ENHANCEMENT OF WASTEWATER TREATMENT WITH MICROBIAL FORMATION OF POLY(β-HYDROXYALKANOATES) FROM ACTIVATED SLUDGES

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Conventional plastics are inherently non-biodegradable and are posing a serious threat to our environment. Polyhydroxyalkanoates (PHAs) and related co-polymers have been discovered and emerged as environmentally-friendly materials. A wide variety of microorganisms can synthesize an optically active polymer of D(-)-3-hydroxybutyric acid as intracellular carbon and energy sources under certain specific production conditions. However, the PHA production cost is too high at present, thus hampering the development of its manufacturing processes and widespread applications. It is know that bacteria indigenous to activated sludge can accumulate large quantities of PHA under environmentally controlled conditions. The main aim of this research project is to investigate the behaviour of the microorganism community from different activated sludge, capable of accumulating PHA by using real wastewater and removing pollutant in wastewater so as to offer a potentially more environmentally-effective method of PHA production.

The microbial communities of 9 activated sludge from different industries in Hong Kong were investigated to confirm their feasibility of PHA accumulation. R2A agar successfully screened out PHA biomasses in the activated sludges. The biomasses were then identified as *Pseudomonas huttiensis*, *Bacillus cereus*, *Pseudomonas putida*, *Bacillus pumilus*, *Bacillus pumilus*, *Sphingopyxis terrae*, *Aeromonas ichthiosmia* and *Yersinia frederiksenii.*

Accord to the community study with shake flask experiments, *Sphingopyxis terrae* and *Aeromonas ichthiosmia* accumulated more than 30% PHB as intracellular

content. *Bacillus pumilus* could accumulate up to 20% PHV as cellular content. SBR application showed that the feeding influent of real wastewater supported the growth of the identified PHA biomasses. Mono strains of *Bacillus pumilus*, *Pseudomonas putida* and *Sphingopyxis terrae* had better BOD and COD removal efficiency than other biomasses. Results showed that increasing the influence concentrations would increase the system stabilization time of bacteria to achieve over 90% COD, BOD and KN removal and also affecting the PHA accumulation capabilities. *Aeromonas ichthiosmia* achieved the highest PHA accumulation. The bacteria accumulated more than 22% PHB and more than 8% PHV of intracellular content when influent concentration of 6,500ppm COD applied. Although increasing of organic loading resulted in higher PHA yield, there was an inhibition phenomenon found in all identified bacteria when 12,000ppm COD applied.

Mixed strains of *Pseudomonas huttiensis* & *Bacillus pumilus* and *Pseudomonas huttiensis* & *Sphingopyxis terrae* achieved 2 days less treatment time than the mono strain to stabilize the system in achieving over 90% COD, BOD and KN removal for the influent concentration ranging from 6,000ppm to 12,000ppm COD. The results suggested that highest PHB production enhancement found by mixing *Pseudomonas putida* with *Yersinia frederiksenii*. A maximum yield of 25% PHB of intracellular contents was produced by mixing these 2 bacteria. It reflected more than 130% PHB accumulation enhancement achieved by mixed strains of *Pseudomonas putida* & *Yersinia frederiksenii*. A decrease in PHA accumulation occurred when 15,000ppm influent COD was applied. The results showed that mixed strains could perform enhancement of pollutant removal and PHA accumulation under a higher organic loading environment than mono strain did.

LIST OF PUBLICATIONS

Related Conference and Journal Papers:

Wang Y.J., **Lam, W.,** Chua, H., Lo, W.H., Yu, H.F. (2005) Integrated process for Microbial Formation of Poly(3-hydroxyalkanoates) from Activated Sludges with Enhancement of Wastewater Treatment. *8th International Conference of the Aquatic Ecosystem Health and Management Society, Restoration and Remediation of Aquatic Ecosystems:Tools, Techniques and Mechanism*. Nanjing-Hangzhou, CHINA.

Lam, W., Chua, H., Lo, W.H., Yu, H.F. (2005) Enhancement Process for Microbial Formation of Poly(β-hydroxyalkanoates from Activated Sludges with Enhancement of Wastewater Treatment – (a). *27th Symposium on Biotechnology for Fuels and Chemicals*. Denver-Colorado, USA

Chan, P.L., Vincent, Y., **Lam, W.,** Yu, H.F. (2006) Production of medium chain

length Polyhydroxyalkanoates by *Pseudomonas aeruginose* with Fatty acids and alternative carbon sources. *Applied Biochemistry & Biotechnology*. **29**:933-941.

Ruan, L., Wang, Y.J., **Lam, W.,** Yu, H.F. (2007) Microcalorimetric Research on Recombinant Escherichia Coli with High Production of Polyhydroxyalkanoates(PHAs). *Journal of Thermal Analysis and Calorimetry.* **89**(3):953-956.

Lam, W., Chua, H., Lo, W.H., Yu, H.F. (2007) Effects of influence COD on PHA production and waste removal by PHA strains isolated from activated sludges. *Proceeding Annual Meeting of Society of Industrial Microbiology.* Denver-Colorado, USA.

Lam, W., Yu, H.F. (2008) Integrated Process of Poly(β-hydroxyalkanoates) for Wastewater treatment from activated sludges with isolated PHAs strains. *Industrial Biotechnology Conference – 2008*. Nanjing-Hangzhou, CHINA.

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ABBREVIATIONS

Acetoacetyl-CoA	Acetoacetyl-coenzyme A
Acetyl-CoA	Acetyl-coenzyme A
BOD	Biological Oxygen Demand
CDW	Cell Dry Weight
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
FTIR	Fourier Transform Infrared
GC	Gas Chromatography
GC-FID	Gas Chromatography Flame Ionization Detector
HB	3-hydroxybutyrate orβ- hydroxybutyrate
HV	3-hydroxyvalerate orβ-hydroxyvalerate
NADH	Nicotinamide Adenine Dinucleotide
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate or Polyhydroxybutyric acid
PHV	Polyhydroxyvalerate
TKN	Total Kjeldahl Nitrogen

CHAPTER 1

INTRODUCTION

1.1 Background

Throughout history, humans have relied extensively on biological materials like wool, leather, silk and cellulose. Today, polymers can be synthetically made and tailored to meet specific needs. Although polymers play a central role both in the natural world and in modern industrial economies, the growing reliance on synthetic polymers has raised a number of environmental and health concerns. For instance, most plastic materials are not biodegradable and are derived from nonrenewable resources. The very properties of durability and strength that make these materials so useful also ensure their persistence in the environment and complicate their disposal (Byrom, 1991). In addition, the synthesis of some polymeric

materials involves the use of toxic compounds or the generation of toxic byproducts (Hong Kong Environmental Protection Department, 2002).

Plastics recycling may be a solution to minimizing plastics waste management. However, there are some constraints due to the practical difficulties in separating plastics waste from waste streams, the relatively low market value of the recycled materials, the high labour cost and the space requirement for their washing and sorting (Hong Kong Environmental Protection Department, 2002). These problems have aroused interests in polymers that are derived from biological precursors or produced by methods of modern biotechnology. The advent of modern biotechnology has fundamentally transformed the way scientists view organisms and the materials they produced. By harnessing the enzymes found in nature, or transforming agricultural or marine feedstocks, a new class of biodegradable, biocompatible and renewable materials is on the horizon (Poillon, 2003). Such biopolymers may prove to have a variety of environmental benefits. Possible applications range from agriculturally or bacterially derived thermoplastics that are truly biodegradable, to novel medical materials that are biocompatible, to water treatment compounds that prevent mineral

buildup and corrosion (Poillon, 2003). In particular, the widely publicized disposal problem of traditional oil-based thermoplastics has promoted the search for biodegradable alternatives (Florence, 2003). Therefore, looking for “environment friendly” plastics is one of the solution to this problem.

1.2 Environmentally Friendly Plastics

Scientists have been interested in wholly degradable plastics include modified starch, composites of starch and other natural polymers, chemically synthesized polyactities and aliphatic polyesters (Maddever, 1989). Much of the interest in biopolymers stems from the growing concern about the environmental impacts of synthetically produced materials. One of the most important characteristics of microbial polyesters is that they are thermoplastic with environmentally degradable properties (Laffery, 1988; Yoshiharus, 1992). In the search of wholly degradable plastics of biological origin, a family of more than 40 poly-hydroxyalkanoates (PHAs) and related co-polymers have been discovered and emerged as environmentally-friendly materials. A wide variety of microorganisms (e.g *Alcaligenes eutrophus* (Linko, 1993), *Bacillus* sp. (Law, 2001), *Knallgas* bacteria (Schlegel, 1961), *Bacillus megaterium* (Findlay, 1983)) can synthesize an optically active polymer of D(-)-3-hydroxybutyric acid and accumulate it as a reserve material. Table 1.1 summarizes some identified bacteria that can produce PHA under certain specific conditions.

Table 1.1: Production of PHA by various bacteria

Reference/Author (Year)	Bacterium used	PHA	Major carbon source
Chan, (2006)	*Pseudomonas aeruginosa*	PHD	Fatty Acids
Wong, (2002)	*Alcaligenes latus*	P(3HB-co-3HV)	Industrial Wastes
Law, (2001)	*Bacillus* species	PHB	Food wastes
Hong, (2000)	Recombinant *Escherichia coli*	P(3HB)	Soy wastes
Wong, (2000)	*Staphylococcus epidermidis*	PHB	Malt wastes
Hideyuki, (1998)	Recombinant *Synechococcus sp.* PCC7942	P(3HB)	Glucose
Lee, (1998)	Recombinant *Escherichia coli*	P(3HB)	Xylose
Liu, (1998)	Recombinant *Escherichia coli*	P(3HB)	Glucose
Kim, (1996)	*Methylobacterium organophilum*	P(3HB)	Methanol
Yoshiyuki, (1996)	*Alcaligenes eutrophus*	P(HB-co-HV)	Butyric acid and valeric acids
Yamane, (1996)	*Alcaligenes latus*	P(3HB)	Sucrose
Kim, (1995)	*Alcaligenes eutrophus*	P(3HB)	Tapioca hydrolysate
Steinbuchel, (1995)	*Chromobacterium violaceum*	P(3HV)	Valeric acid
Tanaka, (1995)	*Alcaligenes eutrophus*	P(3HB)	CO_2
Lee, (1994)	Recombinant *Escherichia coli*	P(3HB)	Glucose
Lee, (1994a)	Recombinant *Escherichia coli*	P(3HB)	Glucose
Kim, (1994a)	*Alcaligenes eutrophus*	P(3HB)	Glucose
Kim, (1994b)	*Alcaligenes eutrophus*	P(3HB-co-3HV)	Glucose and propionic acid
Zhang,n(1994c)	Recombinant *Klebsiella aerogenes*	P(3HB)	Molasses
Page, (1993)	*Azotobacter vinelandii*	P(3HB)	Glucose
Presuming, (1993)	*Pseudomonas oleovorans*	P(3HHx-co-3HO)	n-Octane
Preusting, (1993)	*Pseudomonas oleovorans*	P(3HHx-co-3HO)	n-Octane
Kim, (1992)	*Alcaligenes sp.* SH-69	P(HB-co-HV)	Glucose
Suzuki, (1986)	*Protomonas extorquens*	P(3HB)	Methanol

Poly(3-hydroxybutyrate) (P(3HB)) is an example of poly(3-hydroxyalkanoate). A wide variety of PHA copolymers have been isolated from different environmental samples, such as marine sediments (Findlay, 1983), marine and freshwater cyanobacteria (Capon, 1983) and sewage sludges (Odham, 1986). In 1988, Yoshiharu, Kunioka and co-workers (Kunioka, 1988; Yoshiharu, 1988) found that, P(3HB-co-4HB), a new copolymer of 3- and 4-hydroxybutyrate could be produced by *Alcaligenes eutrophus* from 4-hydroxybutyric acid or γ-butyrolactone:

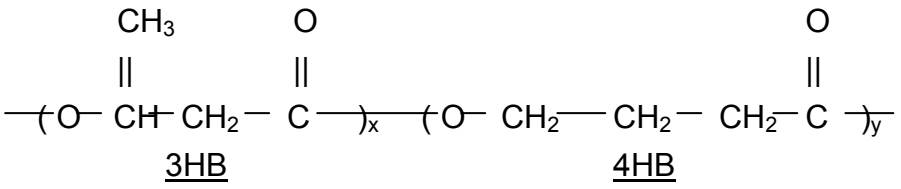


Figure 1.1: P(3HB-co-4HB) (Kunioka, 1988)

These microbial polyesters are thermoplastics with biodegradable and bio-compatible properties and their physical properties can be regulated by varying the compositions of the co-polymers (Yoshiharu, 1990a). The biodegradability of microbial polyesters in environments such as soil, activated sludge and seawater was studied by Kunioka and collaborators in 1989. They studied three polyester films: (i) P(3HB), (ii) copolymer of 91%

3HB and 9% 4HB and (iii) copolymer of 50% 3HB and 50% 3HV. All these films were dept at 30^0C under aeration in an activated sludge obtained from a sewage-treatment plant. The P(3HB-co-9% 4HB) film was completely decomposed after 2 weeks in the activated sludge. The rate of biodegradation of the P(3HB-co-9% 4HB) film was much faster than that of the other films. The P(3HB) and P(3HB-co-21% 3Hv) films were decomposed and disappeared within 8 weeks in seawater. Under aerobic conditions, the final biodegradation products were water and carbon dioxide; while under anaerobic conditions, methane was produced as well (Yoshiharu, 1990a).

P(3HB) and copolymer P(3HB-co-HV) had been traded under the trade name BIOPOL (Davide, 2004). The main reason, that hampered a greater commercialization of PHA was the high cost of production. The price of BIOPOL was as high as $16/kg. It was too high a cost for a bulk plastic material when considering the cost of synthetic plastics such as polypropylene that was less than $1/kg (Lee, 1995). Bacteria indigenous to activated sludge can accumulate large quantities of PHA under environmentally controlled conditions and hence offer a potentially more

environmentally effective method of production if these bacteria are used to produce PHAs from wastewater that acted as carbon sources. This may become an economically feasible alternative source of PHAs in the future. Recently, PHAs were found in the bacterial strains isolated from the activated sludge of wastewater treatment plants (Lam, 2007; Rees, 1992; Satoh, 1992; Yoshiyuki, 1996; Yoshio, 1996).

1.3 Waste Remediation and PHA Synthesis

The effective disposal of sludge that is excessively produced from the waste water treatment plant has become a serious environmental problem in the modern waste water treatment process. Though several methods have been proposed for the effective utilization and volume reduction of the excess sludge, a major part of it is disposed as landfill without reuse (Inoue, 1996). Activated sludge is considered a valuable biomass resource composed of a variety of types and amounts of micro-organisms. Scientists are interested in the production of biodegradable PHAs using activated sludge (Abeling, 1992; Lowell, 1974; Rees, 1992; Satoh, 1992).

In 1992, Satoh and collaborators found that under a biological treatment process, the bacteria in the sludge used the waste substrate for growth and synthesized polyhydroxyalkanoates composed of 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxy-2-methylbutyrate and 3-hydroxy-2-methylvalerate. During the biological treatment process, the polyphosphate accumulating bacteria had an advantage as they could utilize more energy than other microorganisms in uptaking substrates by utilizing

the accumulated polyphosphate. The substrate taken up was accumulated as glycogen or poly(3-hydroxybutyrate) temporarily. The reducing power necessary for the formation of polyhydroxyalkanoates from acetate and/or propionate was shown to be supplied by glycolysis of intracellular carbohydrates. In 1995, Saito and Yoshiharu used the activated sludge after a biological waste treatment process to produce two biodegradable materials, called 3H2MB and 3H2MV. Basically, the morphology strength and biodegradability of copoly-(3-hydroxyalkanoates) generally depended on their molecular microstructure, namely the comonomer composition as well as the different bacteria cultures in the activated sludge.

The role of different microbial populations in different activated sludge is worth investigating. Besides, the mechanism of organic waste reduction in-line with the mechanism of PHA accumulation in the complex substrate of activated sludge should be examined. After understanding the different microbial communities, waste removal activities, mechanism of PHA accumulation and its degradation in activated sludge, the production of PHAs from activated sludge will be greatly improved and such PHAs will be an inexpensive source of biodegradable plastics. Moreover, waste water

treatment efficiency will also be enhanced. All the above mentioned points will be useful in designing a large-scale commercial waste reduction-PHA production facility.

1.4 Objectives

The objectives of this research work are:

(1) to compare the efficiency of different nutrient media for screening potential PHA biomasses from activated sludge;

(2) to investigate the microorganism community of activated sludge from different industries in Hong Kong and their behavior of PHA production;

(3) to investigate the feasibility of PHA accumulation by the isolated PHA biomass from activated sludge with different carbon sources;

(4) to study the feasibility of waste removal and PHA production of activated sludge; and

(5) to investigate the kinetic modeling for optimizing the waste removal and PHA accumulation of activated sludge.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Polyhydroxyalkanoates (PHAs)

The biodegradable plastics polyhydroxyalkanoates (PHAs) involves polyethers of different hydroxycarboxylic acids. They can be accumulated by some microorganisms as a reserve source of carbon and energy to be utilized under unfavorable environmental conditions. Extreme bacterial habitat (eg. atmosphere, medium composition, pH and nutrient substrate deficiency) may stimulate PHA accumulation (Anderson, 1990; Lafferty, 1988; Yoshiharu, 2001). PHAs are similar to the known synthetic polymers in their physical properties, but unlike the latter they can be fully degraded by microorganisms. This makes them an attractive material to the industry of biodegradable polymers despite their rather high price.

A wide variety of prokaryotic organisms such as bacteria and cyanobacteria would accumulate P(3HB) amounting to 30 to 80% of their cellular dry

weight when growth was limited by the depletion of an essential nutrient such as nitrogen, oxygen, phosphorus, sulfur, or magnesium (Anderson, 1990; Yoshie, 2002). For many bacteria, PHAs functions either as a carbon and/or energy reserve or as a sink for excess reducing equivalents. *Rhodospirillum rubum*, *Alcaligenes latus*, *Rohodococcus* sp., *Pseudomonas stutzeri*, *Alcaligenes eutrophus*, *Aermonas* sp., *Aermonas hydrophyla*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Azotobacter vinelandii*, as well as recombinant bacteria, in particular *Escherihia coli*, were identified as PHA producers (Chan, 2006; Kim, 1994a; Lee, 1994; Pages, 1993). Cultivation of these microorganisms was carried out under stringent conditions on different substrates. New strains isolated from the environment would be utilized both as biopolymer producers and unique sources of genetic information to improve similar biodegradable properties of strains commonly used in industry.

P(3HB) is one of the typical examples of PHAs. In 1962, Mulder and collaborators were able to demonstrate that *Sphaerotilis natans* and other "sheath forming bacteria of the *Sphaerotilis leptothrix* group" isolated from sewage were capable of storing large amounts of P(3HB). Lowell and William isolated a heteropolymer by chloroform extraction of activated sludges (Wallen, 1974). The significance of the presence of P(3HB) with respect to the flocculation of activated sewage sludge was the presence of P(3HB) and/or the monomer β-hydroxybutyric acid strongly increases microbial cell surface hydrophobicity. P(3HB) granules together with the monomer possess extremely effective de-emulsification properties with

respect to oil/water emulsions. Thus, the factors of increased cell surface hydrophobicity and de-emulsification properties are synergistic effects due to the synthesis of P(3HB).

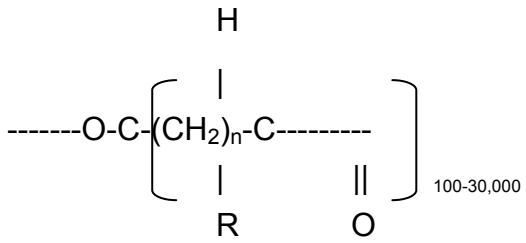
It was shown that many other genera of bacteria were capable of synthesizing P(3HB) and that the P(3HB) from various genera of bacteria had similar physical properties such as infrared absorption and X-ray diffraction patterns (Lafferty, 1988; Lundgren, 1965; Schuster, 1967). It was also found that the capability of the syntheses P(3HB) as an osmotically inert storage material was very widespread in prokaryotic organisms (Dawes, 1973).

Due to the useable properties of P(3HB) that are similar to synthetic polymers, there has been a recognizable shift from the study of cellular morphology and quantitative determination of P(3HB) to the investigation of the biochemistry, synthesis, degradation, and metabolic functions of P(3HB).

2.2 Discovery of Poly-β-hydroxybutyric Acid (P(3HB))

The first example of the microbial copolyesters of 3-hydroxyalkanoic acids, poly(3-hydroxybutyric acid), was isolated from environmental samples in 1974 by Wallen and Rohwedder using chloroform extraction of activated sludges (Yoshiharu, 1990a). The copolyester P(3HA) was present as 1.3% of the dry weight of the activated sludge. In fact, such microbioal Poly(3-hydroxybutyrate) [P(3HB)] was first described in 1925 by Lemoigne, a microbiologist at the Pasteur Institute in Paris (Yoshiharu, 1990a). Since the advent of the use of microscopes in the field of microbiology it had often been described that small “drops of fat” could be observed in many bacterial cells and recognized by many microbiologists (Lafferty, 1988). The composition of these particles was first clarified by Lemoigne. He noticed that when cultures of *Bacillus subtilis* were allowed to autolyze in distilled water, the pH value would decrease due to the formation of an unknown acid that was subsequently found to be identical with β-hydroxybutyric acid. Lemoigne positively identified the source of the monomeric β-hydroxybutyric acid that appeared during the autolysis of *Bacillus megaterium* as the intracellular poly-β-hydroxybutyric acid (Lafferty, 1988). Furthermore, Poly-HB itself was isolated from *Bacillus megaterium* and *Azotobacter chroococcum* and conclusively identifed as such by Lemoigne (Lemoigne, 1926). He isolated the polymer from *Bacillus megaterium* by chloroform extraction and determined the polymer gravimetrically. In addition, he demonstrated that it was polyester of 3-hydroxybutyric acid (Capon, 1983; Lemoigne, 1926; Odham, 1986; Yoshiharu, 1990a). The general chemical

formula is:



R= n-alkyl pendant group of variable chain length

R=methyl	3-hydroxybutyrate; 3HB
R=ethyl	3-hydorxyvalerate; 3HV
R=n-propyl	3-hydroxycaproate; 3HC
R=n-butyl	3-hydroxyheptanoate; 3HH
R=n-pentyl	3-hydroxyoctanoate; 3HO
R=n-hexyl	3-hydroxynonanoate; 3HN
R=n-heptyl	3-hydroxydecanoate; 3HD
R=n-octyl	3-hydroxylundecanoate; 3HUD
R=n-nonyl	3-hydroxydodecanoate; 3HDD

Figure 2.1: The General Chemical structure of PHAs (Kim, 1992)

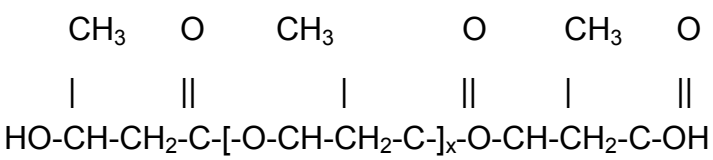


Figure 2.2: The primary structure of P(3HB). The value of x may be extremely variable from 600 to over 35,000 and depends on the organism as well as on the method of separation used to obtain the biopolymer (Yoshiharu, 1990a).

With an R absolute configuration [D(-) in traditional nomenclature] in the chiral center of 3-hydroxybutyric acid, the P(3HB) molecule in the crystalline regions has the conformational structure of a right handed 21 helix (Yoshiharu, 1990a). Unlike other biological polymers such as proteins and polysaccharides, this P(3HB) is thermoplastic with a melting temperature around 180^0C.

Forsythe and co-workers (Forsythe, 1958) were one of the first groups to be able to show that many gram negative bacteria could synthesize P(3HB) and that the presence or lack of this feature could possibly be used as a taxonomic determinant. Williamson and Wilkinson (Williamson, 1958) developed one of the first practical methods for the relatively simple quantitative determination of the P(3HB) content of bacterial biomass. From the methods, cells were treated with an alkaline hypochlorite solution under standard conditions that dissolved all cellular compounds except P(3HB). The concentration of the granules so obtained could be quantitatively determined as “residual turbidity” corresponding to the amount

of intracellular P(3HB). The capability to synthesize an intracellular storage material such as P(3HB) certainly offers a selective advantage to those microorganisms that contain P(3HB) when subjected to adverse conditions such as the lack of utilizable carbon sources (Kim, 1994b; Lafferty, 1988; Lee, 1995; Yoshiharu, 2001). It was summarized earlier that P(3HB) could serve as carbon-containing storage material (Lafferty, 1988; Lee, 1995). P(3HB) was formed when a suitable carbon source was present, but a lack of other essential nutrients such as nitrogen or phosphorus restricted the normal synthesis of cytoplasmic compounds such as proteins and/or nucleic acids. Microbial cells that storted PHAs as a reserve material were better adapted to survive periods of starvation.

2.3 Morphology and Structure

The presence of P(3HB) granules in bacterial cells can be observed with the help of a phase contrast or electron microscope. P(3HB) exists in the cytoplasmic fluid in the form of granules with diameters in the 0.3 – 1.0μm range and generally in spherical shape (Lafferty, 1988; Yoshiharu, 1990a;). Figures 2.3 shows the typical morphology of P(3HB) granules in *Alcaligenes eutrophus.*

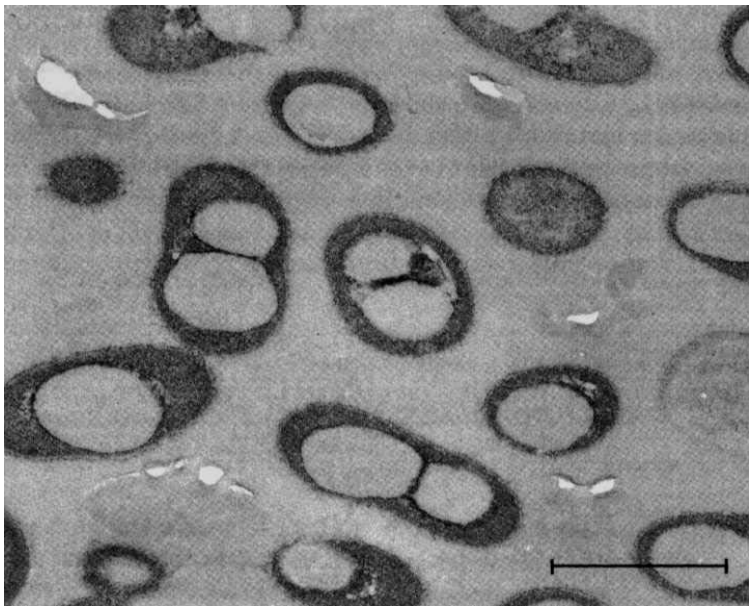


Figure 2.3: Electron micrograph of ultrathin sections of *Alcaligenes eutrophus* containing P(3HB) granules. Dark bar = 1μm (Lafferty, 1988).

Dunlop and Robards (Dunlop, 1973) found that individual P(3HB) granules possess a central core amounting to 50% of the granule volume by using freeze-etching methods. During the freeze-etching process it appeared that the core could be stretched.

Basically, P(3HB) is a macro-molecular polymer of optically active D(-)-3-hydroxybutyric acid and has the primary structure as shown in Figure 2.2. The number of repeating units (x) determined by analytical procedures will greatly depend on a number of various factors. It may reach as high as x = 35,000 (Lafferty, 1988). P(3HB) with a molecular weight of up to 3.39 x 10^6 has been obtained from *Azotobacter vinelandii* by means of extraction of cellular mass with chloroform (Akita, 1976). There are several factors that affect the value of x (Lafferty, 1988; Suzuki, 1988):

(i) the method of isolation (extraction of P(3HB) granules from the biomass);
(ii) the bacterial strain used;
(iii) the type of substrate employed;
(iv) the time of harvesting the cells from a batch culture (i.e. growth curve);
(v) factors that limit growth; and
(vi) fermentation conditions (e.g. temperature, oxygen partial pressure).

2.3.1 Poly(3-hydroxyvalerate)

The presence of Poly(3-hydroxyvalerate) by *A.eutrophus* is statistically random present in terms of commoner distribution in the P(3HB-co-3HV) by Bernoullian model (Yoshiharu, 1990a). The fraction 3HB and 3HV have been determined from the well resolved peaks of the carbonyl resonances in ^{13}C-NMR spectra of P(3HV) samples (Yoshiharu, 1986). The results are summarized as below:

	PHA composition		**Dyad Sequence Distribution**		
Carbon Sources	F_B**(3HB)**	F_V**(3HV)**	F_{BB}	$F_{BV}+F_{VB}$	F_{VV}
Acetic+propionic acids	0.81	0.19	0.67	0.26	0.07
Butyric+pentanoic acids	0.80	0.20	0.64	0.31	0.05
Butyric+pentanoic acids	0.76	0.24	0.57	0.37	0.06
Acetic+priopionic acids	0.68	0.32	0.46	0.43	0.11
Butyric+pentanoic acids	0.65	0.35	0.45	0.40	0.15
Propionic acis	0.59	0.41	0.37	0.44	0.19
Propionic acid	0.57	0.43	0.33	0.46	0.21
Butyric+pentanoic acids	0.42	0.58	0.21	0.41	0.38
Butyric+pentanoic acids	0.39	0.61	0.18	0.40	0.42
Pentanoic acid	0.20	0.80	0.07	0.23	0.70
Pentanoic acid	0.10	0.90	0.03	0.12	0.85

Assuming that the P(3HB-co-3HV) samples are statistically random copolymers that can be described by Bernoullian statistics, the BB, BV, VB and VV dyad fractions (F_{BB}, F_{BV}, F_{VB} and F_{VV}, respectively) can be

expressed with the mole fraction of the 3HV unit in the polymer, F_V, as following equation (1) (Yoshiharu, 1990a).

$$F_{BB} = (1 - F_V)^2$$

$$F_{BV} = F_{VB} = F_V(1 - F_V)$$

$$F_{VV} = F_V^2$$

The observed dyad fractions ((F_{BB}, F_{BV} + F_{VB} and F_{VV}) were compared with the calculated values from Equation 2.1 in Figure 2.4. There is a good agreement between the observed values and the calculated distributions based on the Bernoullian model.

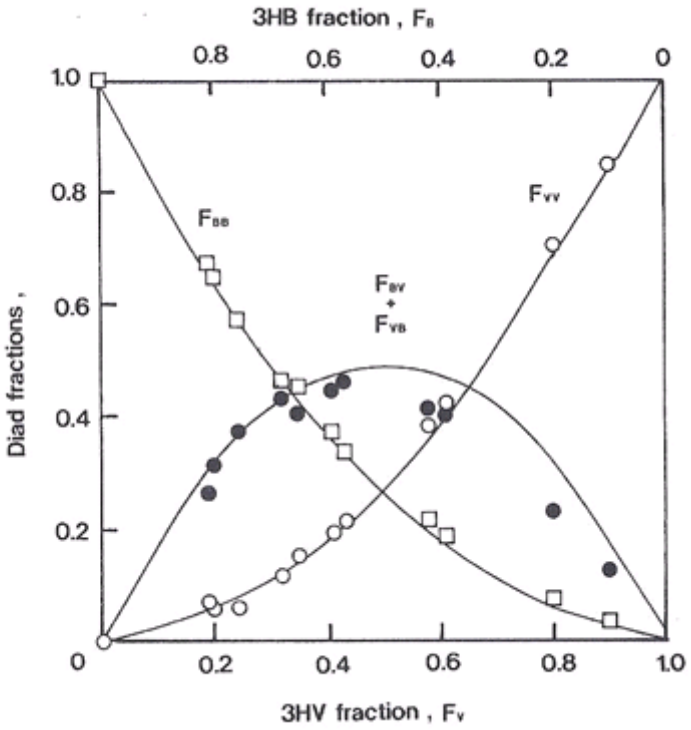


Figure 2.4: Relationship between dyad sequence fractions and mole fraction of 3 HV unit. (□) FBB; (●) FBV + FVB; (○) FVV. Solid lines represent the calculated dyad fractions based on the Bernoullian model (Yoshiharu, 1990a).

2.4 Function of P(3HB)

The role of PHAs as a carbon and energy reserve material in bacteria has been previously reviewed by researchers such as Dawes and Senior (1973), Lafferty, (1988), Merrick, (1978) and Preiss, (1989). They all pointed out that one of the major of an organism from the synthesis of the high-molecular-weight materials (PHA), contained in some 8 to 12 granules per cell, were the ability to store large quantities of reduced carbon without significantly affecting the osmotic pressure of the cell. The possession of PHA frequently, but not universally, retards the degradation of cellular components such as RNA and protein during nutrient starvation. However, there isn't any common pattern of behavior and depending upon the organism, sequential or simultaneous utilization of macromolecules can occur. The significance of the formation of PHA is to enhance the survival of some, but not all, of the bacteria investigated and to serve as a carbon and energy source during periods of starvation.

In addition, Schuster and Schlegel (1967) found that the overall synthesis of P(3HB) in *Alcaligenes* strains was the utilization or removal of superfluous reduction equivalents within the cell. This correlation was particularly important during growth conditions where the oxygen partial pressure was lower than optimal for normal growth. Therefore, the process of intracellular PHA synthesis seemed as an "electron sink". They demonstrated that *Alcaligenes eutrophus* caused the initiation of the synthesis of P(3HB) when growing under chemolithoautotrophic conditions

with a lowering of the oxygen partial pressure. Senior et. al., (1972) observed the same effect of oxygen partial pressure with *Azotobacter beijerinckii* when this organism was grown without a source of reduced nitrogen and with an excess of glucose.

The putative function of PHA in azotobacters was to provide an oxidizable substrate which afforded respiratory protection to nitrogenase under environmental conditions when appropriate exogenous substrate was not immediately available oxidation (Senior, 1971). Stam and his research team (1986) investigated the effect of carbon starvation on P(3HB) degradation in *Rhizobium* sp. strain ORS 571 by using oxygen-limited chemostat cultures of PHB-containing organisms growing on succinate. They observed that for these free-living cultures, the lack of a carbon source did not necessarily result in P(3HB) breakdown and other factors, such as the oxygen concentration and perhaps the nitrogen source, were important; indeed, P(3HB) degradation never occurred under oxygen-limited conditions irrespective of whether exogenous carbon was present. They concluded that there were two possible roles for P(3HB) degradation in *Rhizobium* sp. Strain ORS 571:

(i) protecting nitrogenase when the oxygen concentration in the nodule rises and
(ii) serving as a carbon and energy source during periods of starvation outside the nodule.

2.5 Properties of P(3HB)

Some properties of P(3HB) are related to lipids. This is reflected in its hydrophobicity and the fact that it can be readily stained by lipophilic dyes such as Sudan Black (B) which is not removed from the cells containing P(3HB) even after repeated washing ethanol (Lafferty, 1988). Cells containing large amounts of P(3HB) are stained dark bluish black and remain so after repeated ethanol washing, whereas cells containing little or no P(3HB) appear color less or slightly gray. The use of Sudan Black (B) thus represents a simple quantitative/screening method to differentiate "P(3HB)-present" from "P(3HB)-absent" bacterial colonies on the surface of an agar plate.

P(3HB) is a linear polymer, i.e., it is an aliphatic polyester with alternating carbonyl oxygen and methyl groups along the length of the polymer chain (Hammond, 1995; Lafferty, 1988; Lee, 1995). P(3HB) is, under normal conditions, a relatively un-reactive compound. However, it can be degraded by a number of methods to yield a spectrum of products useful for the synthesis of enantiomerically pure compounds (Seebach, 1987). It has thermoplastic properties and can be extruded and/or pressed. The melting point of P(3HB) is variable and usually lies between 157 and 188^{0}C.

Barham, (1984), found that when absolutely chemically pure P(3HB) is obtained by crystallization from a melt, banded spherulites are formed and the band spacing depends on the temperature of crystallization (T_c). P(3HB)

could be annealed at 160^0C and attained a maximum crystallinity of 86% after 24 hours. There is a logical explanation for the hitherto observed variations of the density and melting points of the P(3HB). Barham and collaborators demonstrated that the method of P(3HB) preparation determines both the morphology and physical properties of P(3HB). Slow cooling from the melted state yields large banded spherulites; rapid quenching leads to formation of a glassy state (T_g =5^0C) and precipitation from a solvent produces lamellar crystals.

Tables 2.1 and 2.2 (Lafferty, 1988) summarize some physical properties and solubility properties of Poly-HB. Table 2.3 lists the properties comparisons between PHB and polyethylene (Glazer, 1995).

Table 2.1: Physical properties of P(3HB) in comparison to other plastics (Lafferty, 1998)

Material	Tensile Strength T_B(N cm^{-2})	Extension to break B (%)	Modulus M (N cm^{-2} x 10^3)
P(3HB)	1,500 – 2,500	8	800 – 1,000
Polyethylene	800 – 3,500	400 – 800	200 – 1,400
Polypropylene	2,100 – 3,700	150 – 400	1,100 – 1,300
Polystyrene	4,000 – 6,500	3 – 5	3,300
Polyvinylchloride	4,000 – 6,000	10 – 50	2,000 – 3,000
Plexiglass	7,000 – 7,600	4	3,000
Polyamide	5,000 – 9,000	50 – 200	1,300 – 3,000

Table 2.2: Solubility properties of P(3HB) (Lafferty, 1998)

***(A) Highly soluble**			
Chloroform	Propylene carbonate	Ethylaceto acetate	
Dichloromethane	Trifulorethanol	Triolein	
Di, tri, tetra-chlorothane	Acetic andride	Acetic acid	
Dichloracetate	1N Sodium hydroxide	Alcohols (> 3 C atoms)	
Ethylene carbonate	Dimethylformamide		
***(B) Fairly soluble**			
Dioxane	Toluene		
Octanol	Pyridine		
***(C)Insoluble**			
Water	Cyclohexanol	Hexane	Tetrahydrofuran
Methanol	Carbontetrachloride	Benzene	Ethyl formiate
Ethanol	Dilute mineral acids	Cyclohexanone	Butyl formiate
1-Propanol	Alkaline hypochlorite	Ethyl acetate	Butyl acetate
2-Propanol	Diethylether	Ethylmethylketone	Valeric acid

**Remark: Solubilities were temperature-dependent.*

Table 2.2 shows that the solubility of P(3HB) in organic solvents has been utilized to extract the polymer from either moist or usually dry microbial biomass. The influent of temperature on the solubility of P(3HB) can often be effectively used to precipitate P(3HB) in the form of a gel from the corresponding solvent by lowering the temperature. However, at higher extraction temperatures, degradation of the molecular weight happens (Lafferty, 1988).

Table 2.3: Comparisons of some properties of Polypropylene and P(3HB) (Park, 2001)

Properties	Polypropylene	P(3HB)
Molecular weight	(2.2 – 7) x 105	(1 – 8) x 105
Melting Point (^{0}C)	171 – 186	171 – 182
Crystallinity (%)	65 – 70	65 – 80
Density (g/cm3)	0.905 – 0.94	1.23 – 1.25
Resistance to UV	Poor	Good
Fexural modulus (GPa)	1.7	3.5 – 4
Tensile strength (MPa)	39	40
Extension to break (%)	400	6 – 8
Solvent resistance	Good	Poor
Oxygen permeability ($cm^3/m^2 \cdot atm \cdot d$)	1,700	45
Biodegradability	None	Good

P(3HB) in Tables 2.1, 2.2 and 2.3 showed similarities in its molecular structure and physical properties to polypropylene that is a widely used polymer. Polypropylene is used in packaging, rope, wire insulation, pipe, fittings and bottles. Both of polypropylene and P(3HB) are isotactic, that is, in each polymer the methyl group attached to the backbone is present in a single configuration throughout the chain. The most notable difference between them is their biodegradability. Polypropylene is highly resistant to biodegradation, whereas PHB is ultimately completely degraded in a variety of environments (Table 2.3). Polypropylene is less dense than water, and when discarded in rivers/oceans, it floats. In contrast, the much higher density of PHB causes it to sink to the bottom sediment layers, where they are degraded.

The molecular weight of PHAs plays a significance role in making PHAs as plastics application. PHAs of a higher molecular weight and low polydispersity are desirable for making thermoplastics (Ward, 2005). PHAs

polymers of medium chain length have the potential to be specialty polymers. They can be applied as coatings, pressure-sensitive adhesives, polymer binding agents in organic-solvent-free paints and a range of medical applications. A good illustrative example of this kind of plastic is copolymer P(HB-co-HV) that has a molecular weight of 600,000 or above. The flexibility and toughness of the material is greatly improved when HV units are incorporated into the polymer. Depending on the HV composition (0-25 mol %), P(HB-co-HV) copolymer shows higher elongation to break and reduction in melting points ranging from 100^0C to 160^0C (Ojumu, 2004). Table 2.4 shows the material properties of some PHAs.

Table 2.4: Material properties of some PHAs (Ojumu, 2004)

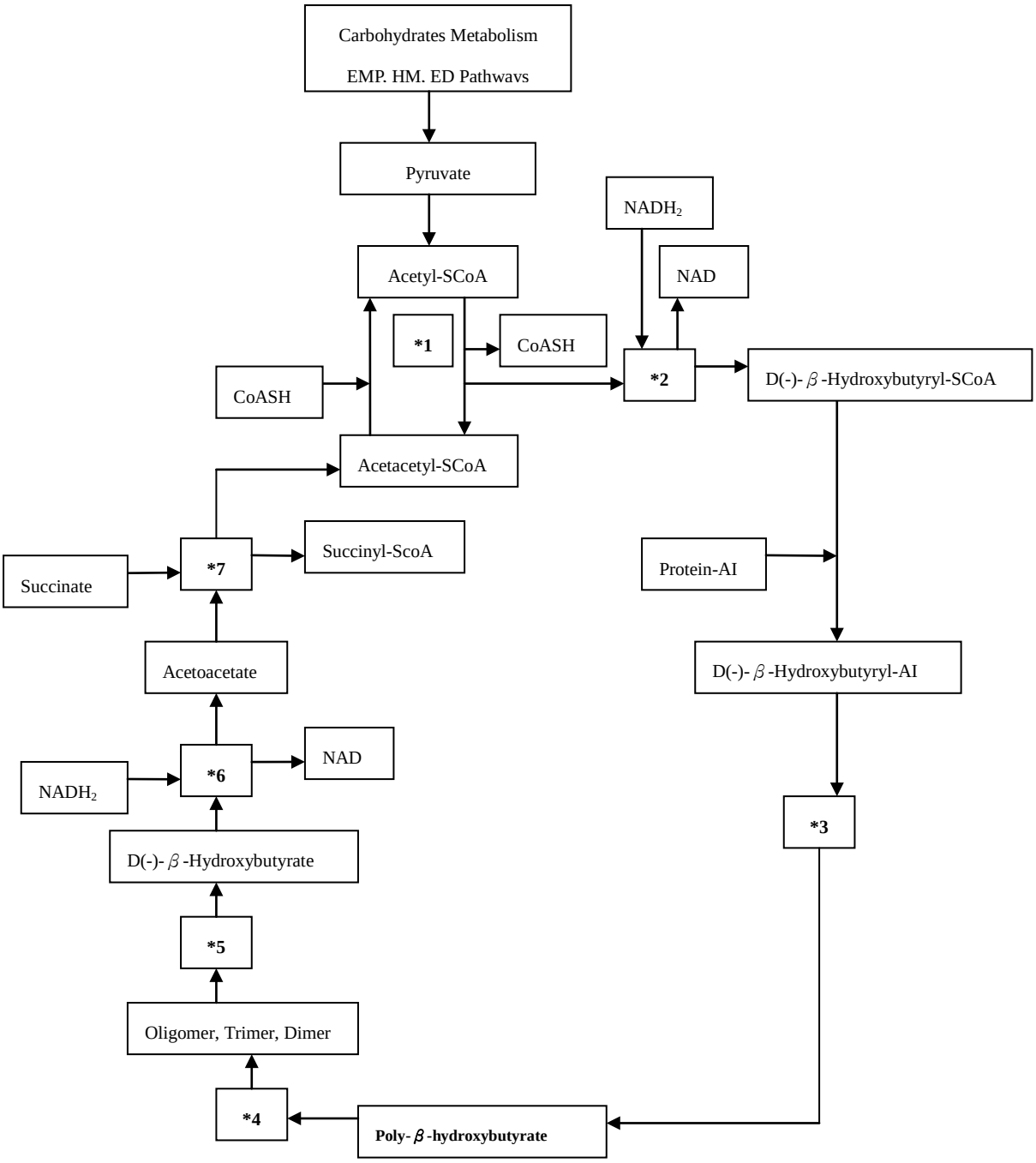
PHAs	Glass transition point (^{0}C)	Melting point (^{0}C)	Tensile strength (MPa)	Crystallinity (%)	Elongation to break (%)
P(HB)	4	177	40	60	5
P(HB-co-10% HV)	6	162	36	69	10
P(HB-co-20% HV)	-1	145	32	53	-
P(HB-co-10% HHX)	-1	127	21	34	400
P(HB-co-15% HHX)	0	115	23	26	760
P(HB-co-17% HHX)	-2	120	20	22	850
P(HB-co-6% HA)	-8	133, 146	17	-	680
PP	-30	130 – 161	29.3	40	400
LDPE	-30	120	15.2	-	620

Remark: PP: Polypropylene; LDPE: Low Density Polyethylene; "-": Data Not Available

2.6 Biosynthesis of PHAs

The first indication as to which intermediate compounds is involved in the synthesis of P(3HB) was described by Lemoigne (Yoshiharu, 1990a). They used *Bacillus megaterium* to determine the effects of various intermediates on the intracellular synthesis of P(3HB). On the basis of the experimental results, it was proposed that pyruvate, acetate, acetoacetate and β-Hydroxybutyrate were intermediates between glucose as the initial substrate and P(3HB) as the final product.

