The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

Selected Activated Sludge for Production of Environment-friendly Bio-plastics

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

December 2012

CERTIFICATE OF ORIGINALITY

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Zhong Dan

ABSTRACT

The environmental problems associated with plastics have become increasingly severe in recent years. Technologically- and economically-feasible solutions are in urgent need in order to ensure sustainable development in the plastic industry. While technical and economic constraints remain with plastic recycling and reuse, attempts to development biodegradable plastics have emerged as a potential solution.

Polyhydroxyalkanoates (PHAs) are a family of specialized polyesters of hydroxyalkanoates (HAs), artificially synthesized or naturally existing as an intracellular carbon reserve by using different bacteria strains, which are potential biodegradable substitutes to conventional petroleum-based plastics.

The significant advantages of PHAs include its biocompatibility with human tissues, biodegradability under natural ambient and thermoplastic properties. Additional beneficial properties, such as zero toxicity and complete recyclability, render PHAs environment-friendly materials to replace conventional plastics. However, it is essential to lower the production costs if PHAs are to be used in large scales.

Production of the various types of PHAs in activated sludge processes specifically designed for treating wastewater has emerged as an economically and

environmentally promising and attractive alternative to conventional pure culture fermentations. This is because large amount of sewage sludge generated from municipal wastewater treatment works has been continuously increasing with rapid urbanization and industrialization.

Conventionally, sewage sludge treatment methods include incineration, composting, land application, landfill, and ocean dumping. However, all these methods have their specific drawbacks, such as the risks of pathogenic proliferation, endotoxins and heavy-metal contaminants spreading from atmospheric, hydrospheric and lithospheric application sites. Consequently, the treatment and disposal of sewage sludge has become a key environmental problem. The method of converting sewage sludge to environment-friendly PHAs is therefore an attractive means for sludge disposal.

Till date, little research has been conducted and published pertaining to the application of optimal parameters in laboratory-scale activated-sludge simulator system and pilot-scale bioprocesses for PHA production.

In this study, several factors affecting the process efficiency and overall economics of PHA production were investigated. In order to examine how these factors contribute to the process efficiency, main focus of the research works was the design of a laboratory-scale activated-sludge simulator system and pilot-scale sequential batch reactor (SBR) system to determine the optimum operating

conditions of activated sludge process for increasing the PHA production yield.

Activated sludge from a conventional municipal sewage treatment works was obtained and conducted for the PHA accumulation by using glucose as the sole carbon sources in a laboratory-scale activated-sludge simulator system. Firstly, the optimal aeration time of 2.5 h and the optimal settling time of 1.0 h were determined, based on the quality of the sludge and treated effluent. Secondly, with the fixed aeration and settling time for the activated-sludge simulator system, the optimal carbon-phosphorus (C:P) ratio was fixed at 300, as determined by observing the PHA accumulation rates.

In the third stage, investigation was directed at evaluation of the optimal values of carbon-nitrogen (C:N) ratio on PHA production yields under the other pre-determined optimal conditions. Results showed that as the C:N ratio increased from 30 to 120, specific polymeric yield increased to a maximum of 0.291 g polymer/g dry cell weight, while specific growth yield decreased with increasing C:N ratio. The highest overall polymer production yield of 0.099 g polymer/g COD consumed was achieved under the C:N ratio of 90. Therefore, a severe nitrogen deficiency triggered the intracellular accumulation of PHAs as a food reserve in the activated-sludge microbial community. Carefully designed sporadic adjustments and feeding patterns of the C:N ratio did not significantly affect the process performance in terms of COD removal efficiency, which

maintained at around 80%. After these three stages of works, the activated sludge was considered to have been selected with a microbial consortium that is best suited for efficient PHA accumulation under the predetermined operating conditions. The aeration and settling times, as physical selection pressures, and the C:P and C:N ratios, as nutrient selection pressures, formed a novel selection procedure for effective establishment of a stable and rigorous sludge suited for PHA accumulation. A relatively high specific polymeric yield of 0.291 g polymer/g dry cell weight indicated that the activated sludge is possibly dominated by hyper accumulators of PHAs such as *Nocardia spp.*, *Alkaligenes spp*. and *Psudomonas spp.*.

In the fourth stage of the study, the pilot-scale SBR system was built for PHA accumulation based on optimal condition determined from the previous laboratory-scale system. The selected activated sludge was used to seed the system. The PHA production yields and COD removal efficiency were studied on a long-term basis. The results showed that the specific growth yield (Yx/s) from the pilot-scale SBR system was higher than that in the laboratory-scale simulator. The specific polymer yield (Yp/x) was 0.237 g polymer/g dry cell weight in pilot-scale, which was less than the 0.259 g polymer/g cell weight observed in the laboratory-scale simulator system. The overall polymer production yield (Yp/s) was 0.092 g polymer / g COD consumed in pilot-scale, which was less than the 0.099 g polymer/g COD consumed in laboratory-scale simulator under the C:N

ratio of 90. The average COD removal efficiency of 83.10% in the laboratory-scale simulator was found to be 2.81% higher than that in the pilot-scale system.

In the final stage, the composition of co-polymeric materials, namely poly-hydroxybutyrate-valerate (PHBV), produced by activated sludge bacteria was controlled by regulating the feed composition, namely the concentration of butyric acid (C_4) and valeric acid (C_5) ratio in the medium, under the established operating conditions of pilot-scale system. The organic feed to PHBV conversion mechanism were studied and mathematically modeled. When butanoic acid, or otherwise known as butyric acid, was used as sole carbon and energy source for fermentation, there was only PHB homo-polymer produced instead of PHBV co-polymers in the culture. On the other hand, the highest 3HV mole fraction 48% in the co-polymer accumulated was when valeric acid was used as sole carbon source. The melting temperature of the PHAs produced by activated sludge decreased from 178.9℃ to 98.5℃, with an increase in the 3HV fraction, indicated that 3HV unit act as defects in the PHBV crystal lattice. Therefore, the composition of the co-polymers, the physic-chemical, thermal and mechanic properties, could be fairly accurately controlled by carefully manipulating the influent organic compositions in the culture medium. The carbon source to PHBV copolymers conversion mechanisms and relations were elucidated.

In conclusion, PHAs including copolymers of PHBVs with industrially applicable properties can be produced at substantially reduced costs using a specifically selected and enriched activated sludge, through a novel procedure, from sewage treatment works. The PHA accumulation process was scaled up in a pilot scale system and the conversion process mechanism was elucidated. The novel use of dimensionless process design parameters, namely Yp/s, Yp/x and Yx/s, served as the process predictive model for the process design, optimization, operation and control. These model parameters were validated in pilot-scale opeartion, and were valuable tools for full-scale industrial opearations. This is a major step ahead towards environment-friendly plastics production and efficient sewage sludge disposal.

LIST OF PUBLICATIONS

Related journal papers:

1. Law Man Chung, Cheng Ka Po*, Kan Chi Wai, Zhong Dan, Chan Yuk Sing Glbert**, Chua Hong. (2012) **An Investigation of Cyanobacteria in Surface Water of China's Yantian Reservoir**

   (Submitted to Environmental Science and Pollution Research)

2. Y.H. Liu,*, D. Zhong, Shirley N. Sin, Gilbert Y.S. Chan, W.H., Onyx. Wai, H. Chua, D. He. (2012) **Study of Pilot-Scale Biosynthesis of Polyhydroxyalkanoates Using Dyeing Activated Sludge.**

   (Submitted to Biochemical Engineering Journal)

3. Y.F.Tsang*, D. Zhong, S.N.Sin, Y.S. Chan, H. Chua (2012) **Bioplastics production from industrial wastewater using recombinant strains.**

   (Submitted to Bioresource Technology)

4. Y.F.Tsang*, D. Zhong, S.N.Sin, Y.S. Chan, H. Chua. (2012) **Recovery of Polyhydroxyalkanoates from Activated Sludge with the Fermentation on Volatile Fatty Acids.**

   (Submitted to Biochemical Engineering Journal)

Related conference papers:

1. Zhong D., Wang Y.J., He D., Sin S.N., Chua H.(2010) **Polyhydroxyalkanoates (PHAs) Production in Activated Sludge fed by Volatile Fatty Acids.** Second International Postgraduate Conference on Infrastructure and Environment,1-2 June 2010, Hong Kong, China.

2. Zhong D., Wang Y.J., He D., Sin S.N., Chua H., Yu P.H.F (2010) **Bio-plastic (PHAs) Production by Using Recombinant Strain.** 1st International Conference on Sustainable Urbanization, 15-17 December 2010, Hong Kong, China.

3. Wang Y.J., Zhong D., He D., Sin S.N., Chua H., Ren N.Q. (2010) **Optimal fermentation type for bio-hydrogen production in continuous-flow acidogenic reactors.** 1st International Conference on Sustainable urbanization, 15-17 December 2010, Hong Kong, China.

4. Zhong D., Tsang Y.F., Wang Y.J., Sin S.N., He D., Chua H. (2010) **Recovery of Polyhydroxyalkanoates from Activated Sludge with the Fermentation on Volatile Fatty Acids.** International Conference on Chemical and Environmental Engineering, 18-20 December 2010, Singapore.

5. Tsang Y.F., Wang Y.J., Zhong D., Sin S.N., He D., Chua H. (2010) **Optimization of Polyhydroxyalkanoates Production from Recombinant Escherichia coli.** International Conference on Chemical and Environmental Engineering, 18-20 December 2010, Singapore.

ACKNOWLEDGEMENT

The author would like to acknowledge the financial support by the RGC Project no. 026515-2 "Selected activated sludge for industrial wastewater treatment and for production of environmental-friendly bio-plastics".

The author would also like to express her sincere gratitude towards her supervisor, Dr CHAN Yuk Sing Gilbert and her co-supervisor Professor H. Chua, for their valuable guidance, comments and support throughout the whole period of this research.

Special thank is also given to Mr. Y. H. Liu for his kind support in laboratory works. Deep appreciation is devoted to former members of the Research Group, namely Dr. Y. F. Tsang and Dr. Y. J. Wang, for their encouragement and help in the past years. Gratitude is also expressed toward staff members in the Wuxi Wastewater Treatment Plant for their effective coordination and assistance in conducting the pilot-scale experiments and field sampling works.

Last but not least, the author would also want to devote her deepest appreciation towards her family and friends in the Polytechnic University for their continuous encouragement and moral support. Without their love, patience and support, successful completion of this work would have been impossible.

