatively stable. It exhibited the highest accumulation of PHAs up to 76% from soybean oil. And the Y p/s was 0.76 g polymers accumulated / g soybean oil used (Kahar et al., 2003).

Recombinant *E. coli* to producing PHAs has been intensively investigated. During the fed-batch culture of recombinant *E. coli*, a large amount of oxygen was necessary to maintain the dissolved oxygen concentration above 20% of air saturation. The ratio of Recombinant *E. coli* uptake to PHAs storage used glucose as carbon source was about 13.4% and 0.058 g polymer accumulated / g substrate consumed under nitrogen limitation but adequate DO conditions (Lee et al., 2001)

Table 7.1 PHAs production yeilds by mixed cultures and pure cultures

Culture Type	**Limitation**	**Extral reqirement**	**PHAs % (Y p/x)**	**Y p/s**
Mixed culture – L1	N		38.1	0.062
Mixed culture – L2	N		48.7	0.070
Mixed culture – P1	N		41.0	0.041
***Azotobacter vinelandii* UWD**		disinfection	79.0	0.003
Pseudomonas aeruginosa	Mg	disinfection	0.27 - 13.4	0.091
Pseudomonas putida	N, O_2	disinfection	21.0	0.15
	P, O_2	disinfection	63.0	0.019
Pseudomonas sp.	N, O_2	disinfection	11.2	0.033

Ralstonia eutropha	N	disinfection	76	-
Recombinant *E. coli*	N	Adequate O_2, disinfection	13.4	0.058

*Mixed culture – L1: Lab-scale experiment with Tai Po STW sample

Mixed culture – L2: Lab-scale experiment with leachate treatment plant sample

Mixed culture – P1: Pilot-scale experiment by mixed culture

Compared with the traditional pure culturation of PHAs, the PHAs% of mixed culture under designed operational condition was not low, in some cases, PHAs% in mixed culture L1, L2 and P1 were much higher than those in pure culture. For instance, PHAs% accumulated by *Pseudomonas sp.* and Recombinant *E. coli* were just 1/3 to PHAs% in mixed culture L1, which was the smallest one in three sets of experiments. Though Y p/s in mixed culture L1, L2 and P1 were smaller than Y p/s of *Pseudomonas aeruginosa* and *Pseudomonas putida*, they were higher than Y p/s in the other bacteria. This was obviously shown that producing PHAs under certain designed condition by mixed culture was strongly competitive to pure culture. The high overall PHAs storage rates as well the high specific PHAs contents achieved by mixed cultures make this process competitive with those based on the use of pure cultures.

7.2.2 Other operating cost between our project and pure culture of PHAs

In pratical application, cost of producing PHAs contained not only the cost of material and carbon source. Fix capital cost, operational cost and maintenance cost were also taken into account.

Mixed cultures L1, L2 and P1 did not require additional and expensive process of environmental disinfection and longer selection time but the pure culturation did. Disinfection process would increase the cost in fixed capital cost.

In addition to disinfection step, some pure culturation required certain circumstances to promote PHAs accumulation. For example, PHAs cultured by recombinant *E. coli* necessarily required a large amount of oxygen. Since the use of a large amount of pure oxygen is economically unfavorable and oxygen transfer is generally poor in a larger scale fermentor, such pure culturation does not have competitive advantages in commercial production.

Some pure cultures required the simultaneous limitation of two or more nutrients, making the operation process complex and maintance cost increased. Generally, a simply process can save a huge amount of time, humman work loading and the

running cost.

In conclusion, assosiated with other operational and maintanance cost, runnin a SBR system with activated sludge was concerned as a superior method to produce PHAs.

CHAPTER 8 CONCLUSIONS

8.1 Conclusions

Results in lab-scale and pilot-scale have shown that producing PHAs in activated sludge under certain condition was a promising alternative approach to explore a biodegradable plastic industrial application.

One desirable goal that use of waste materials as feedstock for production of biomaterials was obtained. The performance of the SBR for production of PHAs by activated sludge was good and steady. Cheaper raw material and its efficient use have direct impaction the final price of the product in market. This demonstrated activated sludge was a good and inexpensive candidate to storing PHAs.