A regulatory biochemical model that incorporates elements of the Tricarboxylic Acid cycle (TCA cycle) to the glyoxylate pathway can account for the intracellular PHAs synthesis (Lafferty, 1988; Louie, 2000). Figure 2.4 shows the general biochemical pathway for intracellular PHA synthesis and degradation (Lafferty, 1988). Figure 2.5 indicates the regulatory influence of PHAs synthesis (Lafferty, 1988).



*Key: 1:β-Ketothiolase (β-Ketoacylthiolase, acetacetyl-CoA thiolase); 2: Acetacetyl-CoA reductase; 3: P(3HB) polymerase [P3(HB) synthetase]; 4: PHB hydrolase; 5: dimer hydrolase; 6:β-Hydroxybutyrate dehydrogenase; 7: Thiophorase (acetacetyl-SCoA thiokinase; Acetoacetate-succinyl-CoA transferase); EMP: Embden-Meyerhof-Parnas pathway; HM: Hexose-Monophosphate pathway; ED: Entner-Doudoroff pathway.

Figure 2.5: General pathway and enzymes involved in the intracellular PHA synthesis and degradation (Lafferty, 1988).

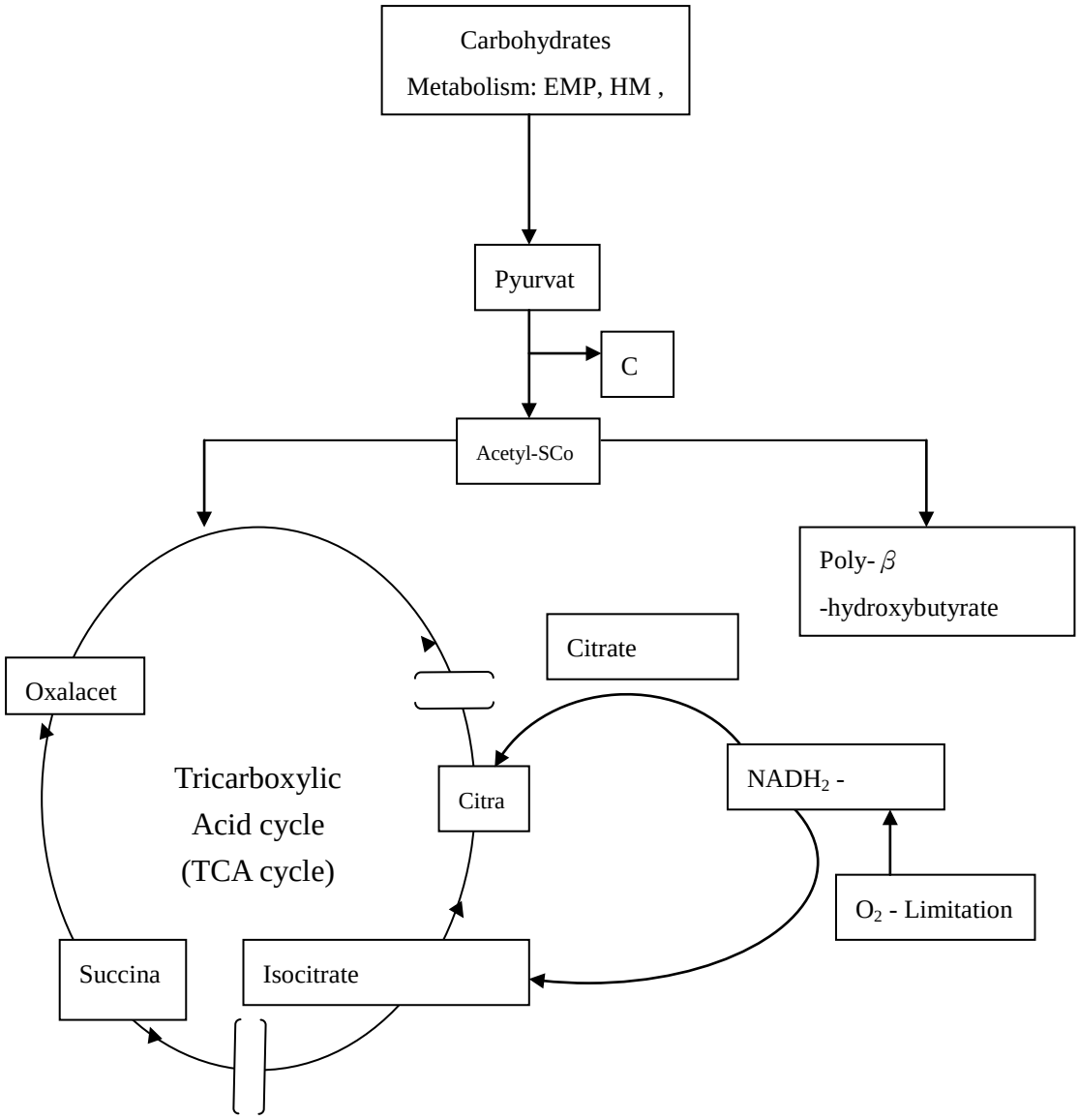


Figure 2.6: Regulatory influence on intracellular PHB synthesis (Lafferty, 1988).

Several researchers demonstrated that there are some specific enzymes that are involved in the synthesis and later utilization of the energy reserve PHA polymers (Anderson, 1990). Generally speaking, the pathway for PHA biosynthesis involves the condensation of two molecules of acetyl-CoA by β-ketothiolase to form acetoacetyl-CoA, which is subsequently reduced by acetoacetyl-CoA reductase to form D-(-)-3-hydroxybutyrl-CoA (i.e. 3HB). The monomeric 3HB is then polymerized to form P(3HB) by PHB synthase (Figure 2.4). The three enzymes that catalyze these reactions are encoded by genes that are organized as an operon in this organism designate PhbA, PhbB and PhbC for the ketothiolase, reductase and synthase, respectively (Anderson, 1990; Lafferty, 1988; Slater, 1988; Yoshiharu, 2001). This polymer is found accumulated in discrete, membrane-bound granules in the bacterial cell (Merrick, 1961; Williamson, 1958).

The percentage of P(3HB) in bacterial cells is normally low, from 1 – 30%, but under controlled fermentation conditions of carbon excess and nitrogen limitation, over-production of polymer can be encouraged to produce yields of up to 80% of the dry cell weight (Dawes, 1973; Ward, 1977). Numerous microbiological species are known to be suitable for the production of PHAs (Anderson, 1990). It was found that glucose is the common carbon source for the production of P (3HB) (Holmes, 1985). However, other substrates such as methanol, sucrose, ethanol and acetic acid can be used by micro-organisms to produce homopolymer (Howells, 1982). It is through various intracellular pathways that PHA-producing organisms are able to convert these substrates to the precursor acetyl-CoA (Figure 2.5). For

examples, Tanaka and his research team (Yoshiharu, 1990b) demonstrated that *Alcaligenes eutropha* can accumulate P(3HB) under autotrophic conditions by cultivating it in an organic medium for exponential growth. The O_2 concentration in the substrate gas (H_2 + CO_2) was below the explosion limit of 6.9%. Through this process, P(3HB) is obtained at a high production rate and concentration.

P(3HB-co-3HV) is typically produced using a combination of glucose and propionate in the growth media (Holmes, 1985). By adjusting the composition of the carbon sources, *Alcaligenes eutropha* can accumulate P(3HB-co-3HV) with up to 95 mol% 3HV content has been achieved (Holmes, P.A., 1985; Howells, E.R., 1982; Yoshiharu, 1990b). In particular, Holmes, P.A. (1985) described the use of *Alcaligenes eutropha* mutanta NCIB 11599 grown under aerobic cultivation conditions to produce the P(3H-co-3HV) copolymer when fed glucose and /or various amounts of propionic acid and other organic acids that lead to the 3HV component. Holmes demonstrated that copolymer can be produced up to 70% of the cell dry weight and with 3HV levels up to 30% of the monomer fraction. In addition, Madden and his research team (1998) found that an alternate feeding of glucose and propionic acid to the phosphate-depleted batch cultures of *Alcaligenes eutropha* produces P(3HB-co-3HV) with thermal properties markedly different from random copolymers of a similar monomer content. Those polymers exhibited a single glass transition and a single melting peak that was significantly higher than expected for a random copolymer. The polymers were found, by solvent fractionation and nuclear

magnetic resonance (NMR), to be a mixture of P(3HB) and a random copolymer, P(3HB-co-3HV). Besides, when compared with random copolymers of similar monomer content, the polymers produced by alternate substrate feeding displayed no improvement in their mechanical properties and possessed similar aging characteristics.

2.6.1 Enzymology of PHA accumulation

In most of the organisms investigated, PHA biosynthesis is from acetyl coenzyme A (acetyl-CoA) by a sequence of three reactions catalyzed by **β**-ketothioalase (acetyl-CoA acetyltransferase), Acetoacdetyl CoA reductase (hydroxybutyryl-CoA dehydrogenase) and poly(3-hydroxbutyrate) synthase (Anderson, 1990; Yoshiharu, 2001).

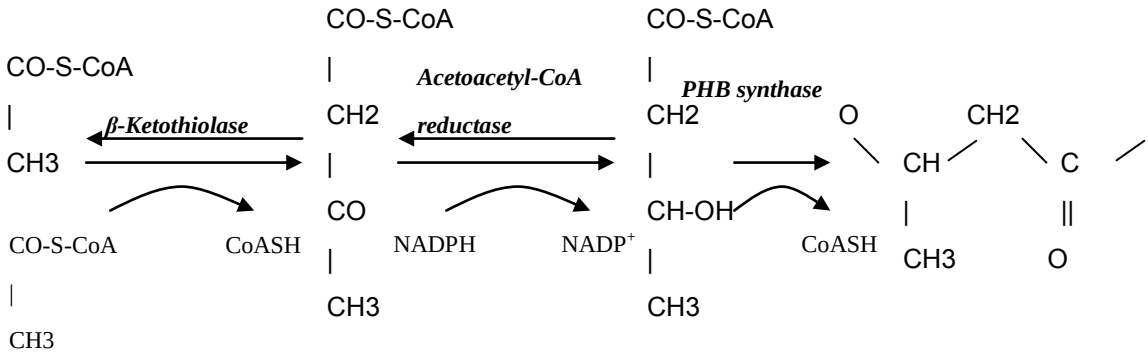


Figure 2.7: Common three steps biochemical pathway to P(3HB) produced found in *Alcaligenes eutropha* (Yoshiharu, 2001).

2.6.1.1 β-ketothiolase

β-ketothiolase has been purified from various PHB-synthesizing bacteria. In 1988, Slater and collaboratos succeeded in cloning two different *Alcaligenes eutrophus* genomic DNA fragments that encoded β-ketothiolase and which were able to express the enzymes in *E.coli*. Subsequently, Haywood, (1988) separated and characterized two thiolases and named them β-ketothiolase A and β-ketothiolase B. They contain different substrate specificities. The activity of β-ketothiolase A is restricted to acetoacetyl-CoA (100% relative activity) and 3-ketovaleryl-CoA (3% relative activity), whereas β-ketothiolase B uses not only these two substrates, but also 3-ketohexanoyl-CoA (17%), 3-ketoheptanoyl-CoA (19%), 3-ketooctanoyl-CoA (10%) and 3-ketodecanoyl-CoA (12%) (Steinbuchel, 1991).

β-ketothiolase A is active only with 4C and 5C 3-ketoacyl-CoAs. It is referred to as a biosynthetic thiolase functioning primarily in PHA biosyntheis. The substrate specificity of 3-ketothiolase A alone can account for the formation of a copolymer containing both 3HB and 3 HV units. β-ketothiolase B with a broad specificity for β-ketoacyl-CoAs ranging from 4C to 16C. This class of enzyme is involved in the degradation of fatty acids and is located in the mitochondria of mammalian, peroxisomes of plant cells and cytoplasm of prokaryotes. (Anderson, 1990; Masamune, 1989). Figure 2.6 shows the enzymatic mechanism of β-ketothiolase in the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. First of all,

an acetyl-S-CoA molecule is attached to form an acetyl-S-enzyme intermediate by an active-site cysteine. Then, another acetyl-CoA is deprotonated by a second cysteine, resulting in an activated acetyl-CoA intermediate that can attack the acetyl-S-enzyme intermediate and form acetoacetyl-CoA (Masamune, 1989). Mechanistic studies suggest that there are three cysteine residues and possibly a histidine residue in or near the active site of the Z.ramigera enzyme, the rate-limiting step for which in the thiolytic direction is the enzymatic deacylation half-reaction and, in the condensation direction, the acetoacetyl-CoA carbon-carbon bond forming step.

2.6.1.2 Acetoacetyl-CoA and Acetoacetyl-CoA reductase

Acetoacetyl-CoA reductase is an (R)-3-hydroxyacyl-CoA dehydrogenase that converts acetoacetyl-CoA into 3-hydroxybutyryl-CoA in the PHB biosynthetic pathway (Figure 2.6). In 1988, Haywood and co-workers found that there were two different acetoacetyl-CoA reductases, namely NADPH-dependent acetoacetyl-CoA reductase and NADH-dependent acetoacetyl-CoA reductase that were present in *Alcaligenes eutrophus*. The NADPH-dependent acetoacetyl-CoA reductase catalyzed the stereoselective reduction of acetoacetyl-CoA formed in the ketothiolase reaction to D(-)3-hydroxybutyryl-CoA. The NADH-dependent acetoacetyl-CoA reductase only formed L(+)-3-hydroxyacyl-CoA but not D(-)-3-hydroxyacyl-CoA, although in the reverse direction the enzyme could

also oxidize the latter. The NADPH-dependent reductase was only active with D(-)-3-hydroxybutyryl-CoA, D(-)-3-hydroxyhexanoyl-CoA, the NADH-dependent reductase used L(+)-3-hydroxyheptanoyl-CoA, L(+)-3-hydroxyoctanol-CoA and L(+)-3-hydroxydecanoyl-CoA as substrates. P(3HB) synthesis from acetyl-CoA using a system reconstituted from 3-ketothiolase, acetoacetyl-CoA reductase and P(3HB) synthase was observed when the NADPH-linked (but not the NADH-linked) reductase was used. This result indicates that the NADH-linked acetoacetyl-CoA reductase is not directly involved in the P(3HB) synthesis but it plays an important role in the conversion of L(+)-3-hydroxybutyryl-CoA to D(-)-3-hydroxybutyryl-CoA (Yoshiharu, 1990a).

It is demonstrated that the cleavage of acetoacetyl-CoA by β-ketothiolase is inhibited by acetoacetyl-CoA (Oeding, 1973), as the binding of CoA to the acetyl enzyme complex is inhibited by the high concentration of acetoacetyl-CoA. In other words, high intracellular concentrations of CoA favours the formation of acetyl-CoA from acetoacetyl-CoA. Activities of TCA cycle maintain a high level of intracellular CoA as TCA cycle acts as a sink for the acetyl residues of acetyl-CoA. Therefore, impairing the protein synthesis stops/inhibits TCA cycle and prevents the cleavage of acetoacetyl-CoA to form acetyl-SCoA. The initial compound for the intracellular synthesis of P(3HB) in prokaryotic cells is acetyl-coenzyme A (Lafferty, 1988). Substrates and ambient conditions which increase the intracellular concentrations of acetyl-coenzyme A and have a positive effect on the synthesis of P(3HB).

2.6.1.3 PHA synthase

PHA synthase links 3-hydroxybutyryl moieties or 3-hydroxyvaleryl moieties to existing polyester molecules by ester bonds (Steinbuchel, 1991). It is absolutely stereospecific for the D(-) stereoisomers and does not react with the L(+) stereoisomers. In 1991, Huisman and collaborators identified that two regions of 54 (residues 180 to 234) and 155 (residues 337 to 492) amino acids that showed approximately 75% identity from *Pseudomonas oleovorans*. Each region included one smaller segment of a particular homology to the *Alcaligenes eutrophus* PHA synthase. Those segments were important for the enzyme structure and for the catalysts of the polymerization reaction. Huisman and collaborators assumed that the segments were involved in the substrate binding and that they defined the specificity of the synthases for hydroxyacyl-CoA thioesters. The polymerization reaction was then summarized in two steps:

(i) an acyl-S-enzyme intermediate was formed in the first reaction and

(ii) the acyl moiety was transferred to the polyester prim (Griebel, 1971).

The polymerization involves two thiol groups, one locating the incoming 3HB monomer and the other locating the growing polymer chain. Condensation occurs through a four-membered transition state, leaving one of the thiol groups vacant for the next monomer. It is presumed that the chain transfer

role performed by the synthase must in some ways control the molecular weight of the polymer produced, which is a characteristic of a given organism.

2.7 Key Factors alter PHA accumulation

The general pathway and enzymes involved in the biosynthesis and degradation of P(3HB) by microorganisms as shown in Figures 2.4 and 2.5 show that there are several factors that possibly regulate PHA accumulation other than Enzymology can alter PHAs accumulation. These factors are shown below:

2.7.1 Nitrogen Factor

Figure 2.6 shows that β-ketothiolase is a key enzyme in PHB metabolism. It plays a key role in both the condensations and cleavage reactions of acetoacetyl-CoA. It has been found that only one product of the condensations reaction, free CoASH, erects an inhibitory effect on the enzyme catalyzing the condensation reaction (Lafferty, 1988, Lee, 1995). Under normal nutrient provided growth conditions, Acetyl-CoA will mainly enter to the tricarboxylic acid cycle (TCA) for energy generation and formation of amino acids (Fig. 2.5), such as aspartate and glutamate. Under citrate synthase reaction, a free CoASH will then be liberated. As a result, the intracellular concentration of acetyl-CoA will be low and the free CoASH will be high. Consequently, the CoASH concentration will inhibit the β-ketothiolase condenzation reaction and of PHB synthesis. On the other hand, if protein synthesis is inhibited, pyruvate and the intermediates of the TCA cycle will not flow into the anabolic pathways.

It has been demonstrated that in the absence of a nitrogen source the intracellular pyruvate concentration is 5 times as high as that under optimum growth conditions (Oeding, 1973). As a consequence, the acetyl-CoA will achieve a high concentration level and the concentration of free CoASH will be low. Therefore, β-ketothiolase will not be inhibited and acetoacetyl-CoA synthesis can proceed unimpaired (Senior, 1973). It will enhance the PHA production.

2.7.2 Molecular oxygen factor

Figure 2.5 shows that oxygen limitation results in an increase in intracellular $NADH_2$/NAD ratio and inhibition of the TCA cycle. Oxygen limitation leads to a rise in the intracellular level of acetyl-CoA. That in turn would result in a pronounced increase in the rate of PHA synthesis. In fact, under an aerobic growth condition, the nicotinamide nucleotides, a metabolite from the TCA cycle, will be re-oxidized. However, if the growth is limited by a shortage of oxygen, the nicotinamide nucleotides cannot be re-oxidized (Eidels, 1970; Senior, 1971). This would result in a decrease in the effectiveness of the TCA cycle, since the activities of the citrate synthase and of the isocitrate dehydrogenase are decreased by NADH (Oeding, 1973). Consequently, the deprivation of oxygen would result in the accumulation of acetyl-CoA and a low concentration of free CoASH in intracellular. It indicates that a lack of oxygen or a low oxygen supply will enhance PHA synthesis.

2.7.3 Carbon source factor

During the degradation of PHA, acetoacetyl-CoA is formed by the activation of acetoacetic acid, which is derived from P(3HB) by hydrolysis and dehydrogenation via the β-hydroxybutyric acid (Hippe, 1967; Merrick, 1964). PHA will be re-utilized and degraded to form acetoacetyl-CoA when there is a lack of carbon or/and energy supply. Then, the intracellular concentrations of acetyl-CoA and free CoASH to be shifted in favour of CoASH and the condensation of two acetyl-CoA to form acetoacetyl-CoA for PHA synthesis are inhibited (Oeding, 1973). This implies that a high carbon and/or energy supply may enhance the PHA production.

2.8 Biological Waste Treatment and PHAs accumulation

With proper analysis and environmental control, almost all types of wastewater can be successfully treated biologically. The major objectives of the biological treatment of wastewater are:

i.) to coagulate and remove the nonsettable colloidal solids;

ii.) to stabilize the organic matter.

Micro-organisms are used to convert the colloidal and dissolved carbonaceous organic matters into various gases and cell tissue. Untreated food wastewater contains sufficient nutrients for bacterial growth and is greatly responsible for the degrading of the aquatic environment, such as re-tide.

The major biological processes for wastewater treatment can be divided into 4 major groups: aerobic processes, anoxic processes, anaerobic processes, combined aerobic, anoxic and anaerobic process (Metcalf, 1991). Cell-Adsorbed Activated Carbon (AAC) and Biological contact Aeration System – Sequencing Batch Reactor (SBR) are two common methods to remove organic materials from food wastewater. Although the activated carbon technology can produce a suitable quality effluent, it is unacceptable for economic and technical reasons (Metcalf, 1991). The use of

Sequencing Batch Reactor to remove organic compounds found in wastewater biologically can provide substantial savings in energy and costs when compared with the activated carbon method (Herzhrun, 1985; Irvine, 1984).

In the SBR, microbial film grown on media surface ingests substances as organic matter, oxygen and trace elements, which is required for biological activity, from the liquid phase with which it is in contact. When these substances reach the microbial film surface, they travel into the film by molecular diffusion, and are then ingested and metabolized by the film microorganisms. The organic substances in the wastewater, which are colloidal or suspended matters, are not able to diffuse into the microbial film directly, and must be hydrolyzed to low molecules on the film surface before they take a course similar to that of lower molecular weight organic substances (Iwai, 1994). In general, the development of an aerobic biofilm can be classified into the following seven steps:

i.) The biofilm is composed of a few aerobic bacteria that are included in a gelatinous matrix;
ii.) Aerobic micro-organisms grow rapidly and the density is an increasing function of the thickness;
iii.) As oxygen depletion begins to occur in the biofilm, an anaerobic zone appears near the solid supporting material;
iv.) Anaerobic and facultative bacteria grow near the solid supporting material as the aerobes decay, causing a decreasing density;

v.) An equilibrium between the anaerobes and aerobes is reached, which means that the density is stabilized;

vi.) The equilibrium is maintained until the substrate concentration is exhausted in the deeper zone, the anaerobe bacteria will begin to decay and parts of the biofilm will finally slough away;

vii.) The newly developed space will be used by the new aerobic bacteria which would start all over again and build new biofilm. Figure 2.7 shows the different steps in the development of the biofilm.

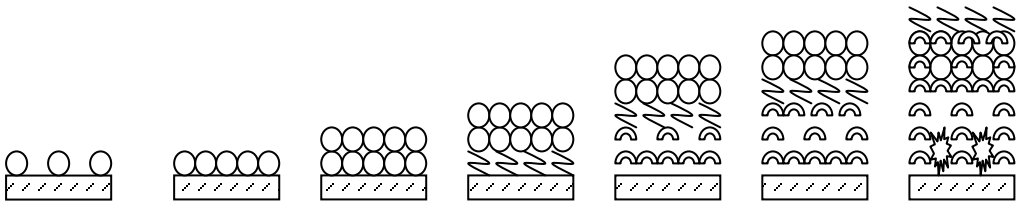


Figure 2.8: The different steps in developing a biofilm, shown as transients of a microbial film (Sorensen, 1993).

▭: Support; ○: aerobic viable microorganisms;

≈: Dead microorganism; ◠ : Anaerobic microorganisms;

✸: Gaseous metabolites

Figure 2.9 shows the reaction in a microbial film:

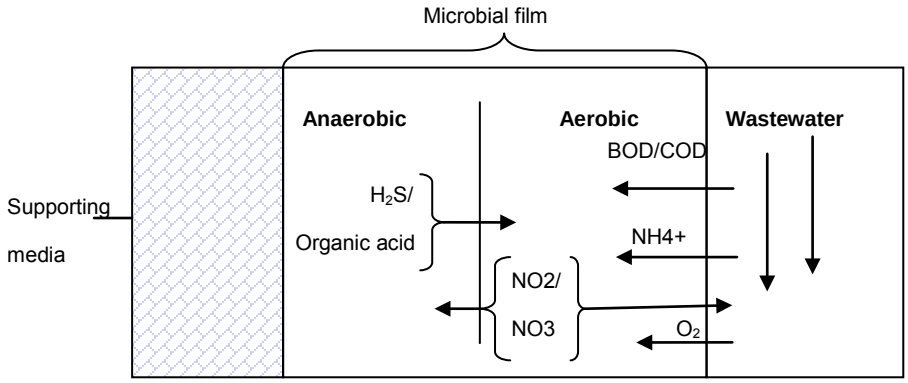


Figure 2.9: Schematic diagram of a microbial film (Iwai, 1994)

In an aerobic zone, pollutant removal mechanisms occur by the following reactions (Metcalf, 1991):

(2.2) organic matter (BOD/COD) + O_2 + nutrient ----------> CO_2, NH_3, cells, NO_3

(2.3) $NH_4 + O_2 + HCO_3$ -----> $C_5H_7NO_2 + NO_2 + H_2O + H_2CO_3$

(2.4) $NO_2 + NH_4 + H_2CO_3 + HCO_3 + O_2$ --> $C_5H_7NO_2 + H_2O + NO_3$

Apart from pollutant removal in the aerobic zone, pollutant removal as well as nitrogen removal also occur in the anaerobic zone by the following reactions:

(2.5) $NO_3 + CH_3OH + H_2CO_3$ -> $C_5H_7NO_2 + N_2 + H_2O + HCO_3$

(2.6) $NO_2 + H_2CO_3 + CH_3OH$ -> $C_5H_7NO_2 + H_2O + N_2 + HCO_3$

When any one of the components of Equations (2.1) to (2.5) is not present, biological reactions will not proceed steadily. Trace nutrients such as nitrogen, phosphorus and trace metals generally will not become limiting factors only if they are contained in wastewater as much as required stoichiometrically in biological reaction, while those will be the limiting factors in the anaerobic zone. Since oxygen cannot get into the anaerobic layer, a condition of oxygen limitation is established. In addition, there is plenty of carbon source contained in the waste that is ready to be treated by a bio-reactor. The plenty carbon sources, nitrogen non-availability and oxygen limitation conditions favor PHA accumulation during the waste treatment process.

Researches are interested in treatment process as favor that PHA accumulation. Uptake mechanisms of acetate, propionate and lactate in an anaerobic aerobic biological phosphate removal process with accumulation of more than 300mgC/L total PHA (including 3HB, 3HV, 3H2MB and 3H2MV) were demonstrated by Satoh's research group in 1992 (Satoh, 1992). The PHA containing 3H2MB and 3H2MV units had been expected to be new biodegradable materials, but their microstructure were not been characterized untill 1996 by Yoshio's research group (Yoshio, 1996). Those new biodegradable materials were isolated and identified from the sludge developed in the anaerobic-aerobic wastewater treatment process that contained propionate as a carbon source. The PHAs were analyzed by ^{13}C NMR spectroscopy.

There is evidence of the PHA storage phenomena in activated sludge process when a real sludge is mixed with influent wastewater (Carucci, 2001; Kang, 2002; Lam, 2009; Law, 2001; Randall, 2002; Yu, 1998). Bacteria isolated from municipal activated sludge by Yu's research group was characterized by DNA sequence alignment and suggested to be closely related to *Bacillus megaterium* (Law, 2001). This screened bacterium on microbial biopolymer production was subjected to fermentor fermentation. The results showed that PHB homopolymer could be produced. Yu's research group isolated *Sphaerotilus natans*, a filamentous bacterium that caused bulking and foaming difficulties in activated sludge (Liu, 2002). *Sphaerotilus natans*, a sheathed bacterium, is considered an excellent candidate for P(HB-co-HV) synthesis from glucose and propionate mixtures. Maximum of 1.59g/L cell growth with 25.2% cellular weight of PHA accumulated by *Sphaerotilus natans* when glucose used as carbon sources. It was demonstrated that HV content decreased from more than 70% HV to 15% HV with decreasing level of carbon sources. Feeding with a mixture of glucose and sodium propionate in different time points was assumed to be a key factor in producing different 3HV contents in the polymer.

The possible correlations between PHA production/form and subsequent aerobic Phosphorous uptake in Enhanced Biological Phosphorus Removal (EBPR) system were observed by Randall and Liu (Randall, 2002). The response of EBPR to PHAs production type might be dependent on the speciation of the bacteria present in the system. Their studies showed that with isovaleric acid as the carbon source, mainly 3HB was resulted during

the treatment process. Moreover, there was a with much greater P uptake than using valeric acid as the carbon source, greater P uptaking resulted in mainly 3 HV accumulation. Their model showed that 100% 3HB would result in the greatest amount of aerobic P uptake for a given amount of PHAs.

In 1998, Yu's research group investigated the use of malt waste from a beer brewery plant as the carbon source for the production of PHAs by microorganisms. The results showed that Alcaligenes eutrophus DSM1124 can convert malt wastes (barley) to bioplastic to up to 70% polymer/cell (g/g) and 32g/L cell dry weight with PHB as the major product. The higher C/N ratio during the waste conversion (deficiency of nitrogen in the medium) would promote the production of polymers by micro-organism (Yu, 1998). In addition, Yu's research group isolated *Staphylococcus epidermidis* from oil wastes and *Staphylococcus epidermidis* showed that it could accumulate PHB to up to 15% of cell dry weight. Different carbon sources, of sucrose, fructose, mannose, xylose, arabinose, galactose, lactose and maltose, were experimented for PHAs accumulation. Fructose was found the most efficient for cell growth and bioplastic production. Moreover, different food wastes, such as sesame oil, ice cream, malt and soya waste, were used as the carbon sources for PHB production during waste treatment process.

Malt waste was found the best carbon source for PHB accumulation (6.93% polymer/cells (g/g)) (Ai, 2000). Copolymers of HB and HV were accumulated by using malt waste and soy waste. The ratio of HB:HV when

wastes of pure glucose, pure fructose, soy wastes and malt waste were used was obtained as 55:45, 20:80, 75:25 and 90:10, respectively (Wong, 2002).

Table 2.5 summarizes the potential PHA production from activated sludge.

Table 2.5: PHA production from activated sludge

Year	Researcher	Carbon Sources	Bacteria involved	Type of PHAs accumulated	Remark
2004	Dionisi, et. al.,	Acetic, Lactic and propionic acids	Mixed cultures of activated sludge	P(3HB-co-3HV)	0.14g PHA/L/hr
2002	Liu et. al.,	Propionic acid and valeric acid	*Sphaerotilus natans*	P(3HB-co-3HV)	25%$_{(max)}$ of dry cell weight PHA produced
2002	Randall and Liu	Synthetic wastewater with glucose, acetic, propionic, valeric, isovaleric and succinic acids	Activated sludge population	3HB, 3HV	2.85mM-C$_{(max)}$ PHB; 2.83mM-C$_{(max)}$ PHV.
2002	Wong, et. al.,	Malt and Soya wastes	*Alcaligenes latus*, *Staphylococcus* spp., and *Klebsiella* spp.	P(3HB-co-3HV)	
2001	Law et. al.,	Soy and Malt wastes	*Bacillus megaterium*	PHB	
2001	Carucci, et. al.,	Wastewater	Mixed culture of activated sludge	PHB	
2000	Wong, et. al.,	Sesame oil waste, ice cream waste, malt and soya wastes	*Staphylococcus epidermidis*	PHB	5.8%$_{(max)}$ dry cell weight PHB accumulated
1998	Yu et. al.,	Malt wastes	*Alcaligenes eutrophus*	P(3HB-co-3HV)	70%$_{(max)}$ dry cell weight polymer accumulated.
1998	Yu et. al.,	Malt wastes	*Alcaligenes eutrophus*	P(3HB-co-3HV)	70%$_{(max)}$ dry cell weight polymer accumulated.
1992	Satoh, et. al.,	Acetate, propionate and lactate	Mixed cultures of activated sludge	3HB, 3HV, 3H2MB, 3H2MV	320 mgC/L PHA accumulated
1992	Rees, et. al.,	Acetate	*Acinetobacter* RA3117; *Acinetobacter* RA3197; *Acinetobacter* RA375	PHB	
1996	Inoue, et. al.,	Artificial waste water	Mixed culture from activated sludge	3HB, 3HV, 2H2MB, 3H2MV	

Activated sludge processes are often operated under non steady-state conditions because of frequent changes in feed flow rate and composition. Moreover, even if the overall process can be considered in steady-state, the biomass growth may still be under dynamic conditions, being continuously recycled among zones with different substrate concentrations (Carcuui, 2001). In fact, the microbial response to dynamic conditions can be different from a simple increase in cell number and include other substrate removal mechanisms like sorption, accumulation and storage. Although Majone's research group showed evidence for aerobic storage under dynamic conditions in laboratory plants with synthetic substrates and ad hoc cultivated cultures, still little is known about COD removal mechanisms in activated sludge plants with real wastewater (Majone, 1999).

2.9 Recombinant gene for PHAs accumulation

In order to achieve PHA accumulation in some high cell density production bacteria, such as *Escherichia coli* and cyanobacteria, recombinant technology was used. Some cyanobacteria have been reported to be able to accumulate PHA (Philippis, 1992), but it was accumulated only intracellularly at a small percent of the dry weight. Hideyuki and collaborators (1998) used *Synechococcus* sp. PCC7942 in their recombinant gene experiment in order to increase the PHB accumulation in the bacteria from 1% to more than 25% yielding. The bacteria were transformed with genes encoding poly-3-hydroxybutyrate(PHB)-synthesizing enzymes from *Alcaligenes eutrophus* by using a new plasmid vector. Under photoautotrophic and nitrogen-starving conditions, the PHB content of the transormant was improved by more than 25%.

Besides, *Escherichia coli* does not usually synthesize PHAs since it lacks any PHA biosynthetic genes. However, it has been reported to accumulate P(3HB) levels approaching 80% of the dry cell weight when it is transformed with plasmids that bear the appropriate biosynthetic genes from *Alcaligenes eutropha* (Fang, 1998; Hong, 2000; Lee, 1998; Yoshiharu, 2001). The major advantage of synthesizing P(3HB) in *Escherichia coli* is that all the genetic engineering principles that apply to this organism can be utilized in optimizing the production of Poly(3HB) and other PHAs. For example, high molecular weights, such as 4-MDa can be achieved in this organism because it does not contain any PHA depolymerase enzymes (Sim, 1997).

There are also potential advantages in terms of ease of extraction of the polymer and purity of the product (Pouton, 1996).

Sang Yup Lee and collaborators (1994) compared the recombinant *Escherichia coli* strains for the synthesis and accumulation of Poly(3HB). They found that the yields of cell mass, true cell mass and P(3HB) varied considerably among the strains. The P(3HB) yield of XL1-Blue (pSYL105) in Luria-Bertani (LB) plus 20g/L glucose was as high as 0.369 g PHB/g glucose. Strains W (pSYL105) and K12 (pSYL105) accumulated PHB to the highest extent (85.6%) with a relatively low true cell mass (0.77g/L). Considerable filamentation of cells accumulating PHB was observed for all strains except for K12 and W. This seemed to be due to either the over-expression of the foreign PHA biosynthesis enzymes or the accumulation of P(3HB).

2.10 Degradation of PHAs

2.10.1 Biodegradation

The major advantage of using PHAs as thermoplastics for the production of disposable articles is its biodegradability. Since microbial formation of PHAs is synthesized by micro-organisms as carbon and energy reserve materials, it is not surprising that many micro-organisms produce enzymes that are capable of depolymerizing/degrading it (Lafferty, 1988; Vert, 1992). Many soil micro-organisms can totally degrade PHAs and utilize them as a source of carbon. The rate of degradation depends on environmental conditions such as moisture level, nutrient supply, temperature and pH (Vert, 1992). Table 2.6 lists some examples for the biodegradation of P(3HB) in different environments.

Table 2.6: Biodegradation of Poly-β-hydroxybutyrate (PHB) in various environments (Lafferty, 1988).

Environment	Period for Dissolution of a 1 mm thick section (weeks)	Average rate of surface corrosion per week (μm/week)	Period for 100% weight loss of 50μm thick packaging films (weeks)
Anaerobic sewage	9	100	0.5
Estuarine sediment	40	10	5
Aerobic sewage	60	7	7
Soil at 25^0C	75	5	10
Sea water at 15^0C	350	1	50

Basically, enzymes that can be used for the depolymerization of PHAs are either intracellular or extracellular (Lafferty, 1988). All naturally occurring bacteria accumulating PHA most probably possess an enzyme system that mobilizes PHAs intracellularly under certain conditions. These intracellular depolymerase are different from the enzymes of microorganisms that use exogenous PHAs in the environment as the sole carbon source for growth (Steinbuchel, 1991). The enzymes that degrade exogenous PHAs are not only synthesized by bacteria but also by moulds and are excreted by the cells to hydrolyse PHA extracellularly (McLellan, 1988). It is evident that the PHAs degrading enzyme systems are rather complex and consist of several components (Steinbuchel, 1991). Whereas a granule associated heat-labile factor, a PHA depolymerase and an activator protein are required in *Bacillus megaterium* for P(3HB) mobilization (Griebel, 1968). While a granule associated heat-labile factor, a P(3HB) depolymerase, an oligomer hydrolase and a heat stable activator protein are required in *Rhodospirillum rubrum* (Gdriebel, 1971; Merrick, 1966). The action of the activator protein could be simulated by mild tryptic treatment in the samples derived from both bacteria. It was identified that 3-Hydroxybutryic acid is the sole or predominant product of P(3HB)-hyrolysis, with the enzyme systems from *Alcaligenes eutrophus* and *Rhodospirillum rubrum* (Hippe, 1967; Merrick, 1966). On the other hand, a less active PHA-mobilizing enzyme system should be more favourable with respect to the yield of PHA during fermentation, and therefore important for the production of PHA.

Production of extracellular PHAs hydrolyases is demonstrated by zones of clearing around colonies on mineral salts agar medium which contains PHA as a carbon source. An alternative detection method is to incubate depolymerase that contains solutes with thin-cast polyester films and follow its dissolution and erosion (Malik, 1978). There are two steps in the extracellular degradation of PHA. First, a PHA depolymerase releases oligomers. Second, an oligomer hydrolase cleaves the dimmer into monomers.

The intracellular degradation of PHB is shown in Figure 2.9. PHB is first degrade into D(-)-3-hydroxybutyric acid by the action of PHB depolymerase. In *Alcaligenes eutrophus*, the sole product of PHB hydrolysis by soluble PHB depolymerase is D(-)-3-hydroxybutyrate (Hippe, 1967). However, the hydrolysis products of soluble PHB depolymrease isolated from *Bacillus megaterium* are a mixture of dimmers and monomers of D(-)-3-hydroxybutyrate (Yoshiharu, 1990a).

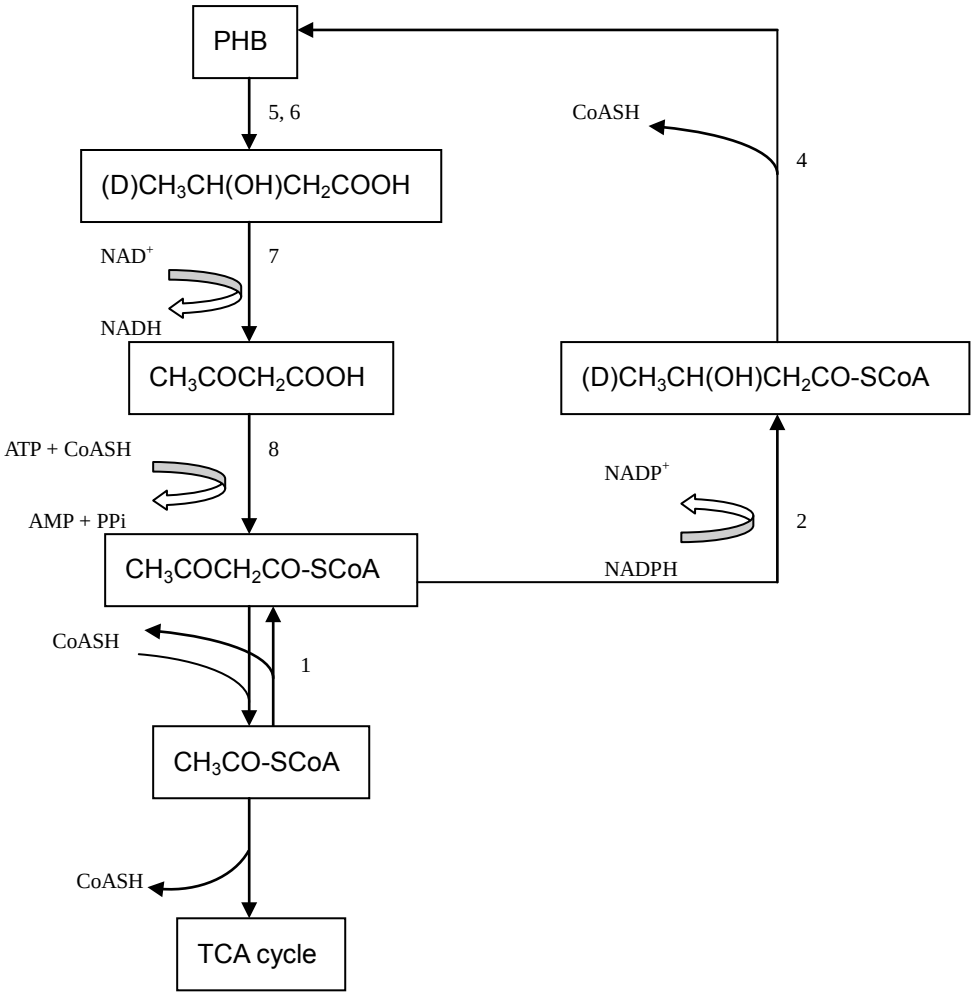


Figure 2.10: Cyclic metabolism of PHB in *Zoogloea ramigera* (Yoshiharu, 1990a).

1: 3-Ketothiolase; 2: NADPH-linked acetoacetyl-CoA reductase; 4: PHB synthase; 5: PHB depolymerase; 6: D(-)-3-hydroxybutyrate-dimer hydrolase; 7: D(-)-3-hydroxybutyrate dehydrogenase; 8: acetoacetyl-CoA synthetase.

Delafield and his research group (1965) identified that *Pseudomonas lemoignei* constitutively produced an extracellular depolymerase to degrade the polymer to a mixture of monomeric D(-)hydroxybutyric acid and the dimeric ester. The dimeric fraction was incorporated by the cell and hydrolyzed by an intracellular dimmer hydrolase. In 1982, Tanio and his research group enriched and purified an extracellular P(3HB) depolymerase from a strain of *Alcaligenes faecalis* grown with P(3HB) as the sole source of carbon. The purified extracellular enzyme had a molecular weigh of 48,000 as determined by gel filtration and 50,000 by polyacrylamide gel electrophoresis. The optimum pH was 7.5. The sole products of the enzymatic hydrolysis of P(3HB) by this enzyme were the dimmer and some minute quantities of the monomer. This enzyme was not constitutive but required P(3HB) in the medium for enzyme formation. However, repression of the secretion of the depolymerase could be avoided by lowering the pH of the medium from 7.5 to 6.9. Therefore, it was apparent that a broad spectrum of bacteria were capable of depolymerizing macromolecular P(3HB).

The bacterial utilization of P(3HB) as a carbon and energy source can be concluded as follows (Yoshiharu, 1990a):

(i) Depolymerization of PHAs, catalyzed by an extracellular depolymerase (hydrolase), and production of dimmer with accompanying traces of monomer, ie. D(-)-3-hydroxybutyrate;

(ii) Incorporation of the dimmer and monomer by the cells; and

(iii) Intracellular hydrolysis of the dimmer catalyzed by an intracellular β-hydroxybutyrate dimmer hydrolase and subsequent incorporation of the monomer into intermediate metabolic pathways.

