THE HONG KONG POLYTECHNIC UNIVERSITY

Department of Civil and Structural Engineering

SYNTHESIS OF POLYHYDROXYALKANOATES (PHA) FROM EXCESS ACTIVATED SLUDGE

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A thesis submitted in partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

June 2004

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own works and that, to the best of my knowledge and belief, it reproduces no material previously published or written nor material which has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Hu Wenfeng

ABSTRACT

This study focused on the polyhydroxyalkanoates (PHA) synthesis from excess activated sludge (EAS), collected from a laboratory-scale sequencing batch reactor or a full-scale municipal wastewater treatment plant (MWWTP), via monitoring and precisely controlling the dissolved oxygen (DO) of the cultural media through an oxidation-reduction potential (ORP) setup instead of conventional DO system from different carbon sources.

No matter what types of EAS was submitted to PHA synthesis, by using fatty acids of shorter chain length including acetate and propionate as carbon sources, the total organic carbon (TOC) removal efficiency was higher than those of longer chain length. When C-even numbered fatty acids such as acetate and butyrate was used as sole carbon source respectively, PHB homopolymer or PHA with dominant 3HB unit was synthesized. Otherwise, PHBV copolymer with 3HV major units was synthesized instead of the PHB homopolymer when C-odd numbered fatty acids, propionate and valerate, were used as sole carbon sources. Increase the concentration of C-odd numbered fatty acids in the cultural medium, may result in the decrease of PHA content in EAS.

The melting temperatures of the PHBV copolymer produced by EAS decreased with the increasing of 3HV molar fraction in the PHBV.

When acetate was used as carbon substrate, the minimum PHA content at ORP -30mV was about 12% (w/w) of cell dry weight (CDW). The maximum

PHA content of 35% (w/w) was achieved when ORP was +30mV. By using propionate as carbon substrate and ORP was +30mV, the PHBV copolymer content, polymer production yield and 3HV molar fraction were 25.8%, 0.38 (g/g) and 78.0 mole%, respectively. Decreasing of ORP from +100mV to -30 mV by adjusting the air flow rate pumped into the cultural broth, equivalent to the DO concentration, resulted in the variation of 3HV mole fraction in the PHBV from 0 mole % to 21 mol% while acetate as carbon source. In other words, these variations were resulted from the changes of DO concentration.

When EAS from full-scale MWWTP was conducted for the PHA production from glucose, the polymer content in percentage of CDW increased with the increasing of ORP levels. The 3HV unit content in the PHBV increased from 11.1 mol% to 78.4 mol% with the decreasing of ORP from +10mV to -20mV by lowering the air flow rate. These results were in accordance with that of acetate and propionate as carbon sources. It was believed that monitoring and controlling the fermentation process by ORP instead of DO provided more sensitive, reliable and precise controll. Slight variation of DO concentration in cultural broth could be observed and realized by ORP system.

A hypothesized biochemical metabolic model by using glucose as sole carbon source was estabolished. It is postulated that TCA cycle and glyoxylate pathway are involved in the PHA accmulation even under anoxic and anaerobic condtions. The most important view in this hypothetic model is that the oxygen is the key exogenous factor to regulate 3HV mole fraction in PHBV copolymer.

These observations and the hypothesized model in this study may provide experimental evidence to that TCA cycle and glyoxylate pathway might be involved in the PHA production and phosphorus removal in the Enhanced Biological Phosphorus Removal process.

PUBLICATIONS ARISING FROM THE THESIS

1. Hu W.F., Chua H., Yu P.H.F. and Low H. (2001). Effect of oxidation reduction potential (ORP) on the accumulation of poly-hydroxyalkanoate from excess activated sludge. *Proceeding for IWA the International Conference in Singapore.*
2. Hu W.F., Sin S.N., Chua H., Chan S.Y., Yu P.H.F. and Lo W. (2004). Synthesis of poly-hydroxyalkanoates from activated sludge under various oxidation-reduction potential values by using glucose as sole carbon source. *Proceeding for 26th Symposium on Biotechnology for Fuels and Chemicals*, B-21. Chattanooga Choo Choo Chattanooga, Tennessee, U.S. (Accepted by professional journal).
3. Hu W.F., Sin S.N., Chua H., Chan S.Y., Yu P.H.F. and Lo W. (2004). Synthesis of poly-hydroxyalkanoate from activated sludge by using short chain fatty acids as carbon sources. (*in press*)
4. Hu W.F., Sin S.N., Chua H., Chan S.Y., Yu P.H.F. and Lo W. (2004). Metabolic pathways of PHA synthesis in activated sludge under various ORPs. (*in press*).

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my Supervisor, Dr. Hong Chua, Professor of the Department of Civil and Structural Engineering, for his unending support and guidance.

I am also thankful to Dr. Peter H.F. Yu, Associate Professor of the Department of Applied Biology and Chemical Technology, for his valuable advice and helpful discussion.

I would also like to thank Mr. K.H. Ng, Mr. W.S. Lam, Mr. C.W. Lau and other technicians in the CSE Department for their endless assistance with the instrumentation techniques and for their friendship that will always be remembered.

I would especially like to thank my family for their continuing support and encouragement for the completion of this study. I also thank my wife, Zhang Xiaoqun and my daughter, Dou-Dou for their support which made my research being more smoothened.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
APHA	American Public Health Association
ATP	Adenosine Triphosphate
C2	Acetic Acid or Acetate
C3	Propionic Acid or Propionate
C4	Butyric Acid or Butyrate
C5	Valeric Acid or Valerate
CDW	Cell Dry Weight
CFU	Colony Forming Unit
CoA or CoASH	Co-enzyme A
DO	Dissolved Oxygen
DW	Dry Weight
EAS	Excess Activated Sludge
EBPR process	Enhanced Biological Phosphorus Removal process
ED pathway	Entner-Doudoroff pathway
EMP pathway	Glycolytic pathway or Embden-Meyerhof-Parnas pathway
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide;

$FADH_2$	Reduced Flavin Adenine Dinucleotide
GAO	Glycogen Accumulating Organism
GC	Gas Chromatography
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
3HA or HA	3-Hydroxyalkanoates
3HB or HB	3-Hydroxybutyrate or β-Hydroxybutyrate
4HB	4-Hydroxybutyrate
3HC or HC	3-Hydroxycaproate
3HD	3-Hydroxydecanoate
HDPE	High-Density Polyethylene
3HHp	3-Hydroxyheptanoate
3HHx	3-Hhydroxyhexanoate
3H2MB	3-Hydroxy-2-methylbutyrate
3H2MV	3-Hydroxy-2-methylvalerate
3HO	3-hydroxyoctanoate
HRT	Hydraulic Retention Time
3HV or HV	3-Hydroxyvalerate or β-Hydroxyvalerate
ICI	Imperial Chemical Industries Ltd.
LDPE	Low-density polyethylene
MCL-PHAs	Medium-chain Length Polyhdroxyalkanoates
MLSS	Mixed Liquor Suspended Ssolid

MWWTP	Municipal Wastewater Treatment Plant
NAD	Nicotinamide Adenine Dinucleotide
$NADH_2$	Reduced Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
$NADPH_2$	Reduced Nicotinamide Adenine Dinucleotide Phosphate
ORP	Oxidation-Reduction Potential
PABER	PHA Accumulating Bacteria Enrichment Reactor
PAO	Poly Phosphate Accumulating Organism
PE	Polyethylene
PET	Poly(ethylene terephthalate)
PHA	Poly-hydroxyalkanoates
P(3HB) or PHB	Poly-(3-hydroxybutyrate)
P(3HB-*co*-3HV) or PHBV	Poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HV) or PHV	Poly-(3-hydroxyvalerate)
PP	Polypropylene
PPR	PHA production reactor
PVC	Polyvinyl Chloride
SCL-PHA	Short-chain Length PHA
SBR	Sequencing Batch Reactor
TCA cycle	Tricarboxylic Acid Cycle or Citric Acid Cycle
TEM	Transmission Electron Microscopy

TOC	Total Organic Carbon
TSS	Total Suspended Solids
VSS	Volatile Suspended solids

CHAPTER 1

INTRODUCTION

Plastics have become an essential part in human's modern society and its demand has increased dramatically. This is because the plastics derived from petroleum are important materials for the manufacturing of industrial and consumer products today. For example, high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) have been wide spread used as construction material, packing material, medical material, electronic appliances and disposal materials. Furthermore, the demand for petroleum-derived plastics will constantly increase. Rudolph (1994) showed that the annual world production of petroleum based and conventional plastics was 100 million tons and might reach 150 million tons by 2000. Yu *et al.* (1998) forecasted that its annual production would increase at 15% per year in the future decades. Let's image: what would happen if the plastics had gone from the human's daily lives?

Unfortunately, plastics derived from petroleum have caused serious environmental problems due to its intrinsic durability and uneasily decomposed in nature by microorganisms. Consequently, plastics can't enter the natural carbon recycling but remain in the environment for years, endangering animals, inhibiting plant growth and contaminating water and soil through the leaching away of

heavy metals and additives. It is estimated that plastics litter kills more than 1 million marine mammals and birds each year around the world (Hankermeyer *et al.* 1992). Hence, plastics derived from petroleum have been among the most environmentally harmful solid wastes (Huang *et al.*, 1991).

In 1999, each day in Hong Kong, an average of 9,335 tons of solid wastes from domestic, commercial and industrial sources are disposed at the landfills of Hong Kong. Of these, approximately 17% of wet weight of the solid wastes is plastic materials (viz. 1,567 tons) (Hong Kong Environmental Protection Department, HKEPD, 2000). On the other hand, over the past two decades, plastics usage and plastic-waste generation have been on an increase of 15% per year and the trend will be continued.

There are several methods to treat plastic wastes and to reduce the environmental impact. The first option is disposing in landfills. The strategic landfills in Hong Kong built in the 1990s can hold more than 140 million tons of waste and will be the major disposal sites for the next two decades. By using this treatment method, plastic takes a lot of space owing to its light-weight, large-volume and non-biodegradable characters (Chua *et al.*, 1995, 1997a). Therefore, a large amount of space is needed in the landfills for their disposal. It is predicted that Hong Kong will not have enough landfill space for disposing of the increasing amount of plastic waste produced, and a significant portion of it will end up as litter in the environment. Furthermore, plastic waste in landfills blocks the infiltration of water and upsets the natural recycling of nutrients and microbes

in the soil structure, consequently causes severely repressed of plant growth and the damage of landfill stability. Additional technical measures and treatments are needed to prevent from soil and water contamination, and emissions of gases. Even worse, it is estimated that waste disposed at landfill sites would be saturated within the next coming fifteen years in Hong Kong (HKEPD, 2000).

The second choice for plastic wastes treatment is incineration. Perhaps, it is the most simply way to treat the plastic wastes. But, plastic wastes are disposed of integrated by incineration and results in hazardous emissions. So, it become necessary to avoid harmful emissions from incineration of plastic wastes by additional technical measures, such as purification of incineration gases and wastewater purification, this leads to residual products that must be further treated as chemical wastes. The emissions from the incineration of plastic wastes consist of CO_2 (contributing to the greenhouse effect), nitrogen oxides and sulphur oxides (contributing to acid rain), dioxin, polyaromatic hydrocarbons and hydrochloric acid. Of these, dioxin is the carcinogen, which is health harmful to the publics. Otherwise, the incineration of waste is accompanied by odour, noise pollution. In Hong Kong, this treatment method by incineration of plastic wastes has been phased out.

Recycling of plastics is the third choice for the reducing of the amount of plastic wastes (Albano and Sanchez, 1999). It is not only the way to meet the need to reduce the volume of plastic waste which is sent to landfills, but also the one of the options to conserve non-renewable petroleum resources. Although the volume

of plastics that are recycled is rapidly increasing each year in the world, only less than 3% of all plastics are now recycled, compared to about 20% of glass, 29% of paper, 16% of steel, and 39% of aluminum. A major reason for so low recycling rate of plastics is that the problem encountered is that the total cost of producing recycled plastics, including the cost for collecting, delivery, resorting and recycling is typically 20% higher than that of original polymer. Moreover, thermosetting plastics that account for nearly 11% of the total volume of plastics cannot be recycled. Otherwise, to realize the excellent performance of plastics and endue them with special features, many kinds of additives are often added into plastic row material during processing procedure. The additives include anti-oxydants, anti-statica, fillers, fire decelerators, pigments and plasticizers (Ma, 2000). These additives make it further complicated and difficult to recycle plastics for generating a product with acceptable quality (Pfeffer, 1992 and Billmeyer, 1971). Thus, recycling is not an effective solution to long-term plastic wastes management till now even though the automatic garbage-sorting systems have been established in many developed countries and regions.

It is very clear that all of the alone options have serious disadvantages and they are not the effective solutions to reduce the environmental impact of plastic wastes. As to see, neither incinerator nor landfill nor recycling could be the long-term solution to plastic wastes. Obviously, the forth option is to manufacture plastics that are degradable. A plastic is defined as "degradable" if the degradation results from the radiation of sunlight (UV) or the action of naturally occurring

microorganisms such as bacteria and fungi in soil, water and sludge (Chua *et al.*, 1997a and Ma, 2000a). In another word, the degradable plastics are made of recyclable sources and will enter the natural carbon recycle after being decomposed or digested instead of retaining in the environment integrallty.

One of the degradable plastics is photodegradable polymer, which is produced by introducing chromophores into the polymer structure by copolymerization (e.g., ethylene and carbon monoxide) or by mixing photosensitive additives (ketone based compounds) with the polymer. Although the development of photodegradable plastic can prevent the release of toxic compound, UV light cannot reach the plastic after it is disposed into landfill, which results in un-completely degradation of plastic wastes. Otherwise, these photodegradable plastics are too expensive to be wide spread used as well as the plastics derived from petroleum.

Another type of degradable plastics is the biodegradable polymers. Microorganisms can decompose degradable plastics partially or completely via using the breakdown of materials as precursors for cell components and energy sources. Various biodegradable plastics have been produced either by incorporating natural polymers into conventional plastics formulations, by chemical synthesis, or by microbial fermentation. One of the biodegradable plastics used today is to incorporate starch or other natural macromolecules into polyethylene (PE) or other plastic resins to form the so-called "starch-plastic" (Maddever *et al.*, 1989). The granular starch content in these starch-plastic

achieve to 70% of the overall weight, which can be degraded and consumed as nutrients by microorganisms in the environment and are mainly used as agricultural mulch, or geo-films, which does not have to be removed after the harvesting seasons (Robert, 1989). It is also used for the manufacturing of garbage bags, packaging materials, sanitary and other disposable products. However, the disadvantage of this type starch-plastic is that the non-natural-macromolecular fraction can't be digested by the microorganisms, and consequently causing a threat to the environment. Furthermore, the portion of embedded starch granules that are not in contact with microorganisms cannot be degraded. Otherwise, it is limited to gain the degradable starch in the formula for increasing the ratio of the biodegradable part due to the influence of starch on the mechanical strength of the plastics. In those points of view, the starch-plastic cannot be classified as fully biodegradable polymers and its usefulness is questionable (Shen *et al.*, 1994).

Among these biodegradable plastics, polyhydroxyalkanoates (PHA) are the other one, which are accumulated by fermentation process of microorganisms and can be fully degraded in the environment. PHA have attracted much attention as candidates for biodegradable polymers, because they possess material properties similar to various petrochemical-based synthetic thermoplastics currently in use, and are completely degraded to water and carbon dioxide (and methane under anaerobic conditions) by microorganisms in various environments (Doi, 1990; Holmes, 1988 and Lee, 1996). The family of more than 40 PHAs, and their copolymeric derivatives, have emerged as very attractive materials for their

complete biodegradability and is the potential alternative on the conventional plastics derived from petroleum (Kumagai *et al.*, 1992). A number of bacteria, including *Ralstonia eutropha*, *Alcaligenes* spp., *Pseudonomas* spp., recombinant *Escherichia coli*, and a number of filamentous genera accumulate these polymers or copolymers to be their intracellular carbon and energy reserves when unfavorable environmental conditions are encountered (Pfeffer *et al.*, 1992). Nutrient-deficient conditions, nitrogen limitation for example, may result in accumulated polymers of up to 75% of dry cell mass (Billmeyer *et al.*, 1971). These extracted and processed polymers have a number of properties that are comparable to commonly used plastics and 100% water resistance. However, widespread application of PHA is hampered by high costs of production. The cost of BIOPOL^{TM} produced from the cultivation of *R. eutrophus* is US$16/kg, and in the meantime, contrarily polypropylene (a conventional plastic) is less than US$1/kg.

Many efforts have been performed for decreasing the costs of PHA as the alternative to conventional plastics. The most of studies on reducing the cost of PHA synthesis were primarily focused on the pure culture of microorganisms or genetically engineered plants to try to lower the costs of the end products. Lee *et al.* (1994) investigated various recombinant *Escherichia coli* using different complex culture media. *E. coli* strain XL1-blue in LB plus 20g glucose/l could accumulate up to 0.369g of polymer/g of glucose, or equivalent to 7g polymer/l. Shirai *et al.* (1994) used a photosynthetic bacteria, *Rhodobacter spheroids*, in a

fed-batch culture with glucose as the sole carbon to achieve a PHA production of 6g/L. Shimizu *et al.* (1992) used a cell-growth phase followed by a separately optimized nutrient-deficient PHA accumulation phase to improve the specific production yield to as high as 0.7g of polymer/g of cell mass. For the production of PHA on a commercial scale, the cost of microbial growth substrates is a critical factor. For the process with recombinant *E. coli*, the cost of carbon source is 30.7% of the total cost. From this viewpoint, several researchers have investigated the possibility of using industrial wastes as the nutrients source for economical PHA production. But the cost of extraction of PHA is still the most costly step. In recent years, microorganisms in excess activated sludge (EAS) have been reported to accumulate PHA as an intermediate metabolic product from the uptake of organic matter in sewage or synthetic cultural medium (Chua *et al.*, 1997a, b; Satoh *et al.*, 1998; Hu *et al.*, 1997). Therefore it is possible to consider coupling a wastewater treatment process with PHA production. This technique is beneficial in several ways. Firstly, it can reduce the quantity of excess sludge, therefore reducing the costs of sludge treatment; secondly, large quantities of biomass harvested from activated sludge wastewater treatment processes were induced to produce PHA instead of producing by pure culture fermentation; thirdly, the facilities for the steam generation and sterilization system for the fermentation process will be not necessary any more, which will significantly reduce the costs of facility construction for PHA production.

CHAPTER 2

LITERATURE REVIEW AND BACKGROUND OF STUDY

2.1. Introduction of Polyhydroxyalkanoates (PHA)

Biodegradable plastics, which can be completely degraded and consumed as the nutrients by microorganisms in environment, have been recognized as a potential environmentally friendly alternatives on the traditional petroleum-derived plastics, especially on the packaging and disposable products (Chua *et al.*, 1995).

Starch-plastic, composted starch or other nature macromolecules with polyethylene (PE) or other plastic resin, is one of those biodegradable plastics which has been commonly used as agricultural mulch, geo-films, garbage bags, packaging, sanitary and other disposable products in local market (Maddever *et al.*, 1989 and HKEPD, 2000). This composite material contains granular starch, accounting for up to 70% of the overall weight, which can be attacked and degraded by microorganisms such as fungi and bacterial resulting in the minimization of plastics impact to environment. However, the residual 30% of the total weight is still not biodegradable and will be remained as a threat to the environment, endangering animals, repressing the growth of plants and even contaminating water and soil via the leaching away of additives. Furthermore, to gain the biodegradability by increasing the proportion of starch in the formulation

is limited due to the affect of the starch on the mechanical strength of the plastics (Shen *et al.*, 1994 and Chua *et al.*, 1995, 1997b). Hence, it is concluded that starch-plastics are not an ideal and final resolution for plastic-waste pollution.

Polyhydroxyalkanoates (PHAs), other kinds of biodegradable plastics, are polyesters of various hydroxyalkanoates (HAs) accumulated as carbon and energy storage compounds in various microorganisms under unbalanced nutrient conditions such as nitrogen, phosphorus, sulphur, oxygen or magnesium shortages in the presence of an excess carbon source (Anderson and Dawes, 1990; Brandl *et al.*, 1990; Doi, 1990a and Lee, 1996a).

They have attracted much attention owing to their unique physical and chemical characters. First of all, they are thermoplastic and/or elastomeric compounds which can be processed with apparatus used by the plastic manufacturing industry. In addition, they are insoluble in water, exhibit a rather high degree of polymerization ranging from 10^5 to almost 10^7 Da, and they are enantiomerically pure chemicals consisting, in general, only of the R-stereoisomer. They are non-toxic and biocompatible and exhibit piezoelectric properties as revealed (at least) for Poly-3-hydroxybutyraet (PHB), and Poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (Poly-(3HB-co-3HV), PHBV)). Several PHAs can be obtained from CO_2 or renewable resources (Doi, 1990a; Ishizaki and Tanaka, 1991 and Walle *et al.*, 1999). Finally, all PHAs are biodegradable and biocompatible; they are hydrolyzed by extracellular PHA depolymerases, and the cleavage products are subsequently utilized as sources of

carbon and energy by many bacteria and fungi (Jendrossek *et al.*, 1996). The most important properties of the PHA is that it is the truly biodegradable matter than the 'starch-plastics', which makes it possible to be a potential alternative to the petroleum-derived plastics in many applications. Otherwise, PHA also represents a class of polymers that have immense potential for medical applications and are, therefore, attracting increasing attention (Table 2.1) (Zinn *et al.*, 2001). The properties of some PHA in comparison with those of PP and PET are listed in Table 2.2 (Sudesh and Doi, 2000).

PHB is the most abundantly appeared derivative of PHA. It was found by Lemoigne (1926) in the cell component of *Bacillus megaterium*. It is now well recognized that PHB is accumulated in a diverse range of microorganisms (Table 2.1, 2.2 and 2.3) (Doi, 1990; Reddy *et al.*, 2003 and Choi and Lee, 1999). Less abundant are PHB's co-polymers such as 3-hydroxyvalerate (3HV). PHAs can be divided into two groups depending on the number of carbon atoms in the monomeric units (Lee *et al.*, 1995). One group of PHAs is short-chain-length PHA (SCL-PHA) consisting of 3-5 carbon atoms, the other group is medium-chain-length PHA (MCL-PHA) consisting of 6-14 carbon atoms. So far, only the homopolyester PHB, the co-polyester PHBV, and PHAs consisting of 3-hydroyxoctanoate, 3-hydroxydecanoate and a few other medium-chain-length 3-hydroxyalkanoates, poly($3HA_{MCL}$), have been characterized and commercialized by fermentative biotechnological processes and revealed various areas application (Lee, 1996b and Steinbüchel, 2001).

Table 2.1. Potential applications of PHA in medicine

(adapted from Zinn *et al.*, 2001)

Type of application	*Products*
Wound management	Sutures, skin substitutes, nerve cuffs, surgical meshes, staples, swabs
Vascular system	Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts
Orthopaedy	Scaffolds for cartilage engineering, spinal cages, bone graft substitutes, meniscus regeneration, internal fixation devices (e.g., screws)
Drug delivery	Micro- and nanospheres for anticancer therpy
Urology	Urological stents
Dental	Barrier material for guided tissue regeneration in periodontitis
Computer assisted tomography and ultrasound imaging	Contrast agents

Table 2.2. Properties of some PHA copolysters in comparison with those of polypropylene and polyethylene terephthalate.

(adapted from Sudesh and Doi, 2000)

Sample	*Melting temperature (°C)*	*Glass-transition temperature (°C)*	*Crystallinity (%)*	*Extension to break (%)*
P(3HB)	177	2	70	5
P(3HB-co-20 mol% 3HV)	145	-1	56	50
P(3HB-co-16 mol% 4HB)	150	-7	45	444
P(3HB-co-11 mol% 3HHx	136	-1	36	400
PP	176	-10	50-70	400
PET	267	69	30-50	100

3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HHx, 3-hydroxyhexanoate; 4HB, 4-hydroxybutyrate; PP, polypropylene; PET, poly(ethylene terephthalate)

Since the discovering of PHA in 1926, PHA then has been studied extensively by biochemists it is generally concluded that bacteria store PHB as an energy reserve in much the same way that mammals accumulated fat. It remained an academic curiosity until W.R. Grace in the United States produced small

quantities for commercial evaluation in the late 1950s and early 1960s several patents were issued as a result of that efforts (Grace and Co. U.S. Pat., 1962, 1965). Commercial interest lay dormant for over a decade until the Imperial Chemical Industries Ltd. (ICI) began a research and development program. This project followed their single-cell protein animal feed project. Thus ICI had the skills in place to run large-scale fermentation processes, and polymer processing technology was available in their plastics division (Holmes, 1985). In the late 1980s, ICI began worldwide commercialization of family of PHBV copolymer, named Biopol®. In 1990, the agricultural and pharmaceutical businesses of ICI, including BIOPOL were spun-off as Zeneca Ltd. Cargill Dow Polymers is marketing its 'EcoPla' resins in Japan, Novamont's starch-based material, 'Mater-Bi', is marketed in Japan by Nippon Gohsei. Mater-Bi has been used in packages of electrical goods, agricultural mulch film and compost. Mitsubishi and Nippon Shokubai under the trade names 'LUNARE ZT' and 'Lunare SE' market 'EnviroPlastic'. 'Bionolle', a thermoplastic aliphatic polyester, is manufactured by Showa Highpolymer and Denko of Japan. It is produced by the polycondensation of glycol with dicarboxylic acids. 'Lacea' is another type of bioplastic manufactured by Mitsui Chemicals, Japan, from fermented starch, derived from a variety of renewable resources, such as corn, beet, cane, and tapioca. Lacea is comparable to polyethylene (PE) in terms of transparency and similar to polystyrene or PE in terms of processability. It also claims good mould resistance, low heat of combustion which is similar to that of paper, superior

stability in processing use and biodegradability superior to that of earlier polylactic acid-based materials. Daicel Chemical Industries of Japan has developed biodegradable blends of two different kinds of material, polycaprolactone and acetyl cellulose resin with the brand name 'Celgreen'. Shimadzu developed a fermentation process for lactic acid and collaborated with Mitsubishi Plastics Industries to develop poly-$_L$-lactic acid. The resins are marketed under the trade name 'Lacty'. The other bioplastics manufactured by Japan based firms are 'Eco-Ware' and 'Eco-Foam' (which are starch-based) and Cardoran and Pulluran (which are based on polysaccharides) (Table 2.3).

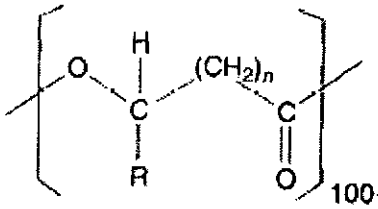
In 1996, Monsanto acquired the BIOPOL business from Zeneca Ltd. Since the acquisition, emphasis at Monsanto has been on producing the polymers in plants and improving their properties for different end-use applications (Asrar and Hill, 2002).

Table 2.3 Manufacturers and the microorganisms, raw materials used for the production of biodegradable plastics (adapted from Reddy *et al.*, 2003)

Microorganisms/raw material	Manufacturer
Ralstonia eutropha (former *Alcaligenes eutrophus* H16)	ZENECA Bio-products, UK (formerly ICI ltd.)
Alcaligenes latus	Biotechnolgische Forschungs gesellschaft mbH (Austria) Petrochemia Danubia
Transgenic plants	Metabolix Inc. (USA) Monsanto (USA) ZENECA Seeds (UK)
Recombinant *Esherichia coli* Starch	Bio Ventures Alberta Inc. (Canada) Warner's Lambert (USA) Fertec,Italy (Ferruzi e Technologia) Biotec (Melitta) Emmerich (Germany) BASF Ludwigshafen (Germany) Bayer/Wolf Walsrode Leverkusen (Germany) Novamont Novara (Italy)
Cheap substrates Bacteria	Polyferm Inc. (Canada) Biocorp (USA) Asahi Chemicals and Institute of Physical and Chemical Research (Japan)

The general structural formulas of PHAs and PHBV are shown in Figure 2.1, Figure 2.2 and Figure 2.3 respectively (Byrom, 1994; Steinbüchel, 1991, 2001; Lee, 1996b and Lee *et al.*, 1999). As illustrated in the figures, the composition of the side chain or group 'R' and value of 'n' determine together the identity of a monomer unit. For PHB, the most common PHAs, 'n' is equal to 1 while R is hydrogen. PHB is composed of monomers with a methyl group as side chain, and the PHV units of PHBV copolymer contain an ethyl group on carbon number 3 (Figure 2.1 and Figure 2.2).

The monomeric composition of PHA depends on the bacterial strain, on the carbon source supplied and the fermentation conditions during the accumulation phase. This will be given more details discussion at next part of this Chapter.



n = 1	R = hydrogen	Poly(3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyoctanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)
	R = methyl	Poly(4-hydroxyvalerate)
n = 3	R =hydrogen	Poly(5-hydroxyvalerate)
	R = methyl	Poly(5-hydroxyhexanoate)
n = 4	R = hexyl	Poly(6-hydroxydodecanoate)

Figure 2.1 General structures of PHAs (adapted from Lee, 1996b)

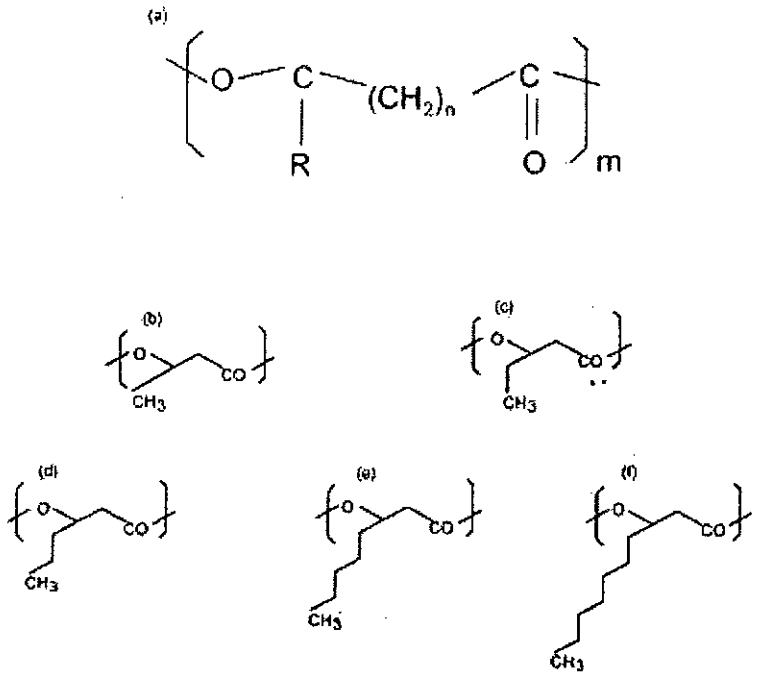
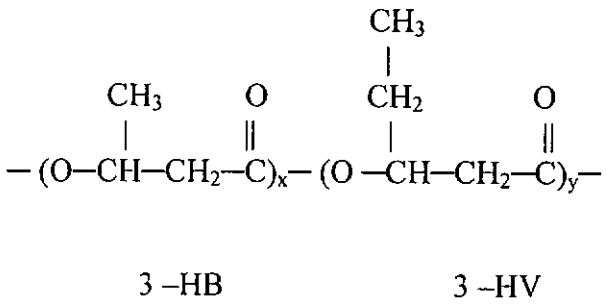


Figure 2.2 Structure of polyhydroxyalkanoates: (a) general structure of PHA; (b) poly(3hydroxybutyrate); (c) poly(3-hydroxyvalerate); (d) poly(3-hydroxyhexanoate); (e) poly(3-hydroxyoctanoate) and (f) poly(3-hydroxydecanoate). (Adapted from Lee *et al.*, 1999)



3 –HB 3 –HV

Figure 2.3 Structure of PHBV (adapted from Doi, 1990)

2.2 Production of PHA by the microorganisms pure culture

2.2.1. The production of PHA by various bacteria

PHAs should be produced biologically in a plant by microorganisms. Most typically, it is a fermentation product where a microorganism lays down PHA during normal or manipulated growth. Manipulation may be achieved by removing or failing to produce, one or more nutrients necessary for cell multiplication. The percentage of PHB in bacteria cells is normally low, from 1 to 30%, but under controlled fermentation conditions of carbon excess and nitrogen limitation, overproduction of polymer can be encouraged to produce yields of up to 90% of cell dry weight (CDW).

More than 300 different microorganisms, wild type or mutated or may have the necessary genetic material introduced into it by the methods of recombinant DNA technology, are known to synthesize and intracellularly accumulate PHAs (Lee, 1996b; Asrar and Hill, 2000 and Sudesh *et al.*, 2000). Amongst these microorganisms, only a few of bacteria have been employed for the production of PHAs by pure cultural process. These included *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) (Byrom 1987; Doi *et al.*, 1989, 1990b and Kim *et al.*, 1994a, b), *Alcaligenes latue* (Hrabak, 1992), *Azotobacter vinelandii* (Page, 1992 and Page *et al.*, 1993), several strains of methylotrophs, *Pseudomonas oleovorans* (Preusting *et al., 1993)*, and, recombinant *R. eutrophus* (Park *et al.*, 1995), recombinant *E. coli* (Lee *et al.*, 1995) and recombinant *Klebsiella*

aerogenes (Zhang *et al.*, 1994). The general strains of bacteria accumulated PHAs and the substrate used are listed in Table 2.4 (Doi, 1990) and Table 2.5.

Table 2.4 The occurrence of PHB in the cell component of various microorganisms (adapted from Doi, 1990)

Acinertobacter	*Gamphosphaeria*	*Rhodobacter*
Actinomycetes	*Haemphilus*	*Rhodospirillum*
Alcaligenes	*Halobacterium*	*Sphaerotilus*
Aphanothece	*Lamprocystis*	*Spirillum*
Aquaspirillum	*Lampropedia*	*Spirulina*
Azospirillum	*Leptothrix*	*Streptomyces*
Bacillus	*Methylobacterium*	*Syntrophomonas*
Beijerinckia	*Methylocytis*	*Thiobacillus*
Caulobacter	*Moraxela*	*Thiocapsa*
Chlorogrexeus	*Mycoplana*	*Thiocystis*
Chlorogloea	*Nitrobacter*	*Thiodictyon*
Chromatium	*Nitrococcus*	*Thiopedia*
Chromobacterium	*Nocardia*	*Thiosphaera*
Clostridium	*Oceanospirillum*	*Vibrio*
Derxia	*Paracoccus*	*Xanthobacter*
Ectothiorhodospira	*Photobacterium*	*zoogloea*
Escherichia	*Pseudomonas*	
Ferrobacillus	*Rhizobium*	

Table 2.5 Production of PHA by bacteria in pure culture from various substrates (modified from Reddy *et al.*, 2003)

Microorganism	Carbon source	PHA	PHA content (%w/v)	Reference
Alcaligenes latus	Sucrose	PHB	50	Yamane *et al.*, 1996
Azotobacter vinelandii	Sucrose	PHB	79.8	Page and Comish, 1993
Az. chroococcum	Starch	PHB	46	Kim and Chang, 1998
R. eutrophus	Gluconate Propionate Octanoate Glucose	PHB PHB PHB PHB	46-85 26-36 38-45 82	Liebergesell *et al.* 1994 Ryu *et a.*, 1997
Bacillus megaterium QMB1551	Glucose	PHB	20	Mirtha *et al.*, 1995
E. coli recombinant	Glucose Glucose Whey Sucrose	PHB PHB PHB PHB	76 77 80 27.5	Lee *et al.*, 1994 Wang and Lee, 1997 Wang and Lee, 1998 Lee and Chang, 1993
K. aerogenes recombinant	Molasses	PHB	65	Zhang *et al.*, 1994
Methylobacterium organophilum	Methanol	PHB	52	Kim *et al.*, 1996
M. rhodesiamum MB1267	Fructose/ Methanol	PHB	30	Ackermann and Babel, 1997
M. extorquens (ATCC55366)	Methanol	PHB	40-46	Borque *et al.*, 1995
P. aeruginosa	Euphorbia and castor oil	PHA	20-30	Eggink *et al.*, 1995
P. denitrificans	Methanol Pentanol	PHV PHV	0.02 55	Yamane *et al.*, 1996
P. oleovorans	Glucanoate Octanoate	PHB PHB	1.1-5.0 50-68	Liebergesell *et al.* 1994
P. putida GPp104	Octanoate	PHB	14-22	Liebergesell *et al.* 1994
P. putida	Palm kernel oil Lauric acid Myristic acid Oleic acid	PHA PHA PHA PHA	37 25 28 19	Tan *et al.*, 1997
P. putida BM01	11-phenoxyun-decanoic acid	5POHV	15-35	Song and Yong, 1996
Sphaerotilus natans	Glucose	PHB PHBV	40 25.2	Takeda *et al.*, 1995 Liu *et al.*, 2002

Bacteria that are used for the production of PHAs can be divided into two groups based on the culture conditions. The first group of bacteria requires the limitation of an essential nutrient such as N, P, Mg, K, O or S for the efficient synthesis of PHA from an excess carbon source. The second group of bacteria does not require nutrient limitation for PHA synthesis, and can accumulate polymer during growth phase. *R. eutrophus*, *Protomonas extorquens*, *Ps. Oleovorans* and many other bacterial belong to the first group, while some bacteria such as *Al. latus*, a mutant strain of *Az. vinelandii*, and recombinant *E. coli* harboring the *A., eutrophus* PHA biosynthesis operon belong to the second group. Therefore, these characteristics should be considered in developing cultivation methods for the efficient production of PHAs. Either fed-batch or continuous cultivation techniques can be used for the production of PHA with high productivity.

For the fed-batch culture of bacteria belonging to the first group, a two-step cultivation method (but not necessarily requiring two fermentor vessels) is most often employed. Cells are first grown to a desired concentration without nutrient limitation, after which an essential nutrient is limited to allow efficient PHA synthesis. During this nutrient limitation stage the residual cell concentration (defined as the cell concentration minus the PHA concentration) remains almost constant, and the cell concentration increases only because of the intracellular accumulation of PHA.

2.2.2. Conditions for the PHA accumulation by bacteria

As mentioned previously, the most abundantly occurring PHA is PHB, which was found in 1926 by Lemoigne (1926) in *B. megaterium*. However, Sudesh *et al.* (2000) reviewed that the effect of the growth conditions on PHA production was first studied by Macrae and Wilkinson in 1958 for strains of *Bacillus cereus* and *B. megaterium* (Macrae and Wilkinson, 1958). They made the important observation that the quantity of PHA accumulated increased while the carbon to nitrogen ratio increased. Their results suggested that, like polyphosphate and carbohydrate reserves, PHA accumulation occurred in response to an imbalance in growth conditions brought about by nutrient limitations. This significant discovery makes begin the investigation into the physiological role of PHA. It was understood later that bacteria synthesis and store PHA when they lack the complete range of nutrients required for cell division but have excess supplies of carbon source. The biosynthesis of PHA was shown to be initiated by a magnesium or sulfate deficiency, as well as by nitrogen, phosphate (Dawes and Senior, 1973 and Repaske R. and Repaske A.C., 1976) and/or oxygen limitations (Ward *et al.*, 1977).

From the first observations, PHA was associated with the sporulation of bacteria such as *B. megaterium* (Lemoigne, 1926; Slepecky and Law, 1961). It was generally observed that PHA is formed before the onset of sporulation, and the rapid utilization of the polymer precedes sporulation. However, PHA is not always associated with sporulation since not all spore formers make the polymer

and not all polymer formers generate spores. To date, it is known that PHAs are synthesized by a wide range of microorganisms. For many bacteria, the polymer, once accumulated, serves as both carbon and energy source during starvation. PHA constitutes an ideal carbon-energy storage material due to its low solubility and high molecular weight, which exerts negligible osmotic pressure to the bacterial cell (Dawes and Senior, 1973). It must be noted that there are also examples of bacteria such as *Al. latus* (Hrabak, 1992; Yamane *et al.*, 1996) and a mutant strain of *Az. vinelandii* (Page and Knosp, 1989; Page and Comish, 1993) and which are known to accumulate PHA during growth in the absence of nutrient limitation. However, nitrogen limitation was shown to further enhance the production of PHA in *A. latus* suggesting that this growth during PHA accumulation is not an efficient process (Lee *et al.*, 1999b).

Senior and Dawes (1971) proposed that PHA also serves as a sink for reducing power and could therefore be regarded as a redox regulator within the cell. It was found that for members of the *Azotobacteria*, the reductive step of PHA synthesis appeared to serve as an electron sink for the reducing power which accumulated when electron flow through the electron transfer chain (ETC, detail description presented in Chapter 4) was affected as a consequence of oxygen limitation (Senior and Dawes, 1973). On the other hand, in the symbiotic nitrogen fixation process, PHA accumulation has been implicated as both an energy source as well as to serve as a regulatory role, controlling the availability of reducing power for the operation of nitrogenase (McDermott *et al.*, 1989).

2.2.3. Metabolic pathways for the PHA synthesis from various carbon sources

So far, we now know that the PHA synthesis enzyme shows broad substrates specificity and therefore a wide variety of monomers can be polymerized. One of the factors that determines the type of PHA constituents is the carbon source. Microorganisms are capable of producing PHA from various carbon sources ranging from inexpensive, complex waste effluents like beet/cane molasses (Page, 1992 and Hängii, 1990), to plant oils (Fukui and Doi, 1998) and its fatty acids (Tan *et al.*, 1997), alkanes (Lageveen *et al.*, 1988) as well as simple carbohydrates. Based on the types of monomer incorporated into PHA, various metabolic pathways have been shown to be involved in the generation of these monomers. The PHA biosynthesis of bacteria can apparently be divided into two major types based on the monomer composition of PHA produced by various wild-type bacteria. *R. eutropha* represents one group while the pseudomonads represent the other major type of PHA biosynthesis. Figure 2.5 summarizes the various metabolic pathways that are known to supply monomer units for PHA biosynthesis.

2.2.2.1. PHA biosynthesis represented by *R. eutropha*.

R. eutropha is among the bacteria that have been extensively studied for the production of PHA. In *R. eutropha*, biosynthetic pathway of PHB consists of three enzymatic reactions catalyzed by three different enzymes (Figure 2.4 and Figure

2.5, Pathway I). PHB is synthesized by the successive action of β-ketoacyl-CoA thiolase (*phb*A), acetoacetyl-CoA reductase (*phb*B) and PHB polymerase (*phb*C) in a three-step pathway while one $NAD(P)H_2$ is oxdized. The genes of the *phb*CAB operon encode the three enzymes. The promoter (P) upstream of *phb*C transcribes the complete operon (*phb*CAB) (Madison and Huisman, 1999).

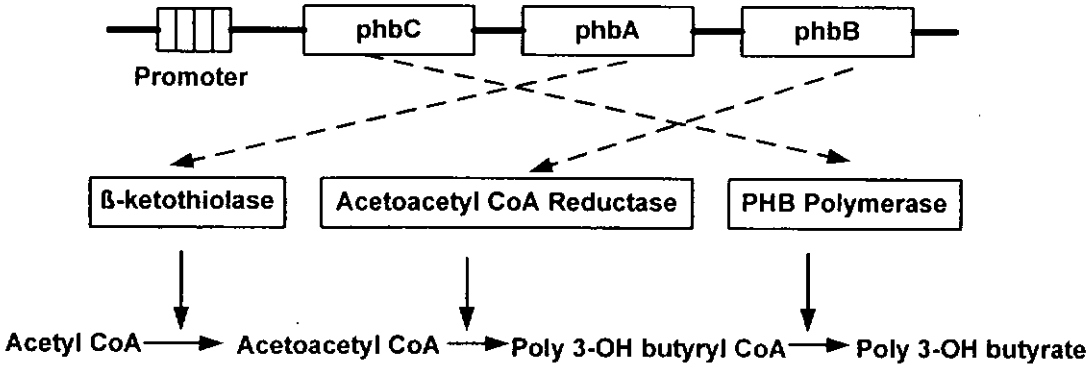


Figure 2.4. Biosynthetic pathway for PHB. PHB is synthesized in a three-step pathway by the successive action of β-ketoacyl-CoA thiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and PHB polymerase (PhbC), while one $NAD(P)H_2$ oxidized. The three enzymes are encoded by the genes of the *phbCAB* operon. A promoter upstream of *phbC* transcribes the complete operon. (adapted from Madison, 1999)

As suggested by Anderson and Dawes (1990), two acetyl-CoA moieties are condensed to acetoacetyl-CoA by a β-ketothiolase (PhaA). The acetoacetyl-CoA then undergoes reduction by an NADPH-dependent reductase (PhaB) which is reduced at position 3. This reaction occurs stereospecifically, which means all resulting of 3-hydroxybutyryl-CoA, are the *R*-configuration [(*R*)-isomer] at position 3 (Figure 2.5, Pathway I). On the other hand, in *R. rubrum* which shares almost similar PHA biosynthesis pathway as *R. eutropha*, the reductase which is an NADH-dependent isoenzyme, gives rise to the (S)-isomer of 3-hydroxybutyryl-CoA. Two enoyl-CoA hydratases then covert the (S)-type to the (R)-type isomer which is the only stereoisomer usually accepted by the polymerizing enzyme, PHA synthase (Moskowitz and Merrick, 1969). The recent genetic analysis of PHA biosynthesis pathways of *A. caviae* had clearly demonstrated the involvement of an (*R*)-specific enoyl-CoA hydratase (PhaJ) in the production of P(3HB-co-3HHx) (Fukui and Doi, 1997; Fukui *et al.*, 1998). Otherwise, the three-step reaction for PHB synthesis takes place only on the surface of the granule. The formation of PhaB is regulated by the intracellular concentration of acetyl-CoA (Steinbüchel and Schlegel, 1991 and Steinbüchel, 2001). Under growth conditions without nutrient restrictions and oxygen limitation (exponential growth phase of batch cultures), acetyl-CoA enters the TCA cycle for energy generation and the biomass growth. In consequence, the pool of free CoASH is enhanced. However, when growth is nutrient limited. e.g., by ammonium or phosphate, the CoASH level is reduced and PHB synthesis is

favored. This is because the key regulatory enzyme in PHB synthesis is 3-ketothiolase (PhaA), which is inhibited by high concentrations of free CoASH (Doi, 1990a). Further, under growth-limiting conditions in the presence of an excess carbon source, citrate synthase is inhibited by high concentrations of $NADH_2$ and acetyl-CoA levels increase, which leads to a decrease in the concentration of free CoASH.

Various carbon sources can be utilized by *R. eutropha* for growth and/or PHA production. Linko and coworkers (1993) reported the successful utilization of lactic acid for the production of PHB, while it is also known that plant oils are an excellent substrate (Fukui and Doi, 1998). *R. eutropha* was also capable of producing the PHB homopolymer from even-carbon numbered n-alkanoates, while odd-carbon numbered n-alkanoates resulted in the accumulation of copolymers with 3HB and 3HV units (Akiyama *et al.*, 1992). Studies of PHA production under autotrophic conditions have resulted in the finding that *R. eutropha* can also utilize carbon dioxide for the production of PHB (Ishizaki and Tanaka, 1991). In addition to the carbon sources mentioned above, *R. eutropha* is also capable of accumulating PHA from specialized carbon sources such as 4-hydroxybutyric acid, g-butyrolactone and 1,4-butanediol which gave rise to the incorporation of 4HB monomers along with 3HB (Doi *et al.*, 1990a and 1990b).

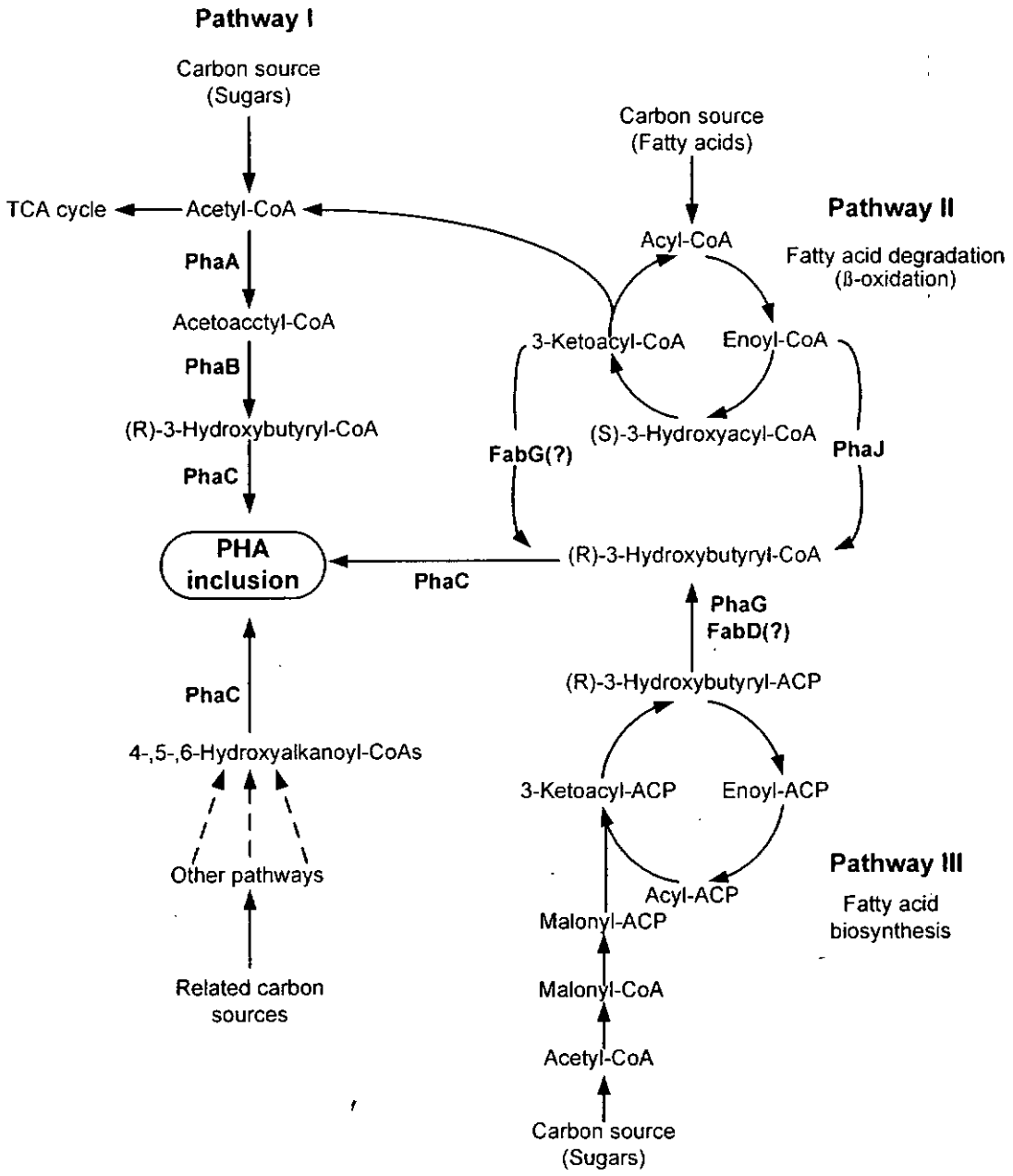


Figure 2.5. Metabolic pathways that supply hydroalkanoate monomers for PHA biosynthesis. PhaA, β-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacel-ACP-CoA transferase; PhaJ, (R)-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA reductase (Adapted from Sudesh *et al.*, 2000)

2.2.2.2. PHA biosynthesis represented by the pseudomonads.

Another type of PHA biosynthesis pathway is exhibited by the pseudomonads belonging to the rRNA-homology-group I which can synthesize medium-chain length PHA (MCL-PHA) from various alkanes, alkanols or alkanoates (Lageveen and Huisman, 1988). The first report on MCL-PHA described the growth and PHA production of *P. oleovorans* on *n*-octane as the only carbon source and ammonium as a growth limiting nutrient (de Smet *et al.*, 1983). The PHA copolymer isolated consisted of 3-hydroxyoctanoate (3HO) and 3-hydroxyhexanoate (3HHx) monomers (de Smet *et al.*, 1983). More organisms were subsequently isolated that are able to produce MCL-PHA, and it appears that all fluorescent pseudomonads belonging to the rRNA homology group 1 are able to synthesize such MCL-PHA.

The range of monomers incorporated into PHA synthesized by these pseudomonads are much wider. Bacteria in this group, convert the 3-hydroxyacyl-CoA substrates of C6-C14 for MCL-PHA synthesis, from the intermediates of fatty acid β-oxidation pathway (Figure 2.5, Pathway II). When *P. oleovorans* was cultivated on *n*-alkane under nitrogen limitation, the monomer composition of the PHA was found to be a reflection of the substrates that were provided (Lageveen and Huisman, 1988). The largest monomer found in PHA, always contained as many carbon atoms as did the *n*-alkane used as a substrate. For C-even substrates, only C-even monomers were found, the smallest was 3HHx. For C-odd substrates, only C-odd monomers were found. Unsaturated

monomers were also incorporated when the carbon sources were 1-alkenes (Lageveen and Huisman, 1988). Specific enzymes such as the enoyl-CoA hydratase (PhaJ) (Fukui *et al.*, 1999) and 3-ketoacyl-CoA reductase (FabG) (Taguchi *et al.*, 1999) are presumably involved in the conversion of fatty acid β-oxidation intermediates into suitable monomers that can then be polymerized by the PHA synthase.

P. oleovorans produces MCL-PHA through β-oxidation by using related carbon substrates. Otherwise, most of the rRNA homology-group I pseudomonads except *P. oleovorans*, can also synthesize PHA containing MCL monomers from unrelated carbon sources such as fructose, glucose and glycerol, or summarized substrates that require fatty acid *de novo* synthesis. These bacteria accumulate PHA containing 3-hydroxydecanoate (3HD) as the predominant monomer from various carbon sources such as gluconate, fructose, acetate, glycerol and lactate (Anderson and Dawes, 1990). In this case, the 3-hydroxyacyl monomers are derived from the *de novo* fatty acid biosynthesis pathway (Huijberts *et al.*, 1994) (Figure 2.5, Pathway III). *Pseudomonads* that have been shown to possess this pathway are *P. aeruginosa*, *P. aureofaciens*, *P. citronellolis*, *P. mendocina* and *P. putida*. Since the fatty acid biosynthesis intermediate is in the form of (R)-3-hydroxyacyl-ACP, an additional biosynthetic step is needed to convert it into the (R)-3-hydroxyacyl-CoA form. An enzyme known as PhaG, which exhibits a 3-hydroxyacyl-CoA-ACP transferase activity, has been shown to be capable of channeling the intermediates of the *de novo* fatty acid biosynthesis

pathway to PHA biosynthesis (Rehm *et al.*, 1998).

When *P. oleovorans* is starved for carbon, the MCL-PHA content decreases in a first order reaction due to the degrading activity delivering organic carbon/energy for cell maintenance through the fatty acid oxidation and TCA-cycle (Zinn *et al.*, 2001).

2.2.2.3. Other metabolic pathways.

Aside from the common 3-Hydroxyalkanoates (3HA) which is oxidized at the third carbon, other HA oxidized at a different carbon have also been frequently reported, such as 4-HA. Besides 4-hydroxybutyrate (4HB), 4-hydroxyvalerate (4HV), 4-hydroxyhexanoate (4HHx), 4-hydroxyheptanoate (4HHp) and 4-hydroxyoctanoate (4HO) are also known to be constituents of PHA. Other known non-3-HAs are 5-HA and 6-HA. Among these non-3-HAs, the incorporation of 4HB as a monomer into PHA has resulted in the production of commercially attractive copolymers (Sudesh *et al.*, 2000 and Zinn *et al.*, 2001).

The known wild-type bacteria having the ability to incorporate 4HB into PHA are *R. eutropha* (Valentin *et al.*, 1995), *A. latus* (Hiramitsu and Doi, 1993), *C. acidovorans* (Saito and Doi, 1994), *R. ruber* (Haywood *et al.*, 1991), *C. testosteronii* (Renner *et al.*, 1996) and *Hydrogenophaga pseudoflava* (Yoon and Choi, 1999). The bacteria from the genus *Comamonas* apparently have the most efficient pathway to supply 4HB monomers, based on the exceptionally high mole fractions of 4HB (above 90%) in the accumulated PHA copolymers (Saito and

Doi, 1994; Renner *et al.*, 1996). However, HA monomers oxidized at the fourth carbon are usually only derived from related carbon sources such as 4-hydroxybutyric acid, 1,4-butanediol and g-butyrolactone by the wild-type bacteria (Saito *et al.*, 1996).

2.2.4. PHA production by recombinant bacteria

Nature PHA-producing bacteria have a long generation time and relatively low optimal growth temperature. These are often hard to lyse and contain pathways for PHA degradation. Bacteria such as *E. coli* are incapable of synthesizing or degrading PHA; however *E. coli* grows fast by using wider range of cheap carbon sources, even at high temperature and is easy to lyse. Fast growth will enable it to accumulate a large amount of polymer. By using cheap carbon sources for PHA accumulation by *E. coli* results in the cost reducing. The easy lysis of the cells also saves the cost of the purification of the PHA granules. Besides that, since *E. coli* does not have an intracellular PHA depolymerase (because *E. coli* is not a natural PHA producer), the synthesized PHA will not be degraded. This is probably one of the reasons that had enabled the production of very high molecular weight PHA using recombinant *E. coli*. (Steinbüchel and Schlegel, 1991; Kusaka *et al.*, 1997; Wang and Bakken, 1998; Madison and Huisman, 1999 and Sudesh *et al.*, 2000).

Table 2.6. PHAs available from recombinant *E. coli*.

(modified from Steinbüchel, 2001)

PHA	*Reference*
Poly(3HB)	Slater *et al.*, 1988; People *et al.*, 1989; Kusaka *et al.*, 1997
Poly(4HB)	Hein *et al.*, 1997; Valentin and Dennis, 1997
Poly(4HV)	Liu and Steinbüchel, 2000
Poly(3HB-co-3HV)	Slater et al., 1992
Poly(3HB-co-4HB)	Hein *et al.*, 1997; Valentin and Dennis, 1997; Liu and Steinbüchel., 2000
Poly(3HB-co-4HV)	Liu and Steinbüchel, 2000
Poly(3HB-co-4HB-co-4HV)	Liu and Steinbüchel, 2000
Poly(3HB-co-3HHx)	Fukui *et al.*, 1999; Taguchi *et al.*, 1999
Poly($3HA_{MCL}$)	Qi *et al.*, 1997; Langenbach *et al.*, 1997

Several quite different PHAs can be obtained from *E. coli* recombinant and these are summarized in Table 2.6.

In summary, *E. coli* offers a well-defined physiological environment for the construction and manipulation of various metabolic pathways to produce a wide range of PHA from cost effective carbon sources.

2.3 Production of PHA by transgenic yeast, the eukaryotes.

The ability to synthesize and accumulate PHA is common among prokaryotes (Dawes and Senior, 1973; Anderson and Dawes, 1990; Brandl *et al.*, 1990 and Steinbüchel, 1991). However, in eukaryotes the polymers have only been detected in low concentrations.

Given the availability of bacterial PHA biosynthesis genes and the development of methods for transferring them to eukaryotic systems, it ought to be possible to equip higher organisms like plants or yeasts (Leaf *et al.*, 1996) with the ability to synthesize PHA. This could be one way of making PHA synthesis cheaper. The first attempts at PHA synthesis in yeasts were undertaken with *Saccharomyces cerevisiae* (Leaf *et al.*, 1996; Carlson *et al.*, 2002 and Breuer *et al.*, 2002).

Leaf *et al.* (1996) reported that the PHB synthase gene of *R. eutrophus*, the prokaryotes, was used to construct a yeast plasmid which enabled expression of the functional synthase enzyme in *S. cerevisiae*. Cells transformed with the synthase plasmid accumulated up to 0.5% of CDW as PHB, with accumulation occurring in the stationary phase of batch growth.