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Acetyl-CoA	Acetyl-coenzyme-A
ADF	Aerobic dynamic feeding
APHA	American public health association
C:N ratio	Carbon-nitrogen ratio
CoA	Aoenzyme-A
COD	Chemical oxygen demand
CSTR	Completely stirred tank reactor
Cu^{2+}	Copper bivalent ion
DCW	Dry cell weight
DO	Dissolve oxygen
EAS	Excessive activated sludge
EBPR	Enhanced biological phosphorus removal
GC	Gas chromagraphy
GAOs	Glycogen accumulating organisms
H_2SO4	Sulfuric acid
HB	Hydroxybutyrate
HKEPD	Hong Kong environment protection department
HPDE	High density polyethylene
HV	Hydroxyvalerate
ISP	Intracellular storage polymer
LOPE	Low density polyethylene
MCL-PHAs	Medium chain length polyhydroxyalkanoates
Mg^{2+}	Magnesium bivalent ion
MLSS	Mass liquor suspended solid
MLVSS	Mixed liquor volatile suspended solid

Mn^{2+}	Manganese bivalent ion
N&P-limitation	Phosphorus and nitrogen-limitation
NaOH	Sodium hydrate
Ni^{2+}	Nickel bivalent ion
N-limitation	Nitrogen-limitation
ORP	Oxidation reduction potential
PAOs	Polyphosphate accumulating organisms
PE	Polyethylene
PET	Poly(ethylene terephthalate)
PGA	Polyglycolide
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxyburate
PHBV	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)
PHV	Polyhydroxyvalerate
P(3HB)	Poly-3-hydroxybutyrate
P(3HV)	Poly-3-hydroxyvalerate
P(HB-*co*-HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P-limitation	Phosphorus-limitation
PLA	Polylactic acid / polylactide
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
RAS	Return activated sludge
SBR	Sequencing batch reactor
SCL-PHAs	Short chain length polyhydroxyalkanoates
SRT	Sludge retention time
SS	Suspended solid
TCA	Tricarboxylic acid

TKN	Total kjeldahl nitrogen
TOC	Total organic carbon
VFAs	Volatile fatty acids
YE	Yeast extract
$Y_{P/S}$	Overall polymer production
$Y_{P/X}$	Specific polymer yield
$Y_{X/S}$	Specific growth yield
3HB	3-hydroxybutyrate
3HBME	3-hydroxybutyrate methyl ester
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
3H2MB	3-hydroxy-2-methylbutyrate
3H2MV	3-hydroxy-2-methylvalerate
4HB	4-hydroxybutyrate

1 INTRODUCTION

Technological advancements have substantially increased the productivity in the industrial and manufacturing sectors, and have, at the same time, generated a number of new materials that have adverse impacts on the environment. Plastics are among these materials that have raised significant concerns in the environmental and public-health aspects. With increasing attention for the environmental quality and consistently high crude oil prices, biopolymers that are sustainable and environment-friendly materials, have received more attention as a substitute for conventional fossil-fuel based plastics. Consequently, the development of biopolymers is one of the most important factors for the new and sustainable economic development, among which are a class of novel materials known as polyhydroxyalkanoates (PHAs) (Chua and Yu, 1999a; Philip et al., 2007).

Chanprateep (2010) predicted that PHAs will have a significant positive impact on global climate scenario since they can be produced from renewable raw materials and are degraded by microorganisms in the natural ecosystem that, in turn, enable carbon dioxide and organic compound recycling in the global environment.

PHAs is undoubtedly one of the potential candidates as an environment-friendly

substitute for conventional petroleum-based plastics, because they have similar material physical and mechanical properties as various thermoplastics and elastomers (Poirier et al., 1995), and complete biodegradability upon disposal under natural environmental conditions (Lee, 1996). Furthermore, these new materials can be produced from biorenewable and biowaste resources (Laycock et al., 2012; Reddy et al., 2003; Taguchi et al., 2012) and will not have an impact on the fossil-fuel reserves.

A life cycle assessment and financial analysis of mixed culture PHAs and the associated biogas production was undertaken based on treating an industrial wastewater. Gurieff and Lant (2007) showed PHAs was preferable to biogas production for treating the specified industrial effluent and also financially attractive when compared to pure culture PHA production. However, it is also essential to lower their production cost if they are to be used as bulk products such as agricultural geofilms, packaging materials and disposable products (Reis et al., 2011).

A potential approach for economical PHA production would be using a mixed culture that can accumulate a high weight percent of intracellular PHA content while growing on inexpensive substrates such as industrial wastes (Punrattanasin, 2001). Therefore, it was first studied by Chua et al. (1996) on the biosynthesis of PHAs by activated sludge using carbon sources and nutrients in industrial waste

waters. It is considered as more environment-friendly and sustainable option, especially when petroleum and common conventional fossil-fuel based plastics as non-sustainable resources are being depleted rapidly (Chen, 2010a).

Production of PHAs in activated sludge treating wastewater represents an economical and environmental promising alternative to pure culture fermentations (Bengtsson et al., 2008). Large amount of sewage sludge generated from wastewater has been on a continuously increasing trend each year with the rapid urbanization and industrialization worldwide, which provides a lavish supply of raw materials for PHA production. However, in-depth investigations on the optimized PHA production process and mechanisms are still lacking.

A variety of wastewater treatment plants will produce large quantities of excess activated sludge (EAS) in modern cities and municipalities. Sewage sludge treatment methods conclude incineration, composting, land application, landfill, and ocean dumping (Hara and Mino, 2008; Khwairakpam and Bhargava, 2009; Renou et al., 2008; Wang et al., 2008). However, all of these methods have its own specific restrictions and potential risks, such as the risk of pathogenic proliferation, and endotoxins and contamination in land application (Liu et al., 2011).

Furthermore, the treatment and disposal of sewage sludge presents substantial

costs for sewage works operation (Barr et al., 2008; Wei et al., 2003). If however, the sewage sludge can be converted into valuable by-products, this may be a potential way for resource recovery. However, explicit analysis on the technical and economic feasibility are still lacking in the published literature.

The current price of PHAs, produced from fermentation or chemical synthetic processes, is much higher than conventional plastics, thus hindering the widespread applications (Braunegg et al., 1998; Choi and Lee, 1997; Reddy et al., 2003). Therefore, more efficient and cost-effective technologies need to be developed in order to enable PHAs to be cost competitive in relation to other products and materials with similar applications.

PHAs are completely biodegradable (Stevens, 2002), water resistant and can perform like elastomeric materials. Therefore, PHAs could be used for parallel applications as conventional plastics, particularly in specialized fields. It is commonly believed that there are three principal areas of applications of PHAs, namely medical and pharmaceutical products, agricultural films and commodity packaging. However, further research on the copolymerization to produce PHAs with wider variety of physical and mechanical properties, thus increasing the areas of applications, are also in urgent need.

The current technical problems faced are that PHA manufacturing is commonly small-scale operations (Braunegg, G. et al., 1998; Serafim et al., 2004).

Technology improvements are necessary to scale up PHA-production operation with optimized conditions and economized costs. Significant cost reduction can possibly be achieved through better understanding of the PHA conversion mechanisms and process influencing parameters, and through pilot-scale studies of the process and improvements on downstream extraction of the end products (Liu, 2009).

1.1 Plastic-Waste Generation and Disposal on the Local and Global Perspectives

Plastic wastes are commonly generated from three sources, namely domestic-consumer activities, trade and industrial activities, and industrial disposal of plastic scrap. The total global capacity of commodity plastic production dramatically increased from 1.5 million tons in 1950 to 245 million tons in 2008, an annual growth rate of 9% (Chanprateep, 2010). Comparatively, Hong Kong produces 9,500 tonnes of municipal waste each day, and plastic materials percentage, close to 20 %, in the municipal-waste is the highest in the world.

Plastics are not easily biodegradable in the natural environment, and are therefore considered to be almost the most environmentally harmful wastes. While plastic wastes that are not appropriately dealt with in the past have become a major source of persistent waste adversely affecting the environment. Plastic wastes that

are treated by means of incineration or landfilling which bring on operational inconvenience to these waste management facilities. Recently, there is more concern on the associated air pollution problems which are associated with the incineration process, including acid gases, carbon dioxide and other toxic gases like dioxins.

Landfills have been the main receptors for plastic wastes in Hong Kong. However, landfill space is rapidly running short to accommodate the increasing amount of non-biodegradable plastic wastes. In addition, plastic wastes in landfills block the infiltration of water and migration of microbial ecosystem. Plant growth on the overlying soil is therefore adversely affected and landfill stability is endangered.

Plastic recycling and reuse, as a solution to mitigate plastic-waste problems, are often hampered by practical difficulties in separating different types of plastic waste from the general municipal waste streams. Furthermore, the low market value of the recycled materials, the high labour cost and the space requirement for plastic waste washing and sorting are among the main constraints (Hu, 2004). With these technical difficulties, it is forecast that a maximum of a mere 25% of total plastic wastes can be recycled over the next decades (HKEPD, 2010). Therefore, development of appropriate biodegradable materials that substitute

conventional plastics are expected to play a major role in the imminent and fast-growing plastic market.

1.2 Background of the Project

Over the past two decades, plastic usage and plastic-waste generation have been on a drastically increasing trend, and are forecast to grow consistently over the next decade. Commonly used packaging plastics and disposable plastic products are generally recognized as environmentally harmful due to their non-biodegradability in the natural environment.

Conventional plastics, such as high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC), have been widely used as various industrial and commercial materials (Hu, 2004). Although conventional plastics have brought about much convenience to human life, they have caused serious environmental problems since they are not readily decomposed by natural strains of microorganisms. Plastics cannot recycle in the natural carbon cycle and remain in the environment for a long time, thus endangering animals, plants, the hydrospheric, lithospheric and atmospheric environments. They have been widely recognized as one of the most environmentally harmful materials (Suresh Kumar et al., 2004).

Plastic has become a significant constituent of the municipal solid-waste stream since the Eighties. In Hong Kong, as in many other advanced cities over the world, an average of 9,114 tonnes of municipal solid wastes from domestic refuse, commercial packaging materials, industrial disposable products and scraps are delivered for disposal at incineration plants and landfills daily, accounting for 20% of gross weight of the municipal waste stream in 2010 (Figure 1.1).