2.10.2 Thermal Degradation

PHB exhibits relatively fast degradation rates at temperatures close to the melting point of the homopolymer (180^0C). The currently accepted degradation mechanism for PHB is a random scission, beta-elimination process (Robert, 2001). The degradation mechanism was proposed by Hammond and Liggat in 1995 as shown in the figure:

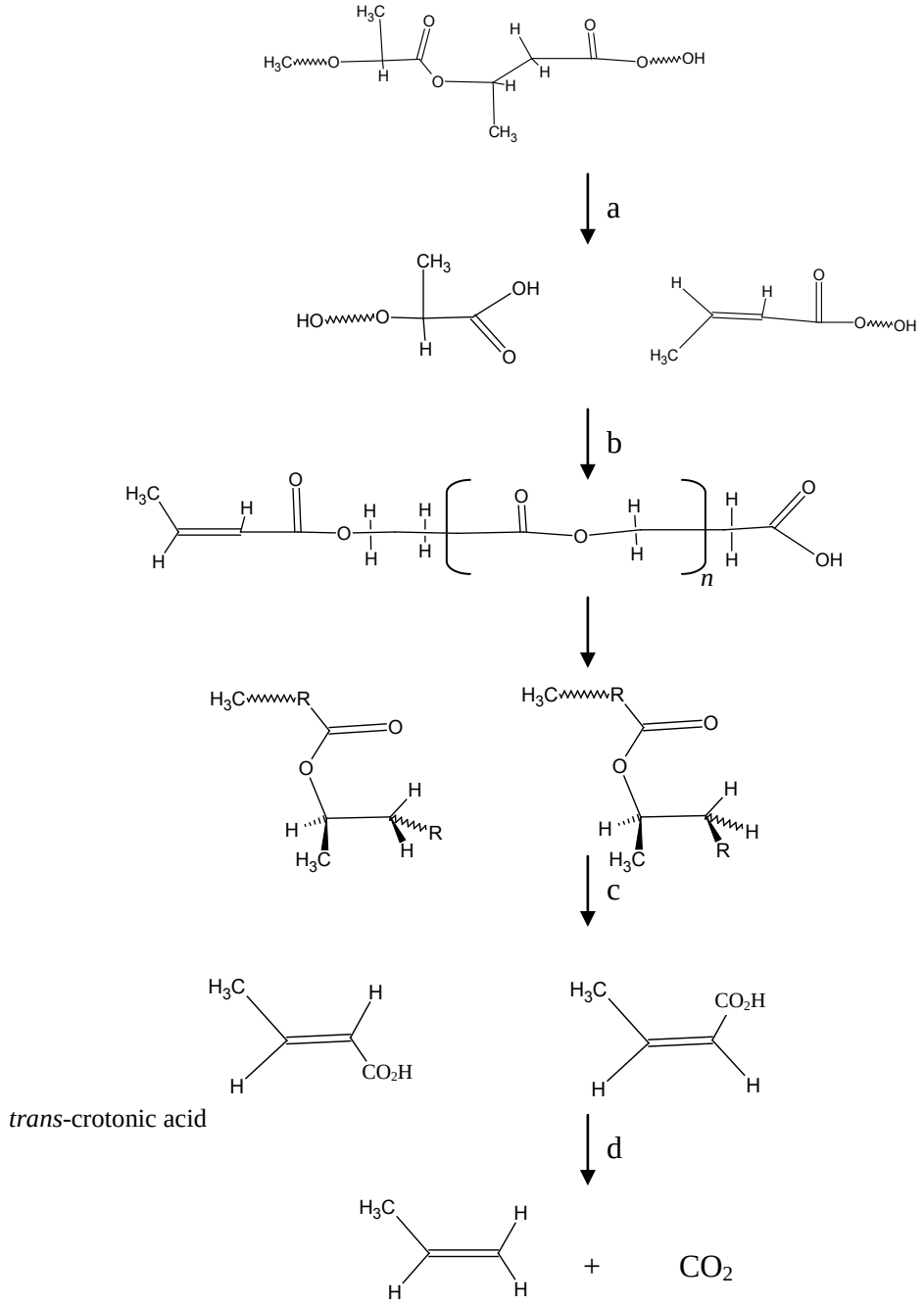


Figure 2.11: Proposed degradation mechanism for polyhydroxybutyrate (Hammond, 1995)

Figure 2.11 shows that a sterically favourable six-membered transition state may be formed which on scission leads to the formation of unsaturated and

carboxylic acid terminated chain fragments. This leads to a reduction in molecular weight and a narrowing of the molecular weight distribution. With the extending of degradation increase, the chain lengths become shorter and volatile oligomeric products begin to be observed (Figure 2.10b). Figure 2.10c shows further degradation that crotonic acid produced. The trans configuration is sterically facoured. It leads predominantly to the formation of the trans isomer. The cis product is a secondary product only seen at higher temperatures. At higher temperatures (> 300^0C), decarboxylation leads to alkene formation (Hammond, 1995) (Figure 2.10d).

2.11 Low Costs of PHAs Production

Possessing of the biodegradable ability of PHAs, PHAs seem to be one of the solution for the plastic pollution problem in the world. PHAs can be used in daily lives only if its production cost is low. PHB and P(3HB-co3HV) are the only two members of PHAs that are in the first stage of commercial development (Lee, 1995). The PHAs production cost ($16/kg) is still too high compared to the cost of synthetic plastics such as polypropylene (less than $1/kg). Historically, about 50% of the total operating costs are for the carbon source (Beom, 2000; Choi, 1999). Moreover, an expensive carbon source is not practical in large scale industrial productions. Isolation and development of a strain that overproduces PHA from a cheap carbon source seem to be more important. Utilization of industrial food waste to produce environmentally friendly bioplastics is one of the good approaches to cut down the costs for PHA production. It has been reported that some recombinant E. coli strains could produce PHB from whey (Lee, 1997; Wong, 2000), molasses (Li, 1990; Liu, 1998), soy waste or malt waste (Yu, 1999).

The utilization of waste materials as substrate is a good alternative to reduce the cost of PHAs production. The most important aspect of producing PHAs is their biodegradable nature that can help 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 PHA.

2.12 Industrial Production of PHA

It is of considerable industrial interest and of environmental importance to evaluate PHA as polyesters for the use in either biodegradable or biocompatible plastics for a wide range of possible applications such as packaging containers, bottles, wrappings, bags, films disposable products, bone replacements, medical sutures, staples and swabs. Table 2.7 is a list of some companies that develop PHA products. ZENECA Bio Products is the sole producer of P(3HB) and P(3HB-co-3HV) under the trade name of BIOPOL.

Table 2.7: Industrial production of PHAs and applications (Lee, 1995).

Company	Areas of interest
Berlin Packaging Corp. (USA)	Marketing, sales, and distribution of ZENECA's BIOPOL. Selling bottles made from BIOPOL to hair care company
Bioscience Ltd. (Finland)	Medical applications of PHAs
BioVentures Alberta Inc. (Canada)	Production of PHA by recombinant Escherichia coli
Metabolix, Inc. (USA)	Production of PHA by transgenic plants. Licensing technology and jointventures
Monsanto (USA)	Production of PHA by transgenic plants (rapeseed and soybean)
Polyferm, Inc. (Canada)	Production of PHA from cheap substrate (hemicellulose). Production of PHA by Pseudomonas cepacia from xylose
ZENECA Bio Products (UK)	Production of P(3HB) and P(3HB-co-3HV) (BIOPOL) by fed-batch culture of Alcaligenes eutrophus
ZENECA Seeds (UK)	Production of PHA by transgenic plants (rapeseed)

In addition its potential as plastics materials, PHA also represents a useful source of stereoregular compounds that can serve as chiral precursors for the chemical synthesis of optically active substances and in particular in the synthesis of certain drugs or insect pheronons (Seebach, 1985). These substances are only biologically active in a correct stereochemical configuration.

For industrial applications, it is desirable to control the incorporation of different repeating units into a polymer in order to produce polyesters with specific material characteristics because their physical and chemical characteristics strongly depend on the copolymer composition. Tailor-made copolymers can be made by the use of controlled conditions for this purpose. If a defined mixture of nutrients for a certain type of microorganisms is supplied for growth, a defined and reproducible copolymer is formed. Biochemically there are two different ways to form polymers in microbes: (i) simultaneous cell growth and PHA formation; (ii) cell growth followed by PHA formation.

For (i), the growth substrate or carbon source as demonstrated by the formation of various PHA copolyesters in bacteria can be metabolized to form microbial biomass and storage polymer simultaneously (Brandl, 1989). During cell growth, part of the polymer forming potential is lost because of its utilization of substrate to maintain the cell's metabolism. For (ii), industrial polymer production micro-organisms are first grown in a carbon source to obtain a large biomass, then the medium is depleted of an essential nutrient

and a polymer forming substrate is added. It is directly converted to polymers and essentially only little growth occurs. This approach is used for large scale PHA production by *Alcaligenes eutrophus* (Kunioka, 1989; Yoshiharu, 1987).

ICI Ltd. evaluated three organisms, namely *Azotobacter* sp., *Methylobacterium* sp. and *Alcaligenes eutrophus*, which collectively embraced a wide range of substrates as the principal contenders for the industrial production of PHB (Anderson, 1990). The *Azotobacter* sp., which could grow on glucose or sucrose with high polymer yields, proved unstable and also synthesized carbohydrate, thereby diverting substrate from PHB production. For *Methylobacterium* sp., which seemed attractive on account of ICI's wide experience in methanol fermentation technology, gave only moderate yields of polymer of low molecular weight that was extracted with difficulty. *Alcaligenes eutrophus* grown heterotrophically became the organism of choice because of its high polymer contents of high molecular weight and the availability of various economically acceptable substrates. (Collins, 1987).

The following metabolic sequence represents the ICI process:

(2.7) PHB_n + glucose + ADP + P_i + 3NAD $\longrightarrow$ PHB_{n+1} + ATP + 3 NADH + $2CO_2$

This equation is energy yielding, and generates ATP and NADH, but since carbon dioxide is eliminated the yield cannot exceed 1 mol of monomer incorporated per mol of glucose. About 2.1g of glucose is required per g of PHB produced, and when a single-stage continuous fermentation is used, energy is also needed for the biomass production (Anderson, 1990). The ICI production process for PHB has also been outlined that, with fed-batch grown *Alcaligenes eutrophus* in a glucose salt medium and with phosphate exhaustion as the growth limiting factor (Byrom, 1987). At the time of growth cessation (about 60 hours), little PHB has been synthesized; glucose is then added to the culture, and in the next 48 hours massive accumulation of the polymer or up to 75% of the total dry biomass occurs. The total fermentation time (growth and accumulation phases) is between 110 and 120 hours.

The recovery of polymer from the biomass is a vital stage in the process. Although large-scale solvent extraction gives high recovery, it is an expensive technique and involves large volumes of solvent and high capital investment in the solvent recovery plant. Polymer separation and purification is accomplished by using hot methanol reflux to remove lipids and phospholipids followed by the extraction of PHB with chloroform or methylene chloride. Cells disintegrated by heat shock are treated with a series o fenzymic and detergent digestive process to solubilize the non-PHB components. The PHB is then washed, flocculated and recovered as a white powder (Anderson, 1990). In addition, PHB is too stiff and brittle for

most applications, so ICI adds a small amount of simple organic acid to the sugar feed stock to make the plastic stronger and more flexible. The average PHB contents are found to be 25-30% of dry weight. The cell was also capable of producing PHV in the PHV to PHB ratio of 0.2.

2.13 PHAs Application

The variations in the physical and mechanical properties of different PHA types offer a wide range of applications with potentially replacement for conventional plastics. Some possible PHA applications are listed in Table 2.8.

Table 2.8: Possible applications of PHAs (Lee, 1990).

- Packaging films, bags and containers
- Biodegradable carrier for long term dosage of drugs, medicines, insecticides, herbicides, or fertilizers
- Disposable items such as razors, utensils, diapers, or feminine hygiene products
- Starting materials for chiral compounds
- Surgical pins, sutures, staples and swabs
- Wound dressing
- Bone replacements and plates
- Stimulation of bone growth and healing by piezoelectric properties
- Blood vessel replacements

2.13.1 Package and derived materials

The most well known application of PHB and poly(3HB-co-3HV) is as substitute for conventional, non-biodegradable plastics for packing purposes and derived products (Yoshiharu, 1990a). Single use bottles for shampoos, cosmetics and biodegrable motor oil have been manufactured from these

biopolyesters by common molding techniques. Containers and cups for food products have been developed similarly and bags have been produced from blown films of the material. It is also possible to use PHB or poly(3HB-co-3HV) in the form of aqueous latex, a dispersion of polymer granules in water, and for coating fibrous materials such as paper or cardboard (van der Walle, 2001). From these PHA coated materials, paper cups, trays for holding foods and drinks were then developed. Due to its outstanding water resistance, the hydrophobic PHA coating protects the hydrophilic paper or cardboard against damages and deterioration caused by moisture of the packaged food or by the environment.

Various other disposable products have been developed from PHAs. Fibers and non-woven fabrics have been manufactured. These materials are very suitable for use in sanitary napkins and diapers (Yoshiharu, 1990a). Other typical single use products include disposable razors and cutlery produced from molded PHAs.

2.13.2 Fishing Lines and Nets

Monofilaments useful for fishing nets, ropes and marine agriculture are produced from Poly(3HB-co-3HV) by a process in which resin is molten for less than 6 min at temperatures about 15^0C higher than the T_m of the polymer, spun at a shear rate above 2 x $10^{-2}s^{-1}$ above the filter layer in the spinneret pack and has a residence time in the pack less than 2 min. to give

monofilaments, which are solidified at T_c around 10^0C for 5-10s and stretched (Yoshiharu, 2001).

The tensile strength of Poly(3HB-co-3HV) increases with decreasing 3HV content. Poly(3HB-co-3HV) with 0-10% 3HV exhibits tensile strength comparable to low density polyethylene and polypropylene. The relatively slow biodegratdation rate of Poly(3HB-co-3HV) in sea water permits a useful lifetime of articles such as fishing nets (Yoshiharu, 1994).

To take advantage of PHAs respective properties, high density polyethylene as the core and Poly(3-HB-co-3HV) as the sheath were melt spun at a 60:40 v/v ratio to give fibers with tenacity 5.4 gd^{-1} and exhibiting a very small amount of adhesion of marine substances on immersion of a net of the fibers in seawater for 6 months (Brandl, 1988).

2.13.3 Medical applications

PHAs have been drawing considerable industrial interest as candidates of biodegradable and/or biocompatible plastics for a wide range of applications. The degradation product of P(3HB), D(-)-3-hydroxybutyrate, is a common intermediate metabolite present in all higher animals (Reusch, 1992). A low molecular weight P(3HB) that consists of 100-20 monomer units which seems to function as a component of an ion channel through cell membranes has been found in prokaryotic and eukaryotic organisms

(Reusch, 1983). In addition, P(3HB) has been detected in relatively large amounts in human blood plasma (Reusch, 1992). Therefore, it is highly plausible that the implanting of P(3HB) in mammalian tissue is toxic.

2.13.3.1 Wound dressing and Scaffold materials

Poly(3HB) and Poly(3HB-co-3HV) can be steam sterilized without losing their properties and are resistant to alcohol (Kledzki, 1994). After sterilization, they may be left in place to aid blood clotting without the rejection problems associated with cotton materials. Swabs, pads, and other articles made from Poly(3HB) that are left in the body by design or by accident will not cause toxemia and will be slowly absorbed by the body. They differ from cotton wool in having little or no tendency to break off small fibers; and even if small pieces are to enter a wound, it is still safe. There is no need to enclose them with a retaining gauze and they can be readily tailored to different sizes at the point of use.

Traditional monofilament surgical suture degrades very slowly and requires a few years to be totally resorbed by the body. The time required for biodegradation is related to the surface area of suture and multifilament sutures resorb much more quickly (Holmes, 1985). In vivo and in vitro assessment of monofilaments of Poly(3HB) and Poly(3HB-co-3HV) for changes in mechanical properties and topography had been done by Miller and Williams (1987). It was found that in vivo biodegradation was observed

only with Poly(3HB) when it had been pre-degraded by 10 Mrad of γ-irradiation before implantation. High temperature in vitro hydrolysis suggested that 3 HV copolymer addition retarded the rate of degradation of Poly(3HB). In contrast, the elastic properties appeared to be relatively unaffected (Miller, 1987). It demonstrated that neither Poly(3HB) nor Poly(3HB-co-3HV) in monofilament form was very biodegradable, although their susceptibility to degradation might be increased by exposure to gamma radiation.

Poly(3HB) was gel-spun into a novel form with one possible application as a wound scaffolding device designed to support and protect a wound against further damage, while promoting healing by encouraging cellular growth on and within the device from the wound surface (Yoshiharu, 2001). The hydrolytic degradation of wool was investigated in an accelerated model of pH 10.6 and 70^0C. The Poly(3HB) wool gradually collapsed during degradation which was characterized by a reduction in T_g, T_m and a fusion enthalpy peak of maximum crystallinity (88%) which coincided with the point of matrix collapse (Foster, 1994).

Absorbable polymeric scaffolds for cell culture and transplantation, tissular reconstruction, and in vivo drug or protein release proposed by salt leaching/solvent casting a Poly (3HB-co-3HV) (Chaput, 1995). Electron microscopy and mercury porosimetry showed highly porous, well-interconnected microstructures with a porosity level of 0.85 and a mean pore diameter of 122μm. Incubation from 1 to 35 days of canine anterior

cruciate ligament fibroblasts in scaffolds had shown a limited proliferation rate (150 x 10^6 cells (g maximal d)$^{-1}$) but high protein synthesis (2.4 ± 0.1 x 10^{-2} ng cell^{-1}day^{-1} at Day 28) (Chaput, 1995).

In fact, surgical removal of bio-tissues is not required after their implantation. Apart from this, PHAs have the potential to be constituents for anti-human immunodeficiency virus drugs, anticancer drugs, antibiotics and vitamins (Ward, 2005).

2.13.3.2 Drug carriers

The hemocompatibility of a material can be evaluated by studying the adsorption of proteins that stimulates the thrombus formation. Films of Poly(3HB-co-3HV) were treated with perfluorohexane and H_2 plasma. It was shown that hydrophobic polymer films adsorb more albumin than fibrogen. Moreover, the amounts of protein adsorbed seem to be mainly a function of the surface roughness of the films – the largest amounts being adsorbed on the rougher plasma-treated films, while the smother perfluorohexane-treated surfaces adsorbed less proteins (Coussot-Rico, 1994).

Poly(3HB-co-3HV) shows low rates of degradation in vitro as well as in vivo. The rate of weight loss generally increases with the copolymer (3HV) content and ranges from 0.15 to 0.30 (in vitro) and to 0.25% day^{-1} (in vivo). Compositional and physicochemical changes in Poly(3HB-co-3HV) materials were rapidly detected during the accelerated hydrolysis, but were much

slower to appear in vivo. The random hydrolytic cleavage of the amorphous part of these polymers might result, in vivo, in the production of small crystalline particles of low-molecular-weight Poly(3HB) that could undergo phagocytosis and biodegradation inside phagosomes (Ciardelli, 1995). In addition, the release rate of the drug aclarubicin from P(3HB) microspheres prepared by a solvent-evaporation process. The release rate of the drug was very slow, and only about 10% of the loaded drug was released after 120 hours in vitro. In order to modify the release rate of the drug, a series of fatty acids and their alkyl esters were mixed with P(3HB). Ethyl and butyl esters of fatty acids with more than 1- carbon atoms were found to effectively enhance the rate of drug release (Juni, 1986).

2.13.3.3 Nanoparticles as potential drug delivery system

In view of the relatively high crystallinity and complete chirality of molecular chains, microbial PHB is unsuitable for the molecular design of specialty polymers such as amphiphilic block copolymers. Yu's research group investigated the new amorphous amphiphilic triblock copolymers of poly(3-hydroxybutyrate)-poly(ethylene glycol)-poly(3-hydroxybutyrate) (PHB-PEG-PHB). These triblock copolymers can form biodegradable nanoparticles with core-shell structure in aqueous solution. (Chen, 2006). It is found that the critical micelle concentrations (CMC) values of these copolymers decreased remarkably with the increase in the PHB block length and the conformation and structure of the PHB-PEG-PHB nanoparticles

were dependent on temperature. Besides, higher drug loading content (DLC) and drug loading efficiency (DLE) were exhibited. These features, together with the simple preparation method without surfactants, make PHB-PEG-PHB nanoparticles very suitable to be delivery carriers.

PHB-PEG-PHB nanoparticles have more superior drug loading properties and shorter biodegradation period in vitro than microbial PHB in the presence of an extracellular enzyme. The cytotoxicity and hemolysis assay of the PHB-PEG-PHB were further investigated. It was found that the PHB-PEG-PHB nanoparticles showed excellent biocompatibility and had no cytotoxicity on animal cells, even when the concentrations of PHB-PEG-PHB nanoparticle dispersions were increase to 120μg/ml (Chen, 2007). It was known that cell adhesion was mediated by the interaction of surface proteins such as integrins with proteins in the extracellular matrix or on the surface of other cells or particles (Lamazi, 1997). The phenomenon of cell adhesion was important in governing a range of cellular functions including cell growth, migration, differentiation, survival and tissue organization, differentiation, survival and tissue organization. Thus the delivery system based on PHB-PEG-PHB nanoparticles should not elicit any generic and chronic inflammatory responses that could ultimately result in failure to achieve normal cell growth and function on the cell particle surface.

It was demonstrated that when PHB-PEG-PHB nanopraticles were injected into the blood, no observational hemolytic activities in the red blood cells were observed (Chen, 2007). This showed that PHB-PEG-PHB nanoparticels were suitable for a wide safety margin in bold-contacting applications and suitable for intravenous administration.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Activated Sludges

There were nine types of activated sludge from different industries in Hong Kong that were used in this research study. Among of them, two came from domestic sludge with different matrix compositions, one came from a piggery farm, one came from Grease Trap, one came from water treatment works and four came from different matrix of food waste sludge were used. Table 3.1 summarizes the types of sludge used in this study and their matrix compositions:

Table 3.1: Different Activated Sludge and their matrix composition

Activated Sludge	Location	Matrix Compositions
Domestic Sludge 1 (DS1)	Tai Po Sewage Treatment Works, Tai Po, N.T.	Mainly Domestic wastes.
Domestic Sludge 2 (DS2)	Shek Wu Hoi Sewage Treatment Works, Yueng Long, N.T.	Domestic wastes with seawater.
Piggery Sludge (PS)	Piggery farm treatment plant, Kwu Tung, Lok Ma Chau, Sheung Shui, N.T	Piggery waste including piggery blood, metabolic wastes.
Oil Sludge (OS)	Greae Trap, Kwai Chung Container Port, Kwai Chung	Food grease and mechanical oily waste.
Purify Bio-filter Sludge (PBS)	Tai Po Water Treatment Works, Tai Po, N.T.	Biological filter sludge.
Food Waste Sludge 1 (FWS1)	Garden Company, Sham Tseng, N.T	Flour, Carbohydrate, eggs waste.
Food Waste Sludge 2 (FWS2)	Vitasoy Treatment Plant, Tuen Mun, N.T.	Milk and Soya bean waste.
Food Waste Sludge 3 (FWS3)	Chinese Restaurant, Shek Kip Mei, Kowloon	Restaurant oily waste sludge.
Mixed Sludge - FWS2 & FWS1 (MS)	FWS2 mixed with FWS1	Milk, soy bean waste sludge with carbohydrate waste sludge.

For Mixed Sludge (MS), FWS1 (Food waste sludge of Garden company) was used as the microbial source for FWS2 (Food waste sludge of Vitasoy treatment plant).

3.1.2 Agar medium

Three agar medium were used throughout the study, they were:

(i) Nutrient Agar (NA);

(ii) Plate Count Agar (PCA); and

(iii) R2A.

Table 3.2: Compositions of different agar medium

Nutrient Agar (NA) (g/L)	**Plate Count Agar (PCA) (g/L)**	**R2A (g/L)**
1. Lab-Lemco Powder (1g) 2. Yeast extract (2g) 3. Peptone (5g) 4. NaCl (5g) 5. Agar (15g)	1. Tryptone (5g) 2. Yeast extract (2g) 3. Glucose (1g) 4. Agar (9g)	1. Yeast Extract (0.5g) 2. Proteose peptone No.3/polypeptone (0.5g) 3. Casamino acids (0.5g) 4. Glucose (0.5g) 5. Soluble starch (0.5g) 6. Dipotassium hydrogen phosphate (0.3g) 7. Magnesium sulfate heptahydrate (0.05g) 8. Sodium pyurate (0.3g) 9. Agar (15g)

3.1.3 Storage Medium (per L)

30 g trytone soya broth, 5 g glucose, 20g skim milk powder, 40 ml glycerol, 1 L distilled water with pH adjusted to 7 and sterilized at 121^0C for 20 min.

3.1.4 Nutrient rich medium (per L)

10 g glucose, 10 g polypeptone, 10 g yeast extract, 10 g meat extract, 1 L distilled water with pH adjust to 7 and sterilized at 121^0C for 20min.

3.1.5 PHA production medium (nitrogen-free medium) (per L)

20 g methanol (C1) or 20 g lactic acid (C3) or 20 g sodium propionic acid (C3_Na) or 20 g glucose (C6) or 20 g sodium octanoate (C8_Na) or 20 g maltose (C12), 4.8 g di-sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), 2.65 g potassium di-hydrogen phosphate (KH_2PO_4), 0.4 g magnesium sulphate

($MgSO_4.7H_2O$), 1 L distilled water with pH adjusted to 7 and sterilized at 121^0C for 20 minutes.

3.1.6 Supplementary trace element (per L)

16.2 g iron III chloride ($FeCl_3.6H_2O$), 13.3 g calcium chloride ($CaCl_2.2H_2O$), 0.1546 g copper sulphate ($CuSO_4.5H_2O$), 0.255 g cobalt chloride ($CoCl_2.6H_2O$), 0.118 g nickel chloride ($NiCl_2.6H_2O$), 0.09 g Chromium (III) Chloride Hexahydrate ($CrCl_2(H_2O)_4Cl.2H_2O$), 1 L of 0.1 M hydrochloric acid and then filtered through 0.25 um filter paper for sterilization.

3.1.7 Buffer solution (per L)

8.5 g sodium chloride (NaCl), 0.75 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), 0.3 g potassium dihydrogen phosphate (KH_2PO_4) and sterilized at 121^0C for 20 minutes.

3.1.8 Esterification solution (per 100 ml)

0.4 g Benzoic acid, 3 ml concentrated sulphuric acid, 97 ml methanol.

3.1.9 Feed Waste

The feed waste was food waste from Garden Company, Sham Tseng, N.T. It contained milk, soya bean, fruit juice, flour, egg, glucose, sucrose, starch and protein as the major constituents (chemical characteristics: BOD: 27,000 mg / L , COD: 30,000 mg/L, SS: 10,000 mg/L, KN: 2,000 mg/L).

3.2 Methodology

In this study, four processes were carried out to investigate the microbial formation of polyester and waste water treatment efficiency. The first process investigated the feasibility of different agar medium for potential PHAs biomass screening. The second process investigated the microbial community of activated sludge from different industries in Hong Kong and their feasibility of PHAs accumulation cultivated with different carbon sources. In the third process, the PHAs production in waste water treatment system. In the fourth process, the induction effect of PHA production among different PHA biomasses during waste water treatment was carried out.

3.2.1 Microbial community of activated sludge and comparison of different agar medium for potential PHAs biomass screening

3.2.1.1 Pretreatment of Feed Waste

The feed waste (3.1.9) collected from Garden Company was sterilized by chlorination of adding excess sodium hypochlorite. It was than

dechlorinated by adding sodium thiosulphate ($Na_2S_2O_3$) solution into the chlorinated feed waste. It was carried out by adding 0.1ml 10% sodium thiosulphate in 100ml feed waste.

3.2.1.2 Sample enumeration and cell screening

Within any population of microorganisms that survive exposure to treatments or environmental stresses, there are exist individual cells that are regarded as sublethanlly injured. Stress treatments that cause injury include heating, refrigeration, freezing, irradiation, high acid or alkali, high levels, preservatives, desiccation, exposure to disinfectants and starvation or nutrient limitation. In this research study, potential PHA bacteria isolated from the sewage/sludge of waste treatment plants. It is, therefore, necessary to encourage the growth of the nutritionally depleted bacteria in the activated sludges samples, from which such bacteria may potentially contain PHA biomass. If the potentially PHA biomasses were hidden or could not be recovered from the sewage/sludge samples during the screening process, the concerned biomass would then be lost.

Numerous strategies have been used to avoid underestimating microbial numbers in samples likely to contain stressed cells. These include (i) avoiding the use of selective media altogether and (ii) formulating the medium to minimize any inhibitor effects on injured target organism. Historically, Nutrient Agar (NA) or Plate Count Agar (PCA) was used for the inoculation of samples to find out the heterotrophic bacteria count (Clesceri, 1998). In the past PHA research, the colonies found were detected by Fourier Transform Infrared spectroscopy (FTIR) to identify whether the potential PHA bacteria were present or not (Hong, 1999; Wallen, 1974). However, NA and PCA are the agar with high nutrient, they may not be suitable for stressed tolerant organisms (e.g. PHA bacteria) presented in the activated sludge/sewage samples.

In this work, different dilutions (10^{-1} - 10^{-7}) of activated sludge samples were enumerated into the three agar medium (3.1.2) (Nutrient Agar, Plate Count Agar and R2A agar) and then incubated at 37^{0}C for 48 hours. Growth colonies were examined by FTIR at wavenumbers from 1728 - 1744 cm^{-1} (Hong, 1999) in order to identify the potential PHA biomass.

3.2.2 PHAs accumulation of activated sludge by shake flask experiment

3.2.2.1 Purification of potential PHA biomass

Nine types of activated sludge from different industries in Hong Kong were used (3.1.1). The potential cells were isolated by FTIR and then purified by streaking the potential cell in another R2A agar. The purified cells were stored in a storage medium (3.1.3) and kept at -70^{0}C for future usage.

3.2.2.2 Microbial formation of PHAs by shake flask set-up

In this work, a two-stage cultivation of potential PHA biomass was employed to stimulate the accumulation of PHAs. The first stage was cultivated the potential cells in a nutrient rich medium (3.1.4). The potential cells from -70^{0}C refrigerator were inoculated in a 100ml sterilized nutrient rich medium (3.1.4) in 250ml baffled conical flasks. The baffled conical flasks were covered with silicon filter sponge for aeration. The potential cells were incubated at 37^{0}C and shaken at 250 rpm for 48 hours. The cells were

harvested by centrifugation and washed twice with buffer solution (3.1.7) in order to remove any residual nitrogen.

The second stage was PHAs accumulation. In this stage, the enriched and harvested cells were transferred to 100ml of different sterilized nitrogen-free medium (3.1.5), i.e. microbial bioplastic production medium containing various carbon sources, namely methanol (C1), lactic acid (C3), sodium propionic acid (C3_Na), glucose (C6), sodium octanoate (C8_Na) and maltose (C12) and 1ml supplementary trace element (3.1.6) in a 250ml shake flask. The potential cells were incubated at 37^0C and shaken at 250 rpm for 48 hours.

3.2.3 Production of PHAs by Wastewater treatment process

3.2.3.1 Construction of Sequencing Batch Bioreactor (SBR)

A bench scale 6L Sequencing Batch Bioreactors (SBR) made of corrugated plates (PVC) (each with surface area of 840 cm^2) and consisted a 6L box (Figure 3.1) was constructed.

Figure 3.1: Immobilized Sequencing Batch Bio-reactor used in our

wastewater treatment and PHA synthesis.

The base of SBR was sloped toward a PVC tube to facilitate the collection of excess sludge and dead microbial cells produced during the process. The reactor was connected to peristaltic pumps and two boxes for substrate feeding and effluent collection. An air compressor was connected to the system to provide oxygen during microbial degradation. Figure 3.2 is a schematic diagram of the SBR configuration.

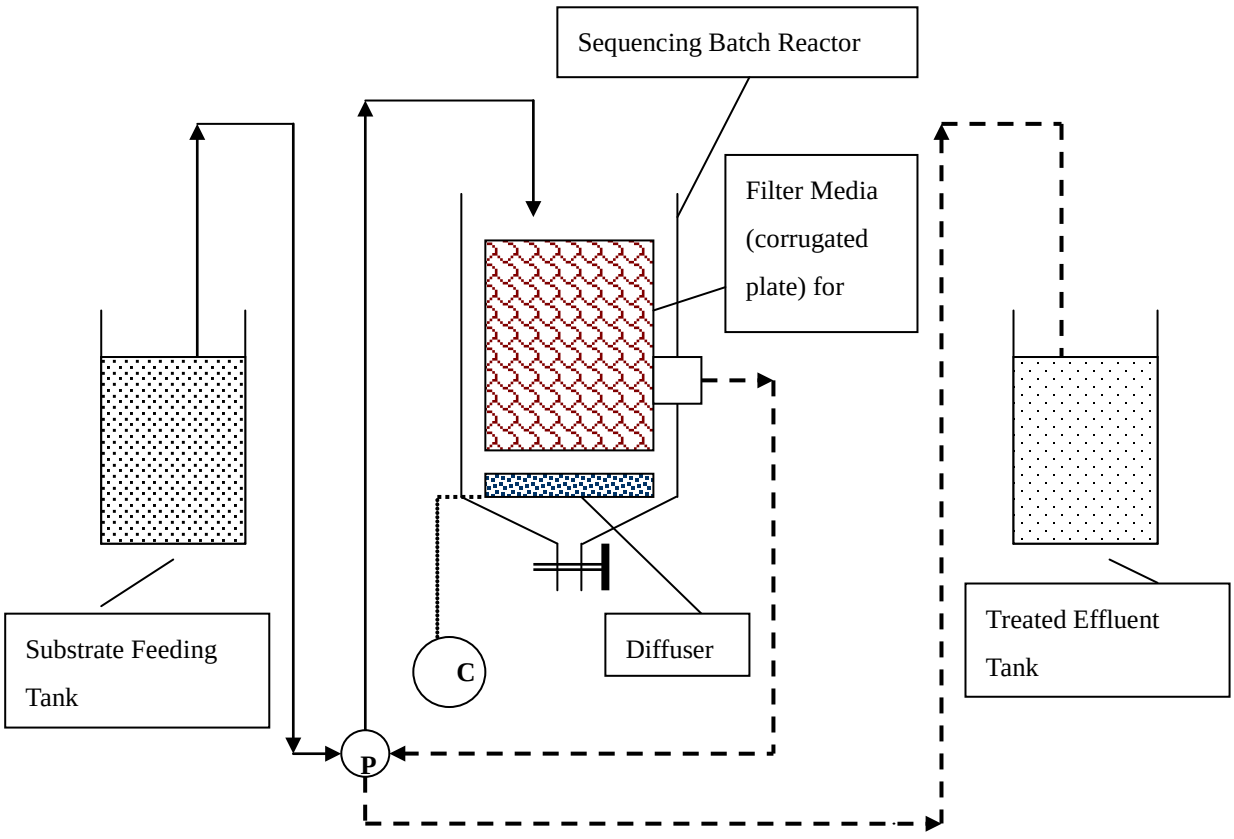


Figure 3.2: The schematic configuration of Sequencing Batch Reactor system. C: Air compressor; P: Peristaltic pump; Filter media:

Corrugated plate; 110m^2/m^3 surface area with 95% void.

3.2.3.2 Operation of SBR

Stage 1: Acclimation

Acclimation was executed by inoculating the confirmed PHAs strains directly into separate identical reactors (6L) and allowing the biofilm to develop with wastewater as the feed substrate. The feed waste (3.1.9) was disinfected by sodium hypochlorite and then dechlorinated by sodium thiosulphate in the ratio of 0.1mL 10% sodium thiosulphate (sodium thiosulphate 100mg/L, disinfected by passing through 0.2um filter) per 100mL wastewater (Clesceri, 1998). During the acclimation stage, the influent organic loading (COD level) was sequentially increased from 500 to 1,000 mg/L by diluting the raw feed waste (3.1.9) with distilled water (sterilized at 121^{0}C for 20 minutes before use) to enable the microbial film to develop. This acclimation stage took 2 months to complete and at least 90% COD removal was achieved.

Stage 2: Operation for Pollutant Removal and PHAs accumulation

After the acclimation period, the influent COD was increased from 1,000mg/L to 15,000mg/L (by diluting the raw wastewater with sterilized distilled water)

with feeding profile as: Day 0 – Day 2: 1,500 ppm COD; Day 3 – Day 4: 3,000 ppm COD; Day 5 – Day 7: 6,000 ppm COD; Day 8 – Day 10: 12,000 ppm COD; Day 11 – Day 13: 15,000 ppm COD. The dosage period for different influent levels depend on the treatability. Parameters of BOD, COD, KN, SS and PHA accumulation were measured after acclimation. During operation, the SBR was operated in 5 steps:

(1) Filling (4hrs): Diluted wastewater starts to feed into the reactor;

(2) Reacting (14hrs): Aeration starts after wastewater feeding. The pollutants of wastewater are degraded by identified strains during this stage. PHAs accumulation may occur in parallel of microbial degradation of wastewater;

(3) Settling (2hrs): Mixing and aeration of the SBR are terminated. The excess sludge and dead cells from the attached medium settle and sink to the bottom of the reactor;

(4) Drawing (4hrs): After the settling period, collection of treated effluent by peristaltic pump starts. The treated wastewater is pumped into the effluent tank. The effluent is analyzed for monitoring of pollutant removal efficiency;

(5) Idling: After the effluent collection, the reactor is ready to be used in the next cycle.

The hydraulic retention time (HRT) was kept to one day (24 hours). The performance of PHAs accumulation was measured in the biofilm developed on the monitoring plates. The pollutants removal efficiencies were monitored after every operation cycle. In addition, the system was run in 2 conditions: operating with individual strains and mixed strains. The microbial film accumulated on the corrugated plates was examined for PHAs monitoring.

3.2.4 Analytical Methods

3.2.4.1 Fourier Transform Infrared Spectrometer (FTIR)

Cell mass was spread on to IR window (ZnSe Disc, Spectratech) and then blot dried. After drying, it was subject to FTIR analysis (Yoshiharu, 1989). A mirror was used to give the reflected infrared signal to the horizontally laid window. With a scan number of 64, resolution of 4 cm^{-1} with a DTGS detector, the examined colonies were recorded at wave numbers from 400 cm^{-1} to 4,000 cm^{-1} using a Bruker VECTOR22. The PHA peaks were observed at wavenumbers about 1728 - 1744 cm^{-1} (Hong, 1999) to isolate potential PHA strains.

3.2.4.2 Dry cell weight (DCW)

Harvesting of cells was performed by centrifugation at 4000 rpm for 20 min. The cells were washed twice with buffer solution (3.1.9). 200 ml of acetone was added to remove the water inside the cells and centrifuged again. Acetone was discarded and freeze dried in the freeze dryer for 72 hours. Dry cell weight can be obtained by weighing the freeze dried cells in a test

tube.

3.2.4.3 PHA content

PHA content was examined by gas chromatography (G.C) method. The freeze dried cell mass samples (~ 20 mg) obtained from shake flask experiments and monitoring plates were extracted by mixing 25mL chloroform, 7.5mL 30% sodium hypochlorite and 17.5mL distilled water in a 100mL conical flask and then shaking this mixture at 37^0C for 1.5 hrs at 250 rpm in an orbital shaker. After extraction, it was centrifuged at 4,000rpm for 20min., and filtered to collect the chloroform layer that contained the extracted PHA. The collected PHA was evaporated to about 5mL and then 45mL of methanol was added to precipitate out the PHA. The precipitated PHA was methanolized by converting the polymer to the methyl esters of the fatty acid repeating units by mixing with 1mL chloroform and 1mL esterification solution (3.1.8), heating at 100^0C for 4 hrs. After heating, 1mL distilled water was added, shaken vigorously for 1 minute and let to stand overnight to allow 2 layers to form. The denser layer that contained the methyl ester was sampled for GC-FID analysis.

3.2.4.4 Gas Chromatography Flame Ionization Detection analysis (GC-FID)

The PHA content in the cells was determined by GC equipped with a Flame Ionization Detector. Benzoic acid in the esterification solution (3.1.8) acted as the internal standard for GC-FID analysis. After stand over-night of extracted PHA content (3.3.3), three layers, namely a upper aqueous layer, a middle cell layer and a lower chloroform with esterified PHAs (organic layer), were observed.

1 µl of the lower organic layer was drawn for GC-FID analysis. It was performed on a Hewlett Packard 5890A Series II Gas Chromatograph equipped with Flame Ionization Detector. Column of DB-5MS with a length of 30 m and 0.25 mm internal diameter with 0.25 um thick film. The instrument conditions and temperature program of the GC-FID were set as follows:

Hydrogen	: 0.1 MPa
Air	: 0.17 MPa
Nitrogen as carrier gas	: 0.16 MPa (10mL/min.)
Detector temperature	: 300^0C
Oven temperature	: 100^0C
Temperature program	: 100^0C (Hold 2min.)
	100^0C → 135^0C (rate: 20^0C/min.; Hold 2min.)
	135^0C → 200^0C (rate: 15^0C/min.; Hold 3min.)

3.2.4.5 Sherlock Microbiological Identification System (MIS)

The Sherlock Microbial Identification System is used to identify bacteria and yeast by Gas Chromatographic (GC) analysis of fatty acid methyl esters (FAME). The Sherlock software, methods and libraries are combined with an Agilent Technologies 6850 or 7890 GC and Agilent ChemStation software for a complete automated microbial identification solution.

The isolated potential PHA bacterial conlonies from the R2A were streaked on the BBL® Trypticase soy broth agar (TSBA) and then incubated at 30^0C for 24 hours. Then the culture was undergone saponification, methylation and extraction. The extractant was then stored into the GC vial and then was subjected to Hewlett-Packard 6890 gas chromatography analysis and compared with their HP A.06.X ChemStation Software which has an extensive databases over 2000 species, including aerobic bacteria, anaerobic bacteria and yeasts.

3.2.4.6 Chemical Oxygen Demand

Chemical oxygen demand (COD) is used as a measure of the oxygen

equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant. From a specific source, COD can be related empirically to BOD, organic carbon or organic matter (Metcalf, 1991). The COD of a waste is in general higher than the waste's BOD because more compounds can be chemically oxidized than can be biologically oxidized. The test is useful for waste removal monitoring and controlling after their correlation has been established. The dichromate reflux method is preferred over procedures using other oxidants because of dichromate's superior oxidizing ability, applicability to a wide variety of samples and ease of manipulation. The oxidation of most organic compounds is 95 - 100% of the theoretical value (Clesceri, 1998). Pyridine and related compounds resist oxidation and volatile organic compounds are oxidized only to the extent that they remain in contact with the oxidant. Ammonia, which presents either in the waste or liberated from nitrogen-containing organic matter, is not oxidized in the absence of a significant concentration of free chloride ions.

2.5 ml sample was added into a culture tube that contained sulphuric acid reagent and digestion solution. The culture tube was then tightly capped

and inverted several time to mix the mixture completely. The tube was placed in a block digester or an oven preheated to 150^{0}C and refluxed for 2 h. After the reflux reaction, it was cooled to room temperature. The absorbance of the culture tube was measured by a Spectrophotometer set at 600 nm. The COD value was calculated by fitting the sample absorbance corresponding to standard curve calibration (for detailed procedures, please refer to Appendix) (Clesceri, 1998).

3.2.4.7 Biochemical Oxygen Demand

The biochemical oxygen demand (BOD) determination is an empirical test to determine the relative oxygen requirements of wastewaters, effluents and polluted waters. BOD monitoring used as:

(i) to determine the approximate quantity of oxygen that will be required to biologically stabilize the organic matter present;

(ii) to determine the size of waste treatment facilities;

(iii) to measure the efficiency of some treatment processes and

(iv) to determine compliance with wastewater discharge permits.

Besides, it may also measure the amount of oxygen used to oxidize reduced

forms of nitrogen unless an inhibitor prevents their oxidation. The sample was suitably diluted with dilution water. In order to ensure meaningful results, the dilution water was “seeded” with a bacterial culture that had been acclimated to the organic matter or other materials that might be present in the wastewater. Such cultures contained large numbers of saprophytic bacteria and other organisms that oxidized the organic matter. The dissolved oxygen value of the diluted sample was measured by a D.O. meter immediately after sample preparation. The sample was then kept in an incubator at 20^0C for 5 days. After the incubation period, the dissolved oxygen value was measured again (for detailed procedures, please refer to Appendix). (Clesceri, 1998).

3.2.4.8 Kjeldahl Nitrogen

Organic nitrogen is defined functionally as organically bound nitrogen in the trinegative oxidation state. It does not include all organic nitrogen compounds. Analytically, organic nitrogen and ammonia can be determined together and have been referred to as “Kjeldahl nitrogen”, a term that reflects the technique used in their determination. Organic nitrogen includes such

natural materials as proteins and peptide, nucleic acids and urea, and numerous synthetic organic materials. 50 ml sample was added into the Kjeldahl flask. 50 mL digestion reagent was then carefully added. A few boiling chips were added and mixed. The mixture in Kjeldahl were heated under a hood or with suitable ejection equipment to remove acid fumes. The mixture were boiled briskly until the volume were greatly reduced and copious white fumes were observed (fumes may be dark for samples high in organic matter). The mixture were continued to be digested for an additional 30 min. As digestion continued, colored or turbid samples became transparent and pale green. The flask and contents were let to be cooled, and then was diluted to 300 mL with water. Sufficient (50 mL/50 mL digestion reagent used) hydroxide thiosulfate reagent was carefully added into the flask to form an alkaline layer at flask bottom. The flask was connected to steamed-out distillation apparatus and was shaked to insure complete mixing. The mixture was distilled and 200 mL distillate was collected below surface of 50 mL indicating boric acid solution. The distillated was titrated with standard 0.02 N H_2SO_4 titrant until indicator turns a pale lavender (for detailed procedures, please refer to Appendix) (Clesceri, 1998).

CHAPTER 4

MICROBIAL COMMUNITY AND POTENTIAL PHA BIOMASS SCREENING FROM ACTIVATED SLUDGE

4.1 Introduction

Activated sludge is a gray-black, flocculent and sticky material formed in a biological wastewater treatment system (Benedict, 1971). In the system, a mixed community of microorganisms in an aerobic aquatic environment derives energy from carbonaceous organic matter in aerated wastewater for the production of new cells, while simultaneously releases energy through the conversion of this organic matter into compounds such as carbon dioxide that contain lower energy. The mixed microbial community in the system obtains energy by converting ammonia nitrogen into nitrate nitrogen. This

consortium of microorganism, the biological component of the process, is collectively known as activated sludge (Benedict, 1971; Jenkins, 1993; Metcalf, 1991).