Breuer *et al.* (2002) successfully established the genes of the PHB synthesis pathway of *R. eutropha* and *Methylobacterium extorquens* in the yeast *S. cerevisiae*. It was found that expression of just the PHA synthase gene in some experiments, and all three PHB genes (i.e., the genes encoding β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase) in others, were detected in *S.*

cerevisiae. Thus, it can be used as a "cell factory" for the production of PHB (Figure 2.6). The maximum amount o polyester accumulated was 6.2% (wt/wt.), and total specific production rate was 0.0035 $g.g^{-1}.h^{-1}$, when all three genes were expressed. These values exceed the values published by Leaf *et al.* (1996), but these results were not yet commercially satisfactory.

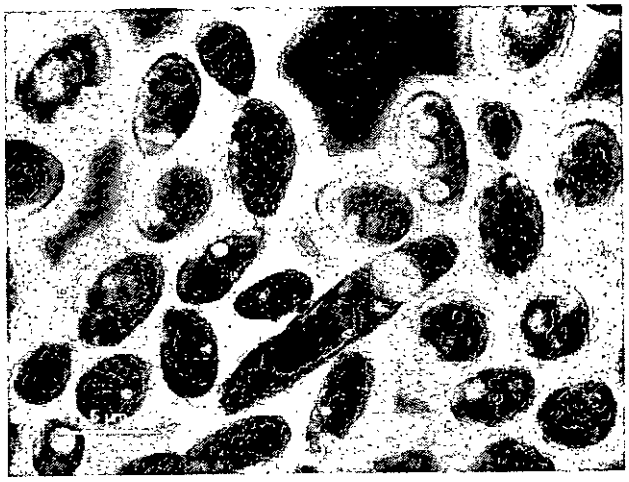


Figure 2.6 Yeasts and its storage compounds (adapted from Breuer *et al.*, 2002)

Poirier *et al.* (2001 and 2002a) has carried out the research of the synthesis of PHA in the peroxisome of *S. cerevisiae* by using intermediated of fatty acid β-oxidation. In his research, *S. cerevisiae* was transformed with the *Pseudomonas aeruginosa* PHAC1 synthase modified for peroxisome targeting by the addition of the carboxyl 34 amino acids from the *Brassica napus* isocitrate lyase. PHA synthase expression and PHA accumulation were found in recombinant *S. cerevisiae* growing in media containing fatty acids. PHA containing even-chain

monomers from 6 to 14 carbons was found in recombinant yeast grown on oleic acid, while odd-chain monomers from 5 to 15 carbons were found in PHA from yeast grown on heptadecenoic acid. The maximum amount of PHA accumulated reached 0.45% of the CDW. Figure 2.6 and Figure 2.7 are the pictures of analyzing the PHA contents of the wild-type and recombinant yeasts by transmission electron microscopy (TEM). As illustrated in Figure 2.7, the wild-type (A) or recombinant yeast expressing the PHA synthase gene (B to D) was used to inoculate media containing 0.1% glucose, 2% pluronic-127, and 0.1% oleic acid and was grown for 4 days before being processed for TEM. Panels C and D are close-up views of panel B. Arrows indicated the presence of PHA inclusions within membrane-bound organelles.

It was also found that *S. cerevisiae* expressing a peroxisomal PHA synthase produces PHA in the peroxisom using the 3-hydroxyacyl coenzyme A intermediates of the β-oxidation of fatty acids present in the media. Thus, the authors concluded that *S. cerevisiae* could be used as a powerful model system to learn how fatty acid metabolism can be modified in order to synthesize high amounts of PHA in eukaryotes, even including plants (Poirier *et al.*, 2001 and 2002a).

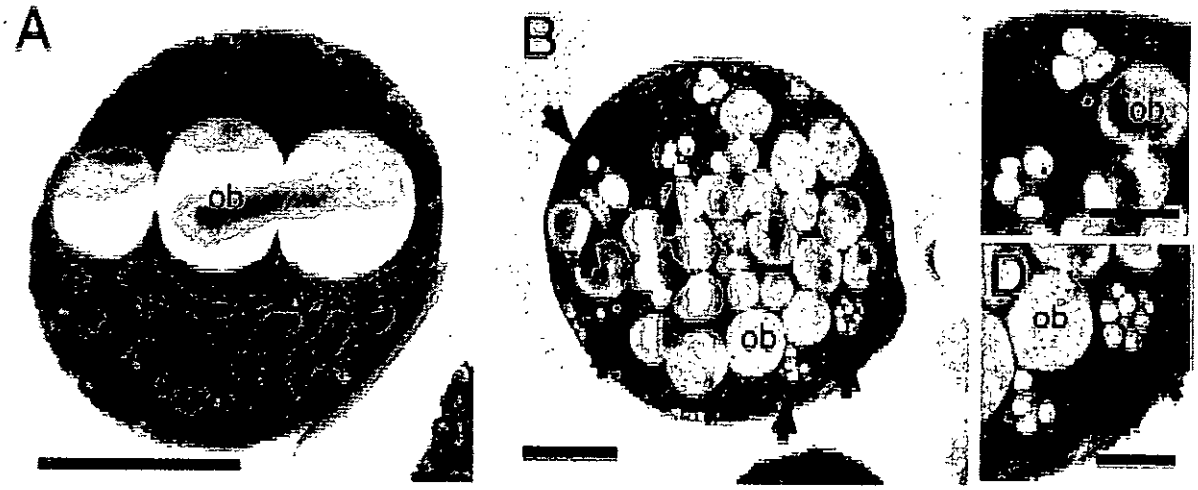


Figure 2.7 Analysis of PHA inclusion in *S. cerevisiae* by transmission electron microscope (TEM). The wild-type (A) or recombinant yeast expressing the PHA synthase gene (B to D) was used to process for TEM analyzing. Panels C and D are close-up views of panel B. Arrows indicated the presence of PHA inclusions within membrane-bound organelles. (ob, oil body; Bars indicate 1μm (A and B) and 0.5 μm (C and D)). (adapted from Poirier *et al.*, 2001).

Carlson *et al.* (2002) has used elementary mode analysis to study a metabolic pathway model of a recombinant *S. cerevisiae* system that was genetically engineered to produce the bacterial storage compound PHB (Figure 2.8). The model includes biochemical reactions from the intermediary metabolism and takes into account cellular compartmentalization as well as the reversibility/irreversibility of the reactions. The reaction network connects the production and/or consumption of eight external metabolites including glucose, acetate, glycerol, ethanol, PHB, CO_2, succinate, and adenosine triphosphate (ATP). Elementary mode analysis of the wild-type *S. cerevisiae* system reveals 241 unique reaction combinations that balance the eight external metabolites. When the recombinant PHB pathway is included, and when the reaction model is altered to simulated the experimental conditions when PHB accumulates, the analysis reveals 20 unique elementary modes. Of these 20 modes, 7 produce PHB with the optimal mode having a theoretical PHB carbon yield of 0.67. Elementary mode analysis was also used to analyze the possible effects of biochemical network modifications and altered culturing conditions. When the natively absent ATP citrate-lyase activity is added to the recombinant reaction network, the number of unique modes increases from 20 to 496, with 314 of these modes producing PHB. With this topological modification, the maximum theoretical PHB carbon yield increases from 0.67 to 0.83. Adding a transhydrogenase reaction to the model also improves the theoretical conversion of substrate into PHB. The recombinant

system with the transhydrogenase reaction but without the ATP citrate-lyase reaction has an increase in PHB carbon yield from 0.67 to 0.71. When the model includes both the ATP citrate-lyase reaction and the transhydrogenase reaction, the maximum theoretical carbon yield increases to 0.84. The reaction model was also used to explore the possibility of producing PHB under anaerobic conditions. In the absence of oxygen, the recombinant reaction network possesses tow elementary modes capable of producing PHB. Interestingly, both modes also produce ethanol. Elementary mode analysis provides a means of deconstructing complex metabolic networks into their basic functional units. This information can be used for analyzing existing pathways and for the rational design of further modifications that could improve the system's conversion of substrate into product.

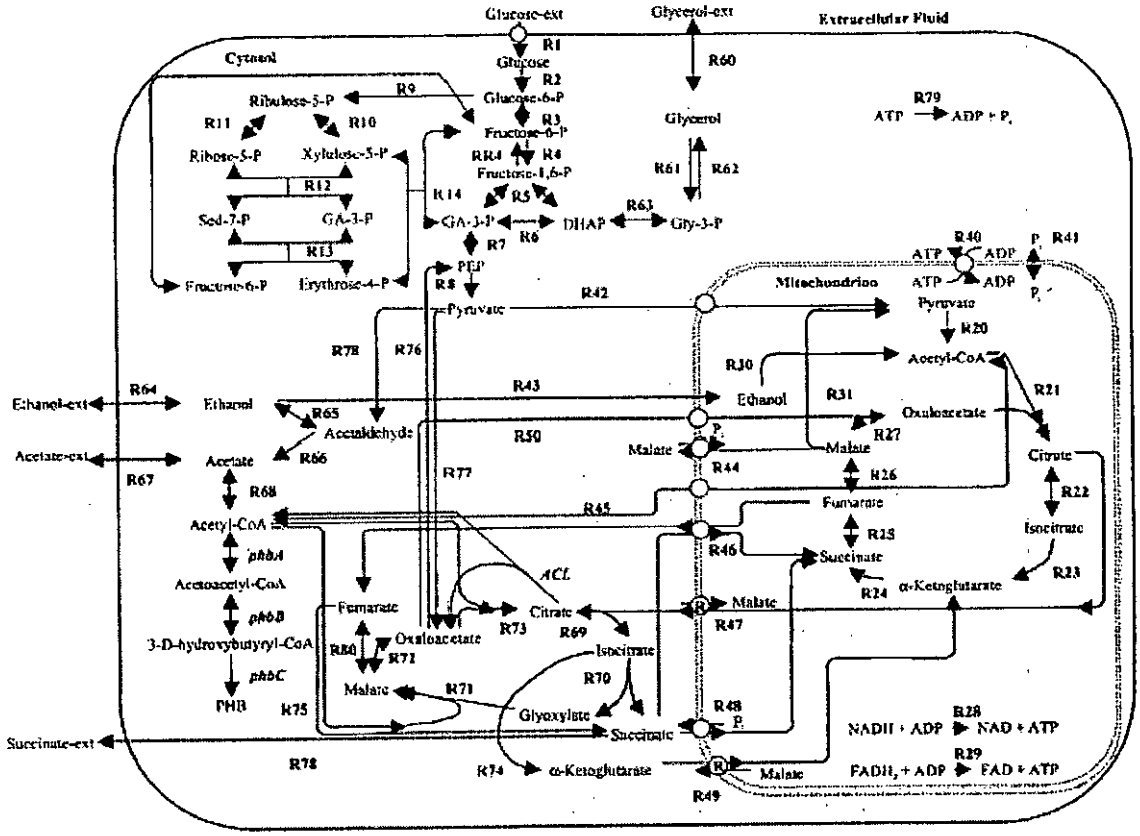


Figure 2.8 Biochemical network model for a recombinant *S. cerevisiae* strain expressing the three-gene PHB pathway (β-ketothiolase [phbA], reductase [phbB], and synthase [phbC]). The model contains 64 reactions, with 28 being reversible, and 67 metabolites, with 8 considered external. The external metabolites include glucose, acetate, ethanol, glycerol, PHB, ATP,CO_2, and succinate. Reactions and metabolites are partitioned between extracellular, cytosolic, and mitochondrial compartments. (adapted from Carlson *et al.*, 2002)

In microorganisms, PHA synthesis usually starts if a carbon source is available in excess, but growth and multiplication are limited by other substrates, e.g. nitrogen, as described previously. Contrarily, in transgenic *Escherichia coli* harboring the three PHB genes, polymer formation is not a consequence of unbalanced growth conditions, but it occurs under growth conditions (Babel *et al.*, 2001). In transgenic PHB-synthesizing yeasts, the genes are induced by galactose during growth, and thus PHA synthesis could be growth-associated, as well as in *E. coli* recombinant.

Using yeasts for synthesizing products offers several advantages: they are traditionally used in biotechnological procedures; yeast cells are larger than bacteria and are, therefore, easier to process; yeast metabolism and nutrition physiology are sufficiently flexible to facilitate such production; wild types are not ecologically harmful.

2.4 The methods for PHA Production by the bacterial pure culture

So far as I know, there are four biosynthetic approaches to produce PHA including *in vitro* via PHA-polymerase catalyzed polymerization, and *in vivo* with batch, fed-batch, and continuous (chemostat) cultivation (Zinn *et al.*, 2001).

2.4.1. In vitro synthesis of PHA

Steinbüchel (2001) has summarized six different systems for the *in vitro* biosynthesis of PHAs employing purified PHA synthases and other enzymes. So far, all PHA synthases employed in these studies were isolated from cells of recombinant strains of *E. coli* since efficient purification procedures for PHA synthases from the original hosts have not been elaborated. PHA synthase of *R. eutropha* has been used to synthesize PHB in one-enzyme systems from 3-hydroxybutyryl-CoA (Gerngross and Martin, 1995). It was observed that PHA granules formed and the granules size could be modulated by the addition of a *phasin*. However, the costs are extraordinarily high due to the use of 3-hydroxybutyryl-CoA as substrate. Otherwise, CoASH released from the substrate inhibited the PHA synthases, and therefore, the reaction does not proceed well or to a large extent. Thus, multiple-enzyme system were developed to use cheaper substrates and to recycle the cofactors, in particular CoASH. Employing a two-enzyme system using purified PHA synthase of *C. vinosum* and propionyl-CoA transferase from *Clostridium propionicum*, acetyl-CoA could be converted into poly(3HB). Recent reports have demonstrated achievements in the

in vitro synthesis of PHB by using a three-enzyme system from yeast that can recycle the expensive CoASH (Jossek *et al.*, 1998a and b).

Much efforts have been performed on research for the *in vitro* production recently because of its advantages over the *in vivo* synthesis (Table 2.7), e.g., production can be controlled through the addition of PHA-precursors and cofactors (Qi *et al.*, 2000). Moreover, isolation of PHA is much easier since the extraction from cells is not necessary anymore. However, the recycling of cofactors appears to be a problematic and expensive procedure. Nevertheless, it was reported that purified polymerase from *R. eutropha* formed granules up to 3 μm in size when exposed to 3-hydroxybutyryl-CoA (Gerngross and Martin, 1995). The molecular weight of the PHA was 10^7 Da.

Advantages and limitations of *in vitro* synthesis and production processes are listed in Table 2.7. *In vitro* engineering of pathways may be a useful strategy to evaluate whether the establishment of a particular pathway in a bacterium by *in vivo* metabolic engineering is feasible. Otherwise, it is thought that *in vitro* system might be a useful tool designed for studying the possible metabolic pathways for PHA accumulation *in vivo* by bacteria and activated sludge co-culture under particular conditions.

Table 2.7. The pros and cons of *in vitro* metabolic engineering for PHA accumulation (adapted from Steinbüchel, 2001)

Advantages and prospects	*Limitations and problems*
• Detailed biochemical studies. Through characterization of the enzyme(s) becomes possible. • Application of non natural substrates. PHAs with new constituents may be produced. • Rapid shifts of substrates applicable. PHAs with a novel order of constituents may be produced (e.g. blockcopolyesters). • Cell-free system. Production is limited by the volume of the reactor and not by the volume of the cytoplasm. Increase of PHA produced per volume is possible. Synthesis may be performed in the presence of compounds or under conditions which are inhibiting cells. No expression required. Amount of enzymes can be easily varied. • Only few components required. Downstream processing will be easier. • Requires less effort than *in vivo* metabolic engineering. Fast evaluation of the feasibility of a strategy and the functionality of a "designed" pathway. • Special applications. *In situ* PHA formation becomes possible.	• Purified PHA synthase is required. • Additional enzymes must be available in a purified stage. • All enzymes must be stable. • Coenzymes must be recycled.

2.4.2. In vivo synthesis of PHA

The synthesis and production of PHA *in vivo* has been and is still mainly investigated and approached using batch pure cultures of the bacteria. Batch cultures are easy to handle and are suited for growth studies and screenings for potential PHA accumulating organisms. Generally, the medium is designed in such a way that one nutrient, mostly nitrogen, limits growth of biomass while other nutrients including the carbon source are in excess. Depending on the microorganism and the substrate(s) the experiments are mainly performed within 24-48 hours. By this time, it is as known that the cells go through a sequence of growth stages, such as lag, exponential growth, PHA production, stationary, and finally decline phases. Concomitantly, the cells perceive a continuous change of their environment due to the ever-changing nutrient concentration caused by the cell metabolism. Since cells that become starved for carbon degrade PHA again, this method rarely gives an indication of the maximum capacity of the cells to accumulate PHA (Zinn *et al.*, 2001).

The fed-batch culture is basically a batch culture that is continuously supplemented with selected nutrients after it enters the late exponential phase. Biopol, a PHBV copolymer that consists of 3HB and 3HV in various molar ratios, was produced by Monsanto in such a fed-batch mode with a mutant of *R. eutropha* that could grow on glucose (Table 2.3). The production process consisted of two main phases. In the first phase, the cells were cultured in a

minimal medium which contained the essential growth nutrients, glucose, and low amounts of phosphate, supporting cell growth to a certain biomass concentration and only minor PHA accumulation (Byrom, 1987). In the second phase, after all the phosphorus was consumed by the cells, PHA accumulation took place instead. PHA accumulation was driven through the continuous addition of glucose and propionic acid to the culture at well defined rates. After 48 hours of feed the PHA consisted of about 80 mol% 3HB and 20 mol% 3HV was obtained. The process was stopped when the PHA content of the cells had reached a desired level, generally between 70 and 80% of the CDW. The fed-batch mode was the culture method conducted since propionic acid at high concentration is toxic to the cells and can inhibit cell growth. The advantage of fed-batch cultures in general is the high cell densities (Lee and Chang, 1995; Kellerhals *et al.*, 1999, 2000) that can be obtained which reduce the costs of PHA production significantly (Lee and Choi, 1999a and Lee *et al.*, 1999b). However, a disadvantage of the process is that the cells grow at a decreasing growth rate when the feed rate and feed concentration are kept constant. The reason is that the added nutrients are consumed by an ever-increasing cell concentration during identical time units. This can lead to unexpected losses in PHA production (Suzuki *et al.*, 1988) and a shift in copolymer composition (Majid *et al.*, 1999).

Finally, the fourth method to produce PHA biotechnologically, the chemostat, is the most controlled cultivation method. In such a system the culture broth is

continuously exchanged with sterile growth medium. According to the theory of Monod (1942) the specific growth rate of the culture can be set by the ratio of feed rate to volume of the culture broth. This allows the determination of the influence of a well-defined growth condition (e.g., nutrient limitation at specific growth rate) on PHA accumulation (Durner *et al.*, 2000; Durner, 2001 and Zinn, 1998). However, to date this method is not yet applied for PHA production on a large scale, although a high PHA productivity can be obtained when appropriate growth conditions are selected (Zinn, 1998). The potential chemostat production can be increased further when two chemostats are connected in sequence. Jung *et al.* (2001) reported that the MCL-PHA productivity in *P.oleovorans* could be increased to a volumetric productivity of 1.06 g l^{-1} h^{-1} and that the PHA content attained 63% (w/w) of the CDW.

2.5 Economics of PHA production by microorganisms fermentation

Unfortunately, the current production costs of PHA are much higher than for petrol-based plastics. It is the major barrier to its commercialization and hence a widespread usage of this high-quality material as a bulk-packaging material cannot be expected in the near future. However, much effort has still been put into the lowering of the PHA cost during recent years. Whereas the price of most commodity plastics derived from petroleum, such as polypropylene, is below US$ 1 per kilogram, the most optimistic cost estimate for the production of PHB or PHBV by bacterial fermentation from *R. eutropha* or recombinant *E. coli* is US$

3-5 per kilogram (Poirier *et al.*, 1999 and Lee, 1996b). Furthermore, a recent study indicates that the energy and carbon costs associated with synthesis of bacterial PHA from corn-derived glucose are higher than for the synthesis of polystyrene (Gerngross, 1999).

It is a prerequisite to standardize all the fermentation conditions for the successful implementation of commercial PHA production systems. The price of the product ultimately depends on the substrate cost, PHA yield on the substrate, and the efficiency of product formulation in the downstream processing (Lee, 1996b). This implies the necessary of high levels of PHA as a percentage of CDW and high productivity in terms of gram of product per unit volume and time (de Koning and Witholt, 1997; de Koning *et al.*, 1997) for the cost controlling of PHA production.

For the purpose of lowering the final price of PHA, it is important to develop a fermentation strategy that allows high PHA productivity. Moreover, the carbon source should be inexpensive since it is the major contributor to the total substrate cost. For the production process of *E. coli* with PHB productivity of 3.2 g/l.h, the cost of the carbon source was as high as 38% of the total operating cost when the production scale was assumed to be 100,000 tons per year (Choi and Lee, 1999a). Table 2.8 summarizes the cost of carbon substrates on the basis of the theoretical yield (Lee, 1996b). Due to their low price, crude carbon substrates such as cane and beet molasses, cheese whey, plant oil and hydrolysates of starch (corn and tapioca), cellulose and hemicellulose, can be excellent substrates for the bacteria

utilizing them. Several bacteria can produce PHB from these inexpensive carbon substrates, but the PHB content and productivity are much lower than those obtainable with purified carbon substrates. The PHA content can be considered as a measurement of the cell's ability to accumulated PHA under certain conditions. High PHA content often results in high PHA yield and is beneficial for the polymer recovery process.

On the other hand, as a novel technology for the PHA production, combined the PHA production process with wastewater treatment process has been proposed (Chua *et al.*, 1997a; Hu *et al.*, 1997; Satoh *et al.*, 1998 and Takabatake *et al.*, 2000). As described by the authors, the cost of PHA produced through this method is lower than that of the methods mentioned above. It is because the sterilization facilities for the pure cultural fermentation are not necessary anymore.

Table 2.8 Cost-balance sheet of substrate and P(3HB) yield

(adapted from Madison and Huisman, 1999)

Substrate	*Substrate price (US$/kg)*	*PHB yield (g PHB/g substrate)*	*Product cost (US$/kg PHB)*
Glucose	0.493	0.38	1.30
Sucrose	0.290	0.40	0.72
Methanol	0.180	0.43	0.42
Acetic acid	0.595	0.38	1.56
Ethanol	0.502	0.50	1.00
Cane molasses	0.220	0.42	0.52
Cheese whey	0.071	0.33	0.22
Hemicellulose hydrolysate	0.069	0.20	0.34

2.6 Production of PHA in transgenic plants

For thousands of years, plants have provided humans with useful polymers, such as cellulose fibers from cotton or flax. In the last decades, however, synthetic polymers derived from petroleum, and in particular plastics, have replaced natural polymers in many aspects of our daily lives. Whereas with the growing awareness, petroleum is a finite resource and that the indestructibility of plastics can be, in many cases, more of a nuisance than a benefit, there has been growing interest in producing biodegradable plastics from renewable resources (Poirier, 1999).

The capability developed in recent years to transfer genes from a number of sources, including bacteria, into plants has led several groups to speculate that PHB could be made in a crop plant. The commercial incentive to accomplish this derives from the fact that the cost of products from crop plants is significantly lower than products from fermentation. This is because transgenic plants could produce PHAs directly from CO_2 and solar energy and, at least theoretically, at costs which are comparable to those of other biopolymers already obtained from plants (see Figure 2.9 and Figure 2.10) (Byrom, 1994; Poirier, 1999 and Steinbüchel, 2001). In addition, as a successful and commercializing technology, to produce PHA from transgenic plants has to meet some requirement. Table 2.9 summarizes and lists the requirements on which the successful generation of transgenic plants suitable for the production of PHAs depends. Most of these requirements have already been met, intensive research is still necessary.

Table 2.9. The requirements of transgenic plants in producing PHAs (modified from Steinbüchel, 2001).

(i)	Availability of bacterial PHA biosynthesis genes.
(ii)	Transfer and functional expression of PHA biosynthesis genes in suitable crops.
(iii)	The transgenes stable over several generations.
(iv)	Single rather than multiple insertions of the heterologous genes into the plant genome.
(v)	Metabolic engineering to provide the PHA biosynthesis enzymes with appropriate intracellular concentrations of substrates.
(vi)	Metabolic engineering to divert from central intermediates substrates others than 3-hydroxybutyryl-CoA for PHA biosynthesis.
(vii)	Sufficiently high PHA content (>15% of plant dry matter).
(viii)	No significant repression of plant growth (<20%) due to PHA accumulation.
(ix)	Sufficient high $\overline{M}_w$ of the PHAs (>650000 g/mol).
(x)	Reliable and simple methods for extraction of PHA from the plant tissues and separation of PHA from residual non-PHA biomass.
(xi)	Measures to prevent uncontrolled release and distribution of PHA biosynthesis genes in the environment.
(xii)	Acceptance by farmers and consumers.
(xiii)	Increasing the value of crops by adding this novel characteristics to plants.
(xiv)	Transgenic plants and its products safe to human, animal and environment.

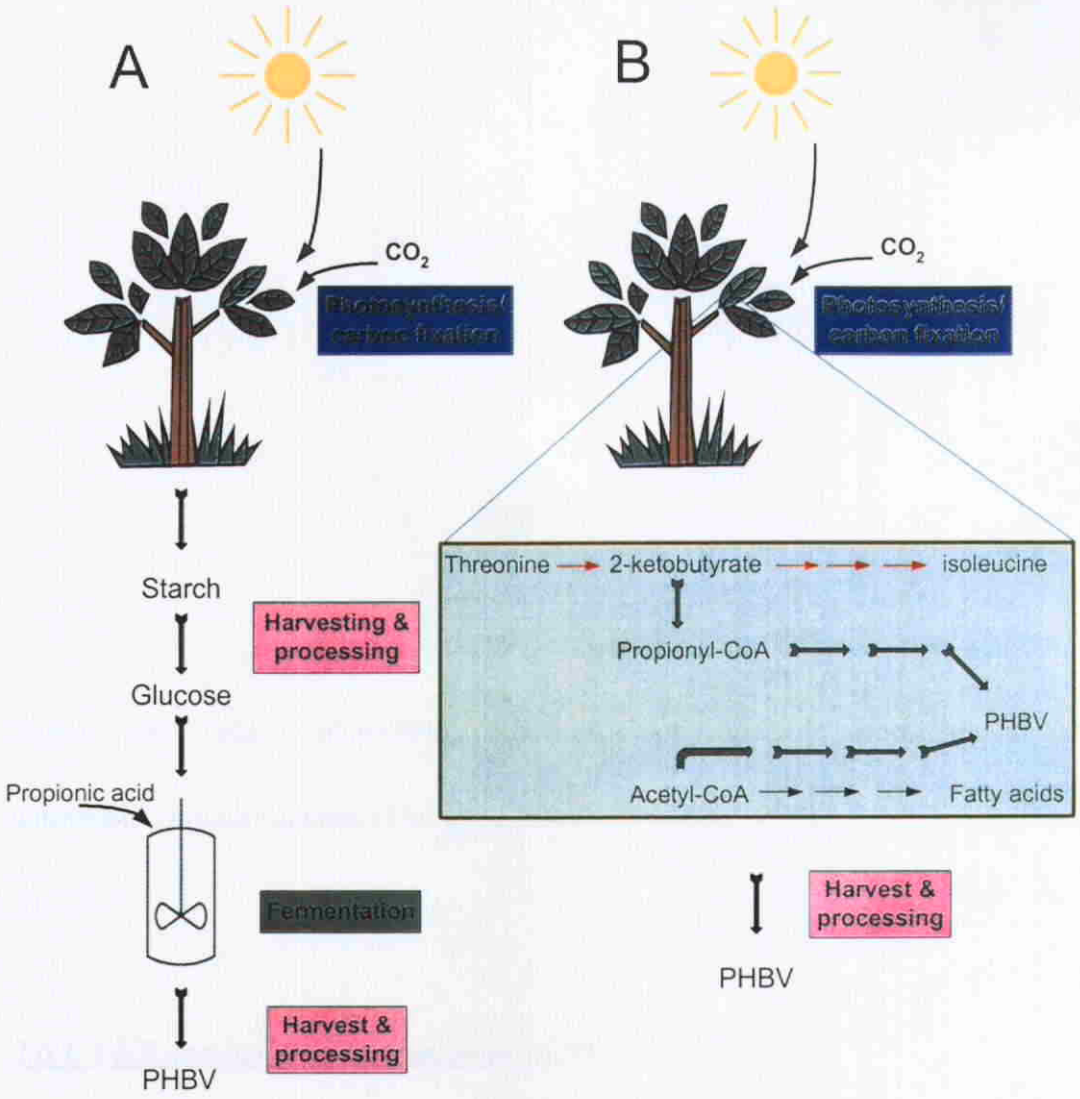
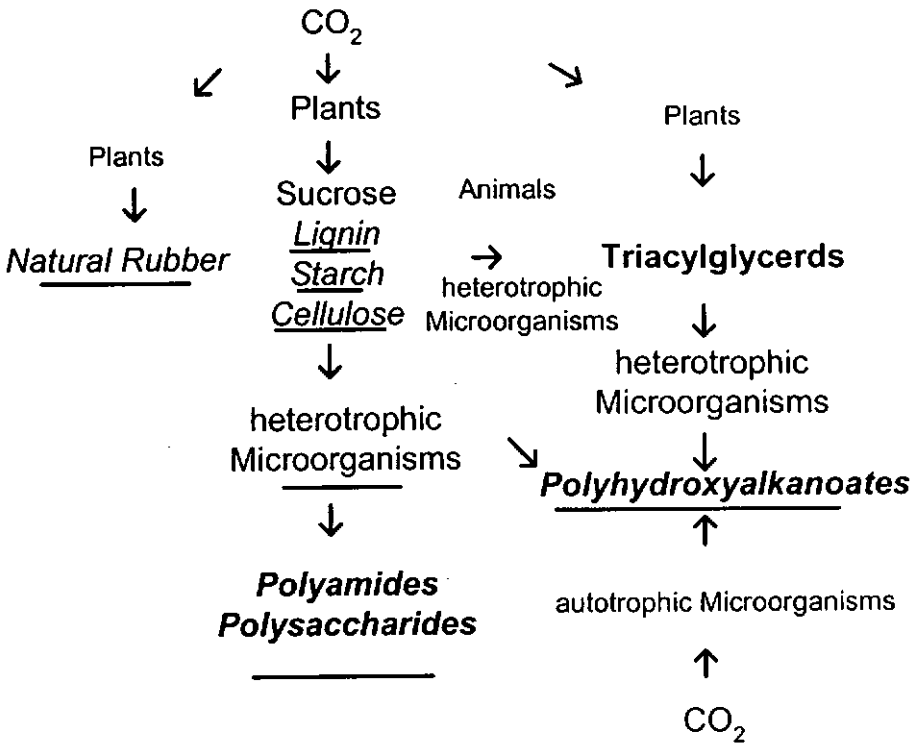


Figure 2.9. (A) Synthesis of PHBV by bacterial fermentation using glucose derived from crop plants. (B) Synthesis of PHBV directly in crop plants. The novel pathway created for the synthesis of PHBV in plants is indicated with large green arrows, and the endogenous metabolic pathways are in red arrows. (adapted from Poirier, 1999)



Figrue 2.10. Major renewable carbon sources available from plants and autotrophic microorganisms (Adapted from Steinbüchel, 2001).

2.6.1. PHB accumulation in transgenic plants

Production of a small amount of PHB in *Arabidopsis* was first demonstrated in 1992 by expressing in the cytoplasm two enzymes from the bacterium *R. eutropha* (Poirier *et al.*, 1992). The yield was later increased from 0.1% to 14% dry weight by expressing the PHB biosynthetic pathway in the plastids (Nawrath *et al.*, 1994).

It is known that, in the bacterium *R. eutrophus*, PHB is derived from acetyl-CoA by a sequence of three enzymatic reactions (Steinbüchle and Schlegel, 1991). The first enzyme of the pathway, 3-ketothiolase (acetyl-CoA acetyl

transferase; E.C. 2.3.1.9), catalyzes the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. Acetoacetyl-CoA reductase (hydroxybutyryl-CoA dehydrogenase; E.C. 1.1.1.36) subsequently reduces acetoacetyl-CoA to D-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of PHB synthase to form PHB. The genes encoding the three enzymes involved in PHB synthesis in *R. eutrophus* have been cloned, and expression of the genes in *E. coli* is sufficient for PHB production. Of the three enzymes involved in PHB synthesis in *R. eutrophus*, only the 3-ketothiolase is also found in the cytoplasm of higher plants, where it is involved in the synthesis of mevalonate the precursor to isoprenoids.

The *phb*B and *phb*C genes, which encoded acetoacetyl-CoA reductase and PHB polymerase, respecitively, were transferred into separate plants using an *Agrobacterium tumefaciens* Ti vector system, and the genes were demonstrated to be integrated into the plant chromosome by southern (DNA) blot. Both genes were transcribed into mRNA as judged by Northern (RNA) blots. The sequencing testing proved that transgenic plants that expressed both the bacterial acetoacetyl-CoA reductase and PHB synthase genes accumulated PHB at different parts of plants and cells (Figure 2.11 and Figure 2.12).

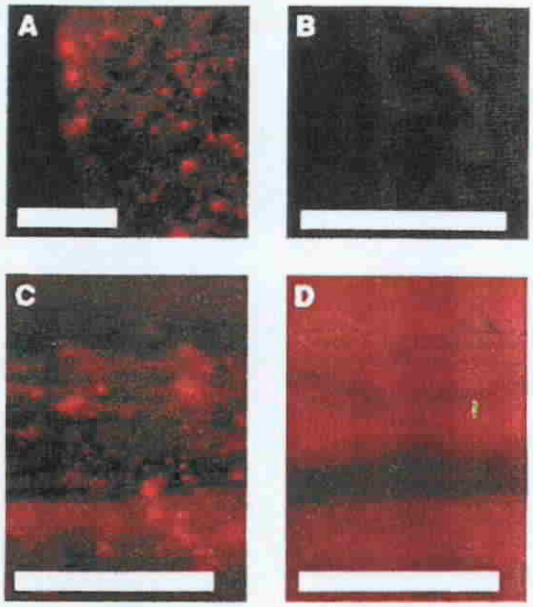


Figure 2.11. Visualization of PHB granules by epifluorescence microscopy of tissues stained with Nile Blue A leaves (A and B) and roots (C and D) from PHB-producing hybrids between RedB-2G (*phb*B+) and S8-1-2C (*phb*C+) (A and C) and from transgenic plants RedB-2G (*phb*B-) that did not produce PHB (B and D). (adapted from Poirier *et al.*, 1992)

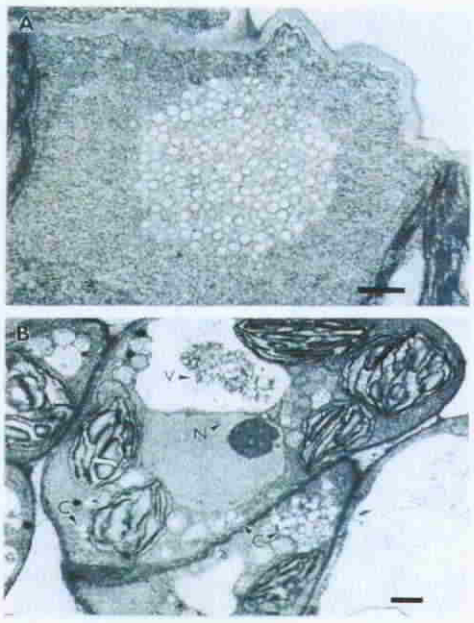


Figure 2.12. Transmission electron micrographas of thin sections from PHB-positive transgenic *A. thaliana* plants. (A) leaf mesophyll cells from a RedB-2D x S8-1-2A F1 hybrid with an agglomeration of granules in the nucleus. (B) Two adjacent mesophyll cells from a cotyledon of a RedB-2C x S8-1-2A F1 hybrid showing electron-lucent granules in the nucleus (N), vacuole (V), and cytoplasm (C). Arrows indicate agglomerations of electron-lucent granules. Bars represent 1 μm. (adapted from Poirier *et al.*, 1992).

Later, these PHB biosynthesis genes were also expressed in agricultural crops such as *Brassica napus* (Valentin *et al.*, 1999). The genes were engineered for plant plastid targeting and expressed using leaf or seed-specific promoters in *Arabidopsis* and *Brassica*. PHA yields in homozygous transformants were 12-13% of the dry mass in homozygous *Arabidopsis* plants and approximately 7% of the seed weight in seeds from heterozygous canola plants (Valentin *et al.*, 1999).

2.6.1.1. Engineering PHB formation in cotton

Gossypium hirsutum, cotton, was studied to accumulate PHB by John and Keller (1996) (Figure 2.13). *R. eutrophas* genes encoding the enzymes, β-ketothiolase (*pha*A), acetoacetyl-CoA reductase (*pha*B), and PHA synthase (*pha*C) catalyze the production of aliphatic polyester PHB from acetyl-CoA. PHB is a thermoplastic polymer that may modify fiber properties when synthesized in cotton. Endogenous b-ketothiolase activity is present in cotton fibers. Hence cotton was transformed with engineered *pha*B and *pha*C genes by particle bombardment, and transgenic plants were selected based on marker gene, b-glucuronidase (GUS), expression. Fibers of 10 transgenic plants expressed *pha*B gene, while eight plants expressed both *pha*B and *pha*C genes. Electron microscopy examination of fibers expressing both genes indicated the presence of electron-lucent granules in the cytoplasm (Figure 2.13). High pressure liquid

chromatography, gas chromatography, and mass spectrometry evidence suggested that the new polymer produced in transgenic fibers is PHB. PHB production ranged from 30 to 3440 μg/g of fiber (0.003-0.344%). Sixty-six percent of the PHB in fibers is in the molecular mass range of 0.6×10^6 to 1.8×10^6 Da. The presence of PHB granules in transgenic fibers resulted in measurable changes of thermal properties. The fibers exhibited better insulating characteristics than wild-type cotton. The rate of heat uptake and cooling was slower in transgenic fibers, resulting in higher heat capacity (Chowdhury and John, 1998). These data show that metabolic pathway engineering in cotton may enhance fiber properties by incorporating new traits from other genetic sources. This may be an important step toward producing new generation fibers for the textile industry. Since the observed changes in insulating properties are small, the authors note that it is likely that PHB production in cotton fibers will need to be increased several folds to be suitable for commercial applications.

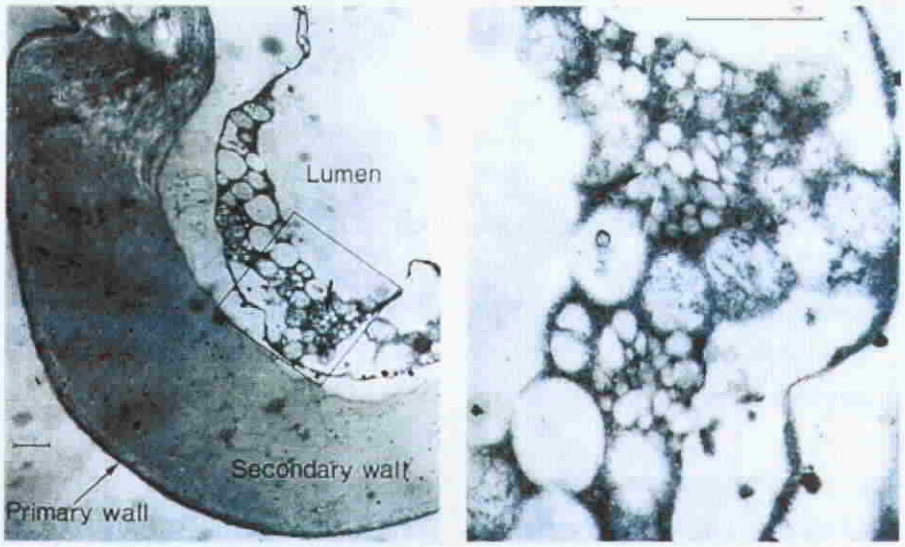


Figure 2.13. PHB granules in transgenic cotton fibers as shown in lucent TEM microscopes. Arrows indicate clusters of granules in the range of 0.15 to 0.3 μm. (Bars = 1 μm.) (adapted from John and Keller, 1996).

2.6.1.2. Engineering PHB formation in tobacco

Several researchers have attempted to produce PHB in the cytosol of tobacco and have obtained yields far lower than the 20-100 μg per gram of fresh weight obtained with cytosolic production in *Arabidopsis*. Nawrath *et al.* (1994 and 1995) transformed tobacco with genes encoding reductase and synthase, but were unable to detect PHB in the resulting transgenic plants. The specific activity of acetoacetyl-CoA reductase in the transgenic tobacco was approximately 100 times lower than in *Arabidopsis* plants transformed with the same genetic constructs, suggesting that expression levels of the enzymes in tobacco may limit PHB

production.

Nakashit *et al.* (1999) has successfully introduced the acetoacetyl-CoA reductase gene (*phb*B) of *R. eutropha* and the PHA synthase gene (phaC(AC)) of *Aeromonas caviae* into tobacco plant, *Nicotiana tabacum,* by *Agrobacterium* mediated transformation method. The resulting transgenic tobacco expressed both introduced genes and the expression of these genes in the cytosol of tobacco was confirmed by enzymatic analysis and western blotting. GC-MS analysis of the chloroform extract of tobacco leaves indicated that the transgenic plant produced biodegradable polyester, PHB. The transgenic tobacco plants producing PHB at less than 10 μg per gram of fresh weight were isolated. GPC analysis indicated that the number-average molecular weights and polydispersity were 32,000 Da. and 1.90, respectively.

Bohmert and Willmitzer (2000) reported the transformation of tobacco plants with a superconstruct containing the chloroplast-targeted *R. eutropha* thiolase, reductase, and synthase genes. The thiolase gene was placed under the control of a salicylic acid-inducible promoter. Up to 50 μg of PHB per gram of fresh cell weight (0.005% PHB per unit of fresh cell weight) was produced in these tobacco plants, significantly lower than yields reported for plastid-targeted PHB production in Arabidopsis.

An attempt to produce PHB upon direct transformation of the plastid genome has also yielded minimal amounts of product. Lossl *et al.* (2000) reported that the transformation of the plastid genome of tobacco with the *R. eutropha*

PHB operon and obtained only 400 ppm dry weight of PHB. Failure to produce higher levels of PHB may be due to the use of the native *R. eutropha* operon, including the *R. eutropha* promoter, regulatory elements, and intergenic spacers, which may not be ideal for transgene expression within the chloroplasts of tobacco.

2.6.1.3. PHB formation in corn's suspension cultures

Transgenic suspension cultures of Black Mexican Sweet maize (*Zea mays* L.) expressing the *R. eutrophus* genes encoding enzymes of the pathway for biosynthesis of PHB were established as a tool for investigating metabolic regulation of the PHB pathway in plant cells (Hahn *et al.*, 1997). Cultures were grown in a 2 l modified mammalian cell bioreactor and in shake flasks. Biomass doubling times for transgenic bioreactor cultures (3.42 ± 0.76 days) were significantly higher than those for untransformed cultures (2.01 ± 0.33 days). Transgenic expression of the bacterial enzymes β-ketothiolase (0.140 units/mg protein) and acetoacetyl-CoA reductase (0.636 units/mg protein) was detected by enzyme assays and immunoblotting. Hydroxybutyrate derivatives were detected by GC at up to 1.5 mg/g fresh weight; however, solvent washing experiments demonstrated that the hydroxybutyrate was primarily nonpolymeric. After the first 2 years of cultivation, reductase activity in the suspension culture decreased to 0.120 units/mg protein. Furthermore, the PHB synthase gene, although initially present, was not detectable after 1.5 years of cultivation in suspension culture.

These facts suggest that transgenic expression of PHB pathway genes in plant cells may not be stable within the suspension cultures. A hydroxybutyrate derivative was detected via gas chromatography even after 4 years of cultivation. Although the method used to prepare samples for gas chromatography (GC) cannot directly distinguish among PHB polymer, hydroxybutyryl-CoA (HB-CoA), and hydroxybutyric acid, solvent washing experiments indicated that most or all of the signal was non-polymeric, presumably HB-CoA. The synthesis of HB-CoA appeared to be linked to substrate growth limitation, with HB-CoA accumulation increasing dramatically and cell growth ceasing upon depletion of ammonium. This suggests a similarity in the regulation of HB-CoA formation in plant cells and prokaryotic cells (Hahn *et al.*, 1997).

Furthermore, Hahn *et al.* (1999) expressed *R. eutropha* genes modified for peroxisomal targeting of *pha*A, *pha*B and *pha*C in black Mexican sweet maize suspension cultures in an effort to produce PHB in the peroxisomes. Individual plasmids containing a 35S promoter and an expression cassette encoding peroxixomally targeted *pha*A, *pha*B, or *pha*C were introduced simultaneously into suspension cultures by particle bombardment. Plants containing all three peroxisomally targeted genes yielded PHB levels of 2 mg/g fresh weight (0.2% PHB), significantly higher than the 0.11 mg PHB/g fresh weight (0.011% PHB) observed in control plants containing untargeted genes. Interestingly, yields of 0.38 and 0.25 mg PHB/g fresh weight were observed in control plants containing peroxisomally targeted and untargeted *pha*C, respectively, suggesting that the

suspension cultures unexpectedly contained a basal amount of R-3-hydroxybutyryl-CoA. Hahn *et al.* (1999) proposed that the PHB is formed from a pool of R-3-hydroxybutyryl-CoA produced from 2-butenoyl-CoA via an R-3-hydroxybutyryl-CoA hydrolase enzyme.

However, PHB has poor physical properties, and is too stiff and brittle for use in most commodity products. But, PHB's poor physical properties have stimulated efforts to synthesize in plants a wider range of PHA copolymers with better characteristics. Some headway was made recently when PHBV copolymer and low amounts of MCL-PHAs copolymers were synthesized in plant or in plant peroxisomes in order to improve the physical and chemical characters of the PHA formed by transgenic plants (Valentin *et al.*, 1999; Slater *et al.*, 1999; Arai *et al.*, 2002 and Mittendorf *et al.*, 1998).

2.6.2. PHBV copolymer and MCL-PHAs accumulation in transgenic plants

2.6.2.1. PHBV accumulation in transgenic plants

Experiments designed to produce PHBV copolymer and MCL-PHAs in plants by engineering pathways for both 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA monomer unit formation were reported by several groups (Table 2.10) (Mittendorf *et al.*, 1998; Valentin *et al.*, 1999; Slater *et al.*, 1999; Arai *et al.*, 2002 and Poirier *et al.*, 2002).

Table 2.10. The PHAs available from transgenic plants.

PHA	*Reference*
Poly(3HB) (PHB)	Poirier *et al.*, 1992; Nawrath *et al.*, 1995; Hahn *et al.*, 1997, 1999; Nakashita *et al.*, 1999; Houmiel *et al.*, 1999; Bohmert and Willmitzer, 2000; Lossl *et al.*, 2000;
Poly(3HB-co-3HV) (PHBV)	Valentin *et al.*, 1999; Slater *et al.*, 1999 Arai *et al.*, 2002
Poly($3HA_{MCL}$) (MCL-PHAs)	Mittendorf *et al.*, 1998 Poirier *et al.*, 1999, 2002 Arai *et al.*, 2002

Valentin *et al.* (1999) introduced PHB biosynthesis genes into agricultural crops, *Brassica napus,* and expressed for the PHB accumulation. The genes were engineered for plant plastid targeting and expressed using leaf or seed-specific promoters in *Arabidopsis* and *Brassica*. He found that PHA yields in homozygous transformants were 12-13% of the dry mass in homozygous *Arabidopsis* plants and approximately 7% of the seed weight in seeds from heterozygous canola plants. When a threonine deaminase was expressed in addition to *bkt*B, *pha*B and *pha*C, a copolyester of PHBV was produced in both *Arabidopsis* and *Brassica*

(Valentin *et al.*, 1999). 0.7-2.3% of CDW of PHBV with 2.3-6.4 mol% HV has obtained. The overall polymer yield observed in canola seeds was slightly higher than the 0.2-0.8% observed in model studies in *Arbidopsis* leaves by Valentin *et al.* (1999). The total HV monomer unit incorporation in canola seeds was, however, lower than the 4-17% obtained in *Arabidopsis* leaves.

Slater *et al.* (1999) obtained similar results upon transforming a multigene vector in which seed-specific expression of *ilv*A, *R. eutropha pha*A, *pha*B, and *pha*C was obtained with the *Lesquerella* hydroxylase promoter. Yields of up to 1.5% total PHBV in *Brassica* seeds with 3 mol% 3HV were observed. Approximately 25% of these plants had low seed recovery, sterility, low vigor, or death of the plant before seed set, suggesting some strain on the plant with the expressed enzymes. Interestingly, attempts to boost the incorporation of 3HV in the polymer by expressing a gene encoding a fully deregulated *Ilv*A failed since transformants containing this gene could not be obtained. The authors suggest that supplementary routes for the conversion of 2-ketobutyrate to propionyl-CoA, or alternative means for generation of propionyl-CoA, will most likely need to be found to improve PHBV production in plants. As mentioned above, the overall PHBV copolymer yields obtained by Valentin *et al.* (1999) and Slater *et al.* (1999) are well below the estimated 15% target required for commercialization.

2.6.2.2.MCL- PHAs formation in plants

MCL-PHAs encompass a large group of PHAs containing 3-hydroxyacid

monomers ranging from 6 to 16 carbons in length. Compared to PHB and PHBV, which are plastics with properties close to polypropylene (PP), MCL-PHAs are considerably softer polymers, having properties similar to elastomers, rubbers and glues (Poirier, 1999).

Plant peroxisomes and plastids are attractive organelles for engineering MCL-PHA formation in that they possess metabolic pathways that proceed through medium chain length 3-hydroxyacyl intermediates. Peroxisomes are a site of fatty acid degradation in plants. Developing seedlings contain a high flux of carbon through medium chain length 3-hydroxy acyl-CoA intermediates when fatty acids are degraded to promote seedling growth. Plastids are the site of plant fatty acid biosynthesis and contain a high flux of carbon through 3-hydroxy acyl groups covalently attached to acyl-carrier protein.

Mittendorf *et al.* (1998) described MCL-PHA formation in *Arabidopsis* leaves using peroxisomal β-oxidation pathways as a source of monomeric units. MCL-PHAs are synthesized in bacteria by using intermediates of the β-oxidation of alkanoic acids. To assess the feasibility of producing MCL-PHAs in plants, *Arabidopsis thaliana* was transformed with the *pha*C1 synthase from *Pseudomonas aeruginosa* modified for peroxisome targeting by addition of the carboxyl 34 amino acids from the *Brassica napus* isocitrate lyase. Immunocytochemistry demonstrated that the modified PHA synthase was appropriately targeted to leaf-type peroxisomes in light-grown plants and glyoxysomes in dark-grown plants. Plants expressing the PHA synthase

accumulated electron-lucent inclusions in the glyoxysomes and leaf-type peroxisomes, as well as in the vacuole. These inclusions were similar to bacterial PHA inclusions. Analysis of plant extracts by GC and mass spectrometry demonstrated the presence of MCL-PHA in transgenic plants to approximately 4 mg per g of dry weight. The plant PHA contained saturated and unsaturated 3-hydroxyalkanoic acids ranging from 6 to 16 carbons with 41% of the monomers being 3-hydroxyoctanoic acid and 3-hydroxyoctenoic acid. These results indicate that the β-oxidation of plant fatty acids can generate a broad range of R-3-hydroxyacyl-CoA intermediates that can be used to synthesize MCL-PHAs.

Arai *et al.* (2002) reported that the PHA synthase gene (*pha*C(AC)) of *Aeromonas caviae* FA440 was modified by adding a peroxisome targeting signal encoding the last 10 amino acids at the carboxyl-terminal of spinach glycolate oxidase. The modified gene was introduced into *Arabidopsis thaliana* plants by *Agrobacterium*-mediated transformation. The transgenic *Arabidopsis* plant expressed the introduced gene and its protein, and it accumulated PHA in its tissues. Gas chromatography-mass spectrometry analysis demonstrated the accumulation of a novel type of PHA, poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate). This strongly suggests that short-chain-length (R)-3-hydroxyacyl-CoAs were generated from intermediates of peroxisomal P-oxidation.

In 2002, Poirier *et al.* (2002b) reported that a PHA synthase from *Pseudomonas aeruginosa* modified at the carboxy-end for peroxisomal targeting

was transformed in *Pichia pastoris*. The PHA synthase was expressed under the control of the promoter of the *P. pastoris* acyl-CoA oxidase gene. Synthesis of up to 1% MCL-PHA per g dry weight was dependent on both the expression of the PHA synthase and the presence of oleic acid in the medium. PHA accumulated as inclusions within the peroxisomes. He concluded that *P. pastoris* could be used as a model system to study how peroxisomal metabolism needs to be modified to increase PHA production in other eukaryotes, such as plants (Figure 2.14).

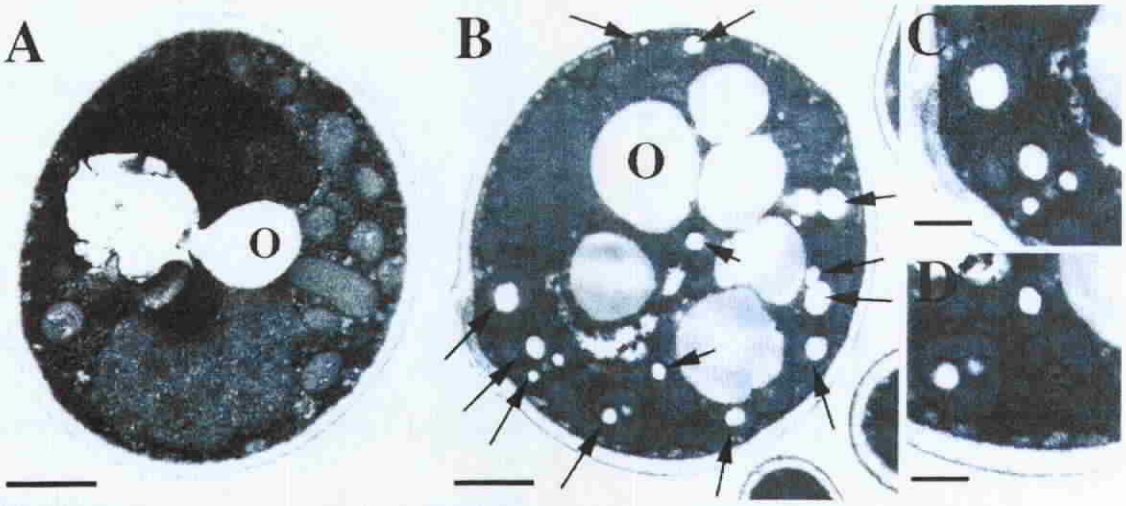


Figure 2.14. Analysis of PHA inclusions in *P. pastoris*. Cells transformed with the control plasmid pTW70 (A) or the plasmid pTW70-PHA (B–D) were inoculated in medium containing 0.1% glucose, 2% Pluronic-127 and 0.1% oleic acid and grown for 3 days before being processed for TEM. C and D are close-up views of B. Arrows indicate the presence of PHA inclusions within membrane-bound organelles. Ob, oil body. Bars indicate 1 μm (A, B) and 0.5 μm (C, D). (adapted from Poirier *et al.*, 2002b).

2.6.3. The metabolic pathway for the PHA formation in plants

Figure 2.15-2.17 illustrate the general pathway for PHA accumulation in the different part of transgenic plants. The production pathway comprises three steps: production of the hydroxyacyl-coenzyme a monomer, polymerization by PHA synthase, and accumulation as submicrometer-size granules.

Pathways for PHA formation in plant plastids are illustrated in Figure 2.15. 3-Hydroxyvaleryl-CoA was produced via a *R. eutropha* thiolase (*Bkt*B)-catalyzed condensation of acetyl-CoA and propionyl-CoA. A two-step pathway designed to convert a portion of the plant's threonine pool to propionyl-CoA was engineered in chloroplasts using a plastid-targeted (Figure 2.15) threonine deaminase (*ilvA* from *Escherichia coli*), to convert threonine to 2-ketobutyrate,and the native plant pyruvate dehydrogenase complex to catalyze the conversion of 2-ketobutyrate to propionyl-CoA. 3-Hydroxybutyryl-CoA was produced from acetyl-CoA using the *R. eutropha* BktB thiolase and PhaB reductase. Yields of 0.2–0.8% leaf dry weight PHBV with 4–17′mol% 3-hydroxyvalerate (3HV) were reported by Valentin *et al.* (1999), whereas PHBV levels of up to 0.84% cell dry weight containing 4% 3HV were obtained by Slater *et al.* (1999). The overall copolymer yields obtained by both studies are well below the estimated 15% target (Slater *et al.*, 1999) required for commercialization. An increased metabolic burden placed on the plant by the *ilvA* gene may account for the low yields observed in plastid-based PHBV production (Slater *et al.*, 1999).