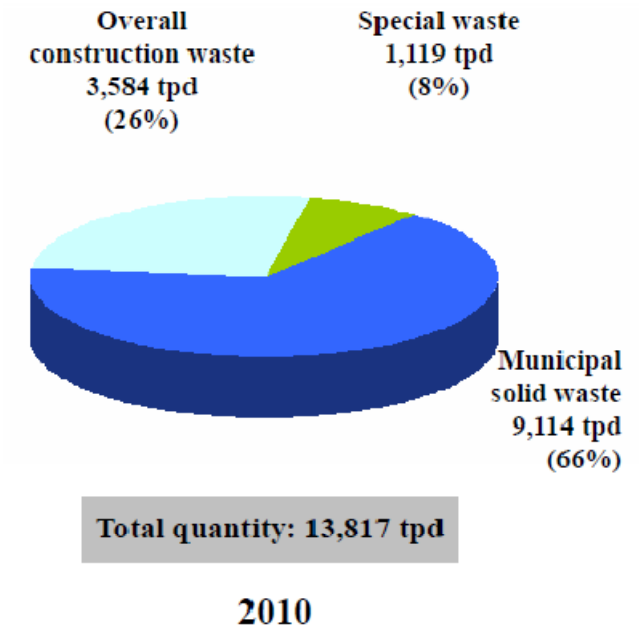


Figure 1.1 Disposal of solid waste at landfills in 2010 (HKEPD, 2010).

As shown in Table 1.1, plastics have accounted for more than one fifth by weight in each kind of waste, including domestic, commercial, and industrial solid waste. Furthermore, an approximate increment of 1 % per year was recorded throughout the past decade resulting in the alarming figures in the year 2010 (Figure 1.2).

This percentage of plastics in municipal waste stream is among the highest in the world.

Table 1.1 Composition of municipal solid waste in 2010 (HKEPD, 2010)

Composition	Average daily quantity (tpd) and percentage by weight				
	Domestic waste	Commercial waste	Industrial waste	Commercial & industrial waste	Municipal solid waste
	(a)	(b)	(c)	(d)=(b)+(c)	(e)=(a)+(d)
Glass	310 (5.1%)	55 (2.4%)	8 (1.3%)	63 (2.1%)	374 (4.1%)
Metals	103 (1.7%)	40 (1.7%)	33 (5.3%)	73 (2.5%)	176 (1.9%)
Paper	1,259 (20.5%)	684 (29.1%)	61 (9.8%)	745 (25.0%)	2,004 (22.0%)
Plastics	1,266 (20.6%)	548 (23.3%)	127 (20.3%)	675 (22.7%)	1,941 (21.3%)
Putrescibles	2,747 (44.8%)	846 (36.0%)	75 (12.0%)	922 (30.9%)	3,668 (40.2%)
Textiles	168 (2.7%)	45 (1.9%)	21 (3.4%)	66 (2.2%)	234 (2.6%)
Wood/Rattan	74 (1.2%)	32 (1.4%)	189 (30.1%)	221 (7.4%)	295 (3.2%)
Household hazardous wastes (HHWs)[1]	75 (1.2%)	25 (1.1%)	8 (1.3%)	33 (1.1%)	108 (1.2%)
Miscellaneous[2]	133 (2.2%)	77 (3.3%)	105 (16.7%)	181 (6.1%)	314 (3.4%)
Sub-total	6,135 (100%)	2,352 (100%)	627 (100%)	2,979 (100%)	9,114 (100%)

As widely recognized, there are limited land-filling areas for disposing of the solid wastes in the highly urbanized Hong Kong, especially for such wastes as non-degradable plastics. Furthermore, in the land-filling area, plastic wastes inhibit plant growth and affect soil stability since it blocks the infiltration of water, adversely influent the vertical and horizontal migration of moisture, and upsets the natural recycling of nutrients and microbial community in soil. Other

treatment methods like incineration, not only cost too much to operate but also runs the risk of emitting toxic gases, like sulphur oxides, dioxins and hydrochloric acid, which are harmful secondary pollutants to the public health and environmental ecosystems.

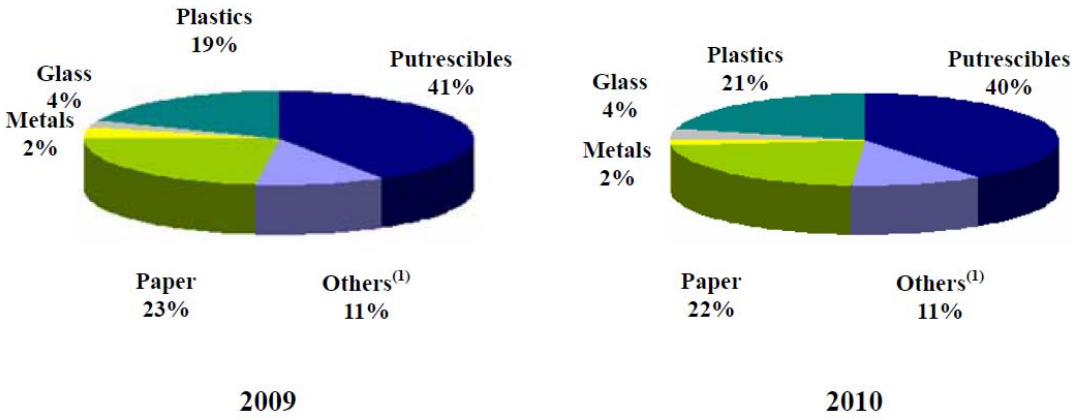


Figure 1.2 Composition of municipal solid waste in 2009 and 2010 – Major waste types (HKEPD, 2010).

1.2.1 Plastic bags

Packaging is a modern and essential technique for protecting food, increasing shelf life and consumer safety. Today, the basic materials of packages include, paper, paperboard, cellophane, steel, glass, wood, textiles and, most important of all, plastics (Avella et al., 2001).

The indiscriminate use of plastic shopping bags is a major and visible environmental problem in Hong Kong. Our environmental surveys on the three major strategic landfills indicate that some eight billion plastic shopping bags are

disposed of at landfills every year. This translates into a seriously alarming figure of more than three plastic shopping bags per person per day, which apparently go beyond the basic needs. In order to alleviate pressure on the environment, the Hong Kong Government has made a number of special measures.

To address the problem of indiscriminate use, after long debates, it had been proposed to introduce an environmental levy of HKD 0.5 on each plastic shopping bag at the retail and end consumer level, with the first phase covering chain or large supermarkets, convenience stores and personal health and beauty product stores. The actual charging of the environmental levy has commenced on 7 July 2009 (HKEPD, 2012). This measure can ease the pressure of the plastic bag, but does not fundamentally solve the whole plastic-associated problem.

1.3 Potential Solutions

As mentioned above, none of the current treatment methods has truly proven to be a reliable long-term solution to plastic wastes. That means we have to find a new way in solving this problem. In order to reduce the environmental impact of plastic wastes, it has huge significance to manufacture plastics, which are degradable. There are already many degradable plastics discovered by the researchers such as biodegradable polymers and photodegradable polymers. Among the family of biodegradable polymers, PHAs have attracted more attention in recent years, because they possess many similar properties and advantages as compared to

conventional plastic material. The significant beneficial properties of PHAs include their biocompatibility, biodegradability and thermoplastic ability as well as other physical and mechanical properties that are essential for widespread applications.

Currently, the main and potential markets are for applications commercial packaging, domestic and industrial disposable products and agriculture geofilms. By 2020, the bio-plastics market in the EU is forecast to increase to 2–5 million tons and to expand to the textile, automotive, and agro-industrial sectors, including many durable applications (Chanprateep, 2010). Although they are useful, widespread application of PHAs is hampered by high production costs. For a long time, much effort has been devoted to reducing the cost of producing, extracting and purifying PHAs. Unfortunately, the effect in cost-reduction is not significant, and the price of these end products are around seven times more expensive than that of conventional synthetic plastics due to high raw material costs, small production volumes, and high processing costs, particularly for downstream purification. To date, none of the methods developed for PHA production is perfect and all are far from ready to be used in large-scale commercial systems.

On the other hand, a large quantity of excess activated sludge is generated daily in municipal and industrial sewage treatment works throughout the world. The

effective disposal of sludge that is excessively produced from the biological processes, namely secondary activated sludge processes, is a technologically-complex and costly system. In this attribute, industrial wastewater and municipal sewage treatment plants have become a serious environmental problem for the excess sludge they generate. Several methods have been proposed for the effective utilization and volume reduction of the excess sludge in the last ten years, while landfilling without reuse remains the main method (Inoue et al., 1996).

However, many methods for the disposal of excess sludge are forecast to be forbidden in the near future due to the environment-harmful side effects, e.g. ground water contamination, and toxic and hazardous gas emission, thus much attention is paid on the exploration of potential technology for sludge reduction.

As generally known, 40 ~ 60% of the total operational cost of an activated sludge treatment plant are spent on sludge handling, treatment, and ultimate disposal of the excess sludge. It is therefore important to develop appropriate environmentally and economically acceptable solutions to reduce excess sludge production from wastewater biological treatment processes (Liu, 2003).

Zhang et al. (2009) and Chua et al., are among the earliest workers, who suggested that excess sludge management should focus on reutilization of sludge as useful resources, such as using it for PHA production. This method is useful

and promising in several ways. Firstly, excess sludge generated from activated sludge processes needs further treatment such as costly anaerobic digestion and disposal by landfill. Recovery of valuable resource from activated sludge and hence reducing its quantity would substantially economize the activated sludge disposal cost. Secondly, large quantities of organic biomass obtained from activated sludge can be used to produce PHAs instead of using pure culture of microorganism or genetically engineered microbial strains, thus effectively and significantly reduce the production cost (Chua et al., 1997b; Hu et al., 1997; Lee, 1996). Thirdly, sterilization facilities and the technologically-complex fermentation processes are not necessary. The advantages of utilizing such novel technique to synthesis of PHAs from activated sludge was also reported by Yu, P H et al. (1999), which can reduce the amount of municipal activated sludge by30 % by weight, therefore substantially reducing the costs of sludge treatment.

Despite the known benefits of PHAs, most of the published research and development works pertaining to PHA production have focused primarily on fermentation processes using pure culture of microorganisms or genetically engineered strains. It is reported that PHA yields could be improved by optimizing process operating parameters and reactor operating conditions. However, process efficiency remains low and the production costs remain relatively high. Till date, little research has been conducted and published pertaining to the application of optimal parameters in laboratory-scale with

specifically selected and enriched activated-sludge and modeling of pilot-scale systems for PHA production.

1.4 Objectives of the Project

1.4.1 General objective

In this study, several essential factors affecting the overall efficiency of PHA production were investigated and their contribution towards process scale-up, optimization and operation were elucidated.

1.4.2 Specific objectives

The specific objectives of the study are six-fold, which are outlined as follows.

1. To investigating the effects of aeration and settling time, and hence the organic loading rate, of an sequencing batch simulator, designed in a laboratory scale, on selecting an enriched activated sludge for PHA production.
2. To investigated the effects of carbon:phosphorus and carbon : nitrogen ratios on selecting an enriched activated sludge for PHA production.
3. To establish a novel selection procedure, integrating the aforementioned physical and nutrient selection pressures, for effective culturing an activated

sludge for stable and efficient intracellular PHA accumulation.