Bacteria, fungi, protozoa, and rotifers constitute the biological mass of activated sludge. In addition, some metazoa, such as nematode worms, may be present. However, the constant agitation in the aeration tanks and sludge recirculation are deterrents to the growth of higher organisms. Which microorganism species will dominate a system will depend on the environmental conditions, process designs, mode of plant operations, and characteristics of the influent wastewater (Water Environment Association, 1987). The microorganisms that are of the greatest numerical importance in activated sludge are bacteria. The preponderance of bacteria living in activated sludge are facultative — able to live in the presence or absence of oxygen, an important factor in the survival of activated sludge when dissolved oxygen concentrations are low or approaching depletion (Jenkins, 1993). Important genera of heterotrophic bacteria include *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Citromonas*, *Flavobacterium*, *Pseudomonas*, and

Zoogloea (Jenkins, 1993). Some researchers (Carucci, 2001; Deinema, 1972; Law, 2001; Yoshio, 1996) reported the findings of different amount of PHAs in samples of activated sludge.

In this research, activated sludge was used as the carbon source because it contains plenty of micro-organisms that offer excellent waste treatment capabilities and potential PHAs biomass. In this section, activated sludges of different matrix were examined to: (i) isolate potential PHAs biomasses in the activated sludge; (ii) evaluate the microbial community of isolated PHAs biomasses; and (iii) evaluate the PHAs accumulation capabilities of the isolated PHAs biomasses.

4.2 Microbial community and potential PHAs biomass screening

The activated sludges of different industries in Hong Kong were inoculated into three agar medium (Nutrient Agar, Plate Count Agar and R2A Agar) with different dilution factors, and then incubated in 37^0C for 48 hours. Their heterotrophic plate count, potential PHAs biomass presumed by FTIR and confirmed by G.C were summarized in the following table:

Table 4.1: Microbial community and biomass screening of activated sludges

[1]Sludge samples	Nutrient Agar (NA)			Plate Count Agar (PCA)			R2A Agar		
	[2]HPC ($x10^5$ cfu/ml)	No. of colonies presumed as PHA biomass by FTIR	No. of colonies confirmed as PHA biomass by G.C.	[2]HPC ($x10^5$ cfu/ml)	No. of colonies presumed as PHA biomass by FTIR	No. of colonies confirmed as PHA biomass by G.C.	[2]HPC ($x10^5$ cfu/ml)	No. of colonies presumed as PHA biomass by FTIR	No. of colonies confirmed as PHA biomass by G.C.
DS1	0.0490 (± 0.0079)	0	0	0.0507 (± 0.0069)	0	0	0.0653 (± 0.0069)	5	2
DS2	0.3200 (± 0.1082)	0	0	0.3133 (± 0.0917)	0	0	0.4900 (± 0.0794)	2	1
PS	18.7 (± 1.87)	5	0	19 (± 0.9165)	3	0	22.3 (± 0.9000)	16	2
OS	2.16 (± 0.1670	0	0	2.22 (± 0.2128)	1	0	29.4 (± 0.1353)	5	1
PBS	340 (± 137)	2	0	353 (± 121)	4	0	510 (± 60.0)	9	7
FWS1	238 (± 73.7)	0	0	237 (± 86.6)	1	0	390 (± 52.0)	7	2
FWS2	103 (± 45.8)	0	0	133 (± 45.8)	0	0	223 (± 75.5)	2	1
FWS3	250 (± 60)	3	0	277 (± 75.5)	6	0	490 (± 84.9)	11	2
MS	291 (± 6.0)	1	0	280 (± 7.5)	5	0	398 (± 4.6)	10	4

*Remarks: [1]DS1=Domestic Sludge 1 (Tai Po Sewage Treatment Works); DS2=Domestic sludge 2 (Shek Wu Hoi Sewage Treatment Works); PS=Piggery sludge (Piggery farm, Kwu Tung); OS=Oil sludge (Grease Trap, Kwai Chung Container Port); PBS=Purify Bio-filter sludge (Tai Po Water Treatment Works); FWS1=Food Waste Sludge 1 (Garden Company); FWS2=Food Waste Sludge 2 (Vitasoy treatment plant); FWS3=Food waste sludge 3 (Chinese Restaurant); MS=Mixed sludge (FWS1+FWS2).

[2] the value in the parentheses were standard deviations.

[3]cfu=colony forming unit.

Among the three agar media, R2A achieved better results in terms of the total number of colonies found, the number of potential PHA biomass presumed by FTIR and the number of PHAs biomass confirmed by GC-FID analysis, for the nine activated sludge of different industries in Hong Kong (Table 3.1). Table 4.1 shows that in general, the number of colonies found in R2A was higher than from either N.A. or PCA. The colony result count

obtained from activated sludge PS (Piggery sludge, Table 3.1) in R2A was 17% higher than in NA or PCA. The higher colony counts in R2A were 77% higher than those in NA or PCA for activated sludge FWS3 (Restaurant oil sludge, Table 3.1). Moreover, colony growth in R2A for activated sludges FWS1 (Food waste sludge of Garden company, Table 3.1) and FWS2 (Food waste sludge of Vitasoy treatment plant, Table 3.1) was at least 60% more than in NA/PCA. This implied that the high nutrient agar N.A and PCA produced a lower bacteria recovery than the R2A. It is because nutritionally rich media NA and PCA supported the growth of fast-growing bacteria but suppressed slow growing or stressed bacteria found in treated wastewater or sludge. On the other hand, low nutrient agar medium in combination with a lower incubation temperature stimulated the growth of stressed and chlorine-tolerant bacteria. In fact, R2A agar has been reported to improve the recovery of stressed and chlorine-tolerant bacteria from drinking water systems (Klei, 1974; Means, 1981,).

The results of potential biomass cells from R2A presumed by FTIR and confirmed by GC-FID were better than that of NA and PCA. On the one hand, the number of presumed PHAs biomass found in R2A for all sludge

samples were larger than that in NA or PCA. On the other hand, all the presumed PHAs biomass in NA and PCA was confirmed as no PHA accumulation by GC-FID analysis. While there was some presumed PHA biomass in R2A were confirmed as PHAs biomass by GC-FID analysis. This better PHA biomass recover performance was mainly due to the two constituents in the R2A (Table 3.2), namely soluble starch and sodium pyruvate. Soluble starch aided the recovery of injured organisms by absorbing toxic metabolic by-products, while sodium pyruvate increased the recovery of stressed cells (Ken, 2001). After waste treatment process, micro-organisms that were in the activated sludge were usually damaged or injured and the bacteria growth was inhibited. Usage of R2A not only stimulated the growth of the stressed bacteria, but also enhanced the total number of colonies formed. Consequently, the probability to screen out potential PHA biomass was increased.

4.3 Feasibility of PHA production by isolated biomass from activated sludges of different Hong Kong industries

There were 22 biomasses isolated from activated sludges of different industries in Hong Kong. Section 3.2 was carried out to confirm the isolated biomasses as PHA synthesis bacteria by GC-FID analysis. Table 4.2 summarizes the performance of PHA production by the isolated biomasses.

Table 4.2: PHAs yielded from activated sludge of different Hong Kong Industries

Biomass code	*Dry Cell Weight (mg/L)	PHB yielding			PHV yielding			PHB:PHV
		mg/g	mg/L	*%	mg/g	mg/L	*%	
DS1 – Domestic Sludge (Tai Po Sewage Treatment Works)								
1.) DS1_1	440 (±7.3)	217	95.5	21.7 (±4.11)	214	94.6	21.4 (±4.13)	1:1
2.) DS1_2	640 (±117)	55.3	35.2	5.53 (±1.09)	11.9	7.55	1.18 (±0.21)	9:2
DS2 – Domestic Sludge (Shek Wu Hoi Sewage Treatment Works)								
1.) DS2_1	2,350 (±419)	281	660	28.1 (±5.58)	0	0	0	PHB
PS – Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui)								
1.) PS_1	810 (±637)	24.1	19.5	2.41 (±0.49)	0.87	0.05	0.09 (±0.02)	28:1
2.) PS_2	3,362 (±617)	62.8	211	6.28 (±1.19)	1.03	0.20	0.10 (±0.02)	60:1
OS – Oily Sludge (Grease Trap – Kwai Chung Container Port)								
1.) OS_1	4,749 (±910)	6.26	29.71	0.63 (±0.11)	0	0	0	PHB
PBS – Purify Bio-filter Sludge (Tai Po Water Treatment Works)								
1.) PBS_1	1,763 (±350)	205	362	20.5 (±3.95)	11.3	0.71	1.13 (±0.21)	18:1
2.) PBS_2	1,330 (±261)	80.0	106	8.00 (±1.43)	5.5	0.27	0.57 (±0.11)	14:1
3.) PBS_3	1,017 (±178)	107	108	10.7 (±2.12)	5.44	0.20	0.54 (±0.11)	20:1
4.) PBS_4	4,896 (±929)	286	1,402	28.6 (±5.69)	17.0	3.72	1.70 (±0.31)	17:1
5.) PBS_5	1,243 (±208)	122	151	12.2 (±2.39)	0	0	0	PHB
6.) PBS_6	1,840 (±338)	310	571	31.0 (±6.08)	0	0	0	PHB
7.) PBS_7	1,838 (±323)	327	601	32.7 (±6.41)	0	0	0	PHB
FWS1 – Food waste sludge 1 (Garden Company – Sham Tseung)								
1.) FWS1_1	420 (±79)	197	82.7	19.7 (±3.87)	18.1	7.60	1.81 (±0.33)	11:1
2.) FWS1_2	760 (±139)	22.4	17.0	2.24 (±0.42)	16.8	12.8	1.68 (±0.31)	4:3
FWS2 – Food waste sludge 2 (Vitasoy treatment plant – Tuen Mun)								
1.) FWS2_1	0	0	0	0	0	0	0	0
FWS3 – Restaurant oily waste sewage (Chinese Restaurant)								
1.) FWS3_1	1,903 (±377)	258	490	25.8 (5.07)	0	0	0	PHB
2.) FWS3_2	2,523 (±491)	319	804	31.9 (±6.21)	0	0	0	PHB
MS – Mixed Sludges (FWS1 & FWS2)								
1.) MS_1	545 (±97)	105	57.5	10.5 (±1.98)	7.04	3.84	0.70 (±0.13)	15:1
2.) MS_2	2,399 (±469)	320	767	32.0 (±6.33)	12.4	298	1.24 (±0.21)	26:1
3.) MS_3	2,982 (±577)	285	849	28.5 (±5.61)	9.06	27.0	0.91 (±0.18)	31:1
4.) MS_4	1,270 (±241)	309	393	30.9 (±5.98)	13.7	17.4	1.37 (±0.29)	23:1

*Remark: the values in the parentheses were standard deviations

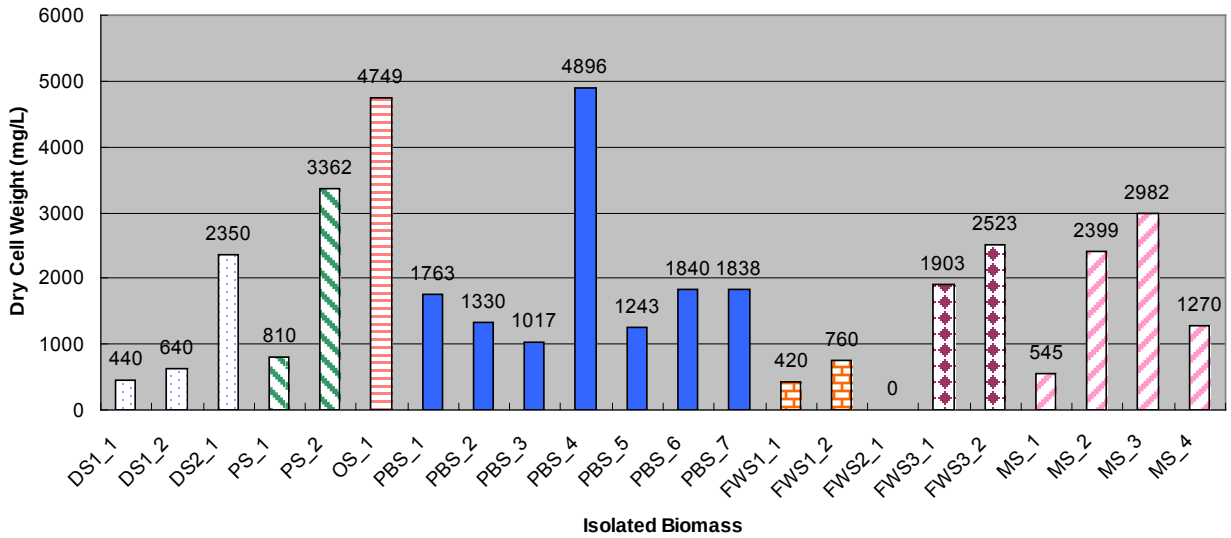
4.3.1 Dry Cell Weight

The net cell growth (dry cell weight) of different isolated biomass ranged from 420 to 4,896 mg/L. The highest net cell growth of 4,896 (± 929) mg/L was obtained from the activated sludge of PBS (Piggery Farm treatment plant). Among the sludge samples, FWS1 (Food waste sludge of Garden company) achieved the lowest net cell growth of 420 (±79) mg/L. The results from Table 4.2 also indicated that the seven isolated biomass of PBS achieved at least 1,000 mg/L dry cell weight gain (from 1,000 to 4,800 mg/L).

It should be noticed that there were no net cell growths in the activated sludge of FWS2 (Food waste sludge of Vitasoy treatment plant). After using FWS1 as the source of bacteria for FWS2 to form mixed sludge (MS), the net cell growth was stimulated. There were four PHA biomasses isolated from MS as confirmed by GC-FID analysis. The net cell growth ranged from 545 to 2,982 mg/L. When compared with the net cell growth of biomasses from FWS1, there was more than 290% net cell growth increase in the mixed sludge samples.

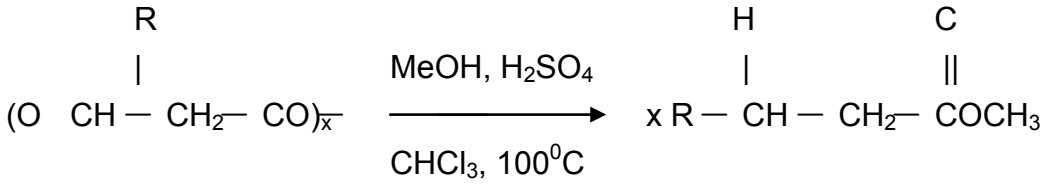
The enhancement of net cell growths in mixed sludge may be due to the matrix effect of both sludge samples (FWS1 and FWS2). Activated sludge FWS1 contained mainly carbohydrate, while FWS2 contained mainly milk and soya bean (plenty of nitrogen sources). By mixing with these 2 sludge samples, a "new nutrient-rich" environment was formed for bacteria growth and consequently achieved a high net cell weight results. Figure 4.1 summarizes the dry cell of the isolated biomasses.

Fig. 4.1: Dry cell weight gained from isolated biomass of different H.K. industries



4.3.2 Microbial formation of PHAs from activated sludge of different HK Industries

Formation of PHAs in isolated biomasses was induced by nutrient limitations. In this study, nitrogen was limited in the microbial bioplastic production medium, as also seen for PHB production in *Alcaligenes* spp., by Kim (1994a and 1994b) and in *Pseudomonas* spp., by Suzuki (1986). In this section, glucose was used as the carbon source in the PHA production medium. The composition of the intracellular polymer products was reacted with sulphuric acid in methanol to methanolize the polymer repeating unit to the methyl ester shown below. The methyl esters were compared with the synthesized branched hydroxyester standards in the GC-FID analysis.



It was found that all the isolated biomasses of activated sludge from different industries, except the biomasses from FWS2 (Food waste sludge of Vitasoy treatment plant), could produce PHA. There were 7 biomasses isolated

from PBS (Purify Bio-filter sludge), and among them, “PBS_1”, “PBS_2”, “PBS_3” and “PBS_4”, could produce PHB and PHV with a PHB:PHV ratio of 18:1, 14:1, 20:1 and 17:1, respectively. Among these 4 biomasses, “PBS_4” achieved the highest PHB yielding of 1,400 mg/L, in the production medium and with 28.6% PHB of cellular weight. It also had performed the highest PHV yielding of 3.7mg/L and with 1.7% PHV of cellular weight. Besides, biomasses isolated from the activated sludge of DS1 (Domestic sludge from Tai Po Sewage Treatment Works), PS (Piggery sludge), FWS1 (Food waste sludge from Garden company) and MS (Mixed sludge of Garden & Vita) could also produce PHB and PHV when glucose was used as the carbon source. A PHB to PHV ratio of 9:2 (5.53% PHB of cellular content; 1.18% PHV of cellular content) could be achieved by the biomass “DS1_2”. Biomass “FWS1_2” produced PHB and PHV in a ratio of 4:3. Biomass “FWS1_1 accumulated 19.7% PHB and 1.8% PHV of cellular weight.

Biomasses “DS2_1”, “OS_1”, “FWS3_2”, “PBS5”, “PBS_6” and “PBS_7” could only produced PHB. Among them, “PBS_6”, “PBS_7” and “FWS3_2” accumulated more than 30% PHB of cellular weight. This study was

comparable to the yield from activated sludge found by Yu, et. al., (1999). The researcher used brewery malt wastes as carbon sources their study. They performed 24 hours fermentation to the bacteria and then obtained about 30% PHB of a dry cell weight.

Figures 4.2 and 4.3 summarize the PHB and PHV yieldings of the typical isolated biomasses from different activated sludge.

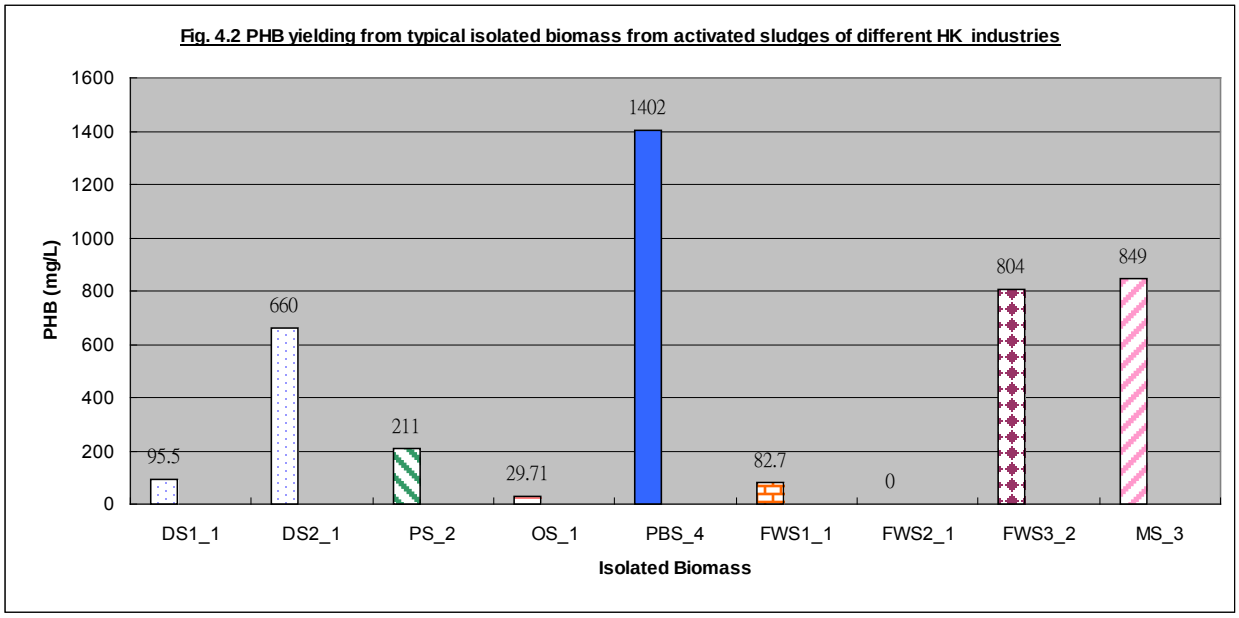


Fig. 4.3 PHV yielding from typical isolated biomass from activated sludges of different HK industries

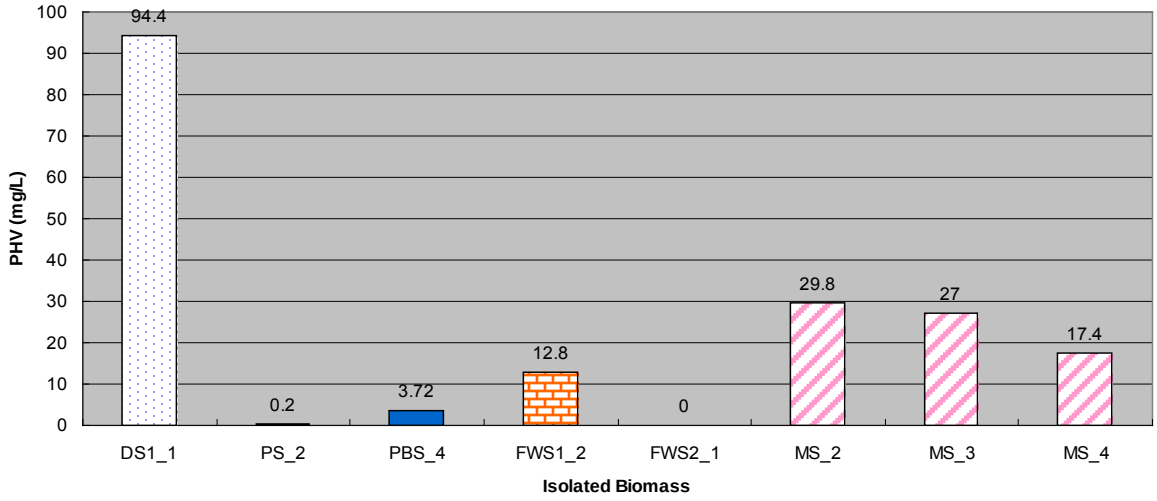


Figure 4.3 shows that the biomass “DS1_1” had the highest cellular content of PHV (21.4 ± 4.13%) and with 95mg/L PHV in the production medium.

Neither PHB nor PHV was found in the biomass “FWS2_1” (Food waste sludge from Vitasoy treatment plant). Once the sludge of FWS1 (Food waste sludge from Garden company) was used as the bacteria source for FWS2 (Food waste sludge from Vitasoy treatment plant), the performance of this mixed sludge was alternated, significantly. First of all, the cell dry weight increased dramatically. There was a net cell weight enhancement of about 290% from 760 mg/L to about 3,000 mg/L. Second, the biomasses isolated from the mixed sludge accumulated both PHB and PHV with the ratio of 15:1, 23:1, 26:1 and 31:1 (PHB:PHV). In addition, PHB production was also enhanced from about 20% to 32% of the cellular weight. The PHV yield of the biomass of the mixed sludge was like the biomass of FWS1, less than 2% of the cellular weight. But, the concentration level of PHV in the production medium was increased from about 13 mg/L to 29.8 mg/L. In fact, the PHB produced in the production medium also increased from 82 mg/L to 850 mg/L by the biomasses of the mixed activated sludge. There was a 930% increment. The enhancement of PHA yield was mainly due to

the cross mixing of both activated sludge. The major constituent of FWS1 (Food waste sludge from Garden company) was carbohydrate, while milk and soyabean waste were the dominant element in FWS2 (Food waste sludge from Vitasoy treatment plant). Once the two activated sludges were mixed, their dominant waste composition mixed leading a high nutrient mixture, the microbial community also cross-mixed, on the other hand. Such a high nutrient mixture would enhance the microbial growth and result in high cell density. Besides, the enhancement of PHB yield by mixed sludge was due to the co-activated sludge induction effect.

4.4 PHA production with different Carbon sources

The PHA production performances of isolated biomasses were different when different carbon sources were used. In this study, methanol (C1), lactic acid (C3), sodium propionic acid (C3_Na), glucose (C6), sodium octanotate (C8_Na) and maltose (C12) were used. The results showed that carbon sources affect the dry cell weight and PHA production capacity. Table 4.3 lists the dry cell weight of isolated biomasses from activated sludge of different Hong Kong industries with different carbon sources in the PHA production medium.

Table 4.3: Summary results of Dry Cell Weight of isolated biomasses

Biomasses	[(1)(2)]Dry Cell Yielding (mg/L)					
	C1	C3	C3Na	C6	C8Na	C12
DS1 – Domestic Sludge (Tai Po Sewage Treatment Works)						
1.) DS1_1	707 (±138)	1,705 (±340)	927 (±183)	440 (±82)	1,194 (±227)	780 (±151)
2.) DS1_2	1,024 (±201)	1,413 (±270)	655 (±122)	640 (±118)	344 (±62)	873 (±164)
DS2 – Domestic Sludge (Shek Wu Hoi Sewage Treatment Works)						
1.) DS2_1	3,970 (±787)	0	1,371 (±279)	2,350 (±463)	0	2,500 (±492)
PS – Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui)						
1.) PS_1	1,842 (±367)	2,168 (±433)	0	810 (±158)	0	855 (±177)
2.) PS_2	1,863 (±366)	566 (±111)	0	3,362 (±670)	0	1,535 (±311)
OS – Oily Sludge (Grease Trap – Kwai Chung Container Port)						
1.) OS_1	1,154 (±218)	1,609 (±317)	0	4,749 (±938)	0	2,903 (±571)
PBS – Purify Bio-filter Sludge (Tai Po Water Treatment Works)						
1.) PBS_1	733 (±143)	2,044 (±410)	662 (±127)	1,763 (±342)	0	1,287 (±249)
2.) PBS_2	367 (±69)	3,161 (±627)	297 (±53)	1,330 (±261)	0	607 (±116)
3.) PBS_3	414 (±75)	1,309 (±255)	0	1,017 (±192)	0	0
4.) PBS_4	172 (±30)	1,119 (±216)	0	4,896 (±971)	0	582 (±112)
5.) PBS_5	453 (±91)	0	454 (±88)	1,243 (±240)	0	457 (±84)
6.) PBS_6	203 (±43)	0	2,244 (±445)	1,840 (±359)	0	1,377 (±271)
7.) PBS_7	190 (±37)	0	2,346 (±461)	1,838 (±362)	0	3,022 (±592)
FWS1 – Food waste sludge (Garden Company – Sham Tseung)						
1.) FWS1_1	550 (±106)	1,380 (±279)	1,415 (±279)	420 (±81)	996 (±192)	1,285 (±249)
2.) FWS1_2	826 (±163)	1,086 (±214)	1,091 (±214)	760 (±148)	1,379 (±270)	1,340 (±255)
FWS2 – Food waste sludge (Vitasoy Treatment plant – Tuen Mun)						
1.) FWS2_1	3,668 (±729)	0	3,289 (±651)	2,520 (±493)	0	2,757 (±543)
FWS3 – Restaurant oily waste sewage (Chinese Restaurant)						
1.) FWS3_1	1,419 (±281)	2,798 (±552)	0	1,903 (±377)	0	3,640 (±719)
2.) FWS3_2	440 (±83)	1,376 (±271)	0	2,523 (±491)	0	6,767 (±135)
MS – Mixed sludges (Vita & Garden)						
1.) MS_1	2,365 (±469)	0	2,940 (±582)	545 (±103)	0	1,846 (±361)
2.) MS_2	782 (±155)	0	585 (±106)	2,399 (±471)	0	1,951 (±382)
3.) MS_3	892 (±175)	0	1,522 (±301)	2,982 (±583)	0	1,165 (±212)
4.) MS_4	857 (±167)	0	1,606 (±311)	1,270 (±241)	0	2,250 (±455)

Remark: [(1)]C1=Methanol; C3=Lactic acid; C3_Na=Sodium propionic acid; C6=Glucose; C8_Na=Sodium octanotate and C12=Maltose.

[(2)]the values in the parentheses were standard deviations.

4.4.1 Dry Cell Weight

Methanol (C1) decreased the net cell content of most of the isolated biomasses when compared with glucose (C6) as carbon source. This was because of the effect of the methanol toxicity on the bacteria community. However, isolated biomasses from sludges DS1 and DS2 (domestic sludge of Tai Po sewage treatment works and domestic sludge of Shek Wu Hoi sewage treatment works, respectively) could withstand the methanol toxicity and their bacterial growths were stimulated. Methanol enhanced biomasses of DS1 and DS2 about 60% and 69% of dry cell weight, representatively, when using results of glucose as reference value. The bacterial growth of activated sludge PBS was the most affected due to the methanol toxicity and there was a decrease of about 90% in dry cell weight.

In general, lactic acid (C3) stimulated biomasses of activated sludges DS1 (Domestic sludge of Tai Po sewage treatment works), PS (Piggery sludge) and PBS (Purify bio-filter sludge). Biomasses of DS1 achieved a dry cell weight of 1,705 (±340) mg/L. Biomass “PS_1” obtained 2,168 (±433) mg/L net dry cell weight in the production medium with lactic acid as the carbon

source. Among these three sludges, biomass “PBS_2” from PBS sludge achieved the highest dry cell weight gain of 3,161 (±627) mg/L. Besides, lactic acid also enhanced the bacterial growth of the activated sludge FWS1 (Food waste sludge of Garden company) and FWS3 (Chinese Restaurant oil sludge). Both of them achieved more than 1,000 mg/L net cell gain. Biomass “FWS3_1” even obtained 2,798 (±552) mg/L net cell weight. On the other hand, the bacteria growth of the activated sludge DS2 (Domestic sludge of Shek Wu Hoi sewage treatment works), FWS2 (Food waste sludge of Vita) and MS (Mixed sludge of Garden & Vita) were inhibited. In addition, biomasses of PBS_5, PBS_6 and PBS_7 were also inhibited by lactic acid.

The bacterial growth of FWS2 (Food waste sludge of Vita) and MS_1 of MS (Mixed sludge of Garden and Vita) was stimulated by sodium propronic acid (C3_Na) and the biomasses of FWS2 achieved dry cell weight of about 3,000 mg/L. Biomasses “PBS_6” & “PBS_7” achieved a 20% enhancement of dry cell weight, while the other biomasses of PBS were inhibited by sodium propionic acid. Sodium propionic acid inhibited the bacterial growth of the piggery sludge (PS) and restaurant oily sludge (FWS3).

Sodium octanoate (C8_Na) did not support the growth of most of the isolated biomasses from different Hong Kong industries, except biomass "DS1_1" of DS1 (Domestic sludge of Tai Po sewage treatment works) and biomasses of FWS1 (Food waste sludge of Garden). They all achieved better dry cell weight of about 1,000 mg/L with at least 80% enhancement when compared with glucose as carbon sources. The finding showed that most of the bacteria community in different Hong Kong industries could not utilize sodium octanoate as their carbon and energy sources.

Maltose (C12) is a crystalline disaccharide. It can be hydrolyzed to glucose by maltase and is an easily digested food for bacterial growth. All isolated biomasses could utilize it as their carbon and energy sources. Biomasses "FWS3_1" and "FWS3_2" (Chinese restaurant oily sludge) achieved the dry cell weight of 3,640 (±719) mg/L and 6,767 (±1,359) mg/L, respectively. There was more or less the same dry cell weight obtained for the other isolated biomasses.

4.4.2 Effect of different carbon sources to the intracellular bioplastic formation

Tables 4.4 and 4.5 summarize the PHB and PHV yield of the isolated biomasses of different activated sludges with different carbon sources in the PHA production medium.

Table 4.4: PHB yield with different carbon source in the microbial bioplastic production medium

Source	[1][2]PHB yielding																	
	C1			C3			C3Na			C6			C8Na			C12		
	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%
DS1 – Domestic Sludge (Tai Po Sewage Treatment Works)																		
1.) DS1_1	10.8	7.61	1.08 (±0.22)	4.64	7.92	0.46 (±0.09)	8.21	7.61	0.82 (±0.155)	217	95.5	21.7 (±4.31)	0	0	0	9.76	7.61	0.98 (±0.21)
2.) DS1_2	7.43	7.61	0.74 (±0.15)	5.47	7.73	0.55 (±0.12)	11.6	7.61	1.16 (±0.231)	55.3	35.2	5.53 (±1.12)	22.1	7.61	2.21 (±0.46)	8.85	7.73	0.89 (±0.18)
DS2 – Domestic Sludge (Shek Wu Hoi Sewage Treatment Works)																		
1.) DS2_1	509	2,023	51.0 (±9.82)	0	0	0	82.3	114	8.33 (±1.61)	281	660	28.1 (±5.54)	0	0	0	768	1,921	76.8 (±14.88)
PS – Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui)																		
1.) PS_1	0	0	0	127	276	12.7 (±2.59)	0	0	0	24.1	19.5	2.41 (±0.50)	0	0	0	0	0	0
2.) PS_2	0	0	0	172	97.2	17.2 (±3.39)	0	0	0	62.8	211	6.28 (±1.27)	0	0	0	64.6	99.1	6.46 (±1.21)
OS – Oily Sludge (Grease Trap – Kwai Chung Container Port)																		
1.) OS_1	0	0	0	0	0	0	0	0	0	6.26	29.71	0.63 (±0.14)	0	0	0	0	0	0
PBS – Purify Bio-filter Sludge (Tai Po Water Treatment Works)																		
1.) PBS_1	101	74.0	10.1 (±1.81)	113	231	11.3 (±2.25)	68.1	45.1	6.81 (±1.33)	205	362	20.5 (±4.01)	0	0	0	780	1,004	78.0 (±14.3)
2.) PBS_2	305	112	30.5 (±5.93)	112	355	11.2 (±2.26)	0	0	0	80.0	106	8.00 (±1.66)	0	0	0	321	195	32.1 (±6.29)
3.) PBS_3	180	74.4	18.0 (±3.52)	0	0	0	75.9	99.4	7.59 (±1.49)	107	108	10.7 (±2.11)	0	0	0	0	0	0
4.) PBS_4	160	27.5	16.0 (±3.35)	0	0	0	0	0	0	286	1,402	28.6 (±5.69)	0	0	0	169	98.6	16.9 (±3.22)
5.) PBS_5	276	125	27.6 (±5.51)	0	0	0	77.4	35.2	7.74 (±1.53)	122	151	12.2 (±2.41)	0	0	0	0	0	0
6.) PBS_6	0	0	0	0	0	0	88.1	198	8.81 (±1.66)	310	571	31.0 (±5.83)	0	0	0	634	873	63.4 (±11.11)
7.) PBS_7	0	0	0	0	0	0	69.7	163	6.97 (±1.38)	327	601	32.7 (±6.47)	0	0	0	339	1,024	33.9 (±6.28)
FWS1 – Food waste sludge 1(Garden Company – Sham Tseung)																		
1.) FWS1_1	159	87.6	15.9 (±3.13)	210	290	21 (±4.1)	326	462	32.6 (±6.49)	197	82.7	19.7 (±3.88)	149	148	14.9 (±2.87)	13.6	17.5	1.36 (±0.29)
2.) FWS1_2	0	0	0	7.11	7.72	0.71 (±0.13)	7.15	7.81	0.72 (±0.15)	22.4	17.0	2.24 (±0.42)	5.79	7.99	0.58 (±0.12)	5.91	7.92	0.59 (±0.12)
FWS2 – Food waste sludge 2 (Vitasoy treatment plant – Tuen Mun)																		
1.) FWS2_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	58.3	161	5.83(±1.12)
FWS3 – Restaurant oily waste sewage (Chinese Restaurant)																		
1.) FWS3_1	90.3	128	9.03 (±1.75)	0	0	0	0	0	0	258	490	25.8 (±5.07)	0	0	0	93.9	342	9.38 (±1.84)
2.) FWS3_2	77.7	34.2	7.77 (±1.54)	0	0	0	0	0	0	319	804	31.9 (±6.32)	0	0	0	645	4,367	64.5 (±11.3)
MS – Mixed sludges (FWS1 & FWS2)																		
1.) MS_1	20.7	49.0	2.07 (±0.36)	0	0	0	23.9	70.2	2.39 (±0.43)	105	57.5	10.5 (±1.96)	0	0	0	148	273	14.8 (±2.91)
2.) MS_2	56.9	44.5	5.69 (±1.18)	0	0	0	15.1	8.82	1.51 (±0.32)	320	767	32.0 (±6.12)	0	0	0	138	269	13.8 (±2.81)
3.) MS_3	149	133	14.9 (±2.86)	0	0	0	44.9	68.3	4.49 (±8.86)	285	849	28.5 (±5.54)	0	0	0	119	139	11.9 (±2.51)
4.) MS_4	104	89.0	10.4 (±2.03)	0	0	0	51.1	82.1	5.11 (±1.02)	309	393	30.9 (±6.11)	0	0	0	100	226	10.0 (±2.23)

Remark: [1]C1=Methanol; C3=Lactic acid; C3_Na=Sodium propionic acid; C6=Glucose; C8_Na=Sodium octanotate and C12=Maltose.
[2]the values in the parentheses were standard deviations.

Table 4.5: PHV yielding with different carbon source in the microbial bioplastic production medium

Source	[1][2]PHV yielding																	
	C1			C3			C3Na			C6			C8Na			C12		
	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%	mg/g	mg/L	%
DS1 – Domestic Sludge (Tai Po Sewage Treatment Works)																		
1.) DS1_1	0	0	0	0	0	0	0	0	0	214	94.4	21.4 (±4.11)	0	0	0	0	0	0
2.) DS1_2	0	0	0	0	0	0	0	0	0	11.9	7.55	1.18 (±0.25)	0	0	0	0	0	0
DS2 – Domestic Sludge (Shek Wu Hoi Sewage Treatment Works)																		
1.) DS2_1	5.87	23.3	0.59 (±0.13)	0	0	0	47.1	64.6	4.71 (±0.89)	0	0	0	0	0	0	27.2	68.1	2.73 (±0.52)
PS – Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui)																		
1.) PS_1	0	0	0	99.2	215	9.92 (±1.92)	0	0	0	0.87	0.70	0.09 (±0.02)	0	0	0	0	0	0
2.) PS_2	0	0	0	97.1	54.9	9.71 (±1.88)	0	0	0	1.03	3.46	0.10 (±0.02)	0	0	0	0	0	0
OS – Oily Sludge (Grease Trap – Kwai Chung Container Port)																		
1.) OS_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBS – Purify Bio-filter Sludge (Tai Po Water Treatment Works)																		
1.) PBS_1	0	0	0	84.8	173	8.48 (±1.61)	40.9	27.1	4.09 (±0.77)	11.	20.0	1.13 (±0.23)	0	0	0	69.9	90.0	6.99 (±1.32)
2.) PBS_2	0	0	0	67.1	212	6.71 (±1.32)	116	34.4	11.6 (±2.27)	5.50	7.65	0.57 (±0.13)	0	0	0	0	0	0
3.) PBS_3	0	0	0	0	0	0	5.95	7.79	0.59 (±0.13)	5.44	5.53	0.54 (±0.12)	0	0	0	0	0	0
4.) PBS_4	0	0	0	0	0	0	0	0	0	17.0	83.0	1.70 (±0.35)	0	0	0	2.28	1.33	0.23 (±0.05)
5.) PBS_5	0	0	0	0	0	0	4.89	2.22	0.49 (±0.01)	0	0	0	0	0	0	0	0	0
6.) PBS_6	0	0	0	0	0	0	5.21	11.7	0.52 (±0.11)	0	0	0	0	0	0	7.73	10.6	0.77 (±0.16)
7.) PBS_7	0	0	0	0	0	0	35.29	82.8	3.53 (±0.68)	0	0	0	0	0	0	4.73	14.3	0.47 (±0.11)
FWS1 – Food waste sludge 1 (Garden Company – Sham Tseung)																		
1.) FWS1_1	10.5	5.79	1.05 (±0.22)	7.06	9.75	0.71 (±0.15)	8.39	11.9	0.84 (±0.18)	18.1	7.60	7.81 (±1.58)	9.0	8.96	0.90 (±0.22)	2.39	3.07	0.24 (±0.05)
2.) FWS1_2	0	0	0	0	0	0	0	0	0	16.8	12.8	1.68 (±0.36)	0	0	0	0	0	0
FWS2 – Food waste sludge 2 (Vitasoy treatment plant – Tuen Mun)																		
1.) FWS2_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FWS3 – Restaurant oily waste sewage (Chinese Restaurant)																		
1.) FWS3_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.) FWS3_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.41	29.	0.44 (±0.10)
V/G – Mixed sludges (FWS1 & FWS2)																		
1.) MS_1	0	0	0	0	0	0	24.2	71.3	2.43 (±0.46)	7.04	3.84	0.70 (±0.16)	0	0	0	11.5	21.2	1.15 (±0.27)
2.) MS_2	0	0	0	0	0	0	10.2	5.97	1.02 (±0.24)	12.4	29.8	1.24 (±0.27)	0	0	0	10.4	20.3	1.04 (±0.22)
3.) MS_3	16.0	14.3	1.60 (±0.33)	0	0	0	43.6	66.3	4.36 (±0.81)	9.06	27.0	0.91 (±0.21)	0	0	0	10.2	11.8	1.02 (±0.23)
4.) MS_4	0	0	0	0	0	0	43.56	70.0	4.36 (±0.84)	13.7	17.4	1.37 (±0.26)	0	0	0	9.33	21.0	0.93 (±0.21)

Remark: [1]C1=Methanol; C3=Lactic acid; C3_Na=Sodium propionic acid; C6=Glucose; C8_Na=Sodium octanotate and C12=Maltose.

[2]the values in the parentheses were standard deviations.

Experimental results (Tables 4.4 and 4.5) showed that methanol (C1) not only exerted the toxicity to the bacterial growth of isolated biomass, but also decreased the PHA production capacity of most of the isolated biomasses. Biomasses isolated from the activated sludge of PS (Piggery sludge), OS (Oily sludge) and FWS2 (Food waste sludge of Vitasoy treatment plant) did not accumulate PHB when methanol was used as the carbon source. Besides, all different isolated biomasses did not accumulate PHV except DS2 (Domestic sludge of Shek Wu Hoi sewage treatment works), “FWS1_1” of FWS1 (Food waste sludge of Garden company) and “MS_3” of MS (Mixed sludge of Vitasoy and Garden company). There was about 1% PHV produced by FWS1_1 and MS_3. However, PHB production by biomasses of DS2 was stimulated. There was an increase of about 200% in the PHB concentration in the production medium and an increase in cellular weight of PHB from 28.1 (±5.54)% to 51 (±9.82)%.

Lactic acid (C3), like methanol, also inhibited the PHA production in most isolated biomasses. The isolated biomasses of the activated sludge of DS2, OS, PBS, FWS2, FWS3 and MS accumulated neither PHB nor PHV. However, the biomass of PS (Piggery sludge) was stimulated to produce PHB

and PHV by lactic acid. There was an increase of about 1,300% in PHB in the production medium. The cellular PHB accumulation also increased from 2.41 (±0.50)% to 12.7 (±2.59)%. Furthermore, PHV produced by the biomass of PS increased significantly from 0.1% to about 10% of the cellular weight.

Compared with glucose as carbon source, sodium propionic acid (C3_Na) decreased the PHB production capacity for most of isolated biomasses. There was only one isolated biomass, “FWS1_1” from FWS1 (Food waste sludge of Garden company), stimulated by sodium propionic acid to produce PHB. It synthesized more than 30% PHB of cellular weight. For PHV accumulation, biomasses of the activated sludge DS2 and MS were enhanced. The isolated biomasses of both activated sludges achieved more than 4% PHV of their cellular dry weight obtained. There was less than 1% PHV produced when glucose was used as the carbon source.

In combination with the dry cell weight obtained (Table 4.3), the isolated biomasses did not utilize sodium octanotate (C8_Na) as their carbon and energy source and did not produce intracellular bioplastics of PHB and PHV except the biomasses from domestic sludge (“DS1_2”) and food waste sludge 1

("FWS1_1" and "FWS1_2"). DS1_2 made use of sodium octanoate to produce 2.21 (±0.46)% cellular content of PHB but there was no PHV accumulated. There was about 14.9 (±2.87)% cellular content of PHB produced by FWS1_1 and less than 1% cellular content of PHV accumulated.

Among the five different carbon sources, maltose (C12) had an enhancement effect on PHB production. The isolated biomasses from DS2 (Domestic sludge of Shek Wu Hoi sewage treatment works) and the biomass "PBS_1" of PBS (Purify Bio-filter sludge of Tai Po water treatment works) accumulated more than 78 (±14.3)% PHB as intracellular resources. More than 60% PHB of cellular weight accumulated by biomass "FWS3_2" of activated sludge FWS3 (Restaurant oily sludge). On the other hand, PHV accumulation of all isolated biomasses was diminished by using maltose as the carbon source. Only "PBS_1" of the activated sludge PBS produced PHV. There was about 6.99 (±1.32)% PHV of cellular content produced by "PBS_1".

Figures 4.4 to 4.8 summarize the typical PHB and PHV yield by the isolated biomass of activated sludge from different industries in Hong Kong with different carbon source in the production medium respectively.