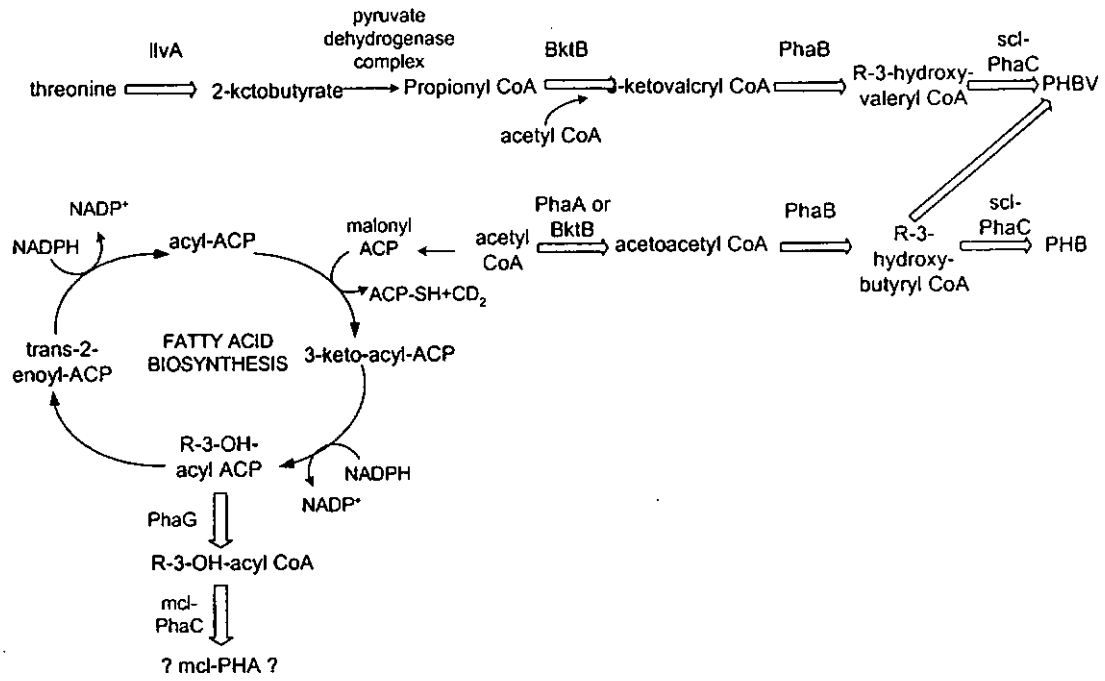


Figure 2.15. Pathways for PHA formation in plant plastids. Solid arrows indicate native plant enzyme activities. Open arrows indicate enzyme activities engineered into the plant for PHA formation. Question marks indicate enzyme activities or products that have not been demonstrated experimentally. The following abbreviations are used: PhaA, *b*-ketothiolase; BktB,a *b*-ketothiolase with substrate specificity for both acetyl and propionyl-CoA; PhaB, reductase; SCL-PhaC, PHA synthase with specificity for short chain length substrates; mcl-PhaC, PHA synthase with specificity for medium chain length substrates; *Ilv*A, threonine deaminase; PhaG, 3-hydroxy acyl ACP-CoA transferase; PHA, poly(3-hydroxyalkanoate), PHB, poly(3-hydroxybutyrate); PHBV, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). (adopted from Snell *et al.*, 2002)

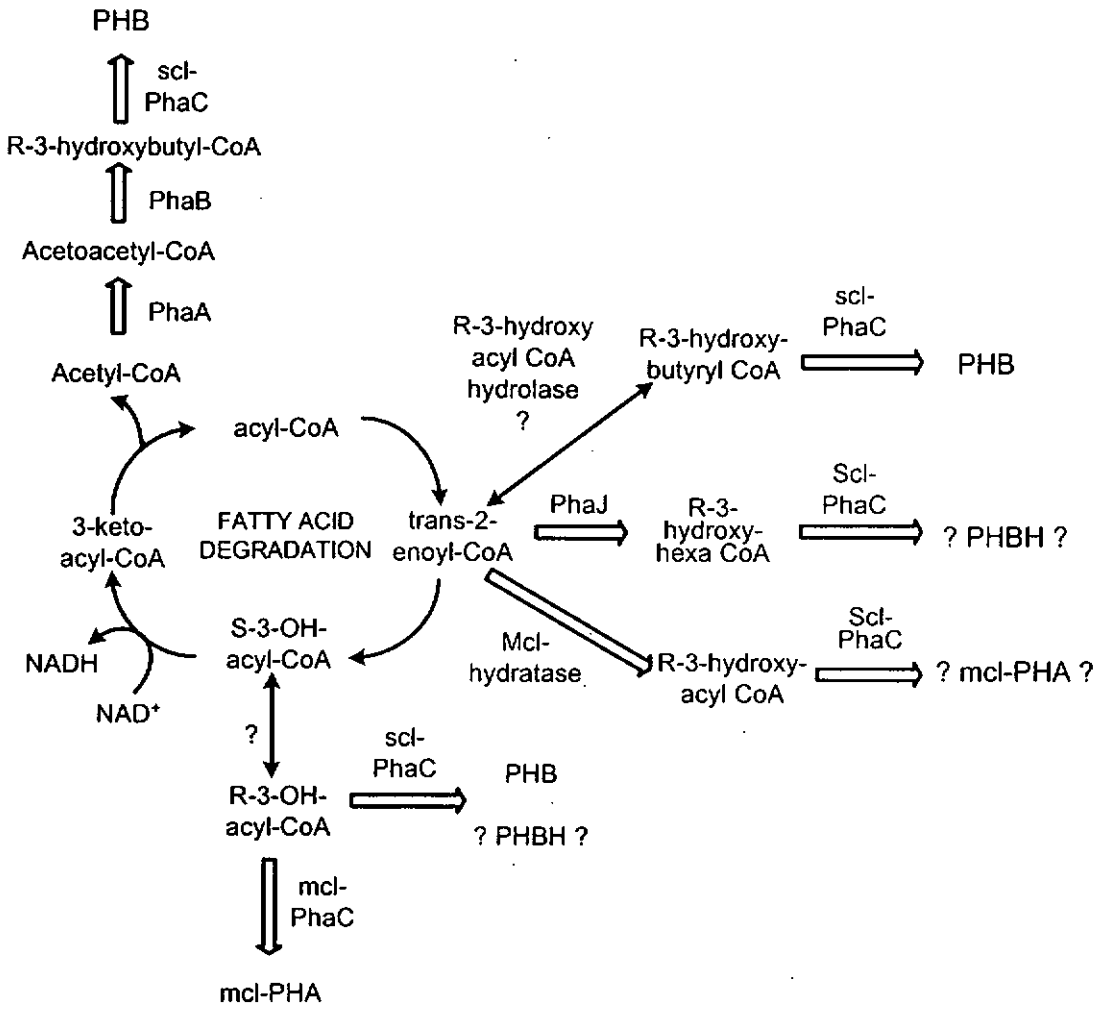


Figure 2.16. Pathways for PHA formation in plant peroxisomes. Solid arrows indicate native plant enzyme activities. Open arrows indicate enzyme activities engineered into the plant for PHA formation. Question marks indicate enzyme activities or products that have not been demonstrated experimentally. The following abbreviations are used: PhaJ,enoyl-CoA hydratase with substrate specificity for short chain length substrates; SCL-PhaC, PHA synthase with specificity for short chain length substrates; MCL-PhaC, PHA synthase with specificity for medium chain length substrates; PHBH, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). (adapted from Snell *et al.*, 2002).

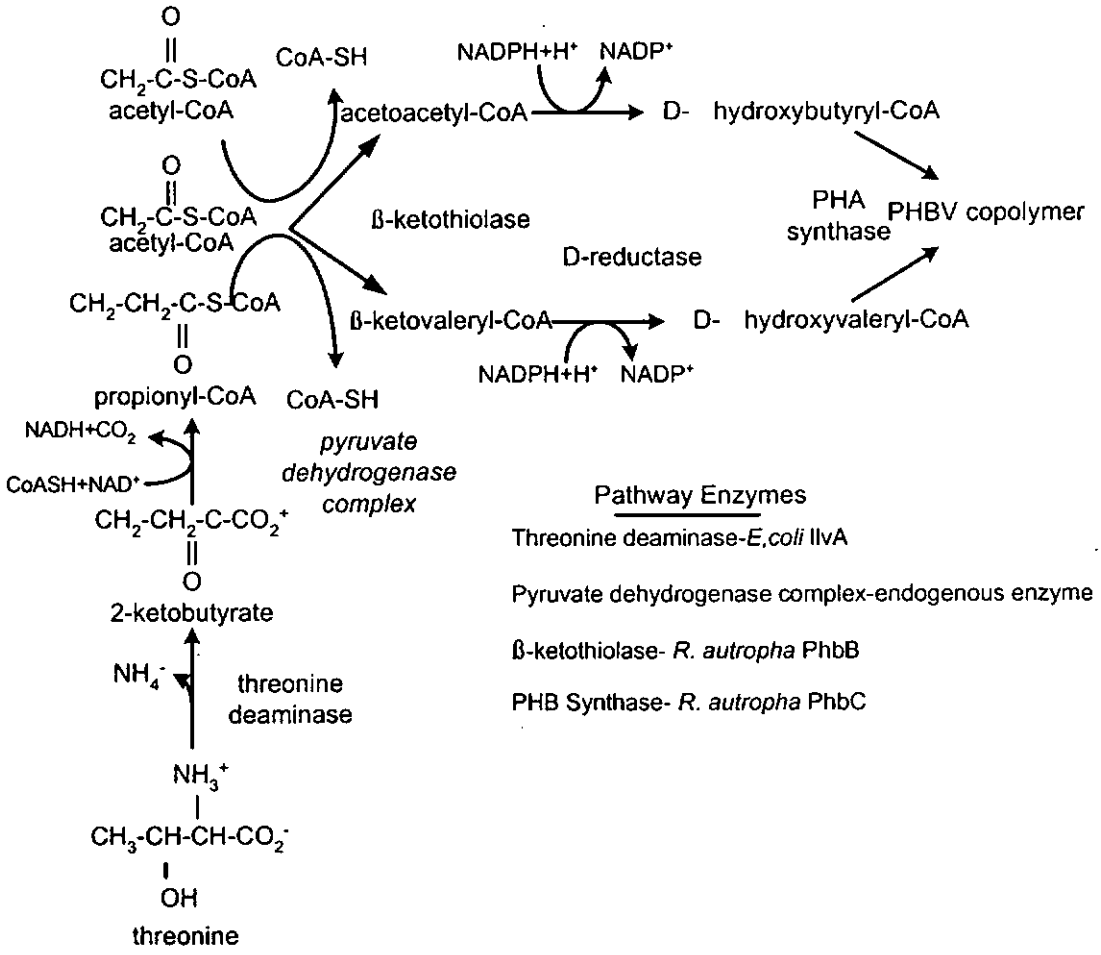


Figure 2.17. A pathway designed to produce PHBV in the plastids of plants. Propionyl-CoA is derived from threonine via threonine deaminase and the pyruvate dehydrogenase complex. Acetyl-CoA is drawn from normal intermediary metabolism. The pathway requires transformation of the plant with four genes (*ilv*A, *bkt*B, *phb*B, and *phb*C), and relies on endogenous pyruvate dehydrogenase. All enzymes encoded by transgenes are targeted to the plastid using chloroplast transit peptides (Adapted from Slater *et al.*, 1999).

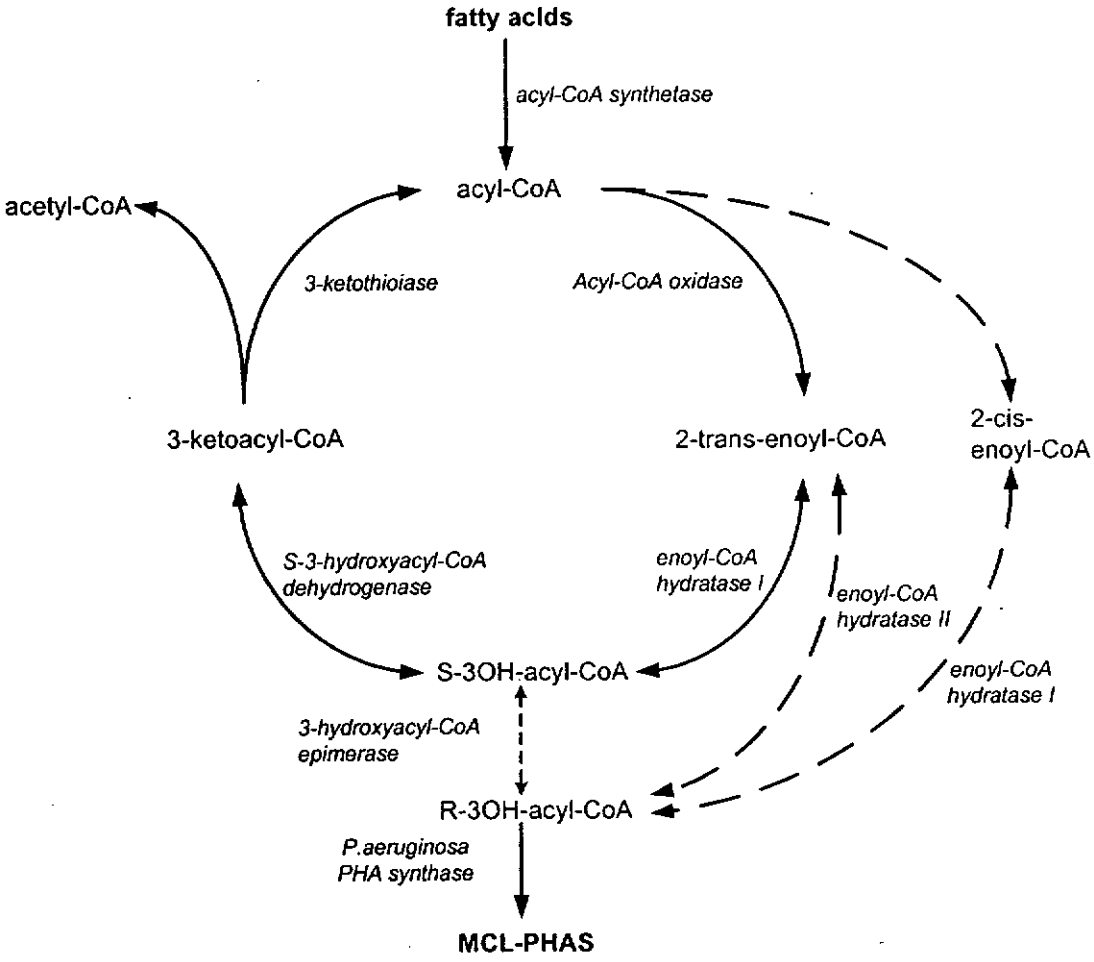


Figure 2.18. Model for the synthesis of MCL-PHAs in transgenic plants. Enzymatic reactions involved in the core β-oxidation cycle are indicated by solid lines, and reactions involved in the oxidation of unsaturated fatty acids with *cis* double bonds at an even carbon are indicated by dashed lines. Synthesis of MCL-PHAs from R-3-hydroxyacyl-CoAs by the PHA synthase is indicated by a bold arrow at the bottom. (adapted from Mittendorf *et al.*, 1998)

Steady progress has been made toward creating transgenic crops for commercialization of PHAs; however, much work still remains to be done. Model plant systems have been successfully used to study PHA formation in plants expressing bacterial genes. Significant additional efforts are needed to use the information obtained from model systems to create plants of commercial interest. Hurdles that will be encountered in these efforts include altered plant phenotypes, low product yields, transgene stability, and difficulties associated with expressing multiple genes simultaneously in one plant. Correcting altered plant phenotypes and low product yields may require a detailed study of the changes in metabolism created upon expression of multiple foreign genes. The metabolic profiling techniques described by Bohmert *et al.* (2000) are a valuable tool for this task. Improved methods for multiple transgene expression may be required to efficiently coordinate expression and prevent gene silencing (Matzke *et al.*, 1994) due to multiple repeated genetic elements. Plastid transformation technologies can simplify the expression of multiple transgenes since the expression of polycistronic operons from a single promoter is feasible from the plastid genome (Hager and Bock, 2000). Direct plastid transformation has the added benefit of gene containment in most crop plants since most plastid genomes are maternally inherited (Daniell *et al.*, 1998). A recently developed nonantibiotic selection method for plastid transformation can eliminate the need for genes encoding antibiotic resistance (Daniell *et al.*, 2001). Although initial attempts to use plastid

transformation for PHB production in tobacco have failed (Lossl *et al.*, 2000), high-level expression of other bacterial proteins upon plastid transformation has been obtained (DeCosa *et al.*, 2001), suggesting that successful expression of PHA genes with this technique is possible and deserves further attention.

It will be still a long time before PHAs can be obtained from agricultural process. However, the concepts have been proved, and it has been demonstrated that PHAs can be synthesized in transgenic plants. Further, not only PHB can be produced but so can the copolyester PHBV and also PHAs consisting of 3HAmcl. At present, the PHA content of the cells is still low, and repression of plant growth even at relative low PHA contents seems a major problem which has to be overcome. In addition, the isolation of the PHAs from the plant material has to be investigated. In principle, and as outlined recently, the costs of the production of PHAs in transgenic plants could be of the same order of magnitude as the costs of sucrose, lipids and starch, if all the prerequisites are met. Under these circumstances, PHAs from transgenic plants would be significantly cheaper than conventional plastics. The acceptance by farmers and consumers will be, most probably, not such a problem as with other transgenic plant products, since these transgenic plants will be used for non-food applications and since they might help to save petroleum resources by providing a substitute for non-biodegradable plastics which are mainly produced from petroleum carbon sources, or by providing new materials (Steinbüchel, 2001).

2.7 Production of PHA by the co-culture of activated sludge

2.7.1 The history of PHA accumulation from activated sludge

Arden and Lockett (1914) established the term "activated sludge" to designate a highly active, acclimatized microbial biomass. Activated sludge is a gray-black, flocculent, sticky material formed during the biological oxidation of sewage in sewage treatment process.

Treatment of wastes with the activated sludge process represents a component of the largest biotechnology industry in the world. Yet activated sludge systems differ substantially from the controlled aerobic fermentations for the large-scale production of economically important metabolites or biomass from microbes (Gray, 1990; Matsui *et al.*, 1991 and Grau, 1992). For example, they are mixed cultures which have to deal with an enourmous diversity of organic compounds entering the system, differing in both their chemical compositions and molecular/particle sizes. Many of these chemicals will have undergone changes in the sewerage system before entering the plant (Nielsen *et al.*, 1992) while some may be unbiodegradable or recalcitrant, and pass through the treatment plant unmodified. Others may originate from industrial sources (Eckenfelder and Musterman, 1992) as xenobiotics and heavy metals and will be selectively toxic to the complex microbial community in the activated sludge process (Tyagi, 1985; Madoni *et al.*, 1996). A detailed description of many of the metabolic mechanisms used for the biodegradation of the substrates in waste materials is given by Slater and Somerville (1979), Painter (1983), Zitomer and Speece (1993) and Ratledge

(1994), and some of the main biochemical events thought to occur are shown in Figure 2.19.

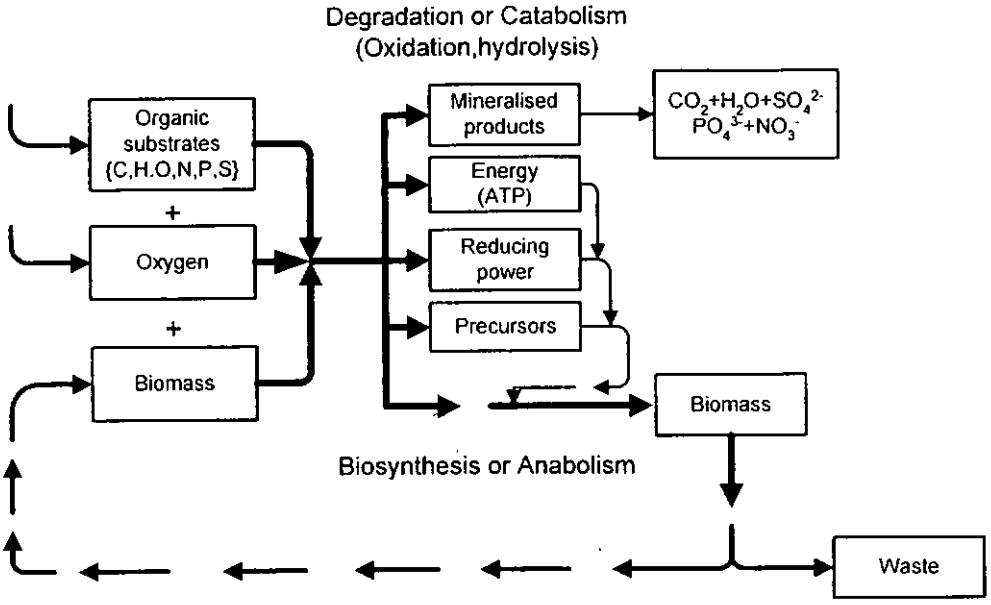


Figure 2.19. Outline of some of the main biochemical events occurring in activated sludge (adapted from Seviour *et al.*, 1999)

The end product of the activate sludge process, the microbial biomass or so called excess activated sludge (EAS), is still a largely unexploited resource in most countries and regions. Although rich in nutrients and valuable metals, and a possible source of food (Slater and Somerville, 1979; Lemmer and Nitschke, 1994b), the EAS is considered a liability which has to be disposed of landfill, often at great expense and inconvenience (Hansen *et al.*, 1993).

Generally, the activated sludge generated from the MWWTP can be divided into four types (Table 2.11). As shown in Table 2.11, the first one, the

conventional sludge is generated from a fully aerobic wastewater treatment process in a Sequencing Batch Reactor (SBR) system. Its main feature is COD removal. The second type of activated sludge is from anaerobic-aerobic alternant process, which is dedicated to phosphorus removal in wastewater so called as Enhanced Biological Phosphorus Removal process (EBPR). Recently, the EBPR process is achieving reputation as an economical and reliable option for the removal of phosphorus from wastewaters.

The third type is from anoxic-aerobic process, and which is dedicated to denitrification and COD removal.

The fourth one is a microphilic-aerobic process proposed by Satoh *et al.* (1998) and modified from anaerobic-aerobic EBPR process. A little amount of oxygen is supplied into the anaerobic zone of the EBPR process. It was reported that the activated sludge acclimatized by this process could accumulate more of PHA in the bacterial cells.

In this study the concept of "activated sludge" will be extended to those of residual biomass including yeast wastes in brewery industry and microbial waste in fermentation plant such as the residue biomass created in amino acid production fermentation process (Table 2.11). It is thought that all of the sludge might be potentially submitted to PHA accumulation, another novel option for the large-scale PHA production.

So far, several literatures and own research have demonstrated that activated sludge generated from the wastewater treatment process possessed of the

capability to accumulate PHA during the wastes treatment procedure, which implied that EAS might be another choice for PHA production from the wastes. The main purposes of wastewater treatment systems are to remove organic pollutants, but it would be very attractive if there were a way to recover the organic pollutants as PHA, and hence resulted in the decrease of PHA price.

Table 2.11. Activated sludge generated in different microbial removal process or residual biomass from industry fermentation.

Activated sludge/residual biomass	*Acclimatized way*	*Operation process*	*Main function*
Conventional sludge	Fully aerobic process	Fully aerobic	COD removal
Anaerobic/aerobic sludge	Alternating of anaerobic and aerobic phases	Anaerobic-aerobic	Enhanced biological phosphorus removal
Anoxic/oxic sludge	Alternating of anoxic and aerobic phases	Anoxic-aerobic	COD removal & Denitrification
Microaerophilic/ aerobic sludge	A limited amount of oxygen is supplied into the anaerobic zone of the anaerobic-aerobic process	Microaero-philic-aerobic	COD removal and PHA accumulation
Brewery yeast	Alcohol Beverage fermentation	Anoxic fermentation	Beverage
Residual biomass in fermentation industry	Microbial fermentation industry	Aerobic or anaerobic fermentation	Metabolic products

In fact, this capability for PHA production by activated sludge has been found for several years. Wallen and Davis (1972) showed that activated sludge usually contains microbial cells, heteropolysaccharides, and polyester. Crabtree *et al.* (1965) implicated PHB as a factor responsible for the flocculation properties of activated sludge. The concentration of PHB in samples of activated sludge biomass from domestic sewage was reported by Deinema (1972) to range from 0.0 to 0.2% on a dry weight biomass. The first example of the microbial copolyesters of PHA was isolated by chloroform extraction of activated sludge from a municipal and grain processing industry wastewater treatment plants in Peoria, Illinois (Wallen and Rohwedder, 1974). The copolyester PHA was identified about 1.3% of the dry weight of activated sludge. On the basis of gas chromatography-mass spectroscopy of a saponified sample, Wallen and Rohwedder (1974) showed that the polymer contained four different monomeric units: 3HB, 3HV (the major component), 3-hydroxycaproate (3HC), and 3-hydroxyheptanoate (3HHp). Further, a wide variety of PHA copolymers have been isolated from different environmental samples, including marine sediments (Findlay and White, 1983), marine and freshwater *Cyanobacteria* (Capon *et al.*, 1983), and sewage sludge (Odham *et al.*, 1986). The PHA copolymer extracted from marine sediments has been demonstrated to contain as many as 11 short-chain 3-hydroxy acids (Findlay and White, 1983).

Dave *et al.* (1996) reported that activated sludge from a petrochemical

wastewater bio-treatment plant contained 28% (w/w) polysaccharides and 1.8% (w/w) PHB.

It has been already demonstrated that the sludge developed in the anaerobic-aerobic two-stage enhanced biological phosphorus removal process produces PHAs with high efficiency (Inoue *et al.*, 1996), which realized PHAs content of more than 30 wt% of the dried weight of sludge under optimum conditions.

Chua *et al.* (1997a, 1997b) and Hu *et al.* (1997) had substantially reduced the production costs by inducing activated sludge bacteria to produce PHA while the fatty acids were used as carbon source. Furthermore, the 3HV molar fraction in the PHBV copolymer could be adjusted by changing the butyric/valeric acids ratios in the medium.

Satoh *et al.* (1998) submitted the activated sludge from a laboratory scale anaerobic-aerobic reactors in the accumulation of PHA. Around 20% under anaerobic conditions and up to 33% under aerobic conditions from the acetate as carbon substrate was abstracted. In order to further increase the PHA content in activated sludge, they introduced a so-called "microaerophilic-aerobic" process, where a limited amount of oxygen was supplied into the anaerobic zone of the anaerobic-aerobic process. Finally, the activated sludge acclimatized in the "microaerophilic-aerobic" process accumulated PHA of as much as 62% of sludge dry weight.

Lemos *et al.* (1998) has devoted attention to understand how different carbon

substrates and their concentration could influence the production of PHA by polyphosphate-accumulating bacteria (PAOs). Acetate, propionate, and butyrate were tested as sole or mixed carbon sources respectively. The compositions of the polymers formed were found to vary with the substrate used. Acetate led to the production of a copolymer of PHBV with the HB units being dominant. With propionate, HV units were mainly produced and only a small amount of HB was synthesized. When butyrate was used, the amount of polymer formed was much lower with the HB units being produced to a higher extent.

Takabatake *et al.* (2000) observed that it was possible to regulate PHA composition formed in activated sludge by utilizing acetate and propionate as carbon sources. The PHA contents of the activated sludge as Mixed Liquor Suspended Solid (MLSS) were achieved up to 57% by using anaerobic-aerobic activated sludge.

Serafim *et al.* (2002) reported that the sludge from a stable and efficient phosphorus removal process was used to evaluate the change in the metabolisms when the reactor operation was modified from anaerobic/aerobic to aerobic dynamic substrate feeding. The change in operational conditions allowed the population to modify the metabolism of phosphorus and PHA accumulation. They found the PHA stored in activated sludge doubled immediately from less than 20% of dry cell weight to around 40%, where the acetate and propionate were used as carbon sources independently.

Hollender *et al.* (2002) observed the effect of different carbon sources acetate,

acetate/glucose or glucose on the EBPR process by experiments under alternating anaerobic-aerobic conditions in one sequencing batch reactor for each carbon source.

As summarized, microorganisms in EAS have been reported to accumulate PHA as an intermediate metabolic product from uptake of organic matter in sewage. Therefore, it is possible to couple a wastewater treatment process with PHA production. This novel technique to produce and recover PHA from activated sludge provided a potentially inexpensive source of biodegradable plastics and, at the same time, reduced the quantity of excess sludge that required further treatment. This could mean a long step towards PHA cost reduction.

2.7.2 *Metabolic pathways for the PHA accumulation in activated sludge*

So far as I know, there isn't a widespread acceptable metabolic model which successfully describes the pathways of PHA synthesis from all kinds of activated sludge. However, noticeably, PHA accumulation from the sludge of EBPR has been investigated by numerous researchers, and different biochemical models have been developed, e.g., by Wentzel *et al.* (1986), Mino *et al.* (1987) and Smolders *et al* (1994), to explain the mechanisms of EBPR process and PHA accumulation under anaerobic condition. In addition, new models and new mechanisms have been suggested in light of these models (Pereira *et al.*, 1996 and Louie, *et al.* 2000). But the fundamental understanding of the process from microbiological and biochemical points of view are still incomplete in the PHA

accumulation from activated sludge including EBPR process.

Biochemical Models of EBPR Metabolism

The major EBPR models, and significant additions to them, are given below in chronological order of their development, with brief explanations (Erdal, 2002):

Comeau-Wentzel Model (1986):

PHA is a reduced energy source and its synthesis therefore requires reducing powers. Partial oxidation of acetyl-CoA through the TCA cycle produces the reducing power. The combination of acetate (acetyl-CoA) and reducing power produce PHA. The proposed stoichiometry of the EBPR process is given by Comeau-Wentzel in Figure 2.20.

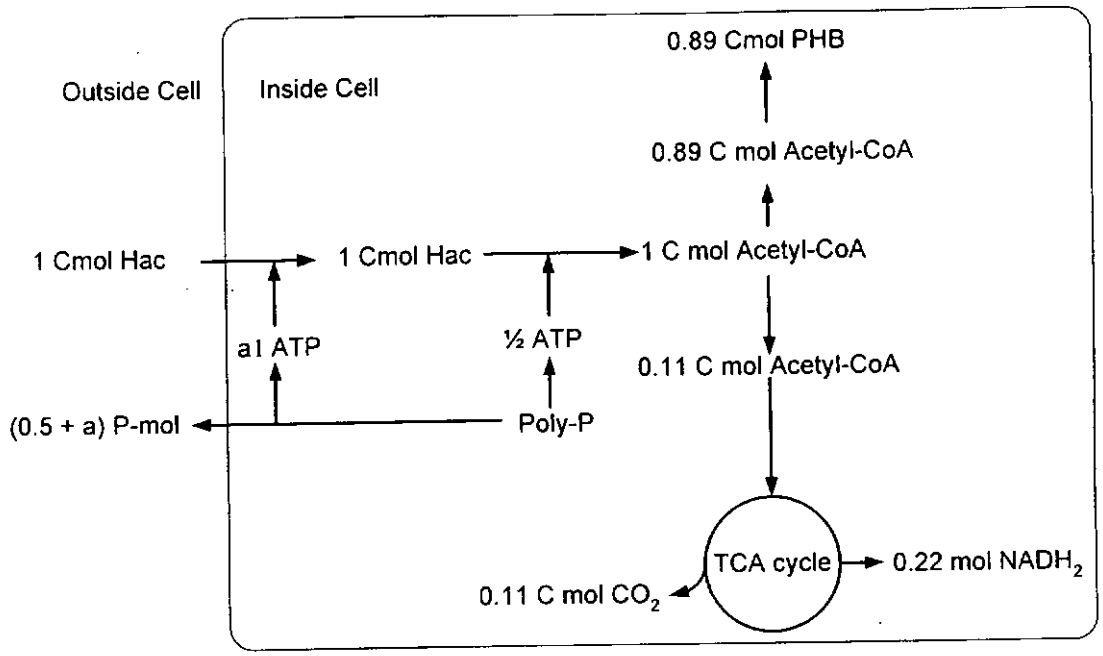


Figure 2.20. Comeau-Wentzed model for PHB accumulation in EBPR process under anaerobic condition (Comeau *et al.*, 1986 and Erdal, 2002).

Mino Model (Mino et al., 1987):

The lack of evidence for the operation of the TCA cycle under anaerobic conditions and the observation of significant changes in intracellular carbohydrate (glycogen) content motivated the development of the Mino model (1987). In this model, reducing power is generated by the degradation of intracellularly stored glycogen (carbohydrate) via the Embden-Meyerhoff Parnas (EMP) pathway. It was assumed that glycolysis of glycogen in addition to the hydrolysis of polyphosphate supplies energy under anaerobic conditions. The model predicts that when six molecules of acetate are taken up, one molecule of glycogen is

consumed, four molecules of 3HB are produced, and three to four molecules of phosphate are released. The proposed model is also given in Figure 2.21.

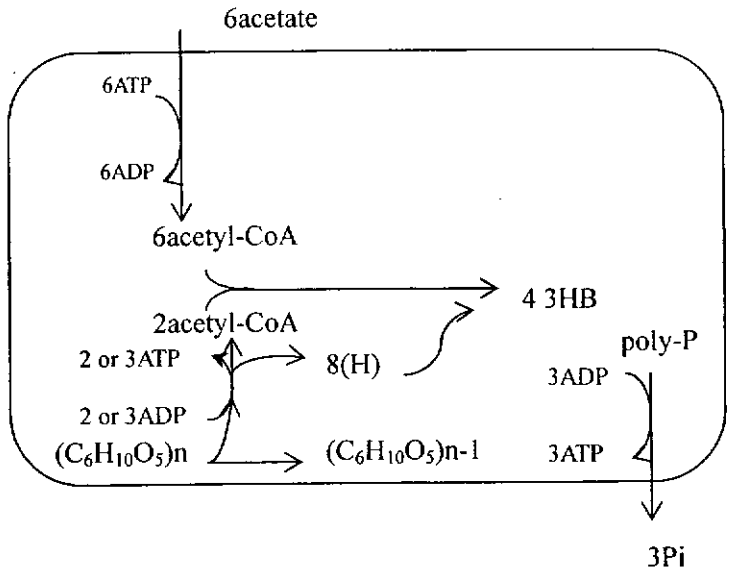


Figure 2.21. Biochemical model of anaerobic uptake of acetate and PHB synthesis by anaerobic-aerobic activated sludge mediated by the hydrolysis of polyphosphate and glycolysis of glycogen.

Otherwise, Satoh *et al.* (1992) demonstrated the formation of very unique PHA containing monomeric units with a methyl branch at α-carbon, 3H2MB and 3H2MV. The formation of these units is reasonably explained by modifying the Mino model, as shown in Figure 2.22. That is, when six molecules of propionate are taken up, six molecules of propionyl-CoA and two molecules of acetyl-CoA are formed as the intermediate, because one molecule of glycogen is consumed. Two molecules of acetyl-CoA and two molecules of propionyl-CoA are condensed, reduced, and polymerized into PHA as 3HV, while the other four molecules of porpionyl-CoA are condensed, reduced and polymerized into PHA

as 3H2MV,

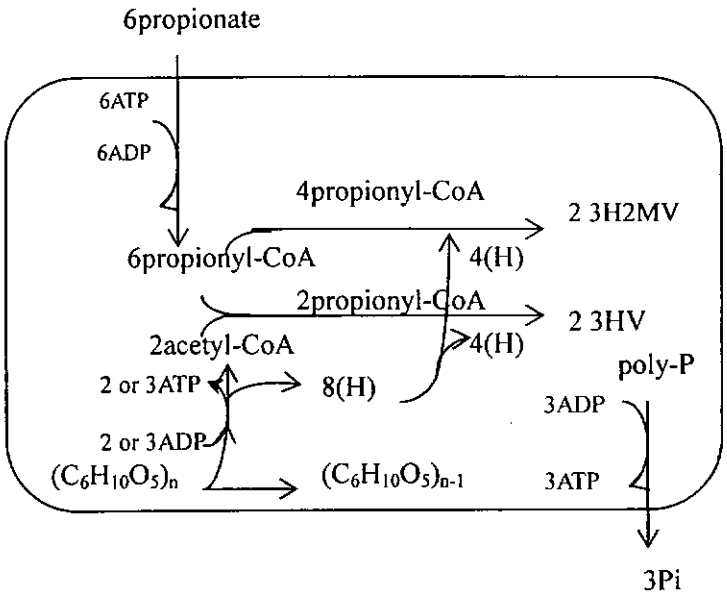


Figure 2.22. Biochemical model of anaerobic uptake of propionate for synthesis of PHA with 3HV units by anaerobic-aerobic sludge. This model is modified from Mino Model.

The Modified Mino Model (Wentzel, 1991):

The only major change from the Mino model is that the modified Mino model postulates the Entner-Doudoroff (ED) pathway for degradation of intracellular glycogen instead of the EMP pathway. This modification was proposed by Wentzel (1991) based upon the results of a single study in which the apparent use of the ED pathway by an *Acinetobacter* was observed.

Observation of Pereira et al. (1996):

Pereira and coworkers (1996) showed that a small portion of labeled acetate was released as CO_2 during an anaerobic batch test experiment. Therefore, their ^{13}C NMR results suggest that at least part of the TCA cycle is still operable under anaerobic conditions, and that some fraction of the reducing power needed is generated through the TCA cycle. The complete pathway of the model is illustrated in Figure 2.23.

Observation of Maurer et al. (1997):

Maurer *et al.* (1997) used a solid state NMR to track carbon flow in EBPR sludge fed with domestic sewage. Although no suggestion was made about the operation of the TCA cycle under anaerobic conditions, they suggested that the ED pathway was used during glycogen breakdown.

Observation of Louie et al. (2000):

More recently, Louie *et al.* (2000) suggested that the glyoxylate pathway is active under anaerobic conditions to provide reducing equivalents and to maintain stable NAD^+/NADH balance. Glyoxylate pathway and TCA cycle are essential for some PHA synthesis (Figure 2.24).

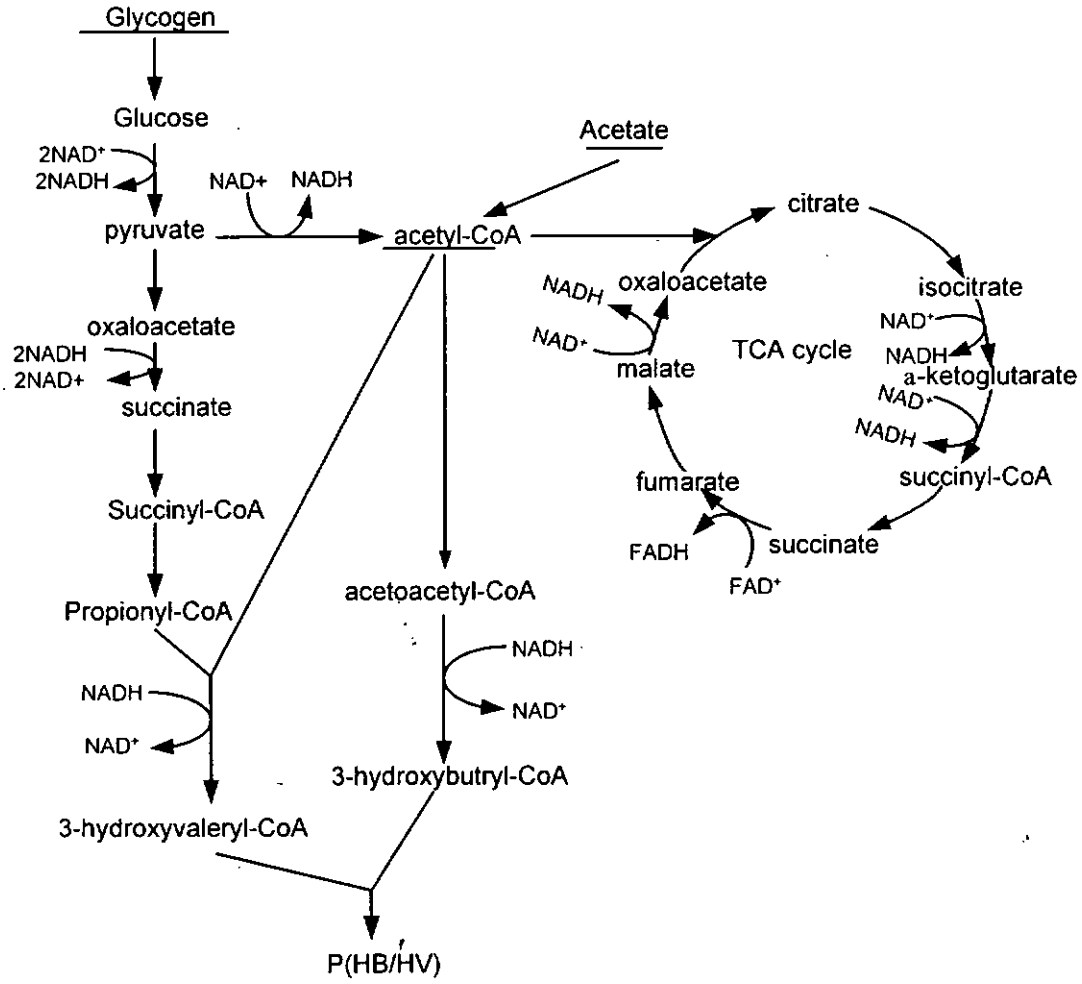


Figure 2.23. The EBPR model for the PHA accumulation under anaerobic condition as proposed by Pereira *et al.* (1996).

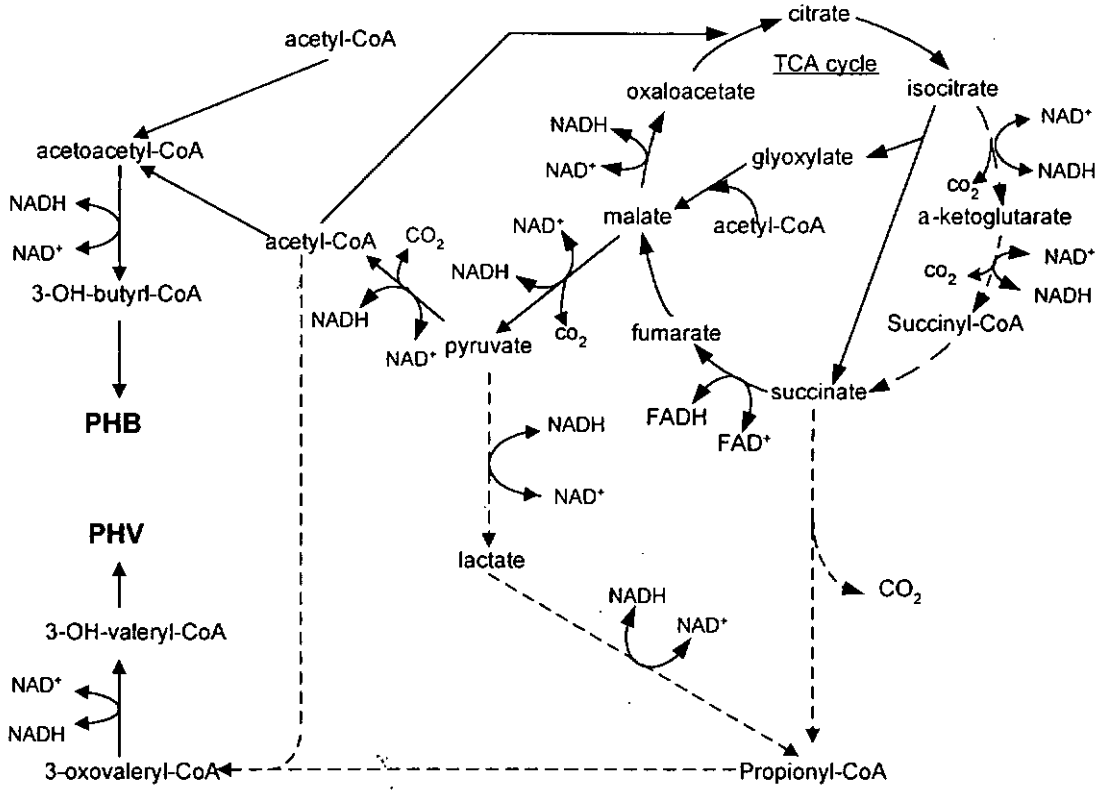


Figure 2.24. A biochemical model for conversion of different metabolites to PHA by EBPR activated sludge under anaerobic conditions (adapted from Louie *et al.*, 2000). It is clearly demonstrated that the early metabolic steps common to the glyoxylate pathway and the TCA cycle are essential for PHA synthesis in EBPR process under anaerobic condition.

Observation of Wang et al. (2002)

Wang *et al.* (2002) proposed a glucose induced EBPR metabolic model. Compared with Bio-P bacteria fed with acetate as the major substrate, lower levels of PO_4-P are released into the medium and 3HV enriched PHA was accumulated. A new biochemical model is presented for the major metabolic activities during the anaerobic condition (Figure 2.25 and Figure 2.26). They concluded that the predominance of 3HV enriched PHA is employed to balance the internal redox during the anaerobic condition. Further, anaerobic metabolism of absorbed glucose under the EMP pathway could produce enough energy for all metabolic activities and prevent the energy derivation from polyphosphate hydrolysis.

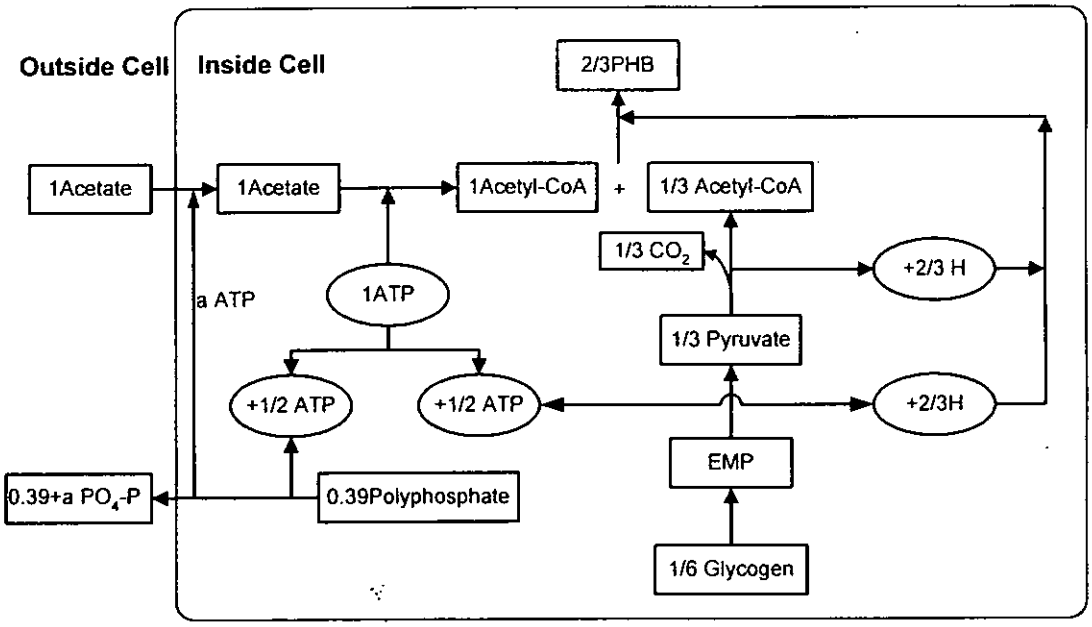


Figure 2.25. The acetate induce EBPR metabolic model under the anaerobic condition proposed by Wang *et al.* (2002)

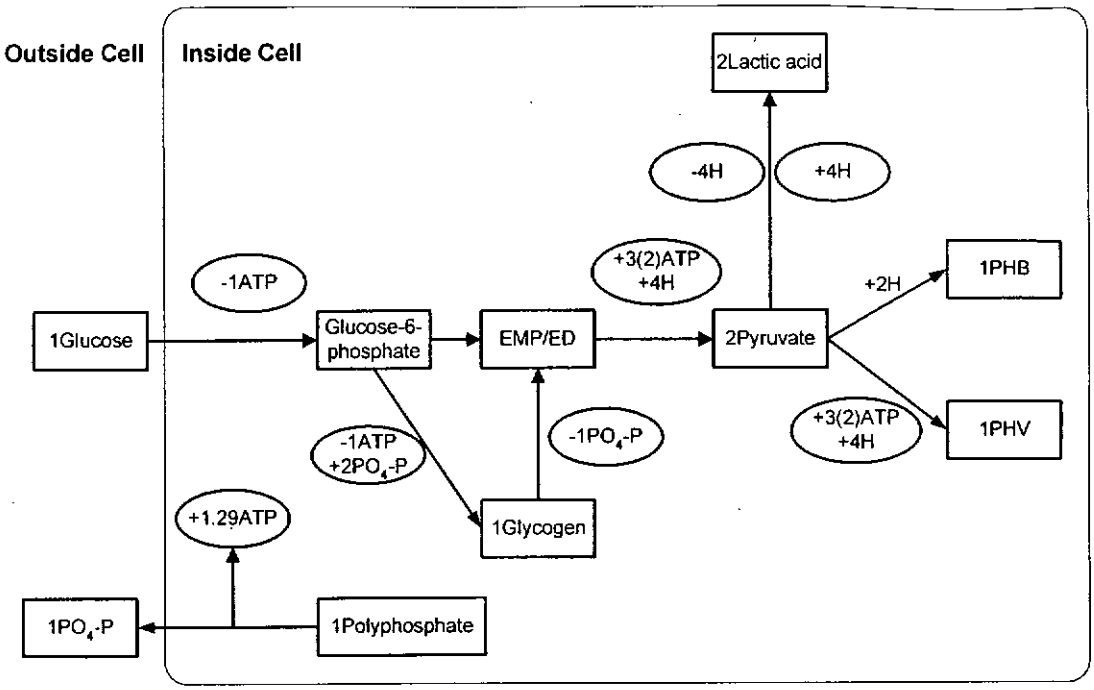


Figure 2.26. The glucose induced EBPR metabolic model under the anaerobic condition proposed by Wang *et al.* (2002).

It is obvious that not all of the biochemical pathways of EBPR have been completely defined yet. In addition to unknown biochemical mechanisms, the effects of other factors that affect the performance of EBPR processes and PHA synthesis are incompletely understood, notably the dissolved oxygen concentration in cultural medium. I believe that no single model account for all PHA synthesis in all the different EBPR facilities. On the other hand, all the models summarized above are just for the anaerobic-aerobic EBPR process. As we know, at least there are four types of activated sludge have been submitted to wastes treatment, which have the ability to accumulate PHA from wastes and

added substrates. The metabolic pathways of the rest types of activate sludge are not clear and need more study. Otherwise, research on the PHA accumulation metabolic pathway is helpful for finding the way to increase the PHA content in activated sludge.

The types of activated sludge and their typical metabolic pathway for organic uptake and PHA accumulation

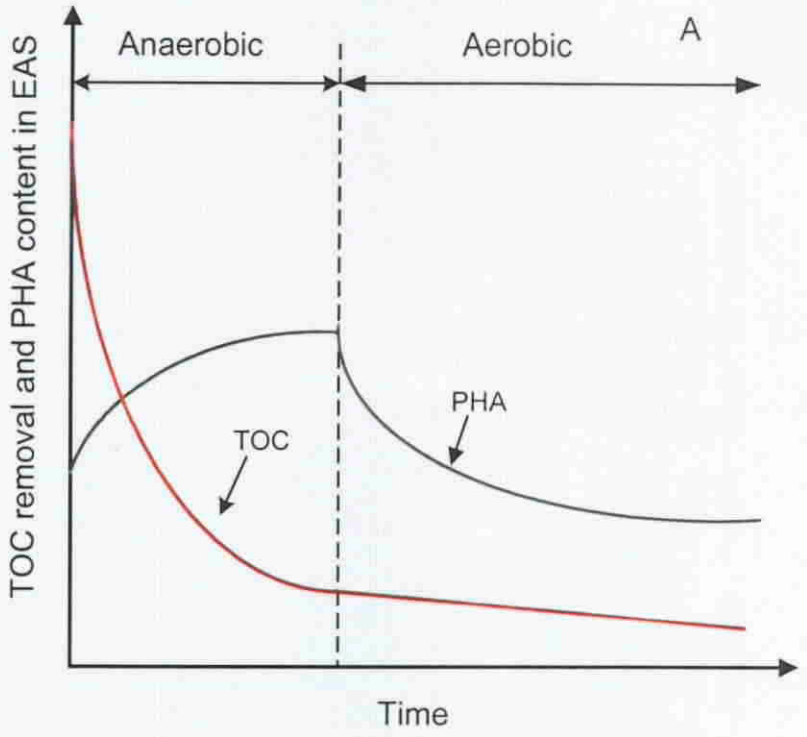
As known, there have been four kinds of activated sludge from laboratory scale bioreactor or full scale MWWTP submitted to PHA production intentionally. The first one was the activated sludge from EBPR process with typical EBPR metabolic characters (Figure 2.27 A). The activated sludge was submitted to anaerobic-aerobic process for the wastewater treatment. The organic substrate was taken up and consumed; PHA was accumulated while phosphate was released in anaerobic phase. The PHA productivity of this kind of activated sludge was relatively low (approximately 10-25% of CDW) (Liu *et al.*, 1996; Satoh et al., 1998 and Randall *et al.*, 2002). In the second one (Figure 2.27 B), the activated sludge was submitted to microaerophilic-aerobic process, and would not take up organic substrate and produce PHA in microaerophilic phase. Under aerobic phase, the activated sludge took up organic substrate for PHA accumulation (Satoh *et al.*, 1999). The PHA production capability of Microaerophilic-aerobic activated sludge was higher (more than 40%) than the first one, the anaerobic-aerobic

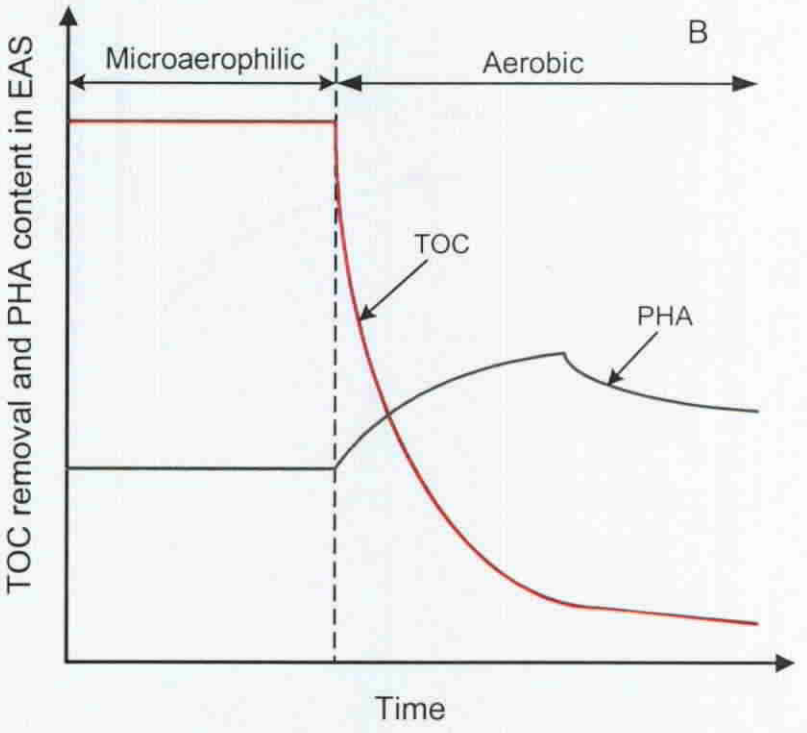
activated sludge (Takabatake *et al.*, 2000).

The Third one, anoxic-aerobic activated sludge, and the fourth one, the aerobic-aerobic activated sludge, were acclimatized and submitted to PHA production by using various fatty acids in this research. Anoxic-aerobic activated sludge was collected from local full-scale MWWTP for denitrification. The organic uptake and PHA production were performed during both of the anoxic and aerobic phase. Most of the organic was consumed at anoxic phase, and the PHA content was kept increase through the whole process (Figure 2.27 C). In our own research, PHA highest content was 35.0% of CDW (Chua *et al.*, 1997a and Hu *et al.*, 1997)) by using acetate as sole carbon source which was lower than the previous reports. So far, it has not been clarified why the difference existed.

Aerobic-aerobic activated sludge was harvested from a laboratory-scale SBR. The organic consumption starts at the beginning of the fermentation. The PHA production is enhanced under unfavorable growth conditions such as nitrogen source absence, the presence of excess carbon source and oxygen-limited condition (Figure 2.27 D).

Any way, no matter what activated sludge will be conducted for the PHA accumulation, it is very important to clarify the types of the typical metabolic characters of the sludge.


A
Anaerobic
Aerobic
TOC removal and PHA content in EAS
PHA
TOC
Time


B
Microaerophilic
Aerobic
TOC removal and PHA content in EAS
TOC
PHA
Time

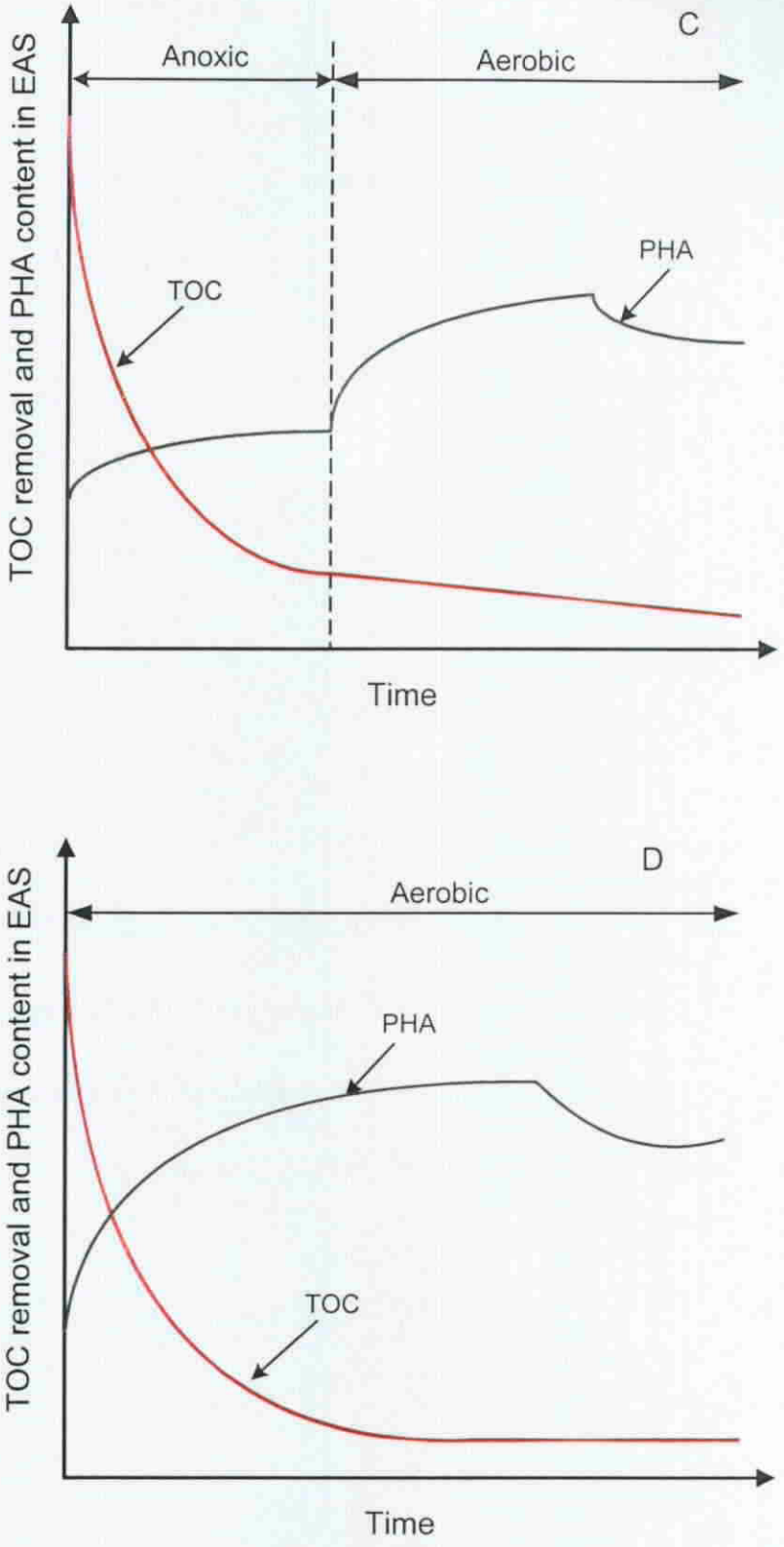


Figure 2.27. The typical metabolisms of TOC removal and PHA accumulation in different activated sludge process under PHA accumulated conditions. (Figure 2.27 A and B were adapted from Takabatak *et al.* (2000))

2.7.3 *To combine PHA production with waste water treatment process in WWTP*

The attempt to produce PHA by activated sludge indicates another way of PHA production. Most of the research on PHA production has been concentrated on pure culture of microorganisms or genetically engineered plants. Using activated sludge, or mixed culture enriched under adequate conditions, may also be a promising option for PHA production.

There is a suggestion proposed for the combination of this novel technic with that conventional MWWTP.

Takabatake *et al.* (2000) proposed a PHA accumulating bacteria enrichment reactor (PABER) for activated sludge acclimatization with high potential of PHA production (Figure 2.28). In PABER, wastewater should be treated simultaneously. The PHA content of activated sludge here is not high. And then, the acclimatized activated sludge in PABER is transferred into PHA production reactor (PPR). In PPR, wastewater with concentrated volatile fatty acids (VFA) is fed as carbon sources for PHA production, and PHA content in activated sludge increases significantly. Finally, PHA is extracted from bacteria and purified to yield biodegradable plastics.