4. To design, fabricate and operate a pilot-scale sequencing batch reactor to assess the long-term stability and reliability of PHA production.
5. To validate the novel dimensionless parameters as effective modeling tools for process design, scale-up, operation and optimization.
6. To study the control of final co-polymeric composition, physical, thermal and mechanic properties in the PHAs through modifications of feed compositions.

2 LITERATURE REVIEW

2.1 Commonly used plastics

Plastics have wide applications in almost every major manufacturing industry, ranging from traditionally automobiles to advanced medicine. These materials are synthetic polymers derived from fossil fuels, and can be chemically manipulated to give rise to a wide range of strengths and shapes (Reddy et al., 2003; Du and Yu, 2002). The earliest man-made plastic was invented by Alexander Parkes, who demonstrated it at the 1862 Great International Exhibition in London (Bellis, 2011), and these new synthetic materials have been extensively used since mid Twentieth Century.

Commonly used plastics consist of a number of broad categories, namely high density polyethylene (HDPE), low density polyethylene (LDPE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) (Krevelen, 1990; Utracki, 1998; Van Krevelen and Te Nijenhuis, 2009).

Each of these polymers has its specific physical and mechanical characteristics, while all polymers, in general, have the following common properties.

1. Polymers can be processed and made to be very resistant to chemicals, including corrosive and oxidizing ones.

2. Polymers are good thermal insulators and are absolute electrical insulators.
3. Polymers are low in relative density, light in weight with varying degrees of mechanical strength.
4. They can be processed in various physic-chemical ways to produce thin fibers, films or very intricate parts of various shapes and sizes.
5. Elastomer and some plastics are very stretchable and flexible.

In general, polymers are materials with an apparently limitless range of characteristics, appearances and colors. They have many inherent properties which can be deeply enhanced by a wide range of additives to substantially expand their scope of applications (Padermshoke et al., 2005).

Since a wide range of plastics can be easily molded in relatively low costs into almost any desired shape, including fine fibers and thin films, these materials have been indispensable to cutting-edge technologies such as surgical sutures, prosthetic limbs, space program and bullet-proof vests. They also have high chemical or even acid resistance and are more or less elastic (Van Krevelen and Te Nijenhuis, 2009); hence popular in many durable, disposal goods, packaging materials and in domestic products such as beverage containers, medical devices and automobiles.

In the recent years, there has been increasing public concern over the adverse

impact and harmful effects of synthetic plastic waste disposed off in the environment (Venkateswar Reddy et al., 2012). There are more than 100 million tonnes of plastics produced yearly (Reddy et al., 2003). Most of them are discarded into landfills, while a small portion of which are illegally discarded into marine environments every year. As these materials are conventionally derived from petroleum, which are very valuable natural resources, and are not easily decomposed in nature by themselves or microorganisms. This may be attributed to the fact that these materials have been present in the environment for a relatively short period of time and natural microorganisms in the ecosystem have not evolved mechanisms or enzymes to disintegrate these materials, rendering their persistency and environmental impact (Mergaert et al., 1992). Therefore, managing and treating plastic wastes has been recognized as a serious problem today.

2.2 Environmental Problems Caused by Plastics

Plastics play an increasingly important role in various domestic, commercial and industrial applications, especially in the areas of packaging and disposable products (Bucci et al., 2005). However, due to their slow biological and photo-degradation in nature, and the predicted rapid exhaustion of the world petroleum reserves within the current century, the use of different types of polymers brings significant environmental problems such as the so called

‘serious white pollution’ that has been drawn increasing attention recently.

Technically, environmental problems directly caused by or associated to plastics can be classified into a few types. Firstly, the raw material of plastics, namely petroleum, which is valuable natural resource, is a non-renewable and limited one. It has been estimated that petroleum would be used up and completely exhausted within a hundred years. The governments of leading countries are carrying out different methods to solve these problems, one of which is to recycle waste plastics. However, these efforts have not been very successful owing to the difficulties in collecting plastics wastes and the high cost in cleaning and sorting that make it unwelcome in the market. Therefore, the accumulation of petrochemically derived plastic waste in the environment has become more severe year by year.

In general sense, there are three treatment methods for the conventional plastic wastes, which are disposing in landfills, incineration, and recycling and reuse. However, each one of these methods has its specific drawbacks.

For disposal of plastics in landfills, it is predicted that highly urbanized municipalities such as Hong Kong will not have enough land area to do this. And another problem is that the plastic waste in landfills, due to their non water permeability, blocks the infiltration of water, upsets the natural nutrient and soil structure and inhibits the health of microbial ecosystems (Reddy and Mohan,

2012b). In such circumstances, the general stability of the landfill is adversely affected.

For plastic waste disposal through incineration, it may be the simplest and most convenient way to deal with these wastes. However, there are severe consequences. In addition to the expensive capital and operating costs, it is potentially hazardous due to the inevitable emission of hazardous gases under fluctuating incineration temperatures. These stag gases consist of CO_2 which is known to contribute towards the green house effect and global warming, nitrogen oxides and sulphur oxides that will result in acid rain, highly toxic dioxins, polyaromatic hydrocarbons and hydrochloric acid. Among these by-products, dioxins are known to be carcinogenic, which pose great harm to human health (Gandini, 2011). Taking these environmental and public health factors into consideration, incineration has been opposed by many in municipalities such as Hong Kong.

For recycling and reusing plastic wastes, it has been faced with technical difficulties in collecting and sorting the wide variety of plastics and there are also changes in the plastics formulations in these materials resulting in impurities in the recycled plastic resins and thus limiting its further application range. The cost of whole process of recycle and reuse is often is higher than the original raw polymeric resins (Bucci et al., 2005; Yu et al., 2006). So it is obvious that the

above options have serious disadvantages and not effective to reduce the environmental impact given rise by plastic wastes.

Furthermore, it has been the interest of many researchers in the last century to develop physic-chemical methods to degrade plastic wastes. Plastics can be degraded by three different processes, either independently or in combination, namely (i) light or high energy radiation (photodegradation), (ii) heat decomposition, and (iii) microbial degradation (Kalia et al., 2000). However, the first two methods are unreasonably expensive while the third on is highly inefficient due to the very slow degradation rates.

Biodegradable Polymers as Environment-Friendly Substitutes for Conventional Plastics

Due to the aforementioned problems, developing environment-friendly substitutes for conventional petroleum-derived plastics has become very important and has aroused much interest among researchers. In recent years, with the high-pace development of large economies, such as China, substantial living standard improvements inevitably resulted in environmental pollution caused by disposal of plastic packaging materials and disposable products. This is the drive for the development of packaging materials that are biologically degradable. In particular, the disposable packaging products, such as garbage bags, shopping bags, lunch boxes and other product protective and filler materials, are areas that

degradable plastics are in urgent needs.

Developing "environment-friendly" substitutes for conventional petroleum-derived plastics has been recognized as one feasible solution to plastic waste problem. There has been great interest in developing biodegradable plastics that have properties of conventional plastics in various physical and mechanical aspects. Biopolymers are a category of materials that are renewable, largely biodegradable and can have very similar properties to conventional polyolefin polymers. These materials possess all desirable properties as conventional plastics and can be produced from agriculture or biotechnological processes (Steinbüchel, 2001; Lee et al., 1995).

Although environment-friendly substitutes, such as bio-plastics, are potential solution to overcome the environmental problems associated with the non-degradability of plastics. Unfortunately, several commercial applications of biodegradable polymers are still not yet market viable and fully competitive compared with most other conventional thermoplastics, due primarily to their compromised mechanical performances and higher production costs. These biomaterials have to be further optimized in order to have acceptable properties by blending conventional plastic formulations with other suitable compounds or by selecting the appropriate micro matrices.

Degradable plastics, as a new type of plastic, was first developed in the 1970s

(Gandini, 2011; Wong et al., 2005). In those days, the main development was focused on light-degradable or photodegradable plastics, particularly for industrial and commercial packaging products. In the 1980s, many studies were concentrated on biodegradable plastics and the use of renewable resources, such as plant starch and cellulose, animal chitin as raw materials instead of mineral oils to produce biodegradable plastics. However, widespread applications of these materials were often hampered by their relatively poor properties. These were improved by combining with conventional plastics such as polyethylene or polypropylene. This would in turn generate materials that were not completely degradable (Reis et al., 2003; 2011), and the associated environmental problems are not truly solved.

Following this, it was the main drive to develop fully biodegradable plastic materials. Along this trend, a family of more than 40 polyhydroxyalkanoates (PHAs) and related co-polymers have been discovered and quickly emerged as potential environment-friendly materials (Lam, 2010; Chua et al., 1997a; 1997b; Chen, 2009). Around the 90s, the starch filling in the commonly used plastics as biodegradable plastics were widely used among Asian countries. However, these were not completely degradable and caused unexpected environmental problems. These added on the urgency for the need of completely biodegradable plastics, such as the PHAs that are of biological origin. Currently, various types of biodegradable plastic films have been developed, but these are

still in its infancy due to the stability of the production process and the relatively high cost.

According to Scaffaro et al. (2012) and Harding et al. (2007), the numerous biodegradable polymers can be divided into three classes, namely (1) novel polymers that are derived from renewable sources, such as starch, proteins, etc., (2) intracellular biodegradable polyesters, such as PHAs, polylactic acid (PLA), and (3) aliphatic polyesters, such as polysaccharides, different copolymers and/or special blends of the above (Kalia et al., 2000). These observations agreed more or less with those reported by Chanprateep (2010), in that the wide range of biopolymers can also be classified into four basic groups based on their characteristics, constituting components and origins.

1. The first group consists of polymers that are generated directly from/by living organisms, namely microbes.

2. The second group is a family of polymers that are produced by polymerization of monomers that either exist in nature or are derived from materials that exist in nature.

3. The third group of polymers contain various combinations of monomers from renewable resources with a blend of petrochemical-derived monomers on a molecular level.

4. The fourth group includes polymers produced from specific blends of different renewable resources and petroleum-based materials, such as blends of starch and polyvinyl alcohol, on a macro levels.

On the other hand, these novel materials can also be categorized by their biodegradation process and biodegradability, into two classes, namely completely biodegradable plastics and bio-collapse plastics.

Furthermore, from the preparation and production methods, these materials can be divided into (1) generated from biological fermentation synthesis, (2) generated from chemical synthesis, and (3) produced from natural plant and animal polymers.