Fig. 4.4: PHA yielding from isolated biomass from different HK industries by using *Methanol (C1)* as carbon source

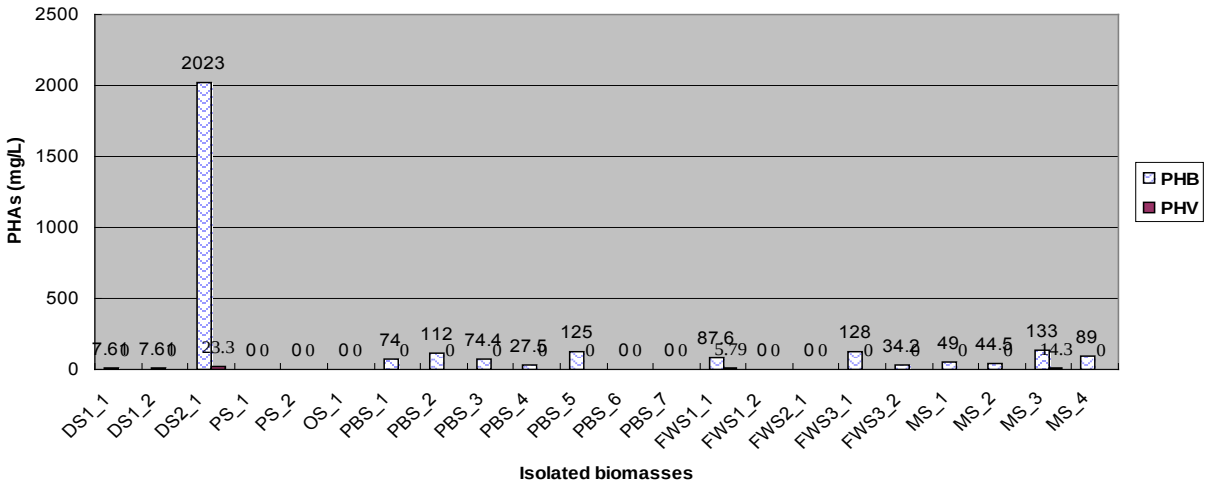


Fig. 4.5: PHA yielding from isolated biomass from different HK industries by using *Lactic Acid (C3)* as carbon source

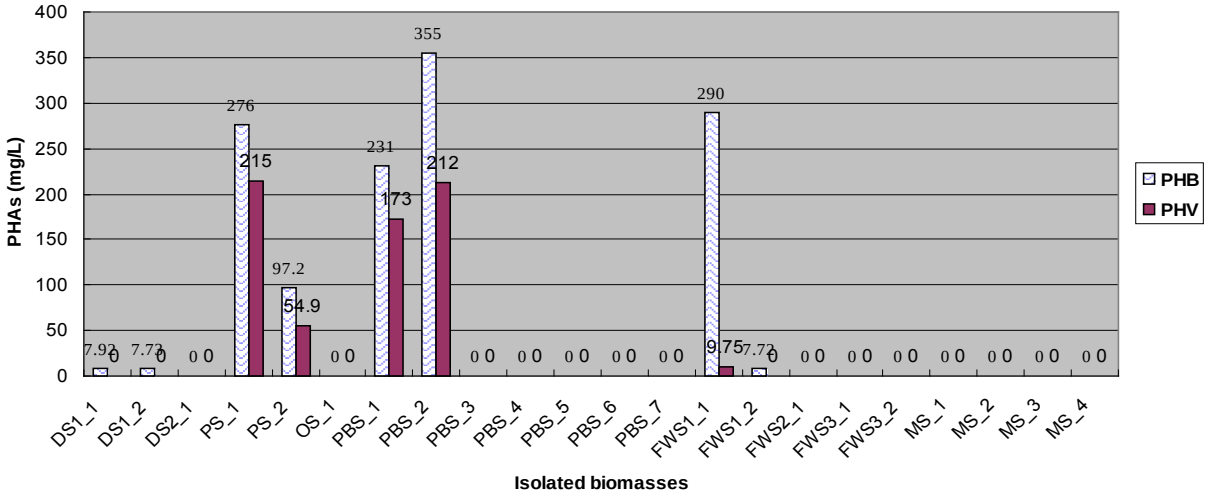


Fig. 4.6: PHA yielding from isolated biomass from different HK industries by using *Sodium Propionic acid (C3_Na)* as carbon source

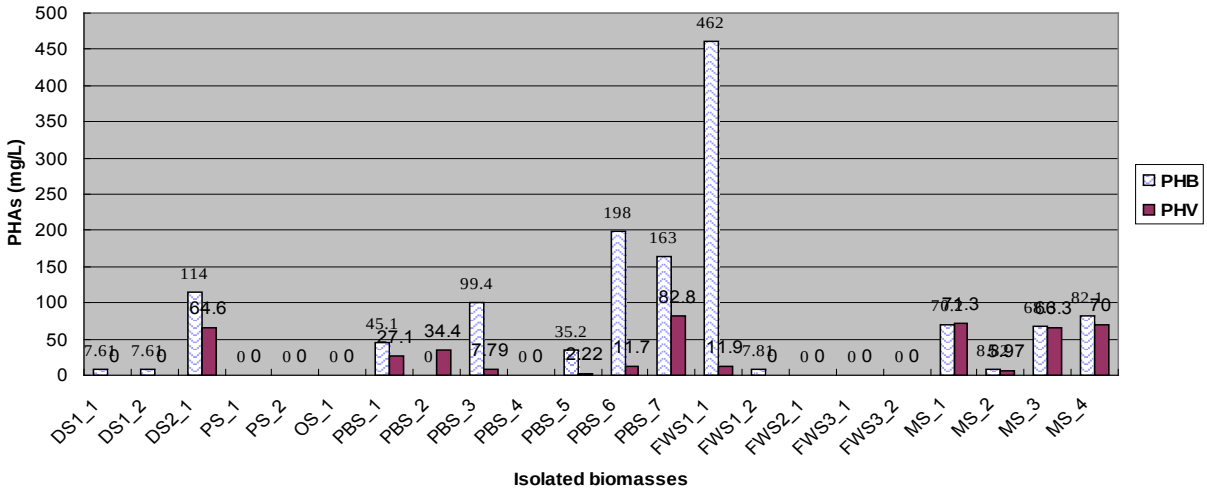


Fig. 4.7: PHA yielding from isolated biomass from different HK industries by using *Sodium propionic acid (C8_Na)* as carbon source

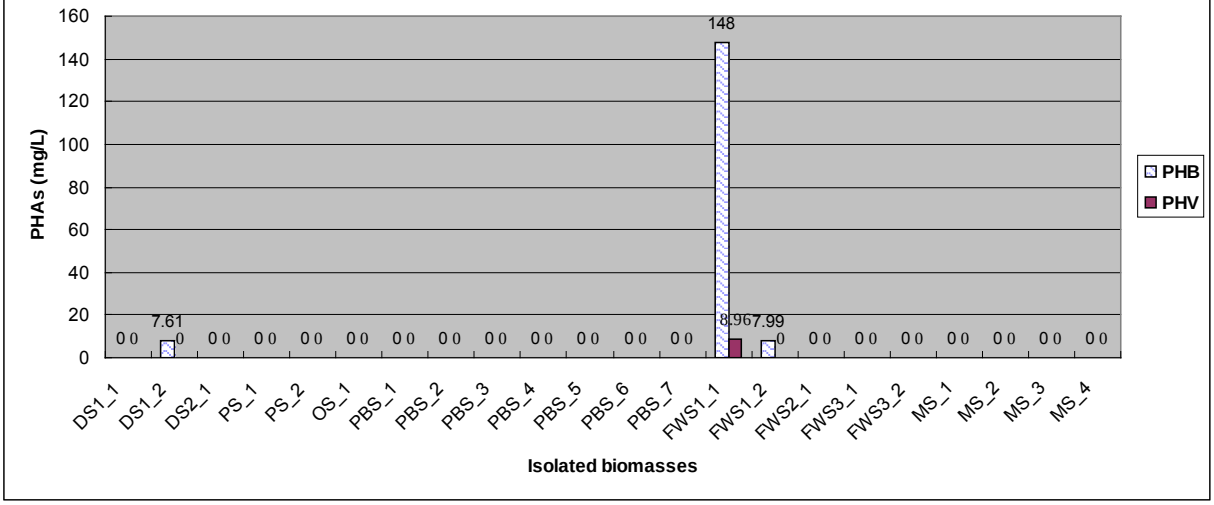
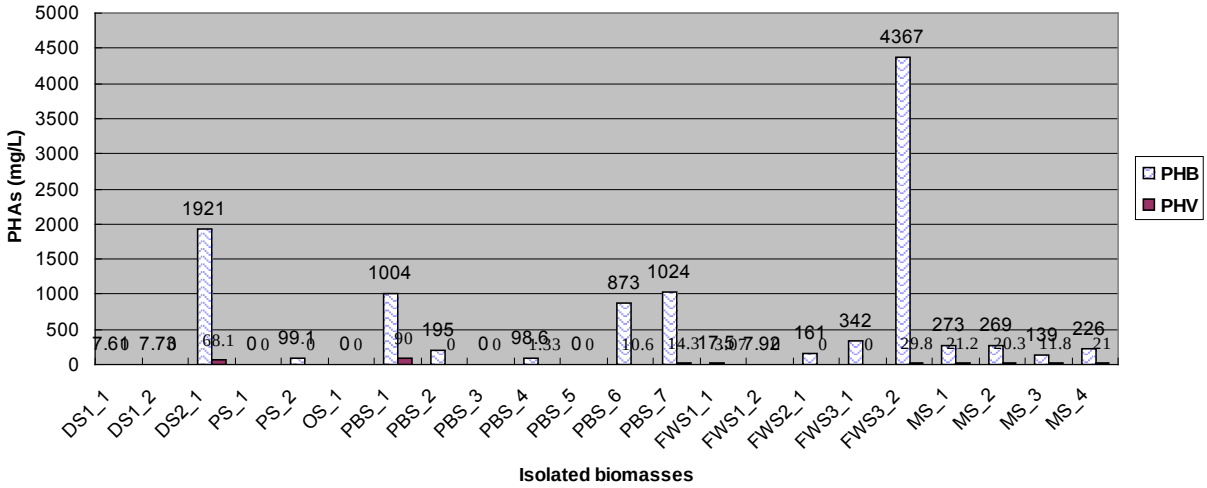


Fig. 4.8: PHA yielding from isolated biomass from different HK industries by using *Maltose (C12)* as carbon source



There was no PHB produced by isolated biomass “FWS2_1” of activated sludge FWS2 (Food waste sludge from Vita company) when using methanol, lactic acid, sodium propionic acid, glucose or sodium octanotate as the carbon source. It only produced PHB with 5.8% of cellular weight when maltose was used as the carbon source. However, when FWS1 (Food waste sludge of Garden company) mixed with FWS2 (Food waste sludge from Vitasoy treatment plant), PHB was obtained when maltose, sodium propionic acid, glucose or maltose was used. It implied that cross culture mixing would induce the capacity of PHA production within the mixed bacterial community. This finding could help to improve biological waste treatment. As mentioned before, there were no biomasses found when FWS2 used individually. When lack of biomass accumulation, biological treatment could not perform well. Once the bacteria growth was stimulated and enhanced by the induction of FWS1, the efficiency of biological treatment may increase.

4.5 Discussion

Potential PHAs biomass was isolated from activated sludges in this study. Results showed that R2A achieved better microbial recovery. With the function of starch and sodium pyruvate in R2A, the stressed and injured cells were recovered. Soluble starch aided the recovery of injured organisms by absorbing toxic metabolic by-products while sodium pyruvate increased the recovery of stressed cells. No potential PHA biomasses could be isolated by N.A. and PCA. There were twenty-two potential PHA biomasses recovered by R2A.

When glucose was used as the sole carbon source, among the isolated potential PHA biomasses, PBS_4 and OS_1 achieved the most DCW results at about 4,800 mg/L. 14 out of 22 isolated potential PHA biomasses produced both PHB and PHV while others produced PHB only. Biomasses of “PBS_6”, “PBS_7”, “FSW3_2” and “MS_2” synthesized more than 32% PHB of cellular content. In general, all isolated biomasses accumulated PHB ranged from about 2% to 30% of cellular content. This level of PHB yielded dry cell weight basis from food waste sludge and bio-filter sludge in this research is

comparable to the yield from activated sludge we obtained using brewery malt wastes as carbon sources by researchers (Yu, 1998). Biomasses of PS accumulated the highest PHV yield at 9.9% of cellular content. The PHV content produced by other biomasses varied from 0.7% to 7% of their cellular content.

Among the six different carbon sources used, methanol (C1) and sodium octanoate (C8_Na) showed toxicity to most of the isolated biomasses. In addition, the PHAs accumulations were also diminished by these 2 carbon sources. On the other hand, lactic acid stimulated biomasses of PS.

CHAPTER 5

IDENTIFICATION OF ISOLATED POTENTIAL PHA BIOMASSES FROM DIFFERENT INDUSTRIAL ACTIVATED SLUDGES OF HONG KONG

5.1 Introduction

Eight selected PHAs biomasses from different activated sludge of Hong Kong were further analyzed for their whole cell fatty acid profile by Sherlock Microbiological Identification System (MIS) in using Gas Chromatographic analysis of Fatty Acid Methyl Esters (GC-FAME) by GC-FID.

Analysis of short chain fatty acids (volatile fatty acids, VFAs) has been routinely used in the identification of bacteria. The fatty acids between 9 and 20 carbons in length have been used to characterize the genera and species of bacteria. With the advent of fused silica capillary columns (which allow

recovery of hydroxy acids and resolution of many isomers), it has become practical to use gas chromatography of whole cell fatty acid methyl esters to identify a wide range of organisms.

5.2 Identification by MIS

Clinical and environmental laboratories worldwide have used the Sherlock Microbial Identification System (MIS) since 1991. The system can identiy bacteria and yeast by analyzing the unique cellular fatty acid patterns present in the cell membrane. The fatty acids are extracted from the microbe, converted to their corresponding Fatty Acid Methyl Esters (FAMEs) and then separated with a GC (http://www.midi-inc.com/pages/microbial_id.html).

A summary of comparison among the conventional taxonomic bacteria identification and MIS are listed as below table.

Table 5.1: Summary comparison of conventional identification methods and MIS

Conventional Bacteria Identification	Sherlock Microbial Identification System (MIS)
1.) Operation Cost > 10 USD/sample	**1.) Operation Cost** ~ 6 USD/sample
2.) Sample preparation Complicated in using different chemical and bio-chemical reactions for sample preparation, incubation and identification.	**2.) Sample preparation** Simply chemical extraction method and identification facilitated by GC analysis.
3.) Turn Around Time More than 1 weeks for different bio-chemical test incubation and identification	**3.) Turn Around Time** Within 1 day
4.) Chemical/Reagents usage More than ten chemical/solvent/agar to be used.	**4.)Chemical/Regents usage** Around three – four chemical/regents/agar to be used.
5.) Identification verification Results based on the grain stain, morphology, chemical and biochemical reactions to identify the samples.	**5.) Identification verification** Limited to the built-in instant libraries.
6.) Investment cost Less than 20,000 USD	**6.) Investment cost** More than 80,000 USD

5.3 Similarity Index

The Similarity Index of the MIS is a numerical value which expresses how closely the fatty acid composition of an unknown strain is compared with the

mean fatty acid composition of the strains used to create the library entry or entries listed as its match. According to the MIDI System manual, strains with a similarity of 0.500 or higher is considered good library comparisons. If the similarity index is between 0.300 and 0.500, it may be a good match but an atypical strain. Values lower than 0.300 suggest that the system does not have the species in the database, but indicate most closely related species.

From the SIM index results found that, most of the similarity index is more than 0.6. It indicated that the fatty acid profiles of isolated strains are highly matched with the pattern stored in the instant libraries. Table 5.2 summarized the Similarity (SIM) Index of the selected eight biomasses:

Table 5.2: Bacteria Identification

Sources of Activated sludges	**Biomass Code**	**SIM index**	**Identified Strain Name**
Domestic Sludge (Tai Po Sewage Treatment Works)	DS1_1	0.775	*Bacillus pumilus*
Domestic Sludge (Shek Wu Hoi Sewage Treatment Workds)	DS2_1	0.804	*Bacillus pumilus*
Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui)	PS_2	0.895	*Yersinia-frederiksenii*
Purify Bio-filter Sludge (Tai Po Water Treatment Works)	PBS_4	0.723	*Pseudomonas putida*
Food Waste sludge 1(Garden Company)	FWS1_1	0.582	*Pseudomonas huttiensis*
Food Waste sludge 1 (Garden	FWS1_2	0.648	*Bacillus cereus*

company)			
Mixed Sludge	MS_2	0.807	*Sphingopyxis terrae*
Mixed Sludge	MS_4	0.846	*Aeromonas ichthiosmia*

Figure 5.1 to 5.8 shows the results of strains identification by MIS system.

E067289.84A [1340] Natalie-25July06-3

Volume: DATA2 File: E067289.84A Seq Counter: 6 ID Number: 1340
Type: Samp Bottle: 4 Method: TSBA50
Created: 7/29/06 2:24:21 AM
Sample ID: Natalie-25July06-3

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.716	5.251E+8	0.029	----	6.992	SOLVENT PEAK	----	< min rt	
2.101	144	0.019	----	7.742		----	< min rt	
5.596	614	0.026	1.016	12.614	13:0 ISO	0.43	ECL deviates 0.000	Reference -0.004
6.890	1123	0.032	0.992	13.620	14:0 ISO	0.76	ECL deviates 0.001	Reference -0.002
7.408	1107	0.037	0.985	13.998	14:0	0.74	ECL deviates -0.002	Reference -0.004
8.370	78798	0.040	0.975	14.624	15:0 ISO	52.46	ECL deviates 0.001	Reference -0.001
8.508	29872	0.040	0.973	14.714	15:0 ANTEISO	19.86	ECL deviates 0.001	Reference -0.001
9.594	388	0.034	0.965	15.389	16:1 w7c alcohol	0.26	ECL deviates 0.002	
9.990	3832	0.042	0.962	15.627	16:0 ISO	2.52	ECL deviates 0.000	Reference -0.002
10.210	953	0.039	0.961	15.759	16:1 w11c	0.63	ECL deviates 0.002	
10.608	5320	0.044	0.958	15.998	16:0	3.48	ECL deviates -0.002	Reference -0.003
11.284	2231	0.044	0.955	16.390	ISO 17:1 w10c	1.46	ECL deviates 0.002	
11.698	18452	0.047	0.953	16.631	17:0 ISO	12.01	ECL deviates 0.001	Reference -0.001
11.858	8313	0.047	0.952	16.723	17:0 ANTEISO	5.41	ECL deviates 0.000	Reference -0.002

ECL Deviation: 0.001 Reference ECL Shift: 0.003 Number Reference Peaks: 9
Total Response: 151004 Total Named: 151004
Percent Named: 100.00% Total Amount: 146424

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.775	Bacillus-pumilus-GC subgroup B

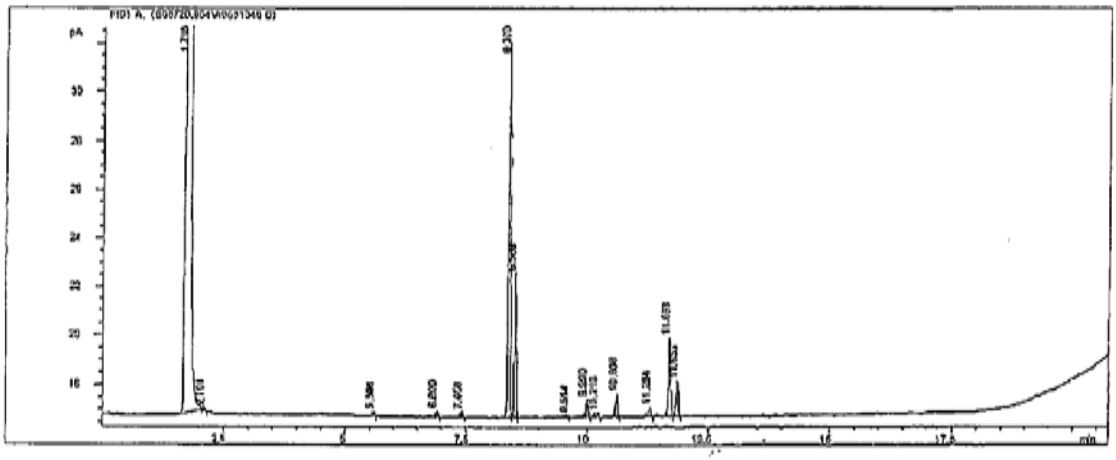


Figure 5.1: Chromatograph and SIM index of *Bacillus pumilus* (DS1_1)

E067289.84A [1341] Natalie-25July06-4 Pag

Volume: DATA2 File: E067289.84A Seq Counter: 7 ID Number: 1341
Type: Samp Bottle: 5 Method: TSBA50
Created: 7/29/06 2:48:56 AM
Sample ID: Natalie-25July06-4

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.044	549	0.043	----	3.737		----	< min rt	
1.713	5.266E+8	0.030	----	6.990	SOLVENT PEAK	----	< min rt	
1.998	349	0.023	----	7.541		----	< min rt	
2.100	123	0.016	----	7.739		----	< min rt	
5.507	673	0.036	1.016	12.614	13:0 ISO	0.33	ECL deviates 0.000	Reference -0.003
6.550	1967	0.037	0.992	13.619	14:0 ISO	0.93	ECL deviates 0.000	Reference -0.002
7.411	2045	0.036	0.985	13.999	14:0	0.96	ECL deviates -0.001	Reference -0.002
8.372	109397	0.040	0.975	14.624	15:0 ISO	50.72	ECL deviates. 0.001	Reference 0.000
8.510	42612	0.040	0.973	14.714	15:0 ANTEISO	19.73	ECL deviates 0.001	Reference 0.000
9.600	978	0.042	0.965	15.391	16:1 w7c alcohol	0.45	ECL deviates 0.004	
9.991	6046	0.042	0.962	15.626	16:0 ISO	2.77	ECL deviates -0.001	Reference -0.001
10.210	2953	0.043	0.961	15.758	16:1 w11c	1.35	ECL deviates 0.001	
10.609	10061	0.046	0.958	15.998	16:0	4.59	ECL deviates -0.002	Reference -0.003
11.284	3370	0.044	0.955	16.390	ISO 17:1 w10c	1.53	ECL deviates 0.002	
11.437	722	0.039	0.954	16.478	Sum In Feature 4	0.33	ECL deviates 0.002	17:1 ISO I/ANTEI B
11.700	25509	0.044	0.953	16.631	17:0 ISO	11.56	ECL deviates 0.001	Reference 0.000
11.860	10525	0.044	0.952	16.724	17:0 ANTEISO	4.77	ECL deviates 0.001	Reference 0.000
----	722	---	----	----	Summed Feature 4	0.33	17:1 ISO I/ANTEI B	17:1 ANTEISO B/I I

ECL Deviation: 0.002 Reference ECL Shift: 0.002 Number Reference Peaks: 9
Total Response: 216857 Total Named: 216857
Percent Named: 100.00% Total Amount: 210245

Matches:

Library	Sim Index	Entry Name
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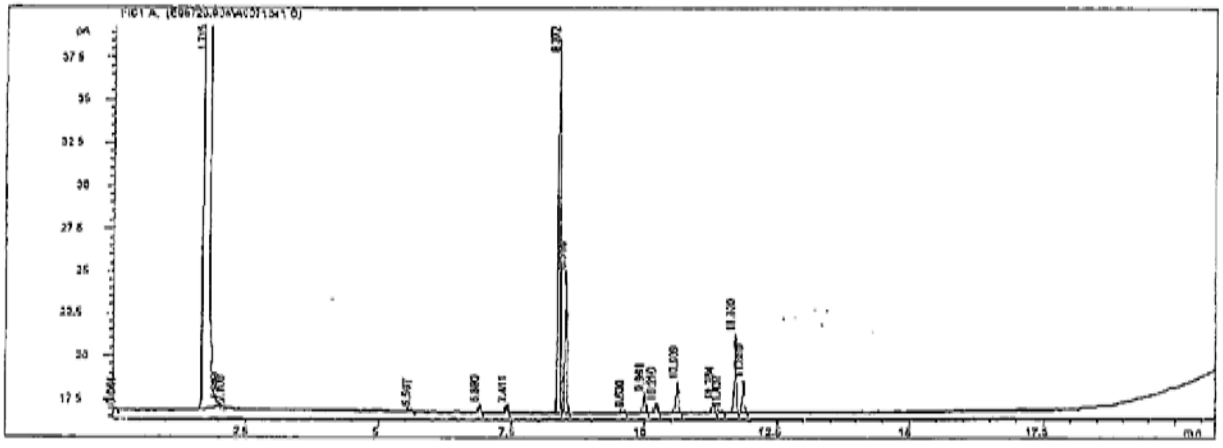

Figure 5.2: Chromatograph and SLIM index of *Bacillus pumilus* (DS2_1)

Volume: DATA2
Type: Samp
Created: 6/23/07 6:59:44 AM
Sample ID: Yin-22Jun07-14

File: E076230.35A
Bottle: 47

Seq Counter: 16
Method: TSBA50

ID Number: 1470

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.521	4.676E+8	0.025	----	7.015	SOLVENT PEAK	----	< min rt	
1.870	155	0.017	----	7.757		----	< min rt	
3.521	773	0.026	1.087	10.922	Sum In Feature 2	0.29	ECL deviates -0.006	unknown 10.928
4.433	15289	0.027	1.033	12.000	12:0	5.47	ECL deviates 0.000	Reference 0.000
6.825	2159	0.046	0.967	13.999	14:0	0.72	ECL deviates -0.001	Reference -0.002
8.309	2826	0.036	0.949	15.001	15:0	----	ECL deviates 0.001	
9.096	23878	0.039	0.943	15.487	Sum In Feature 2	7.79	ECL deviates -0.001	14:0 3OH/16:1 ISO I
9.632	90671	0.040	0.940	15.819	Sum In Feature 3	29.49	ECL deviates -0.003	16:1 w7c/15 iso 2OH
9.781	740	0.040	0.939	15.911	16:1 w5c	0.24	ECL deviates 0.002	
9.926	94138	0.040	0.938	16.001	16:0	30.58	ECL deviates 0.001	Reference -0.001
10.076	1208	0.060	----	16.089		----		
11.268	1433	0.046	0.935	16.793	17:1 w8c	0.46	ECL deviates 0.001	
11.429	26984	0.044	0.934	16.888	17:0 CYCLO	8.73	ECL deviates 0.000	Reference -0.001
11.619	1310	0.040	0.934	17.000	17:0	0.42	ECL deviates 0.000	Reference -0.001
12.515	622	0.042	0.934	17.517	16:0 3OH	0.20	ECL deviates -0.002	
12.920	737	0.038	----	17.751		----		
13.044	41212	0.043	0.935	17.822	18:1 w7c	13.33	ECL deviates -0.001	
13.206	538	0.033	0.935	17.916	18:1 w5c	0.17	ECL deviates -0.003	
13.350	656	0.036	0.935	17.999	18:0	0.21	ECL deviates -0.001	Reference -0.002
13.493	2568	0.040	0.935	18.081	11 methyl 18:1 w7c	0.83	ECL deviates 0.000	
14.114	1000	0.041	----	18.440		----		
14.814	2619	0.041	0.939	18.845	Sum In Feature 7	0.85	ECL deviates -0.001	un 18.846/19:1 w6c
14.914	615	0.039	0.939	18.902	19:0 CYCLO w8c	0.20	ECL deviates 0.000	Reference 0.000
----	24651	---	----	----	Summed Feature 2	8.08	12:0 ALDE ?	unknown 10.928
----	-----	---	----	----		----	16:1 ISO I/14:0 3OH	14:0 3OH//16:1 ISO I
----	90671	---	----	----	Summed Feature 3	29.49	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c
----	2619	---	----	----	Summed Feature 7	0.85	un 18.846/19:1 w6c	19:1 w6c/.846/19oy
----	-----	---	----	----		----	19:0 CYCLO w10c/19w6	

ECL Deviation: 0.002
Total Response: 309148
Percent Named: 99.05%

Reference ECL Shift: 0.001
Total Named: 306203
Total Amount: 291582

Number Reference Peaks: 7

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.895	Yersinia-frederiksenii

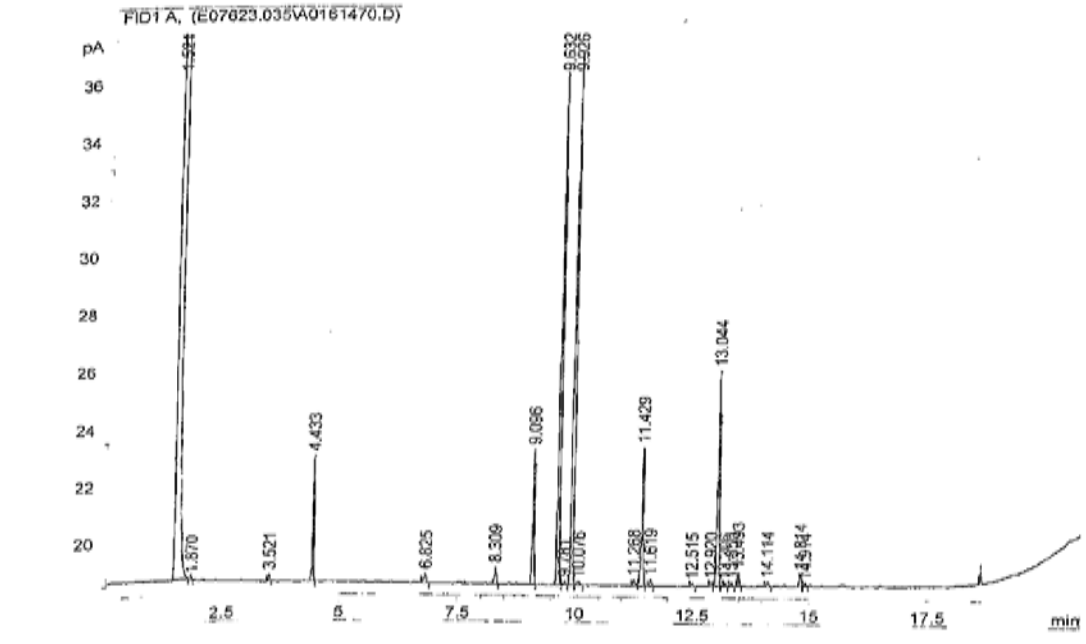

Figure 5.3: Chromatograph and SIM index of *Yersinia frederiksenii* (PS_2)

E067289.84A [1339] Natalie-25July06-2 Page 1

Volume: DATA2 File: E067289.84A Seq Counter: 5 ID Number: 1339
Type: Samp Bottle: 3 Method: TSBA50
Created: 7/29/06 1:59:40 AM
Sample ID: Natalie-25July06-2

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.715	5.421E+8	0.030	----	6.989	SOLVENT PEAK	----	< min rt	
2.130	1225	0.023	----	7.797		----	< min rt	
2.395	405	0.024	----	8.312		----	< min rt	
2.555	528	0.023	----	8.624		----	< min rt	
2.719	1999	0.024	----	8.943		----	< min rt	
2.861	171	0.020	----	9.220		----		
2.949	472	0.029	1.153	9.391	8:0 3OH	0.12	ECL deviates -0.001	
3.253	3791	0.030	----	9.983		----		
3.594	5232	0.028	----	10.471		----		
4.145	2056	0.029	----	11.194		----		
4.356	32329	0.031	1.055	11.422	10:0 3OH	7.47	ECL deviates 0.000	
4.891	12754	0.035	1.034	12.000	12:0	2.89	ECL deviates 0.000	Reference -0.001
5.294	1446	0.032	----	12.350		----		
5.452	1323	0.046	1.020	12.488	unknown 12.484	0.30	ECL deviates 0.004	
6.288	24329	0.036	1.002	13.179	12:0 2OH	5.34	ECL deviates 0.002	
6.440	3128	0.035	0.999	13.290	12:1 3OH	0.69	ECL deviates 0.002	
6.668	22679	0.035	0.996	13.456	12:0 3OH	4.95	ECL deviates 0.002	
7.163	737	0.040	----	13.818		----		
7.410	1687	0.038	0.985	13.999	14:0	0.36	ECL deviates -0.001	Reference -0.003
9.410	611	0.038	----	15.277		----		
9.760	395	0.043	0.984	15.488	Sum In Feature 2	0.08	ECL deviates 0.000	14:0 3OH/16:1 ISO I
10.311	132942	0.051	0.960	15.819	Sum In Feature 3	27.97	ECL deviates -0.003	16:1 w7c/15 iso 2OH
10.612	119370	0.044	0.958	16.000	16:0	25.07	ECL deviates 0.000	Reference -0.001
12.148	10547	0.046	0.951	16.891	17:0 CYCLO	2.20	ECL deviates 0.003	Reference 0.002
13.788	105982	0.050	0.943	17.823	18:1 w7c	21.94	ECL deviates 0.000	
14.095	2344	0.044	0.944	17.997	18:0	0.48	ECL deviates -0.003	Reference -0.003
15.682	608	0.045	0.938	18.903	19:0 CYCLO w8c	0.13	ECL deviates 0.001	Reference 0.002
----	395	----	----	----	Summed Feature 2	0.08	12:0 ALDE ?	unknown 10.928
----	-----	----	----	----		----	16:1 ISO I/14:0 3OH	14:0 3OH/16:1 ISO I
----	132942	----	----	----	Summed Feature 3	27.97	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c

ECL Deviation: 0.002 Reference ECL Shift: 0.002 Number Reference Peaks: 6
Total Response: 484935 Total Named: 470890
Percent Named: 97.10% Total Amount: 456291

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.723	Pseudomonas-putida-biotype A

E067289.84A [1339] Natalie-25July06-2 Page 2

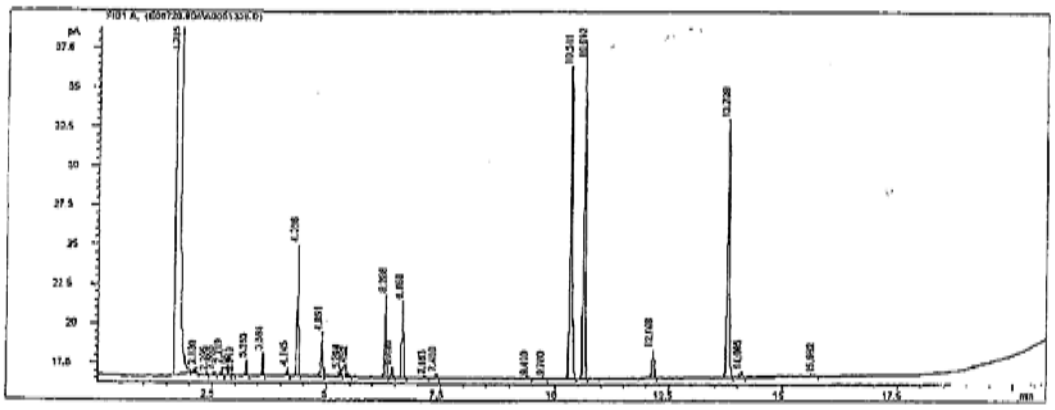

Figure 5.4: Chromatograph and SIM index of *Pseudomonas putida* ((PBS_4)

Volume: DATA2 File: E076152.12A Seq Counter: 9 ID Number: 1431
Type: Samp Bottle: 8 Method: TSBA50
Created: 6/15/07 8:21:09 AM
Sample ID: Yin-14Jun07-11

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.519	4.701E+8	0.025	****	7.019	SOLVENT PEAK	****	< min rt	
1.862	187	0.020	****	7.751		****	< min rt	
2.915	421	0.021	1.132	9.999	10:0	0.47	ECL deviates -0.001	Reference -0.001
3.223	352	0.024	****	10.474		****		
3.929	1423	0.027	1.055	11.423	10:0 3OH	1.49	ECL deviates 0.001	
4.427	7358	0.028	1.031	12.001	12:0	7.54	ECL deviates 0.001	Reference 0.003
6.107	2868	0.033	0.987	13.454	12:0 3OH.	2.81	ECL deviates 0.000	
6.819	471	0.030	0.976	14.000	14:0	0.46	ECL deviates 0.000	Reference 0.004
9.624	39936	0.039	0.952	15.818	Sum In Feature 3	37.76	ECL deviates -0.004	16:1 w7c/15 iso 2OH
9.916	24566	0.040	0.950	15.999	16:0	23.19	ECL deviates -0.001	Reference 0.002
12.018	1632	0.042	0.944	17.236	16:0 2OH	1.53	ECL deviates 0.003	
12.510	946	0.038	0.943	17.520	16:0 3OH	0.89	ECL deviates 0.001	
13.039	22362	0.046	0.942	17.825	18:1 w7c	20.93	ECL deviates 0.002	
13.343	1616	0.045	0.942	18.001	18:0	1.51	ECL deviates 0.001	Reference 0.002
14.805	840	0.041	0.940	18.848	Sum In Feature 7	0.78	ECL deviates 0.002	un 18.846/19:1 w6c
15.223	667	0.033	0.939	19.091	18:1 2OH	0.62	ECL deviates 0.002	
****	39936	***	****	****	Summed Feature 3	37.76	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c
****	840	***	****	****	Summed Feature 7	0.78	un 18.846/19:1 w6c	19:1 w6c/.846/19cy
****	*****	***	****	****		****	19:0 CYCLO w10c/19w6	

ECL Deviation: 0.002
Total Response: 105459
Percent Named: 99.67%

Reference ECL Shift: 0.002
Total Named: 105106
Total Amount: 100662

Number Reference Peaks: 5

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.582	Pseudomonas-huttiensis

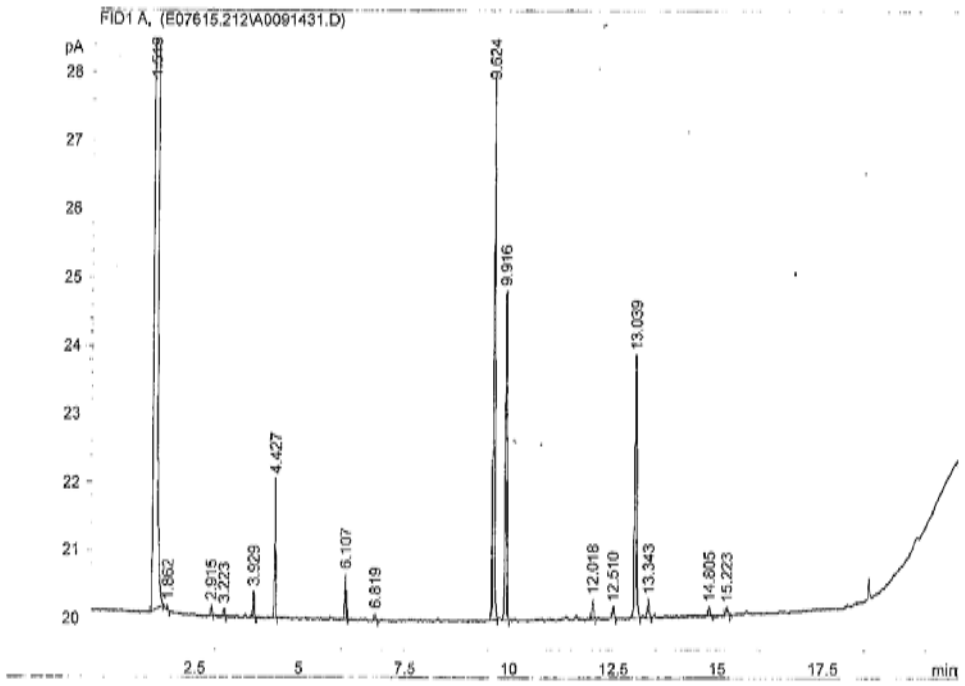

Figure 5.5: Chromatograph and SIM index of *Pseudomonas huttiensis* (FWS1_1)

Volume: DATA2 File: E067289.84A Seq Counter: 4 ID Number: 1338
Type: Samp Bottle: 2 Method: TSBA50
Created: 7/29/06 12:49:30 AM
Sample ID: Natalie-25July06-1

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.717	5.173E+8	0.029	---	7.034	SOLVENT PEAK	---	< min rt	
1.914	1874	0.020	---	7.416		---	< min rt	
1.994	321	0.024	---	7.572		---	< min rt	
2.044	415	0.023	---	7.668		---	< min rt	
4.502	1373	0.032	1.048	11.607	12:0 ISO	2.17	ECL deviates -0.002	
5.572	8595	0.034	1.016	12.614	13:0 ISO	13.16	ECL deviates 0.000	
5.674	1294	0.035	1.014	12.702	13:0 ANTEISO	1.98	ECL deviates 0.000	
6.865	6044	0.038	0.992	13.619	14:0 ISO	9.04	ECL deviates 0.000	
7.385	2073	0.040	0.985	13.998	14:0	3.08	ECL deviates -0.002	
8.348	15075	0.040	0.975	14.622	15:0 ISO	22.14	ECL deviates -0.001	
8.487	3253	0.041	0.973	14.713	15:0 ANTEISO	4.77	ECL deviates 0.000	Reference -0.015
8.928	385	0.038	0.970	15.001	15:0	---	ECL deviates 0.001	
9.578	1612	0.039	0.965	15.393	16:1 w7c alcohol	2.34	ECL deviates 0.006	
9.736	1998	0.041	0.964	15.488	Sum In Feature 2	2.90	ECL deviates 0.000	14:0 3OH/16:1 ISO I
9.969	5860	0.046	0.962	15.627	16:0 ISO	8.50	ECL deviates 0.000	Reference -0.015
10.198	713	0.037	0.961	15.764	16:1 w11c	1.03	ECL deviates 0.007	
10.357	6740	0.045	0.960	15.860	Sum In Feature 3	9.75	ECL deviates 0.008	15:0 ISO 2OH/16:1w7c
10.590	2753	0.043	0.958	16.000	16:0	3.98	ECL deviates 0.000	Reference -0.014
10.966	616	0.037	0.956	16.217	15:0 2OH	0.89	ECL deviates -0.002	
11.268	3322	0.044	0.955	16.391	ISO 17:1 w10c	4.78	ECL deviates 0.003	
11.395	1868	0.042	0.954	16.464	ISO 17:1 w5c	2.69	ECL deviates 0.003	
11.685	4026	0.046	0.953	16.630	17:0 ISO	5.78	ECL deviates 0.000	Reference -0.009
11.847	730	0.051	0.952	16.723	17:0 ANTEISO	1.05	ECL deviates 0.000	Reference -0.008
---	1998	---	---	---	Summed Feature 2	2.90	12:0 ALDE ?	unknown 10.928
---	---	---	---	---		---	16:1 ISO I/14:0 3OH	14:0 3OH/16:1 ISO I
---	6740	---	---	---	Summed Feature 3	9.75	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c

ECL Deviation: 0.003 Reference ECL Shift: 0.012 Number Reference Peaks: 5
Total Response: 67946 Total Named: 67946
Percent Named: 100.00% Total Amount: 66746

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.648	Bacillus-cereus-GC subgroup B

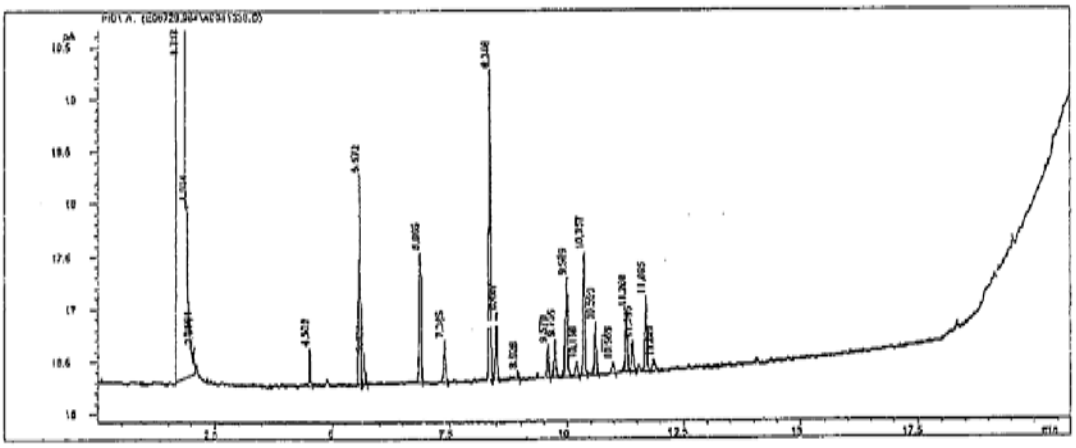

Figure 5.6: Chromatograph and SIM index of *Bacillus cereus* (FWS1_2)

E067289.84A [1343] Natalie-25July06-6

Volume: DATA2 File: E067289.84A Seq Counter: 9 ID Number: 1343
Type: Samp Bottle: 7 Method: TSBA50
Created: 7/29/06 3:38:17 AM
Sample ID: Natalie-25July06-6

MS_2

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.717	5.129E+8	0.029	----	6.989	SOLVENT PEAK	----	<min rt	
2.000	355	0.024	----	7.541		----	<min rt	
8.952	6010	0.041	0.970	15.001	15:0	----	ECL deviates 0.001	
9.293	979	0.043	0.967	15.206	14:0 2OH	1.18	ECL deviates 0.003	
10.311	6841	0.043	0.960	15.821	Sum In Feature 3	8.20	ECL deviates -0.001	16:1 w7c/15 iso 2OH
10.458	788	0.043	0.959	15.909	16:1 w5c	0.94	ECL deviates 0.000	
10.610	6019	0.048	0.958	16.000	16:0	7.20	ECL deviates 0.000	Reference -0.002
10.991	7145	0.046	0.956	16.221	15:0 2OH	8.53	ECL deviates 0.002	
11.333	600	0.042	0.955	16.418	ISO 17:1 w9c	0.72	ECL deviates 0.002	
11.984	5612	0.048	0.952	16.794	17:1 w8c	6.67	ECL deviates 0.002	
12.104	35748	0.047	0.951	16.864	17:1 w6c	42.46	ECL deviates 0.004	
12.340	5088	0.046	0.950	17.000	17:0	6.04	ECL deviates 0.000	Reference 0.001
12.572	833	0.048	----	17.131		----		
12.747	543	0.043	0.949	17.230	16:0 2OH	0.64	ECL deviates -0.003	
13.790	13297	0.048	0.945	17.824	18:1 w7c	15.69	ECL deviates 0.001	
14.240	1027	0.041	0.943	18.081	11 methyl 18:1 w7c	1.21	ECL deviates 0.000	
15.625	433	0.036	0.938	18.872	Sum In Feature 7	0.51	ECL deviates 0.005	19:0 CYCLO w10c/19w6
----	6841	----	----	----	Summed Feature 3	8.20	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c
----	433	----	----	----	Summed Feature 7	0.51	un 18.846/19:1 w6c	19:1 w6c/.846/19cy
----	----	----	----	----		----	19:0 CYCLO w10c/19w6	