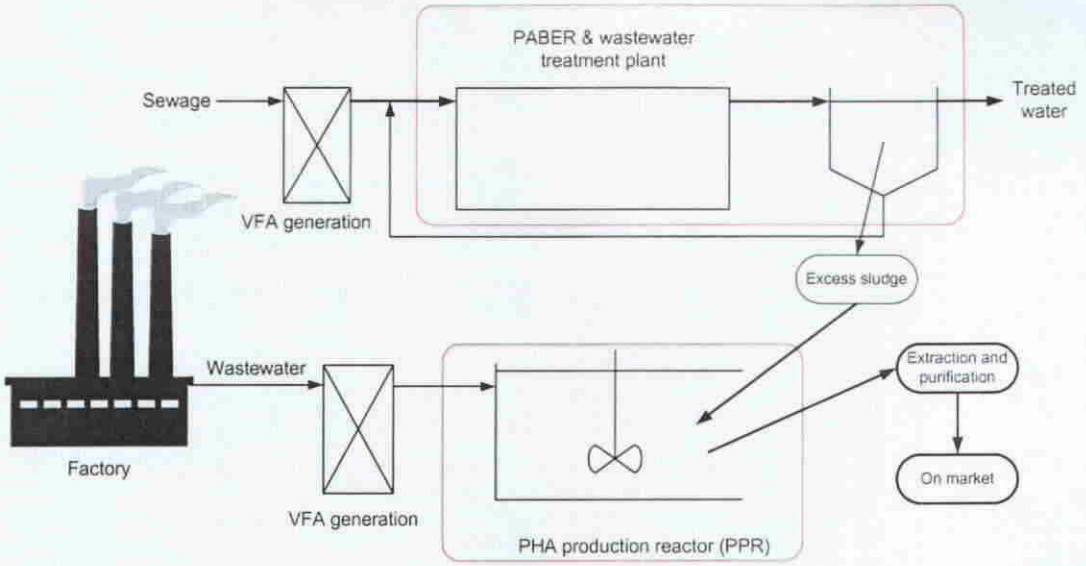


Figure 2.28. A proposed PHA production system by activated sludge combined with a MWWTP (adapted from Takabatake *et al.*, 2000) (VFA, volatile fatty acid).

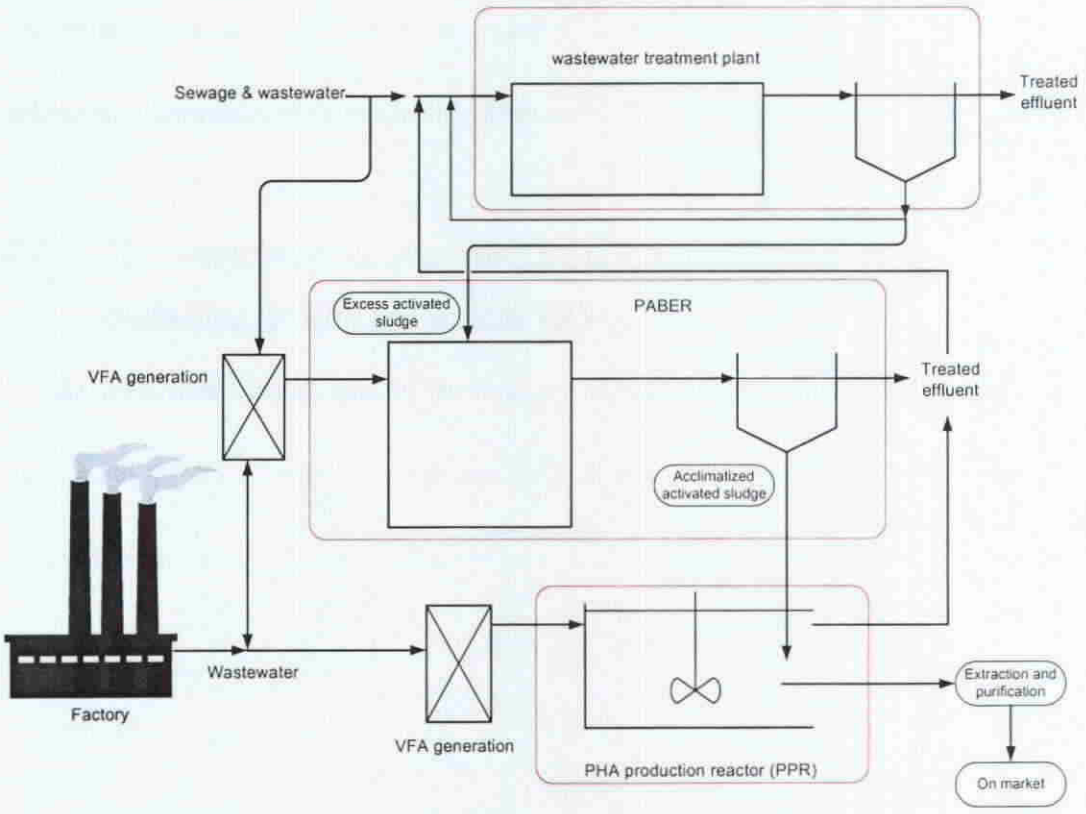


Figure 2.29. A modified PHA production system by activated sludge combined with a MWWTP. This system is consisted of three parts, Wastewater treatment plant; PABER for activated sludge acclimatization and PHA production reactor.

In my opinion, the step of activated sludge acclimatization is necessary for any types of activated sludge, and can be assembled behind the wastewater treatment procedure. The activated sludge submitted to PHA production is collected and transferred from the excess activated sludge in the secondary sedimentation tank in the MWWTP. The wastewater with carbon substrate is the nutrients for the acclimatization. During the PHA production stage, the carbon sources for the PHA accumulation include sewage, wastewater, anaerobic digested excess activated sludge with concentrated fatty acids or added substrates intentionally. Finally, the effluent from the PABER and the supernatant from PPR are return back the wastewater treatment plant for further treating. Therefore, the schematic diagram can be revised as Figure 2.29.

2.7.4 The importance of dissolved oxygen and ORP in monitoring and controlling the PHA synthesis by EAS

As described above, one of the favored conditions for PHA accumulation by bacteria is oxygen limitation. On the other hand as Satoh *et al.* (1998) reported that microaerophilic-aerobic process accumulated more PHA than the original anaerobic-aerobic process, when little amount of oxygen was supplied into the reactor. Thus it was thought that the dissolved oxygen (DO) in the cultural broth plays a very important role in the PHA production by activated sludge. The PAOs and GAOs in sludge are very sensitive to oxygen even under a low concentration. The variation of DO concentration could result in changes of microbial community or metabolic pathway. So, it is decided that a more precise method to

adjusting DO in the cultural broth have to be employed to take place the traditional way of DO monitoring and controlling system. Furthermore, measuring a low DO concentration is unreliable and the terms of anoxic and anaerobic are only qualitative descriptions, ORP monitoring is more flexible and reliable for process controlling under such conditions (Yu R.F. *et al.*, 1997). Therefore, the oxidation-reduction potential (ORP) system was taken into account for the fermentation process monitoring and controlling instead of DO system in this research.

Oxidation and reduction systems play a key role in the life processes of all living organisms. Hence, it is revealed that both theoretical and experimental studies of oxidation and reduction processes in animals, plants, and microorganisms have been pursued ever since Priestley discovered and isolated oxygen (Hewitt, 1950).

The ORP has been used as a control parameter in several fermentation processes. Actually, it is an expression of the sum of all oxidoreduction processes in fermentation, in particular the metabolic processes in the cells. The main determinant of the ORP is the presence in the cell of reversible oxidoreduction couples and irreversible reductors coupled with the action of free oxygen and hydrogen (Rabotnowa, 1963).

The ORP is a function of pH, dissolved oxygen concentration, equilibrium constant, and ORP of a number of compounds dissolved in the medium (Ishizaki *et al.*, 1974). ORP is a highly sensitive and instantaneous on-line instrumentation in fermentation process. The ORP value was found to be correlated to the logarithm of DO concentration with a linear relationship (Peddie *et al.*, 1990). The importance of the ORP can be seen clearly in various aerobic processes. Thus, in the transformation of L-sorbose to 2-keto-L-gluconic acid by a mutant strain of *Pseudomonas* (Tengerdy, 1961), the ORP was shown to indicate the oxygen demand of the culture. In biosynthesis of the antibiotics levorin A and levorin B by *Actinomyces levoris,* Sukharevich *et al.* (1970) found that compound A was produced at a high ORP, and compound B at low values. Similar results were found in amino acid fermentation by *Corynebacterium glutamicum* (Radjai et al., 1984), in which the maximum lysine yield was obtained when there was a moderate oxygen deficiency, as expressed by an ORP between -230 and –210 mV. At conditions of more severe oxygen deficiency (-275 < E_h < -225 mV), the largest yields of homoserine and valine were found. It was also found that the ORP minimum of the broth was influenced by the agitation rate (Radjai *et al.*, 1984). Monitoring of ORP was also performed in fermentations with *Proteus vulgaris, Clostridium paraputrificium,* and *Candida utilis* (Balakireva *et al.*, 1974; Jacob, 1970, 1974) as well as *Lactobacilus sanfrancisco* (Stolz *et al.*, 1993) and *Lactoccocus lactis* (Vonktaveesuk *et al.*, 1994), etc. In xylitol production with recombinant *Saccharomyces cerevisiae* containing the xyl-1 gene of *Pichia*

stipatis, the xylitol yield on substrate increased with increased xylose activity, as measured by a high value of ORP (Hallborn *et al.*, 1994). In acetone/butanol fermentation by *Clostridium acetobutylicum*, the ORP was measured in batch and in continuous fermentations. A correlation between the ORP and a switch from acidogenic to solventogenic metabolism was also reported (Penguin *et al.*, 1994).

In recent years, much attention has been paid to ORP measurements and the results have been particularly rewarding in anaerobic bioprocesses (Beck and Schink 1995; Heppner *et al.*, 1992; Peck and Chnoweth 1992). Also, a few reports have related the relevance of ORP measurements for cell physiology and its interpretation in eucaryotic cell cultivation (Huang and Sinskey 1991), and in hybridoma cultures in which oscillations in ORP were related to changes in physiology of viable cells (Eyer and Heinzle, 1996; Higareda *et al.*, 1997).

Although the ORP level in a fermentation broth is reflected in the measurements of ORP, it is not possible to draw any general conclusion regarding the relationship between the two. In any given fermentation, the role of the ORP should be studied and its possible application for a heuristic process control should be investigated (Berovič and Cimerman, 1982 and Berovič, 1999). Although measurements of ORP have been published in several articles, its control or its use for any type of process control is rare. A constant level of ORP in various bioprocesses was noted by Lengel and Nyri (1961).

In a *Bacillus lichenoformis* cultivation, glucose was added to control the ORP

(Kjærgaard and Joergensen 1976, 1979). In a continuous production of xylanase by *Bacillus amyloliquefaciens* the redox level was controlled by the dilution rate and by the agitation rate (Memmert and Wandrey 1987). The most important factor to affect the ORP is DO concentration. Therefore, by adjusting the DO concentration in cultural broth will result in the variation of ORP.

2.8. Conclusion of literature review

PHA is thought to be an environmental friendly biodegradable material and as an alternative to the conventional petrochemical plastics in order to minimize the environmental impact of plastics wastes.

So far, widespread application of the PHA is hampered by its high production cost. Therefore, substantial reduction in PHA production cost is necessary before widespread applications in packaging and disposable products are possible.

Five methods submitted to PHA production have been discussed, which including the bacterial pure culture, transgenic yeast pure culture, transgenic plants, *in vitro* synthesis and activated sludge co-culture. To combine PHA production with wastes treatment in the MWWTP is a novel technique and tracts more and more attention so far. It has been found that the characters of PHA accumulation and production by activated sludge, particularly by the excess activated sludge (EAS), are very similar to the bacterial pure cultures. The EAS uses fatty acids such as acetate, propionate, butyrate and valeric as carbon substrates to synthesis PHA directly without decomposition of carbon skeleton.

The 3HV unit fraction in PHBV copolymer is adjustable by the carbon sources composition and substrate to co-substrate ratios. However, the PHA content in the sludge is still lower than that of pure culture which is a essential factor to the cost reduction. Otherwise, in spite of great efforts on the metabolic pathways for PHA synthesis by activated sludge, the mechanisms for the PHA production by EAS are not understood very well till today. Much effort focused on the anaerobic-aerobic EBPR process for the PHA production and several models have been proposed. But no single model accounts for all PHA synthesis in all the different activated sludge process facilities. Therefore, much effort should be devoted for the further understanding of PHA accumulation pathways by the EAS. It is very significant for increasing the PHA content and hence reducing the cost of the PHA production.

The attempt to produce PHA by activated sludge proposes another way for PHA production. Most of the research on PHA production has been concentrated on pure culture of microorganisms or genetically engineered plants. Using activated sludge, or mixed culture enriched under adequate conditions, may also be a promising option for PHA production. Further investigation is needed.

2.9. The main objectives of this research

The EAS collected from different source such as laboratory-scale SBR with fully aerobic process and full-scale MWWTP will be submitted to PHA synthesis

in order to investigate the difference between these two types of activated sludge in the PHA production.

Fatty acids such as acetate, propionate, butyrate and valerate will be used as sole and complex carbon sources to investigate the PHA synthesis pathways by the EAS. And these results will be compared with that of bacterial pure culture so to understand the metabolic pathways for PHA synthesis.

Simultaneously, an on-line ORP monitoring and controlling system will equipped with the fermentation setup instead of the DO system, to try to monitor DO variation at low concentration and provide the precise controlling of the DO concentration. It is thought a little bit changes of DO will result in the variety of PHA content and its composition. Only ORP system can provide such sensitive monitoring and controlling features.

For further reducing the cost of PHA production, a cheaper carbon source, e.g. glucose will be supplied as sole carbon source instead of fatty acid to accumulate PHA by EAS collected from full-scale MWWTP. The metabolic pathways will also be investigated and discussed in this study.

CHAPTER 3

MATERIALS AND METHODS

3.1. Operation of Sequencing Batch Reactor (SBR)

A laboratory scale SBR with 12-L effective volume and 1.5 days hydraulic retention time (HRT) was inoculated with activated sludge from the MWWTP in Stanley (Hong Kong) (Figure 3.1). The SBR was performed daily for three consecutive cycles. Each cycle included of 8 hours period: 2 hour for anaerobic stage, 4 hour for aerobic stage, and settled 1 hour, another total 1 hour for drawing effluent and refilling of synthetic wastewater. The reactor was fed with a synthetic wastewater containing reconstituted milk at 1.92 g/L, or equivalent to 1200 mg/L in terms of total organic carbon (TOC). Trace elements solution was also added (Table 3.1). The SBR was equipped with peristaltic pumps for influent feeding, effluent withdrawing and excess activated sludge (EAS) collection. The air compressor was employed for aeration. The procedures of the reactor operation, such as feeding, aerating, settling and withdrawing, were controlled automatically by timers, and temperature was maintained at 25°C ± 2°C in a temperature-controlled room. When realized the steady state after 25 days operation, TOC removal efficiency was about 98%. Residue TOC in the effluent was about 24 mg/l. 7-8 gram (dry weight, DW) of EAS could be harvested for the further PHA accumulation fermentation.

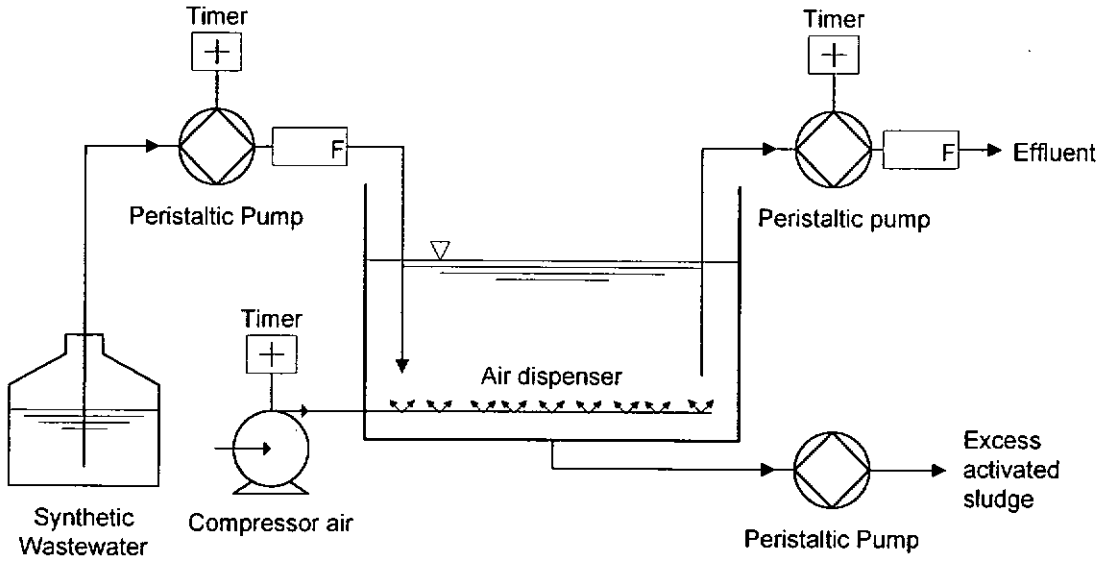


Figure 3.1 The scheme of Sequencing Batch Reactor (SBR). The SBR system equipped with peristaltic pumps for influent feeding and effluent withdrawing, air compressor for aeration and timers for automatically controlling.

Table 3.1. Composition of trace elements of the synthetic waster water for SBR operation

Compound	*Concentration (mg/l)*
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	20
Ferric chloride ($FeCl_3 \cdot 6H_2O$)	28.4
Manganese chloride ($MnCl_2 \cdot 2H_2O$)	0.3
Aluminium sulphate ($Al_2(SO_4)_3 \cdot 18H_2O$	2.2
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	40
Sodium silicate ($Na_2SiO_3 \cdot 5H_2O$)	4
Thiamine hydrogen chloride ($C_{12}H_{17}N_4OSCl \cdot HCl$)	8
Hydrogen borate (H_3BO_3)	4
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	2
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	2
Ammonium molybdenum oxide ($(NH_4)_6Mo_2O_{24} \cdot 4H_2O$)	2
Potassium dihydrogen phosphate (KH_2PO_4)	0.0037

3.2. Collection and pretreatment of EAS from SBR or local MWWTP

Two types of EAS were collected for PHA accumulation. One was harvested from laboratory scale SBR system as mentioned above, and the other was collected from the sedimentation tank in local MWWTP.

At each batch experiment, about 7-8 gram (dry weight) of EAS from the SBR was harvested periodically, washed with distilled water twice to remove any residual nitroeneous matters, and then centrifuged at 1000 × g for 15 minutes at 4 °C to remove the supernatant. Subsequently, the residual biomass pellet of EAS was resuspended with liquid cultural medium and then inoculated into the jar fermenter with cultural medium for the PHA accumulation. Meanwhile, the total suspended solids (TSS) and volatile suspended solids (VSS) of the cultural medium with seeded EAS were also measured as the initial cell dry weight (CDW).

For the purpose of study and understanding the way to accumulate PHA by real EAS from MWWTP, the EAS was collected from the returned sludge at the sedimentation tank. After separating the sludge from residual supernatant, the EAS was then seeded in the jar fermenter with cultural medium for the batch experiments of PHA accumulation. In the same way, the initial cell density (presented as CDW, g/l) of the seeded cultural broth was measured according to the method of TSS and VSS measurement just after the inoculation of EAS.

3.3. The procedures and external conditions of fermentation

The fermentation process was performed in a computer-controlled automatic jar fermenter of 3 l working volume equipped with an ORP meter, air compressor and magnetic stirrer (Bioengineering Model ALF, Ruti/Switzerland) (Figure 3.2, Figure 3.3 and Figure 3.4). The computer-controlling unit could be used to pre-set the fermentation conditions and record the variation of the parameters throughout the fermentation process. The air compressor would compress air and then let the compressed air into the cultural broth to maintain the dissolved oxygen (DO) or oxidation-reduction potential (ORP) at preset levels. The flow-rate of the compressed air could be adjusted by regulating the flow meter.

The ORP meter equipped for the ORP monitoring and controlling is a Cole-Parmer product, pH/ORP CONTROLLER MODEL 5656-00 with feedback sensor (Figure 3.2).

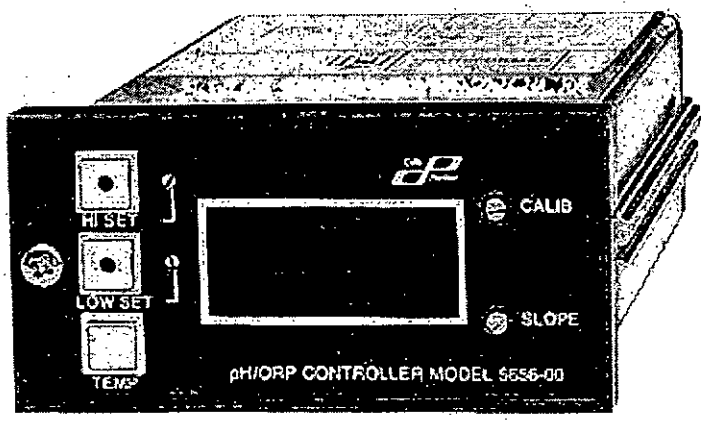


Figure 3.2. Cole-Parmer, pH/ORP Controller Model 5656-00, equipped with the fermenter for monitoring and controlling ORP level of the cultural broth.

For each batch experiment, the jar fermenter was filled with 3 l proper cultural medium containing carbon source and other nutrients, and then adjusted the pH to the designed level. This was a multi-bacteria co-culture process, thus the sterilization of the cultural medium and the accessories of the fermentation system was not necessary any more. Subsequently, about 7 to 8 g (average of 6 g) pretreated EAS from laboratory SBR or MWWT was inoculated and mixed well. By this time, the first cultural broth sample had to be collected for the initial cultivation parameters analysis, initial CDW and PHA content for instance. The fermentation system was operated at 350 rpm stirring speed, 30±1°C for 24 or 48 hours. The temperature was kept by the water jacket automatically. The pH was stabilized at 6.8-7.0 by dosing 2M NaOH. When the experiment was carried out under the ORP monitoring and controlling procedure, the pH was stabilized by a phosphate buffer solution instead to avoid possible effects on the stabilization of ORP. The buffer solution contained 1.794 g/l of sodium di-hygrogen phosphate and 12.350 g/l of di-sodium hydrogen phosphate resulting in an initial pH of 7.5 of the cultural medium.

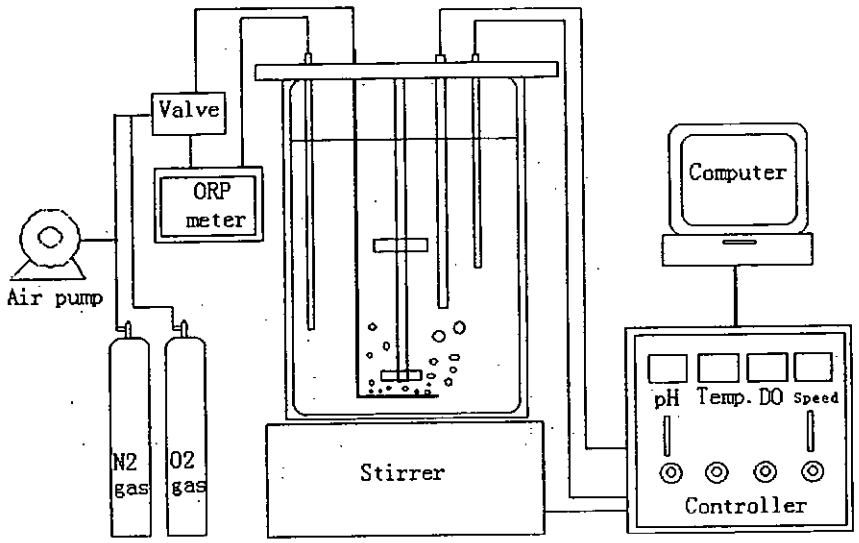


Figure 3.3. Schematic diagram of fermentation setup including Magnetic stirrer; Glass jar reactor; pH meter equipped with a pH sensor; ORP meter equipped with an ORP sensor; Temperature and computer-Controller unit. This system operated with 350 rpm of stirring speed, temperature and pH automatically controlling at 30±1°C and average of 6.8 respectively for 24 or 48 hours fermentation.

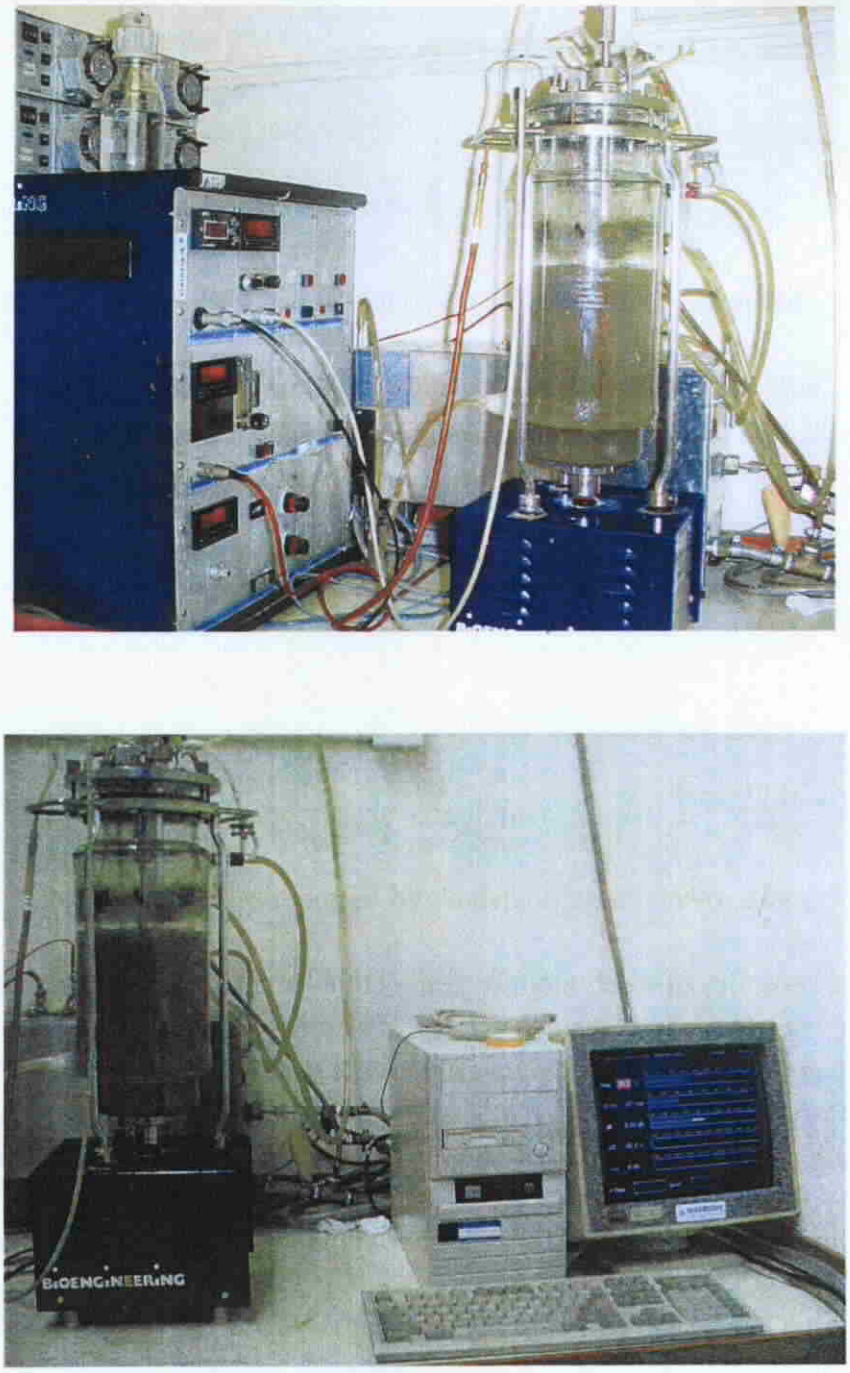


Figure 3.4 The graphs of the computer-controlled Bioengineering Model ALF, Ruti/Switzerland automatic jar fermenter equipped with Computer Central Controller, ORP meter, Air Compressor and Magnetic Stirrer.

3.4. Cultural media for PHA accumulation from EAS

3.4.1. Media for PHA accumulation by SBR formed EAS where butyric, valeric and butyric/valeric acids as sole or complex carbon sources.

A carbon-source-free and nitrogen-free basal medium including supplementary trace minerals and growth factor with formulation was added and listed at Table 3.3. At each batch experiment, the proper carbon sources would be added and dissolved in the basal medium as the fermentation medium.

In this part of experiment, the butyric (C4) and valeric (C5) acids were used as sole or complex carbon sources. The reason for choosing C4 and C5 as carbon sources in the medium was because copolymer formation from C5 followed different mechanisms. It has reported by Anderson *et al.* (1990) that C5 could be incorporated into the polymer as a 3HV unit without decomposition of the carbon skeleton by *R. eutrophus*. Hence it was also investigated whether the copolymer of PHBV with higher mole% 3HV produced by EAS when using butyric and valeric acids as carbon sources. On the other hand, the capability of regulating the 3HV mole fraction in the PHBV copolymer by adjusting the C4/C5 ratio was also studied. Six batch experiments with different C4/C5 ratios were conducted, and the carbon source composition in each batch culture were listed in Table 3.2.

Due to over 0.1% of acids in the medium were inhibitory and toxic to the bacteria in pure culture and EAS co-culture (Kim *et al.*, 1992, Park *et al.*, 1994 and Satoh *et al.*, 1992), periodical feeding batch culture model was performed by adding butyric acid and/or valeric acid at 12 hours interval in the 48 hours culture

process. Therefore, the initial organic acids concentration was 1 g/l, and 1 g/l of organic acids would be fed again after each 16-hours fermentation, which resulted in a total of 3 g/l of organic acids being added. The initial pH of the medium was adjusted to 7.0 by dosing 2M NaOH solution, and then the pH of cultural broth through fermentation process was maintained automatically at the range of 6.8-7.0 by the pH controlling system equipped with the fermentation setup.

This part of research was mainly to verify whether the EAS formed in laboratory scale SBR accumulate PHA by using short chain fatty acid, butyric acid and valeric acid, as carbon sources, and meanwhile, the capability of 3HV/3HB mole ratios regulation by adjusting the C4/C5 ratio was also investigated.

Table 3.2 Carbon sources composition in the media with butyric and valeric acids being the source of carbon.

Media	*Butyric/valeric acids (C4/C5)*		*Content Butyric & Valeric acids in medium (g/l)*		*Total TOC (mg/l)*
	g/g	*mole/mole*	*Butyric acid (C4)*	*Valeric acid (C5)*	
Medium 1	100:0	34:0	3.0	0.0	1636
Medium 2	80:20	27:5	2.4	0.6	1661
Medium 3	60:40	20:11	1.8	1.2	1686
Medium 4	40:60	13:17	1.2	1.8	1712
Medium 5	20:80	6:23	0.6	2.4	1738
Medium 6	0:100	0:29	0.0	3.0	1764

3.4.2. *Media for PHA accumulation by EAS from SWWTP where acetate, propionate and acetate/propionate as sole or complex carbon sources.*

A carbon-source-free and nitrogen-free basal medium including supplementary trace minerals and growth factor with formulation was added and listed at Table 3.3. At each batch experiment, the proper carbon sources would be added and dissolved in the basal medium as the fermentation medium.

To refer to the basal medium (Table 3.3), sodium acetate (C2) and sodium propionate (C3) were added into the basal medium as sole or complex carbon sources respectively. In separate batch cultures, the C2 to C3 mole ratios in the medium were adjusted to 100:0, 80:20, 50:50, 20:80 and 0:100, respectively. Initial TOC of the media was about 1500 mg/l at each batch culture no matter what the varied C2/C3 ratio was, with initial pH of 7.0. The purpose for PHA accumulation with different C2/C3 ratios was to try to prove that the composition of the PHA from EAS co-culture could be adjustable just like the bacterial pure cultures by regulating the composition of carbon sources. The carbon sources compositions of these media were listed in Table 3.4.

Due to the reason described above, a feed batch mode of carbon sources has been conducted by adding 1/4 of total acetate and/or propionate at each 12 hours interval during the 48 hours culture process, to avoid possible bacterial growth inhibition. For example, if total 5.13 gram/l of acetate was used as sole carbon source, it would be added by four times during 48 hours fermentation (1.28 gram/l

per time), thus the initial concentration of acetate was 1.28 gram/l, and the residual of acetate would be added once every 12 hours to avoid possible growth suppression. The initial pH was about 7.0 and maintained through at the range of 6.8 to 7.0 automatically by adding 2M of NaOH solution via the pH controlling system equipped in the fermentation setup.

Table 3.3. Composition of nutrients and trace elements in the basal medium

Compound	*Concentration in the medium (per liter)*
K_2HPO_4	4.1g
Na_2HPO_4	1.8g
$MgSO_4 \cdot 7H_2O$	0.2g
$FeCl_3 \cdot 6H_2O$	16.2mg
$CaCl_2 \cdot 2H_2O$	10.3mg
$MnCl_2 \cdot 2H_2O$	0.6mg
H_3BO_3	0.3mg
$ZnSO_4 \cdot 7H_2O$	0.08mg
$NiCl_2 \cdot 6H_2O$	0.07mg
$Na_2MoO_4 \cdot 2H_2O$	0.05mg
$CuSO_4 \cdot 5H_2O$	0.02mg
$CoCl_2 \cdot 6H_2O$	0.4mg

Table 3.4. Carbon source composition in the media with acetate and propionate being sole or complex source of carbon.

Media	*Acetate/Propionate (C2/C3) (mole/mole)*	*Acetate & Propionate in medium (g/l)*		*TOC (mg/l)*
		Acetate (C2)	*Propionate (C3)*	
Medium 1	100:0	5.13	0	1501
Medium 2	80:20	3.72	1.09	1496
Medium 3	50:50	2.05	2.40	1500
Medium 4	20:80	0.73	3.42	1492
Medium 5	0:100	0	4.00	1500

3.4.3. Medium for PHA accumulation by MWWTP formed EAS at different ORP levels where glucose being the sole carbon source.

This medium mainly contented 4 g/l of glucose as carbon sources, 0.058 g/l of NH_4Cl as nitrogen source, resulting in a C/N mass ratio of 96 which provided the highest overall polymer production yield of polymer per g of carbonaceous substrate consumed (Chua *et al.*, 1997a, b; Ma *et al.*, 2000b). The other nutrients, trace elements and growth factors were added and the concentration in the medium listed as Table 3.5.

The initial pH of the medium was regulated to 6.8-7.2 by adding 0.2M of NaOH solution. Instead of using a pH controlling system, pH was stabilized by a phosphate buffer containing 1.794 g/l of sodium dihygrogen phosphate and

12.350g/l of di-sodium hydrogen phosphate resulting to an initial pH of 7.5. The pH of the cultural broth was successfully stabilized between 5.8 and 7.6.

3.4.4. Media with addition of metabolic inhibitor where glucose serves as the carbon source.

To test whether the oxidation of succinate to fumarate was a necessary part of the process resulting in PHA accumulation in TCA cycle under anoxic and anaerobic conditions, 50 mg/l of malonate (malonic acid, $COOHCH_2COOH$) was added to inhibit that step in the pathway (Quastel, 1963 and Louie *et al.*, 2000). The concentrations of glucose and trace elements in this medium were the same as described above and listed in Table 3.5.

Table 3.5. Composition of the mineral nutrients, trace elements and growth factors in the fermentation medium where glucose as sole carbon sources.

Compounds	*Concentration in medium (per liter)*
K_2HPO_4	3.0 g
$MgSO_4 \cdot 7H_2O$	0.2g
$FeCl_3 \cdot 6H_2O$	28.4mg
$CaCl_2 \cdot 2H_2O$	40.0mg
$MnCl_2 \cdot 2H_2O$	0.3mg
H_3BO_3	0.4mg
$ZnSO_4 \cdot 7H_2O$	2.0mg
$Al_2(SO_4)_3 \cdot 8H_2O$	2.2mg
$Na_2MoO_4 \cdot 2H_2O$	0.05mg
$CuSO_4 \cdot 5H_2O$	0.2mg
$CoCl_2 \cdot 6H_2O$	0.4mg
Thiamine hydrogen chloride	0.8mg

3.5. Analytical methods

3.5.1. Observation of biopolymer accumulation under a microscope by Sudan Black staining

A loop of activated sludge is smeared on the slide, dried in air. Then to take the slide passes through a flame in a process called—Heat Fixing. This process bonds the cells onto the slides and killed many organisms that may still be alive. Then a drop of Sudan Black B solution is covered the smeared area and stayed for 10 min. To decolour the stained area with Xylene for about few seconds, and then rinsing in tap water to remove excess Sudan Black solution. After blot drying, the slide is stained again with Safranine solution for 1 min and is allowed air drying. Observation can be taken under microscope. Blue granules represent the presence of PHA granules and the rest part of the cell is in red color (Gomez *et al.*, 1996)

3.5.2. Total Organic Carbon (TOC)

In this study, the analysis of initial and residual carbon source concentration in the media or cultural-broth supernatants was taken by measuring TOC instead of reduction sugar with a Shimadzu TOC-5000A TOC Analyzer equipped with ASI-5000A Auto Sampler (Figure 3.5). The procedure of analysis was according to the method suggested by American Public Health Association (APHA) (1992; Standard code: 4500). The data of TOC could indicate the initial, residual and

final concentrations of carbon source in the cultural broth, and hence calculating the TOC removal efficiency performed by the bacteria in EAS.

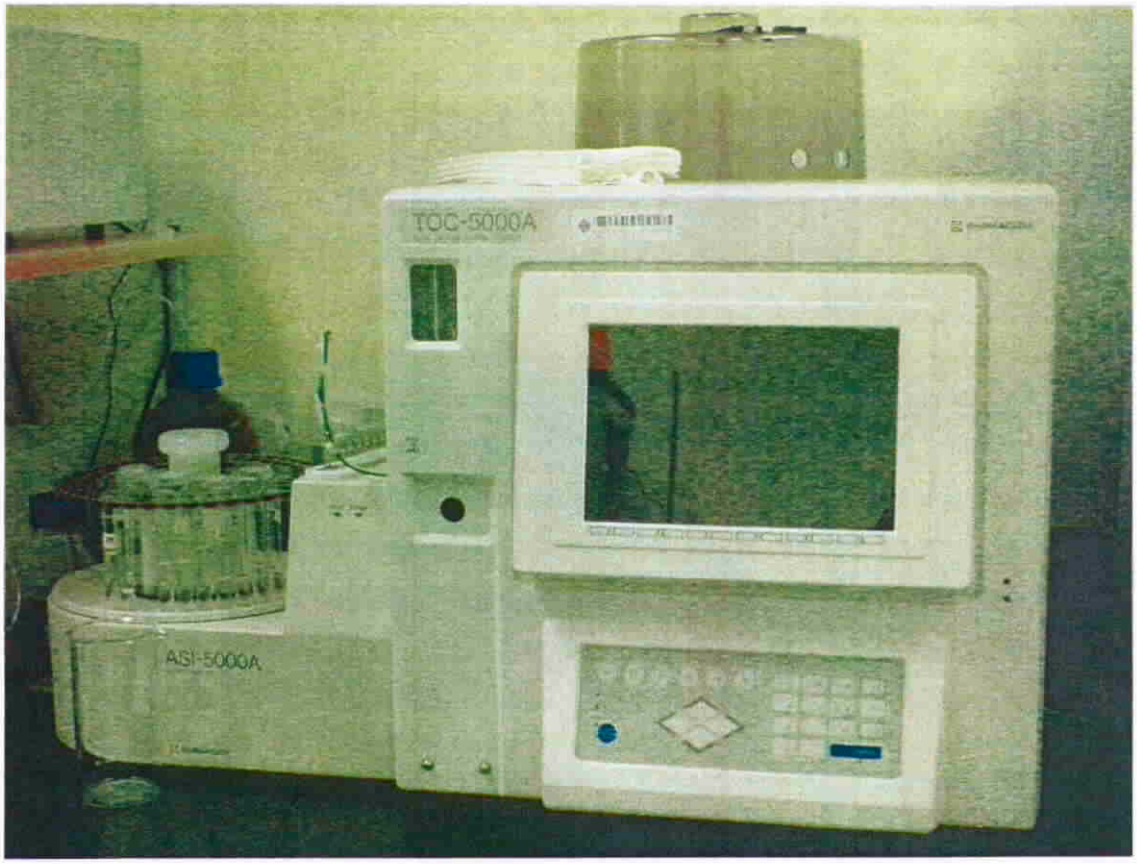


Figure 3.5 Shimadzu TOC-5000A TOC Analyzer equipped with an ASI-5000A Auto Sampler. TOC of the media and cultural-broth supernatants was measured according to the method presented by APHA (1992) (Standard code: 4500).

3.5.3. pH measurement:

The pH of media and cultural broth was measured by an Oakton Benchtop pH/Ion 2100meter pH meter (Figure 3.6).

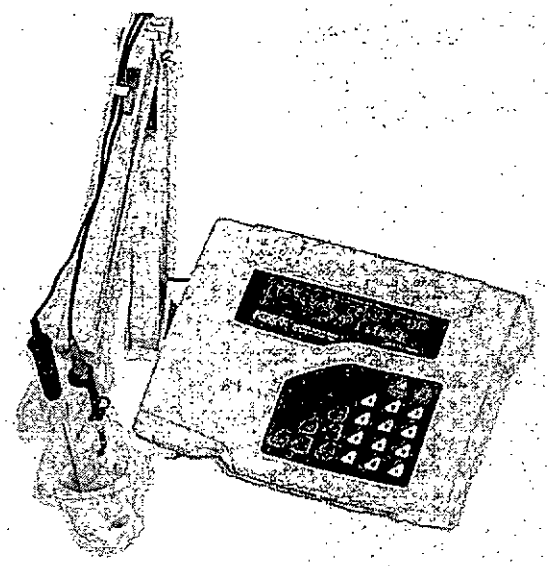


Figure 3.6. An Oakton Benchtop pH/Ion 2100meter pH meter employed for pH measurement.

3.5.4. Cell Dry Weight (CDW)

To measure the biomass in EAS and cultural broth, the CDW was determined according to the APHA (1992) standard method (Standard code: 2540D). The cultivation broth was periodically sampled and analyzed for CDW. 50 ml of mixed liquor of the activated sludge samples or the cultivation broth was taken out, and filtered by using a pore size of 0.45 μm filter paper which was pre-weighted. After drying for 2 hours at 105 °C, the filter paper together with the biomass was weighted to obtain CDW. The quantity of CDW in EAS collected from SBR or MWWTP would be referred to guide the inoculation volume of EAS into the jar bioreactor. As described above, at each batch fermentations, 7-8 gram of the initial EAS would be inoculated into the jar bioreactor with 3 l working volume, which resulted in the initial average biomass of 3 gram/l. CDWs of the cultural broth were measured periodically in order to analyze the growth of the bacteria during the fermentation process, and how many nutrients, carbon sources or nitrogen source for example were consumed.

3.5.5. Polymer analysis

3.5.5.1. Gas Chromatography method

1 liter of EAS from SBR, MWWTP or fermentation broth was centrifuged at 3000 rpm (1000 x g) for 15 minutes. After centrifugation, the supernatant was sucked off with a syringe and the EAS in the bottom of centrifuge tubes were

collected, frozen and lyophilized. A 15 mg of lyophilized EAS was combined with 1 ml chloroform and 1 ml of esterification solution, which is consisted of 3 ml 98% H_2SO_4 and 0.29 g benzoic acid dissolved in 97 ml methanol as an internal standard. DL-3-Hydroxybutyric acid (HB) sodium salt dissolved in acidified methanol was used as the standard. Samples and standards were heated for 4 h at 100 °C in Pyres test tubes (15 ml) with Teflon lined caps to convert the polymer to the methyl esters of the fatty acid repeating units. After cooling, 1 ml of distilled water was added and then was mixed well by vortexing. The samples were left overnight to induce phase separation. Then, two phases were observed. 1 μl of lower organic phase was injected by split injection into a G.C. Hewlett-Packard 5859 Series II Gas Chromatograph (Figure 3.7), using a Supelco (10% Carbowax 20M with 80/100 in mesh size Chromosorb WAW) Packed Column with 6 feet in length. The fractionated products were detected by flame ionization (30 ml/min. of hydrogen, 20 ml/min. of air and 1 ml/min. of auxiliary nitrogen). Nitrogen was chosen as the carrier gas. The inlet, oven and detector temperatures were 260°C, 135°C and 300°C respectively (Braunegg *et al.*, 1978 and Jan *et al.*, 1995).

PHA purity and its composition could be determined by this gas chromatograph method, and the quantity of PHA was calculated from a calibration curve using different concentration of PHA standards. To recognize the retention time of the peaks firstly and then compare the peak areas with the standard curves, the components and the amount of the PHA in the samples could be identified and estimated.

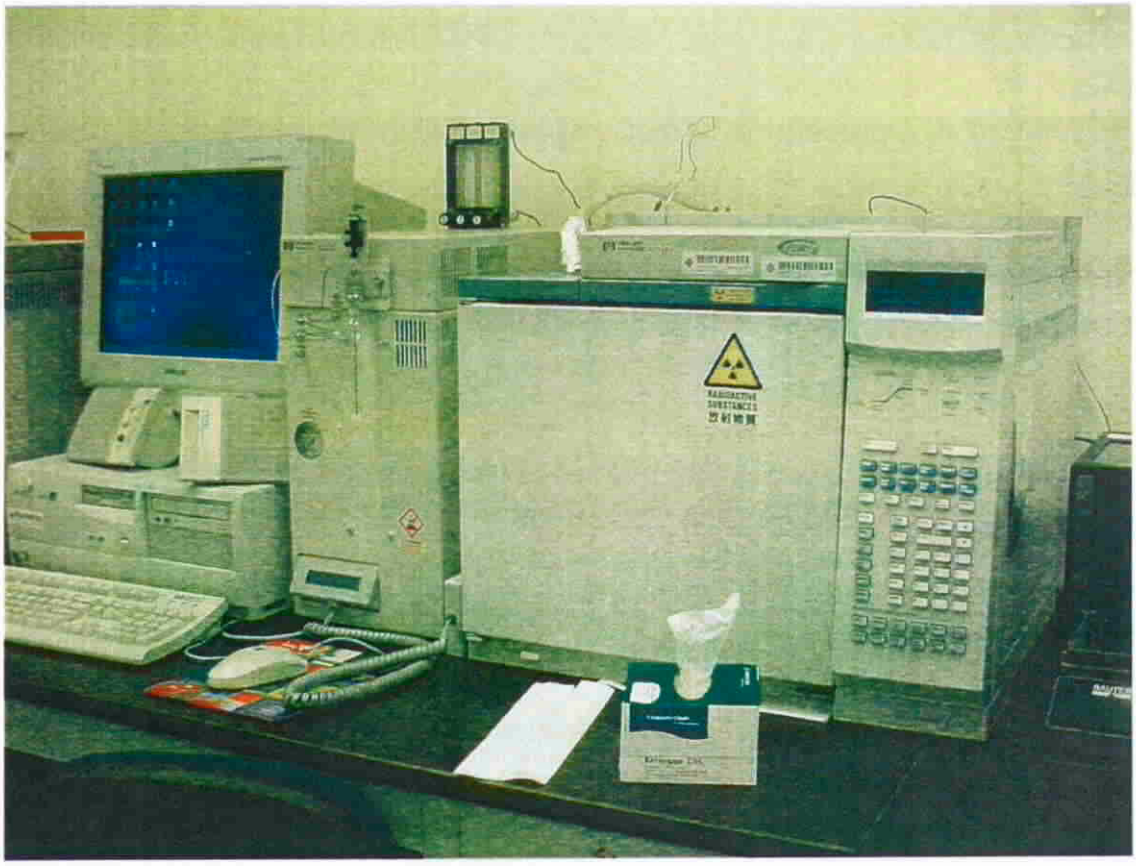


Figure 3.7 the Hewlett-Packard 5859 Series II Gas Chromatograph for PHA analysis. A Supelco (10% Carbowax 20M with 80/100 in mesh size Chromosorb WAW) Packed Column with 6 feet in length was conducted for the performance. The pre-treated samples were detected by flame ionization (30 ml/min. of hydrogen, 20 ml/min. of air and 1 ml/min. of auxiliary nitrogen).

3.5.5.2 Extraction of PHA

When the fermentation process for PHA accumulation was finished, the EAS biomass in 3 l of fermentation broth was harvested by centrifugation at 3000 rpm (1000 × g) for 20 min. The residual sludge pellets was washed twice with distilled water, and then frozen drying. It was then weighted. About eight grams of EAS biomass powder was treated by 100 ml 30% sodium hypochloride and 100 ml chloroform. The mixture was agitated in a shaker at 250 rpm at 37°C for 1 hour. After the treatment, the dispersion was centrifuged at 3000 rpm (1000 × g) for 20 min. The three separate phases were obtained. The upper phase was the hypochlorite solution, the middle phase contained non-PHA cell material (NPCM) and undisrupted cells, and the bottom phase was the chloroform layer containing PHA. Firstly, the hypochlorite solution phase was removed with a pipet, and then chloroform layer was obtained by filtration. Then, the PHA material was precipitated by mixing methanol with the concentrated chloroform (methanol : chloroform = 9:1). Finally, the white precipitate was filtered by simple filtration and then dried by evaporation (Doi, 1990a; Hahn *et al.*, 1994 and Cromwick *et al.*, 1996).

3.5.6. Determination of melting temperature of the PHA.

Melting point of the extracted PHA was determined with a Fisher Electrothermal Digital Melting Point Aapparatus Model IA9100 (Figure 3.8). The temperature

increase rate program was set at 10 °C/min from room temperature to 200°C. Detail operation procedure of the melting point measurement was presented in Appendix AA.

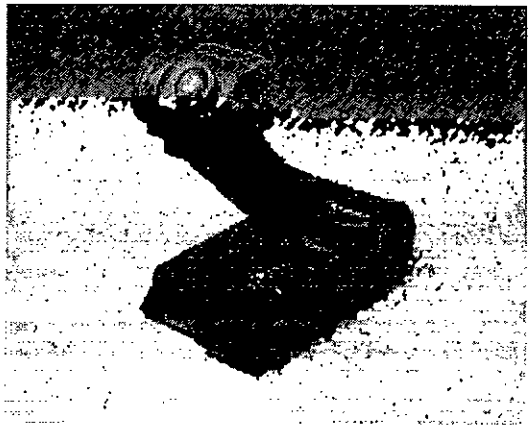


Figure 3.8 Fisher Electrothermal Digital Melting Point Apparatus Model IA 9100.

3.6. Oxidation-Reduction Potential (ORP) monitoring and controlling of the cultural broth

ORP is the electromotive force developed when oxidizers or reducers are present in aqueous solution. In this research, an on-line ORP monitoring and controlling system with intermittent nitrogen, air or oxygen feeding program has been equipped with the fermentation setup instead of the DO control system. The ORP levels during the cultural process were monitored through out the cultural process. Meanwhile, in order to optimize the PHA accumulation from EAS, the ORP was regulated and maintained at different levels by controlling the gas

supply procedure (nitrogen, oxygen and air). The set ORP levels were -100mV, -30mV, -20mV, -10mV, 0mV, +10mV, +20mV and +30 mV respectively.

To get the initial negative ORP, the media or cultural broth might be heated at 100°C for 10 minutes at first, as well as sparging with nitrogen gas to remove the dissolved oxygen in the broth and the oxygen on the top of the jar bioreactor to avoid the surface transfer of oxygen. If the actual ORP were lower than the set level, the air or pure oxygen gas would be pumped into the broth or increasing the gas-flow rate to get or maintain the ORP at designed point. Contrarily, if the ORP were higher than preset value, gas-flow rate would be lowed or nitrogen gas would be flowed into the broth if necessary. Any variation of the ORP could be adjusted back to the designed level automatically by changing the kinds of gases flowed into the broth or gas-flow rate.

Since the pH and chemicals such as NaOH and HCl can affect the value and stabilization of ORP, the phosphate buffer solution was employed for pH maintenance instead of adding NaOH or HCl. The phosphate buffer solution consisted of 1.794 g/l of Sodium Di-hydrogen phosphate and 12.350 g/l of Di-Sodium Hydrogen Phosphate resulting in an initial pH of 7.5. The pH was successfully kept at the average of 6.8 during the period of fermentation process.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 The synthesis of PHA by the EAS acclimatized with synthetic wastewater while butyric, valeric and butyric/valeric acids as carbon sources.

Introduction

Recently, many efforts have been performed in optimizing the PHA production process and reducing costs. Lee *et al.* (1994) investigated various recombinant *E. coli* using different complex culture media. *E. coli* strain XL1-blue in LB plus 20 g glucose/L could accumulate up to 0.369 g PHA/g glucose, or equivalent to 7 g PHA/L. Shirai *et al.* (1994) used a photosynthetic bacterium *Rhodobacter spheroides* in a fed batch culture with glucose as the sole carbon to achieve a PHA production of 6 g/L. Shimizu *et al.* (1992) used a cell growth phase followed by a separately optimized nutrient-deficient PHA accumulation phase to improve the specific production yield to as high as 0.70 g PHA/g cell mass.

To produce co-polymers with improved mechanical properties, Doi *et al.* (1988) and Ishihara *et al.* (1996) demonstrated the accumulation of a variety of PHA co-polymers in a pure culture of *Alcaligenes eutrophus* with different compositions of butyric and valeric acids in the culture medium. The

co-polymeric synthesis pathway had also been traced.

So far, these results resulted from the efforts can't meet the marketing needs yet in the cost reduction of PHA production.

In recent years, much attention has been devoted to accumulate the PHA by activated sludge as a byproduct of wastewater treatment process. In fact the capability of PHA production by activated sludge has been found several years before (more detail in Chapter 2). Therefore, it is thought that PHA synthesis from EAS might be another option for the industrial production of PHA.

The production of PHA by activated sludge including EAS may be an inexpensive way of producing a highly increased value product and also a way to decrease the quantity of excess sludge for further treatment, whereas first of all we have to identify the difference of all kinds of activated sludge existed so far.

Generally, the activated sludge generated from the municipal wastewater treatment plant could be divided into four types as shown in Table 2.11. In this study the concept of "activated sludge" will be extended to those of residual biomass including yeast wastes in brewery industry and microbial waste in fermentation plant such as the residue biomass created in amino acid production fermentation process (Table 2.11). The information on the production of co-polymers from this extended activated sludge is not completely elucidated in particularly the metabolism mechanisms are not yet revealed.

In this study, the activated sludge from a conventional SBR was obtained and conducted for the PHA accumulation by using butyric acid (C4), valeric acid (C5) or butyric (C4)/valeric (C5) acids as sole and complex carbon sources respectively.

The main objectives of this study

In this part, the EAS applied to PHA production was harvested from laboratory scale conventional SBR process acclimatized with synthetic wastewater. Two aspects were investigated. The one was that the possibility to introduce the activated sludge bacteria in a conventional food-processing wastewater treatment process to produce PHA by using butyric acid (C4), valeric acid (C5) or butyric (C4)/valeric (C5) acids as sole and complex carbon sources independently. The other was that the regulation of 3HB to 3HV mole ratio of PHBA copolymer from EAS by changing the composition of carbon sources in the medium, hence resulted in the regulation of the thermal property of the PHA. The metabolic pathways for the PHA production by EAS under these conditions have also been discussed.

By the way, each batch experiment was performed in a jar fermenter filled with 3 l proper medium seeded 7-8 g (CDW) EAS from laboratory scale SBR, 30°C ± 1°C for 48 hours. A feed batch model has been conducted, thus the initial total fatty acid concentration in the medium was 1g/l, other 1g/l of substrates would be added after every 12 hours fermentation to avoid possible growth

inhibition. Further detail of the methods and medium are referred to Chapter 3.

4.1.1 Cell growth, TOC removal and PHA production under various C4/C5 ratios

Bacteria in activated sludge are known to accumulate internal storage polymers, a variety of PHAs, and some isolated microorganisms can also form PHAs in the pure cultural conditions (Stanier *et al.*, 1959; Wallen and Davis, 1972; Wallen and Rohwedder, 1974; Zevenhuizen and Ebbink, 1974; Wong, 2001 and Liu *et al.*, 2002). In this research, the activated sludge collected from a laboratory scale SBR has been conducted directly and to be acclimatized by using C4 and C5 as carbon sources to accumulate PHA.

Table 4.1 listed the biomass, TOC removal efficiency, polymer content, product yields, final 3-hydroxyvalerate (3HV) fraction and thermal property (melting temperature, T_m) of the polymer after 48-h fermentation under six different carbon sources ratios.

The initial cell mass concentration in the fermenter was about 2.5 g/l, whereas the final cell mass was varied within the range from 2.30 to 3.01 g/l after the 48 hours culture process. Obviously, Table 4.1 illustrated that there was no significant change in biomass observed under every fermentation process with various C4/C5 ratios. When C4 was used as sole carbon source, the final cell mass concentration was increased around 20% compared to the initial concentration of average of 2.5 g/l. It is estimated that the C4 was partially converted for the cell

growth.

But when C5 was introduced into the cultural media, the cell mass concentration were either maintained at constant or decreased (Table 4.1). Maximum of 8% of cell mass decline was observed while total of 3g/l C5 was used as sole carbon source in the medium. In other case, the variation of cell mass concentration was less than 3% which can be recognized that the cell mass maintained constant and no net cell growth occurred under these conditions. This could be recognized as the result of absence of nitrogen source for the cell growth. Thus most of the substrates including C4 and C5 were transported into the cell and then converted into PHA copolymer.

To understand the C4 and C5 metabolism pathway for PHA conversion after being transported into cells in activated sludge, a biosynthetic pathway modified from Doi *et al.* (1989) was shown in Figure 4.6. Originally, this pathway was illustrated by Doi (1989) to demonstrate the PHBV production in *Ralstonia eutrophus* (formerly known as *Alcaligenes eutrophus)* from butyric acid and valeric acid. They observed that the PHB synthesized from ^{13}C-labeled butyric acid as the sole carbon source for *R. eutrophus* without decomposition of the butyric acid skeleton into acetyl-CoA. Butyric acid is metabolized to acetoacetyl-CoA in the β-oxidation cycle by the catalyzetion of specific enzymes. Most of the acetoacetyl-CoA must be hydrogenated by reduced $NADPH_2$ into D-3-hydroxybutyryl-CoA, which is then directly incorporated into PHB under catalysing of polymerase. A similar synthesis pathway of 3HV from valeric acid

has been found by Anderson *et al.* (1990). He wrote that C5 could be incorporated into the polymer as a 3HV unit without decomposition of the carbon skeleton. When activated sludge from a petrochemical waster biotreatment plant was submitted to an nitrogen free medium with butyrate as sole carbon source by Dave *et al.* (1996), the PHB content reached a 2-fold increasing in dry cell weight. So, it summarized that PHA production by using butyric acid and valeric acid as sole or complex carbon sources are the inherent feature not only for *R. eutrophus* pure culture but also the co-culture of activated sludge. Lemos *et al.* (1998) conducted the polyphosphate-accumulating activated sludge to accumulate PHA under anaerobic condition by using acetate, propionate and butyrate as sole carbon source independently. It was found that when butyrate was used as substrate, the PHA content was about 110 mg/g VSS (Volatile Suspended Solids). Therefore, the pathway demonstrated in Figure 4.6 consisting of the PHBV production from butyric acid and valeric acid respectively adapted and modified from the bacterial pure culture could be presented partly the similar way in activated sludge. Furthermore, the pyruvate-propionyl-CoA pathway (Wentzel *et al.*, 1986; Mino *et al.*, 1987; Smolders *et al.*, 1994; Pereira *et al.*, 1996; Maurer *et al.*, 1997 and Louie, *et al.* 2000) for the anaerobic-aerobic sludge in EBPR process has also been combined with the modified biosynthetic pathway (Figure 4.6).

It can be seen from the modified biosynthetic pathway, a fraction of acetoacetyl-CoA derived from C4 enters the TCA cycle under balanced growth conditions in the presence of excess oxygen and nitrogen for energy generation

and amino acids production (Doi, 1990; Lee and Choi, 1999), which results in the cell growth.