Before lab-scale and pilot-scale study, a preliminary study helped to find out the significant variables influencing on the output. Usually, there is a long list of variables that may affect the process. It immediately became obvious that a

complete factorial with all the factors and combinations required an unreasonable number of runs. To address this problem, Plackett-Burman design was adopted. Its analysis stated that C:N ratio was the key variable during the PHAs optimization process. Therefore, this variable became the main object in lab-scale and pilot-scale study. Also, from the Plackett-Burman design results, it was suggested Feast/Famine to be 1/3 and SRT designed as 10 days. Oxygen concentration and pH were not significant factors and would be without control in both lab-scale and pilot-scale study.

During the first part of lab-scale study, C:N was adjusted in different ratio to investigate the performance of PHAs yield and best value of production. A parameter Y p/s played a significant role in evaluating the cost of PHAs production. Less carbon used was associated with a higher profit obtained. So a larger Y p/s indicated that more PHAs accumulated in the cell and less carbon/raw material used. The optimized C:N point with the maximum PHAs content was 100:1 in both two groups of studies. Under C:N ratio of 100:1, the maximum PHAs content production from Tai Po wastewater treatment sample and refuse transfer station leachate sample were 14.78% and 18.47%, respectively.

Bacillus sphaericus, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* were identified in two groups of lab-scale study in Part two, Stage I. These bacteria had been proved to have abilities of storing PHAs by previous research.

The last part of Stage I study was about effect of carbon sources on monomer compositions of PHAs. Results were shown that PHBV was accumulated when valeric acid or glucose was used. No PHV but PHB solo appeared only when butyric acid was used as solo carbon source. It demonstrated that the monomeric unit composition can be controlled by carbon substrate.

Not only success in lab-scale production, pilot-scale processing is successful also. The pilot-scale study provides a detailed demonstration of the principle of enhanced PHAs accumulation via nitrogen-deficiency stimulation in mixed culture in large scale, paving the way to exploit the opportunity to refine and optimize the PHAs productive process. Y p/s reached a maximum 0.041 polymer accumulated / g COD consumed under C:N ratio of 100:1. It is becoming obvious that low nitrogen environments promote PHA accumulation within mixed cultures in either lab-scale or pilot-scale system.

The comparison between lab-scale and pilot-scale results shown that the Y p/x, Y p'/x' and Y p"/x" were all increasing as C:N ratio increased. Their maximum were 0.386, 0.533 and 0.526 g polymer accumulated / g biomass with C:N ratio from 60:1 to 140:1 among three sets of experiment, respective. And the Y p/s, Y p'/s' and Y p"/s" obtained their maximum point under the same C:N ratio (C:N = 100:1) in three groups of systems.

8.2 Suggestions for future research

Based on the findings from this study, some suggestions for future research work are proposed.

Limitation or partial limitation of a single nutrient, like P, as used for commercial production, may not promote massive PHA production in activated sludge biomass compared to the simultaneous limitation of two or more nutrients. To obtain higher cellular PHA accumulation, study of combining two or more nutrients

Kinetics of analysis can provide a comprehensive understanding of the optimized

system. The modified model could be used as a guide for the further design and operation of industrial-scale systems.

As mentioned in Chapter 5 bacterial identification works, *Micrococcus luteus* and *Micrococcus lylae* both appeared in two groups of systems, which may have potential capacities of storing PHAs. These may draw an attention for study these bacterium. They can be tested and verified whether they have abilities to produce PHAs further. And if they succeed in accumulating PHAs as intracellular energy, what kind of PHAs will be obtained during process will be a contribution and enrich the study about different composition of PHAs.

Recovery process affected the final percentage of PHAs to manufacture commercial products. A high efficiency recovery rate will further reduce the cost of production. Most current study and experimental guide were just shown how PHAs was extracted in lab-scale. Improvement of the efficiency of PHAs recovery and making it possible to extract PHAs in large scale will be a useful extension work in biodegradable plastic industrial production.

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