As discussed previously, one common and relatively inexpensive method to prepare biodegradable plastics is to incorporate starch granules or other natural macromolecules into conventional PE or other plastic resins. These are commonly used in all aspects of life, such as garbage bags, packaging and filling materials, sanitary and other disposable products. However, such biodegradable plastics are only partially biodegradable (Klemchuk, 1990). Normally, the incorporated starch granules or any other natural components should not exceed 30 % by weight, otherwise the material properties will be compromised. One main environmental problem associated with this is that the residual petroleum-based plastics portion (usually more than 70 % by weight) remains as

broken undegradable pieces in natural soil layers and landfills, creating additional pollutional problems (Chua et al., 1997a). The non-natural and chemically synthetic macromolecular fraction remains a threat to the environment, ecosystems and human health. Furthermore, the portion of embedded starch granules that are not exposed to the atmosphere and moisture cannot be effectively degraded, thus further lowering its biodegradability. Increased biodegradability by increasing the proportion of starch beyond 30 weight % in the formulation would adversely affect the mechanical properties of the plastic and thus hampering its applications. As a result, these plastics have a number of physical constraints, including intrinsic thermal and mechanical weaknesses, and as a result, they are now rarely utilized in large industrial scales (Chen, 2010b; Yu et al., 2006).

In the so-called starch plastics, the allosteric disorder of starch molecules forms thermoplastic starch resins, which are commonly added with a very small amount of plasticizers and other functional additives. The starch content can then go beyond 30% by weight. Furthermore, the small amounts of other substances and additives are also non-toxic and can be completely biodegraded. So the whole starch plastic is the almost fully degradable. Almost all plastic processing methods can be used in the processing of the whole starch plastic (Liu et al., 2009). Full starch plastic is considered to be the one of the most prospective fully biodegradable plastic, with the relatively high cost (more than

five times higher than conventional plastics) of production left as the main drawback. Complexity of the production in additive formulation and processing are also added disadvantages.

Therefore, utilization of microbial fermentation method to produce fully biodegradable plastics remains the better choice. Many selected species and genera of microorganisms have the ability to accumulate these macromolecules as their intracellular food reserves under specific ambient conditions. Other microorganisms in the natural environment are capable of degrading these macromolecules that are used to form biodegradable plastics under the action of the enzymes (Mergaert et al., 1992). This is because these macromolecules are accumulated as food reserves, which are meant to be readily degraded in the first place (Huisman et al., 1989). As a result, the important advantages of this series of novel materials over petrochemical plastics are that they are naturally formed, 100 % renewable, biocompatible with human tissues, non-toxic and non-hazardous to the environment (Verlinden et al., 2007).

Two of the most promising bio-based plastics, namely PLA and PHAs, have received much attention as potential replacements for other polymeric materials since late Twentieth Century (Chanprateep, 2010). Over the past decades, the main uses of PLA have been limited to medical applications such as implant devices, tissue scaffolds and internal surgical sutures, due to its high cost, low

availability and limited molecular weight and hence limited property variations (Lim et al., 2008). The costs of these materials remained around 6-7 times higher than that of conventional plastic resins, despite the various advancements in the production technology (Huisman et al., 1989; Kalia et al., 2000). About 80 % of the production cost is due to the upstream fermentation and the remaining 20 % is due to downstream extraction and purification (Chen, 2010a).

Figure 2.1 shows the biopolymer existing in a recyclable ecosystem involving biological, biochemical, artificial chemical syntheses and natural biological degradation. The entire ecosystem consists of a number of components, namely upstream process for bio-refinery and bioprocesses such as microbial fermentation factory and manufactory for bio-polymeric production. Representative biopolymers under this category are PHA and PLA (Taguchi et al., 2012). Other biodegradable plastics, namely polyglycolides (PGA), have also been gaining much attention. They are all thermoplastics and biodegradable polyesters, which are also mainly applied in the medical field, again due to the relatively high costs. The shelf life of such products, which is dependent on the degradation times of PLA and PGA, range from a few months to a few years depending on the formulation and the molecular weight (Reddy et al., 2003).

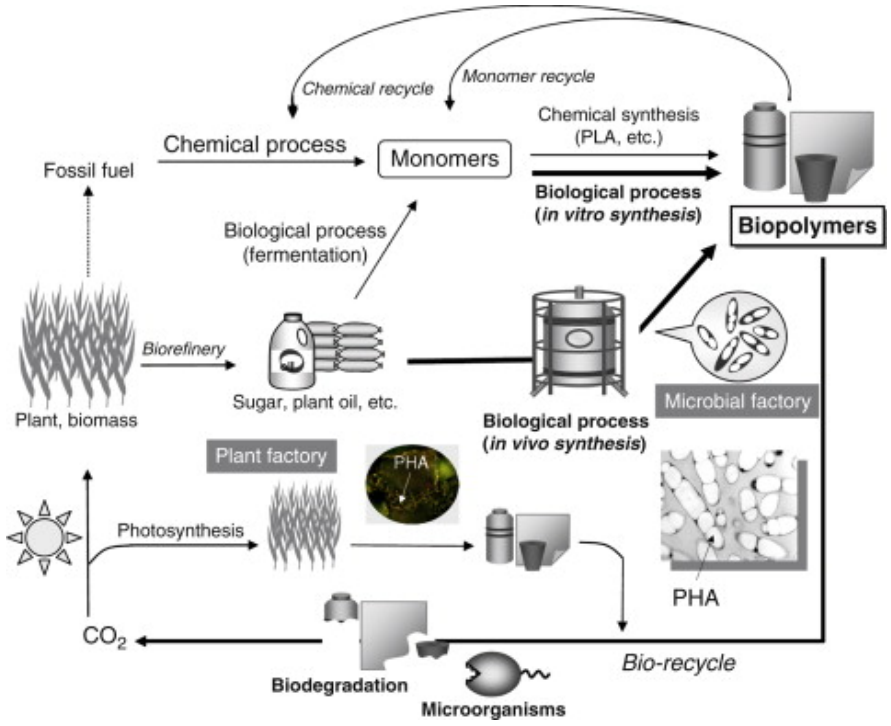


Figure 2.1 Biopolymer recyclable ecosystem involving biological and/or chemical syntheses and biological degradation (Taguchi et al., 2012).

The status and level of advancement of research and development of degradable plastics in China is basically in sync with the developed world. However, the earlier degradable plastics research began in the production of agricultural mulch film (Figure 2.2), since China is a large agricultural country and mulch consumption ranks the first in the world. In order to solve the severe environmental problem associated with of crop plantation, biodegradable plastics have been widely used (Yu et al., 2003). These eco-friendly materials are mainly used as agricultural mulch, or geo-films, for seedlings, which are not physically removed at the end of growing seasons. Some biodegradable plastics, after a series of biochemical changes and decomposition, they can eventually

remain in the top soil and be utilized as an alternative fertilizer or soil conditioner (Chen, 2003; Avella et al., 2001). The somewhat less stringent requirements on material purity rendered the production costs slightly lower, standing at around 5 times higher than that of conventional plastics (Md Din et al., 2006; Sato et al., 1998). However, this is still too expensive for widespread applications on domestic, commercial and industrial packaging materials and disposable products.

Figure 2.2 Agricultural mulch films.

As shown in Table 2.1, the relevant global policies and legislations that have been designed and enacted stating specific requirements for biodegradable/compostable plastics for various usages. This indicated that biodegradable plastics, with biological origins, are beginning to be taken seriously in many parts of the world.

Although it has many advantages, the high cost of commercial production for these bio-plastics contrasting with the low-cost production of petrochemical-derived plastics result in biodegradable polymers being left unused in a very long period of time over the decades. As analyzed previously, depending on the fluctuation of the oil prices, biodegradable plastics produced through pure-culture fermentation processes, namely PHAs, ranges from 5 to 7 times of conventional plastics, hence hampering widespread applications, namely as packaging materials and disposable domestic products (Haywood et al., 1990; Chua et al., 2003). It was particularly noted that the relatively high production costs was attributed 80 % to the upstream mono-culture fermentation process and 20 % to the downstream organic extraction and purification processes.

Table 2.1 The global policies and measures in different countries (Chanprateep, 2010)

Countries	National policies and measures
Germany	German Packaging Directive has been in force (2005). The compostable packaging will be exempt from the requirements in § 6 of the Directive
France	A law for the promotion of French agriculture has been in force (2006) stating a requirement for biodegradability of disposable retail carry bags by 2010
Italy	Markets in Florence had been charging €0.10–0.20 per plastic bag (2009)
Ireland, Scotland, Denmark and Sweden	These countries have already imposed levies and taxes on non-degradable plastic bags
UK	In 2003, county Durham has been charging Ecotax per plastic bag
US	San Francisco: in March 2007, the San Francisco Board of Supervisors approved first-in-the nation legislation that outlaws the use of non-biodegradable plastic bags in large supermarkets within 6 months and large chain pharmacies in about a year
Canada	Toronto City Council: retailers will be required to charge a minimum of 5 cents for each plastic retail shopping bag that customers take (2008)
Japan	Law on Promoting Green Purchasing and Law on Recycling have been in force in 2001
India	Plastic is officially banned in Ladakh
Australia	Thin non-biodegradable plastic shopping bags have been prohibited in South Australia from 4 May 2009
Bangladesh	From the beginning of January 2002, the Bangladesh government is banning the use of plastic bags in Dhaka

2.3 Development of PHAs

2.3.1 Historical Development of PHA Production

PHAs are polyesters that can be produced through chemical synthetic or fermentation processes using renewable sources, which can be synthesized by specific microorganisms, namely bacteria and possibly fungi, as intracellular carbon reserves and energy storage materials (Haywood et al., 1989; Haywood et al., 1988; Scaffaro et al., 2012). This PHA accumulation process is usually initiated in response to a variety of nutritional and micro environmental stress conditions (Byrom, 1994; Doi, 1990; Lee and Chang, 1995; Linko et al., 1993; Steinbüchel, 1991; Ward and O'Connor, 2005; Yamane, 2004). It is of great interest to elucidate the mechanisms for PHA production for lowering the cost, thus enabling large-scale applications and feasible replacement for conventional plastics.

The bacteria that are found to be capable of PHA accumulation included *Alcaligenes spp., Pseudomonas spp., recombinant Escbericbia coli* and a number of filamentous genera such as *Nocardia spp.* and *Gordonia spp*. (Chen, 2003; Chua et al., 1997a; 1997b). Haywood et al. (1990) utilized *Pseudomonas spp. strain* NCIMB 40135 to accumulate PHAs from a wide range of carbon sources (C2 to C6). However, PHAs from this accumulation process contained longer-chain monomer units (hence limiting the property varieties), and the yield

was only 16.9% (wt/wt) under nitrogen limitation in a continuous culture. The *Alcaligenes eutrophus* can accumulate a short-chain copolymer including 3HB and 3HV, thus improving on the physical and mechanical properties (Anderson et al., 1990). A study investigating the production and characterization of biodegradable polymers from *Pseudomonas oleovorans* and *Rhodospirillum rubrum* obtained the results that oxygen concentration and oxidation reduction potential (ORP) affected cell growth, polymer content and cell yield (Fuller and Lenz, 1989; Hu, 1999).