As shown in Table 4.1, when C4 was used as sole carbon source, the total TOC removal was 98.0%. However, total TOC removal efficiency decreased from 98.0 to 65.0% when valeric acid in the medium was increased from 0 (i.e. C4/C5= 34:0 mol/mol) to 100 mol% (i.e. C4/C5=0:29 mol/mol), while the PHA content in cell dry weight (CDW) decreased from 40% to 18%. When valeric acid was used as sole carbon source, the TOC removal efficiency decreased to a minimum value of 65.0% (Table 4.1, Figure 4.1 and Figure 4.3). These results were in agreement with that reported by Ishihara *et al.* (1996) for a pure culture of *R. eutrophus* with butyric and valeric acids as the carbon sources. Ishihara *et al.* (1996) observed that when butyric acid concentration in their medium was maintained at a constant value while valeric acid concentration was increased, cell growth and fatty acids consumption were substantially affected. Liu *et al.* (1996) reported a similar observation when glycogen-accumulating population in anaerobic-aerobic activated sludge was submitted to accumulate PHA under anaerobic condition, where butyrate and valerate were used as independent carbon sources. The butyrate and valerate uptakes were about 58.7 mg-C/g TSS and 29.2 mg-C/g TSS (Total Suspended Solids) respectively. It is concluded that no matter what microorganisms conducted (pure culture or co-culture by EAS), the substrate removal efficiency decrease with the increasing carbon chain length of the acids taken up (Lemos *et al.*, 1998; Randall and Liu, 2002).

Table 4.1 Production of PHB and PHBV by activates sludge with different carbon sources ratios of butyric (C4) and valeric (C5) acids

C4/C5 ratios		*C4 and C5 in medium (g/l)*		*Biomass (g/l)*	*Total TOC (mg/l)*	*TOC Removal (%)*	*Polymer content (wt%)*	***$Y_{p/s}$ (g/g)*	*3HV fraction (mol%)*	**T_m (℃)*
g/g	*mol/mol*	*C4*	*C5*							
100:0	34:0	3.0	0.0	3.01	1636	98.0	37.0	0.69	0	178
80:20	27:5	2.4	0.6	2.45	1661	96.0	40.0	0.61	12	144
60:40	20:11	1.8	1.2	2.52	1686	95.0	35.0	0.55	30	133
40:60	13:17	1.2	1.8	2.30	1712	90.2	24.0	0.35	35	127
20:80	6:23	0.6	2.4	2.50	1738	83.4	18.0	0.32	51	109
0:100	0:29	0.0	3.0	2.30	1764	65.0	22.0	0.44	54	99

** T_m, melting temperature of PHA; ** Yp/s = polymer production yield (g polymer/g carbon), which was calculated as the polymer accumulated divided by the TOC consumed.*

Figure A Residual TOC (C4/C5=34mol:0mol First Run)

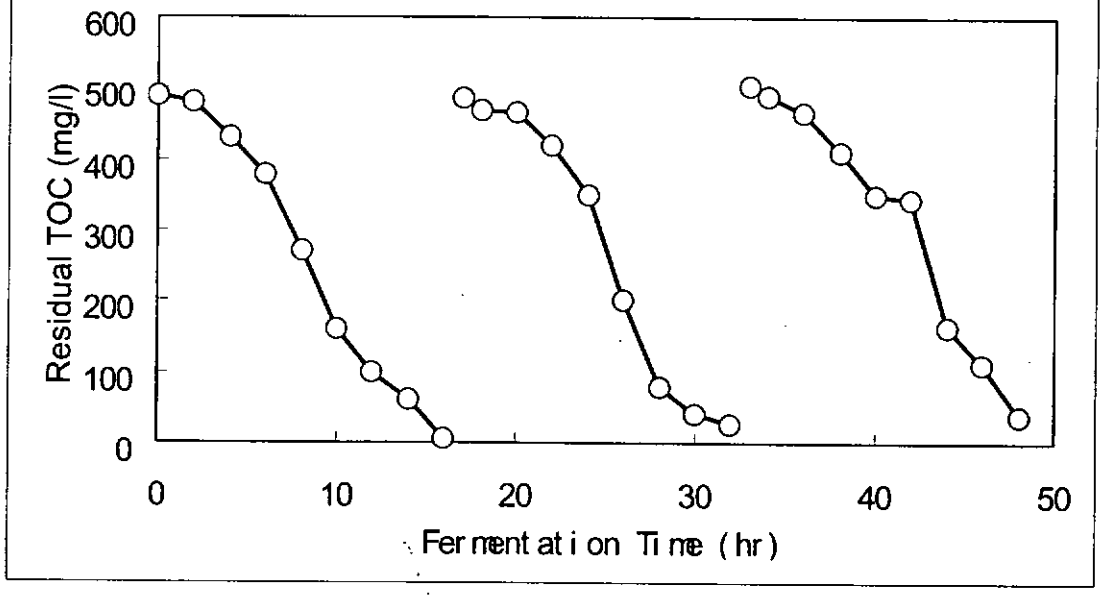


Figure B Residual TOC (C4/C5=27mol:5mol Second Run)

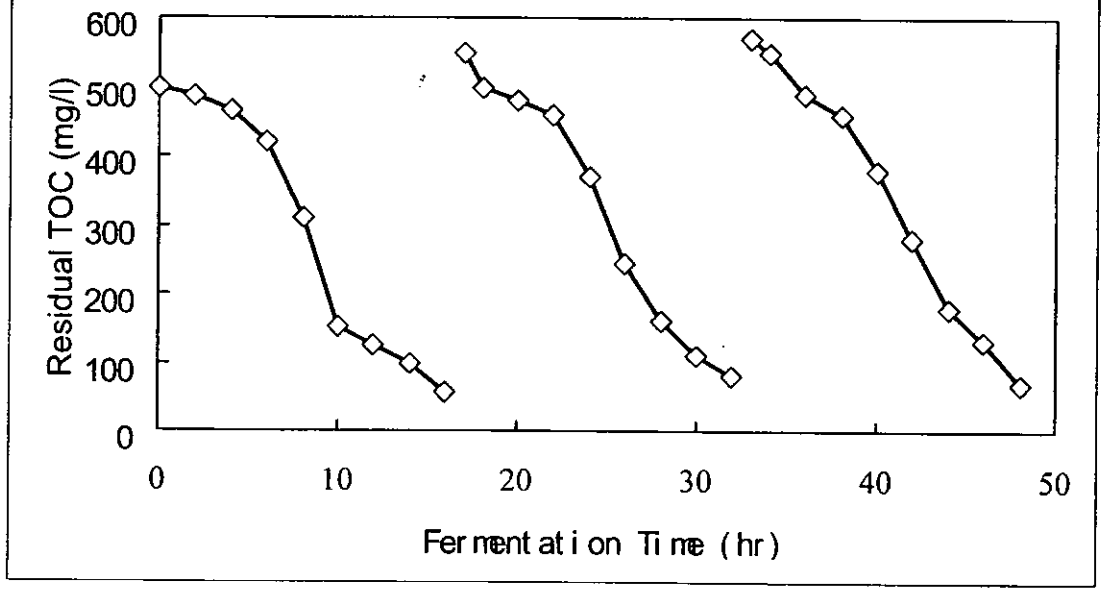


Figure C Residual TOC (C4/C5=20mol:11mol. Third Run)

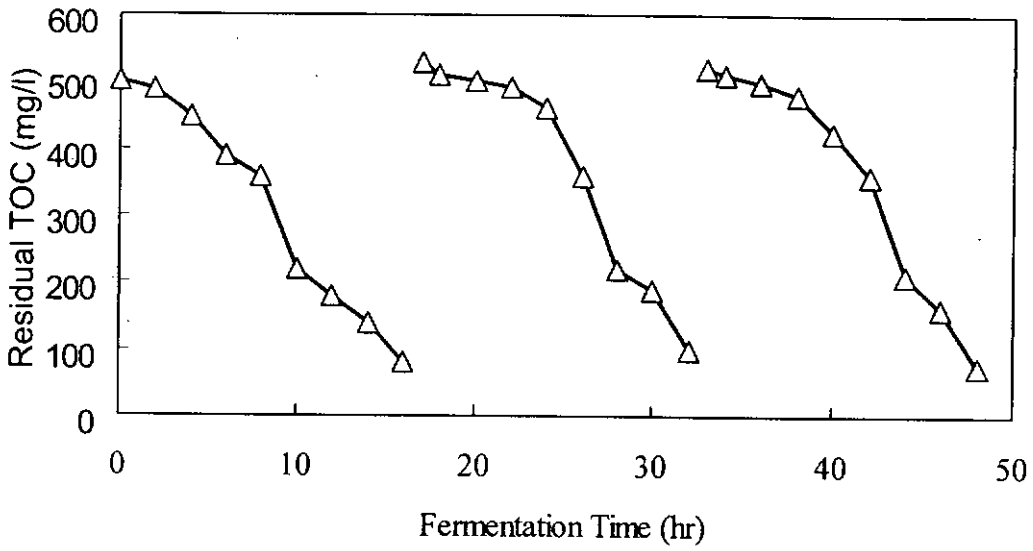
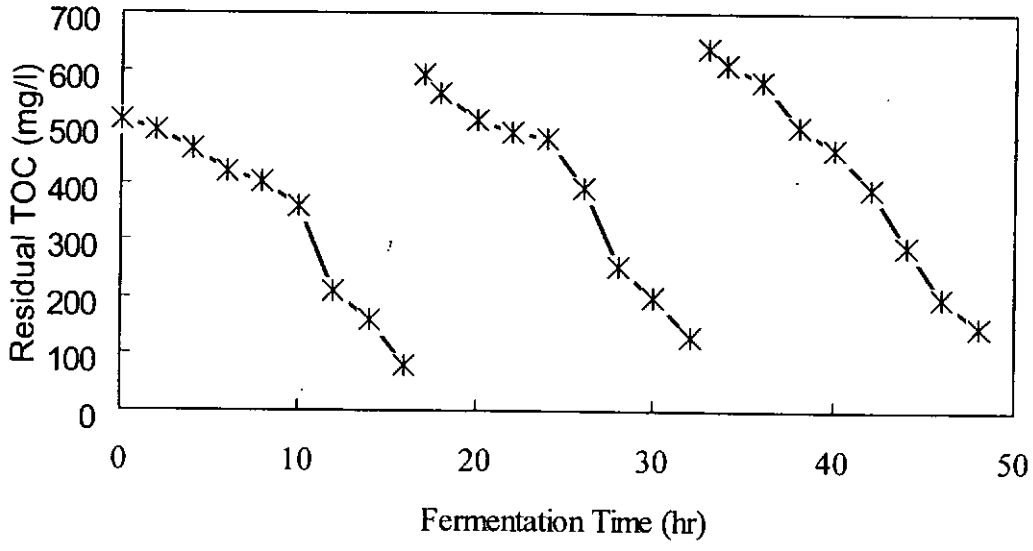
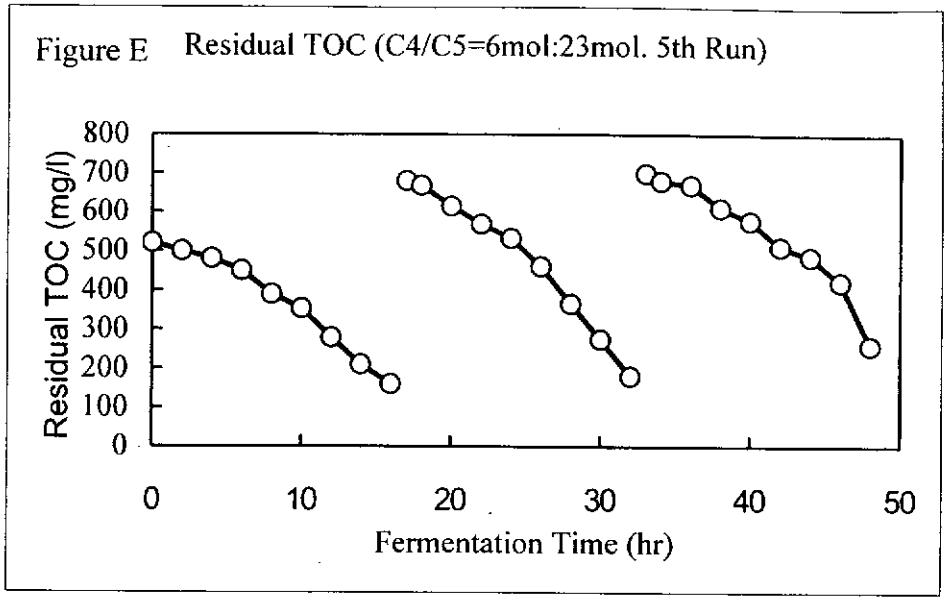


Figure D Residual TOC (C4/C5=13mol:17mol. 4th Run)





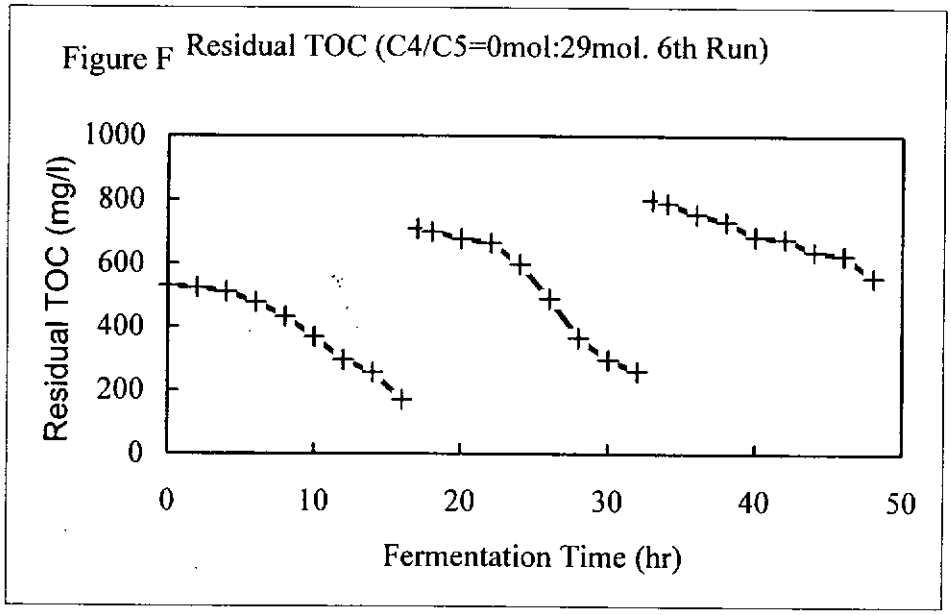


Figure 4.1. The time courses of the TOC removal at different C4/C5 molar ratios in a fed-batch fermentation model. Figure (A), C4/C5 = 34mol/0mol, C4 (butyric acid) was used as sole carbon source; Figure (B), C4/C5 = 27mol/5mol; Figure (C), C4/C5 = 20mol/11mol; Figure (D), C4/C5 = 13mol/17mol; Figure (E), C4/C5 = 6mol/23mol and Fiugure (F), C4/C5 = 0mol/29mol, C5 (valeric acid) was used as sole carbon source.

An increase in valeric acid content in the medium from 0 to 100 mol % resulted in a decline in PHA production yield, $Y_{P/S}$, from 0.69 to 0.32 g-polymer/g-TOC consumed. The PHA content in the cells also decreased from 40 to 18 wt %, which indicated that the production of polymer was inhibited by valeric acid.

These observations were in contrast to those reported by Ishihara *et al.* (1996) and Yamane (1993) that the yield and polymer content remained unchanged despite the variation in valeric acid concentration in the media. The range of $Y_{p/s}$ in the activated sludge, between 0.69 and 0.32 (g/g), was lower than the theoretical yield of PHBV from fatty acids of 0.98 g-PHBV/g-fatty acids by *Rhodospiril rubrum* (Yamane, 1993). However, it was closer to the theoretical yield of 0.65 g-PHBV/g-fatty acids by *A. eutrophus* (Yamane, 1993), which is a common genus in activated sludge. Moreover, when butyrate was used as sole carbon source in an anaerobic-aerobic phosphate-accumulating mixed culture during anaerobic period, the yield of polymer PHA produced per carbon consumed ($Y_{p/s}$: mg polymer/mg COD) was 0.21 (Lemos *et al.*, 1998). The PHA polymer production is however lower than that observed in this research.

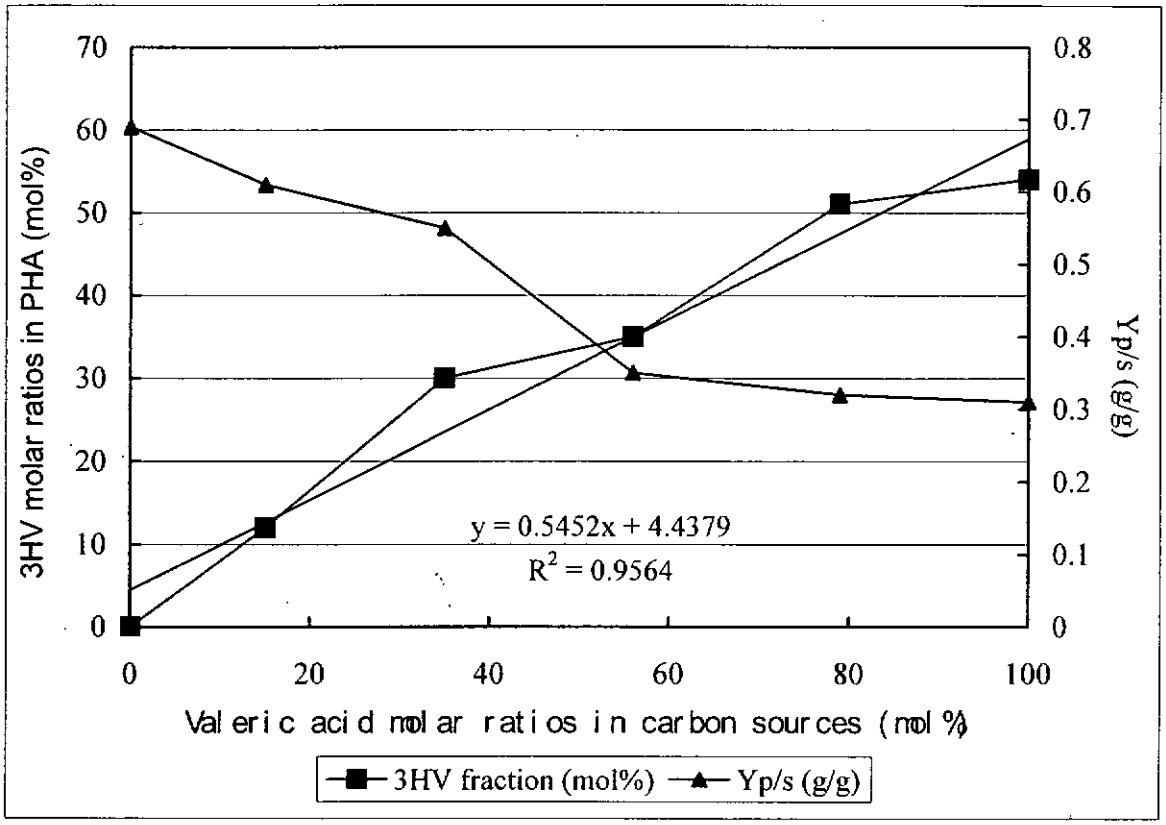


Figure 4.2. The final PHA production yield and the 3HV molar rations in PHA formed by EAS when the valeric acid (C5) used as carbon source at different molar percentage in addition with butyric acid (C4).

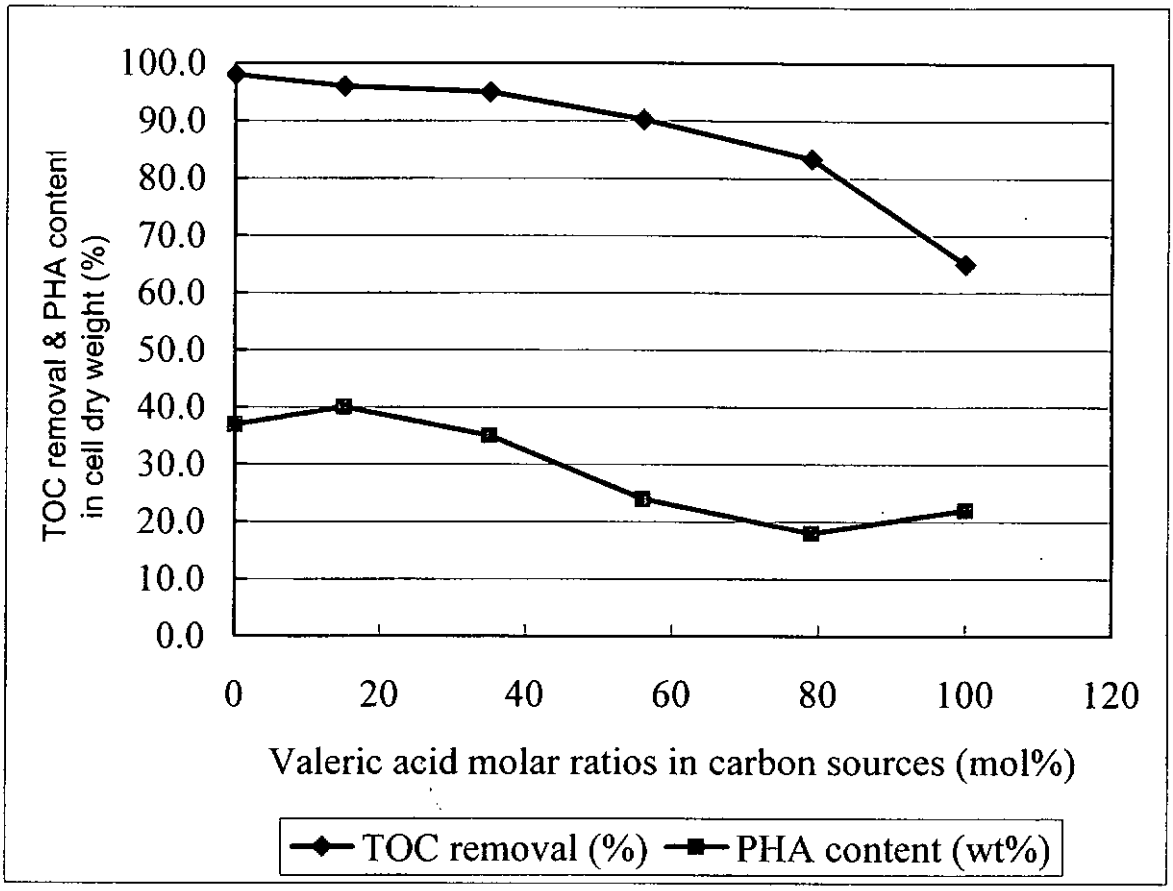


Figure 4.3 TOC removal and PHA content at different valeric acid molar ratios in cultural media.

The wide range of yields in the activated sludge attributed to the complex microbial species in the ecosystem of the sludge, which gives rise to widely varied metabolic pathways from fatty acid to polymer in different microbial species. On the other hand, Dave *et al.* (1996) described that when activated sludge was incubated under nitrogen- and phosphorus-limiting conditions, selective overgrowth of *Bacillus* spp. From 5 to 80% (cell count) was observed. It is therefore believed that the

variation of valeric acid concentration in medium caused the changes in the balance of microbial species, and hence the prevailing metabolic pathways in activated sludge during the 48 hours incubation. This, in turn, resulted in the wide ranging of $Y_{P/S}$ values.

During the PHA accumulation fermentation process, the EAS sample were collected and observed under microscopy (1000 × magnification) by staining with Sudan Black. It was found that some of bacterial cells in EAS filled with inclusions of PHA granules stained in dark-blue. Most of the cells with PHA granule were in the shape of bacillus and coccus. The size of PHA granules correlated with the time course of cultivation and the kinds of carbons sources supplied. Noticeably, it was observed that under the conditions of excess fermentation (more than 48 hours), the PHA granules disappeared gradually with the time course. It was indicated that the PHA granules could be reused by the cells as storage energy and carbon sources by the end of fermentation process.

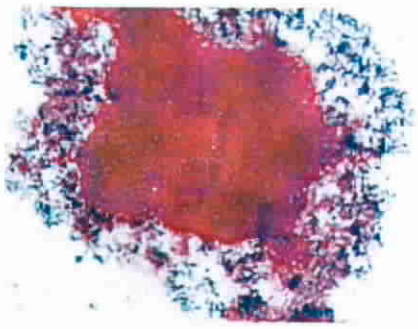


Figure 4.4. The optical micrographs of the EAS samples.

4.1.2 Effects of C4/C5 ratios on PHA composition

When butyric acid was used as sole carbon source, there was only PHB homo-polymer produced instead of PHBV co-polymer (Table 2 and Figure 4.5). On the other hand, the highest 3HV mole fraction of 54 mol% in the co-polymer accumulated was when valeric acid was used as sole carbon source (Figure 4.5). The mole fraction of 3HV in the accumulated co-polymer increased proportionately with the valeric acid concentration in medium. Figure 4.2 illustrates a linear relationship between 3HV mole fraction in the co-polymer and the valeric acid concentration in the medium. Linear regression was conducted using 3HV as dependent variable and valric acid molar concentration as the independent variable, hence the equations could be presented as $y = 0.5452x + 4.4379$, $R^2 = 0.9564$ and $R = 0.978$ (where "y" stands for 3HV mole fraction and "x" stands for valeric acid mole concentrations in media). These results from activated sludge were in agreement with that observed in pure cultures of *A. eutrophus* (Doi *et al.*, 1988; Ishihara *et al.*, 1996 and Ho, 1997).

Liu *et al.* (1996) observed that when butyrate was used as sole carbon source in an anaerobic-aerobic activated sludge without biological phosphorus removal during anaerobic phase. They found that the 3HB and 3HV molar fractions were 37 mol.% and 18.3 mol.% respectively. Furthermore, 42.5 mol.% of 3HC (3-hydroyxhexanoate) and 1.6 mol.% of 3H2MV were observed too. When valerate was used as sole carbon source, the 3HB and 3HV molar fractions were 13.8 mol.% and 76.2 mol.% respectively. Otherwise, remaining of 6.7 mol.% of 3H2MV (3-hydroxy-2-methyl-valerate) and 3.2 mol.% 3H2MB (3-hydroxy-2-methyl-bytrate) were also obtained.

Lemos *et al.* (1998) have conducted a similar experiment by using butyrate as independent substrate in EBPR sludge under anaerobic condition, PHA copolymer was accumulated with 3HB and 3HV molar percentage of 59.68 mol.% and 40.32 mol.% respectively, where 3HB was slightly higher than 3HV. In the control experiment without addition of butyrate, no net production of 3HB and 3HV units was observed. Comeau *et al.* (1987) reported approximately the same values of production of 3HB of 52 mol.% and 3HV of 42 mol.% when using butyrate as carbon substrate in a biological dephosphatation system. To compare the results from the above three authors and that observed in this research, the uniform observation was that PHA copolymer with 3HV as main unit was achieved when using valerate as sole carbon source; the different part of the results was that when butyrate was used as sole carbon source the PHA copolymer with 3HB as main unit addition to 3HV unit and other units. It was thought that the factors caused this difference were the variety of the activated sludge conducted for the PHA accumulation and PHA conversion conditions. The above reports suggested that the experiments were carried out by using anaerobic-aerobic activated sludge from EBPR process during anaerobic phase. Contrarily, the EAS in this paper was aerobic-aerobic activated sludge from a laboratory scale SBR conducted for the PHA accumulation under aerobic condition. The presence and absence of oxygen during the fermentation process would cause significant variation of the metabolisms pathway for PHA production from different activated sludge.

In the case of oxygen presence, it can be seen from the modified metabolic

pathway in Figure 4.6, the EAS from aerobic-aerobic SBR will transport the C4 and C5 into the cell and then convert them for PHBV copolymer production without decomposition. Otherwise, part of the substrates will be converted to acetyl-CoA to enter TCA cycle for energy generation and cell growth. When C4 is used as sole carbon source, only PHA with 3HB as dominant unit without 3HV will be produced. When C5 is used as sole carbon source, most of C5 will be converted to 3HV unit for PHBV copolymer production; otherwise, part of 3-ketovaleryl-CoA derived from C5 will be decomposed to propionyl-CoA and acetyl-CoA, and then the acetyl-CoA will form 3-ketobutyryl-CoA for the 3HB formation. Therefore, when C5 was used as sole carbon source, the 3HV-enriched PHBV copolymer was formed with the rest of 3HB unit.

The activated sludge from anaerobic-aerobic EBPR process will take up C4 and C5 as substrates for the PHBV copolymer production by using the energy and reducing power generated from the glycogen and poly-p, and the composition of C4/C5 ratios in medium will regulate the 3HB/3HV molar ratios in the copolymer. When C4 is used as sole carbon source, during the aerobic phase, most of C4 will be converted to acetyl-CoA to enter TCA cycle for energy production, glycogen and Poly-P generation as energy and carbon reserves. During the anaerobic phase, most of the C4 is converted to 3HB and simultaneously glycogen produced in the aerobic phase will be decomposed as glucose, and then to pyruvate-propionyl-CoA pathway for 3HV formation, and finally, PHBV copolymer with 3HB dominant unit addition to 3HV unit produced. When C5 is used as sole carbon source, during the aerobic

phase, C5 will be converted to acetyl-CoA to enter TCA cycle for ATP production and glycogen synthesis. During the anaerobic phase, most of the C5 will be converted to 3HV without decomposition, and simultaneously glycogen will be reused to form acetyl-CoA and then 2 molecular of acetyl-CoA to form one molecular 3HB for the PHBV copolymer production. Certainly, the PHBV formed under this condition would be predominant with 3HV unit coupled with the rest of 3HB unit.

Anyway, the metabolic pathway for PHA accumulation by the mixed culture of activated sludge must be complicate than that of any other pure culture, but at least it can be concluded that these results indicate that the 3HV mole fraction of the PHBV co-polymer accumulated in activated sludge could be controlled by adjusting the valeric acid concentration in the medium.

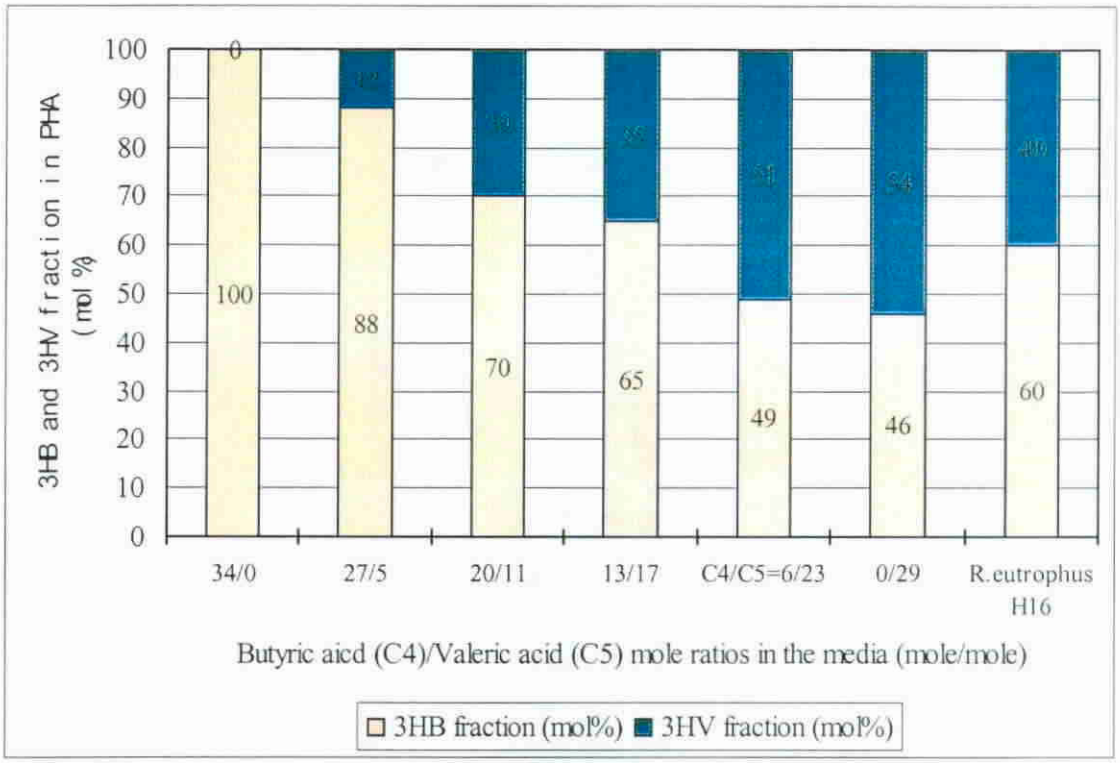


Figure 4.5. The 3HB and 3HV molar fractions of the PHA formed at different butyric/valeric acids (C5) mole ratios. *R. eutrophus* H16 (the formerly known as *Alcaligenes eutrophus*) is just a reference set adapted from Doi *et al.* (1987).

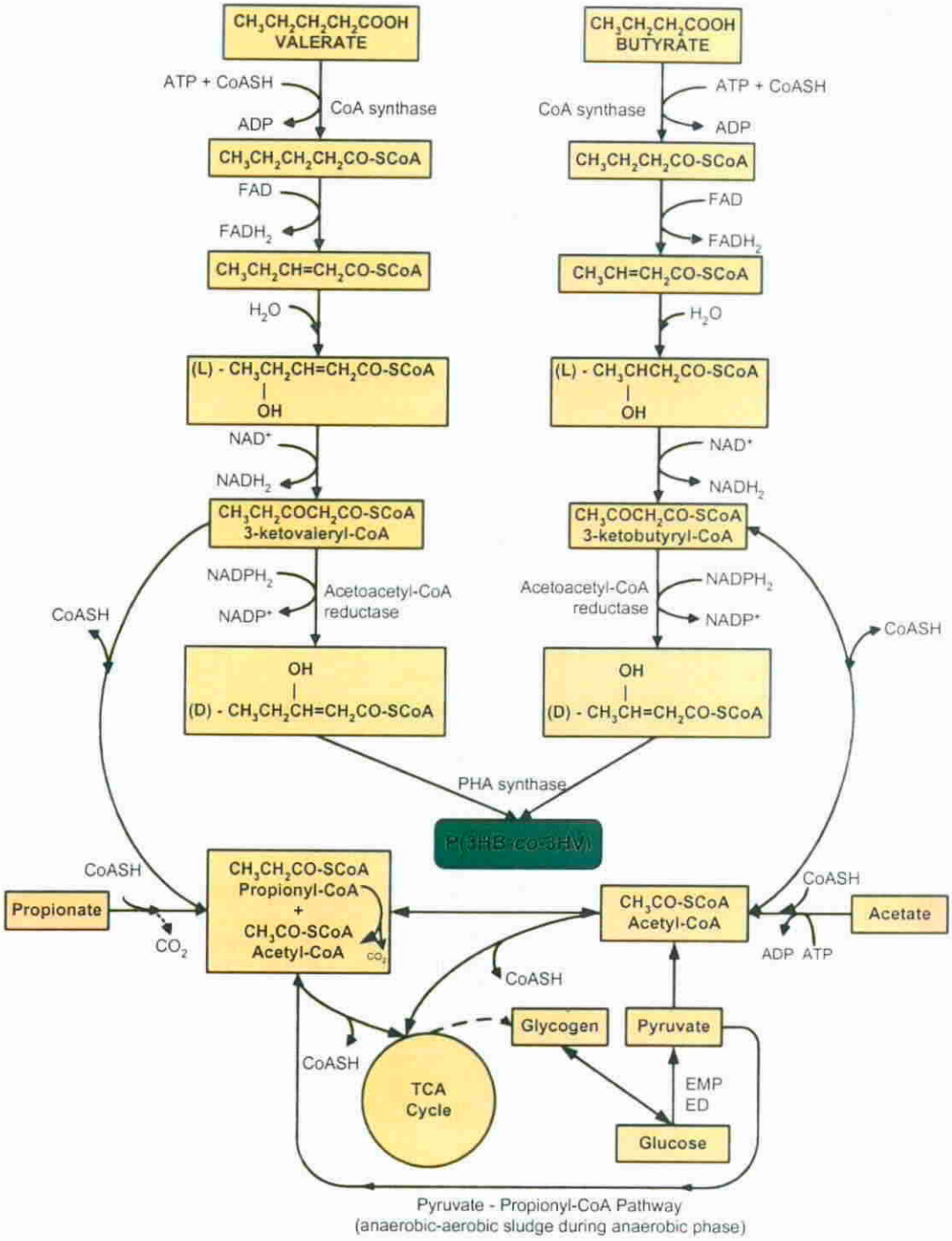


Figure 4.6. Biosynthetic pathway to PHBV copolymer from activated sludge by using butyric and valeric acids as sole and complex carbon sources. (modified from Doi *et al.*, 1989).

4.1.3 Thermal property of PHA from activated sludge

After fermentation, the EAS was then harvested for the PHA extraction by the method in accordance with Chua *et al.* introduced (1997a and 1997b). The extracted PHA was illustrated below and then submitted to test the melting temperature.

The melting temperature, T_m, of the co-polymer accumulated in the activated sludge with different medium composition ranged from 99 to 178°C (Table 4.1). The co-polymers with maximum and minimum values of *T*m were obtained when butyric acid and valeric acid were respectively used as the sole carbon sources in the medium. Increase of 3HV monomeric units in the PHBV co-polymer formulation resulted in a close to proportionate decrease of polymer melting temperature (Figure 4.7). The trends of T_m and 3HV molar fraction could be described as flowed through linear regression:

$$y = -1.198x + 167.08$$

where "y" stands for T_m, °C,

and "x" stands for the 3HV molar ratio in the PHA copolymer.

In the meanwhile, $R^2 = 0.9591$ and $R = 0.979$ which indicated that the T_m is related to the 3HV molar ratio in PHA with a closed linear relationship.

The melting temperature of the PHA produced by EAS from SBR decreased with an increase in the 3HV fraction, indicating that 3HV unit act as defects in the PHBV crystal lattice. Otherwise, the infrared spectrums of the PHA produced by activated sludge were identical with that of the standard samples including PHB and PHBV. Such defects are also known to have adverse effects of such mechanical properties as tensile, compressive, flexural and shear stresses of the materials. These results agreed with the published thermal properties of polymers and co-polymers of PHA. Intracellular polymers of PHA are highly crystalline thermoplastics with a melting temperature *T*m) around 180°C for PHB homo-polymer and a range from 96 to 160°C for PHBV co-polymers depending on the fraction of 3HV units (Doi *et al.*, 1990). Hence, the 3HV molar fraction of the PHBV copolymer accumulated in activated sludge, and its thermal and mechanical properties, were controlled by adjusting the valeric acid concentration in the medium.

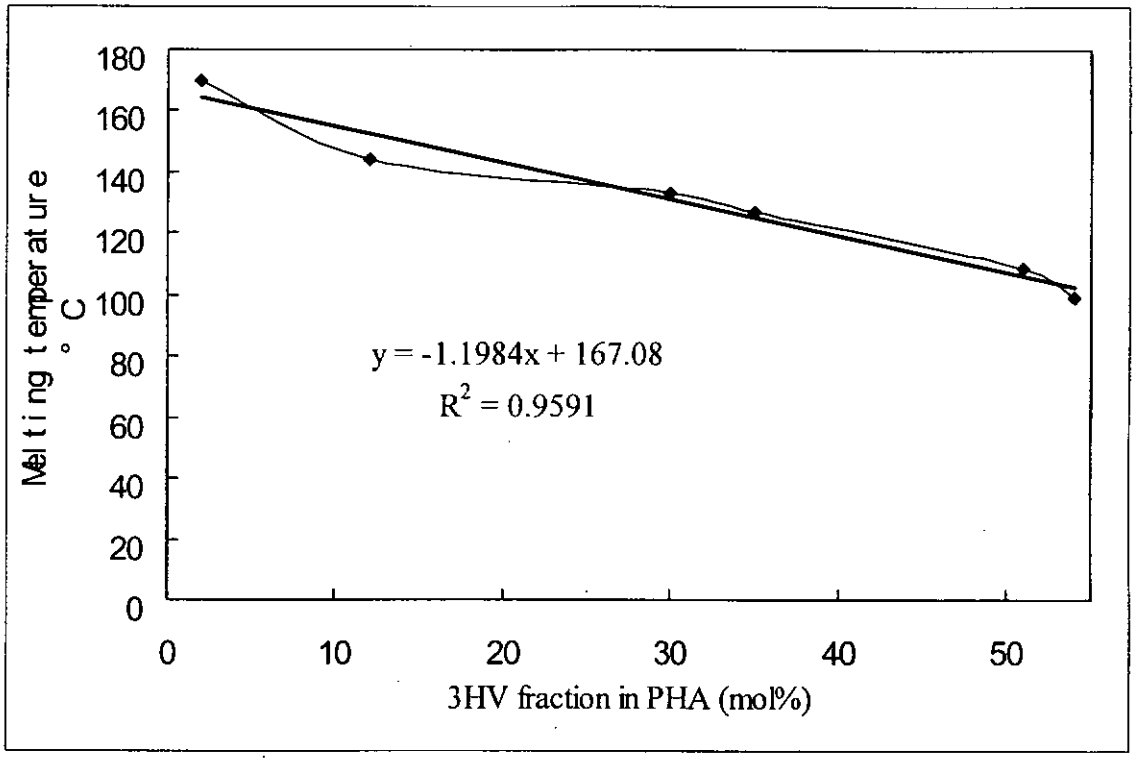


Figure 4.7 The relationship between melting temperature of the PHA accumulated by EAS under different C4/C5 ratios and the 3HV molar fraction in the PHA.

4.1.4 Variation of microbial community structures after PHA accumulation

It was observed that the main bacterial genus in the activated sludge that accumulated the polymers was rod shaped bacteria. *Alcaligenes* spp was the main group existed in the cultural broth after 48 hours cultivation for the PHA accumulation. This results agreed well with that described by Dave *et al.* (1996). When activated sludge was incubated under nitrogen- and phosphorus-limiting conditions, selective overgrowth of *Bacillus* spp. from 5 to 80% (cell count, CFU/ml) was observed (Dave *et al.*, 1996). *Bacillus* spp. are known to synthesize and accumulate PHB (Chen *et al.*, 1991). Thus, the observed increase in PHA content may

be attributed to the selective growth of *Bacillus* spp. present in the sludge. The detailed bacteriological analysis of activated sludge conducted for the PHA production revealed the presence of about 3×10^8 CFU/ml bacteria consisting of *Acinetobacter*, *Aeromounas*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, etc.

It can be concluded that, butyric acid and valeric acid could be transported into cells in the EAS from conversional aerobic-aerobic activated sludge SBR as sole or complex carbon sources without nitrogen source under aerobic condition, and then most of the substrates were converted directly into 3HB and 3HV units for PHBV copolymer production without decomposition of carbon skeleton. Moreover, when butyric acid and valeric acid were used as sole carbon source independently, PHA with 100 mol% of 3HB unit and 3HV-enriched PHBV copolymer were synthesized respectively. Contrarily, when butyric acid was used as sole substrate for PHA production by the anaerobic-aerobic activated sludge from EBPR process during anaerobic phase, PHBV copolymer with dominant of 3HB and rest of 3HV units was obtained. This difference resulted from the variety of activated sludge and the presence or absence of oxygen. Therefore, for explaining these observations in this paper and previous documents, a modified metabolic pathway has been proposed.

On the other hand, the 3HB/3HV ratios in the PHBV copolymer formed by the EAS could be adjusted by altering the butyric/valeric acids composition in the cultural media. 3HV molar fraction in the copolymer presented a close linear relationship with the valeric acid mole concentrations in the media. Further, the melting temperatures of

the PHBV copolymer formed from EAS by using butyric and valeric acid as sole or complex carbon source are decreasing with the increasing of 3HV molar fraction in the copolymer. The melting temperatures of the copolymer could then be regulated via adjusting the butyric and valeric acids compositions in the media.

4.2 Synthesis of PHA while acetate, propionate and acetate/propionate as sole and complex carbon sources under various ORP by the EAS from MWWTP

Introduction

Among the various biodegradable plastics developed, poly-hydroxyalkanoates (PHA) have been paid much attention owing to their similar material properties to conventional plastics and complete biodegradability (Steinbüchel, 2001). In the early 1990s, the production and application of PHA flourished and commercial products manufactured from PHA has been developed. However, widespread application of PHA is hampered by high production costs. Although much effort has been devoted to develop a process for economically producing PHA the price of end products is still higher to average 3-5 times than that of conventional synthetic plastics.

Recently, microorganisms in EAS have been reported to accumulate PHA as an intermediate metabolic product from the uptake of organic matter in sewage (Chua *et al.*, 1997a, 1997b; Hu *et al.*, 1997). It is become possible to couple a wastewater treatment process with PHA production.

Notablely, PHA accumulation from the sludge of EBPR has been investigated by numerous researchers, and different biochemical models have been developed, e.g., by Wentzel *et al.* (1986), Mino *et al.* (1987) and Smolders *et al* (1994), to

explain the mechanisms of EBPR process and PHA accumulation. In addition, new models and new mechanisms have been developed in light of these models (Pereira *et al.*, 1996 and Louie, *et al.* 2000). But the fundamental understanding of the process from microbiological and biochemical points of view are still incomplete in the PHA accumulation from activated sludge including EBPR process.

The fermentation process has not yet studied and optimized for the production of PHA by EAS. In general, for example, to establish the PHA accumulation from EAS instead of pure culture in practice, the cultural medium and the fermentation conditions have to be designed and optimized. Some achievements have been reported.

By controlling the carbon:nitrogen (C:N) ratio in the reactor liquor, PHA was synthesized with a yield of 0.11 g of polymer/g of carbonaceous substrate consumed from activated sludge (Chua *et al.*, 1997a, 1997b and 1999; Ma *et al*, 2000). However, it was demonstrated that biomass growth was restricted under nitrogen-deficient conditions, which favors PHA accumulation. In prolonged nitrogen-deficient conditions in wastewater treatment, PHA production continued; however, cell growth and organic removal efficiency was adversely affected. Contrarily, the results from Takabatake *et al.* (2000) indicated that nitrogenous compounds did not depress PHA productivity in the case of activated sludge process. When nitrogenous compounds, i.e. $(NH_4)_2SO_4$, peptone adn yeast extract, coexistented in carbon sources, the conversion of activated sludge to intracellular

carbohydrates and protein was higher than that in the case of non-coexistence. The nitrogenous compounds promoted the productivity of structural materials, and meanwhile PHA productivity in each runs was almost the same level of PHA contend 20%-27%. Therefore, cell growth and PHA accumulation should and can be considered simultaneously to achieve a mutual benefit when designing wastewater treatment systems.

As known, the synthesized PHA is the byproduct of EBPR process from wastewater by anaerobic-aerobic activated sludge. The bacteria in the sludge of EBPR could be divided into two parts. The one is polyphosphate-accumulating organisms (PAOs) the other is the glycogen-accumulating organisms (GAOs). Generally, under anaerobic condition PAOs take up the carbon sources, short chain fatty acids or their salts, and store them in the form of PHA (mainly 3HB unit) with simultaneous phosphorus release to the external medium. Energy for this process comes from the break-down of intracellular polyphosphate, glycogen consumption and substrate degradation in the tricarboxylic acid cycle (TCA) (Pereira *et al.*, 1996 and Mino *et al.*, 1998). Under aerobic condition, PHA previously accumulated in anaerobic stage is metabolized as the energy source for anabolic precursors and microorganism growth, synthesis of polyphosphate with phosphate uptake from the medium. The theory above is hard to explain the phenomena described by Satoh *et al.* (1998 and 1999), because the microaerophilic-aerobic activated sludge did not take up organic substrates under

this condition, but the PHA content in the sludge increased than that in the anaerobic conditions.

GAOs are the competing group of bacteria to PAOs in EBPR, and they that use glycogen as energy source for acetate uptake and storage, and obtain the needed reducing power through glycolysis. GAOs have similar features to PAOs except they do not accumulate excess phosphorus and they tend produce PHV from acetate under anaerobic condition.

Hence, it was believed that the dissolved oxygen (DO) in the cultural broth play a very important rule in the PHA production by activated sludge. The PAOs and GAOs in sludge were very sensitive to oxygen even under a low concentration. The variation of DO concentration could result in changes of microbial community or metabolic pathway. So, it is decided that a more precious method to regular DO in the cultural broth have to be employed to take place the traditional way of DO monitoring and controlling system.

On the other hand, when PHA in PAO-enriched sludge has been verified to be co-polymers composed of mainly 3HB and 3HV, a lower quantity of 3H2MB and 3H2MV was produced (Satoh *et al.*, 1992). The composition of PHA is dependent on the carbon source. When acetate is the only carbon source available in the anaerobic phase, the 3HB unit is the major unit in the PHA formed. 3HV is formed by conversion of glycogen to propionyl-CoA via the succinate-propionate pathway and subsequent reaction with acetyl-CoA (Pereira *et al.*, 1996 and Holender *et al.*, 2002), which reminds us that the propionate may be a proper

carbon source for forming 3HV unit in the PHA copolymer. Thus it is important to select the acetate and propionate as carbon source to understand the way of PHA production by EAS from MWWT.

To take these previous own results and literatures' reports into account, the carbon source should be selected and the concentration of DO in the cultural broth have to be controlled preciously for the purpose of composting the cultural medium and optimizing the fermentation conditions. Therefore, in this study, an open, complex and dynamic system to monitor and control the PHA synthetic process has been established.

The most important part of the control system is an on-line ORP monitoring system with intermittent nitrogen and oxygen feeding program equipped with the fermentation setup (Figure 3.2, Figure 3.3 and Figure 3.4), which is a highly sensitive and instantaneous on-line instrumentation in fermentation process. The variation of ORP in the cultural medium would be monitored and controlled to regulate the PHA production instead of DO concentration.

ORP is the electromotive force developed when oxidizers or reducers are present in aqueous solution. Compared with DO-based regulation, which can only show dissolved oxygen concentrations in cultural broth, ORP regulation is more in turn with the process dynamics, since many biological substances, such as enzymes, vitamins, and most metabolic processes correlate strongly with ORP values (Lu and Ukita, 2000; Li and Bishop, 2002). ORP reflects the amount of materials such as dissolved oxygen, organic substrate, activity of organisms etc.

The ORP value is correlated to the logarithm of DO concentration with a linear relationship (Peddie *et al.*, 1990 and Chang *et al.*, 1994). It was reported that controlling the full-scale activated sludge by measuring ORP showed that such a control strategy can not only remove carbonaceous and nitrogenous with good performance, but also conserve 20% of the energy cost (Charpentier *et al.*, 1987). Since measuring a low DO concentration is unreliable and the terms of anoxic and anaerobic are only qualitative descriptions, ORP monitoring is more flexible for process regulation under such circumstances (Yu R.F. *et al.*, 1997).

The on-line ORP monitoring strategy has been proved to be very practical for process control of activated sludge process, sludge digestion, biological nutrient removal, chemical oxidation/reduction and biological fermentation process (Peddie *et al.*, 1990; Lo *et al.*, 1994; Chang *et al.*, 1994, 2002 and Saby *et al.*, 2003).

As mentioned previously, many factors could affect the ORP level and cause the variation of ORP in the cultural media such as dissolved oxygen, chemical concentration, pH and the biomass content in cultural broth (VSS of activated sludge) etc. In this study, only DO concentration in cultural broth was required to be regulated preciously during the process of PHA accumulation and PHA composition, it was expected that the ORP monitoring and regulation instead of DO will work well.

In addition, the shortcoming about the research conducted in 4.1 was that the

EAS formed in a laboratory scale aerobic-aerobic SBR. For example, the EAS was acclimatized not with sewage but with synthetic wastewater which contained milk and other mineral elements. In actual case, activated sludge is fed with real wastewaters containing various organic compounds. In this study, the EAS collected from MWWT instead of the one from laboratory scale SBR would be submitted to the PHA production. On the other hand the substrates used for the PHA production would be selected as acetate (C2) and propionate (C3) instead of C4 and C5.

Main objectives of this study

The main objectives of this work is to develop an on-line ORP-based monitoring and controlling system with a jar fermenter, to find out the relationship between the ORP level of the cultural broth and the quantity of PHA accumulated and PHA composition from the co-culture of EAS and realize the precisely controlling of DO concentration, and thus allowing optimization of both PHA production and PHA composition. For this purpose, firstly, to maximize the production of PHA under different ORP levels by using acetate (C2) as sole carbon source will be carried out. Secondly, another carbon source, propionate (C3), will be introduced to investigate the possibility of controlling the PHA composition by changing the C2 to C3 ratios under optimized ORP condition. Finally, the EAS collected from MWWTP in Hong Kong instead of the one from laboratory scale SBR will be conducted to the PHA production. Furthermore, the

metabolic pathway of PHA production from MWWTP real EAS from C2 and C3 will also be discussed.

4.2.1 TOC removal efficiency and biomass growth

The TOC removal efficiency, together with polymer content, product yields, final 3HV molar fraction and thermal property of the polymer after 48 hours fermentation at five carbon sources ratios, were listed in Table 4.2. The initial cell mass concentration in the bioreactor was about 3.0 g/l, final cell mass was maintained within the range of 3.0 to 3.2 g/l during the culture process. Since the bacteria in EAS growing under aerobic conditions only, it could be considered that cell mass concentration remained approximately constant during the periods of the cultivation for PHA accumulation (Pereira *et al.* 1996). The TOC removal efficiency was 98.2% when acetate was as sole carbon source, while, it was decreased to 90.4% when propionate as sole carbon source. Thus, there was not big variation of TOC removal causing by the changes of carbon sources.

Table 4.2. Productivity of PHA by EAS which was collected from full-scale MWWTP where acetate (C2) and propionate (C3)[a] were used as sole or complex carbon sources

C2 to C3 (mol/mol)	*C2 and C3 (g/l)*		*TOC removal (%)*	*Polymer content (wt%)*	$Y_{p/s}$[b] *(g/g)*	*3HV fraction (mol%)*	T_m *(℃)*
	C2	*C3*					
100:0	5.13	0.00	98.2	35.0	0.48	8.0	168
80:20	3.72	1.09	98.8	33.0	0.45	22.0	140
50:50	2.05	2.40	95.6	28.0	0.39	55.0	108
20:80	0.73	3.42	96.3	26.1	0.36	70.0	98
0:100	0.00	4.00	90.4	25.8	0.38	78.0	95

[a] *The total TOC (total organic carbon) of the cultural medium was 1500 mg/l added in a fed batch mode once every 12 hours in order to avoid possible growth inhibition caused by C2 and C3. ORP was kept at +30 mV.*

[b] $Y_{p/s}$ *= Polymer production yield (g polymer/g carbon), which was calculated as the polymer accumulated divided by the TOC consumed.*

4.2.2. PHA production under various ORP levels

During the fermentation process, the ORPs dropped immediately at the beginning of the process and kept decrease to less than -30mV, the preset lowest point of ORP. Therefore, air or pure oxygen gas had to be supplied for keeping the ORPs at the preset levels.

Acetate as sole carbon source

- Productivity of PHA

PHA accumulated by the EAS from full scale MWWTP by using sodium acetate as sole carbon source under varied ORP levels was shown in Figure 4.8.

It is illustrated that PHA content in the EAS increased with the increasing of ORP values. It also demonstrated that the minimum PHA content at ORP -30mV was about 12% (w/w) of EAS dry weight. The maximum PHA accumulation of 35% (w/w) was achieved when ORP was maintained at +30mV, and PHA content in the EAS declined to 28% of dry weight when, ORP was kept at +100mV. The PHA production yield, $Y_{p/s}$ (g PHA/g TOC consumed), was 0.48. The calculation of PHA production yield could be written as below:

$$Y_{p/s} = \frac{g\ PHA\ produced}{g\ TOC\ Consumed}\ (g/g)$$

These results agreed very well with that of descriptions by Satoh *et al.* (1998)

and Serafim *et al.* (2002).

Serafim *et al.* (2002) has applied the activated sludge from a stable and efficient phosphorus removal EBPR process to evaluate the change in the metabolisms when the reactor operation was modified from anaerobic/aerobic to aerobic dynamic substrate feeding. The change in operational conditions allowed the population to modify the metabolism of phosphorus and PHA accumulation. It was found that under aerobic dynamic feeding condition where acetate was used as sole carbon source, phosphorus accumulation was almost hindered while the amount of PHA accumulation increased significantly. The amount of PHB accumulated by the population under anaerobic/aerobic conditions was lower than 20% dry weight. The storage capacity was however immediately and significantly improved when the reactor operation was modified to aerobic dynamic feeding conditions, reaching a maximum value of 42% of PHA contend (cell dry weight).

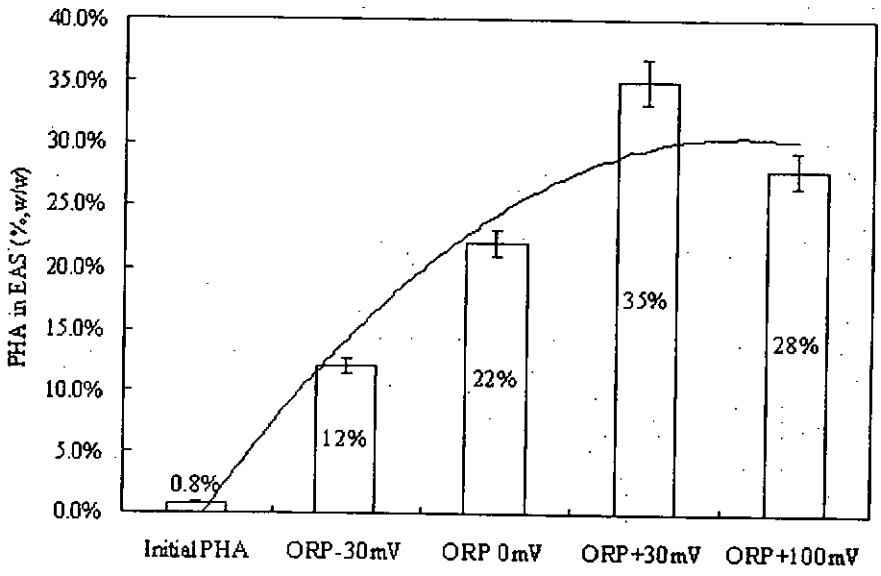


Figure 4.8. PHA contents in EAS accumulated under various ORP values where acetate (C2) was used as sole carbon source. Data is the means of triplicate results and error bars show standard deviations.

PHA is known to play a very important role in EBPR from wastewater by anaerobic-aerobic activated sludge process. Under anaerobic condition, the activated sludge uptakes organic substrates and accumulates it as PHA. Energy for this process comes from the break-down of intracellular polyphosphate, glycogen consumption and substrate degradation in the tricarboxylic acid cycle (TCA) (Pereira *et al.*, 1996; Sudiana *et al.*, 1999 and Jeon *et al.*, 2000). Under aerobic condition, PHA previously accumulated in anaerobic stage is metabolized as the energy source for anabolic precursors and microorganism growth, synthesis of polyphosphate with phosphate uptake from the medium. But this theory

mentioned above can not explain the phenomena found in this study, because the activated sludge does not take up organic substrates under oxygen limited condition as described.

As observed, the increase of PHA production with the increase of ORP value, even under the oxygen-limited condition which is probably due to the supply of energy by oxidation of organic substrate- acetate. Under the anaerobic conditions, while the ORP maintained at low level (–30mV, for example), the energy source is limited by the amount of polymers such as polyphosphate and glycogen. Polyphosphate and glycogen are the limited factors for the PHA accumulation under anaerobic conditions. When energy sources are exhausted, the PHA accumulation will be stopped. Adjusting the ORP to higher value of +30mV, an oxygen-limited condition, EAS takes up organic substrate such as acetate to get energy except polyphosphate and glycogen by oxidative degradation of some part of the organic substrate to meet the need for the PHA accumulation.

However, when ORP was kept at +100mV, the PHA content decreased. This result did not agree with Serafim *et al.* (2002) reported. It was believed that the PHA stored in cells could be reused as carbon and energy sources under aerobic condition, where the declining of PHA content in EAS was resulted. On the other hand, as illustrated in Figure 4.10, 3-ketothiolase, one of the three key regulatory enzymes for PHB synthesis (Oeding and Schlegel, 1973 and Doi, 1990a), is inhibited by high concentration of free CoASH. Under balanced growth conditions with the presence of excess oxygen and nitrogen source, acetyl-CoA

submitted to the TCA cycle for energy generation and biomass growth. In consequence, the concentration of free CoASH is high and resulted in the repression of PHA synthesis (Lee *et al.*, 1995 and 1999; Steinbüchel, 1991 and 2001). Therefore, supplying of pure oxygen for keeping ORP at +100mV resulted in the presence of excess oxygen in cultural medium, and consequently inhibited the PHA synthesis which induced the declining of PHA content in EAS.