ECL Deviation: 0.002
Total Response: 84954
Percent Named: 99.02%

Reference ECL Shift: 0.002
Total Named: 84121
Total Amount: 85906

Number Reference Peaks: 2

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.807	Sphingopyxis-terrae (Sphingomonas)

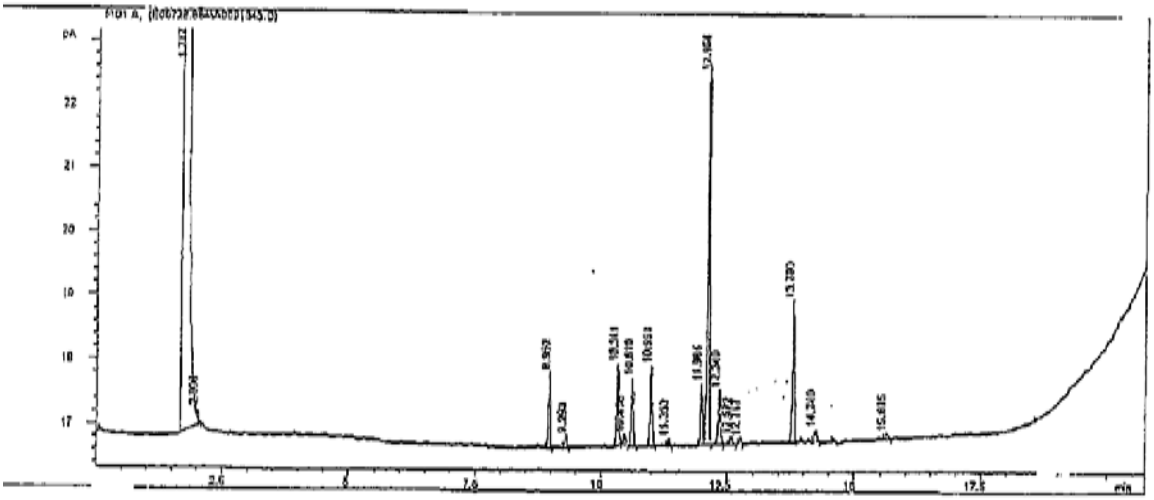

Figure 5.7: Chromatograph and SIM index of *Sphingopyxis terrae* (MS_2)

Volume: DATA2 File: E067289.84A Seq Counter: 8 ID Number: 1342
Type: Samp Bottle: 6 Method: TSBA50
Created: 7/29/06 3:13:40 AM
Sample ID: Natalie-25July06-5

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.716	5.261E+8	0.029	----	6.987	SOLVENT PEAK	----	< min rt	
2.105	160	0.021	----	7.743		----	< min rt	
3.319	1110	0.031	----	10.078		----		
3.910	2209	0.029	1.075	10.919	Sum In Feature 2	0.99	ECL deviates 0.005	12:0 ALDE ?
4.466	423	0.028	1.050	11.540	unknown 11.543	0.18	ECL deviates -0.003	
4.892	12350	0.031	1.034	12.000	12:0	5.31	ECL deviates 0.000	Reference 0.000
5.600	1527	0.031	1.016	12.614	13:0 ISO	0.64	ECL deviates 0.000	Reference -0.001
6.047	831	0.034	1.006	13.001	13:0	0.35	ECL deviates 0.001	Reference 0.001
6.666	490	0.037	0.996	13.454	12:0 3OH	0.20	ECL deviates 0.000	
6.892	400	0.033	0.992	13.618	14:0 ISO	0.17	ECL deviates -0.001	Reference -0.001
7.160	659	0.041	----	13.814		----		
7.413	9370	0.042	0.985	13.999	14:0	3.84	ECL deviates -0.001	Reference -0.001
8.138	2654	0.047	0.977	14.504	unknown 14.502	1.08	ECL deviates 0.002	
8.372	2228	0.039	0.975	14.623	15:0 ISO	0.90	ECL deviates 0.000	Reference 0.000
8.511	370	0.034	0.973	14.714	15:0 ANTEISO	0.15	ECL deviates 0.001	Reference 0.001
8.950	2080	0.042	0.970	14.999	15:0	----	ECL deviates -0.001	
9.243	1241	0.064	----	15.176		----		
9.594	693	0.037	0.965	15.387	16:1 w7c alcohol	0.28	ECL deviates 0.000	
9.762	17706	0.044	0.964	15.488	Sum In Feature 2	7.09	ECL deviates 0.000	14:0 3OH/16:1 ISO I
9.991	1745	0.043	0.962	15.626	16:0 ISO	0.70	ECL deviates -0.001	Reference -0.001
10.311	90199	0.043	0.960	15.818	Sum In Feature 3	35.99	ECL deviates -0.004	16:1 w7c/15 iso 2OH
10.610	39460	0.043	0.958	15.998	16:0	15.72	ECL deviates -0.002	Reference -0.002
10.842	2964	0.044	0.957	16.133	15:0 ISO 3OH	1.18	ECL deviates -0.001	
11.334	4502	0.044	0.955	16.418	ISO 17:1 w9c	1.79	ECL deviates 0.002	
11.700	4998	0.044	0.953	16.631	17:0 ISO	1.98	ECL deviates 0.001	Reference 0.000
11.863	703	0.040	0.952	16.725	17:0 ANTEISO	0.28	ECL deviates 0.002	Reference 0.001
11.982	4656	0.046	0.952	16.794	17:1 w8c	1.84	ECL deviates 0.002	
12.104	958	0.039	0.951	16.865	17:1 w6c	0.38	ECL deviates 0.005	
12.337	2361	0.045	0.950	17.001	17:0	0.93	ECL deviates 0.001	Reference 0.000
13.788	44261	0.047	0.945	17.825	18:1 w7c	17.38	ECL deviates 0.002	
14.095	1683	0.046	0.944	17.999	18:0	0.66	ECL deviates -0.001	Reference -0.003
----	19915	---	----	----	Summed Feature 2	8.08	12:0 ALDE ?	unknown 10.928
----	-----	---	----	----		----	16:1 ISO I/14:0 3OH	14:0 3OH/16:1 ISO I
----	90199	---	----	----	Summed Feature 3	35.99	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1 w7c

ECL Deviation: 0.002
Total Response: 252753
Percent Named: 98.81%

Reference ECL Shift: 0.001
Total Named: 249743
Total Amount: 242626

Number Reference Peaks: 13

Matches:

Library	Sim Index	Entry Name
TSBA50 5.00	0.846	Aeromonas-ichthiosmia A/hydrophila

E067289.84A [1342] Natalie-25July06-5

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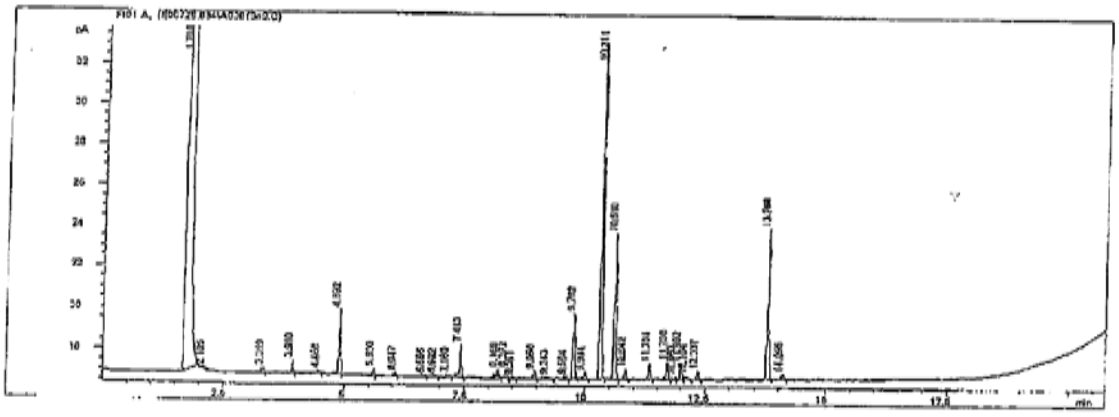


Figure 5.8: Chromatograph and SIM index of *Aeromonas ichthiosmia* (MS_4)

CHAPTER 6

WASTEWATER TREATMENT AND PHA SYNTHESIS: MONO STRAIN

6.1 Introduction

Micro-organisms have a vital role to play in both the aerobic and anaerobic treatment of wastewater. They degrade organic materials present in the wastewater and convert the resultant products into microbial biomass, humic materials, and waste gases such as carbon dioxide, nitrogen compounds and methane (Grady, 1999; Metcalf, 1991). Biopolymers are produced by many different types of bacteria. In environmental biotechnology and commodity production, there are biologically-derived polyesters known as polyhydroxyalkanoates (PHAs) which represent a potentially sustainable replacement of fossil fuel-based thermoplastics. Synthesis of PHAs, which serves as bacterial carbon and energy storage reserves, is currently estimated to be accomplished by over 300 different bacterial species in the form of

cytoplasmic granules (Lee, 1996). Although PHAs represent an opportunity to reduce the reliance on fossil fuel and solid waste quantities, current commercial production practices, which produce PHAs with pure microbial cultures grown on renewable but refined feedstoks (e.g. glucose) under sterile conditions, are not sustainable (Chan, 2006; Gerngross, 1999; Lee, 1995). However, recognizing the apparent propensity of wild microbial identified from activated sludge to synthesize biopolymer (Law, 2001; Liu, 2002; Rees, 1992; Wong, 2000), production of PHAs would be theoretically a natural extension of the wastewater treatment regime. While there is an abundant evidence of aerobic/anaerobic storage under dynamic waste treatment conditions in laboratory plants with synthetic substrates and ad hoc cultivated cultures (Majone, 1999), there is little knowledge about waste parameters removal and biopolymer accumulation mechanisms by bio-reactors with real wastewater.

In this section, the identified isolated PHA biomasses from different activated sludge of some Hong Kong industries were used in wastewater treatment with real wastewater feeding. Their corresponding waste pollutant removal efficiency was compared. The intra-cellular bioplastic accumulation was also studied.

6.2 Evaluation of SBR process by using Mono strain for wastewater treatment and PHAs synthesis

In order to evaluate the individual performance of the seven bacteria identified in waste removal and PHAs synthesis under SBR conditions, batch tests were performed using real feeding waste from Garden Company (Sham Tseng, N.T., Kowloon) as the only carbon source. The seven identified bacteria, *Aeromonas ichthiosmia*, *Bacillus cereus*, *Bacillus pumilus, Pseudomonas huttiensis, Pseudomonas putida, Sphingopyxis terrae, and Yersinia frederiksenii* were inoculated into the SBR separately. Their waste treatability and PHAs accumulation activities were evaluated. Typical profiles of the waste removal of COD, BOD, KN and PHA accumulation corresponding to different COD influence concentrations are presented in Figures 6.1 – 6.7.

6.2.1 *Aeromonas ichthiosmia*

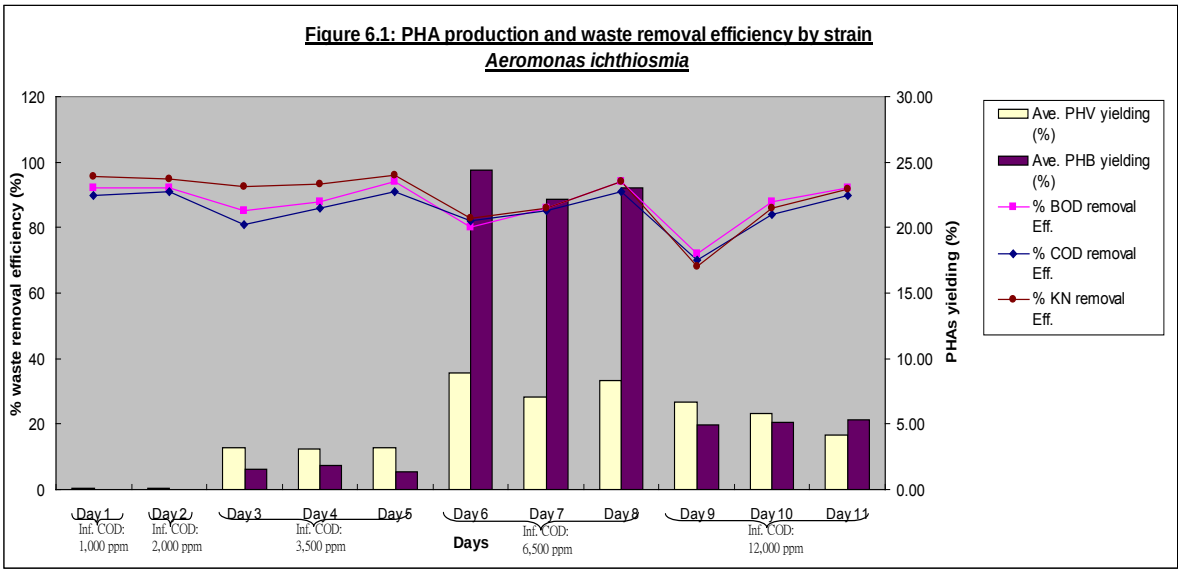
Figure 6.1 shows that when the influence of organic loading was below 3,500ppm COD, there was no PHB accumulated by *Aeromonas ichthiosmia*. It took 1 day for *Aeromonas ichthiosmia* to achieve COD and KN removal

efficiency of above 90%. The BOD removal efficiency performed at 1,000ppm and at 2,000ppm COD influence concentrations was above 90%. This indicated that the carbon source concentration at 2,000ppm was not enough for *Aeromonas ichthiosmia* to accumulate PHAs (Figure 6.1). Once the organic influence reached 3,500ppm, about 1.6% PHB and 3% PHV of cellular content accumulation were observed.

With the increase of the COD influence loading to 3,500ppm, it took 3 days for *Aeromonas ichthiosmia* to stabilize the waste utilization capabilities for having over 90% waste parameter removal. It was only after feeding for 3 days that over 90% of COD and BOD could be removed. The increment of organic loading exerted no effect to the KN removal efficiency. It took 1 day to perform above 90% KN removal. The optimum PHA yield of about 22% PHB and 8.3% PHV of cellular content was achieved when 6,500ppm COD feeding was applied. Although the organic loading was increased to 12,000ppm, it took 3 days for *Aeromonas ichthiosmia* to stabilize the waste removal performance so as to have COD removal efficiency of above 90%. A significant drop of PHA content was observed at 12,000ppm of COD influence. There were only 5% PHB (decreased by 77%) and 5.5% PHV (decreased by 39%) of cellular

content accumulated at that organic loading.

Figure 6.1: PHA production and waste removal efficiency by strain *Aeromonas ichthiosmia*

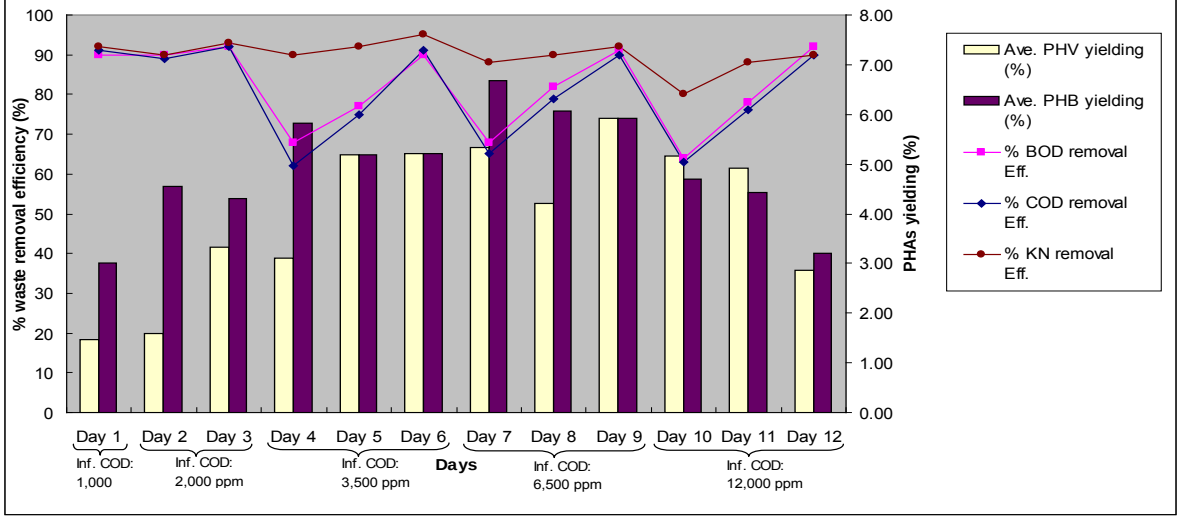


6.2.2 *Bacillus pumilus*

Figure 6.2 indicates that the capabilities of *Bacillus pumilus* for PHAs accumulation at 1,000 ppm COD influence were about 3% of PHB and 1.5% of PHV of cellular content. It took only 1 day for *Bacillus pumilus* to reach above 90% COD, BOD and KN removal efficiency. With the increase of the organic loading from 1,000ppm to 6,500ppm, the time needed to stabilize the system in order to have same waste removal efficiency as at 1,000ppm COD influence increased from 1 day to 3 days. A maximum of about 6.2% PHB and 5.2% PHV of cellular content were accumulated under 6,500ppm COD influence (Figure 6.2).

A significant drop in PHA content was observed at 12,000ppm COD influence. There were only about 4.1% PHB (decreased by 34%) and 4.3% PHV (decreased by 17%) of cellular content accumulated at that organic loading. Although there was as a drop of PHA content, the pollutant removal capabilities were not affected. It took *Bacillus pumilus* 3 days to achieve 90% removal of COD and BOD. KN removal efficiency gradually decreased from 2 days at 6,500ppm COD influence to 3 days at 12,000 COD influence.

Figure 6.2: PHA production and waste removal efficiency by strain *Bacillus pumilus*

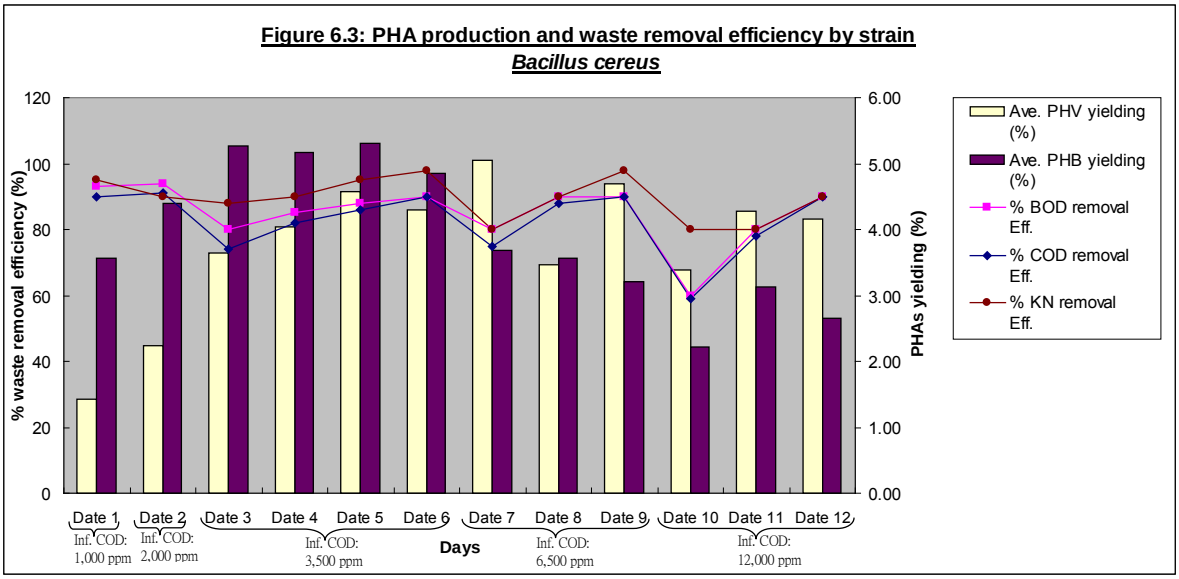


6.2.3 *Bacillus cereus*

Figure 6.3 shows that *Bacillus cereus* started accumulating about 3.0% PHB and 1.4% PHV of cellular content and with 1 day needed to achieve above 90% pollutant removal, including BOD, COD and KN at 1,000ppm COD influence. At 3,500ppm COD influx, it took 4 days to achieve 90% BOD and COD, and 98% KN removal. The highest PHA yields of 5.1% PHB and 4.1% PHV of cellular content were obtained at that concentration of the organic loading.

Figure 6.3 shows that, PHAs synthesis was found by *Bacillus cereus* with the organic loading ranging from 1,000ppm to 3,500ppm COD influence. Within this COD influence range, *Bacillus cereus* favored PHB synthesis to PHV yielding. When the organic loading increased to 6,500ppm COD, it took three days to achieve above 90% BOD and COD removal as well as 98% KN removal efficiency. Meanwhile, the PHA content decreased to 3.4% of PHB and 4.4% PHV of cellular content. The PHAs further reduced to about 2.7% PHB and 3.9% PHV of cellular content at 12,000ppm COD influx. At high COD influence level (6,500ppm – 12,000ppm), *Bacillus cereus* favored PHV yielding to that of PHB (Figure 6.3).

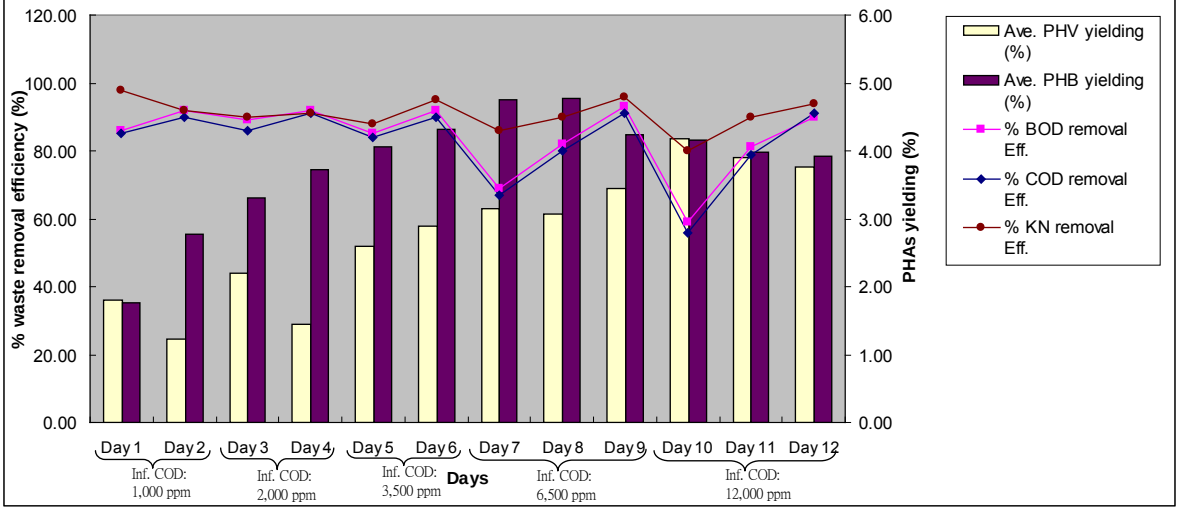
Figure 6.3: PHA production and waste removal efficiency by strain *Bacillus cereus*



6.2.4 *Pseudomonas putida*

It took *Pseudomonas putida* 2 days to achieve above 90% COD and BOD removal when 1,000 ppm COD was fed (Figure 6.4). About 2.3% of PHB and 1.5% of PHV of cellular content were accumulated. With the influence organic loading increased from 1,000ppm to 2,000ppm COD level, the pollutant removal efficiency of *Pseudomonas putida* maintained of 2 days to have above 90% COD, KN and BOD removal efficiency. The PHA content increased to 3.5% PHB and 1.8% PHV of cellular content. With the increase of the organic loading to 6,500ppm COD, PHA accumulation was enhanced and a maximum yield of 4.6% PHB of cellular content was obtained at 6,500ppm COD influence. When 12,000ppm COD influence was reached, a drop in PHB accumulation was observed (decreased by 13%). Meanwhile, PHV accumulation was enhanced and increased from 1.5% to 3.9% of cellular content. It took three days for *Pseudomonas putida* to stabilize and achieve above 90% COD and BOD removal when 6,500ppm and 12,000ppm COD influence was applied. It took 2 days to stabilize the system to have above 90% KN removal over the COD influence ranging from 3,500ppm to 12,000 ppm.

Figure 6.4: PHA production and waste removal efficiency by strain *Pseudomonas putida*



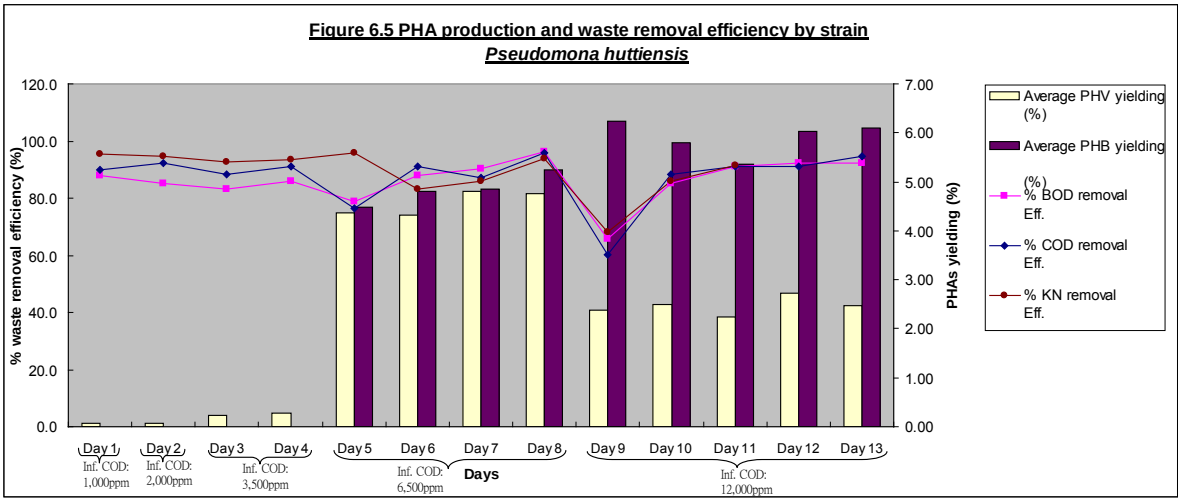
6.2.5 *Pseudomona huttiensis*

Figure 6.5 shows that there was no PHB accumulation in the COD influence concentrations range from 1,000ppm to 3,500ppm. Only a small amount of PHV (0.06% to 0.25%) was found within this COD influence concentration range. There was a significant enhancement of PHB and PHV accumulation when COD influence increased to 6,500ppm. About 4.8% PHB and 4.6% PHV of cellular content were accumulated. The continuous enhancement of PHB accumulation showed when increasing of COD influence concentration used. A maximum yielding of 5.9% PHB of cellular content was obtained when COD influence reached 12,000ppm. Although PHB was continuously enhanced over the COD influence range from 6.500ppm to 12,000ppm, 4.6% PHV of cellular content was the maximum PHV yield for *Pseudomonas huttiensis* at 6,500ppm COD influence. There was about a decrease of 47% in PHV accumulation when 12,000ppm COD influence was applied.

Figure 6.5 shows that *Pseudomonas huttiensis* favored PHB synthesis when sufficient carbon content was provided (i.e. 6,500ppm COD influence). It took 4 days for *Pseudomonas huttiensis* to have above 90% COD removal efficiency

at 6,500ppm COD influence. For this COD influence concentration, it took 3 days for *Pseudomonas huttiensis* to have above 90% BOD and KN removal. With COD influence increased to 12,00ppm, it took 5 days to have above 90% COD removal, 3 days for above 90% BOD removal and 4 days for above 90% KN removal.

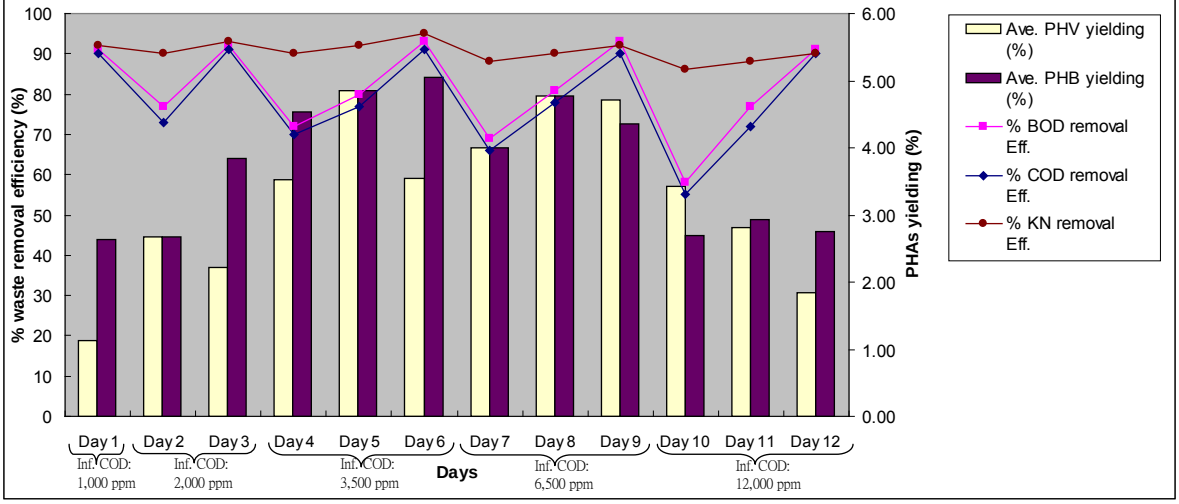
Figure 6.5 PHA production and waste removal efficiency by strain *Pseudomona huttiensis*



6.2.6 *Sphingopyxis terrae*

Figure 6.6 shows that about 2.6% PHB and 1.1% PHV of cellular content were accumulated by *Sphingopyxis terrae* when 1,000ppm COD was used as the feeding substrate. The time needed to stabilize the system to achieve above 90% COD removal efficiency increased from 1 day to 3 days when the feeding substrate concentration increased from 1,000ppm to 3,500ppm COD. It took 3 days for Sphingopyxis terrae to stabilize the system to have above 90% COD and KN removal efficiency. PHB accumulation increased from 2.6% to 4.8% at 3,500ppm COD influence. The PHB accumulation decreased from 4.8% to 2.8% when COD influence concentration at 6,500ppm and 12,000ppm. A maximum of 4.5% PHV of cellular content was obtained at 6,500ppm COD feeding. PHV accumulation dropped to 2.7% when the feeding concentration increased from 6,500ppm to 12,000ppm.

Figure 6.6: PHA production and waste removal efficiency by strain *Sphingopyxis terrae*

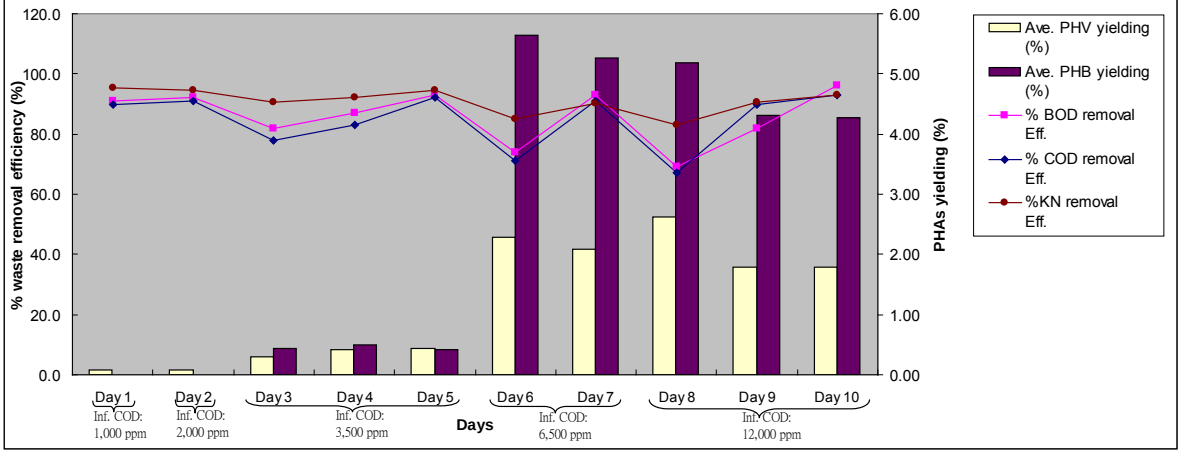


6.2.7 *Yersinia frederiksenii*

Figure 6.7 shows that *Yersinia frederiksenii* started to accumulate PHAs at 3,500ppm COD influence. About 0.5% PHB of cellular content was obtained. A small amount of PHV (about 0.4% of cellular content) was obtained at that COD feeding level. There was a significant PHA accumulation enhancement when the COD feeding concentration increased to 6,500ppm. About 5.5% PHB and 2.2% PHV of cellular were accumulated. A decrease of PHA accumulation occurred when the COD feeding increased from 6,500ppm to 12,000ppm. Only about 4.3% PHB and 1.8% PHV were accumulated.

Three days were needed for *Yersinia frederiksenii* to stabilize the system to achieve over 90% COD and BOD removal efficiency when the COD influence concentration increased from 2,000ppm to 12,000ppm. Two days of stabilization were needed to achieve 90% KN removal efficiency at 6,500ppm and 12,000ppm COD influence concentration. In general, increasing the COD concentration of feeding would favor the PHB accumulation to PHV accumulation by *Yersinia frederiksenii* (Figure 6.7).

Figure 6.7: PHA production and waste removal efficiency by strain *Yersinia frederiksenii*



6.3 Discussion

The feasibility of using isolated bacteria from different activated sludge of industries to synthesize PHAs and wastewater treatment depended on some economical areas such as the carbon sources, technical pre-treatment methods, cultivation condition, system stabilization time, waste treatability and PHA synthesizability.

The experiment results in this section show that the feeding from Garden Company, Sham Tseng, N.T. supported the growth of isolated PHA bacteria and PHA synthesis. In addition to acting as culture medium, the typical pollutant parameters (i.e. COD, BOD and KN) of the feed wastes were removed by the bacteria at the same time. Furthermore, the feed wastes were free of charge.

In considering waste treatability, *Aeromonas ichthiosmia*, *Bacillus cereus*, *Pseudomonas huttiensis* and *Yersinia frederiksenii* performed better BOD and COD removal than *Bacillus pumilus*, *Pseudomonas putida* and *Sphingopyxis terrae* at COD influence concentrations ranging from 1,000ppm to 2,000ppm COD. *Aeromonas ichthiosmia*, *Bacillus cereus*, *Pseudomonas huttiensis* and

Yersinia frederiksenii removed over 90% COD and BOD within one day. *Bacillus pumilus*, *Pseudomonas putida* and *Sphingopyxis terrae* required two days for stabilization to achieve the same removal efficiency. All of the isolated bacteria performed over 90% KN removal within one day in COD influence concentrations ranging from 1,000ppm to 3,500ppm COD.

Increasing the organic loading would increase the system stabilization time of bacteria to achieve over 90% COD, BOD and KN removal. At 12,000ppm COD influence, three days were required for all bacteria to stabilize the system. Among the isolated bacteria, *Pseudomonas putida* and *Yersinia frederiksenii* performed better in KN removal. It took 2 days for *Pseudomonas putida* to stabilize the system at 12,000ppm COD influence. It took *Pseudomonas huttiensis* 4 days, the longest among the isolated bacteria, to achieve over 90% KN removal at 12,000ppm COD influence.

The results of the bacteria biosynthesis of PHAs by the isolated bacteria show that the feed waste used had the high potential as the culture media for this bio-technology. *Aeromonas ichthiosmia* achieved the highest PHA accumulation (Figure 6.1). It accumulated more than 22% PHB and more than

8% PHV of cellular content. The experimental results found are comparable to other researchers findings (Law, 2001; Wong, 2000; Yasushi, 1992; Yu, 1998). *Pseudomonas putida* showed the worst PHA synthesis capabilities among the seven bacteria. A maximum of 4.6% PHB and 3.2% PHV of cellular content were accumulated. Other isolated bacteria accumulated PHB in the range of 4.8% - 6.2% and 3.9% - 5.2% of PHV.

There was a PHA accumulation inhibition phenomenon found in all isolated bacteria at corresponding COD influence concentration. Most of the isolated bacteria achieved the maximum PHA yield at 6,500ppm COD influence, a decrease of PHA accumulation occurred when 12,000ppm COD influence was applied as the carbon source. It was because although high organic loading provided high carbon concentration source, concentration of KN in the waste feeding was also proportionally higher than other influence with lower organic loading. The high nitrogen content resulted in a high concentration of free CoASH. The CoASH inhibited the β-ketothiolase condensation reaction. This inhibition led to a decrease of PHA accumulation (Figures 2.4 and 2.5).

CHAPTER 7

WASTEWATER TREATMENT AND PHA SYNTHESIS: MIXED STRAINS

7.1 Introduction

Historically Mixed microbial-based engineered biological systems have been utilized principally for the remediation and/or treatment of anthropogenic-derived pollution. It is, appropriately referred to as environmental biotechnology, and its potential to synthesize commodities and provide services beyond waste treatment has been recognized (Rittmann, 2006). In environmental biotechnology and commodity production there are the biologically-derived polyesters known as polyhydroxyalkanoates (PHAs), which represent a potentially sustainable replacement of fossil fuel-based thermoplastics. Synthesis of PHAs, which serve as bacterial carbon and energy storage reserves, is currently estimated to be accomplished by over 300 different bacteria species in the form of cytoplasmic granules (Lee, 1996).

The apparent propensity of the wild microbial population to synthesize polymer

means theoretically the commercial production of PHAs be a natural extension of the wastewater treatment regime. While there is abundant evidence of PHA accumulation under dynamic conditions in laboratory plants with synthetic/real waste substrates and ad hoc cultivated cultures (Beun, 2002; Caruccci, 2001; Chan, 2006; Law, 2001; Michele, 1999; Satoh, et. 1992), there is still little knowledge about pollutant removal and PHA accumulation by mixed PHAs producing bacteria from real wastewater as carbon sources under a dynamic conditions. In fact, PHA synthesis was associated with the empirical wastewater treatment process known as Enhanced Biological Phosphorus Removal (EBPR) (Satoh, 1992), although biological synthesis of PHA in full scale EBPR facilities, estimated at upwards of 4% (w/w), falls short of the quantities needed for commercial exploitation. Nevertheless, waste stream utilization in PHA productions is recognized as a sustainable way for process integration exists.

Mono strain integrated PHAs synthesis and wastewater treatment process for municipal wastes were studied in Chapter 6. In this Chapter, mixed strains were studied to evaluate the optimization of PHA synthesis and waste removal efficiency.

7.2 Mixing with *Bacillus pumilus*

7.2.1 *Bacillus pumilus* and *Aeromonas ichthiosmia*

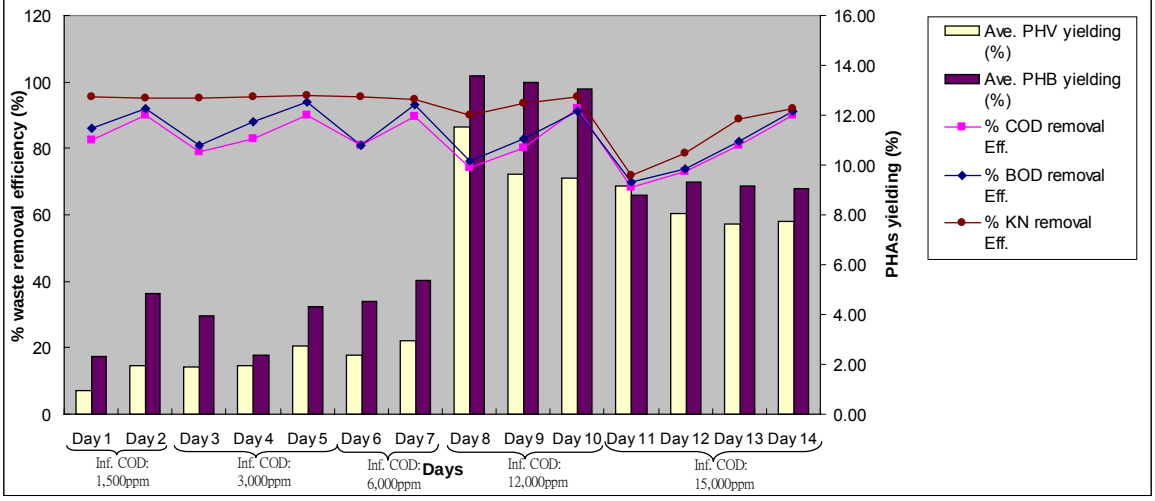
In this section, identified *Bacillus pumilus* from Domestic Sludge of Shek Wu Hoi Sewage Treatment Works, and *Aeromonas ichthiosmia* from Mixed sludge were mixed into a Sequencing Batch Reactor for study.

After mixing *Bacillus pumilus* with *Aeromonas ichthiosmia*, their pollutant removal performance showed as the same capabilities as mono strain did over the influent concentration ranging from 1,000ppm to 6,000ppm COD (Figures 6.1 and 6.2). Figure 7.1 shows that three days were needed to stabilize the system by the mixed strains so as to achieve over 90% COD and BOD removal for the influent concentrations ranging from 3,000ppm to 12,000ppm COD. Capabilities of mixed strains to remove over 90% KN at 12,000ppm COD influent is better than mono strain by 2 days advancement. Similar to *Bacillus pumilus* (Figure 6.2), the mixed strains started accumulating PHAs at 1,500ppm COD influent. About 3.6% PHB and 1.5% PHV of cellular content were accumulated. There was an increasing of PHAs accumulation when the

organic loading increased from 1,500ppm COD to 6,000ppm COD (Figure 7.1). PHB increased from about 3.6% to 5.0% of cellular content and PHV increased from about 1.5% to 2.7% of cellular content. A maximum of 13.3% PHB and 10.2% PHV were obtained when 12,000ppm COD influent was applied. A drop in PHA accumulation was observed when the organic loading reached 15,000ppm COD. For the mono strain experiment, the PHA dropping phenomena occurred when 12,000ppm COD influent applied with less than 5% PHB and PHV accumulated. It took the mixed strains 4 days to stabilize the system to have over 90% COD, BOD as well as 90% KN removal when 15,000ppm COD influent applied.

There was about 40% PHB accumulation decreased by mixed strains when compared the maximum yield of PHB produced by mixed strain to mono strain. The mixed strains increased 23% PHV accumulation when compared to maximum PHV yields accumulated by mono strain. The capability of PHB accumulation by mixing *Bacillus pumilus* (Figure 6.2) and *Aeromonas ichthiosmia* (Figure 6.1) of withstanding high waste feed concentration was enhanced. Organic loading up to 12,000ppm COD without PHA accumulation decrease by the mixed strains was demonstrated.

Figure 7.1: PHA production and waste removal efficiency by mixed strains: *Aeromonas ichthiosmia & Bacillus pumillus*



7.2.2 *Bacillus pumilus and Sphingopyxis terrae*

Identified *Bacillus pumilus* from Domestic Sludge of Shek Wu Hoi Sewage Treatment Works and *Sphingopyxis terrae* from Mixed sludge were mixed into a Sequencing Batch Reactor for study.

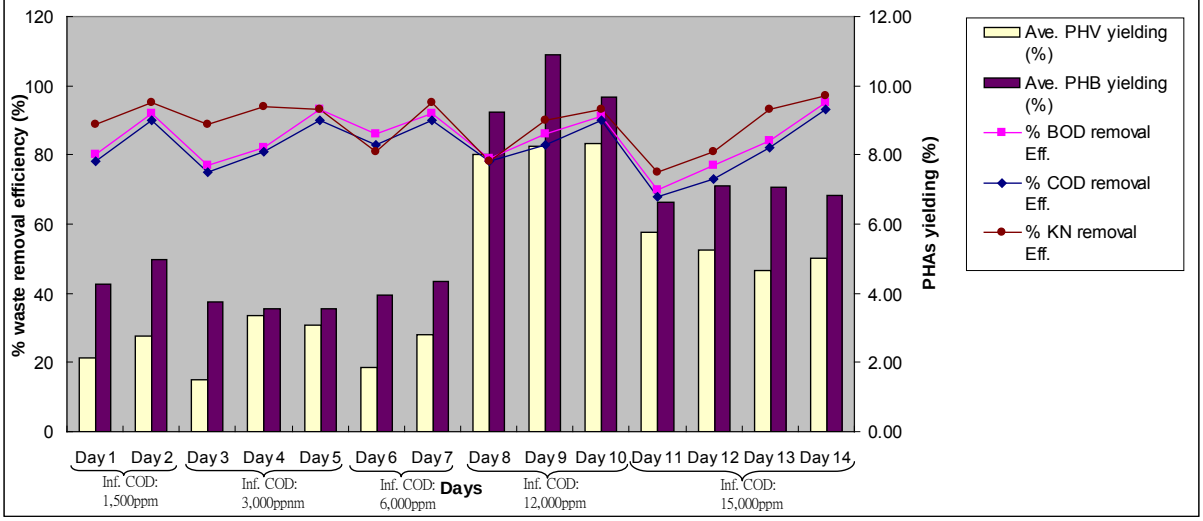
The pollutant removal efficiency trends of the mixed strains and of individual strains looked similar (Figure 6.2 and Figure 6.6). It took mixed strains of *Bacillus pumilus* & *Sphingopyxis terrae* two to three days to achieve over 90% COD and BOD removal for the COD influent range from 1,500ppm to 12,000ppm. Within this range, over 90% KN removal was achieved by the mixed strains after a two-day stabilization. Mixed strains of *Bacillus pumilus* and *Sphingopyxis terrae* accumulated about 4% PHB and 1-3% PHV when 1,500ppm to 6,000ppm influent COD was applied (Figure 7.2).