The homopolymer of 3-hydroxybutyrate (3HB), Polyhydroxyburate (PHB) was first discovered in the middle of the 1920s (Lemoigne, 1926), and was left basically untouched for several years. Since the discovering of more varieties and usages of PHAs in 1926, these materials have then been studied extensively by biochemists (Hu, 2004). Chanprateep (2010) reported that the history of commercialized PHAs and their applications dated back to 1959 when W. R. Grace and Company produced PHB in the U.S. for possible commercial applications. But this ended in a failure due to the extremely low production efficiency and a lack of suitable down-stream purification methods in those days.

Wallen and Rohwedder (1974) on the other hand discovered that PHAs contained other 3-hydroxyalkanoates in addition to 3HB in 1974. This new material melts at 97-100°C, whereas PHB melts at 160-170°C. Due to the discovery of numerous

new PHAs with a wide variety of physical and mechanical properties in the early 1990s, the production and applications of PHAs flowered and many commodities were manufactured from PHAs, while the cost remained high.

After many practices and trials, PHAs have received significant concern due to their similar physical material properties and processibility to conventional plastics and complete photo- and biodegradability (Braunegg et al., 1998; Lee and Choi, 1999; Song et al., 1999). They can be produced by technologically improved and more efficient bacterial fermentation processes (Van Wegen et al., 1998; Chen, 2010b), and the downstream extraction and purification processes were also fairly well established. PHAs can be entirely bio-degraded within a few months by a variety of bacteria and a wide range of other microorganisms in the natural ecosystem, such as prokaryotes and eukaryotes (Brandi et al., 1995). It is different from petroleum-derived plastics which take several decades or even longer to degrade. This biodegradation of PHAs results in carbon dioxide and water under aerobic conditions, which are then returned to the environment (Suriyamongkol et al., 2007). On the other hand, under anaerobic or anoxic conditions, the degradation products and intermediates are carbon dioxide and methane in an approximately 25:75 volume ratio (Reddy et al., 2003). However, the degradation rates have to be controllable considering the required shelve life of the products made from PHAs. This warrants further research and development work in order to improve the applicability of these novel materials.

It was found that PHAs stored in microbial cells can be utilized as carbon and energy sources under aerobic condition. Acetyl-CoA submitted to the TCA cycle for energy generation and biomass growth when excess oxygen and nitrogen sources on the condition of balanced growth and favorable ambient conditions. As a result, the concentration of free CoASH is high and caused the repression or inhibition of PHA synthesis (Lee et al., 1995). High concentration of pure oxygen lead to the excess oxygen supply in cultural medium, and ultimately prohibited the PHA synthesis, which is favorable under low ORPs and Dos, that brought on the declining of PHA content in Excessive Activated Sludge (EAS) (Hu et al., 2005).

The process for production of PHAs at laboratory-scale commonly includes three stage: (1) acidogenic fermentation or organic acid-forming process to convert wastewater organic matters to volatile fatty acids (VFAs), (2) an activated sludge system operating under feast/famine conditions to enrich the microbial ecosystem for PHA accumulating and producing organisms, and (3) accumulation of PHAs in batch or fed-batch cultures (Bengtsson et al., 2008).

2.3.2 Biosynthesis of PHAs

PHA biosynthesis has been studied in great depth in recent years. PHA synthesis is a normal way for bacteria to store carbon and energy intracellularly under unfavorable conditions, such as a deficiency of nutrient supplies (Wong et al.,

2004). These polyesters can be accumulated as the bacterial growth are subject to conditions nitrogen, phosphorous and potassium deficiency (Shang et al., 2003). Furthermore, metabolic engineering techniques are also being extensively investigated and explored to introduce new metabolic mechanisms and pathways to broaden the utilizable substrate range, to enhance intracellular PHA synthesis and accumulation, and to produce novel PHAs with wide ranging properties (Din et al., 2006).

Generally speaking, there are three steps for PHA biosynthetic pathway. The first step is β-Ketoacyl-CoA thiolase catalyzed. Acetoacetyl-CoA reductase is the second step in the PHB biosynthesis pathway by converting acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The third step is the polymerase actions in PHA biosynthetic pathway.

Biosynthesis of different types of PHAs depends on the chosen microorganisms, fermentation conditions and different carbon sources. For example, PHB, a common PHAs, can be produced when glucose is used as a carbon sources for bacteria, but growing the bacteria on valeric acid leads to production of PHV (Chua et al., 1999; Yu et al., 1999).

For example, the intracellular presence and relative proportions of different types of PHAs, and their copolymeric compositions are dependent on the molecular characteristics and types of carbon substrate available. The microorganisms can

take up such organic substrates as acetate and activate it to form acetyl-CoA. Acetyl-CoA is then consumed for the subsequent synthesis of PHB by condensation to acetoacetyl-CoA, reduction to hydroxybutyl-CoA and finally polymerization to PHB (Figure 2.3).

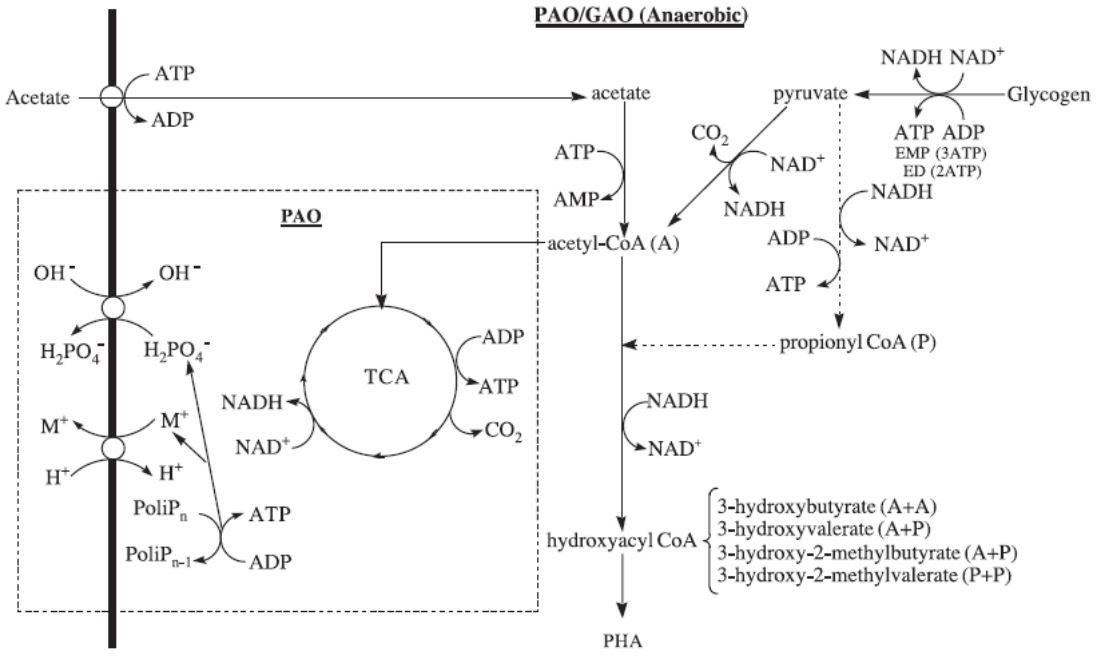


Figure 2.3 PHA production metabolism in PAO/GAO system (Salehizadeh and Van Loosdrecht, 2004).

Many researchers have concentrated their effort on investigating the intracellular production and accumulation of PHAs by mixed cultures when the microbial culture is subjected to an intermittent carbon supply. Different feeding and operational patterns in an aerobic SBR influences the interspecies composition and physiological state of the biomass which in turn affects the substrate removal pathway (Çığgın et al., 2011). Intermittent feeding causes a strong increase of

substrate uptake rate. Under dynamic conditions, growth of biomass and storage of polymer occur at the same along with excess external substrate. When all the external substrate is exhausted, the previous stored intracellular polymers can be spontaneously utilized as carbon and energy sources (Figure 2.4).

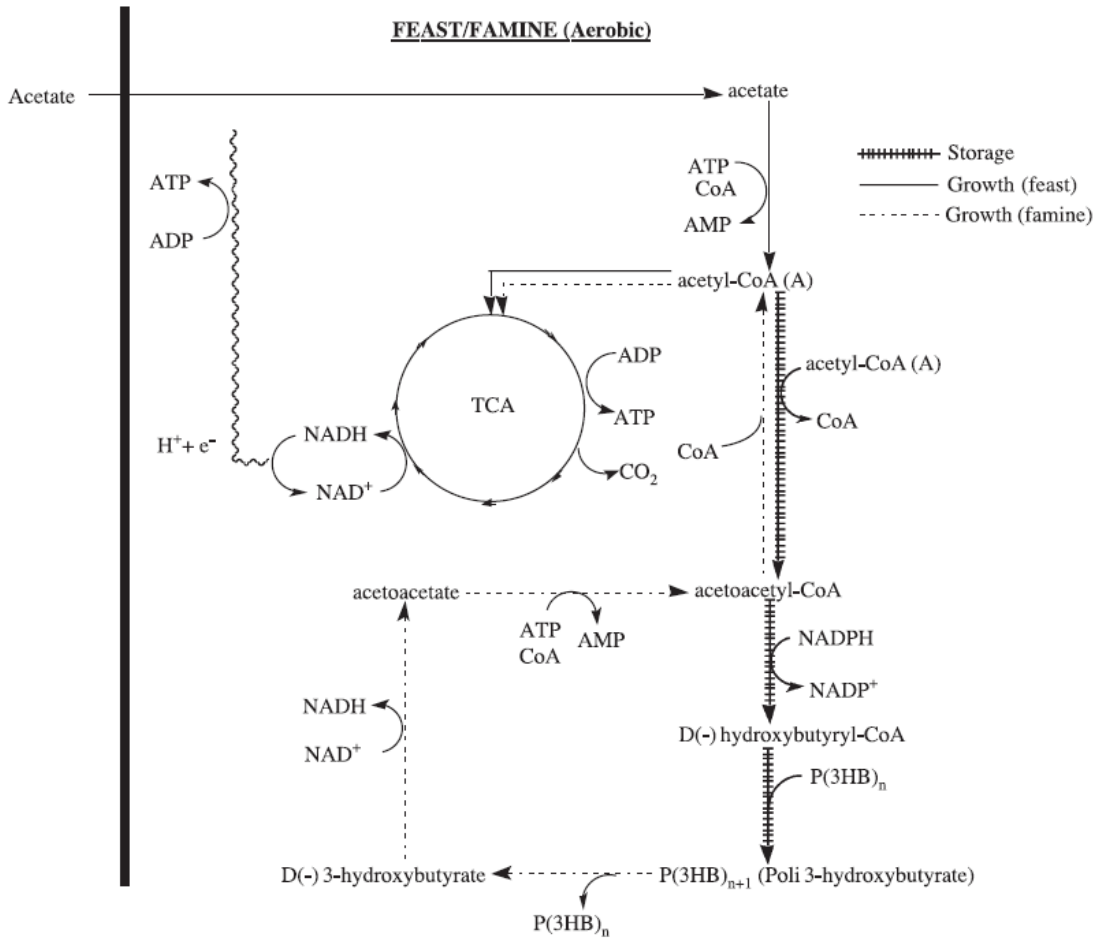
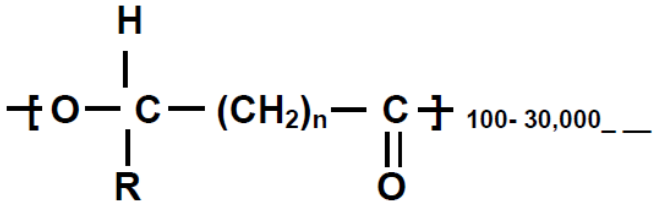


Figure 2.4 PHA production pathways in feast/famine conditions (Salehizadeh and Van Loosdrecht, 2004).