- *3HV molar fraction in PHA under various ORP levels where acetate (C2) as sole carbon source.*

It can be seen from Table 4.2, when C2 was used as sole carbon source, the 3HB-enriched PHBV copolymer with only 8% mole fraction of 3HV unit was produced instead of PHB homopolymer as mentioned in the previous part of this Chapter where butyric acid was used. This result agreed with the reports by Satoh *et al.* (1992), Liu *et al.* (1996), Lemos *et al.* (1998), Satoh *et al.* (1998), Takabatake *et al.* (2000) and Randall *et al.* (2002).

Satoh *et al.* (1992) observed that when acetate was used as sole carbon source, 87% of the PHA produced were 3HB, 11% were 3HV, and the remaining 2% was 3H2MB (3-hydroxy-2-methylbutyrate) and 1% 3H2MV (3-hydroxy-2-methylvalerate). When propionate as carbon source they obtained 3% 3HB with the rest as 3HV (43%), 3H2MV (50%), and 3H2MB (6%) (Satoh *et al.*, 1992).

Liu *et al.* (1996) found that when the GAOs population from a EBPR process without biological phosphorus removal was conducted for acetate uptakes under

anaerobic condition, the PHA increase to 13.2% of TSS with 66.7 mol% of 3HB unit and 26.4 mol% of 3HV unit. Even under aerobic condition, Radall *et al.* (2002) found that PHBV copolymer with 3HB as dominant unit according with 3HV unit obtained when acetate as carbon source. Takabatake *et al.* (2000) conducted the activated sludge in the PHA accumulating bacteria enrichment reactor. This was a modified SBR which was operated in the anaerobic-aerobic mode with a 6 hour cycle: 30 min for decantation, 30 min for nitrogen purging, 1 hour for anaerobic conditions, 3 hour for aerobic condition, and 1 hour for sedimentation. The acclimated activated sludge then was submitted to PHA production under aerobic condition. Consequently, PHBV copolymer with predominant 3HB and only 3% molar fraction of 3HV unit has been obtained when acetate was used as sole carbon source.

At the same time it has been observed during this research that the decreasing of ORP levels from +100mv to -30 mV resulted in the variation of 3HV mole fraction in the PHBV copolymer formed by the EAS from the range of 0 mole % to 21 mol% (Figure 4.9). In other words, the variation of 3HV molar fraction in the PHBV copolymer accumulated by the EAS resulted from the changes of DO concentration in cultural broth even acetate as sole carbon source. These observations have never been mentioned and described by other literatures before. In the pure culture for PHA production from acetate as sole carbon substrate, only PHB homopolymer accumulated by the strains of *R. eutropha* (Anderson and Dawes, 1990; Doi, 1990). Serafim *et al.* (2002) found that there was only PHB

homopolymer produced when reactor operation was changed from anaerobic-aerobic conditions to aerobic dynamic substrate feeding process while C2 was used as sole carbon source. Moreover, when the reactor operation was changed back to anaerobic-aerobic conditions, PHBV copolymer with predominant 3HB unit was accumulated (Serafim *et al.* 2002). This was a closer observation to my own. However, the authors did not find, mention and discuss this evidence that oxygen might play a very important role to regulate the 3HV molar fraction in PHBV copolymer. This is a new challenge to that of currently existed biosynthetic pathways on the accumulation of PHA. It is because the existed biosynthetic pathways can't explain these observations very well.

It is speculated that there are two possible explanations for these observations. Firstly, the changes of ORP (i.e. DO in this research) became a selecting force and resulted in the selected growth of bacteria in the EAS, and then lead to different metabolic pathways for the accumulation of PHA with different 3HV mole fraction. Secondly, there must be some other regulation mechanisms for the synthesis of 3HV unit in PHBV copolymer which can be affected by the DO concentration when using acetate as sole carbon substrate. Actually, the first one was unreasonable and second option was my favor. Therefore, a hypothetic metabolic pathway for PHA production from EAS by using acetate as sole carbon source has been suggested and shown in Figure 4.13.

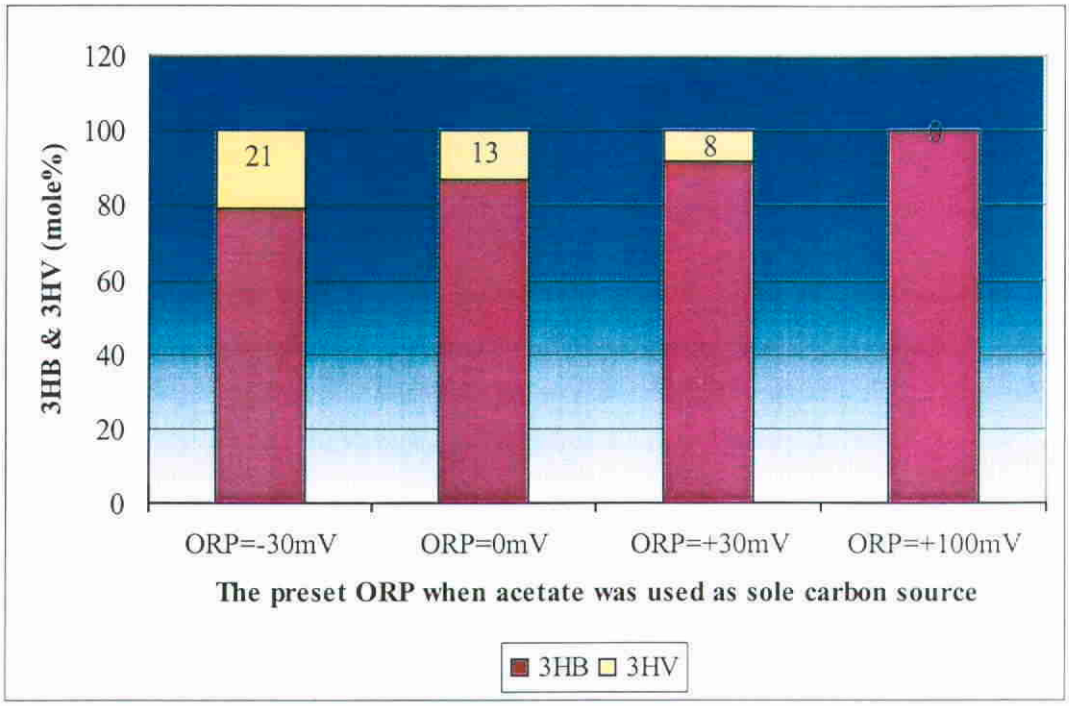


Figure 4.9. 3HB and 3HV molar fractions in the PHBV copolymer produced by the EAS under various presetting ORP levels when acetate (C2) was used as sole carbon source.

This hypothetic metabolic pathway was modified from Doi (1990), and combined with recent research works such as succinate-propionate pathway, EBPR metabolic models and my own creation (Comeau *et al.*, 1986 and 1987; Mino *et al.*, 1987, 1994 and 1998; Wentzel *et al*, 1986 and 1991; Satoh *et al.*, 1992; Pereira *et al.*, 1996; Maurer *et al.* 1997 and Louie *et al.*, 2000).

For understanding the proposed model further, the previous models involved in the hypothetic metabolic model are reviewed and summarized below:

The Comeau-Wentzel Model (1986):

PHA is a reduced energy source and its synthesis therefore requires reducing powers. Partial oxidation of acetyl CoA through the TCA cycle produces the reducing power. The combination of acetate (acetyl CoA) and reducing power produce PHA.

The Mino Model (Mino et al., 1987):

The lack of evidence for the operation of the TCA cycle under anaerobic conditions and the observation of significant changes in intracellular carbohydrate (glycogen) content motivated the development of the Mino model (1987). In this model, reducing power is generated by the degradation of intracellularly stored glycogen (carbohydrate) via the Embden-Meyerhoff Parnas (EMP) pathway.

The Modified Mino Model (Wentzel, 1991):

The only major change from the Mino model is that the modified Mino model postulates the Entner-Doudoroff (ED) pathway for degradation of intracellular glycogen instead of the EMP pathway. This modification was proposed by Wentzel *et al.* (1991) based upon the results of a single study in which the apparent use of the ED pathway by an *Acinetobacter* was observed.

The Succinate-propionate pathway (Satoh et al., 1992)

Satoh *et al.* (1992) first proposed the succinate-propionate pathway as a possible pathway for the conversion of internal glycogen to propionyl-CoA, which is needed for 3HV synthesis. In this theory, acetate is taken up by the cell and converted via acetyl-CoA to 3HB units. Internal glycogen is converted to Acetyl-CoA and partly to propionyl-CoA in order to adjust the intracellular redox balance.

The model proposed by Pereira et al. (1996):

Pereira and coworkers (1996) showed that a small portion of labeled acetate was released as CO_2 during an anaerobic batch test experiment. Therefore, their ^{13}C NMR results suggest that at least part of the TCA cycle is still operable under anaerobic conditions, and that some fraction of the reducing power needed is generated through the TCA cycle.

The model proposed by Maurer et al. (1997):

Maurer *et al.* (1997) used a solid state NMR to track carbon flow in EBPR sludge fed with domestic sewage. Although no suggestion was made about the operation of the TCA cycle under anaerobic conditions, they suggested that the ED pathway was used during glycogen breakdown.

The model proposed by Louie et al. (2000):

More recently, Louie *et al.* (2000) suggested that the glyoxylate pathway is active under anaerobic conditions to provide reducing equivalents and to maintain stable NAD+/NADH balance. More details are presented in Chapter 2, Literatures Review.

As shown in the present study and previous research, the most important part of the hypothetic pathway is that the TCA cycle and glyoxylate pathway are involved in the model even under oxygen-limited and anaerobic conditions. Generally, it is thought that only under aerobic condition the TCA cycle functions completely. However, recent research works suggested that TCA cycle and glyoxylate pathway were fully or incompletely involved in the bacterial metabolisms of the activated sludge and PHA production even under anaerobic condition (Pereira *et al.*, 1996; Maurer *et al.* 1997 and Louie *et al.*, 2000). Thus, in this model in Figure 4.13, the TCA cycle and glyoxylate pathway were combined with the Doi's model proposed for the PHA production from *R. eutropha* pure culture (Doi, 1990a).

It is obvious to be seen in the hypothetic metabolic model, all the C2 are transferred into acetyl-CoA, and then part of acetyl-CoA are directly converted to acetoacetyl-CoA for PHB production. Others of acetyl-CoA flow into TCA cycle and glyoxylate pathway for energy and reducing power generation and cell growth. Otherwise, as we known completely functioning of TCA cycle must be in the presence of excess oxygen; in contrarily, only incomplete of TCA cycle

functions under anoxic and anaerobic conditions (Comeau *et al.*, 1986 and 1987; Pereira *et al.*, 1996; Maurer *et al.* 1997 and Louie *et al.*, 2000). Furthermore, in the absence of oxygen or oxygen-limited conditions, the step from succinate to fumarate in TCA cycle will be stopped which is indicated by the black bar in Figure 4.13. This is because the oxidation of succinate to fumarate in the TCA cycle requires a terminal electron acceptor with a redox potential ($E^{0'}$) more positive than that of fumarate/succinate couple (+32 mV). In this case, only O_2 (O_2/H_2O, $E^{0'}$= +818 mV) appears to meet these conditions (Thauer, 1988). Therefore, under oxygen-limited (anoxic) and anaerobic conditions, succinate derived from the front part of TCA cycle or glyoxylate pathway cannot be oxidized to fumarate, and consequently is converted to propionyl-CoA as the precursors of 3HV synthesis. When ORP increased from -30mV to +100mV, i.e. alternating the anaerobic or oxygen-limited conditions to aerobic condition, complete TCA cycle is functioning and there is no net succinate accumulated for the conversion of propionyl-CoA, and consequently no 3HV unit will be synthesized, and hence PHB homopolymer will be produced instead of PHBV copolymer. More details of the description and explanation about the biochemical principles of the hypothetic metabolic pathways will be demonstrated in the next part of this chapter.

It can also be seen from Figure 4.13, the production of glycogen from fatty acids (C2 and C3, for example), and then the glycolysis of glycogen were also taken into account. Comeau *et al.* (1987) reported the accumulation of PHV in

addition to 3HB after the anaerobic uptake of various short chain organic acids. On the other hand, based on the observation that glycogen in the activated sludge decreased in the anaerobic uptake of acetate, Arun *et al.* (1988) and Mino *et al.* (1987) suggested the significance of glycolysis as the supplier of reducing power in the anaerobic substrate uptake. Satoh *et al.* (1992 and 1996) identified that the sink of carbon in the anaerobic uptake of acetate and propionate was PHA composed of 3HB, 3HV, 3H2MB (3-hydroxy-2-methylbutyrate), and 3H2MV (3-hydroxy-2- methylvalerate). They measured the amount of substrate being taken up, PHA in sludge increased, carbohydrates in sludge decreased, and phosphate released. The observed stoichiometric relations among the metabolic substances were in good correspondence with the model, which assumes that glycolysis of glycogen supplies the reducing power necessary for the conversion of acetate or propionate into PHA. Thus the generation of glycogen from C2 and C3, and then glycolysis of glycogen were involved in the hypothetic metabolic pathways (Figure 4.13).

Propionate as sole carbon source

- *Productivity of PHA*

As shown in Table 4.2, when propionate (C3) was used as the sole carbon source (total of 4g/l) and ORP was kept at +30mV, the PHBV copolymer content, polymer production yield and 3HV molar fraction were 25.8%, 0.38 (g/g) and 78.0 mol%, respectively. Moreover, the TOC removal efficiency was 90.4%. On

the other hand, as illustrated in Figure 4.10, the initial PHA was 0.8% CDW; when ORPs were preset at -30mV, 0mV, +30mV and +100mV, the PHA contents in the EAS were 8.0%, 18.9%, 25.8% and 22.5% CDW, respectively. The PHA content in EAS was increased with the increasing of ORPs. Finally, when the ORP was +100mV, the PHA content was declined compared to that of ORP +30mV. As suggested in the previous report while C2 as sole carbon source, the presence of excess oxygen during the period of +100mV ORP (i.e. pure oxygen was supplied), resulted in the balanced growth conditions, and acetyl-CoA was submitted to the TCA cycle for energy generation and biomass growth. In consequence, the concentration of free CoASH was become higher. The key enzyme for PHA synthesis, 3-ketothiolase, was inhibited by high concentration of free CoASH, and resulted in the repression of PHA synthesis

All these observed results coincide with the observations while C2 as carbon substrate. However, the results are lower or smaller than that of C2 as sole carbon source presented previously. Furthermore, these results agreed well with the point that the increase of carbon skeleton length of fatty acid results in the declining of the performance of activated sludge.

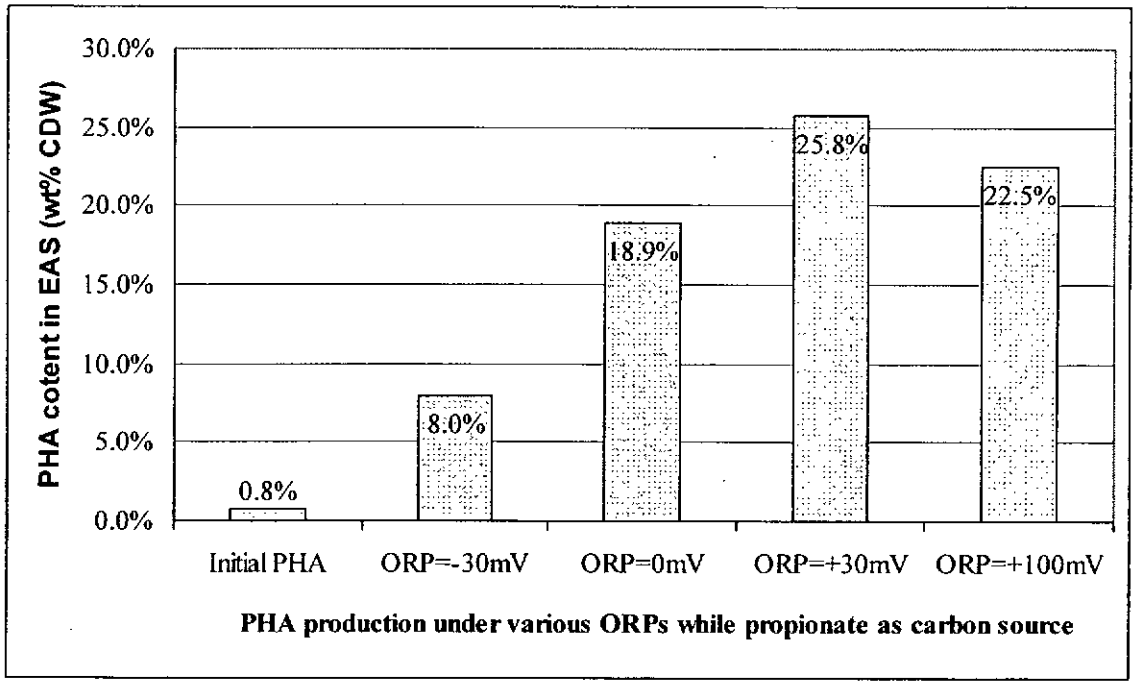


Figure 4.10 PHA contents in EAS accumulated under various ORPs where propionate (C3) was used as sole carbon source.

Actually, these results were also agreed well with other observations reported in the literatures.

When propionate was used as the carbon source, the PHBV copolymer was obtained with 3% 3HB, and the rest as 3HV (43%), 3H2MV (50%), and 3H2MB (6%) from EBPR process under anaerobic condition (Satoh *et al.*, 1992).

Liu *et al.* (1994 and 1996) reported that PHBV copolymer with 3HB, 3HV and 3H2MV units were synthesized when using propionate as sole carbon source by the glycogen-accumulation population from EBPR process under anaerobic

condition. The mole fractions of 3HB, 3HV and 3H2MV were 86.4, 1.3 and 10.9 mol%, respectively. The PHA content was 9.3% of TSS lower than that of 13.2% while acetate as carbon substrate.

Lemos *et al.* (1998) observed that polymer composition was also affected by the carbon source used by a phosphate-accumulating population mixed culture during the anaerobic phase. In fact, acetate consumption leads to the production of a copolymer of 3HB and 3HV repeating units with 3HB being predominant of 75.25 mol%. While using propionate, the opposite behavior, i.e. the occurrence of the 3HV units, were of 71.95 mole %. Moreover, the production yield of copolymer, $Y_{p/s}$, obtained when using acetate and propionate were 0.97 and 0.61, respectively.

19% of PHA content of MLSS was obtained when C3 was used as sole carbon source, and 3HV mole fraction was about 84% by the acclimatized activated sludge under aerobic condition (Takabatake *et al.*, 2000).

Randall *et al.* (2002) obtained PHBV copolymer with average of more than 2.0 mM-C of 3HV and about 0.2 mM-C of 3HB unit.

Serafim *et al.* (2002) reported that when propionate as carbon source, the PHA stored under anaerobic/aerobic around 20% dry cell weight doubled instantly to 40% when the conditions changed to aerobic dynamic feeding process. The PHA was consisted of more than 25% CDW of 3HV and less than 10% of 3HB units, which in turn of approximate 72 % (w/w) of 3HV fraction.

R. eutropha H16 produced PHBV copolymer with approximately 40% of 3HV

content (Doi *et al.*, 1987) when C3 was used as a carbon substrate whereas activate sludge can produce more than 70 mol% of 3HV unit as mentioned by own observation and literatures. This means that activated sludge accumulates the copolymers of 3HB and 3HV with wider 3HV fraction than *R. eutropha* pure culture when propionate is used as carbon sources.

- *The 3HV molar fraction in PHA under various ORPs while propionate (C3) as carbon source*

It can be seen from Figure 4.11, when C3 was submitted as sole carbon source to PHA production by EAS, the 3HV fractions in PHBV copolymer were decreased slightly from 83.2 mol% to 70.5 mol% with the increasing of ORPs from -30mV to +100mV, and in turn of the increasing of 3HB molar fraction from 16.8 mol% to 29.5 mol%. These results are in accordance with that of C2 as sole carbon source and have not been reported in literatures. However, the effect of DO concentration on the 3HV molar fraction in copolymer was not efficient as that of C2 as sole carbon source. Obviously, there was a little bit variation of 3HV molar fraction in the PHA copolymer resulted from the changes of DO concentration in cultural broth via regulation of ORP levels. Otherwise, notably no matter how much ORP was, the 3HV was the dominant unit in the PHBV copolymer while C3 as sole carbon source. The reasonable answer and explanations for these observed phenomena could be found from the hypothetic metabolic model in Figure 4.13.

After entering the cells, part of C3 will be directly converted into propionyl-CoA as 3HV precursor; otherwise, other part of C3 will be decarboxylated and converted to acetyl-CoA simultaneously for PHV and PHB synthesis. On the other hand, the acetyl-CoA derived from C3 will flow into TCA cycle and gloxylate pathway for energy and reducing power generation and biomass growth. Noticeably, when C3 was used as carbon source the propionyl-CoA, the one of the 3HV precursors, was converted directly and independently from C3 and was not through the TCA cycle and glyoxylate pathway in the absence of oxygen. Therefore, the effect of ORPs (i.e. DO concentration) on the 3HV fraction in copolymer is diminished even under anoxic and anaerobic conditions.

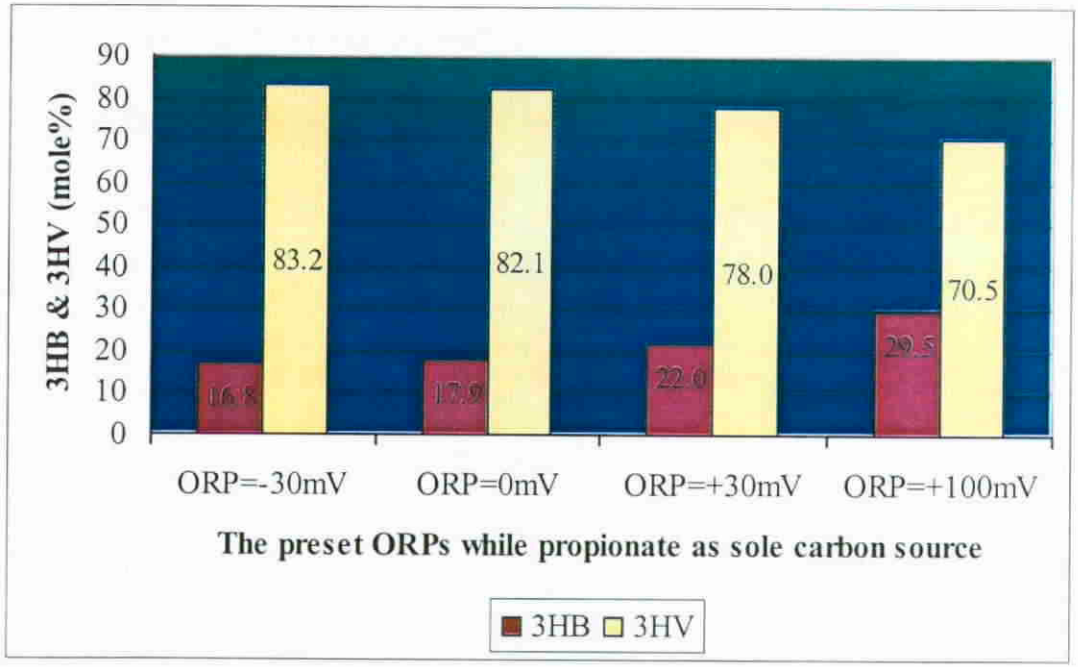


Figure 4.11. 3HB and 3HV molar fraction in PHA copolymer from EAS under various preset ORPs when propionate (C3) was used as sole carbon source.

4.2.3 Effects of C2/C3 ratios on PHA composition when acetate and propionate was used as complex carbon source where ORP was maintained at +30mV

As indicated in Table 4.2 and Figure 4.12, when C2 and C3 were used as complex carbon sources, an increase of C3 in the medium from 0 to 100 mol% (i.e. from 0 to 1.09, 2.40, 3.42 and 4.00g/l respectively) were resulted with a decline in polymer production yield, $Y_{p/s}$, from 0.48 to 0.38 g-polymer/g-TOC consumed. It was close to the theoretical yield of 0.65 g-PHBV/g-fatty acids by *Alcaligenes eutrophus* (Yamane, 1993), a common genus in activated sludge. The PHA productivities were lowered by the gain of the inclusion of propionate in carbon source of the medium which was in accordance with that of observation by Satoh *et al.* (1992, 1998) and Takabatake *et al.* (2000). The polymer content in the EAS also decreased from 35.0% to 25.8 wt%.

The infrared spectrums of the PHA produced by EAS were found identical with that of standard samples including PHB and PHBV.

The maximal 3HV molar fraction of 78.0 mol% achieved when C3 was used as sole carbon source. The molar fraction of 3HV in the co-polymer increased proportionately with the C3 concentration in medium. This result agreed with the early observation (Hu *et al.*, 1997) where butyric and valeric acids were used as carbon sources. Takabatake *et al.* (2000) found the similar results. It is demonstrated that C3 could serve as the precursors for 3HV units in the PHBV

co-polymer as well as the pure culture described by Choi *et al.* (2003). The PHBV co-polymer accumulated in EAS with wide range of 3HV molar fractions (8 mol% to 78 mol%) could be produced and regulated by controlling the C3 concentration in the medium. These results coincided with that of Satoh *et al.* (1992), Mino *et al.* (1987) and Sudiana *et al.* (1999) reported.

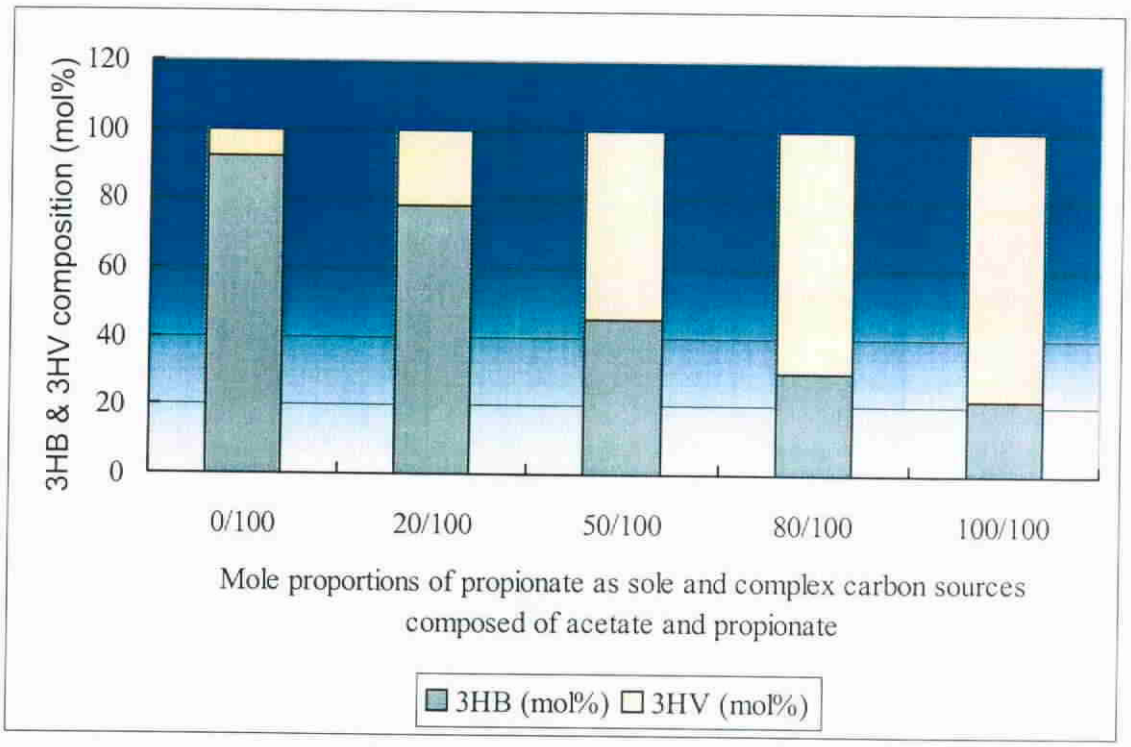


Figure 4.12. Composition of 3HB and 3HV in the PHA accumulated in batch experiments with acetate and/or propionate as sole and complex carbon sources, where the ORPs were maintained at +30mV.

Sudiana *et al.* (1999) described that under anaerobic condition, the PHA composition in both high and low P sludge is also well predicated theoretically. In

the case of the high P sludges or PAO enriched sludge acetate was converted to 3-hydroxybutyrate as predicted by Mino *et al.* (1987). In the case of low P sludge or GAO enriched sludge a certain amount of 3 hydroxyvalerate was produced in addition to 3-hydroxybutyrate unit. The ratio of 3HB/3HV was very close to the theoretical prediction (Satoh *et al.*, 1992; Liu *et al.*, 1994). In the theory, acetate is taken up by the cell and converted via acetyl-CoA to 3HB units. Internal glycogen is converted to acetyl-CoA and partly to propionyl-CoA in order to adjust the intracellular redox balance. Satoh *et al.* (1992) first proposed the succinate-propionate pathway as a possible pathway for the conversion of internal glycogen to propionyl-CoA for 3HV synthesis. The present result is close to the relevant theory and the proposed mechanism is likely to be functioned.

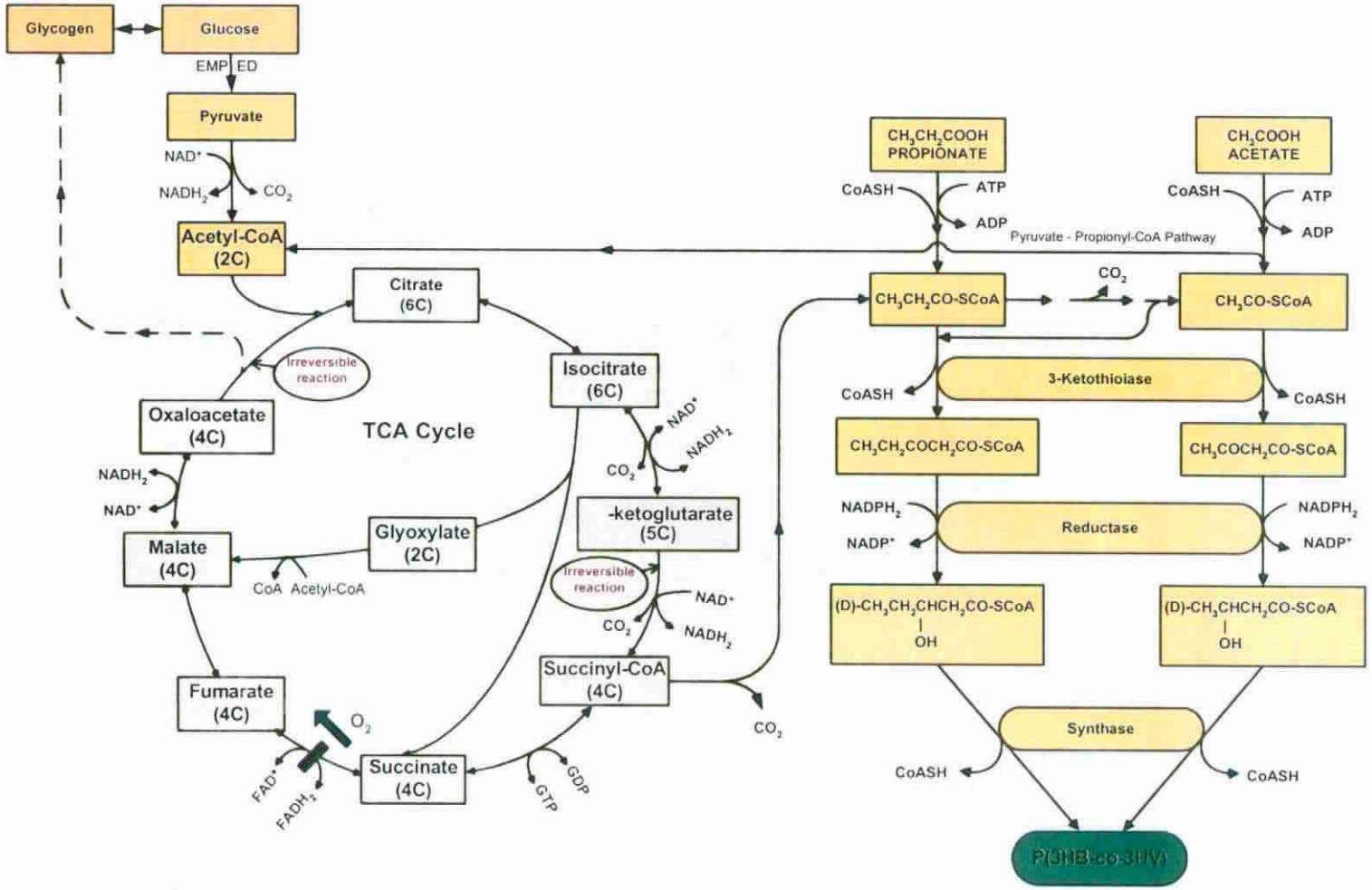


Figure 4.13. PHA accumulation pathway from EAS with acetate and propionate as sole or complex carbon sources. The TCA cycle, glyoxylate pathway and succinate-propionate pathway were combined with Doi model, and the oxygen play very important role for the 3HV fraction regulation in PHA (modified from Doi, 1990).

4.2.4 Thermal property of PHA from activated sludge

After fermentation by using C2 and C3 as complex carbon source (C2/C3: 80:20, mole/mole), the EAS was collected for the extraction of PHA by the method in accordance with that described in Chapter 3 (Chua *et al.*, 1997a, Hu *et al.*, 1997 and Yu *et al.*, 1998). The PHA harvested is demonstrated in Figure 4.14.

Figure 4.14. The PHA extracted from the EAS after 48 hours fermentation where acetate and propionate were used as complex carbon sources with the substrates molar ratio of C2/C3=80:20.

Subsequently, the extracted PHA was collected for further analysis, melting temperature for instance.

The melting temperature, T_m, of the PHA accumulated in the EAS with different medium composition ranged from 95 °C to 168 °C (Table 4.2).

The PHAs with maximum and minimum values of T_m were obtained when C2 and C3 were respectively used as the sole carbon sources in the medium. In these two cases, the 3HV molar fractions were respectively 8.0% and 78%. Increase of 3HV molar fraction in the PHBV formulation resulted in decrease of polymer melting temperature. The melting temperature of the PHA produced by EAS by using C2 and C3 as sole and complex carbon sources decreased with an increasing in the 3HV fraction (Figure 4.15), indicating that 3HV unit acts as defect in the PHBV crystal lattice. These results agreed with the published thermal properties of polymers of PHA are highly crystalline thermoplastics with a melting temperature around 180 °C for PHB homo-polymer and a range from 96 °C to 160 °C for PHBV co-polymers depending on the fraction of 3HV units (Doi *et al.* 1990) and previous report where butyrate and valerate as carbon substrates. Therefore, the 3HV molar fraction of the PHBV co-polymer accumulated in EAS, with thermal and mechanical properties being controlled by adjusting the C2 to C3 ratio in the media.

It can be seen from Figure 4.15, the relationship between T_m of the PHA and 3HV molar fraction was close to a linear relationship. The linear regression equation can be presented as: $y = -1.0125x + 168.98$, and $R^2 = 0.9623$, where "y" stands for T_m, "x" stands for the 3HV molar fraction. This observations are in accordance with that of butyrate and valerate as carbon substrates reported in the paragraph of 4.1.

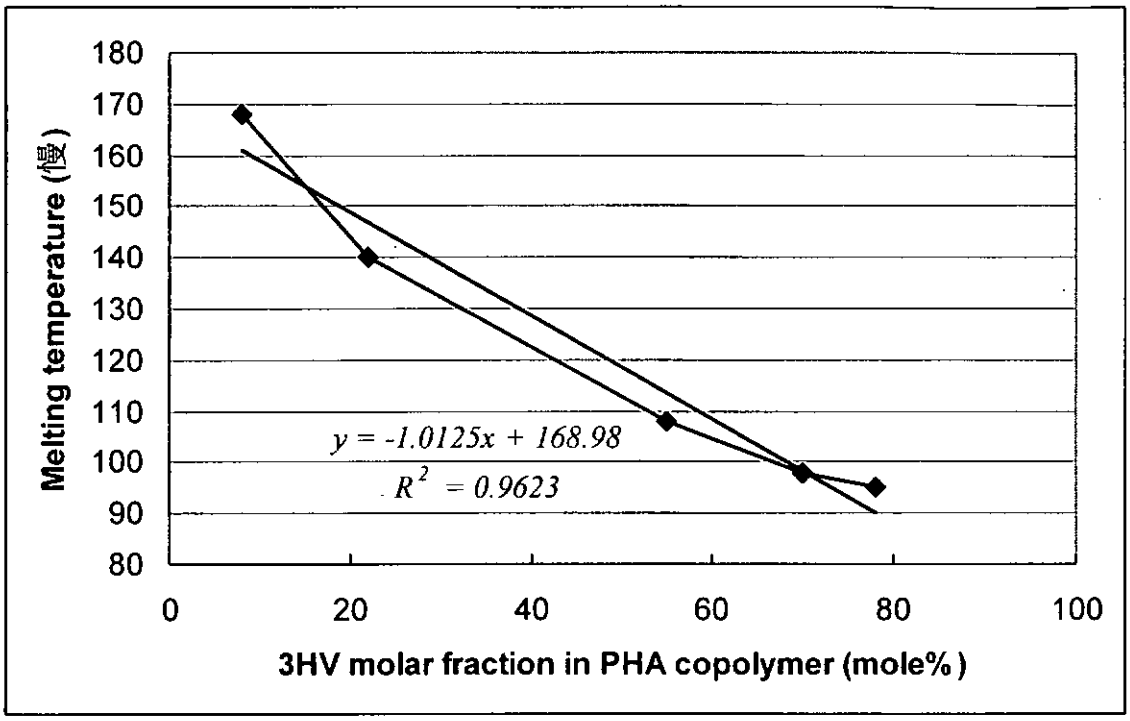


Figure 4.15. Melting temperature, T_m, of the PHA with different 3HV molar fraction. The PHAs were extracted from the EAS by using acetate and propionate as sole or complex carbon sources.

4.2.5 Variation of microbial community structures after fermentation

After 48 hours fermentation and acclimation, it was observed under microscope with 1000 × magnification that the main bacterial genus in the EAS with the inclusion of PHA polymer were cocci and bacilli. On the other hand, the PHA granules were clearly observed inside the bacterial cells after Sudan Black staining.

The cocci were 0.9-2.0 μm in diameter, appearing in pair, tetrad or aggregates; Sudan Black staining and Gram staining positive. There were not flagella observed. Bacilli were rod shape single cells, 2.5-3.5 μm in size, Sudan Black and Gram

Staining positive. Flagella were observed with motile ability. More detail morphology identification proved that *Alcaligenes* spp was the main group existed in the cultural broth after 48 hours cultivation for the PHA accumulation. This result agreed well with that described by Dave *et al.* (1996) and Chua *et al.* (1997a). When activated sludge was incubated under nitrogen- and phosphorus-limiting conditions, selective overgrowth of *Bacillus* spp. from 5 to 80% (cell count) was observed (Dave *et al.*, 1996). Satoh *et al.* (1996) described that if a mixed culture of activated sludge was cultivated under a sequencing anaerobic and aerobic condition and fed it with organic substrates only in the anaerobic phase of the sequence, the PAOs bacteria usually get dominant in the culture. This is because the PAOs microorganisms have an advantage in the competition toward organic substrates under anaerobic conditions.

Therefore, it is concluded that the fermentation process for PHA accumulation by the EAS is an acclimation procedure and selected overgrowth occurring through out the periods. Summarizing the two parts observation by using C4/C5 and C2/C3 as sole and complex carbon sources, the main bacterial genus are cocci and bacilli with the capability of PHA accumulation.

4.3 Synthesis of PHA by the EAS collected from full-scale MWWTP when glucose was used as sole carbon source under different ORPs

Introduction

Hollender *et al.* (2002) observed the effects of different carbon sources, acetate, acetate/glucose or glucose on the EBPR process by experiments under alternating anaerobic-aerobic conditions in SBR for each carbon source. It was revealed that the glucose was consumed completely within the first 30min of the anaerobic phase whereas acetate degradation was slow and incomplete. Phosphate was released independently of the carbon source during the whole anaerobic phase. The highest phosphate release (27 mg P/l) and PHA storage (20mg C/g dry matter (DM)) during the anaerobic phase as well as the highest polyphosphate (8 mg P/g DM) and glycogen storage (17 mg C/g DM) during the aerobic phase were observed with acetate. In contrast to other investigations, glycogen storage did not increase with glucose as substrate but was significantly smaller than with acetate. The PHA composition was also influenced strongly by the carbon source. The PHV portion of the PHA was maximal 17% for acetate and 82% for glucose. Due to the strong influence of the carbon source on the PHA concentration and composition, PHA storage seems to regulate mainly the phosphate release and uptake.

In general, PAOs are known to consume short chain VFAs and are unable to utilize glucose, if glucose is not converted to short chain VFAs by other organisms (Randall *et al.*, 1994). Glucose promotes the growth of glycogen-accumulating

organisms (GAOs) although GAOs were reported to be able to grow even in acetate-fed sludge (Cech and Hartman, 1990; Satoh *et al.*, 1994, 1996). The metabolism of GAOs seems to be similar to that of PAOs, except that glycogen serves as the intracellular energy pool for anaerobic substrate uptake instead of poly-p (Mino *et al.*, 1994; Satoh *et al.*, 1994; Liu *et al.*, 1996). As a result PHA is synthesized without energy deficit but with a net production of one mole of ATP in these GAOs. In contrast, several studies reported that good phosphorus removal efficiency could be achieved if glucose or a mixture of glucose and peptone are supplied as carbon source (Fukase *et al.*, 1984; Nakamura *et al.*, 1991; Carucci *et al.*, 1995, 1999). However, glucose decrease was not associated with the release of orthophosphate (Carucci *et al.*, 1995; Jeon and Park, 2000). Recently, Jeon and Park (2000) proposed a metabolic model which postulates the accumulation of glucose as glycogen, conversion of glucose to lactate and the formation of a lactate polymer by one population. The conversion of lactate to PHAs with a consumption of poly-P is performed by the PAOs.

Therefore, the carbon sources play the very important role in the production and the composition of PHA (3HB/3HV ratio) from the anaerobic-aerobic activated sludge in EBPR process. In fact as mentioned in the paragraph 4.2, there are four typical activated sludges are used for the wastewater treatment, and each of them performs a typical metabolic pathway in organic consumption and PHA accumulation. So far as I know, there is not literature to report the PHA production by activated sludge from glucose. Only fatty acids such as acetate, propionate, butyrate and

valerate have been used for the purpose of PHA accumulation from activated sludge (Chua *et al.*, 1997a, b; Hu *et al.*, 1997; Satoh *et al.*, 1998, 1999; Takabatake *et al.*, 2000 and Serafim *et al.*, 2002). In the previous parts of this paper, the four fatty acids have been submitted to the PHA production by activated sludge from laboratory-scale SBR or full-scale MWWTP. In this part research, glucose will be used as sole carbon source for the PHA production by EAS from a full-scale MWWTP in Hong Kong. Otherwise, the effects of ORPs on the PHA production and EAS performance are also investigated. Finally, the typical metabolic pathway for glucose consumption and PHA accumulation by the activated sludge will be proposed at the same time.

The main objectives of this study

The feasibility of using glucose as substrate to form PHA in EAS collected from local full-scale MWWTP in Hong Kong will be studied. The effects of ORPs on the PHA production and EAS performance are also investigated. In order to establish a biochemical metabolic model for PHA accumulation from EAS by using glucose as single carbon source, the metabolic pathway of glucose under various ORPs will be proposed and discussed based on the previous research. In this research, the ORPs will be preset at -20mV, -10mV, 0mV and +10mV, respectively.

4.3.1 Cell growth and glucose consumption under various ORPs

During the fermentation process, the ORPs dropped immediately at the beginning

of the process and even declined to the preset lowest level of -20mV. Thus, air or pure oxygen gas had to be supplied for keeping the ORPs at the preset level.

After 48-hours fermentation, the net cell growth was 1.73g, 3.19g, 6.69g and 7.27g when ORP was maintained at -20mV, -10mV, 0mV and +10mV respectively. The higher the ORP value caused the faster rate of consumption of substrate by the microorganisms, thereby resulting in faster growth rate of microorganisms. Oxygen supplied (for instance, ORP was increased from –20mV to +10mV) process or under oxygen-limited condition the organic substrate uptake and consumption are more efficient than that of anaerobic process as well as biomass conversion rate. As a result, the increasing of ORP, i.e. the gain of DO, led the increase of net EAS growth (Figure 4.16).

This fact could be proved by the TOC removal efficiency at different ORP levels observed. After 24-hours operation, the TOC removal efficiency was 43.4%, 41.9% 57.4% and 66.2%. At the end point of the fermentation, TOC removal kept rising to 65.9%, 71.1%, 81.5% and 96.0%, while ORP was kept at -20mV, -10mV, 0mV and +10mV, respectively. It was thought that when more oxygen was flowed into the cultural broth, the microorganisms consumed more carbonceaous substances (glucose) for PHA accumulation and cell growth in a faster rate (Figure 4.17 and Figure 4.18).

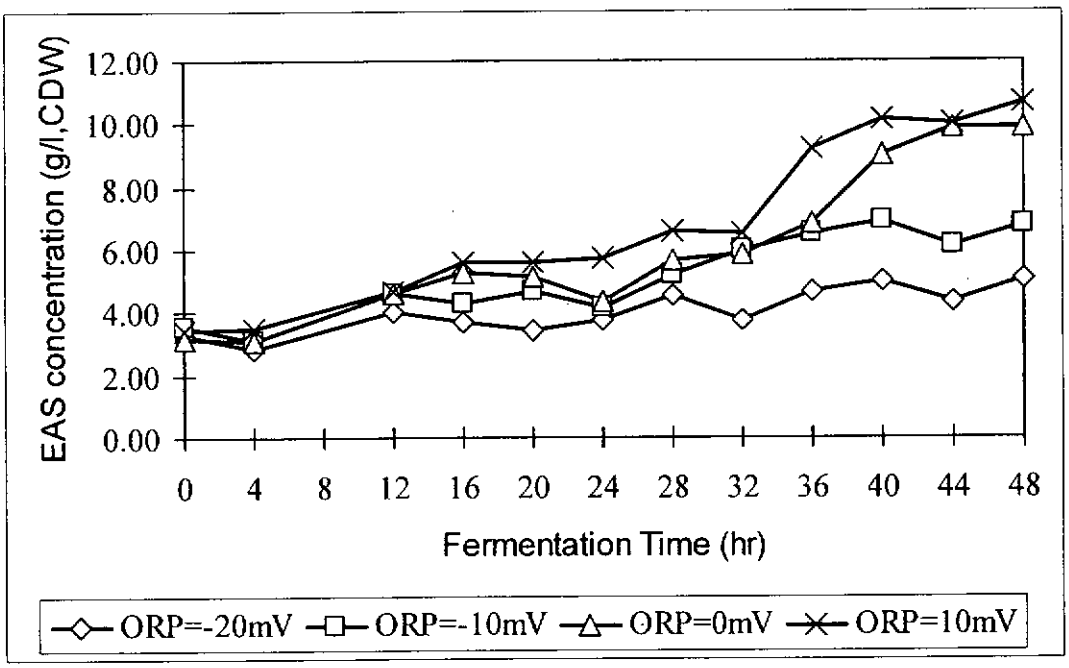
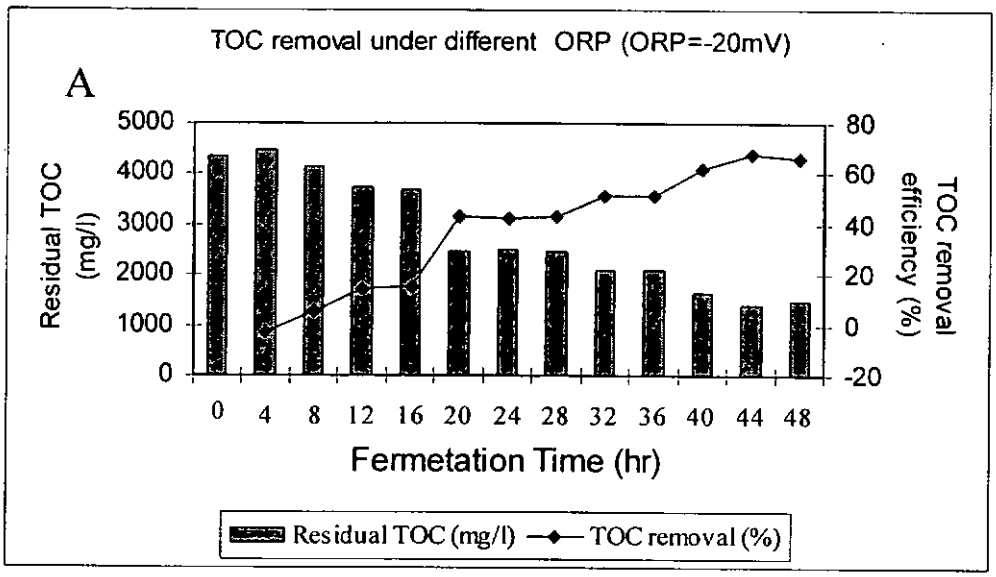
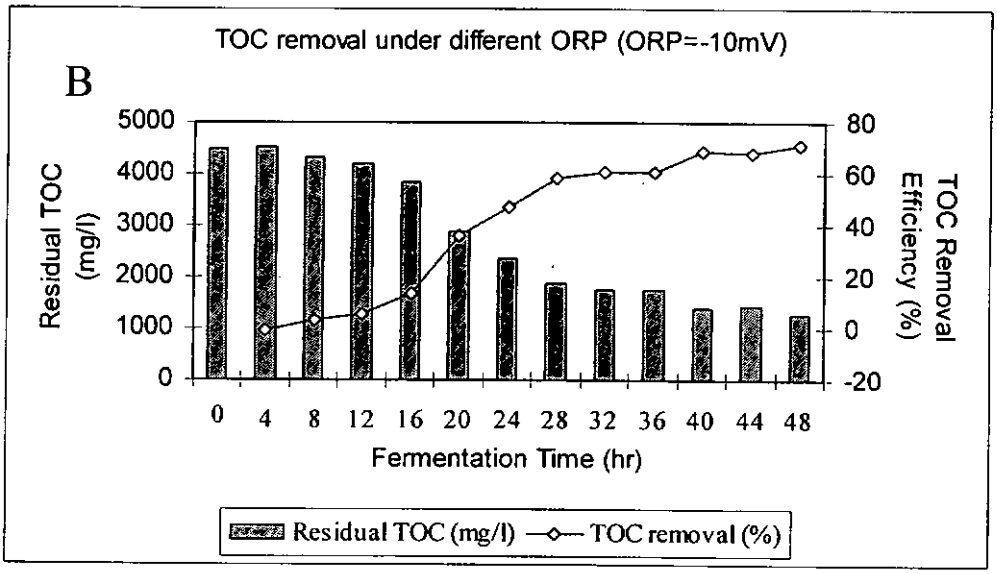
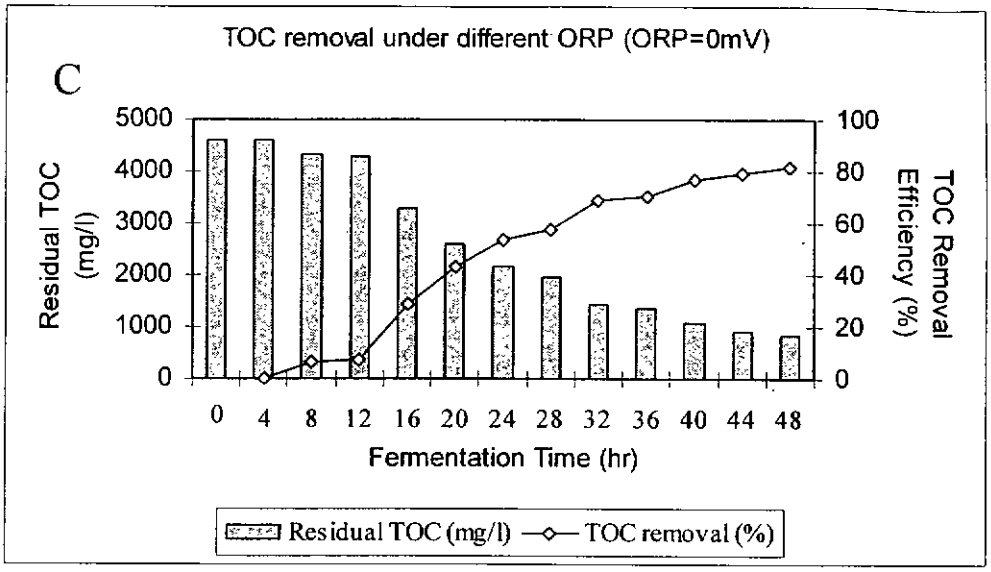


Figure 4.16 Time course of cell growth under various ORP levels. The data is the means of triplicate results obtained.


TOC removal under different ORP (ORP=-20mV)
A
Residual TOC (mg/l)
5000
4000
3000
2000
1000
0
80
60
40
20
0
-20
TOC removal efficiency (%)
0 4 8 12 16 20 24 28 32 36 40 44 48
Fermetation Time (hr)
Residual TOC (mg/l)
TOC removal (%)


TOC removal under different ORP (ORP=-10mV)
B
Residual TOC (mg/l)
5000
4000
3000
2000
1000
0
80
60
40
20
0
-20
TOC Removal Efficiency (%)
0 4 8 12 16 20 24 28 32 36 40 44 48
Fermentation Time (hr)
Residual TOC (mg/l)
TOC removal (%)



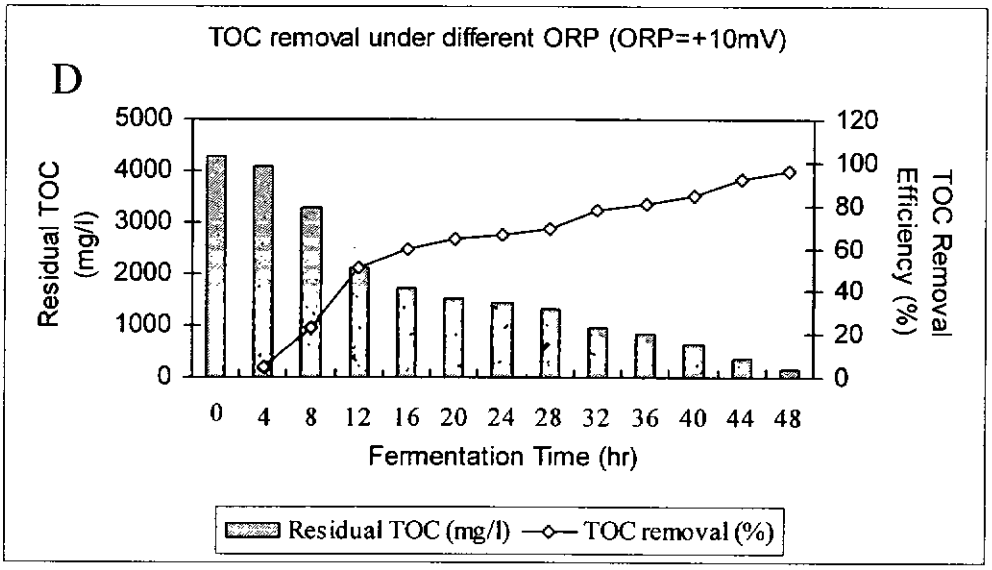


Figure 4.17 The time courses of residual TOC in cultural broth and TOC removal efficiency under various ORP levels where A, ORP=-20mV; B, ORP=-10mV; C, ORP=0mV and D, ORP=+10mV, respectively.

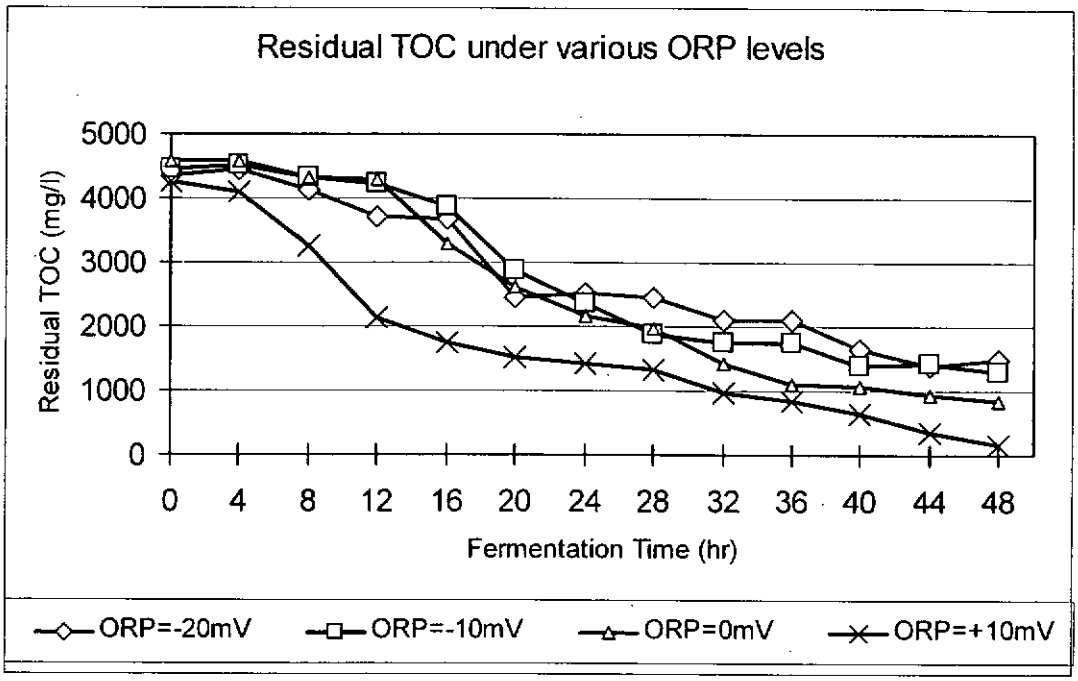


Figure 4.18. Residual TOC under various ORPs. It can be seen that higher ORP preset resulted in more TOC consumed by the bacteria in the EAS.

4.3.2 PHA production under various ORPs

As shown in previous sections, the ORPs were dropped immediately at the beginning of the process and even declined to the preset lowest level of -20mV. Thus, air or pure oxygen gas had to be supplied for keeping the ORPs at the preset level. On the other hand, the ORPs in this part of research were preset respectively at -20mV, -10mV, 0mV and +10mV instead of the lowest of -30mV and highest +100mV as conducted in the previous research where fatty acids as carbon substrates. This was because it was found by using glucose as carbon source and fed initially with about 3 gram/l of EAS, the ORPs dropped significantly and it was hard to maintain the ORP

to +100mV only by supplying the compressed air and even pure oxygen gas.

Results indicated that the polymer accumulation and polymer content in percentage of CDW increased with the increasing of ORP levels from -20mV to +10mV (Figure 4.20). The initial PHA content in EAS was about 2.5%. After 24-houres fermentation, the maximum PHA accumulation in the EAS of 8.34% of CDW was achieved when ORP was maintained at +10mV. PHA content in the EAS declined to 5.33% when ORP was kept at 0mV. The minimum PHA accumulation was 3.93% when ORP was kept at –20mV. After 48-hours operation, the PHA content were 5.55%, 6.28%, 8.33% and 11.15% of CDW while ORPs were kept at -20mV, -10mV, 0mV and +10mV respectively. Therefore, it was suggested that PHA content in the EAS increased with the increasing of ORP value. The presence of oxygen resulted in more glucose consumed for the cell growth, energy generation and PHA accumulation.