In the mono strain experiment, both *Bacillus pumilus* (Figure 6.2) and *Sphingopyxis terrae* (Figure 6.6) performed maximum PHA accumulation at 6,500ppm influent COD and a drop of PHA accumulation was observed when 12,000ppm influent COD was applied. After mixing these two bacteria, there

was a significant enhancement in PHA accumulation when 12,000ppm influent COD was applied. About 9.9% PHB and 8.2% PHV were produced by the mixed strains in the 12,000ppm COD feeding. A decrease in PHA accumulation also occurred with a higher organic loading at 15,000ppm influent COD. About 6.9% PHB (31% decreased) and 5.2% PHV (37% decreased) of cellular content were accumulated at this influent COD concentration. PHA accumulation inhibition for mono strain was appeared when 12,000ppm influent COD applied.

Compared the PHA accumulation of mixed strains to mono strain studies, there was at least 59% PHB and 58% PHV of cellular content of the maximum yielding enhanced by mixed strains.

Figure 7.2: PHA production and waste removal efficiency by mixed strains: *Sphingopyxis terrae & Bacillus pumillus*



7.2.3 *Bacillus pumilus and Pseudomonas putida*

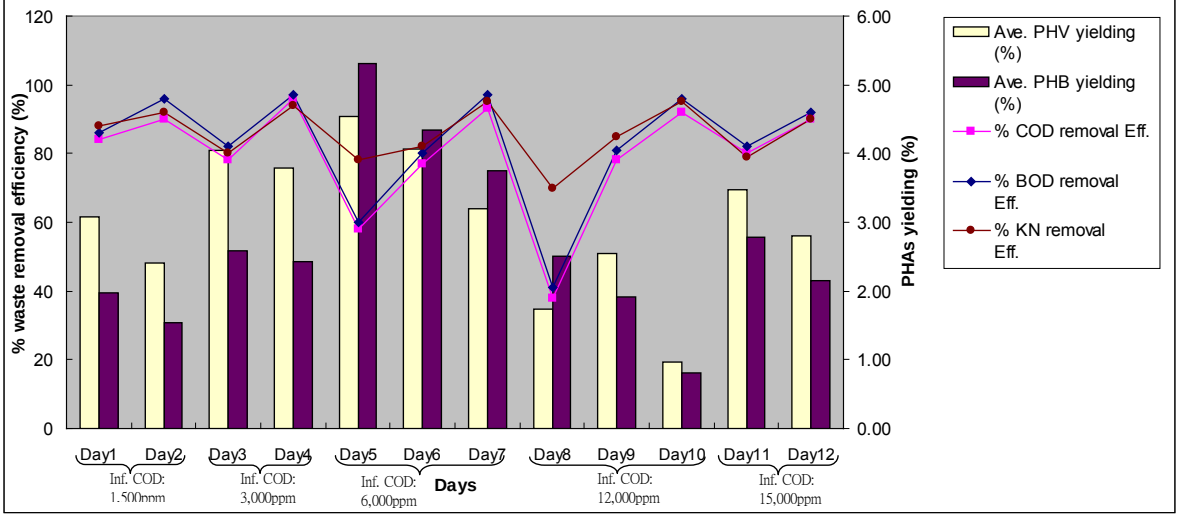
Identified *Bacillus pumilus* from Domestic Sludge of Shek Wu Hoi Sewage Treatment Works and *Pseudomonas putida* from Purify Bio-filter Sludge (Tai Po Water Treatment Works) were mixed into a Sequencing Batch Reactor for study.

The capabilities of the mixed strains of *Bacillus pumilus* & *Pseudomonas putida*

to have over 90% COD, BOD and KN removal were the same as mono strain when influent COD concentrations ranging from 6,000ppm to 12,000ppm. Mixed strains needed two days to stabilize the system to achieve over 90% COD, BOD and KN removal with 15,000ppm COD feeding.

Figure 7.3 shows that the PHA accumulation performance of the mixed strains and the mono strain of *Pseudomonas putida* (Figure 6.4) was similar with the forming having a maximum of PHA yield of 4.5% PHB and 3.9% PHV of cellular content produced at 6,000ppm influent COD. Similar to the mono strain, PHB accumulation of the mixed strains decreased when 12,000ppm influent COD was applied.

Figure 7.3: PHA production and waste removal efficiency by mixed strains: *Pseudomonas putida & Bacillus pumilus*



7.2.4 *Bacillus pumilus and Bacillus cereus*

Identified *Bacillus pumilus* from Domestic Sludge of Shek Wu Hoi Sewage Treatment Works and *Bacillus cereus*, from Food Waste sludge 1 (Garden company) were mixed into a Sequencing Batch Reactor for study.

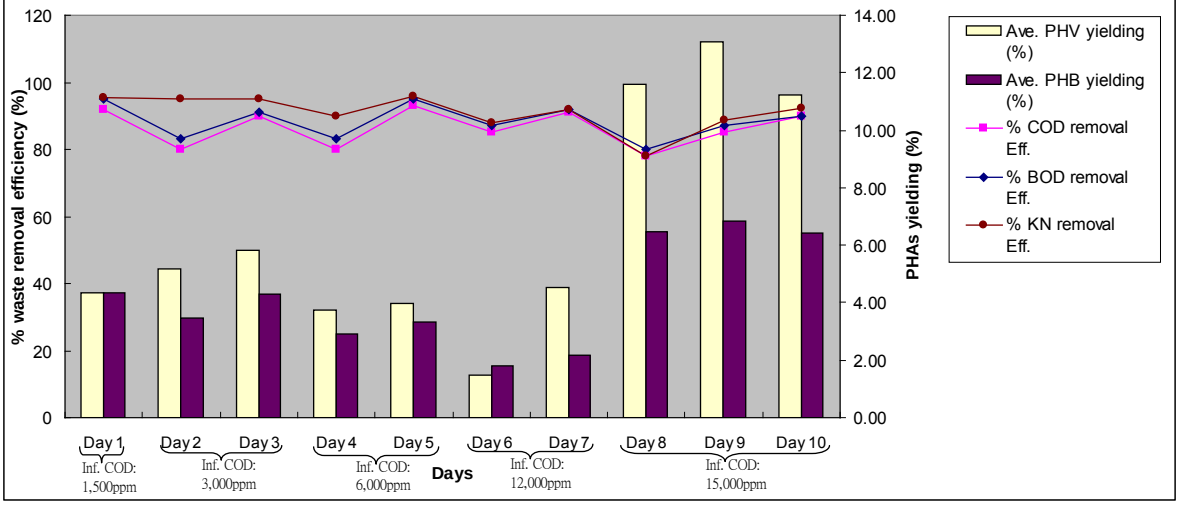
Figure 7.4 shows that waste treatment efficiency of the mixed strains of *Bacillus pumilus* and *Bacillus cereus* was enhanced. Only two days were needed for the mixed strains to stabilize to have over 90% COD and BOD removal with influent COD ranging from 3,000ppm to 12,000ppm. KN removal efficiency was also enhanced by the mixed strains; it took only one day to achieve over 90% KN removal with 1,500ppm to 6,000ppm influent COD. For 12,000ppm influent COD, the mixed strains only took 2 days to stabilize the system to achieve over 90% KN removal. The mono culture needed three days to achieve the same pollutant removal efficiency.

There was a significant PHV accumulation enhancement by the mixed strains. About 12% PHV of cellular content was accumulated when 15,000ppm influent COD was applied (Figure 7.4). A maximum of 6.6% PHB accumulation was

also obtained at this influent COD.

Comparing to the mono strain studies of PHA accumulation (Figure 6.2 and 6.3), the mixed strains could at least increase 6.5% PHB and 130% PHV accumulation at the maximum yielding point. In addition, the PHA accumulation inhibition of mixed strains occurred at a higher influent COD concentration than that of mono strain. 15,000ppm influent COD influent could be applied to the mixed strains to obtain the maximum PHB and PHV yielding.

Figure 7.4: PHA production and waste removal efficiency by mixed strains: *Bacillus pumilus & Bacillus cereus*



7.2.5 *Bacillus pumilus and Pseudomonas huttiensis*

Identified *Bacillus pumilus* from Domestic Sludge of Shek Wu Hoi Sewage Treatment Works and *Pseudomonas huttiensis* from Food Waste sludge 1 (Garden company) were mixed into a Sequencing Batch Reactor for study.

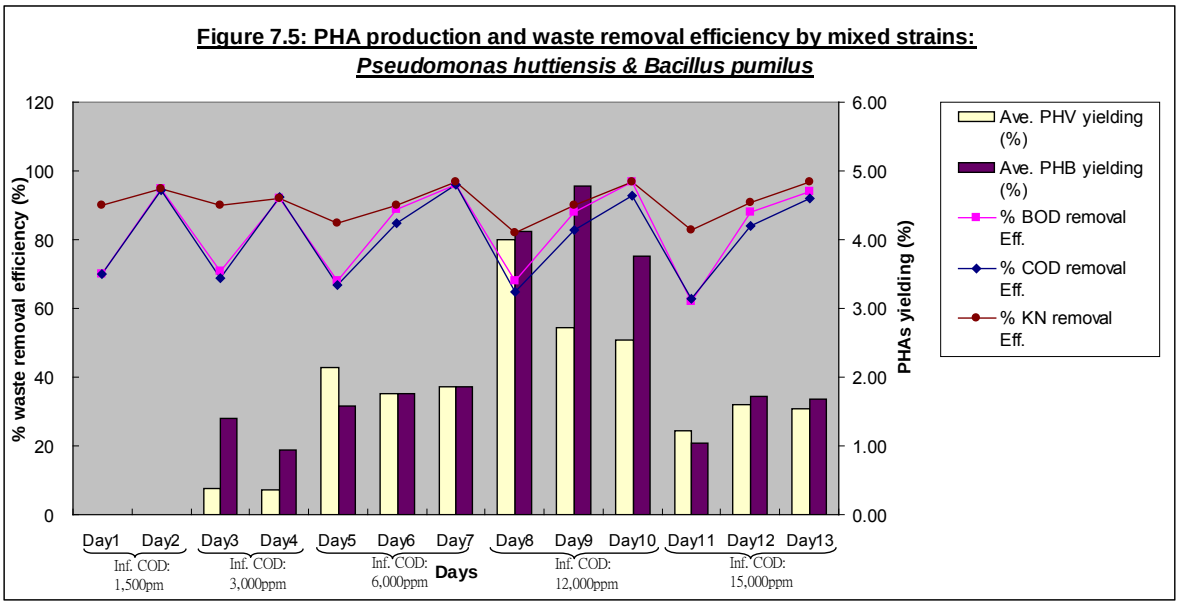
Figure 7.5 shows that the mixed strains of *Bacillus pumilus* and *Pseudomonas huttiensis* had the same waste treatment capabilities as the mono strain of *Bacillus pumilus* (Figure 6.2) at the influent COD range from 1,000ppm to 12,000ppm. They were better than the mono strain of *Pseudomonas huttiensis* (Figure 6.5), which needed four days to stabilize the system to achieve over 90% COD removal at 6,500ppm and 12,000ppm influent COD. The stabilization time needed for the mixed strains to have over 90% COD and BOD at 6,000ppm influent COD was 2 days and at 12,000ppm influent COD was 3 days. In addition, two days of stabilization were needed for the mixed strains to have over 90% KN removal when the influent COD ranging from 6,000ppm to 15,000ppm.

The PHA accumulation of the mixed strains was inhibited. A maximum of

4.2% PHB and 3.1% PHV of cellular content was accumulated at 12,000ppm influent COD (Figure 7.5). A decrease in PHA accumulation was observed when a higher influent COD was applied (i.e. 15,000ppm COD). About 1.5% PHB (65% decreased) and 1.5% PHV (52% decreased) of cellular content were obtained at 15,000ppm influent COD.

Compared the PHA accumulation of mixed strains to mono strain studies, the mono strains achieved higher PHB and PHV yielding than mixed strains. There was a decrease of at least 29% of PHB and 33% of PHV in the mixed strains studies.

Figure 7.5: PHA production and waste removal efficiency by mixed strains: *Pseudomonas huttiensis & Bacillus pumilus*



7.2.6 *Bacillus pumilus and Yersinia frederiksenii*

Identified *Bacillus pumilus* from Domestic Sludge of Shek Wu Hoi Sewage Treatment Works and *Yersinia frederiksenii* from Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui) were mixed into a Sequencing Batch Reactor for study.

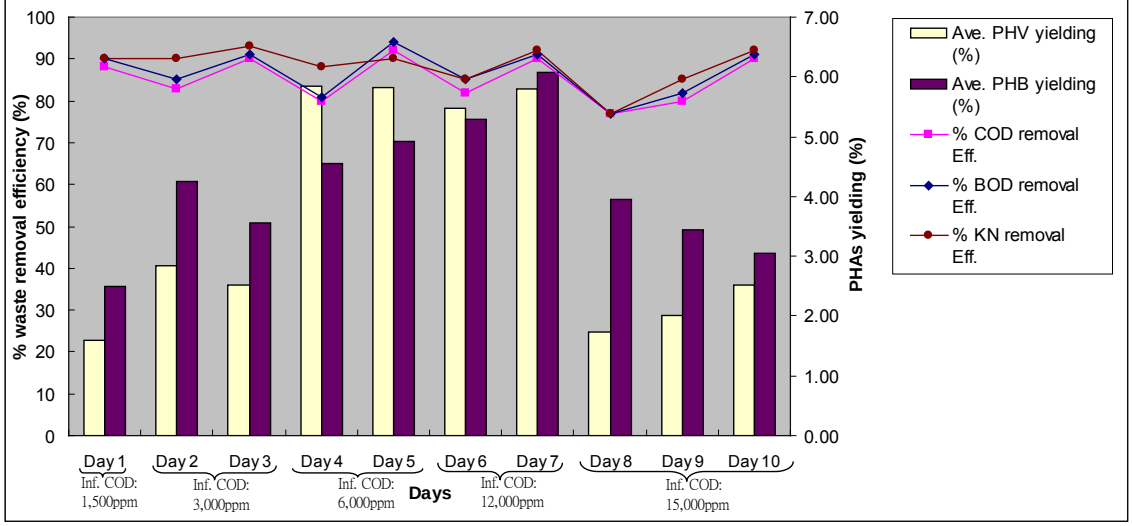
Figure 7.6 shows that the waste treatment capabilities of the mixed strains of *Bacillus pumilus* and *Yersinia frederiksenii* were better than the mono strain of *Bacillus pumilus* (Figure 6.2). But the mixed strains performed same pollutant removal efficiency as mono strain of *Yersinia frederiksenii* (Figure 6.7). The mixed strains took one day to achieve over 90% COD, BOD and KN removal at 1,500ppm and 3,000ppm influent COD. Two days were needed to stabilize the system to have the same pollutant removal efficiency when 6,000ppm and 12,000ppm influent COD were applied.

At 1,500ppm influent COD as the carbon source, 2.5% PHB and 1.6% PHV of cellular content were accumulated. The PHA accumulation increased with increasing organic loading to up to 12,000ppm. At that level of influent COD, a

maximum of 5.7% PHB and 5.8% PHV of cellular content was accumulated. When a higher organic loading was applied (i.e. 15,000ppm COD), both PHB and PHV accumulation decreased. About 3.5% PHB (39% decreased) and 2.1% PHV (63% decreased) of cellular content can be accumulated by the mixed strains at 15,000ppm influent COD (Figure 7.6).

Compared to the mono strain PHA accumulation experiment, there was no enhancement effect found in the mixed strains on PHB accumulation. Nevertheless, about 12% of PHV accumulations enhanced by the mixed strains. The mixed strains demonstrated a better PHV producing capability with a higher organic loading tolerance.

Figure 7.6: PHA production and waste removal efficiency by mixed strains: *Bscillus pumilus & Yersinia frederiksenii*



7.3 Mixing with *Bacillus cereus*

7.3.1 *Bacillus cereus* and *Aeromonas ichthiosmia*

Identified *Bacillus cereus* from Food Waste sludge 1 (Garden company) and *Aeromonas ich*thiosmia from Mixed Sludge were mixed into a Sequencing Batch Reactor for study.

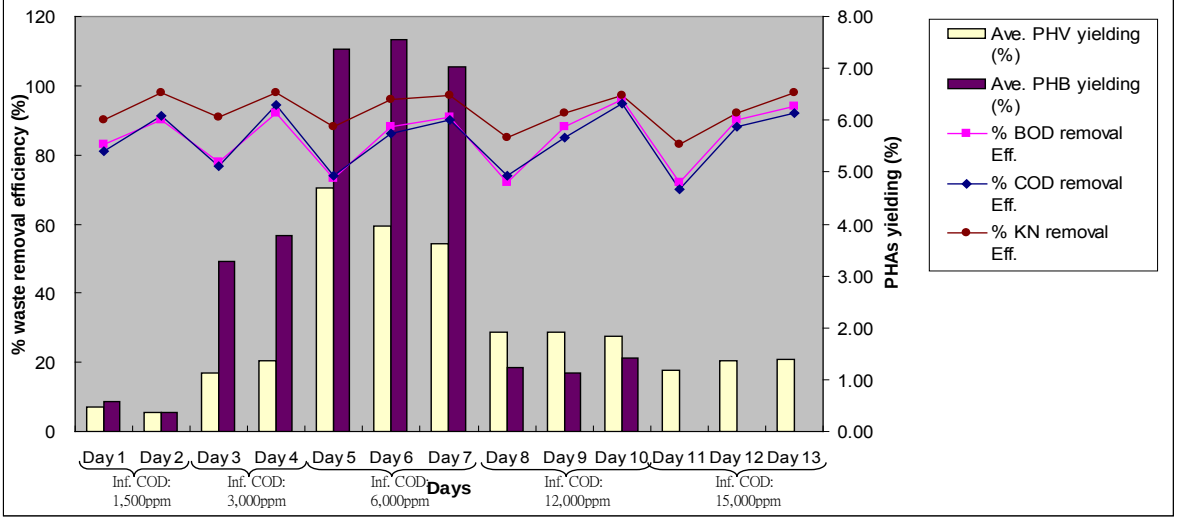
Figure 7.7 shows that the mixed strains of *Bacillus cereus* & *Aeromonas ichthiosmia* had the same pollutant removal capacities as mono strain (Figure 6.1 and 6.3) at 6,000ppm and 12,000ppm influent COD. Three days were needed system stabilization to achieve over 90% COD and BOD removal by mixed strains. Over 90% KN removal efficiency was achieved within two days of stabilization. The mixed strains started to accumulate 0.47% PHB and 0.43% PHV at 1,500ppm influent COD (Figure 7.7). The PHA accumulation increased when the concentration of COD feeding increased from 1,500ppm to 6,000ppm. A maximum of about 7.4% PHB and 4.1% PHV of the cellular content was accumulated at 6,000ppm influent COD. The PHA of the mixed culture decreased to 1.3% PHB and 1.9% PHV of cellular content when the

COD feeding further increased to 12,000ppm. There was no PHB accumulation found when 15,000ppm influent COD was applied. The PHV content decreased to 1.3% of cellular content at that influent COD level.

Figure 7.7 shows that organic loading lower than 6,000ppm COD was favourable for PHB accumulation. At higher organic loading, amount of PHV accumulated by the mixed strains was larger than PHB. No PHB was found at 15,000ppm COD but 1.3% of PHV was synthesized at that COD influent level.

Compared to the mono strain PHA production studies (Figure 6.1 and 6.3), both PHB and PHV accumulation by the mixed strains was inhibited. There were about 67% decrease in PHB and 50% decrease in PHV. Mixed strains demonstrated an inhibitory effect on PHA accumulation.

Figure 7.7: PHA production and waste removal efficiency by mixed strains: *Aeromonas ichthiosmia & Bacillus cereus*



7.3.2 *Bacillus cereus* and *Sphingopyxis terrae*

Identified *Bacillus cereus* from Food Waste sludge 1 (Garden company) and *Sphingopyxis terrae* from Mixed Sludge were mixed into a Sequencing Batch Reactor for study.

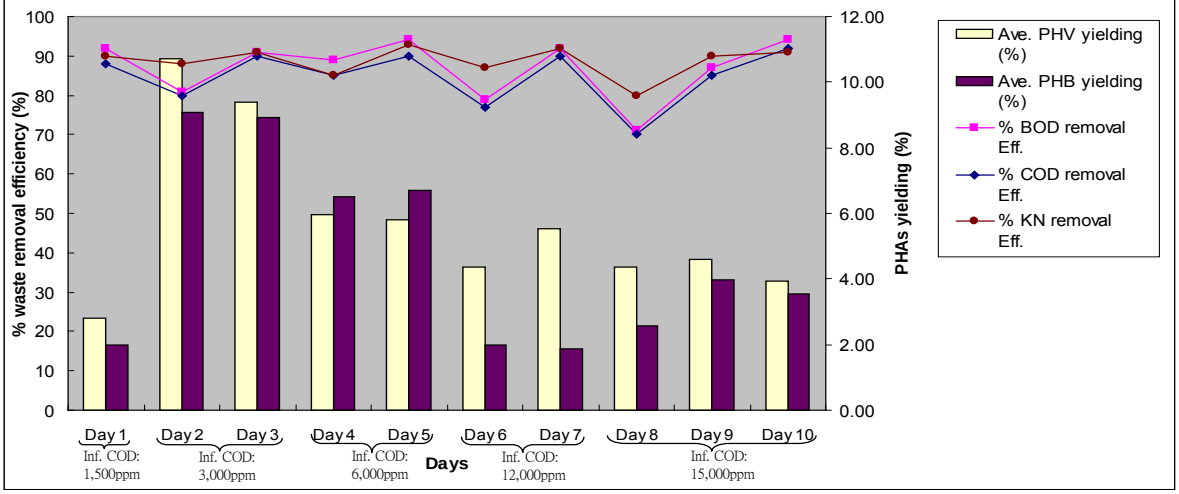
The mixed strains of *Bacillus cereus* and *Sphingopyxis terrae* showed a better waste treatment efficiency and PHA accumulation than the mono strain. Figure 7.8 shows that two days were needed to stabilize the system to achieve over 90% COD, BOD and KN removal within the organic loading range of 3,000ppm to 12,000ppm COD. The mixed strains started to accumulate PHAs at 1,500ppm influent COD with 2.0% PHB and 2.8% PHV of cellular content found. A maximum of 9.0% PHB and 10.0% PHV were accumulated by the mixed strains at 3,000ppm influent COD.

The PHA accumulation dropped with increasing influent COD concentration influent (Figure 7.8). When 15,000ppm influent COD was applied, three days were needed by the mixed strains to stabilize the system to have over 90% COD and BOD removal. Only about 3.4% PHB and 4.3% PHV of cellular

content were accumulated at 15,000ppm influent COD.

Compared to the PHA accumulation of mono strain (Figure 6.3 and 6.6), the mixed strains showed one day advance to achieve over 90% COD, BOD and KN at the organic loading from 1,500ppm to 12,000ppm COD. The mixed strains enhanced PHB accumulation by 73% and PHV accumulation by 120%.

Figure 7.8: PHA production and waste removal efficiency by mixed strains: *Sphingopyxis terrae & Bacillus cereus*



7.3.3 *Bacillus cereus* and *Pseudomonas putida*

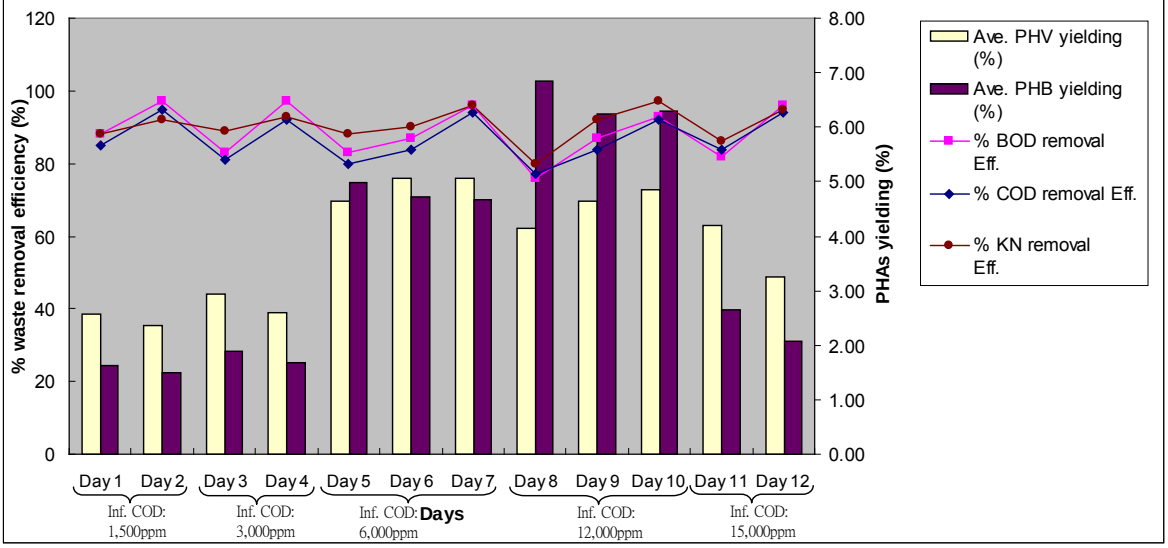
Identified *Bacillus cereus* from Food Waste sludge 1 (Garden company) and *Pseudomonas putida* from Purify Bio-filter Sludge (Tai Po Water Treatment Works) were mixed into a Sequencing Batch Reactor for study.

Figure 7.9 shows that mixed strains of *Bacillus cereus* and *Pseudomonas putida* showed same waste treatment capabilities as mono strain (Figures 6.3 and 6.4). A stabilization time of two days were needed by the mixed stains to achieve over 90% KN removal with the organic loading from 1,500ppm to 12,000ppm COD. Three days were needed to stabilize the system to achieve over 90% COD and BOD removal. PHA accumulation began at 1,500ppm influent COD. Both PHB and PHV accumulation increased when the organic loading increased from 1,500ppm to 12,000ppm influent COD. PHB increased from 1.6% to a maximum yield of 6.5% of cellular content at 12,000ppm influent COD. PHB decreased to 2.4% of cellular content if higher COD influent (i.e. 15,000ppm) was applied (Figure 7.9). The same increasing trend of PHV accumulation was observed for influent COD ranging from 1,500ppm to 6,000ppm. PHV increased from 2.5% to 4.9% of cellular weight at 6,000ppm

influent COD. It dropped to 3.8% of cellular weight when the organic loading increased to 15,000ppm COD.

Compared to the PHA accumulation of mono strain, similar waste treatment capabilities were observed by the mixed strains. The mixed strains enhanced PHB accumulation by 25% and PHV accumulation by 11%.

Figure 7.9: PHA production and waste removal efficiency by mixed strains: *Pseudomonas putida & Bacillus cereus*



7.3.4 *Bacillus cereus* and *Pseudomonas huttiensis*

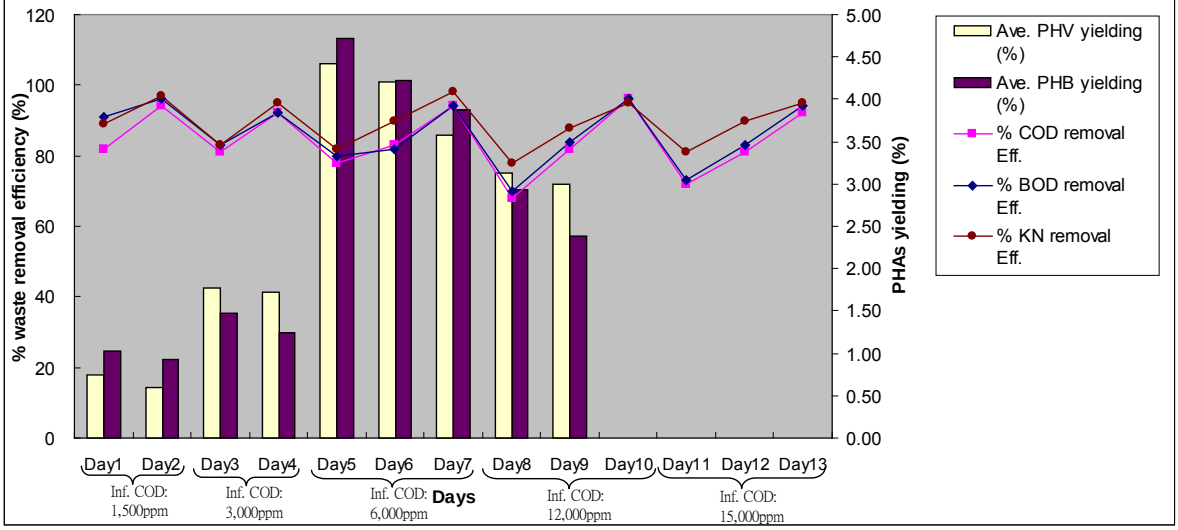
Identified *Bacillus cereus* from Food Waste sludge 1 (Garden company) and *Pseudomonas huttiensis* from Food Waste sludge 1 (Garden Company) were mixed into a Sequencing Batch Reactor for study.

Figure 7.10 shows that two days were needed for the mixed strains of *Bacillus cereus* & *Pseudomonas huttiensis* to stabilize the system to achieve over 90% KN removal with 1,000ppm to 6,000ppm influent COD. Three days were needed to achieve over 90% COD as well as over 90% BOD removal when the organic loading ranged from 6,000ppm to 15,000ppm COD. About 1.0% PHB and 0.7% PHV of cellular content were accumulated at 1,500ppm influent COD. A maximum of 4.3% PHB and 4.1% PHV of cellular content accumulated by the mixed strains at 6,000ppm influent COD. Both accumulation of PHB and PHV decreased when higher organic loading was applied (i.e. 12,000ppm COD) (Figure 7.10). At 15,000ppm influent COD, PHA accumulations were inhibited. No PHB or PHV accumulation by the mixed strains was found.

The waste treatment capabilities of the mixed strains were similar to that of the

mono strain (Figures 6.3 and 6.5). At least 17% PHB and 6% PHV accumulation were inhibited by the mixed culture.

Figure 7.10: PHA production and waste removal efficiency by mixed strains: *Pseudomon huttiensis & Bacillus cereus*



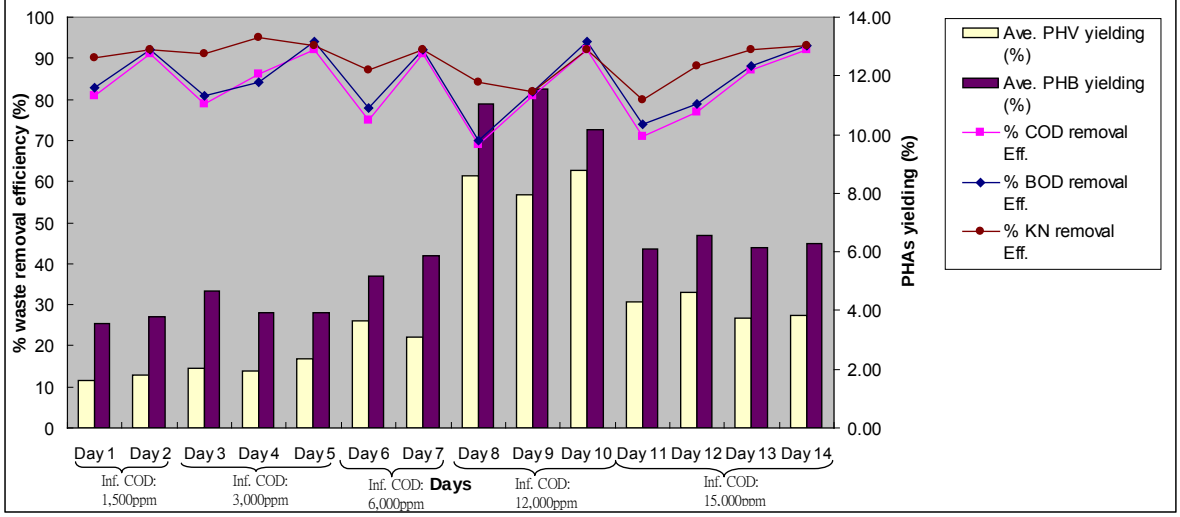
7.3.5 *Bacillus cereus* and *Yersinia frederiksenii*

Identified *Bacillus cereus* from Food Waste sludge 1 (Garden company) and *Yersinia frederiksenii* from Food Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui) were mixed into a Sequencing Batch Reactor for study.

Figure 7.11 shows that the waste treatment trend of the mixed strains of *Bacillus cereus* and *Yersinia frederiksenii* was similar to the mono strain of *Yersinia frederiksenii* (Figure 6.7). Three days were needed to stabilize to achieve over 90% COD and BOD removal with the organic loading at 3,000ppm and 12,000ppm COD. Two days were needed to achieve the same pollutant removal efficiency at 6,500ppm influent COD. About 3.7% PHB and 1.7% PHV of cellular content were accumulated at 1,500ppm influent COD. The accumulation of PHB and PHV increased when the organic loading increased from 1,500ppm to 12,000ppm COD. A maximum of about 10.9% PHB and 8.4% PHV of cellular content were obtained when 12,000ppm influent COD was applied.

A decrease in PHB and PHV accumulation by the mixed strains were observed when higher influent COD was applied (i.e. 15,000ppm COD) (Figure 7.11). About 6.3% PHB and 4.1% PHV was accumulated at 15,000ppm influent COD. Four stabilizing days were need to achieve over 90% COD and BOD removal at 15,000ppm influent COD. Three days were needed to achieve over 90% KN removal when 12,000ppm and 15,000ppm influent COD was applied. The mixed strains had waste treatment capabilities similar to the mono strain of *Yersinia frederiksenii* (Figure 6.7). The PHA accumulation was stimulated by the mixed strains. At least 98% PHB and 90% PHV accumulation of mixed strains were enhanced. Apart from PHA accumulation enhancement, the inhibitory of PHA accumulation to the mixed strains occurred at a higher influent COD concentration than that of mono strain.

Figure 7.11: PHA production and waste removal efficiency by mixed strains: *Yersinia frederiksenii & Bacillus cereus*



7.4 Mixing with *Sphingopyxis terrae*

7.4.1 *Sphingopyxis terrae* and *Aeromonas ichthiosmia*

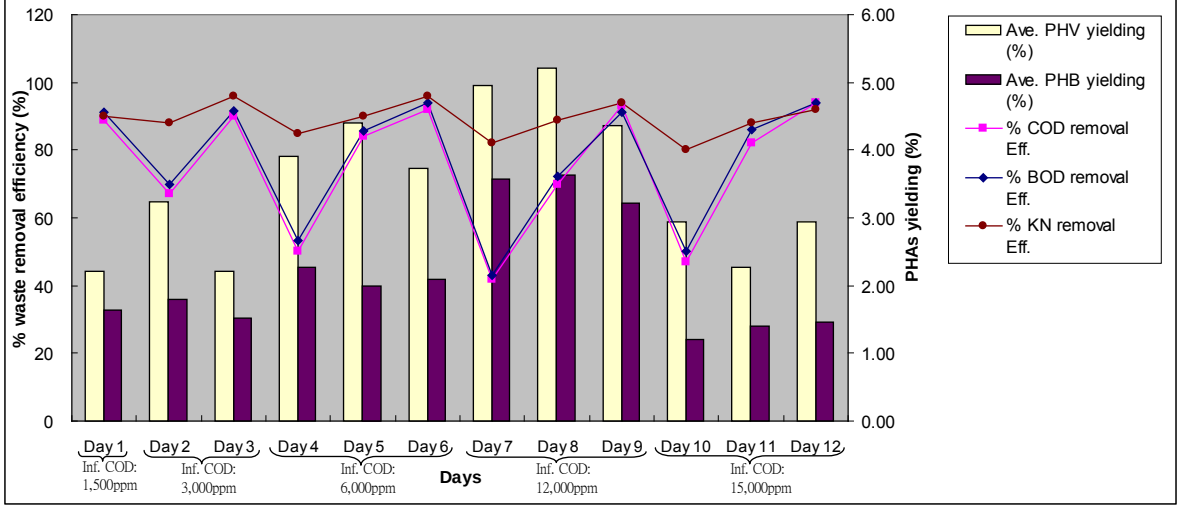
Identified *Sphingopyxis terrae* and *Aeromonas ichthiosmia* from Mixed Sludge were mixed into a Sequencing Batch Reactor for study.

Figure 7.12 shows that mixed strains of *Sphingopyxis terrae* and *Aeromonas ichthiosmia* had a better waste treatment efficiency than the mono culture (Figures 6.1 and 6.6) with an organic loading at 3,000ppm COD. It took two days to stabilize the system to achieve over 90% COD, BOD and KN removal by the mixed strains. With the increasing of influent COD concentrations, the treatment efficiency of the mixed strains were similar to mono culture (Figure 6.1 and 6.6). The mixed strains performed an increasing of PHA accumulation from 1.6% to 3.5% PHB and 2.2% to 4.8% PHV of cellular content over the COD influent range of 1,000ppm to 12,000ppm (Figure 7.12). When 15,000ppm COD influent was applied, the accumulation of both PHB and PHV was inhibited. About 1.4% PHB and 2.7% PHV of cellular content were found under that influent concentration. The mixed strains needed three days to

stabilize the system so as to have over 90% COD, BOD and KN removal at 15,000ppm influent COD.

Compared to the mono strain, the mixed strains had better waste treatment efficiency at 3,000ppm influent COD feeding. The same waste treatment performance was achieved for the influent COD range from 6,000ppm to 12,000ppm COD. At least 27% PHB and 6% PHV accumulation were inhibited by the mixed strains. In addition, after mixing the two strains, yield of PHV production was higher than that of PHB in the studies.

Figure 7.12: PHA production and waste removal efficiency by mixed strains: *Aeromonas ichthiosmia & Sphingopyxis terrae*



7.4.2 *Sphingopyxis terrae & Pseudomonas putida*

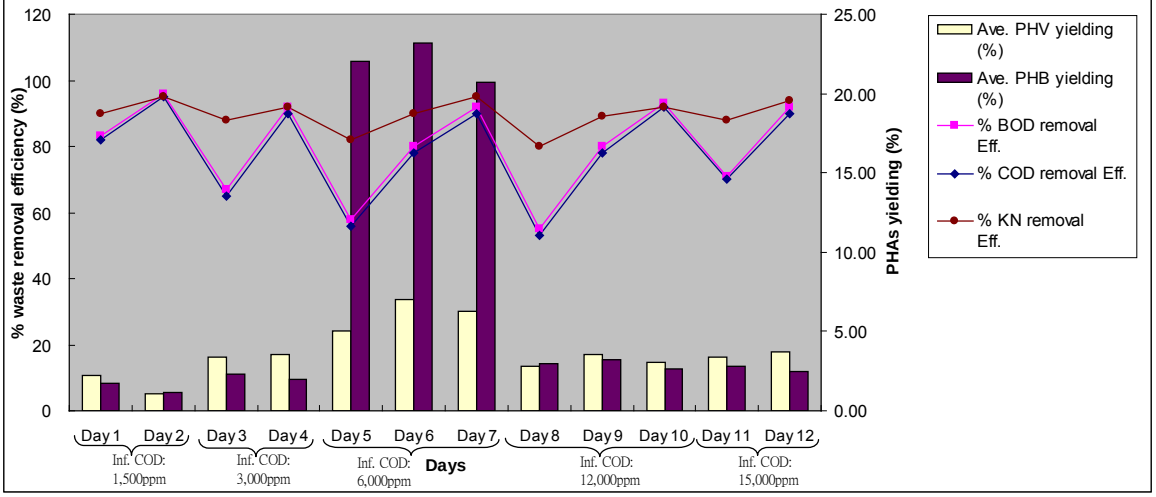
Identified *Sphingopyxis terrae* from Mixed Sludge and *Pseudomonas putida*, from Purify Bio-filter Sludge (Tai Po Water Treatment Works) were mixed into a Sequencing Batch Reactor for study.

Figure 7.13 shows that mixed strains of *Sphingopyxis terrae* and *Pseudomonas putida* had a waste treatment trend similar to that of the mono strain of *Pseudomonas putida* (Figure 6.4). It took two days to stabilize the system to achieve more than 90% COD, BOD and KN removal at 3,000ppm COD feeding. With increasing organic loading from 3,000ppm to 12,000ppm influent COD, three days were needed to achieve over 90% COD and BOD removal and two days were needed to achieve over 90% KN removal. Within the organic loading range of 1,000ppm to 3,000ppm, PHB accumulation increased from 1.4% to 2.1% of cellular content. PHV accumulation increased from 1.6% to 3.5%. There was a significant PHA accumulation increment when 6,000ppm influent COD applied. It was observed that PHB accumulation increased from 2.1% to 21.9% (ten fold increment) of cellular weight. Besides, PHV increased from 3.5% to 6.1% of cellular weight (Figure 7.13). A decrease of PHA

accumulation by the mixed strains occurred when the organic loading increased from 12,000ppm to 15,000ppm. The accumulation of both PHB and PHV was inhibited. About 2.6% PHB and 3.5% PHV of cellular content were obtained at 15,000ppm influent COD. On the other hand, the mixed strains performed better waste treatment with one day advance to achieve over 90% COD, BOD and KN at 15,000ppm than 12,000ppm influent COD.

Compared to PHA accumulation by mono strain, mixed strains enhanced at least 350% PHB and 35% PHV accumulation. Below 12,000ppm influent COD, the mixed strains had a similar waste treatment trend to that of the mono strain of *Pseudomonas putida* (Figure 6.4). There was an improvement in waste treatability with higher organic loading. One day advance of having over 90% COD, BOD and KN removal was achieved when 15,000ppm influent COD was applied.

Figure 7.13: PHA production and waste removal efficiency by mixed strains: *Pseudomonas putida & Sphingopyxis terrae*



7.4.3 *Sphingopyxis terrae* and *Pseudomonas huttiensis*

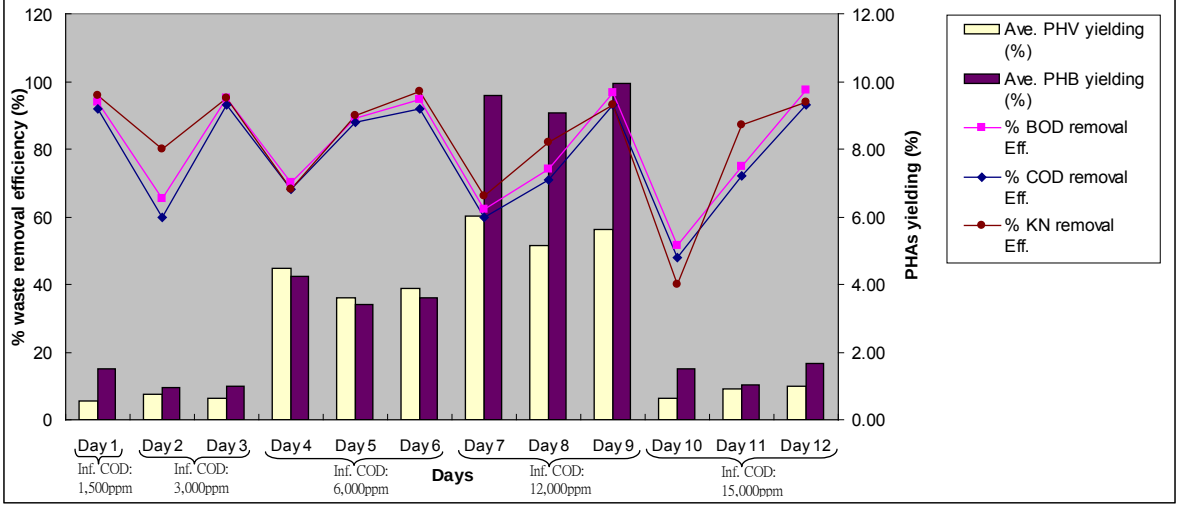
Identified *Sphingopyxis terrae* from Mixed Sludge and *Pseudomonas huttiensis*, from Food Waste sludge 1 (Garden Company) were mixed into a Sequencing Batch Reactor for study.

Figure 7.14 shows that the mixed strains of *Sphingopyxis terrae* and *Pseudomonas huttiensis* had a better waste treatment capability for the influent range from 6,500ppm to 12,000ppm COD than the mono strain of *Pseudomonas huttiensis* (Figure 6.5). Two days advanced were achieved for

having over 90% COD, BOD and KN removal over the influent range from 6,500ppm to 12,000ppm COD. About 1% PHB of cellular content was accumulated by the mixed strains when 1,500ppm or 3,000ppm COD influent was applied. There was about 0.7% PHV of cellular content accumulated in the same organic loading concentration. PHA accumulation increased continuously and a maximum of 9.5% PHB and 5.6% PHV of cellular content were obtained at 12,000ppm influent COD (Figure 7.14). The PHA accumulation capability of the mixed strains decreased when the concentration of the influent COD further increased to 15,000ppm. About 1.4% PHB and 0.9% PHV of cellular content can only be produced at 15,000ppm influent COD. PHA inhibitory to the mixed strains occurred at 15,000ppm influent concentration. The mixed strains needed 3 days to stabilize the system to achieve over 90% COD, BOD and KN removal when 15,000ppm influent COD was applied.