2.3.3 Structure of PHAs

PHAs can be divided into three groups depending on the number of carbon atoms in the molecular structures (Lee et al., 1995).



n = 1 R = hydrogen 3-hydroxypropionate

R = methyl 3-hydroxybutyrate

R = ethyl 3-hydroxyvalerate

R = propyl 3-hydroxyhexanoate

R = pentyl 3-hydroxyoctanoate

R = nonyl 3-hydroxydodecanoate

n = 2 R = hydrogen 4-hydroxybutyrate

R = methyl 4-hydroxyvalerate

n = 3 R = hydrogen 5-hydroxyvalerate

R = methyl 5-hydroxyhexanoate

n = 4 R = hexyl 6-hydroxydodecanoate

Figure 2.5 General structure of PHAs.

The following Figure 2.6, Figure 2.7 and Figure 2.8 show the chemical and molecular structures of PHB, Polyhydroxyvalerate (PHV) and Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)(PHBV), respectively.

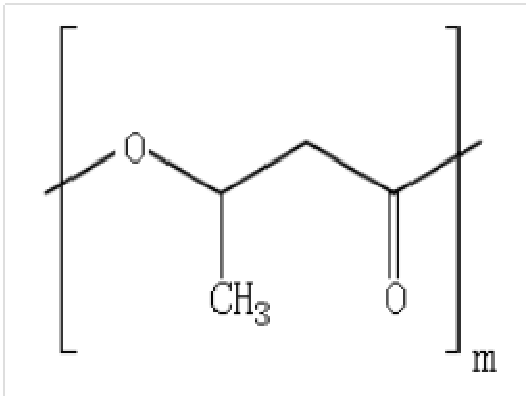


Figure 2.6 Structure of poly-3-hydroxybutyrate, PHB (Adapted from Wikipedia, 2012).

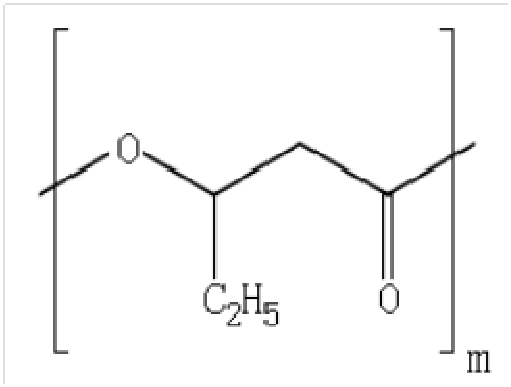


Figure 2.7 Structure of poly-3-hydroxyvalerate, PHV (Adapted from Wikipedia, 2012).

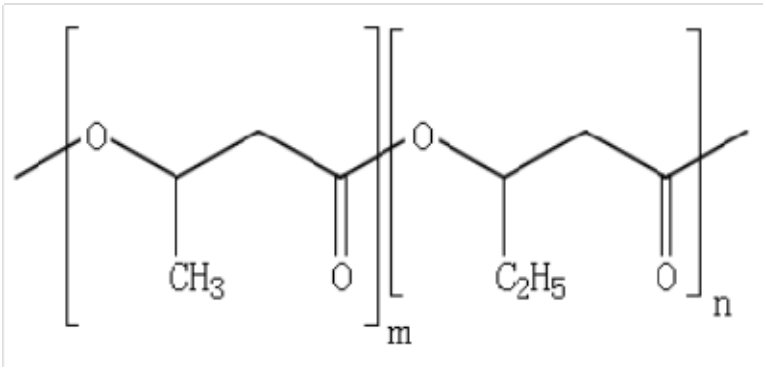


Figure 2.8 Structure of poly-3-hydroxybutyrate-co-hydroxyvalerate, PHBV (Adapted from Wikipedia, 2012).

2.3.4 Properties of PHAs

The different members in the PHA family are generally non-toxic, biocompatible to human tissues, and biodegradable thermoplastics with a high degree of polymerization. They are also highly crystalline, optically active and isotactic, piezoelectric and insoluble in water, which make them highly competitive and comparable with polypropylene and the petrochemical-derived plastic (Reddy et al., 2003).

The most important property distinguishes PHAs from common plastics is its biodegradability in the natural environment. Biodegradation process dependents on many factors, including such parameters as microbial characteristics (including biomass concentration, population diversity, enzyme activities), substrate conditions, and a series of environmental physic-chemical factors (including pH, temperature, moisture content, availability of electron acceptors and carbon and energy sources), all of which affect the acclimation period and rate of the microbes to the substrate (Boopathy, 2000).

There are many methods to classify biopolymers. Most commonly, according to the number and arrangement of carbon atoms in the monomer units of PHAs, they can be broadly divided into two groups (El-Hadi et al., 2002; Philip et al., 2007; Sanchez et al., 2003): the short chain length PHAs (3-5 carbon atoms) with its monomers containing HB and/or HV and medium-chain-length PHAs (6-14

Carbon atoms) (C4 and/or C5) (Yao et al., 1999). The chain length of PHAs determines their different physical, mechanical and thermal properties: for example, SCL-PHAs exhibit hard and brittle properties, while MCL-PHAs exhibit soft and elastic mechanical properties. A number of copolymers of SCL-PHAs and MCL-PHAs have useful and flexible physical and mechanical properties. This is why they are the most potentially suitable materials for industrial applications and future development.

Many different monomeric units have been identified as constituents of the storage PHAs, which creates a possibility for producing different types of biodegradable polymers with an extensive range of properties, and hence affecting their degradation capability and ultimate applications (Madison and Huisman, 1999).

PHAs have a variety of physical, mechanical, thermal and biodegradability properties depending on the molecular and polymeric structures (See Figure 2.9 and Figure 2.10).

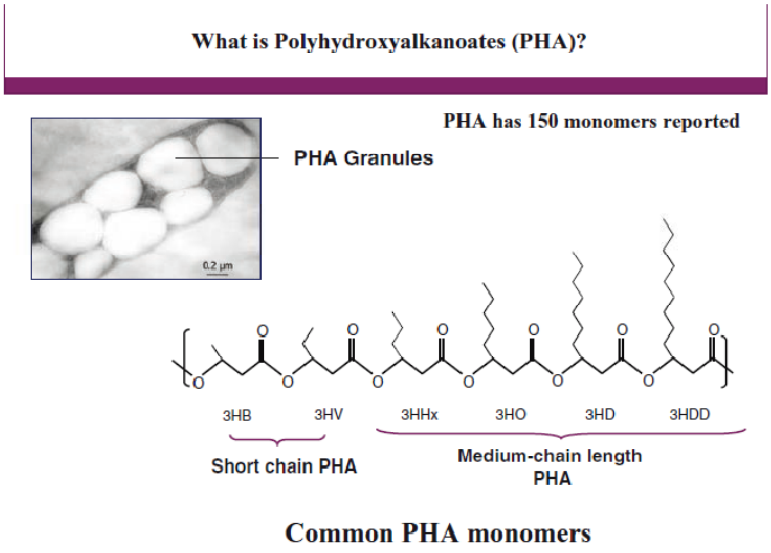


Figure 2.9 Common PHA monomer structures. Short-chain-length monomers: 3-hydroxybutyrate(3HB), 3-hydroxyvalerate(3HV). Medium-chain-length monomers:3-hydroxyhexanoate(3HHx),3-hydroxyoctanoate(3HO),3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) (Chen, 2010a).

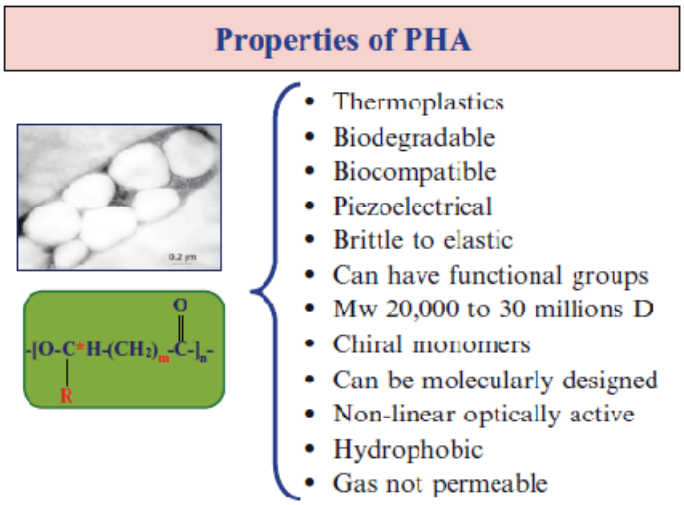


Figure 2.10 Common properties of PHA (Chen, 2010a).

A summary of the physical, mechanical and thermal properties of some more established and commercialized PHAs is presented in Table 2.2.