After 48 hours fermentation, the PHA in the EAS was extracted according to the method introduced by Chua *et al.* (1997a and 1997b). Figure 4.19 shows the PHA polymer harvested from the EAS.

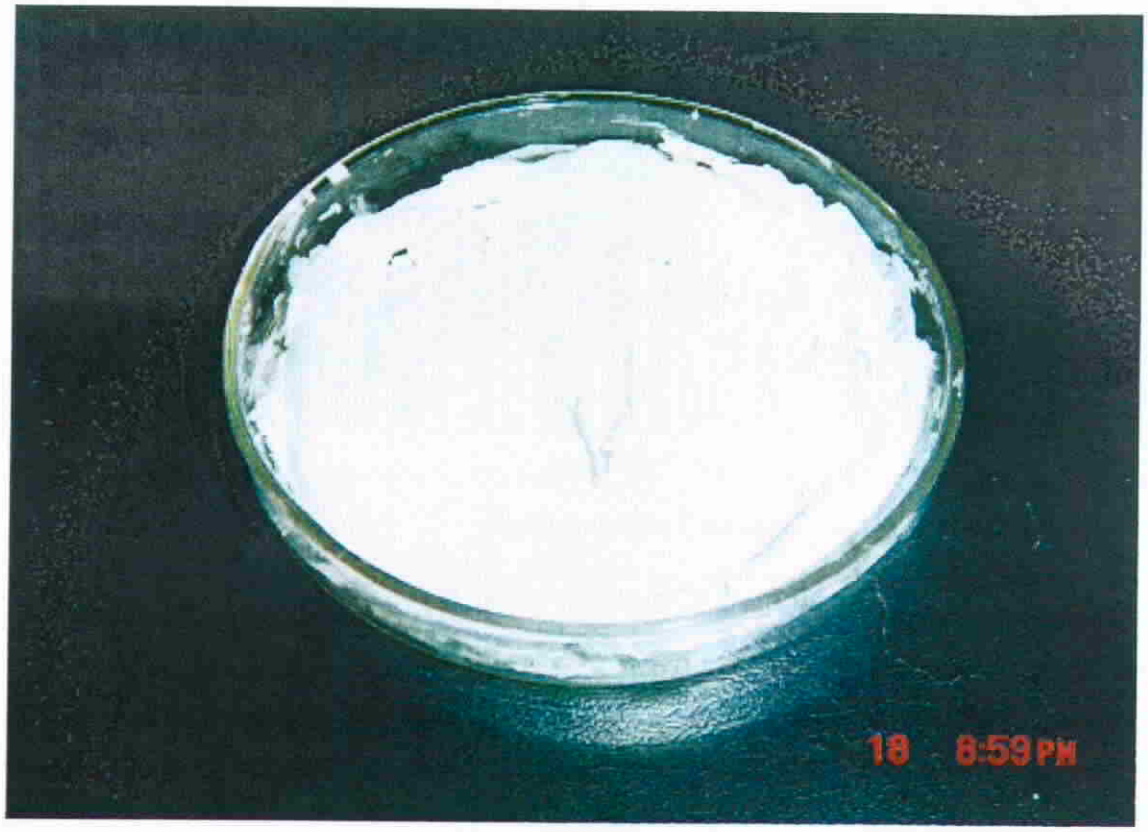


Figure 4.19. PHA extracted from EAS after 24 hours fermentation by using glucose as sole carbon source, ORP was maintained at +10mV.

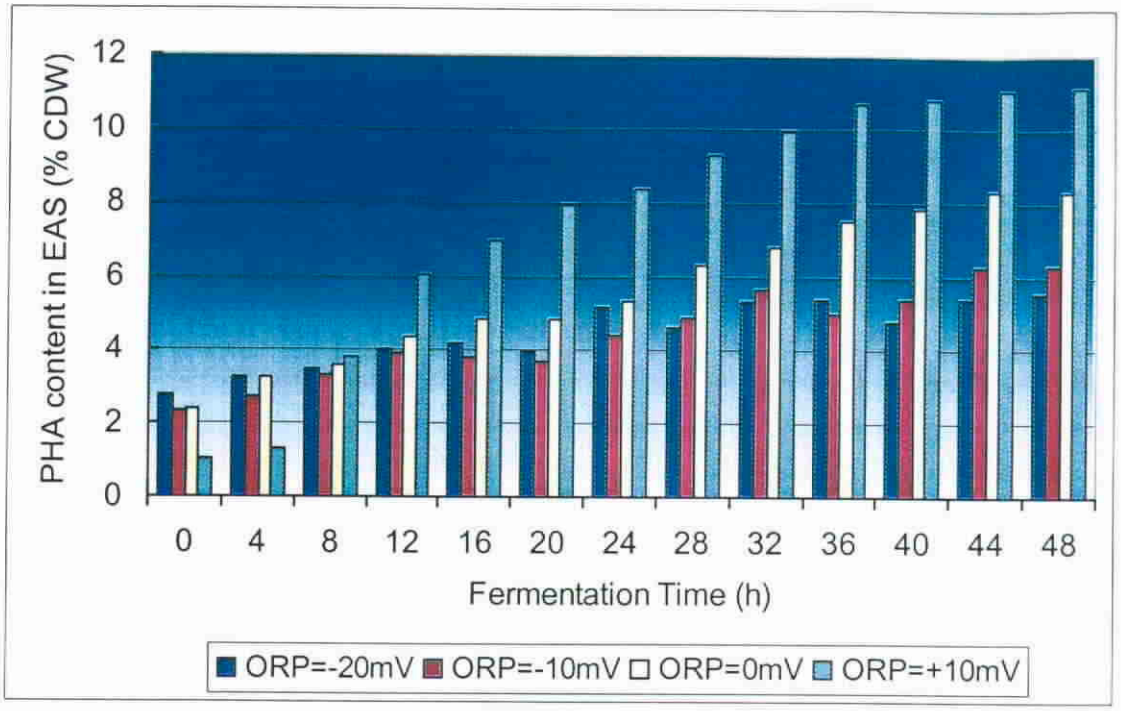


Figure 4.20. Time courses of PHA accumulation from EAS under various ORPs. The data is the means of triplicate results obtained.

It was reported that ORP is correlated to the logarithm of dissolved oxygen (DO) concentration with a linear relationship (Peddie *et al.*, 1990). ORP is a highly sensitive and instantaneous on-line instrumentation in fermentation process. ORP reflects the amount of materials such as dissolved oxygen, organic substrate, activity of organisms etc. Actually, in this study, the ORP was regulated only by controlling the air flow rate purged into the bioreactor, which meant that, the ORP value reflected the concentration of DO in the cultural broth. The reason of the increase of PHA production by the gain of ORP, even under oxygen-limited condition is probably due to the supply of energy by oxidation of organic substrate, glucose. Under the anaerobic condition, the energy source is limited by the amount of polymers such as

polyphosphate and glycogen. The energy source of polyphosphate and glycogen limit the PHA accumulation under anaerobic conditions. When energy sources are used up, the PHA accumulation will be stopped. By adjusting the ORP value to a higher level which in fact increasing the concentration of DO, microorganisms in EAS can take up organic substrate such as glucose to get more energy except polyphosphate and glycogen via oxidative degradation of some part of organic substrate to satisfy the need for the PHA synthesis (Satoh *et al.*, 1999; Hu *et al.*, 2001).

The polymer production yield ($Y_{P/S}$) under different ORP levels was analysised. $Y_{P/S}$ (g PHA/g carbon) was calculated as the polymer accumulated divided by the TOC consumed (Figure 4.21). The maximum polymer production yield of 0.31 was achieved by keeping the ORP level at +10mV. When the ORP was maintained at 0 mV, the $Y_{P/S}$ declined to 0.24. Further reducing of ORPs (-10mV and –20mV) resulted to 0.16 to 0.07 of $Y_{P/S}$ respectively.

These results and observations were coincided with that observed by using acetate and propionate as carbon sources.

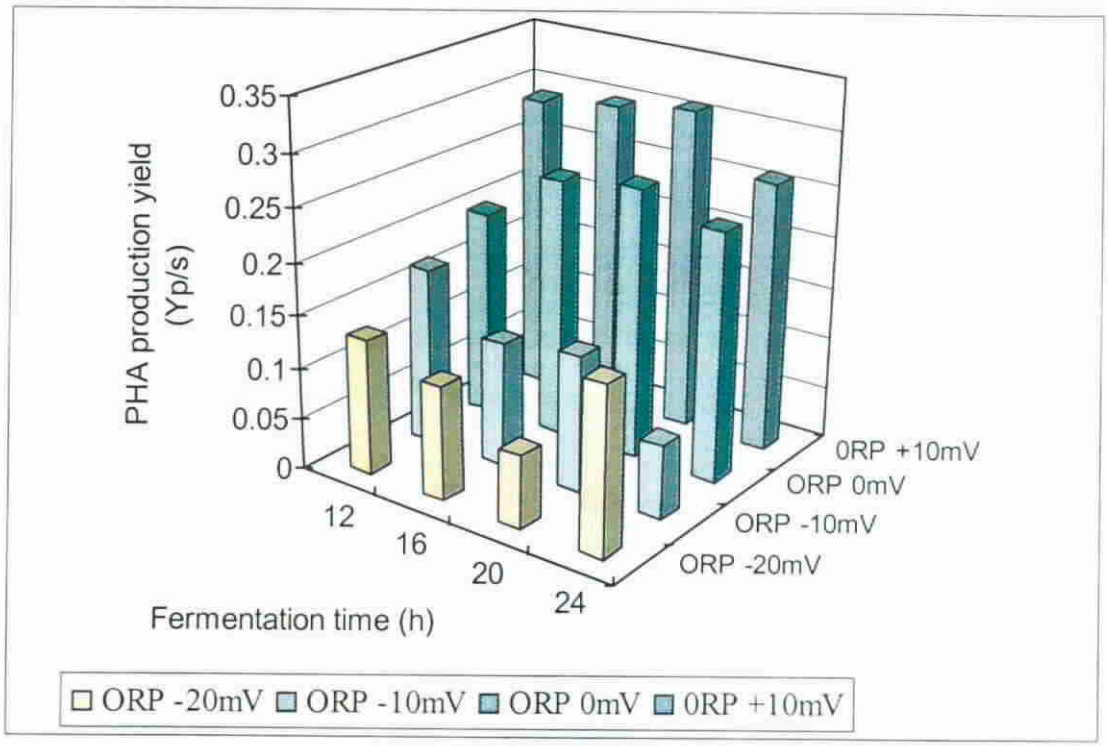


Figure 4.21 Time courses of the yields of PHA accumulation from EAS ($Y_{p/s}$) under various ORP values.

4.3.3 Effects of ORP on PHA composition

According to the results of GC analysis, the PHA produced by EAS was identical with that of the standard samples including PHBV (poly-3-hydroxybutyrate-co-3-hydroxyvalerate). The polymers formed by EAS under different ORP conditions were recognized as PHBV co-polymer.

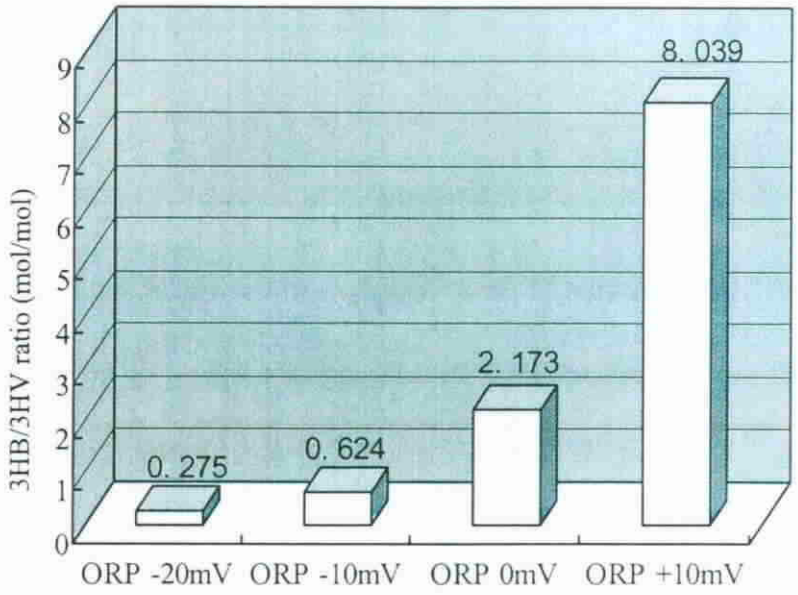


Figure 4.22. The variation of HB/HV molar rations of PHA co-polymer from EAS under different ORP levels, while glucose was used as sole carbon source.

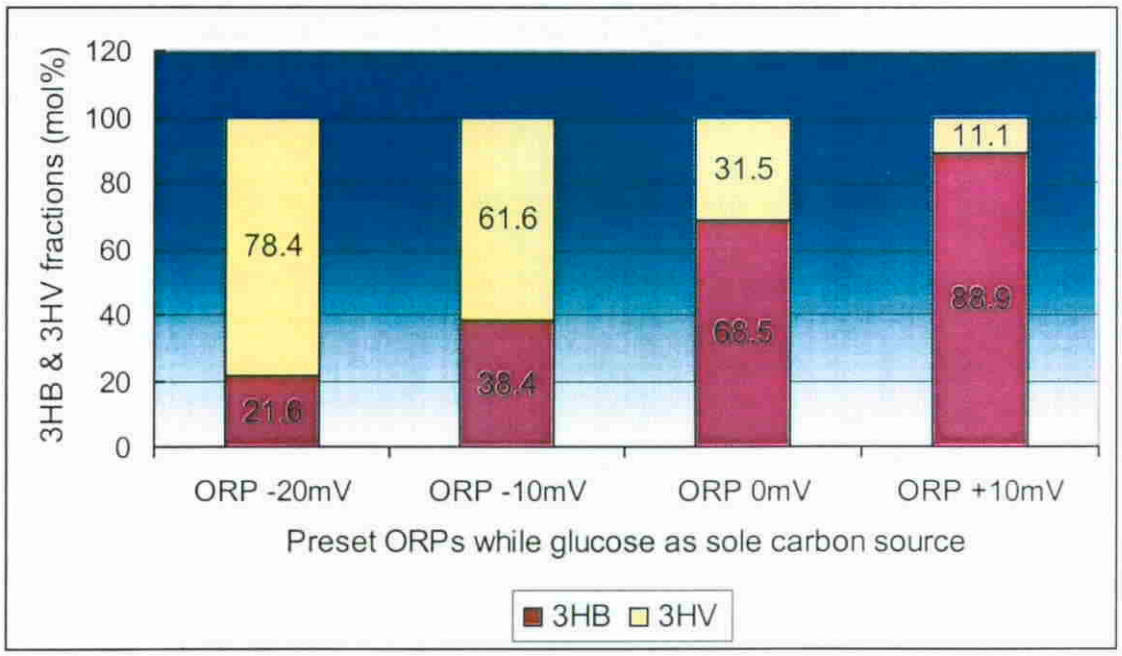


Figure 4.23. 3HB and 3HV molar fractions in the PHBV copolymer accumulated by EAS by using glucose as sole carbon source under various preset ORPs.

In addition, as illustrated from Figure 4.22 and Figure 4.23, 3-hydroxybutyrate (3HB) unit fraction to 3-hydroxyvalerate (3HV) unit fractio (3HB/3HV) mole ratios increased with that of increasing of ORP values. The maximum HB/HV mole ratio was 8.03 (3HB 88.93 mol%, 3HV 11.07 mol%), when the ORP level was maintained at +10 mV. The HB/HV value then declined with the decreasing of the ORP level. When ORPs were kept at 0 mV and -10mV, the 3HB/3HV ratios were 2.173 and 0.624, which in turn the 3HV molar fractions were 31.5 mol% and 61.6 mol% respectively. It reached the minmum of 0.275 (3HB 21.60 mol%; 3HV 78.40 mol%) when the ORP was kept at –20 mV (Figure 4.22 and Figure 4.23). These results are in accordance with that of acetate and propionate as carbon sources presented previously.

Actually, similar results have been revealed by other authors where the glucose was used as sole carbon source in EBPR process (Wang *et al.*, 2002 and Sudiana *et al.*, 1999). They found that under anerobic condition, when glucose was used as sole carbon source the PHA was composed mainly of 3HV instead of 3HB unit.

It is known that PHB is the most abundant form of PHA accumulated when acetate is used as the dominant organic substrate in EBPR processes (Dawes, 1986; Mino *et al.*, 1987 and Sudiana *et al.*, 1999). Under anaerobic condition, in the case of the high P sludges or PAO enriched sludge acetate was converted to PHA, and the 3HB/3HV ratio reached to 95:5 or equivalent to 19. However, in the case of low P sludge or GAO enriced sludge a certain amount of 3HV unit was produced in addition

to 3HB unit, and the 3HB/3HV ratio was 84:16 or equivalent to 5.25 (Sudiana *et al.*, 1999).

Wang *et al.* (2002) has conducted the glucose as the dominant substrate to induce and maintain a successful EBPR process. However, compared to the convertional EBPR process using acetate as the dominant substrate, it was found that the PHA produced was composed mainly of 3HV instead of 3HB. The 3HV accounted for around 83% of the total PHA accumulated at the end of the anaerobic stage. Sudiana *et al.* (1999) also reported independently that the analysis of PHA composition revealed that EBPR sludge accumulated PHA composed of almost 100% 3HV unit by using glucose as sole carbon source. Only in a few cases they have observed the formation of 3HB. It was suspected this might be due to re-adsorption of fermented substances like acetate or lactate and its conversion to 3HB. However, the verification of re-adsorption of fermented metabolities was not yet available (Sudiana *et al.*, 1999; Wang *et al.*, 2002).

The results observed in my own research that the variation of 3HB/3HV ratios in PHA accumulated by EAS could be resulted from the changes of ORP levels or equivalent DO concentration in the cultural broth surprised us very much, and have never been reported before. In general, either in pure coulutre of single microbial strain or co-culture of activated sludge, the 3HB/3HV ratios in the PHA are maintained constant, and the variation caused only by the types of substrate or co-sbustrate used as carbon sources, C/N ratio, C/P ratio and the different microbe trains submitted for the PHA accumulation (Holmas, 1988; Inoue *et al.*, 1996; Chua

et al., 1997a, b; Hu *et al.*, 1997, 2001; Hong *et al.*, 2000; Takabatake *et al.*, 2000 and Ma *et al.*, 2000b). These results implied that ORP or equivalent DO in cultural medium might act as an exogenous factor to determine the ratio of 3HB/3HV of PHA copolymer synthesised by EAS, and subsequentially to control the PHA's physical and mechanical properties. Otherwise, 3HB/3HV ratios, and hence the mechanical properties of PHA could be regulated and maintained through controlling the DO concentration via the ORP monitoring and regulation procedures.

In this research, these results and phenomena have been observed twice while respectively using fatty acids (acetat and prionoate) and glucose as carbon sources under the ORP monitoring and controlling conditions. It was thought that monitoring and regulating the fermentation process by ORP instead of DO provided more sensitive and reliable control. A little bit variation of DO concentration in cultural broth could not be observed and realized by DO system, but ORP system could do it. This why the phenomena was found so later and only being observed under the ORP monitoring and regulating conditions.

After being accepted that DO was the exogenous factor to affect the 3HV molar fraction in PHA copolymer, two of the explanations were proposed. The first one was that oxygen could be a exogenous force which resulted in the selected overgrowth of the bacteria in EAS. The selected bacteria converted organics such as fatty acids and glucose into PHA copolymer with 3HB or 3HV as major units. The second one was that the presence or absence (or the quantity) of oxygen in cultural broth would resulted in the changes of metabolic pathway for the PHA accumulation and causing

3HV molar fraction variation. The second explanation is more reasonable than the first one, I thought. Therefore, in the next part of this chapter, a hypothesized biochemical metabolic model by using glucose as sole carbon source was estabolished to try to explain and understand these phenomena.

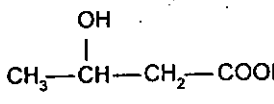
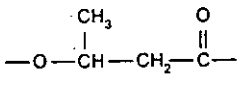
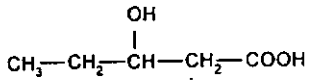
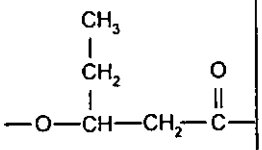
The hypothesises of biochemical metabolic models for the PHA production from EAS are consisted of two parts. The first part is an anaerobic (or facultative condition) biochemical model, where ORP is maintained at low levels (-20mV and –10mV, for example), which means that the PHA production process was performed under oxygen-limited condition. The second part is an aerobic biochemical model in which the ORP was maintained at higher levels (0mV or 10mV, for example), and the air (oxygen) was kept flowing into the cultural broth. On the other hand, the cell net growth is also taken into account (Figure 4.24).

4.3.4. Hypothesized biochemical metabolic model for the PHA accumulation from EAS by using glucose as sole carbon source.

At first, let's review and describe the experimental conditions and the metabolic pathways for the formation of PHA in the activated sludge under anaerobic condition.

The primary monometric units of PHA formed in the activated sludge are listed in Table 4.3, which will make the hypothetic models below be easily understood.

Table 4.3 Primary monometric units of PHA formed in the EAS

PHA	Free Acid	Unit	Precursors
3-hydroxybutyrate (3HB)			2 acetyl-CoA
3-hydroxyvalerate (3HV)			1 acetyl-CoA + 1propionyl-CoA

It is under the anaerobic condition that the bacteria in activated sludge establish the mechanism of organic substrate uptake and PHA accumulation. Therefore, most of the metabolic pathways are summarized from the anaerobic-aerobic process for EBPR performed by several researchs. Compared with the anaerobic phase in EBPR process, there are different conditions

employed in my research works. This research was conducted by monitoring and regulating the ORP levels via controlling the DO concentration in cultural broth. When ORP was maintained at –20mV or –10mV, the nitrogen gas was purged into the jar fermenter firstly for 10 minutes in order to remove DO in the medium, and keep the ORP at the designed volume. Subsequently, the ORP dropped after 4 hr fermentation caused by the substrate consumption, PHA accumulation and cell growth. When ORP was lower than the designed level, -20mV, the air had to be flowed into the cultural broth to maintain the ORP at constant level. Otherwise, the pathway of PHA formation in the bacteria of EBPR is assumed that the external substrate is utilized for the synthesis internal carbon reserves and no biomass growth occurs under anaerobic condition (Wang *et al.*, 2002). In contrast, the biomass in this research was gained during the fermentation process owing to the limitation supply of oxygen which induced the glucose fermentation and precursor synthesis of cell construction. Finally, the difference between EBPR and this research is that the phosphate buffer solution has been used for keeping the pH constant instead of NaOH. The phosphate in cultural medium might cause effects on the cell growth, PHA accumulation and metabolic pathway. The data indicated that when 1 M NaOH solution was used for the pH maintenance, the glucose-consumed rate was higher than that of phosphate buffer solution used (data not shown). On the other hand, it was observed that the variation of ORP level (i.e., DO concentration) in the cultural medium caused the changes of HB/HV ratio of the PHA copolymer formed by EAS while glucose was used as

sole carbon source. Under anaerobic conditions, the major unit of PHA was 3HV instead of 3HB under aerobic condition. This result has never been reported. It is known that the factors which cause the variation of HB/HV ratio in the PHBV copolymer include the substrates as carbon sources and the microorganisms strains used for the PHA production. For example, the acetate, propionate, acetate/propionate ratio, butyrate, valerate, and butyrate/valerat ratio used for sole or mixed carbon source result in the variation of 3HB/3HV ratio in PHA copolymer. These facts have been verified by the previous researches.

Based on the batch experimental results described above, and take all factors into account, a biochemical model is hypothesized to predict the fate of glucose transported into the cells and the major metabolic activities by the EAS bacteria during the anaerobic and aerobic phase (Figure 4.24).

As illustrated in Figure 4.24, the TCA cycle, Glyoxylate pathway, EMP/ED pathway and PHBV synthetic pathway are all involved in this hypothetic model. This hypothetic metabolic model is consisted of five parts. The first part is the glycolysis of glucose oxidized through EMP or ED pathway to pyruvate. The second part is pyruvate decarboxylation to acetyl-CoA to form the PHB or acetyl-CoA enters the TCA cycle. The third part is lactic acid fermentation where pyruvate as electro acceptor under anaerobic condition, and then lactic acid for the further conversion to 3HV. The fourth part is the succinate-propionate pathway to synthesis PHV from pyruvate to propionyl-CoA under anoxic or anaerobic conditions. The fifth part is the TCA cycle and glyoxylate pathway. The most

important view in the hypothetic model is that the oxygen is the key factor to regulation of 3HV mole fraction in PHA copolymer.

The detail explanation, analysis and description of substrate metabolisms, ATP generation, redox balance, 3HV and 3HB synthesis in this hypothetic metabolic model have been demonstrated as follows.

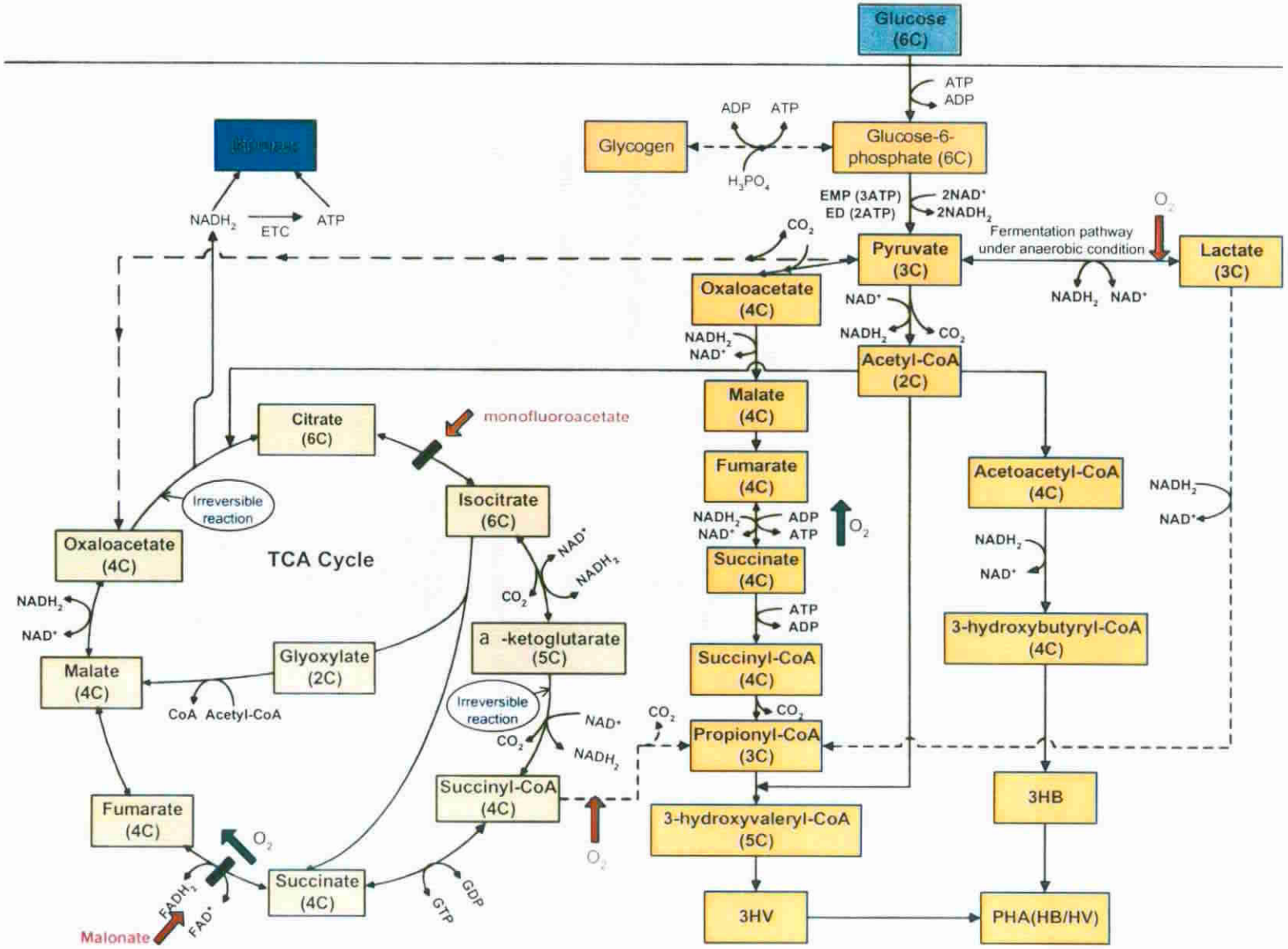


Figure 4.24. A hypothesized chemical metabolic pathway for PHA accumulation from EAS under anaerobic, anoxic and aerobic conditions. This hypothetic metabolic model is consisted of five parts, glycolysis of glucose (or glycogen) to pyruvate; pyruvate to propionyl-CoA pathway for PHV synthesis; pyruvate decarboxylation to acetyl-CoA to form the PHB; lactic acid fermentation from pyruvate to form PHV; the TCA cycle and glyoxylate pathway respectively.

4.3.4.1 Glucose uptake

According to Gottschalk (1986), glucose is phosphorylated during the transportation into the cytoplasm of the bacteria in EAS and 1 mole ATP is consumed for transferring 1 mole of glucose into the cell:

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}$$

4.3.4.2. Glycogen accumulation and degradation

Glycogen is considered the common storage carbon and energy source for many bacteria in EAS and accumulated under favored conditions, nutrient enriched and aerobic conditions for instance. But glycogen would be hydrolyzed as nutrient and energy source under starving and anaerobic conditions. With the addition of glucose into the jar fermenter under anaerobic condition, an increase of glycogen arises from the conversion of phosphorylated glucose (Dawes and Senior, 1973). Subsequently, the glycogen oxidized to pyruvate via EMP or ED pathway and continuous convert to PHA during the anaerobic phase (Mino *et al.*, 1987; Liu *et al.*, 1994, 1996; Pereira *et al.*, 1996 and Wang *et al.*, 2000). It is therefore assumed in the hypothetic metabolic model that glycogens hydrolyzed back to glucose-6-phosphate which is then utilized to synthesize PHA. The metabolic pathway of glycogen synthesis proposed by Dawes and Senior (1973) is summarized as below:

$$\text{glucose-6-phosphate} + ATP + (C_6H_{10}O_5)_n + H_2O \rightarrow$$

$$(C_6H_{10}O_5)_{n+1} + ADP + 2H_3PO_4.$$

The degradation of glycogen (Dawes and Senior, 1973) is summarized as below:

$$(C_6H_{10}O_5)_n + H_3PO_4 \rightarrow \text{glucose-6-phosphate} + (C_6H_{10}O_6)_{n-1}.$$

4.3.4.3. Glycolysis of glucose and glycogen, pyruvate and ATP production

There are two possible pathways for the glycolysis reaction of the phosphorylated glucose derived from glucose or the glycogen: the Embden-Meyerhof-Parnas (EMP) pathway or the Entner-Douderoff (ED) pathway (Mino *et al.*, 1998).

EMP pathway is the most important pathway for glucose metabolism and the majority of microbes utilize it for the catabolism of carbohydrates such as glucose and fructose, or the glucose monomeric unit derived from polysaccharides, glycogen in the activated sludge and starch for example. The products of this pathway are 2 mole of pyruvate, forming 3 mole of ATP and 2 mole reduced form of $NADH_2$ for one mole glucose consumed (Figure 4.26).

The EMP pathway can be summarized in following equation:

$$\text{glucose-6-phosphate} + 3ADP + 2NAD + 2H_3PO_4 \rightarrow$$

$$2CH_3COCOOH\ (\text{Pyruvate}) + 3ATP + 2NADH_2 + 2H_2O.$$

Otherwise, bacteria in the activated sludge might also use ED pathway for glucose metabolism. Besides the glucose, the carbohydrate can be used for the metabolism through ED pathway include fructose and the glucose monomeric unit of polysaccharides which is the same as described in the EMP pathway. 2 mole pyruvate, 2 mole of ATP and 2 mole $NAD(P)H_2$ are produced from each mole glucose-6-phosphate converted (Figure 4.27). The ED pathway is summarized in the following equation:

$$\text{glucose-6-phosphate} + 2ADP + H_3PO_4 + 2NAD \rightarrow$$

$$2CH_3COCOOH\ (\text{pyruvate}) + 2ATP + 2NADH_2\ (\text{or } NADH_2 + NADPH_2) + 2H_2O$$

To compare the two equations above, it is shown that one mole less of ATP is produced from each mole metabolized glucose-6-phosphate in the ED pathway than the EMP pathway. However, the same amount of 2 mole reducing equivalents ($NADH_2$) is produced from the ED pathway as from the EMP pathway. In addition, glycolysis of glucose-6-phosphate to pyruvate through EMP and ED pathway could be performed under both aerobic and anaerobic conditions.

ATP generated from the glycolysis can be used to drive the uptake of substrates or to drive the synthetic reactions, the PHA production for example. $NADH_2$ can be used to produce energy via oxidative phosphorylation (ETC) or as a source of H^+ for reduction reactions in PHA accumulation. This pathway is located in the cytoplasm of microbes and can function either aerobically (in the presence of oxygen) or anaerobically (in the absence of oxygen).

4.3.5. PHA formation from pyruvate

Pyruvate formed by glycolysis of glucose-6-phosphate can be further metabolized in the presence of oxygen to generate ATP via the citric acid cycle (TCA cycle) and Electron-Transport Chain (ETC), or can be used as terminal electron acceptor for synthesis of other metabolic intermediates and end products in the absence of oxygen. As illustrated in the hypothesized metabolic model, pyruvate is decarboxylated to form acetyl-CoA, and subsequently acetyl-CoA enters the TCA cycle for the further metabolism or transformed in the glyoxylate pathway to produce both malate and succinate. In the meantime, part of acetyl-CoA is assumed as both of the PHBs' and PHVs' precursor for PHB and PHV synthesis respectively. On the other hand, the pyruvate is converted to lactate by the fermentation pathway under anoxic and anaerobic conditions, where the pyruvate is the terminal electron acceptor. The lactate could be converted to propionyl-CoA under anaerobic condition, another PHV precursor.

Actually, pyruvate can be converted to oxaloacetate where 1 mole ATP consumed with combining of 1 mole carbon via using biotin as the carbon carrier. This reversible reaction is involved in the succinate-propionate pathway (Gottschalk, 1986), or in term of the pyruvate-propionyl-CoA pathway modified by Zeng *et al.* (2002) for PHV production (Figure 4.25). Further discussion has been performed in the TCA cycle, the next part of this paragraph.

4.3.5.1. PHB formation from pyruvate

As described previously, after the beginning of fermentation, the PHB content increased in the EAS, which was arisen from the conversion of glucose into pyruvate and then to acetyl-CoA, as the precursor of PHB synthesis (Doi *et al.*, 1989). It is assumed that part of the end product of glycolysis from glucose, pyruvate, was utilized for PHB synthesis after being converted to acetyl-CoA molecules. On the other hand, part of acetyl-CoA is transformed in the glyoxylate pathway to produce both malate and succinate. The malate is then oxidized to oxaloacetate to sustain the cycle of the glyoxylate pathway and form an equivalent of the $NADH_2$ necessary for PHB synthesis. The precursor for PHB formation is 2 acetyl-CoA (Table 4.26). The pathway of PHB synthesis from pyruvate is demonstrated in the hypothetic metabolic model and summarized (Dawew and Senior, 1978) as follows:

- The formation of acetyl-CoA (a precursor of PHV) from pyruvate is summarized as below according to Gaudy (1978)

$$CH_3COCOOH \text{ (Pyruvate)} + CoASH + NAD^+ \rightarrow$$

$$CH_3COSCoA \text{ (Acetyl-CoA)} + CO_2 + NADH_2$$

- 3HB unit formation by coupling of 2 acetyl-CoA, and then the formation of PHB.

$$2CH_3COSCoA(Acetyl\text{-}CoA) + (C_4H_6O_2)_n + NADH_2 \rightarrow$$

$$(C_4H_6O_2)_{n+1} + 2CoASH + NAD^+$$

- PHB formation from pyruvate via coupling the upper two equations as below:

$$2CH_3COCOOH\ (pyruvate) + NAD + (C_4H_6O_2)_n \rightarrow$$

$$(C_4H_6O_2)_{n+1}\ (PHB) + 2CO_2 + NADH_2$$

It should be noticed that, as shown in the hypothetic metabolic model (Figure 4.24) and the equations listed above, it is clear that 1 mole of 3HB is formed from 2 mole of pyruvate, and meanwhile, 2 mole CO_2 and 1 net mole reduced $NADH_2$ were generated during this conversion process.

4.3.5.2. PHV formation from pyruvate (pyruvate---propionyl-CoA pathway)

As mentioned in the previous part of this chapter, the experimental results showed that 3HV unit was the predominant form of PHA that was accumulated when ORPs were maintained at -20 and -10 mV respectively, where glucose was used as sole carbon source. The 3HV molar fraction in PHA decreased from 78.4 mol% to 11.07 mol% with the increasing of ORP from –20mV to +10mV. Table 4.26 illustrated that the PHV formation requires two precursors in equal amounts: acetyl-CoA and propionyl-CoA. It is assumed that the formation of propionyl-CoA is through the pyruvate-propionyl-CoA

pathway (Figure 4.25) (Zeng *et al.*, 2002), modified from the former succinate-propionate pathway (Satoh *et al.*, 1992 and Filipe *et al.*, 2001) and will be expended the excess $NADH_2$ generated from the glycolysis pathway or PHB production to enable the redox balance in the bacteria of EAS.

- Propionyl-CoA formation through the pyruvate-propionyl-CoA pathway could be summarized as follow:

$$CH_3COCOOH \text{ (Pyruvate)} + 2NADH_2 + CoASH \rightarrow$$

$$CH_3CH_2COSCoA \text{ (Propionyl-CoA)} + H_2O + 2NAD^+$$

- Acetyl-CoA formation from pyruvate is summarized in the following equation.

$$CH_3COCOOH \text{ (Pyruvate)} + CoASH + NAD^+ \rightarrow$$

$$CH_3COSCoA \text{ (Acetyl-CoA)} + CO_2 + NADH_2$$

- The formation of PHV since acetyl-CoA and propionyl-CoA are the precursors for PHV formation, the formation of PHV can be derived by coupling the upper two equations:

$$2CH_3COCOOH \text{ (Pyruvate)} + 2NADH_2 + ATP + (C5H_8O_2)_n \rightarrow$$

$$(C_5H_8O_2)_{n+1} + H_2O + 2NAD^+ + CO_2 + ADP + H_3PO_4$$

This final equation shows that for each mole of PHV unit formation from pyruvate, 2 mole of $NADH_2$ are consumed for 2 mole of pyruvate reacted.

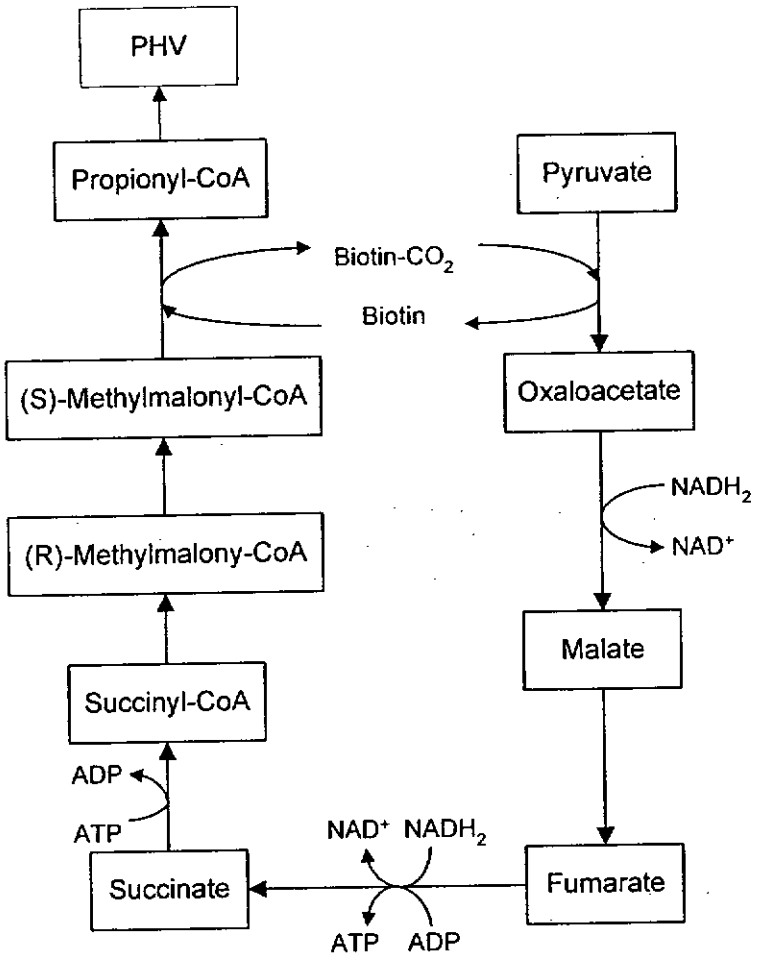


Figure 4.25. Pyruvate-propionyl-CoA pathway (the former succinate-propionate pathway suggested by Satoh *et al.* (1992)) for PHV production by the glycogen accumulation organisms in anaerobic-aerobic activated sludge under anaerobic condition (adopted from Zeng *et al.*, 2002).

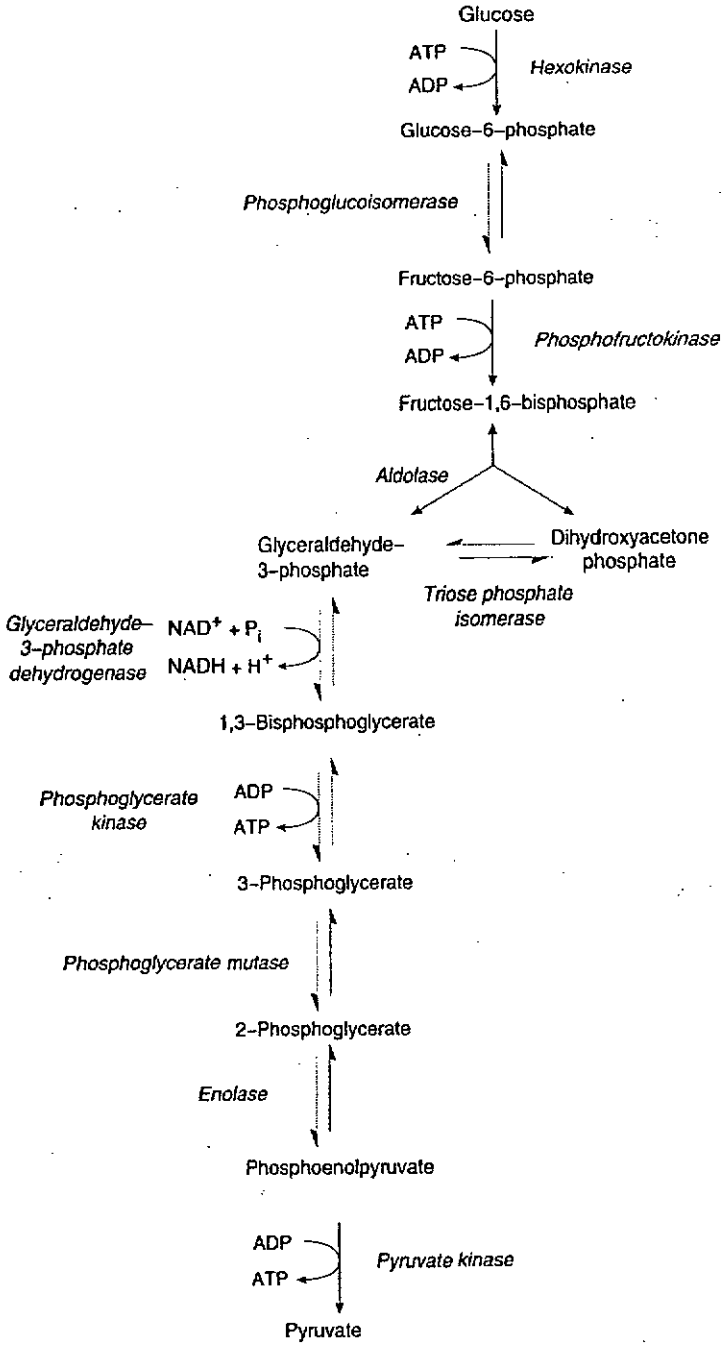


Figure 4.26 The Embden-Meyerhof-Parnas (EMP) pathway or so-called glycolytic pathway. Most microbes utilize the EMP pathway for the catabolism of carbohydrates such as glucose and fructose. This pathway is located in the cytoplasm of microbes and functions either in the presence or absence of oxygen.

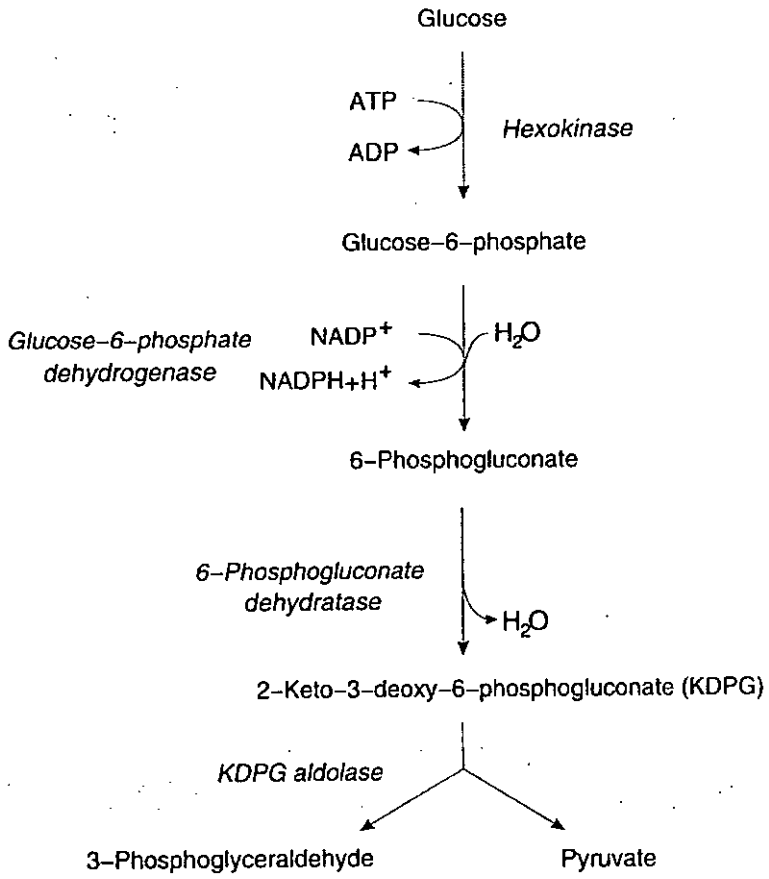


Figure 4.27 The Entner-Doudoroff (ED) pathway. The bacterial genera *Pseudomonas, Rhizobium* and *Agrobacter* substitute the ED pathway for the glycolytic pathway from glucose, fructose and even glycogen in activated sludge.

4.3.6. Lactic acid fermentation and PHV production from lactic acid

A major product in the catabolic pathways from glucose to pyruvate described previously is $NADH_2$. In the presence of oxygen, $NADH_2$ can be further oxidized through ETC to generate ATPs. However, in the absence of oxygen, $NADH_2$ must be oxidized back to NAD^+ for the purpose of electron balance. Many microbes utilize derivatives of pyruvate as electron and H^+ acceptors and this allows $NADH_2$ to be re-oxidized to NAD^+. This process may lead to an increase in ATP synthesis, an important factor for organisms growing in the absence of oxygen. Such pathways are commonly termed fermentation reactions. Lactic acid fermentation is a common type of fermentation characteristic of lactic acid bacteria and some *Bacillus* species in activated sludge under anoxic and anaerobic conditions.

In this research, it was found that when glucose was used as sole carbon source, the pH of cultural broth dropped rapidly and hence 0.5M NaOH solution had to be added for keeping the pH constant. Even through the phosphorus buffer solution has been employed for the pH maintenance, the pH varied from initial of 7.2 to the end point of 6.5. It was estimated that fermentation from pyruvate to organic acids has been taken placed in the PHA accumulation process from EAS under oxygen-limited (anoxic or anaerobic) condition. Lactic acid is likely the one produced during the process. Therefore, the fermentation of lactic acid from pyruvate and then to synthesis the PHV was involved in the hypothetic metabolic model.

The fate of this synthetic lactate was mainly for the PHA production, which based on the following observed facts from other researchers.

Satoh *et al.* (1992) detected that when lactate was fed as external substrate, PHA was also produced under the anaerobic condition, therefore, it is assumed that after being converted back to pyruvate, lactate was used to accumulate PHA by bio-P bacteria. When lactate was added into the anaerobic phase of EBPR process, it seem to have been taken up by microorganisms in the very short time of initial 5 minutes, and gradually converted to PHA in the following 2 to 3 hours (Satoh *et al.* 1996). In 1996, Liu *et al.* described that lactate was taken up concurrently with a consumption of cellular glycogen when it was added into an anaerobic-aerobic activated sludge in EBPR without biological phosphorus removal. The composition of PHA produced from this substrate consisted of mainly 3HV unit of 82.6 mol% followed by 10.3 mol% 3HB, mainly from the glycogen-accumulating sludge under anaerobic condition (Liu *et al.*, 1996). On the other hand, when glucose was used as sole carbon source, lactic acid was detected in the medium during the anaerobic reaction in EBPR process and resulted in the synthesis of PHA (Wang *et al.*, 2002). These supplied or synthetic lactate could either be reduced to propionyl-CoA, or converted to acetyl-CoA via pyruvate decarboxylation (Louie *et al.*, 2000) for the production of PHB, PHV and PHBV copolymer.

To take these previous works and my own observation into account, lactic acid is assumed to be a fermentation product from the added glucose or glycogen stored inside the bacterial cells in the PHA accumulation process by EAS under anoxic and anaerobic conditions. The lactic acid will be converted to propionyl-CoA under anoxic and

anaerobic conditions; or be converted to acetyl-CoA via pyruvate under aerobic and anoxic conditions for entering the TCA cycle or production of 3HB, the PHV precursor, for the PHA copolymer synthesis (Figure 4.24).

As mentioned previously, the lactic acid accumulation through the fermentation pathway of pyruvate happened only under anoxic or anaerobic conditions, where the pyruvate was the terminal electron acceptor. Obviously, in the presence of oxygen this reaction, lactic acid fermentation from pyruvate, can be stopped completely by the presence of excess oxygen and results in the interruption of lactate production and the 3HV synthesis from propionyl-CoA, and hence the PHV synthesis from lactate will also be diminished. Lactate formed previously during anaerobic phase will also be converted into pyruvate and then to be turned to actyle-CoA and subsequently enters TCA cycle or become the precursors of PHB, the 3HV mole fraction in PHA copolymer will be decrease and contrarily the 3HB mole fraction will be increased (Figure 4.24). From this point of view, it is estimated that the decreasing of 3HV mole fraction to certain extent in PHA copolymer from EAS is arisen from this inhibition of lactic acid fermentation caused by the increasing of DO concentration through ORP monitoring and regulation process in the cultural broth. This is the first assumed key pathway might be interrupted by the presence of oxygen, and consequently causes the variation and decreasing of 3HV mole fraction in PHA copolymer (indicated by red arrow in Figure 4.24).

The summarized chemical equations of the lactic acid fermentation from pyruvate

and PHV production from lactic acid were listed as follows.

- The formation of lactic acid from pyruvate is summarized at a reversible equation below according to Wang *et al.* (2000):

$$CH_3COCOOH \text{ (Pyruvate)} + NADH_2 \leftrightarrow$$

$$CH_3CHOHCOOH \text{ (Lactic acid)} + NAD^+.$$

- The formation of acetyl-CoA (a precursor of PHV) from pyruvate is summarized as below according to Gaudy (1978)

$$CH_3COCOOH \text{ (Pyruvate)} + CoASH + NAD^+ \rightarrow$$

$$CH_3COSCoA \text{ (Acetyl-CoA)} + CO_2 + NADH_2$$

- The formation of propionyl-CoA (another precursor of PHV) from lactate is summarized in the following equation (Louie *et al.*, 2000 and own research):

$$CH_3CHOHCOOH \text{ (Lactic acid)} + CoASH + NADH_2 \rightarrow$$

$$CH_3CH_2COSCoA \text{ (propionyl-CoA)} + 2H_2O + NAD^+$$

- The formation of PHV from acetyl-CoA and propionyl-CoA. Since acetyl-CoA and propionyl-CoA are the precursors for PHV formation, the formation of PHV

from these two precursors is summarized as below:

$$CH_3COSCoA\ (Acetyl\text{-}CoA) + CH_3CH_2COSCoA\ (Propionyl\text{-}CoA) +$$

$$(C_5H_8O_2)_n + NADH_2 \rightarrow (C_5H_8O_2)_{n+1}\ (PHV) + NAD^+ + 2CoASH$$

- The formation of PHV from lactate directly under anoxic and anaerobic conditions by combining the upper four equations was demonstrated as below:

$$2CH_3CHOHCOOH\ (Lactic\ acid) + (C_5H_8O_2)_n \rightarrow$$

$$(C_5H_8O_2)_{n+1}(PHV) + 2H_2O + CO_2\ .$$

It can be seen from this combined equation, two chemical reactions of dehydration and decarboxylation occurred for the PHV formation from lactate directly under anoxic and anaerobic conditions by EAS. Furthermore, coupling the two conversion reactions of lactated to propionyl-CoA and lactate to pyruvate, then to acetyl-CoA for the PHV synthesis has achieved the perfect balances of redox and energy source.

4.3.7. TCA cycle and glyoxylate pathway

Although energy is obtained from the breakdown of glucose to pyruvate by EMP or ED pathway, a significantly greater yield can be achieved in the presence of oxygen from the further oxidation of pyruvate to CO_2 via the TCA cycle (Figure 4.24 and Figure 4.29) also known as the citric acid cycle. Pyruvate does not enter this pathway

directly; it must first undergo conversion into acetyl-CoA:

$$\text{Pyruvate} + NAD^+ + CoA \rightarrow \text{Acetyl-CoA} + NADH_2 + H_2O$$

This reaction is catalyzed by pyruvate dehydrogenase, a large complex containing three enzymes. Acetyl-CoA can also be produced by the catabolism of lipids, amino acids and fatty acids as well as a wide range of carbohydrates. ATP can be formed from $NADH_2$ by oxidative phosphorylation (ETC). Theoretically, 1 mole of glucose oxidized aerobically via EMP pathway and TCA cycle and then to through the ETC pathway, 38 net ATPs will be produced. Otherwise, this pathway is also an important source of carbon skeletons for use in biosynthesis.

TCA cycle enzymes are widely distributed in most microbes and other microorganisms. Functional and complete cycles are found in most aerobic microbes, algae, fungi and protozoa. However, in facultative organisms (those that can grow in the presence or absence of oxygen) the complete TCA cycle will only be functional in the presence of oxygen. Although it is generally believed that the TCA cycle does not function anoxically, several studies have demonstrated that most of the TCA enzymes are active under anoxic conditions (Matsuo, 1985; Osborn *et al.*, 1986). Many anaerobic organisms have an incomplete cycle for the production of synthetic precursors, the PHA production for example (Comeau *et al.*, 1986; Wentzel *et al.*, 1986; Pereira *et al.*, 1996 and Louie *et al.*, 2000).

The hypothetic metabolic model in this paper (Figure 4.24) assumed audaciously that TCA cycle (full cycle or part of it) is involved in the PHA production by EAS under aerobic, anoxic or even anaerobic conditions. On the other hand, as indicated by the green arrow in the figure, the presence of oxygen plays a key role to maintain the fully circulating of TCA cycle in the step from succinate to fumarate. The absence of oxygen will cause the interruption of this step, which results in the accumulation of succinate. The accumulated succinate will be converted to succinyl-CoA and then propionyl-CoA instead of further reduction to α-ketoglutarate. Consequently, a large amount of propionyl-CoA will be converted into 3HV units, and then resulted in the significantly increasing of 3HV fractions in PHA copolymer.

This is the second hypothetic key reaction that might be affected by oxygen concentration and results in the variation of 3HV fraction in PHA formed from EAS.

4.3.7.1. TCA cycle and glyoxylate pathway are involved in the PHA accumulation by EAS under aerobic, anoxic and even anaerobic conditions.

First of all, it has to be confirmed that TCA cycle and glyoxylate pathway are fully or partly involved in the PHA accumulation by activated sludge even under anoxic and anaerobic conditions.

By this time, only a few researchers mentioned that incomplete TCA cycle involved in the PHA production in EBPR under anaerobic condition for the energy,

reducing power supporting and the production of PHA. The idea that the TCA cycle functions under anaerobic conditions to oxidize a part of the acetate to CO_2 and to generate reducing power in the form of $NADH_2$ was first proposed by Matsuo (1985) and later by Osborn *et al.* (1986), Comeau *et al.* (1986) and Wentzel *et al.* (1986).

The conversion of acetate, a favorable substrate for EBPR and other activated sludge, to PHA requires reducing power, because PHA is a more reduced compound than acetate. The acetyl-CoA molecules arisen from pyruvate decarboxylation that can either be directly used for PHB synthesis (Doi *et al.*, 1989) or transformed in the glyoxylate pathway to produce both malate and succinate. The malate is then oxidized to oxaloacetate to sustain the cycle of the glyoxylate pathway and form one mole $NADH_2$ necessary for PHB synthesis (Louie *et al.*, 2000). In addition, the pathway of the succinate is more complicated but the succinate must be metabolized because continued accumulation of succinate will inhibit the glyoxylate pathway. One of the important common ways to consume the excess succinate is to convert it into fumarate and then to malate in order to sustain the TCA cycle and glyoxylate pathway (Herman and Bell, 1970).

Pereira *et al.* (1996) incubated ^{13}C labeled acetate with a PAO-enriched sludge under anaerobic conditions (without nitrite or nitrate) and found that a small fraction of the labeled carbon in acetate was released as CO_2. Also based on a redox balance considerations, they concluded that the reducing power generated in the observed degradation of glycogen was insufficient to account for the PHA production. They

provided strong evidence that a small fraction of acetate is metabolized through the TCA cycle under anaerobic conditions supplying a minor part (30%) of the reducing power for PHA formation. This is the first and reliable experimental result indicating the possible functioning of the TCA cycle in the anaerobic phase of the EBPR process.

Liu *et al.* (1996) reported that when pyruvate, succinate, malate, fumarate and oxaloacetate the most key intermediates except citrate and α–ketoglutarate in the TCA cycle were added into the anaerobic-aerobic sludge in an EBPR process SBR under anaerobic condition respectively, it was observed that these intermediates were taken up concurrently with a consumption of cellular glycogen. Except pyruvate, the composition of PHA produced from these substrates all consisted of mainly 3HV unit followed by 3HB by the glycogen-accumulating sludge under anaerobic condition. 3HV mol percentage varied from 76.5mol% to 91.8 mol%. The 91.8 mol% of 3HV followed with 5.2 mol% of 3HB have been received when α–ketoglutarate was added. 83.8 mol%, 83.4mol% and 83.2mol% of 3HV fraction in PHA have been observed from fumarate, succinate and malate, respectively. Compared with the other intermediates in the TCA cycle, the maximum 3HB mol ratio of 19.4 mol% followed with 76.5mol% 3HV fraction has been achieved from oxaloacetate.