Compared to the PHA accumulation in mono strain studies (Figures 6.5 and 6.6), there was enhancement of at least 61% PHB and 21% PHV accumulation by the mixed strains.

Figure 7.14: PHA production and waste removal efficiency by mixed strains: *Pseudomonas huttiensis & Sphingopyxis terrae*



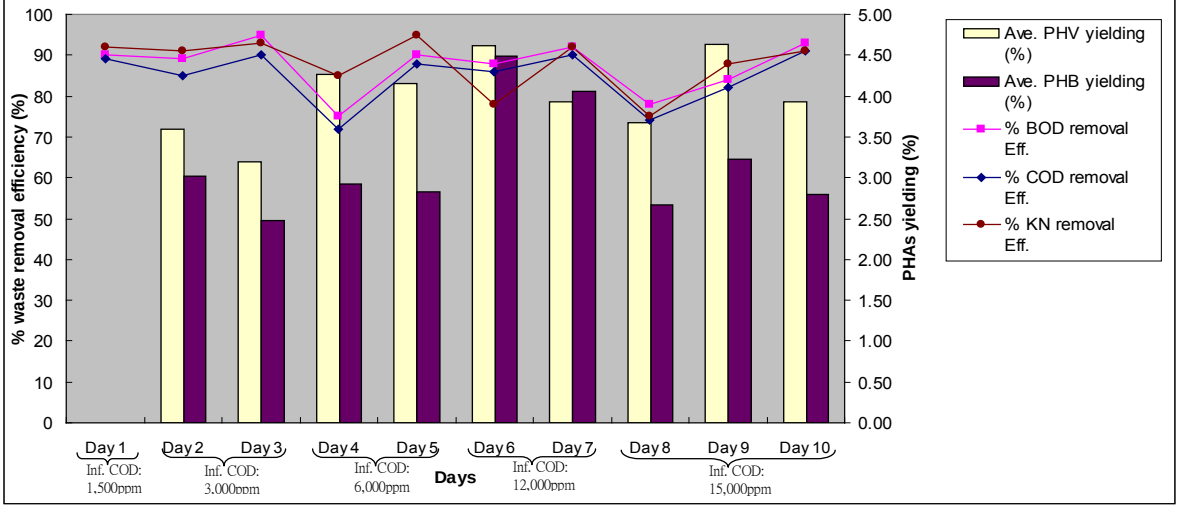
7.4.4 *Sphingopyxis terrae* and *Yersinia frederiksenii*

Identified *Sphingopyxis terrae* from Mixed Sludge and *Yersinia frederiksenii* from Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui) were mixed into the a Sequencing Batch Reactor for study.

Compared to the mono strain (Figures 6.6 and 6.7), the mixed strains of *Sphingopyxis terrae* and Yersinia *frederiksenii* shown in Figure 7.15 had an advantage in waste treatment efficiency. It took two days to stabilize the system to have over than 90% COD, BOD and KN removal over the organic

loading range from 3,000ppm to 12,000ppm COD. For the mono strain, three days were needed to stabilize the system within the same organic loading range. The Mixed strains started to accumulate PHAs at 3,000ppm influent COD with 2.8% PHB and 3.4% PHV of cellular content obtained. A maximum of 4.3% PHB and 4.3% PHV of cellular content was accumulated when 12,000ppm influent COD was applied (Figure 7.15). A decrease of PHA accumulation occurred when the organic loading increased to 15,000ppm COD. About 2.9% PHB and 4.1% PHV were obtained in that influent concentration. Besides, three days were needed to stabilize the system to achieve over 90% COD, BOD and KN removal. Compared to the mono strain in waste treatment efficiency and PHA accumulation, waste treatment efficiency by the mixed strains was enhanced. But at least 10% PHB and 4% PHV accumulation were inhibited by the mixed strains.

Figure 7.15: PHA production and waste removal efficiency by mixed strains: *Yersinia frederiksenii & Sphingopyxis terrae*



7.5 Mixing with *Pseudomonas putida*

7.5.1 *Pseudomonas putida* and *Aeromonas ichthiosmia*

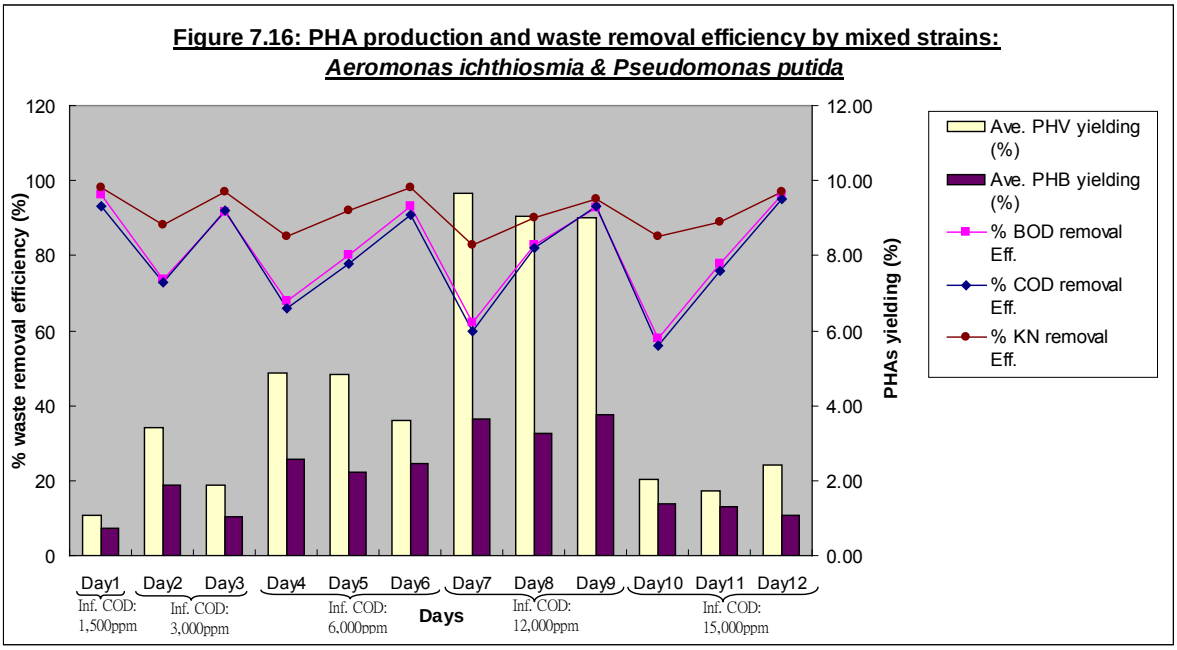
Identified *Pseudomonas putida* from Purify Bio-filter Sludge (Tai Po Water Treatment Works) and *Aeromonas ichthiosmia* from Mixed Sludge were mixed into a Batch Reactor for study.

Figure 7.16 shows that the mixed strains of *Pseudomonas putida* and *Aeromonas ichthiosmia* had a waste treatment trend similar to that of the mono strain to have over 90% COD, BOD and KN removal over the organic loading range from 1,500ppm to 12,000ppm. The mixed strains needed different time to stabilize the system when different concentrations of influent COD applied, one day for 1,500ppm COD, two days for 3,000ppm COD, three days for 6,000ppm and 12,000ppm, to have over than 90% pollutant removal. 0.7% PHB and 1% PHV of cellular content were accumulated at 1,500ppm influent COD. The PHAs accumulation increased with increasing the concentration of influent COD. A maximum of 3.6% PHB and 9.2% PHV of cellular content obtained when influent 12,000ppm COD was applied (Figure 7.16). The

organic loading of 15,000ppm COD exerted an inhibition effect on PHA accumulation to the mixed strains. Only about 1.3% PHB and 2.1% PHV of cellular content could be obtained at 15,000ppm influent COD.

Compare to the results obtained in mono strain studies (Figure 6.1 and 6.4), the mixed rains had waste treatment capabilities similar to that of mono strain of *Pseudomonas putida* (Figure 6.4). PHB accumulation by the mixed strains was inhibited by at least 21% of cellular content. PHV accumulation by the mixed strains was stimulated and enhanced by at least 10% of cellular content. The high organic loading at 15,000ppm inhibited the PHA accumulation. In addition, the mixed strains favored PHV accumulation during the experiments.

Figure 7.16: PHA production and waste removal efficiency by mixed strains: *Aeromonas ichthiosmia & Pseudomonas putida*



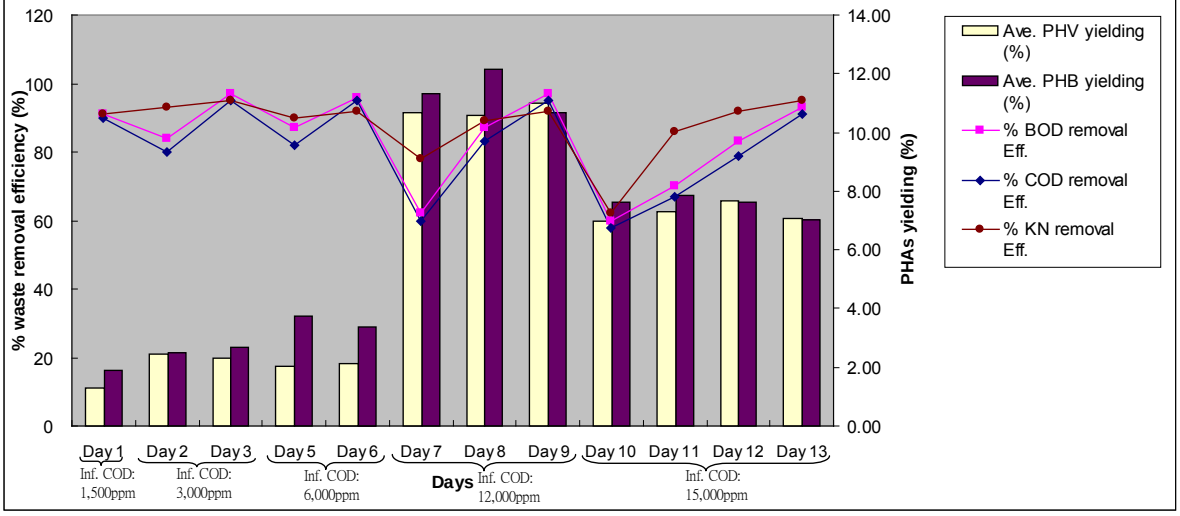
7.5.2 *Pseudomonas putida & Pseudomonas huttiensis*

Identified *Pseudomonas putida* from Purify Bio-filter Sludge (Tai Po Water Treatment Works) and *Pseudomonas huttiensis* from Food Waste sludge 1 (Garden Company) were mixed into a Sequencing Batch Reactor for study.

Figure 7.17 shows that two days were needed for the mixed strains of *Pseudomonas putida* and *Pseudomonas huttiensis* to stabilize the system to achieve over 90% COD as well as BOD removal at the COD influent range from 3,000ppm to 6,000ppm. The mixed strains achieved over 90% KN removal by one day at the influent COD range from 1,500ppm to 6,000ppm. Three days for stabilization were needed to achieve over 90% COD and BOD removal when 12,000ppm influent COD was applied. PHA accumulation by the mixed strains continuously increased to the maximum yield at 12,000ppm COD. A maximum of 11.4% PHB and 10.8% PHV of cellular content was produced when 12,000ppm influent COD was applied. A higher organic loading at 15,000ppm exerted an inhibitory effect on PHA accumulation (Figure 7.17). PHB and PHV accumulation decreased to 7.5% and 7.3% of cellular content, respectively.

The mixed strains had a better waste treatment efficiency with one day advance at the organic loading below 6,000ppm than the mono strain. The results showed there were enhancement effects on PHA accumulation by the mixed strains. At least 93% PHB and 134% PHV of cellular content accumulation enhanced by the mixed strains.

Figure 7.17: PHA production and waste removal efficiency by mixed strains: *Pseudomonas huttiensis & Pseudomonas putida*



7.5.3 *Pseudomonas putida & Yersinia frederiksenii*

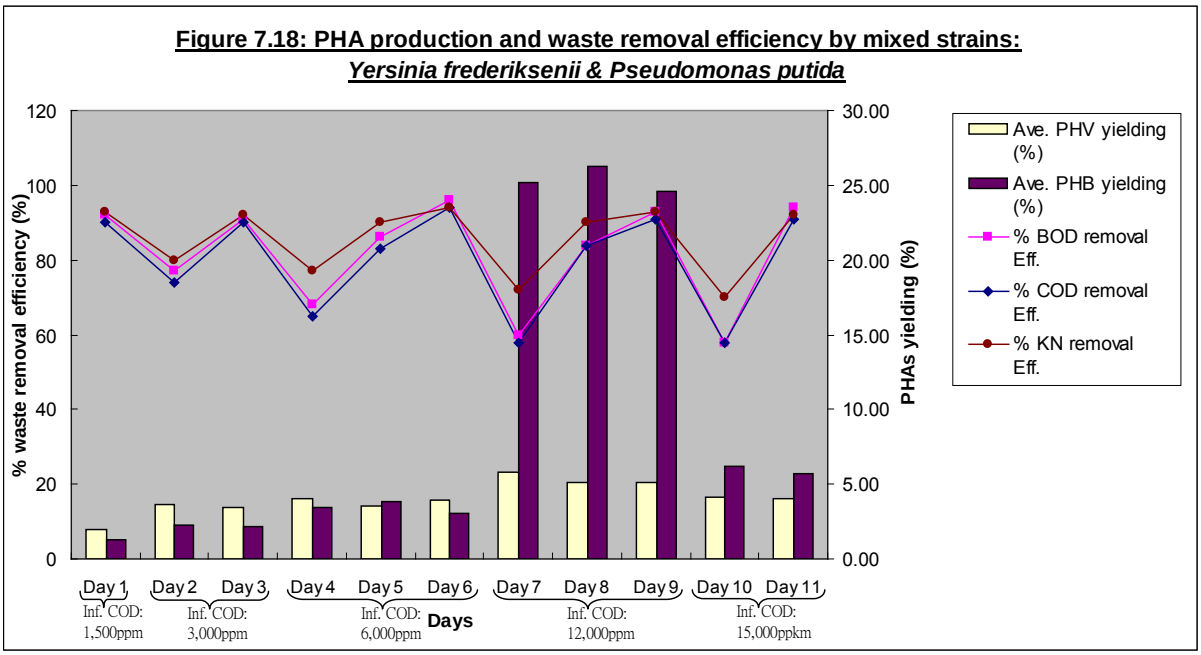
Identified *Pseudomonas putida* from Purify Bio-filter Sludge (Tai Po Water Treatment Works) and *Yersinia frederiksenii* from Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui) were mixed into a Sequencing Batch Reactor for study.

Figure 7.18 indicates that the mixed strains of *Pseudomonas putida* and *Yersinia frederiksenii* had a waste treatment trend similar to that of the mono strain of *Pseudomonas putida* (Figure 6.4) at the organic loading range from 1,500ppm to 12,000ppm. The mixed strain needed different stabilization time to stabilize the system when different concentrations of influent COD were applied, one day for 1,500ppm, two days for 3,000ppm, three days for 6,000ppm and 12,000ppm influent COD, to have over 90% COD and BOD removal efficiency. Two days were needed for the mixed strains to stabilize the system to achieve over 90% KN removal at 3,000ppm to 12,000ppm influent COD. When the organic loading as high as 12,000ppm COD, PHA accumulation by the mixed strains was stimulated significantly. A Maximum of 25.3% PHB and 5.4% PHV of cellular content was obtained (Figure 7.18). At

15,000ppm influent COD, the PHA accumulation capability of the mixed culture was inhibited. Only about 5.9% PHB (76% decreased) and 4.1% PHV (24% decreased) of cellular content was produced.

The mixed strains had a waste treatment efficiency trend similar to that of *Pseudomonas putida* (Figure 6.4) at the range of 1,500ppm to 12,000ppm influent COD. The mixed strains stimulated PHA accumulation by at least 350% PHB and 68% PHV of cellular content were enhanced.

Figure 7.18: PHA production and waste removal efficiency by mixed strains: *Yersinia frederiksenii & Pseudomonas putida*



7.6 Mixing with Pseudomonas huttiensis

7.6.1 Pseudomonas *huttiensis* and *Aeromonas ichthiosmia*

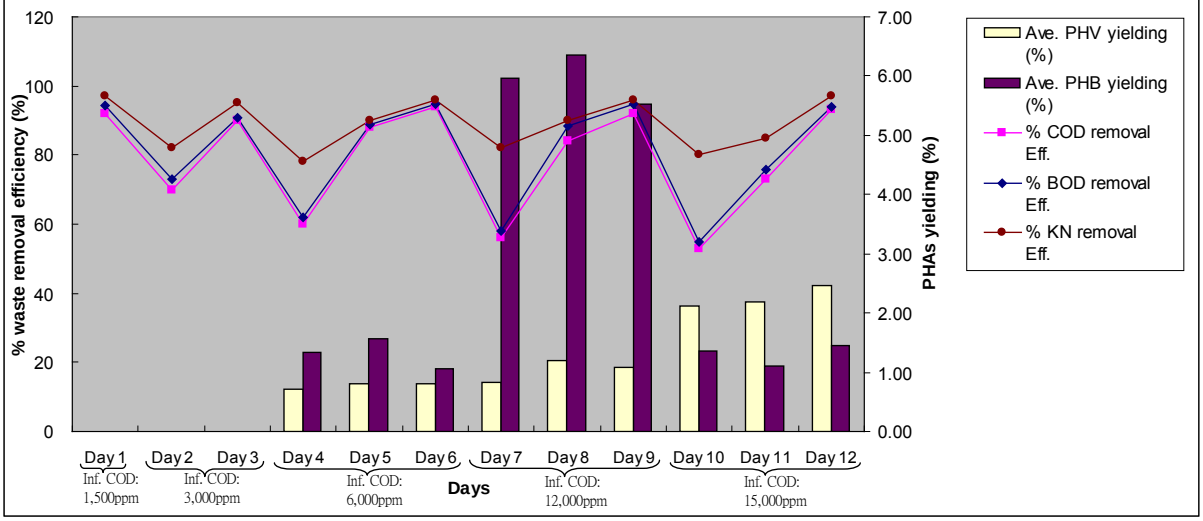
Identified *Pseudomonas huttiensis* from Food Waste sludge 1(Garden Company) and *Aeromonas ichthiosmia* from Mixed Sludge were mixed into a Sequencing Batch Reactor for study.

Figure 7.19 indicates that the mixed strains of *Pseudomonas huttiensis* and *Aeromonas ichthiosmia* need two days to stabilize the system to have over 90% COD, BOD and KN removal at 3,000ppm influent COD. A stabilization time of three days was needed at 6,000ppm and 12,000ppm influent COD for the mixed strains to achieve the same waste removal efficiency as at 3,000ppm influent COD. Neither PHB nor PHV was synthesized at 1,500ppm and 3,000ppm influent COD by the mixed strains. About 1.3% PHB and 0.8% PHV of cellular content were accumulated at 6,000ppm influent COD influent (Figure 7.19). A maximum of 6.0% PHB of cellular content was accumulated by the mixed strains when 12,000ppm influent COD was applied. PHB accumulation decreased to about 1.3% of cellular content when 15,000ppm influent COD was

applied. 2.2% PHV was accumulated at this influent concentration.

Compared to the results obtained by mono strain (Figure 6.1 and 6.5), waste treatment capacity of the mixed strains was not stimulated. PHA accumulation was inhibited by the mixed culture. At least 73% PHB and 50% PHV of cellular content accumulation were inhibited.

Figure 7.19: PHA production and waste removal efficiency by mixed strains: *Aeromonas ichthiosmia & Pseudomonas huttiensis*



7.6.2 *Pseudomonas huttiensis* and *Yersinia frederiksenii*

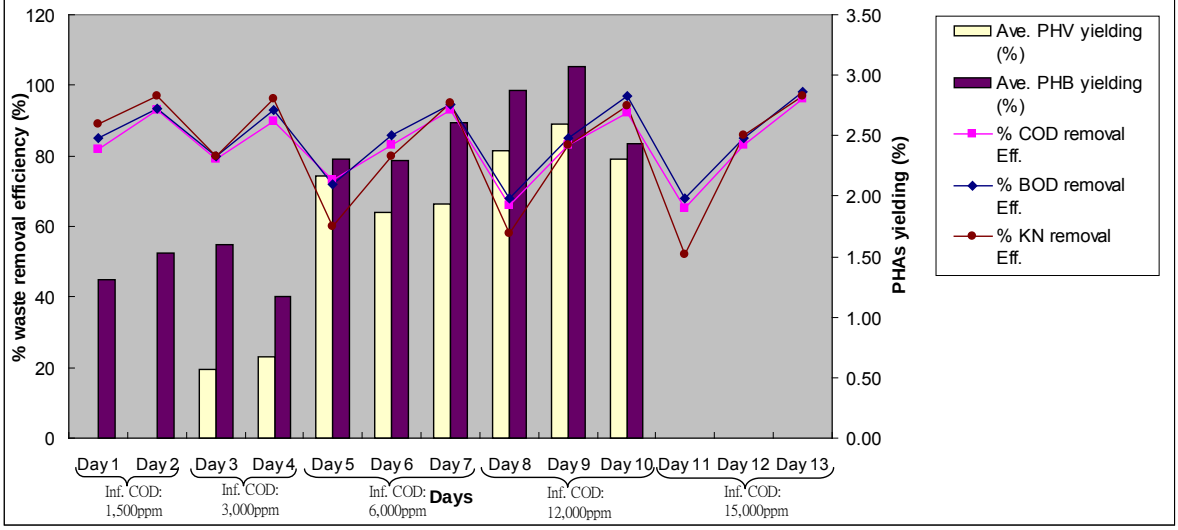
Identified *Pseudomonas huttiensis* from Food Waste sludge 1(Garden Company) and *Yersinia frederiksenii* from Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui) were mixed into a Sequencing Batch Reactor for study.

Figure 7.20 shows that the mixed strains of *Pseudomonas huttiensis* and *Yersinia frederiksenii* took three days to stabilize the system to achieve over 90% COD, BOD and KN removal at the organic loading of 6,000ppm and 12,000ppm COD. Two days were needed for the mixed strains to stabilize the system to achieve the same waste treatment efficiency at the organic loading below 3,000ppm. 1,500ppm influent COD influent was unable to initiate accumulation of PHV by the mixed strains. Only 1.4% PHB was accumulated when 1,500ppm influent COD was applied. PHA accumulation increased continuously with increasing organic loading. About 0.6% PHV of cellular content was produced at 3,000ppm influent COD (Figure 7.20). A maximum of 2.8% PHB and 2.4% PHV of cellular content were accumulated at 12,000ppm influent COD. PHA accumulation by the mixed strains was

stopped when 15,000ppm influent COD was applied.

Compared to the mono strain (Figures 6.5 and 6.7), there were no significant changes in the waste treatment efficiency of the mixed strains. The results showed that at least 49% PHB accumulation was inhibited and no changes in PHV accumulation performance was found in the mixed strains.

Figure 7.20: PHA production and waste removal efficiency by mixed strains: *Yersina frederiksenii & Pseudomon huttiensis*



7.7 Mixing with *Yersinia frederiksenii*

7.7.1 *Yersinia frederiksenii* and *Aeromonas ichthiosmia*

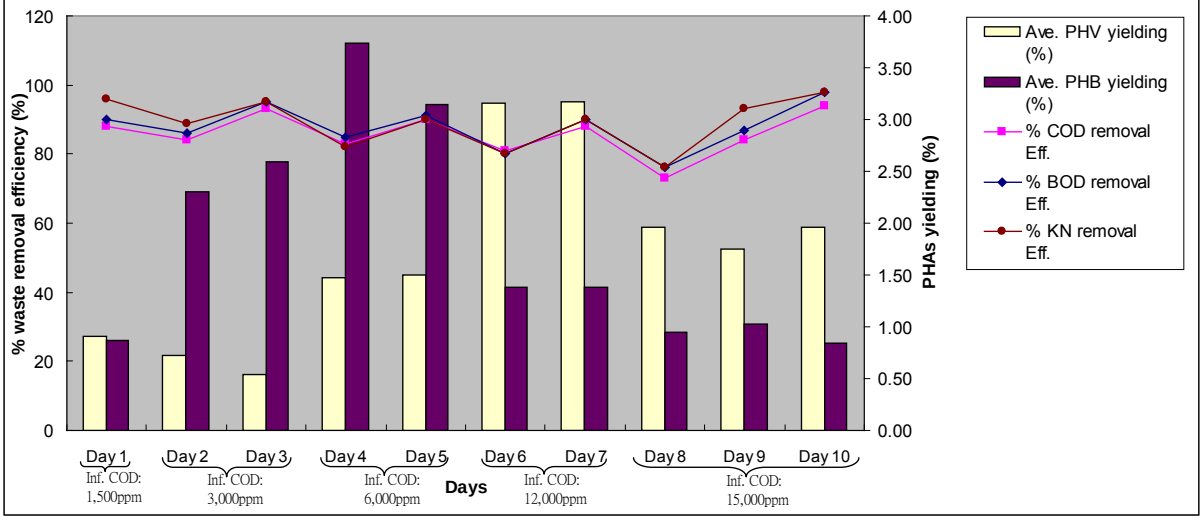
Identified *Yersinia frederiksenii* from Piggery Sludge (Piggery Farm treatment plant – Kwu Tung, Lok Ma Chau, Sheung Shui) and *Aeromonas ichthiosmia* from Mixed Sludge were mixed into a Sequencing Batch Reactor for study.

Figure 7.21 shows that the mixed strains of *Yersinia frederiksenii* and *Aeromonas ichthiosmia* had steady waste treatment efficiency. It took two days to stabilize the system to achieve over 90% COD, BOD and KN removal at the organic loading range of 3,000ppm to 12,000ppm COD. The mixed strains started to accumulate PHAs at 1,500ppm influent COD. A maximum accumulation of about 3.4% PHB of cellular content was obtained at 6,000ppm influent COD. When higher than 6,000ppm influent COD was applied, PHB accumulation was inhibited. The PHB accumulation decreased to about 0.9% at 15,000ppm influent COD. PHV accumulation continuously increased to up to 3.1% of cellular content at 12,000ppm influent COD. Similar to PHB accumulation, accumulation of PHV was also inhibited when 15,000ppm

influent COD was applied. About 1.9% PHV of cellular content could only be produced at that COD influent level.

Compared to the results obtained by mono strain (Figure 6.1 and 6.7), the waste treatment efficiency by mixed strains was enhanced with one day advance than the mono strain. On the other hand, at least 38% PHB accumulation was inhibited by the mixed strains. There was about 40% of PHV accumulation enhanced by mixed strains.

Figure 7.21: PHA production and waste removal efficiency by mixed strains: *Aeromonas ichthiosmia & Yersinia frederiksenii*



7.8 Discussions

PHAs can be synthesized by identified bacteria and their yields can be controlled by varying the influent COD concentration levels. From this section of experiments, mixed strains also had different PHA accumulation performance corresponding to the influent COD concentration levels. An Inhibitory and synergistic effect on PHA accumulation and waste treatment capability was achieved by different mixed strain combinations.

Most of the mixed strains did not affect much on the waste treatment capabilities. They had the same waste treatment capabilities as the mono strain did. Only the mixed strains of *Pseudomonas huttiensis* & *Bacillus pumilus* and *Pseudomonas huttiensis* & *Sphingopyxis terrae* achieved a significant waste treatment enhancement. The System stabilization time of the mixed strains has two days advancement than the mono strain. For PHAs accumulation enhancement, the mixing of PHA biomasses would create a synergy effects among the biomasses used. The mixing strains of *Bacillus pumilus* & *Aeromonas ichthiosmia* and *Sphingopyxis terrae* & *Bacillus cereus* resulted in more than 20% PHAs accumulation enhancements. More than 50% PHA accumulation enhancement was found when *Bacillus pumilus* was

mixed with *Sphingopyxis terrae* (more than 58% enhancement) or *Bacillus cereus* (more than 130% PHV production enhancement). Mixing strains of *Bacillus cereus* with *Sphingopyxis terrae* would have produce over 70% PHB and over 120% PHV production enhancement. In addition, mixing strains of *Bacillus cereus* with *Yersinia frederiksenii* resulted in over 90% PHB and PHV production enhancement. Mixing *Pseudomonas putida* with *Yersinia frederiksenii* or *Pseudomonas huttiensis* resulted in the highest PHB and PHV accumulation enhancement, respectively. There were more than 350% PHB accumulation enhancement with *Yersinia frederiksenii*. More than 130% PHV production was enhanced by *Pseudomonas huttiensis*.

PHA production inhibition was also found in all combinations of mixed strains. Most of the isolated bacteria performed the maximum PHA yields at 12,000ppm influent COD. A decrease in PHA accumulation occurred when 15,000ppm influent COD was applied as the carbon source. Compared to the mono strain, mixed strains could function waste treatment and PHAs accumulation under a higher organic loading environment without any performance inhibition. The high nitrogen content also exerted an unfavorable condition to the mixed strains in PHA production.

CHAPTER 8

CONCLUSIONS

8.1 Microbial community studies and PHA biomass screening from Activated Sludge

Microbial screening of nine activated sludge of different Hong Kong industries was conducted by using NA, PCA and R2A agar. The results suggested that R2A acted as the favourable medium for the recovery of potential PHA biomasses from the activated sludges. R2A consists of soluble starch and sodium pyruvate. Soluble starch aids the recovery of injured organisms by absorbing toxic metabolic by-products and sodium pyruvate increases the recovery of stressed cells.

There were 22 potential PHA biomasses presumed by FTIR and then further

confirmed by GC-FID as PHA biomasses. Among the six different carbon sources used in the PHA production studies, methanol and sodium octanoate showed toxicity to most of the isolated biomasses. Glucose was shown as the most favourable carbon source for PHA accumulation by the isolated PHA biomasses. Biomasses of PBS_6, PBS_7, FWS3_2 and MS_2 achieved over 30% PHB accumulation. The highest PHV accumulation was achieved by the biomass of PS which accumulated 9.9% PHV of intracellular content.

Among the 22 isolated PHA biomasses, 8 typical biomasses with high PHA yield were identified by Sherlock Microbiological Identification System (MIS) in using Gas Chromatographic analysis of Fatty Acid Methyl Esters (GC-FAME) by GC-FID. The eight biomasses, DS1_1, DS2_1, PS_2, PBS_4, FWS1_1, FWS1_2, MS_2 and MS_4 were identified as *Bacillus pumilus*, *Yersinia frederiksenii*, *Pseudomonas putida*, *Pseudomonas huttiensis*, *Bacillus cereus* and *Sphingopyxis terrae* and *Aeromonas ichthiosmia*.

8.2 Wastewater treatment and PHA synthesis: Mono strain

The results showed that the real wastewater from Garden Company supported the growth of isolated PHA bacteria and PHA synthesis. The real waste water could act as a culture medium for PHA synthesis. The identified bacteria could also remove the pollutants of COD, BOD and KN in the wastewater.

When considering the pollutant removal capabilities, *Aeromonas ichthiosmia*, *Bacillus cereus*, *Pseudomonas huttiensis* and *Yersinia frederiksenii* had better BOD and COD removal than *Bacillus pumilus*, *Pseudomonas putida* or *Sphingopyxis terrae* at the influent concentrations ranging from 1,000ppm to 2,000ppm COD. *Pseudomonas huttiensis* and *Yersinia frederiksenii* achieve over 90% COD and BOD removal within 1 day. All of the isolated bacteria performed over 90% KN removal within one day in the influent concentrations ranging from 1,000ppm to 3,500ppm COD. Increasing the influent COD concentrations would increase the system stabilization time of bacteria to achieve over 90% COD, BOD and KN removal. When 12,000ppm influent COD was applied, three days were needed by the bacteria to have over 90%

COD, BOD and KN removal.

The results of the bacteria biosynthesis showed that *Aeromonas ichthiosmia* achieved the highest PHA accumulation. A maximum of 22% PHB and 8% PHV of intracellular content were accumulated by *Aeromonas ichthiosmia* when 6,500ppm influent COD was applied. Increasing the influent COD concentration would increase the amount of PHA accumulation by bacteria. PHA accumulation inhibition was found in most bacteria when 12,000ppm influent COD was applied. The inhibition was caused by the high KN concentration in the real influent waste. The high nitrogen content resulted in a high concentration of free CoASH. The CoASH inhibited the β-ketothiolase condensation reaction. This inhibition led to a decrease in PHA accumulation.

8.3 Wastewater treatment and PHA synthesis: Mixed Strains

Varying of the influent COD concentrations would affect the PHA accumulation by mixed strains and the pollutant removal efficiency. Mixed strains of *Pseudomonas huttiensis* & *Bacillus pumilus and Pseudomonas huttiensis* & *Sphingopyxis terrae* achieved a significant waste treatment enhancement. They achieved 2 days advancement than the mono strain in having over 90% COD, BOD and KN removal.

Most of the isolated bacteria produced the maximum PHA yields at 12,000ppm influent COD. PHA production could be enhanced by mixing the strains of *Bacillus cereus* with *Sphingopyxis terrae.* This combination would have performed over 70% PHB and over 120% PHV production enhancement. In addition, the mixing strains of *Bacillus cereus* with *Yersinia frederiksenii* resulted in over 90% PHB and PHV production enhancement.

Mixing *Pseudomonas putida* with *Yersinia frederiksenii* or *Pseudomonas huttiensis* resulted in the highest PHB and PHV accumulation enhancement,

respectively. There was more than 350% PHB accumulation enhancement with *Yersinia frederiksenii*. More than 130% PHV production was enhanced by the mixed strains of Pseudomonas putida & *Pseudomonas huttiensis*.

PHA production inhibition was also found in all combinations of mixed strains. A decrease in PHA accumulation occurred when 15,000ppm influent COD was applied. Compared to the mono strain, the mixed strains could perform waste treatment and PHA accumulation under a higher organic loading environment without any performance inhibition. The high nitrogen content at 15,000ppm influent COD also exerted an unfavorable condition to the mixed strains in PHA production.

8.4 Overall Achievement

In the study, potential PHA biomass has been successfully isolated from different activated sludges of Hong Kong Industries. The confirmed PHA biomasses were identified as *Bacillus pumilus*, *Yersinia frederiksenii*, *Pseudomonas putida*, *Pseudomonas huttiensis*, *Bacillus cereus* and *Sphingopyxis terrae* and *Aeromonas ichthiosmia*. The shaking experiment showed that glucose was the widely adopted carbon sources for the identified bacteria in synthesizing PHA. *Sphingopyxis terrae* achieved the 32% PHB accumulation, the highest PHB yield produced among other identified bacteria in shaking experiments. When considering a low cost PHA production, wastewater treatment process is a potential inexpensive method. The results showed that the real wastewater from Garden Company supported the growth of isolated PHA bacteria and PHA synthesis. The real wastewater could act as a culture medium for PHA synthesis. The identified bacteria could also remove the pollutants of COD, BOD and KN in the wastewater. The mono strain results demonstrated that the identified PHA bacteria could accumulate PHA with maximum yield of about 22% PHB and 10% PHV in a SBR system. The results were satisfactory that the maximum COD, BOD and KN reduction

of the wastewater reached over 90% at influent COD concentration level of 15,000ppm. There was synergistic effect in PHA accumulation by mixed strains experiments. Up to about 300% PHB increment was showed by mixing *Pseudomonas putida* with *Yersinia frederiksenii* when compared to the PHA accumulation by mono strain studies. In addition, mixed strains strategy achieved a significant waste treatment enhancement with 2 days advancement than the mono strain in having over 90% COD, BOD and KN removal. The high nutrient inhibition effect to PHA accumulation also been improved by mixed strains from inhibition level at 12,000ppm influent concentration to 15,000ppm influent concentration. From the results known that the role of the identified bacteria that proliferate in response to pilot scale of SBR together with an understanding of the mechanism of PHA accumulation and waste removal, high yields of PHA from activated sludge are moved closer to realization. These PHA will provide a potentially inexpensive source of biodegradable plastics and, at the same time, improve wastewater treatment efficiency and reduce the quantity of excess sludge requiring treatment. Besides, the employed strains in this PHA research have been isolated from activated sludge, the knowledge of PHA accumulation by these organisms in their native will be helpful for engineering future large scale commercial production facilities.

In addition, the production of PHA from activated sludge not only solving (space problem of disposing sludge waste) imminent problem of the 2,000 tons/day solid waste in Hong Kong but also can produce the environmental friendly bioplastics for environmental protections.

8.5 Recommendations

8.5.1 Elimination of PHA accumulation inhibition

Although increasing of influence COD concentrations would increase the PHA accumulation, inhibition of PHA accumulation occurred when high nutrient content was applied. Further investigation for optimization of PHA produced in SBR system by optimizing the C:N ratio of influence would be important.

8.5.2 Continuously Mode of SBR system

In this study, the PHA is produced by bacteria under conditions of fed-batch mode of SBR, it is interesting to investigate the PHA accumulation under a condition of continuous feeding mode of SBR system. By investigating the

best hydraulic retention time for continuously mode, optimization of wastewater treatment and PHA accumulation would be more cost-effective.

8.5.3 Recombinant technology

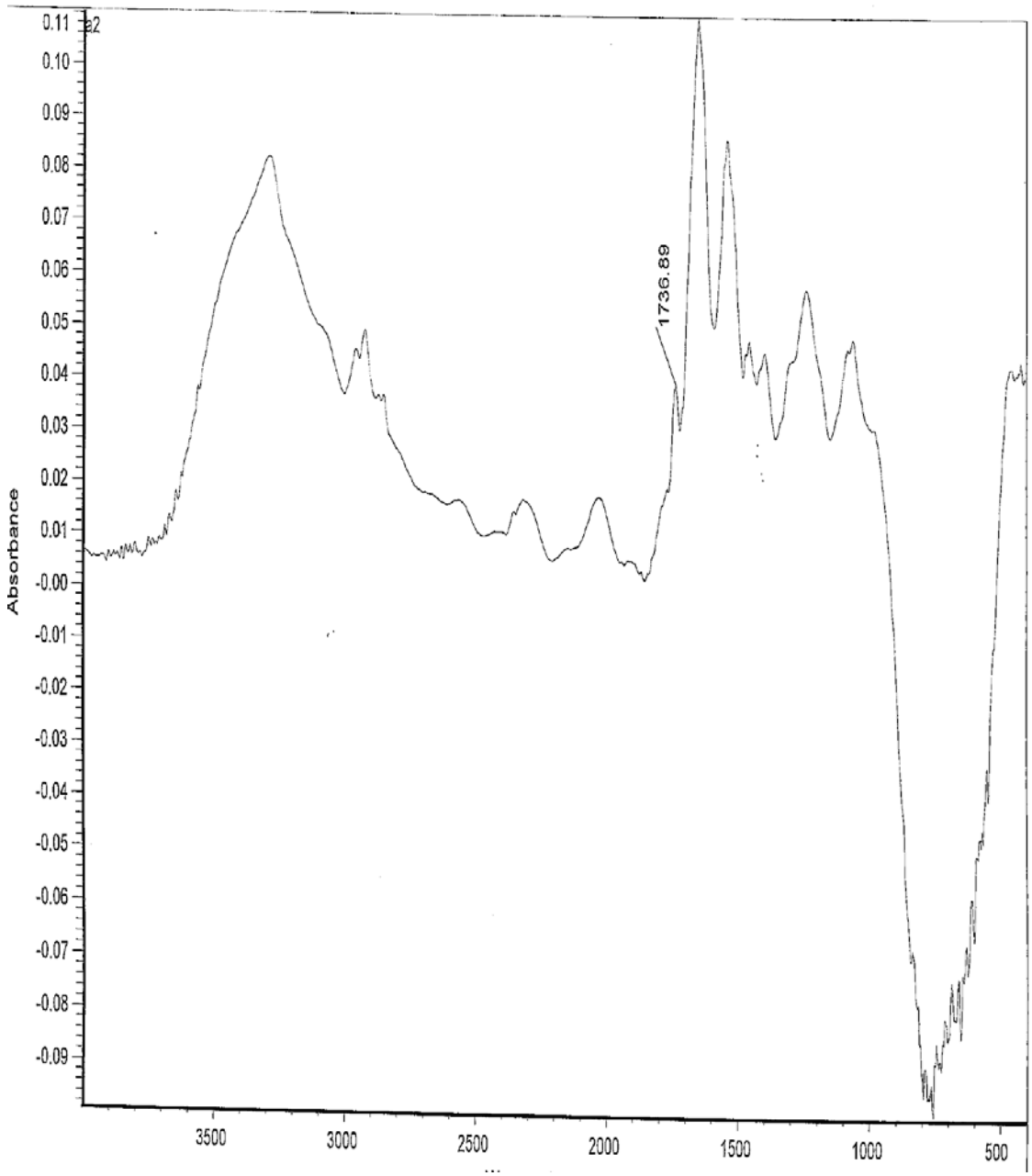
Although recombinant gene expression for PHA accumulation is not part of this research, it is recommend that detailed information of recombinant technology to involve in the pilot SBR system for enhancing waste treatment efficiency and PHA accumulation. As high as 85% PHB could be accumulated by recombinant gene (Lee, 1994). Optimization of the PHA production yield and enhance wastewater treatment capacities will be necessary in further study.

8.5.4 Further Enhancement of PHA production

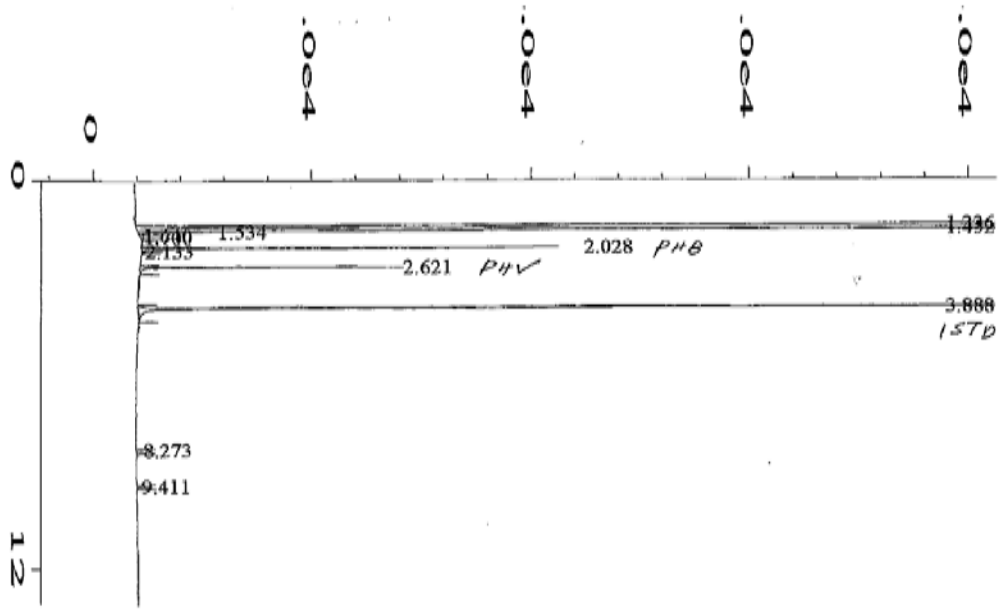
Although a synergy effect has demonstrated by mixed strains experiments, it is recommended to have a through study about the chemical matrix of the waste feeding and sludge composition including nutrient contents of nitrogen (organic and inorganic), phosphorus, ammonia and trace elements. By understanding such original compositions, further change of nutrient ratio may change the condition for PHA accumulation. A optimum conditions should be further studied.

APPENDIX

Appendix 1: FTIR spectrum of potential PHA biomass



Appendix 2: Chromatograph of standard PHB and PHV



Area Percent Report

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Operator : LamWai
Instrument : ANALYZER1
Sample Name :
Run Time Bar Code:
Acquired on : 09 Sep 07 02:37 PM
Report Created on: 09 Sep 07 02:50 PM

Page Number : 1
Vial Number : 1
Injection Number :
Sequence Line :
Instrument Method: LW_MCLM.MTH
Analysis Method : LW_MCLM.MTH

Sig. 1 in C:\HPCHEM\1\DATA\LAMWAI\TEST.D

Pk#	Ret Time	Area	Height	Type	Width	Area %
1	1.336	348657	524605	BV	0.016	12.7623
2	1.452	2200904	1351139	VV	0.026	80.5621
3	1.534	5418	3645	VV	0.022	0.1983
4	1.640	470	157	VV	0.050	0.0172
5	1.700	453	160	VV	0.040	0.0166
6	2.028	31843	20295	BV	0.026	1.1656
7	2.133	381	183	VV	0.029	0.0139
8	2.621	18601	12162	BB	0.024	0.6809
9	3.888	124042	86124	BB	0.022	4.5405
10	8.273	744	338	BB	0.034	0.0272
11	9.411	422	221	BB	0.030	0.0154

Total area = 2731934

Appendix 3: Activated sludge (DS1) sampling site of Tai Po Sewage Treatment Works, Tai Po, N.T.

Appendix 4: Activated sludge (PS) sampling site of Piggery farm treatment plant, Kwu Tung, Lok Ma Chau, Sheung Shui, N.T.

Appendix 5: Activated sludge (OS) sampling site of Grease Trap, Kwai Chung Container Port, Kwai Chung

Appendix 6: Activated sludge (FWS1) sampling site of Garden Company, Sham Tseng, N.T.

Appendix 7: Activated sludge (FWS2) sampling site of Vitasoy Treatment Plant, Tuen Mun, N.T.

Appendix 8: Activated sludge (FWS3) sampling site of Chinese Restaurant, Shek Kip Mei, Kowloon.

Appendix 9: Activated sludge (MS) sampling site of Mixed Sludge (FWS1 & FWS2).

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