Louie *et al.* (2000) had conducted detailed investigation on the role of the TCA cycle and the glyoxylate pathway in anaerobic accumulation of PHA by EBPR activates sludge. It was observed that feeding of pyruvate combined with acetate resulted in accumulation of PHB and PHV, in approximately a 1:1 ratio. Citrate

addition did not result in PHA accumulation but both PHB and PHV were formed when citrate combined with acetate was fed to the EBRP sludge. Succinate or succinate combined with acetate stimulated 2-fold higher accumulation of PHV than PHB. Furthermore, treating the EBRP sludge by adding different nutrients combined with α-ketoglutarate and monofluoroacetate, the common inhibitors of steps in the TCA cycle significantly impaired PHA accumulation and implied that these steps can be important for PHA synthesis.

According to the methods described by Weitzman and Dunmore (1969), Danson and Weitzman (1973), excess α-ketoglutarate (50mg/l) was used to block the conversion of citrate to isocitrate to test the necessity of this step in TCA cycle for the PHA synthesis. When acetate was fed together with α-ketoglutarate to anaerobic EBPR activated sludge the net accumulation of PHB and PHV decreased over 90% compared to feeding with pure acetate. Similar results were observed when monofluoroacetate (50 μM) was added. Since monofluoroacetate also blocks the conversion of citrate to isocitrate (Morrison and Peters, 1954; Quastel, 1963). Louie *et al.* (2000) concluded that the combined results imply that this part of the TCA cycle is required to synthesize PHA from acetate and other nutrients (Louie *et al.*, 2000). The locations of the steps that the inhibitors blocked the reaction have been indicated by the black bar in Figure 4.24 and Figure 4.29.

The preferential PHV synthesis in these results indicates that the biochemistry of PHA synthesis is more complicated than simply reducing and combining two 3HB

precursors to reoxidize the $NADH_2$ (or $FADH_2$). Instead, it appears to involve pathways that favor the endogenous production of the propionyl-CoA or hydroxyvaleryl-CoA precursors that are necessary to produce PHV. Therefore, the two major known pathways of propionyl-CoA synthesis including reduction of pyruvate produced in the glycolytic pathway or decarboxylation of succinate formed by the TCA cycle and the glyoxylate pathway were involved in the hypothetic metabolic model for PHA accumulation by EAS under aerobic, anoxic and even anaerobic conditions where glucose was used as sole carbon source.

4.3.7.2. Oxygen plays a very important role in the regulation of 3HV mole fraction in PHA copolymer via TCA cycle.

As mentioned in previous descriptions, the 3HV mole fraction in the PHA copolymer formed by the EAS was decreased from 78.40 mol% to 11.07 mol% when the ORP was regulated from the range of –20 mV to +10 mV, where the glucose was used as sole carbon source. In fact, the variation of ORP level was resulted from the changes of gas flow pumped into the jar bioreactor, and subsequently caused the variation of DO concentration in the cultural broth.

On the other hand, during the research periods, it was found that when the ORP was not adjusted back by air flow during the fermentation (the nature ORP) process, the ORP and the DO declined immediately after the beginning of cultivation of EAS with glucose as sole carbon source. The PHA content to the EAS dry weight

(percentage of the dry cell weight) gained with the fermentation time. However, only the weight of 3HV unit in the PHA copolymer was increased with the time course, at the meantime, 3HB unit was kept at approximately constant level (data not shown). A similar result has been demonstrated in a figure by Wang *et al.* (2000), but it was a pity to that there was no any detail analysis or explanation on the results obtained by the authors. It was thought that the reason for 3HV unit accumulation alone in the PHA copolymer was the consumption of oxygen immediately at the beginning of the fermentation process, and consequently resulted in the fermentation condition from aerobic to anoxic and finally to anaerobic conditions, unfavorable conditions for the cell growth and PHB production but it was favored for PHV accumulation.

All these observed evidences from the literature reports and own experiments strongly implied that there is some relationship between the 3HV formation in PHA copolymer from EAS and the DO concentration of the cultural medium. Accordingly, it was postulated in the hypothetic metabolic model that oxygen might play a very important role to regulate the 3HV production via TCA cycle, glyoxylate pathway and others.

Usually, the TCA cycle is linked with respiration and operates only under aerobic or anoxic conditions. The oxidation of succinate to fumarate in the TCA cycle requires a terminal electron acceptor with a redox potential ($E^{0\prime}$) more positive than that of fumarate/succinate couple (+32 mV). Only O_2 (O_2/H_2O, $E^{0\prime}$= + 818 mV), NO_3^-

(NO_3^-/NO_2^-, $E^{0'}$ = + 433 mV) and NO_2^- (NO_2^-/N^{2-}, $E^{0'}$= + 970 mV) appear to meet these conditions (Thauer, 1988). In this research, only O_2 was supplied for the ORP regulation. The presence of oxygen results in the step of oxidation succinate to fumarate in TCA cycle (green arrow in Figure 4.24 and Figure 4.29) working well. But the absence of oxygen under anoxic and anaerobic conditions will induce the failure of this step, and then results in the accumulation of succinate. Otherwise, as mentioned previously, part of the acetyl-CoA molecules arisen from pyruvate decarboxylation can be transformed into the glyoxylate pathway to produce both malate and succinate. The malate is then oxidized to oxaloacetate to sustain the cycle of glyoxylate pathway and form one mole $NADH_2$ necessary for PHB synthesis (Louie *et al.*, 2000). In addition, the pathway of the succinate is more complicated but the succinate must be metabolized because continued accumulation of succinate will inhibit the glyoxylate pathway. One of the important common ways to consume the excess succinate is to convert it into fumarate and then to malate in order to sustain the TCA cycle and glyoxylate pathway (Herman and Bell, 1970). This conversion step from succinate to fumarate is an oxygen-depending reaction. The absence of oxygen often results in the accumulation of succinate. In order to sustain the glyoxylate pathway, the accumulated succinate has to be converted to propionyl-CoA for 3HV synthesis, another common way to consume the excess succinate generated both from TCA cycle and glyoxylate pathway under anoxic and anaerobic conditions by the bacteria in activated sludge.

As described before, the accumulated succinate will be enforced to convert to

succinyl-CoA and then propionyl-CoA through decarboxylation reaction instead of further reduction to α-ketoglutarate. This is because the step of conversion α-ketoglutarate to succinyl-CoA is an irreversible step in TCA cycle, as illustrated in Figure 4.24 and Figure 4.29. Consequently, a large amount of propionyl-CoA will be forced to convert into 3HV units, and then resulted in the significantly increasing of 3HV fractions in PHA copolymer.

To test if the oxidation of succinate to fumarate were a necessary part of the process resulting in 3HV significant accumulation in PHA copolymer, malonate, the metabolic inhibitor, was used to suppress the step in the TCA cycle as an alternative to the absence of oxygen according to the methods reported by Quastel (1963) and Louie *et al.* (2000). In addition, this experiment performed to directly examine the potential role of the oxygen in TCA cycle and glyoxylate pathway for the synthesis of PHA copolymer with 3HV unit majority. For this experiment, the ORP was maintained at +10mV (with oxygen supply) and glucose as the sole carbon source.

As expected, after 24 hrs cultivation, with the addition of malonate and glucose into the cultural medium (treated group), the EAS from local wastewater treatment plant was observed strongly PHV synthesis. The 3HV mole fraction in the PHA copolymer was 83.01 mol%, higher than that of keeping ORP at –20 mV (anaerobic condition). Otherwise, the PHA content was 4.32% of cell dry weight, lower than the control group without the addition of malonate. Meanwhile, in the control group,

without the addition of malonate, the PHA content in EAS was 8.9% CDW with 3HV mole fraction of 11.07 mol% (Figure 4.28). Theoretically, interruption of the oxidation from succinate to fumarate should completely inhibit the PHA synthesis. Succinate is normally oxidized to fumarate, generating 1 equivalent of $FADH_2$. However, in this case, these didn't happen.

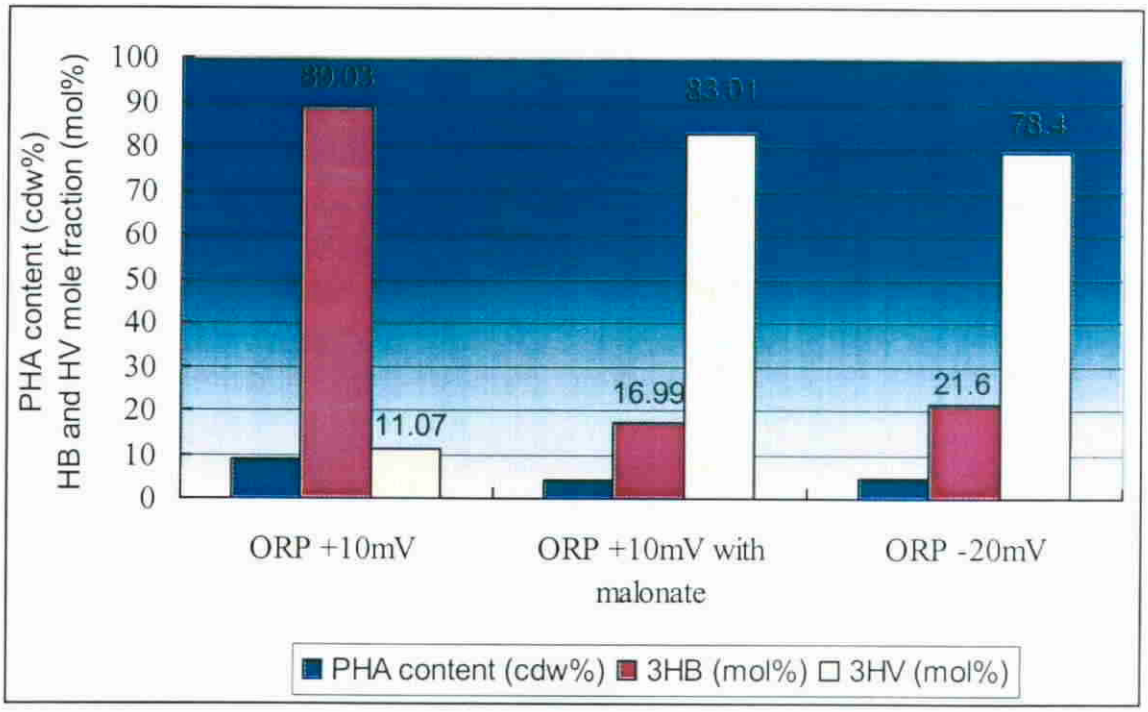


Figure 4.28. The comparison trial for 3HV and 3HB mole fraction in PHA copolymer with or without the addition of malonate (50mg/l). Malonate is an inhibitor to the step of succinate oxidation to fumarate in TCA cycle.

Louie *et al.* (2000) has presented a similar report. They observed that when succinate and malonate were added simultaneously to the anaerobic EBRP activated sludge, the observed PHV/PHB ratio was 2.1, which indicated that succinate oxidation to fumarate was a significant step in the PHA synthetic pathway. When malonate was fed together with citrate and acetate the net accumulation of PHA was also diminished.

All the observed results suggested that even though the oxidation of succinate to fumarate was important for both sets of nutrients the specific blockage of succinate oxidation to fumarate by malonate (black bar in Figure 4.24 and Figure 4.29) may enforce the decarboxylation of succinate to propionate for the 3HV production, and cause the enhanced 3HV-enriched PHA synthesis. In addition, if the blockage were caused by the absence of oxygen, the succinate generated both from the TCA cycle and glyoxylate pathway would be also forced for the 3HV-enriched PHA copolymer accumulation in EAS. Furthermore, there are two options for the decarboxylation of succinate to propionyl-CoA for PHV production. One is that the citrate by synthesizing oxaloacetate with acetyl-CoA is decarboxylated and oxidized to α-ketoglutarate, and then to succinyl-CoA. Finally, the succinyl-CoA is converted by decarboxylation to propionyl-CoA for 3HV synthesis. The other option is that the pyruvate is converted to oxaloacetate, and then oxaloacetate is oxidized to malate, to fumarate, succinate, succinyl-CoA, and finally to propionyl-CoA. In fact, the second option is actually the pyruate-propionyl-CoA pathway mentioned again and again in this paper. The

common characteristics of the two options are that they perform only under anoxic or anaerobic conditions and result in the conversion of succinate to propionyl-CoA, the 3HV precursors, and enhance the PHV production. Comparison the two assumed pathways in TCA cycle, there are big difference existed between them. First, there are two oxidation and decarboxylation reactions occurred in the citrate-propionyl-CoA pathway, and generates 2 $NADH_2$ and releases 3 CO_2. However, the pyruate-propionyl-CoA pathway consumed 2 $NADH_2$ and one ATP, and only 1 CO_2 released (Figure 4.24 and 4.32).

Therefore, it can be concluded that oxygen plays a very important role for the synthesis of PHV in TCA cycle and glyoxylate pathway. Under aerobic condition, the succinate generated from TCA cycle and glyoxylate pathway is oxidized to fumarate and contributed to sustain the full cycling of the TCA cycle and glyoxylate pathway. In the absence of oxygen, the oxidation of succinate to fumarate is blocked without the higher redox potential (O_2/H_2O), the succinate generated from incomplete TCA cycle or glyoxylate pathway is forced to convert to propionyl-CoA as the precursors of PHV, and enhance the production of 3HV-enriched PHA copolymer.

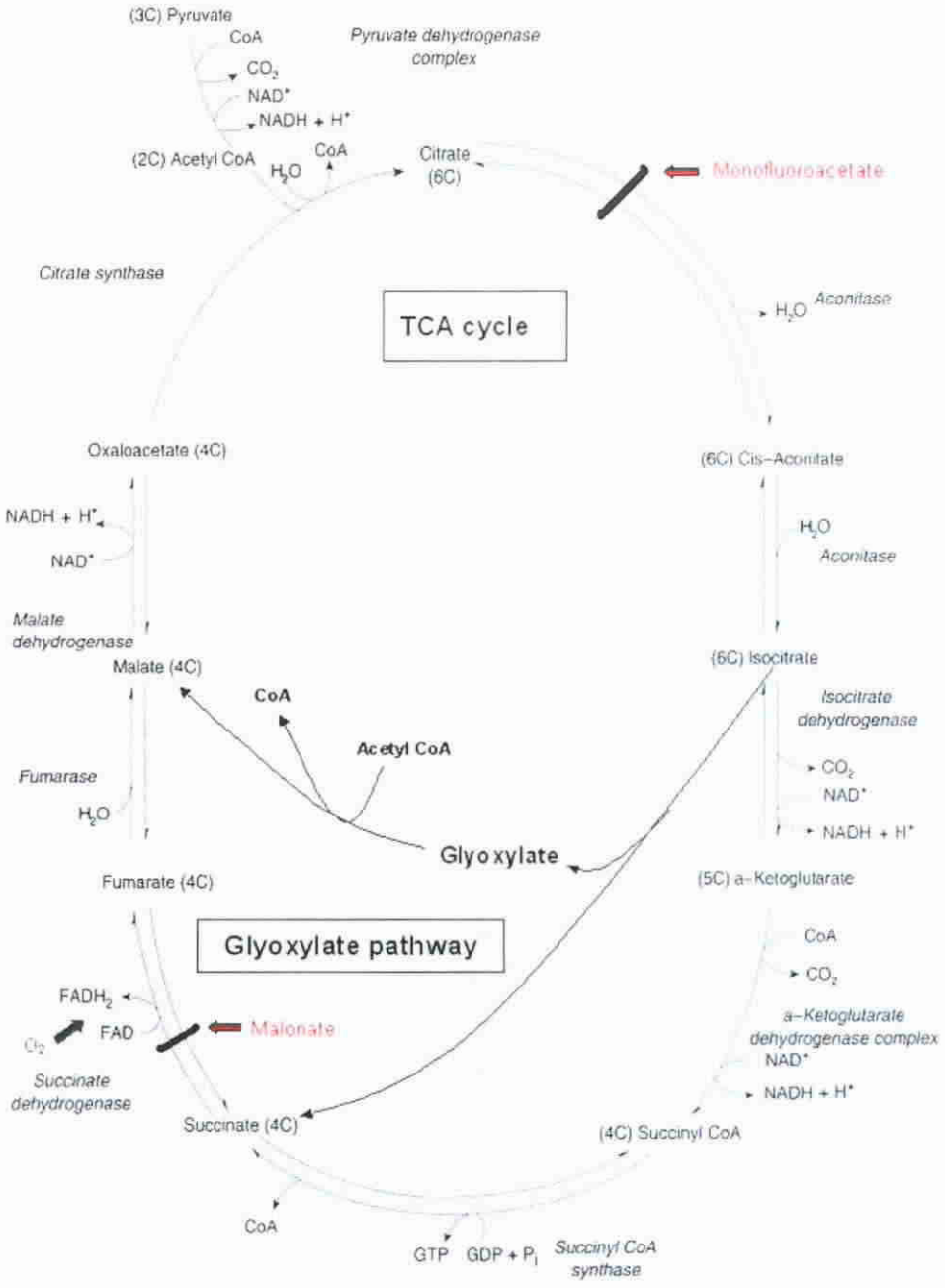


Figure 4.29 Tricarboxylic Acid Cycle (TCA cycle) and glyoxylate pathway. The black bars indicate that the steps were interrupted by the inhibitors in the TCA cycle by malonate and monofluoroacetate respectively. The green arrow with the O_2 means that the steps will be realized at the presence of oxygen.

4.3.7.3. PHV synthesis via the TCA cycle and glyoxylate pathway.

By integrating the analysis of the observed evidences with the hypothetic metabolic models, PHV synthesis through TCA cycle and glyoxylate pathway could be summarized as followed equations.

4.3.7.3.1. Glyoxylate pathway for the PHV synthesis

- Synthesis of isocitrate from the combination of acetyl-CoA with oxaloacetate.

$$CH_3COSCoA(\text{Acetyl-CoA}) + COOHCOCH_2COOH(\text{oxaloacetate}) + H_2O \rightarrow$$

$$COOHCH_2CHCOOHCHOHCOOH\ (\text{isocitrate}) + CoASH$$

- Isocitrate breakdown to succinate and glyoxylate, and then synthesized the malate for the further cycling.

$$COOHCH_2CHCOOHCHOHCOOH\ (\text{isocitrate}) \rightarrow$$

$$COOHCH_2CH_2COOH(\text{Succinate}) + CHOCOOH(\text{Glyoxylate})$$

$$CHOCOOH(\text{Glyoxylate}) + CH_3COSCoA(\text{Acetyl-CoA}) + H_2O \rightarrow$$

$$COOHCH_2CH(OH)COOH(\text{Malate}) + CoASH$$

- Succinate generated from glyoxylate pathway being converted into succinyl-CoA, and then to propionyl-CoA by the decarboxylation.

$$COOHCH_2CH_2COOH(Succinate) + CoASH + ATP \rightarrow$$

$$COOHCH_2CH_2COSCoA(Succinyl\text{-}CoA) + ADP + H_2O$$

$$COOHCH_2CH_2COSCoA(Succinyl\text{-}CoA) \rightarrow$$

$$CH_3CH_2COSCoA\ (propionyl\text{-}CoA) + CO_2$$

- PHV synthesis by combining the propionyl-CoA from glyoxylate pathway with acetyl-CoA from the oxidation and decarboxylation of pyruvate.

$$CH_3COSCoA\ (Acetyl\text{-}CoA) + CH_3CH_2COSCoA\ (Propionyl\text{-}CoA) +$$

$$(C_5H_8O_2)_n + NADH_2 \rightarrow (C_5H_8O_2)_{n+1}\ (PHV) + NAD^+ + 2CoASH$$

4.3.7.3.2. PHA synthesis from TCA cycle.

As mentioned above, there are two options for the PHV production from TCA cycle. The one is from the acetyl-CoA to citrate by combining one molecular of oxaloacetate, to isocitrate, to α-ketogluarate, succinyl-CoA and then to propionyl-CoA by decarboxylation (the so called acetyl-CoA-propionyl-CoA pathway); the other is pyruvate-propionyl-CoA pathway.

Acetyl-CoA -Propionyl-CoA Pathway

- Synthesis of isocitrate from the combination of acetyl-CoA with oxaloacetate.

$$CH_3COSCoA(Acetyl\text{-}CoA) + COOHCOCH_2COOH(oxaloacetate) + H_2O \rightarrow$$

$$COOHCH_2CHCOOHCHOHCOOH\ (isocitrate) + CoASH$$

- Oxidation and decarboxylation of isocitrate to α-ketogluarate and 1 $NADH_2$ produced.

$$COOHCH_2CHCOOHCHOHCOOH\ (isocitrate) + NAD^+ \rightarrow$$

$$COOHCH_2CH_2COCOOH(\alpha\text{-}ketogluarate) + NADH_2 + CO_2$$

- Oxidation and decarboxylation of α-ketogluarate to Succinyl-CoA.

$$COOHCH_2CH_2COCOOH(\alpha\text{-}ketogluarate) + NAD^+ \rightarrow$$

$$COOHCH_2CH_2COSCoA(Succinyl\text{-}CoA) + NADH_2 + CO_2$$

- Decarboxylation of succinyl-CoA to propionyl-CoA under anoxic and anaerobic conditions (the step from succinate to fumarate was blocked in the absence of oxygen).

$$COOHCH_2CH_2COSCoA(Succinyl\text{-}CoA) \rightarrow$$

$$CH_3CH_2COSCoA\ (propionyl\text{-}CoA) + CO_2$$

- PHV synthesis by synthesizing the propionyl-CoA from TCA cycle under

anoxic and anaerobic conditions with acetyl-CoA from the oxidation and decarboxylation of pyruvate.

$$CH_3COSCoA\ (Acetyl\text{-}CoA) + CH_3CH_2COSCoA\ (Propionyl\text{-}CoA) + (C_5H_8O_2)_n + NADH_2 \rightarrow (C_5H_8O_2)_{n+1}\ (PHV) + NAD^+ + 2CoASH$$

Pyruvate-propionyl-CoA Pathway

This pathway has been demonstrated at the prior part of this paper.

4.3.8. Comparison of Pyruvate-propionyl-CoA pathway with succinate-propionyl-CoA in TCA cycle for the synthesis of PHA with 3HV major unit under anoxic and anaerobic conditions

The presence of a glycogen accumulating population and its abilities of substrate uptake and storage in anaerobic-aerobic activated sludge of EBPR process was investigated by Liu *et al.* (1996). When pyruvate, succinate, malate, fumarate and oxaloacetate the most key intermediates except citrate and α–ketoglutarate in the TCA cycle were added into the sludge at anaerobic phase respectively, it was observed that these intermediates were taken up concurrently with a consumption of cellular glycogen. Except for pyruvate, the composition of PHA produced from these substrates all consisted of mainly 3HV unit followed by 3HB. 3HV mole fraction varied from 76.5mol% to 91.8 mol%. The 91.8 mol% of 3HV followed with 5.2 mol%

of 3HB was received when α–ketoglutarate was added. 83.8 mol%, 83.4mol% and 83.2mol% of 3HV fractions in PHA have been obtained by the addition of fumarate, succinate and malate respectively. Compared with the other intermediates in the TCA cycle, the maximum 3HB mol ratio of 19.4 mol% followed with 76.5mol% 3HV fraction has been achieved from oxaloacetate. It is very interesting that the more close to the Succinyl-CoA of the intermediates in the TCA cycle added for PHA accumulation, the more 3HV mole fraction in the PHA were synthesized (Figure 2.30). On the other hand, when glucose and lactate were added into the activated sludge independently, the PHAs with major 3HV unit mole fractions of 87.0 mol% and 82.6 mol% obtained respectively.

The conventional models, the succinate-propionate pathway (or the pyruvate-propionyl-CoA pathway, modified by Zeng *et al.*, 2000) for example, cannot account for the effect of the intermediates and inhibitors observed by the literature reports and own experimental results. Because these conventional pathways were designed for the PHA accumulation by activated sludge only under anoxic and anaerobic conditions, and hence the involvement of TCA cycle for this process was of cause not considered. The Pyruvate-propionyl-CoA pathway is the only wide spread accepted pathway for the PHA copolymer with 3HV unit accumulation by activated sludge during the anoxic and anaerobic phase. To add the intermediates of TCA cycle and glyoxylate pathway into the anaerobic phase of anaerobic-aerobic activated sludge resulted in the PHV-enriched PHA accumulation (Liu *et al.*, 1996; Louie *et al.*, 2000).

In addition, the regulation of ORP level by controlling the DO concentration caused the variation of 3HV mole fraction in PHA from EAS by using glucose as sole carbon source described in this paper. These results are serious evidences that can't find the answers and explanations from the pyruvate-propionyl-CoA pathway or the former one, succinate-propionate pathway. The results also suggested that these substrates were firstly converted into intermediates or precursors of the acetyl-CoA or propionyl-CoA producing pathway, and then synthesized into different PHA monomers. Accordingly, production of PHA with 3HV as the major unit from glucose and lactate also indicated the participation of an unknown pathway from pyruvate to propionyl-CoA for the redox balance under anaerobic conditions. Therefore, the new hypothetic metabolic model has been established.

In the hypothetic metabolic model, the pyruvate-propionyl-CoA pathway has also been involved. But in fact, in my opinion, either pyruvate-propionyl-CoA and succinate-propionate pathway is just a reversal steps of TCA cycle from pyruvate to oxaloacetate, malate, fumarate to succinate, and then to propionyl-CoA for 3HV synthesis under oxygen-limited or anaerobic conditions. If the fact could be accepted that the TCA cycle were involved in the PHA production from activated sludge even under anoxic and anaerobic conditions, the pyruvate-propionyl-CoA pathway could be recognized as a complement pathway in TCA cycle and performed only under the absence of oxygen. Therefore, the hypothetic metabolic model demonstrated in Figure 4.24 could be revised and simplified as illustrated in Figure 4.31.

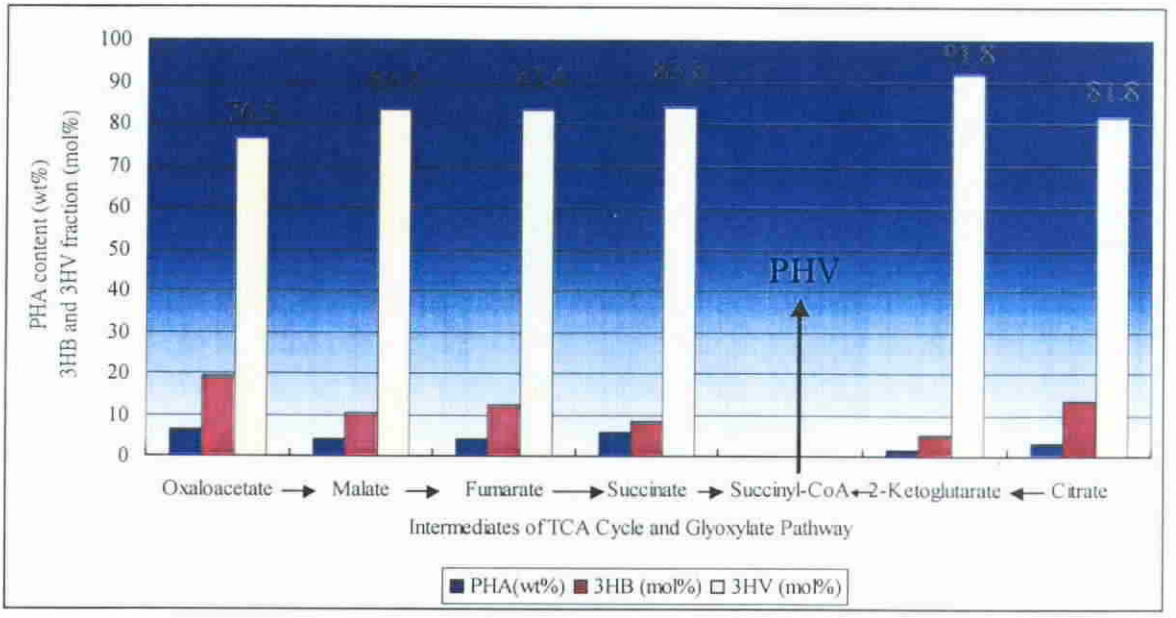


Figure 4.30. The PHA with 3HV major unit accumulation when the intermediates associated with the TCA cycle was added into the anaerobic-aerobic activated sludge under anaerobic condition (the Original data was adapted from Liu *et al.* (1996)).

4.3.9. Electron-transport chain (ETC)

$NADH_2$ and $FADH_2$ formed by the catabolism of organic molecules are used to produce ATP by the action of an electron-transport chain (ETC) that is composed of a series of electron carriers which transfer electrons to a terminal electron acceptor such as oxygen. This final reduction is performed by a terminal oxidase. Oxygen is not the only useful electron acceptor. In bacteria, electron transport occurs in the inner cell membrane, but in algae, fungi and many protozoa, electron transport and oxidative phosphorylatio occur in the inner membrane of the mitochondria. Most bacterial ETC are different to that found in mammalian mitochondria (Figure 4.24) and many like *E. coli* are branched. Some chains are short and this reduces their capacity for ATP production. Electrons can enter bacterial ETC at various points and this increases the number of substrates that can be used for ATP synthesis.

All electron-transport pathways function in a similar manner, i.e. requiring a series of oxidation and reduction reactions. The oxidation of a molecule involves the loss of electrons, and reduction involves the addition of electrons. Since electrons are conserved in chemical reactions, oxidation must be coupled with reduction (redox reactions). The oxidation-reduction potential of a compound is a measure of its affinity for electrons. Redox potentials are measured relative to hydrogen; thus, a positive redox potential indicates that the compound has a greater affinity for electrons than has hydrogen and would accept electrons from hydrogen. A negative redox potential indicates a lower affinity and thus the molecule would donate electrons to hydrogen.

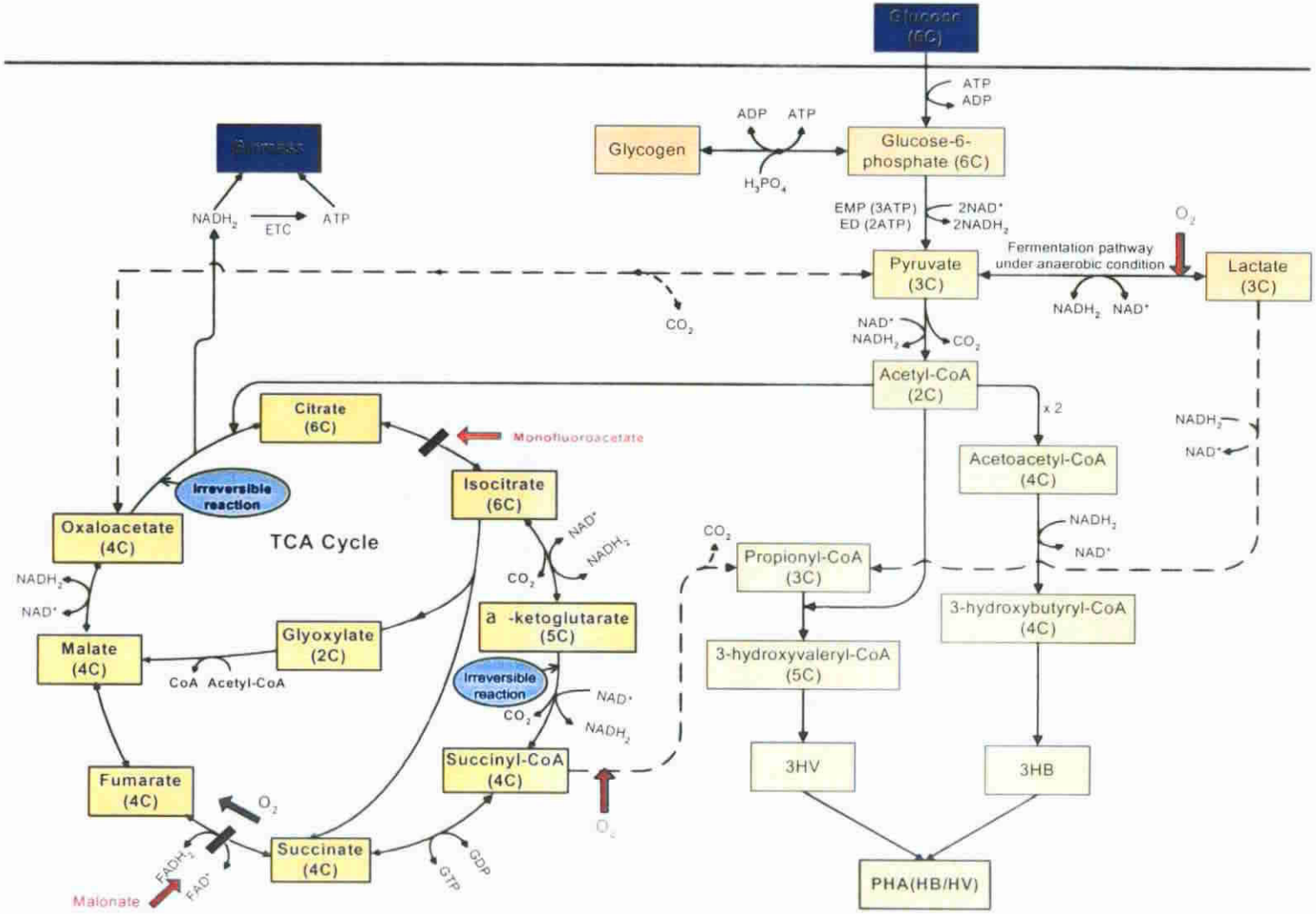


Figure 4.31. A revised and simplified hypothetic metabolic model for PHA production from EAS under aerobic, anoxic and anaerobic conditions.

The difference in redox potential between $NADH_2/NAD^+$ (-320mV) and O_2/H_2O (+820mV) drives the movement of electrons through a series of electron carriers which are arranged to accept electrons from a carrier with a more negative redox potential and donate to the next carrier which has a more positive redox potential. Energy is released as the electrons move between carriers. If the energy release is large this can be coupled with the movement of H^+ across the membrane, which can generate ATP. The number of sites where this can occur depends on the difference between the redox potential of the substrate and the terminal electron acceptor.

4.3.10. $NADH_2$ and ATP balance in the hypothetic metabolic models

The analysis, explanation and description of the hypothetic metabolic models have been demonstrated as above. The fate of substrates, the chemical reactions and the PHA production with the energy and redox balance have also been illustrated in the models and equations. At this part, the major subject is to further discuss the $NADH_2$ and ATP balance in the hypothetic metabolic models. Based on the analysis above, the metabolic models are composed of five and actually four major parts of reactions. What ever, two important metabolic principles have to be considered here to evaluate the hypothesized bio-chemical models for glucose metabolism. The first principle is the redox balance and second one is that microorganisms seldom engage metabolic activities without purpose (Wang *et al.*, 2000; Liu *et al.*, 1996).

The first principle is that bacteria need to maintain redox balances during the various metabolic reactions. It is known that an organic compound is oxidized biochemically by losing electrons in the form of hydrogen atoms. Consequently, oxidation is synonymous with dehydrogenation (Bailey and Ollis, 1986). Figure 4.24 and 4.32 also show the production and consumption of electrons during the aerobic and anaerobic metabolism of glucose.

As shown in Figure 4.24, the production of pyruvate through the EMP or the ED pathway generates 4 mole of electrons per mole of glucose-6-phosphate consumed. The production of PHB from pyruvate will also generate two mole of electrons per mole of 3-HB formed. The generated electrons must be removed in order to maintain the redox balance of the bio-P bacteria. However, under the anaerobic condition, there are no external ultimate electron acceptors (i.e., O_2). Therefore, the extra electrons must be consumed by an internal mechanism. Figure 4.24 indicates that the formation of PHV can become a significant sink of reducing power by the pyruvate-propionyl-CoA pathway under anaerobic condition. Four moles of electrons will be consumed for the formation of 1 mole of 3HV from pyruvate. It can be seen from Figure 4.24 that, in order to balance the electrons produced from the EMP pathway, all of the generated pyruvate should be utilized for the synthesis of PHV. In our batch experiments, it was detected that 3HV was the predominant unit of accumulated PHA by EAS. Over 80 mol% units of the total accumulated PHA under the anaerobic condition were 3HV where ORP was kept at -20mV. The experiments results were consistent with the first

principal of redox balance analyzed above.

Under anoxic condition, the $NADH_2$ generated from the glucose glycolysis through EMP or ED pathway, uncompleted TCA cycle and from the formation of PHB could be consumed in third ways. The first way is that $NADH_2$ is sunk for PHV accumulation; the second one, the reducing power would be further oxidized by O_2 as the terminal electro acceptor via ETC to generate ATPs; and the third one, the extra reducing power would be used for the biomass growth. Consequently, the pathway to form PHV from pyruvate would be partially inhibited, which caused the decreasing of the amount of 3HV unit. Therefore, the PHB/PHV ratios in the PHA accumulated by EAS declined with the supply of the amount of oxygen when the ORP was maintained at higher levels, 0 mV and +10 mV in this research, for instance.

From this point of view of redox balance, it is found that by comparing "Citrate-propionyl-CoA pathway", "pyruvate-propionyl-CoA pathway" and Glyoxylate pathway, under anoxic and anaerobic conditions, the pyruvate-propionyl-CoA pathway and glyoxylate pathway will consume the extra reducing power generated from glucose glycosis and PHB formation for PHV synthesis; contrarily, the citrated-propionyl-CoA pathway will not only sink the extra reducing power but also generate 2 mole of $NADH_2$ for each mole 3HV unit synthesis. Therefore, pyruvate-propionyl-CoA pathway and glyoxylate pathway are the effective pathways for the consuming of extra reducing power for the PHV production under anoxic and anaerobic conditions. Otherwise, if oxygen were supplied, the citrate-propionyl-CoA pathway will work well and the extra

reducing power will be sunk for ATP generation and biomass production.

The second principle for metabolic reactions is that microorganisms seldom engage metabolic activities without purpose. Figure also shows the ATP balance during the anaerobic condition. The transport of glucose into the bacterial cells includes the phosphorylation of glucose which requires 1 mole of ATP to transport 1 mole of glucose. The phosphorylated glucose has two alternative pathways: (1) entering the glycolysis pathway by which 3 mole of ATP (through the EMP pathway) or 2 mole of ATP (through the ED pathway) are generated per mole of glucose-6-phosphate consumed; or (2) synthesizing glycogen where 1 mole of ATP is consumed per mole of unit glycogen synthesized. There is no ATP requirement for PHB formation from pyruvate. However, 1 mole of ATP is required for the formation of 1 unit of PHV from pyruvate. It is observed that the cell could maintain the ATP requirements if glucose were metabolized through the EMP pathway alone, since the 3 mole of ATP generated from the glycolysis of 1 mole of glucose-6-phosphate could cover the energy requirements for all other anaerobic metabolisms. According to the second principle, the microorganisms would not seek energy from another source if they could derive enough energy from metabolizing glucose alone. When limited oxygen was supplied, part of reducing power would be further oxidized through ETC for ATPs generation. These ATPs would be used for further substrate transporting and biomass growth.

CHAPTER 5

CONCLUSION

5.1. PHA synthesis from excess activated sludge by using butyric acid, valeric acid, acetate and propionate as sole or complex carbon sources.

5.1.1. Organic consumption and cell growth

It was observed that no matter what types of EAS (collected from laboratory-scale SBR or full-scale MWWTP) was submitted to PHA accumulation, when short chain length fatty acids such as acetate and propionate are used as sole carbon source, the total TOC removal is higher than that of longer chain length fatty acids. The total TOC removal efficiency decreases with the increasing of the carbon skeleton length of the fatty acids as carbon source for PHA production.

It is concluded that just like the same of single strain pure culture, when EAS is conducted for the PHA formation, the substrate removal efficiency decreases with the increasing of carbon chain length of the fatty acids consumed. More quantity of oxygen supplied for EAS fermentation results in the improvement of TOC consumption. Under the nitrogen free condition, it is thought cell growth is stopped and biomass in the cultural medium is maintained constant.

5.1.2. PHA accumulation at different butyric/valeric acids and acetate/propionate ratios

When butyric acid and valeric acid were used as sole or complex carbon substrates without nitrogen source under aerobic condition, the fatty acids could be transported into bacterial cells in the EAS, and then most of the fatty acids were converted directly into 3HB and 3HV units for PHBV copolymer production without decomposition of carbon skeleton. Moreover, even carbon numbered fatty acids such as acetate and butyrate were used as sole carbon source respectively, PHB homopolymer or PHA with dominant 3HB unit were synthesized. When odd carbon numbered fatty acids such as propionate and valerate were used as sole carbon source, PHBV copolymer with 3HV major units were accumulated instead of the PHB homopolymer. Increasing the concentration of longer chain length fatty acids including propionate and valerate, or the C-odd fatty acids, may resulted in the decrease of PHA content in EAS. These results coincide with that of pure culture of *R. eutropha*.

5.1.3. 3HV molar fraction in PHA copolymer and carbon source composition in medium.

When butyric acid and valeric acid were used as complex carbon sources in the butyric/valeric acids ratios of 100/0, 80/20, 60/40, 40/60, 20/80 and 0/100, the 3HV molar fraction were 0, 12 mol%, 30 mol%, 35 mol%, 51 mol% and 54 mol%, respectively. Therefore, the 3HB/3HV ratios in the PHBV copolymer formed by the EAS could be adjusted by altering the butyric/valeric acids composition in the cultural media. Otherwise, 3HV molar fraction in the copolymer presented a close linear relationship with the valeric acid mole concentrations in the media.

The minima 3HV mole fraction of 8.0% has been obtained while acetate was as sole carbon source. The maximal 3HV molar fraction of 78.0 mol% achieved when propionate was used as sole carbon source. The molar fraction of 3HV in the co-polymer increased proportionately with the propionate concentration in medium. When propionate concentrations were 0, 1.09, 2.40, 3.42 and 4.00g/l, 3HV molar fractions of the PHA copolymer accumulated in the EAS were 8.0 mol%, 22.0 mol%, 55.0 mol%, 70.0 mol% and 78.0%, respectively. These results agreed with the early observation while butyric and valeric acids were used as carbon sources. It was demonstrated that propionate could serve as the precursors for 3HV units in the PHBV co-polymer as well as the pure culture described by Choi *et al.* (2003). The PHBV co-polymer accumulated in EAS with wide range of 3HV molar fractions (8.0 mol% to 78.0 mol%) could be produced and

regulated by controlling the propionate concentration, as a co-substrate, in the medium.

It is concluded that 3HV molar fraction in the PHBV copolymer increases with the increasing of the concentration of C-odd fatty acids as co-substrate for PHA synthesis from EAS. The PHBV copolymer accumulated in EAS with wide range of 3HV molar fraction can be obtained and adjusted by controlling the C-odd fatty acids concentration as the co-substrate in the medium.

5.1.4. Melting temperature of PHA

The melting temperatures of the PHBV copolymer formed by EAS by using fatty acids as sole or complex carbon sources were decreasing with the increasing of 3HV molar fraction in the copolymer. 3HV molar fraction in PHA produced at different butyric/valeric acid ratios were 0 mol%, 12 mol%, 30 mol%, 35 mol%, 51 mol% and 54 mol%, which resulted in the melting temperature of 178°C, 144°C, 133°C, 127°C, 109°C and 99°C, respectively. A similar result was observed by using acetate and propionate as sole and complex carbon sources.

Simultaneously, it was found that the melting temperature presented a close linear relationship with 3HV mole fraction in PHA. These results were agreed well with the observations of pure culture of *R. entropha*. Thus, the melting

temperatures of the copolymer could be regulated via adjusting the fatty acids compositions as carbon sources in the media.

5.1.5. PHA production under various ORPs while acetate and propionate as carbon sources.

By using acetate and propionate as sole or complex carbon sources, the PHA contents in the EAS increased with the increasing of ORP values.

When acetate was used as sole carbon substrate, the minimum PHA content at ORP -30mV was about 12% (w/w) of CDW. The maximum PHA accumulation of 35% (w/w) was achieved when ORP was maintained at +30mV. PHA content in the EAS declined to 28% of EAS dry weight when ORP was kept at +100mV. The PHA production yield, $Y_{p/s}$ (g PHA/g TOC consumed), was 0.48.

When propionate was used as sole carbon substrate and ORP was kept at +30mV, the PHBV copolymer content, polymer production yield and 3HV molar fraction were 25.8%, 0.38 (g/g) and 78.0 mole%, respectively. On the other hand, when ORPs were preset at -30mV, 0mV, +30mV and +100mV, the PHA contents in the EAS were 8.0%, 18.9%, 25.8% and 22.5% CDW, respectively. The PHA content in EAS was increased with the increasing of ORPs. Finally, when the ORP was +100mV, the PHA content was declined compared to that of ORP

+30mV. As suggested in the previous report while acetate as sole carbon source, the presence of excess oxygen during the period of +100mV ORP (i.e. pure oxygen was supplied), resulted in the balanced growth conditions, and acetyl-CoA was submitted to the TCA cycle for energy generation and intermediates synthesis. In consequence, the concentration of free CoASH was become higher. The key enzyme for PHA synthesis, 3-ketothiolase, was inhibited by high concentration of free CoASH, and resulted in the repression of PHA synthesis

5.1.6. 3HV molar fraction of the copolymer and ORPs while acetate and propionate as carbon sources.

It has been observed that the decreasing of ORP levels from +100mv to -30 mV resulted in the variation of 3HV mole fraction in the PHBV copolymer formed by the EAS from the range of 0 mole % to 21 mol% while acetate as sole carbon source. In other words, the variation of 3HV molar fraction in the PHBV copolymer was caused by the changes of DO concentration in cultural broth. Generally, it is thought that there is only PHB homopolymer produced in the pure culture when acetate as sole carbon substrate by the strains of *R. eutropha* under aerobic condition. However, the PHA copolymer with 3HV unit synthesized from the activated sludge has been found under the anoxic and anaerobic conditions

(ORP, -20mV) by using acetate as carbon substrate. This observation strongly implied that DO concentration might be the determine factor for regulating the 3HV fraction. These observation and suggestion were proposed for the first time in this research and have never been mentioned and described by other literatures before.

It is believed that there are two possible explanations for these observations. Firstly, the changes of ORP (i.e. DO) is become a selecting force and results in the selected overgrowth of bacteria in the EAS, and then leads to different metabolic pathways for the PHA accumulation with different 3HV mole fraction. Secondly, there must be some other regulation mechanisms for the synthesis of PHBV copolymer with 3HV unit from acetate which might be affected by the DO concentration when using acetate as sole carbon substrate. Actually, the second one is more reasonable and believable. Therefore, a hypothetic metabolic pathway for PHA production from EAS by using acetate as sole carbon source has been proposed.

Furthermore, similar result has been observed while propionate was used as sole carbon substrate under various ORPs. But the effects of DO on the 3HV mole fraction seemed to be slighter than that of acetate as sole carbon source.

This hypothetic metabolic pathway was modified from Doi's (1990) model for

the PHA production by *R. eutropha*, and combined with recent research works such as succinate-propionate pathway, EBPR metabolic models and my own creation.

One part of the hypothetic pathway is that the TCA cycle and glyoxylate pathway are fully or incompletely involved in the model even under oxygen-limited and anaerobic conditions. Another part of the model is the generation of glycogen from acetate and propionate through TCA cycle, and then glycolysis of glycogen results in the formation of propionyl-CoA for 3HV synthesis under anoxic and anaerobic conditions. The most important is that it is postulated oxygen plays a key role for the regulation of metabolic pathway.

5.2. PHA synthesis from activated sludge collected from full-scale SWWTP by using glucose as carbon source under various ORPs.

5.3.1. Organic consumption and cell growth under various ORPs

After 24-hours operation, the TOC removal efficiency was 43.4%, 41.9% 57.4% and 66.2%, respectively. At the end point of the fermentation, TOC removal kept rising to 65.9%, 71.1%, 81.5% and 96.0%, while ORP was kept at -20mV, -10mV, 0mV and +10mV, respectively. After 48-hours fermentation, the net cell growth was 1.73g, 3.19g, 6.69g and 7.27g when ORP was maintained at

-20mV, -10mV, 0mV and +10mV respectively. The higher the ORP value caused the faster rate of consumption of substrate by the microorganisms, thereby resulting in faster growth rate of microorganisms. Oxygen supplied (for instance, ORP was increased from –20mV to +10mV) process or under oxygen-limited condition the organic substrate uptake and consumption are more efficient than that of anaerobic process as well as biomass growth rate. It was thought that when more oxygen was flowed into the cultural broth, the microorganisms consumed more carbonceaous substances (glucose) for PHA accumulation and cell growth in a faster rate.

5.2.2. PHA production under various ORPs while glucose as sole carbon source

The polymer content in percentage of CDW increased with the increasing of ORP levels from -20mV to +10mV. The PHA content were 5.55%, 6.28%, 8.33% and 11.15% of CDW while ORPs were kept at -20mV, -10mV, 0mV and +10mV respectively. Therefore, it was suggested that PHA content in the EAS increased with the increasing of ORP value. The presence of oxygen resulted in more glucose consumed for the cell growth, energy generation and PHA production. The maximum polymer production yield of 0.31 was achieved by keeping the

ORP level at +10mV. When the ORP was maintained at 0 mV, the $Y_{P/S}$ declined to 0.24. Further reducing of ORPs (-10mV and –20mV) resulted to 0.16 to 0.07 of $Y_{P/S}$ respectively. These results and observations were all accordance with that observed by using acetate and propionate as carbon sources.

5.2.3. 3HV molar fraction in copolymer and ORPs while glucose as carbon source.

It was observed that 3HB/3HV mole ratios in PHBV copolymer increased with that of increasing of ORP values. The maximum HB/HV mole ratio was 8.03 (3HB 88.93 mol%, 3HV 11.07 mol%), when the ORP level was maintained at +10 mV. The HB/HV ratio then declined with the decreasing of the ORPs. When ORPs were kept at 0 mV and -10mV, the 3HB/3HV ratios were 2.173 and 0.624, which in turn the 3HV molar fractions were 31.5 mol% and 61.6 mol% respectively. It reached the minmum of 0.275 (3HB 21.60 mol%; 3HV 78.40 mol%) when the ORP was kept at –20 mV. These results are in accordance with that of acetate and propionate as carbon sources under various ORPs.

These observations have never been reported before. In general, either in pure coulutre of single microbial strain or co-culture of activated sludge, the 3HB/3HV

ratios in the PHA are maintained constant, and the variation caused only by the substrate or co-sbustrate used as carbon sources, C/N ratio or C/P ratio, or the microbe strains conducted for the PHA accumulation. These results implied that ORP or equivalent dissolved oxygen in cultural medium can act as an exogenous factor to determine the ratio of 3HB/3HV of PHA copolymer synthesised by EAS, and subsequentially to control the PHA's physical and mechanical properties. Otherwise, 3HB/3HV ratios, and hence the mechanical properties of PHA could be regulated and maintained through controlling the DO concentration via the ORP monitoring and regulation procedures.

Acturally, in this research, these results and phenomena have been observed twice while respectively using fatty acids (acetat and prionoate) and glucose as carbon sources under the ORP monitoring and regulation conditions. It suggested that monitoring and regulating the fermentation process by ORP instead of DO provided more sensitive and reliable controlling. Slight variation of DO concentration in cultural broth could not be observed and realized by DO system, but ORP system could do it. This why the phenomena was found only under the ORP monitoring and regulating conditions.

After being accepted that DO was the exogenous factor to affect the 3HV molar fraction in PHA copolymer, it was assumed that the presence or absence (or the quantity) of oxygen in cultural broth would resulted in the changes of metabolic pathway for the PHA accumulation and causing 3HV molar fraction variation. A hypothesized biochemical metabolic model by using glucose as sole carbon source can there be established to try to explain and understand these phenomena. Furthermore, a revised metabolic model combiant the previous model by using fatty acid to produce PHA was also proposed.

5.2.4. A modified metabolic pathway for organic substrate uptake and PHA production by activated sludge.

This hypothetic metabolic model consists of five parts. The first part is the glycolysis of glucose oxidized through EMP or ED pathway to pyruvate. The second part is pyruvate decarboxylation to acetyl-CoA to form the PHB or acetyl-CoA enters the TCA cycle. The third part is lactic acid fermentation where pyruvate as electro acceptor under anaerobic condition, and then lactic acid for the further conversion to 3HV. The fourth part is the succinate-propionate pathway to synthesis PHV from pyruvate to propionyl-CoA under anoxic or anaerobic conditions. The fifth part is the TCA cycle and glyoxylate pathway. The most

important view in this hypothetic model is that the oxygen is the key extrogenous factor to regulation of 3HV mole fraction in PHA copolymer.

All these observed evidences implied that there is strong relationship between the 3HV formation in PHA copolymer from EAS and the DO concentration of the cultural medium. Accordingly, it was postulated in the hypothetic metabolic model that oxygen might play a very important role to regulate the 3HV production via TCA cycle, glyoxylate pathway and others.

Usually the TCA cycle is linked with respiration and operates only under aerobic or anoxic conditions. The oxidation of succinate to fumarate in the TCA cycle requires a terminal electron acceptor with a redox potential ($E^{0'}$) more positive than that of fumarate/succinate couple (+32 mV). Only O_2 (O_2/H_2O, $E^{0'}$= + 818 mV), NO_3^- (NO_3^-/NO_2^-, $E^{0'}$ = + 433 mV) and NO_2^- (NO_2^-/N^{2-}, $E^{0'}$= + 970 mV) appear to meet these conditions. In this research, only O_2 was supplied for the ORP controlling. The presence of oxygen results in the step of oxidation succinate to fumarate in TCA cycle (green arrow in Figure 4.25 and Figure 4.30) performed. The absence of oxygen under anoxic and anaerobic conditions, however will induce the failure of this step, and then resulted in the accumulation of succinate. Otherwise, as mentioned previously, part of the acetyl-CoA molecules arisen from pyruvate decarboxylation that can be transformed in the

glyoxylate pathway to produce both malate and succinate. The malate is then oxidized to oxaloacetate to sustain the cycle of the glyoxylate pathway and form one mole $NADH_2$ necessary for PHB synthesis (Louie *et al.*, 2000). In addition, the pathway of the succinate is more complicated but the succinate must be metabolized because continued accumulation of succinate will inhibit the glyoxylate pathway. One of the important common mechanisms to consume the excess succinate is to convert it into fumarate and then to malate in order to sustain the TCA cycle and glyoxylate pathway (Herman and Bell, 1970). This conversion step from succinate to fumarate is oxygen-depending reaction as explained above; the absence of oxygen will result in the accumulation of succinate. In order to sustain the glyoxylate pathway, the accumulated succinate has to be converted to propionyl-CoA and then results in 3HV synthesis as carbon sink.

As described, the accumulated succinate will be converted to succinyl-CoA and then propionyl-CoA through decarboxylation reaction instead of further reduction to α-ketoglutarate. This is because the step of conversion α-ketoglutarate to succinyl-CoA is an irreversible step in TCA cycle, as illustrated in Figure 4.25 and Figure 4.30. Consequently, a large amount of propionyl-CoA will be forced to convert into 3HV units, and then resulted in the significantly

increasing of 3HV fractions in PHA copolymer.

Malonate, the metabolic inhibitor, was conducted to suppress the step in the TCA cycle as an alternative to the absence of oxygen to directly examine the potential role of the oxygen in TCA cycle and glyoxylate pathway for the synthesis of PHA copolymer with 3HV unit majority. The ORP was maintained at +10mV (with oxygen supply) and glucose as the sole carbon source.

After 24 hrs cultivation, when malonate and glucose were simultaneously added into the medium (treated group) inoculated with EAS from local MWWTP, strong PHV synthesis was observed. The 3HV mole fraction in the PHA copolymer was 83.01 mol/%, higher than that of keeping ORP at –20 mV (anaerobic condition). Nonetheless, the PHA content was 4.32% of cell dry weight, lower than the control group without the addition of malonate. Meanwhile, in the control group, without the addition of malonate, the PHA content in EAS was 8.9 cdw% with 3HV mole fraction of 11.07 mole%.

The present experimented results and literatures review have indicated that even though the oxidation of succinate to fumarate was important for both sets of nutrients the specific blockage of succinate oxidation to fumarate by malonate may force the decarboxylation of succinate to propionate resulting in 3HV production, and cause the enhanced 3HV-enriched PHA synthesis. In addition, if

the blockage were caused by the absence of oxygen, the succinate generated both from the TCA cycle and glyoxylate pathway would be also forced for the 3HV-enriched PHA copolymer accumulation in EAS.

Therefore, it can be concluded that oxygen plays a very important role for the synthesis of PHV in TCA cycle and glyoxylate pathway. Under aerobic condition, the succinate generated from TCA cycle and glyoxylate pathway is oxidized to fumarate and contributed to sustain the full cycling of the TCA cycle and glyoxylate pathway. In the absence of oxygen, the oxidation of succinate to fumarate is blocked without the higher redox potential (O_2/H_2O), the succinate generated from incomplete TCA cycle or glyoxylate pathway would be forced to convert to propionyl-CoA as the precursors of PHV, and enhance the production of 3HV-enriched PHA copolymer.

The observations and the hypothesized model presented in this study has provided experimental evidence to prove that TCA cycle and glyoxylate pathway are involve in the PHA production and phosphorus removal in the anaerobic-aerobic EBPR process.

5.3. Limitation of the research

The limitation of this study is the absence of analysis and measurement equipments such as high sensitive DO meter, CO_2 meter for the detecting of DO concentration and oxygen consumption rate under various ORPs. These data would help me identify and verify the hypothetic metabolic pathways. By using ^{13}C labeled carbon source, the metabolic pathway of carbon by the EAS could be traced, and which would result in the establishment of stoichiometry of varied carbon sources for the PHA synthesis by EAS under oxygen limitation conditions.

5.4. Suggestions for further research

5.4.1. The effects of ORP on the PHA production from EAS.

In this research, it was proved that when ORP levels in the cultural medium sere accurately controlled, changes in metabolic pathway for PHA production from EAS would be resulted. Only DO was regulated during fermentation process for ORP controlling in this research. Actually, other factors can also induce the variation of ORP and hence resulting in the verity of PHA synthesis. The effects of these factors including biomass density, pH, substrates concentration and chemicals on PHA synthesis by EAS should be taken into account.

5.4.2. The metabolic pathway for PHA accumulation.

In this research, it has been found that oxygen plays a very important role to the PHA synthesis, particularly to the 3HV molar fraction in PHBV copolymer. More experimental evidence should be observed if metabolic inhibitors except malonate are employed for the study of chemical reaction between the intermediates of TCA cycle and glyoxylate pathway.

On the other hand, the stoichiometry of this hypothesized model such as carbon flow, ATP and reducing power balance may need further investigation.

The ^{13}C labeled carbon sources such as acetate, propionate and glucose are useful tool for the further investigation of metabolic pathway.

5.4.3. Optimization of fermentation conditions and cultural medium

Complex and cheaper carbon sources such as mixed fatty acids from the digestion of excess activated sludge and sewage, molasses should be taken into account for the PHA production by EAS. The possible effect of pH, phosphorus, initial concentration of carbon source, and the feeding mode on the accumulation of PHA from EAS are worthy to be studied.

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Appendix AA

Procedures for Determination of Melting Point with Electrothermal Digital Melting Point Apparatus Model IA 9100.

1. One end of 2 mm diameter of the capillary melting point tubes were sealed with a buchen burner.

2. The sample was crushed into a very fine powder.

3. A small amount of the fine sample powder was pushed into the open end of the capillary tube.

4. In order to make a well-compacted sample powder about 1/4 inch long at the closed end of the capillary tube, the capillary tube was dropped through a larger glass tube abut 24-inches long to hit the closed end on the bench top for several times.

5. The capillary tube was inserted into the electrothermal digital melting point herter and then the heating rate was set at 10 °C/min from room temperature to 200°C.

6. A 6x magnifier viewing lens was used for observing when the sample in the capillary tube was melted and then melting temperature was recorded as an approximate melting point.

7. After recording the first rapid heating run, the heater was cooled several degrees below the temperature of first melting.

8. Repeated step from 3 to 6, a fresh capillary tube containing the same was inserted into the melting point heater.

9. The capillary tube was heated gently with the melting temperature being recorded.