Table 2.2 The physical, mechanical and thermal properties of some commercialized PHAs (Chanprateep, 2010)

	PHB		PHB copolymers		PHBV		PHBH
	Biomer240	Biomer P226	MirelP1001	MirelP1002	Biocycle100	Biocycle24005	Kaneka
Application grade	Injection mold		Injection mold		Extrusion and injection		Foam mold
Physical properties							
Melt flow rate (g/10 min)	5–7	9–13			10–12	15–25	5–10
Density (g/cm^3)	1.17	1.25	1.39	1.30	1.22	1.20	1.2
Crystallinity (%)	60–70	60–70			50–60		
Mechanical properties							
Tensile strength (MPa)	18–20	24–27	28	26	30–40	25–30	10–20
Elongation (%)	10–17	6–9	6	13	2.5–6	20–30	10–100
Flexural strength (MPa)	17	35	46	35			
Flexural modulus (GPa)			3.2	1.9			0.8–1.8
Thermal properties							
Melting temperature (°C)					170–175		
VICAT softening point (°C)	53	96	148	137			120–125

A summary of short-chain-length PHA production by various genera and species of bacteria is presented in Table 2.3.

Table 2.3 Summary of short-chain-length Polyhydroxyalkanoate (PHA) production by various bacteria. P(3HB) Poly(3-hydroxybutyrate), P(3HV) poly(3-hydroxyvalerate), YE yeast extract, CSL corn steep liquor (Choi and Lee, 1999).

Strain	PHA	Fermentation strategy	Substrate	Time (h)	Cell concentration (g/l)	PHA concentration (g/l)	PHA content (%)	Productivity ($g\ l^{-1}\ h^{-1}$)	Reference
Ralstonia eutropha	P(3HB)	Fed-batch	Glucose	50	164	121	76	2.42	Kim et al. 1994a
	P(3HB)	Fed-batch	Glucose	74	281	232	82	3.14	Ryu et al. 1997
	P(3HB)	Fed-batch	Tapioca hydrolysate	59	106	61	58	1.03	Kim and Chang 1995
	P(3HB/3HV)	Fed-batch	Glucose + propionic acid	46	158	117	74	2.55	Kim et al. 1994b
Alcaligenes latus	P(3HB)	Fed-batch	Sucrose	18	143	71.4	50	3.97	Yamane et al. 1996
	P(3HB)	Fed-batch	Sucrose	20	111.7	98.7	88	4.94	Wang and Lee 1997a
Azotobacter vinelandii	P(3HB)	Fed-batch	Glucose + fish peptone	47	40.1	32	79.8	0.68	Page and Comish 1993
Azotobacter chroococcum	P(3HB)	Fed-batch	Starch	70	54	25	46	0.35	Kim and Chang 1998
Methylobacterium organophilum	P(3HB)	Fed-batch	Methanol	70	250	130	52	1.86	Kim et al. 1996
Recombinant *E. coli*	P(3HB)	Fed-batch	Glucose[a]	42	117	89	76	2.11	Lee et al. 1994
	P(3HB)	Fed-batch	Glucose + YE + CSL + casein hydrolysate	41	112	81	72.3	1.98	Lee and Chang 1994
	P(3HB)	Fed-batch	Glucose	49	204.3	157.1	77	3.2	Wang and Lee 1997b
	P(3HB)	Fed-batch	Sucrose[a]	48	124.6	34.3	27.5	0.71	Lee and Chang 1993
	P(3HB)	Fed-batch	Whey	49	87	69	80	1.4	Wong and Lee 1998
Recombinant *Klebsiella aerogenes*	P(3HB)	Fed-batch	Molasses	32	37	24	65	0.75	Zhang et al. 1994

[a] Fed-batch culture in complex medium

2.3.5 PHB and PHBV

The generic term 'PHAs' is essentially referring to a huge family of polymers and copolymers including PHB, PHV, 3H2MB (3-hydroxy-2-methylbutyrate) and 3H2MV (3-hydroxy-2-methylvalerate).

The most commonly studied members of the PHAs family are PHB that can be produced in high yield by specialized fermentation processes with a variety of bacterial strains. It is an example of a microbial polyester (Morikawa and Marchessault, 1981). PHB as a biodegradable thermoplastic of considerable commercial importance first aroused intense attention decades ago (Anderson and Dawes, 1990; Dawes, 1988).

It has many properties similar to PE, which are thermoplastic process ability, complete resistance to water, and complete biodegradability by bacteria and fungi (Lee and Gilmore, 2005). Their rapid biodegradability in natural environment renders them extremely desirable substitutes for conventional synthetic plastics (Harding et al., 2007; Kumagai and Doi, 1992; Reddy et al., 2003). It was also the first homopolymer PHAs to be discovered (Chen, 2010a).

PHB has characteristics that increase crystallinity, stiffness and brittleness of the end product (Rhu et al., 2003). Copolymers of PHB and other monomers are easily formed when mixed substrates consisting of more than two types of

organics are used, such as a mix of glucose and valerate (Chua et al., 1999; Yan et al., 2005; Yu et al., 1999). Its copolymers called hydroxyvalerate (HV) with varying ratios are also widely used. The other copolymers of hydroxybutyrate (HB) with hydroxylvaleric acid hold less crystalline, more readily processable and more flexible than PHB by itself. Their diverse properties such as piezoelectricity, relative density, optical activity, physical processibility, natural origin, biodegradability, biocompatibility, and thermoplasticity make them suitable for a variety of applications in health and other specialized industries.

However, PHB homopolymer is a tough, highly crystalline, rigid, stiff and brittle material. Incorporation of a number of different monomeric units, other than HB, in the polymer chain, can generally result in specialized copolymers with improved mechanical properties. And this structure seems to be similar in various copolymers (Lemos et al., 2006; Padermshoke et al., 2004; Padermshoke et al., 2005).

It has been found that PHB is an amphiphilic lipid ubiquitous in cellular membranes of bacteria, plants and animals. Different sources, such as natural isolates, recombinant bacteria, plants and other methods are being researched to exert an influence on the quality, quantity and economics of PHB production.

The natural distribution and existence of PHB in human plasma can be detected using chemical and immunological methods (Reusch et al., 1992). In

microorganisms, PHB can serve as an intracellular energy and carbon storage product acting as glycogen in mammalian tissues.

Another important property of PHB is that it is bio-compatible with human tissue, presenting it a suitable material for surgical sutures, fractured-bone supports and other medical and surgical applications. Furthermore, it has been found to have low toxicity, to some extent because it degrades in vivo to HB, a normal constituent of human blood, which means it is a metabolite normally present in blood. They have been used to support cell growth in vitro, guide tissue growth inside the body, and also found to be fully biocompatible to several cell lines, including articular cartilage chondrocytes, osteoblasts and epithelial cell (Wang, 2007).

However, the costs of PHB production remain high, about seven to ten times that of traditional plastics (depending on the prices of petroleum), and its applications are restricted to very specialized areas (Shirai et al., 1994). Normally, pure homo-polymers of PHB is stiff and brittle. But when adding other monomers in the polymer, the resulting copolymers has elasticity and flexibility substantially increased thus making the polymer with properties similar to PP (Lee, 1996).

For specific reasons mentioned above, an extensive survey of the chemical and physical properties of PHAs is conducted as follows.

There are some properties of PHB shown as follows,

1. PHB is insoluble in water and relatively resistant to hydrolytic degradation, but soluble in organic solvents such as chloroform and other chlorinated hydrocarbons;
2. PHB shows good oxygen permeability;
3. PHB has good ultraviolet resistance but has poor resistance to acids and bases;
4. PHB is biocompatible and hence is suitable for medical application;
5. PHB has a melting point around 175℃ and a glass transition temperature around 15℃;
6. PHB has a tensile strength of 40 MPa, which is close to that of PP;
7. PHB sinks in water which facilitates its anaerobic biodegradation in sediments;
8. PHB is non-toxic.

It is generally known that the PHB content of the end product of a typical fermentation process may be increased as the carbon sources with even numbers of carbon groups are used, such as often used substrate acetate and butyrate (Saito et al., 1995).

In recent years, PHBV has emerged as a new generation of PHB-based materials. Compared with the wide range of copolymers of PHB, PHBV or MCL-PHAs,

they are less stiff, but still retaining most of other mechanical properties of PHB. Carrasco et al. (2006) investigated that PHB decomposition starts at 246.3°C, while the value for PHBV is 260.4°C, which shows that the presence of valerate in the chain increases the thermal stability of the polymer. Degradation rate of PHBV can be easily adjusted by altering the copolymer composition. It is also indicated that different PHA copolymers could be produced with distinct polymer properties with the use of different types of food wastes as the C sources (Yu et al., 1998).

In the typical PHA metabolic pathway that is current known and published, bacteria produce acetyl-coenzyme-A (acetyl-CoA), which is converted into PHB by three specific biosynthetic enzymes (Figure 2.11).

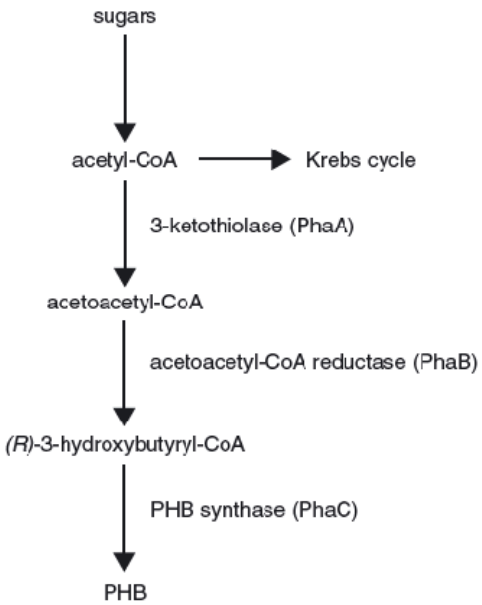


Figure 2.11 Metabolic pathway to PHB (Verlinden et al., 2007).

Wen et al. (2010) noticed that the PHB/PHA ratio increased with the increasing P limitation in the substrate. This indicated that activated sludge biomass tends to produce more PHB than PHV under the stress of P limitation. However, in-depth investigation on this has not been conducted yet. From the following Table 2.4, the maximum percentage PHA accumulation was observed when the influent C:P was equal to 750.

Table 2.4 PHB and PHV production at different C:P weight ratios (%) (Wen et al., 2010)

Running time	C:P = 100			C:P = 160			C:P = 250		
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA
Anaerobic beginning	7.14	3.08	0.70	11.07	2.04	0.84	13.67	4.18	0.76
Anaerobic end	8.51	3.14	0.73	9.41	1.79	0.84	22.14	4.34	0.84
Aerobic beginning	8.63	2.72	0.76	15.05	1.75	0.89	17.28	3.47	0.83
Aerobic for 2 hr	3.71	1.62	0.70	13.82	1.39	0.91	12.90	3.29	0.79
Aerobic end	1.84	0.56	0.77	NA	NA	NA	8.79	1.34	0.87
Running time	C:P = 500			C:P = 750					
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA			
Anaerobic beginning	7.56	0.51	0.94	NA	NA	NA			
Anaerobic end	9.05	0.64	0.93	18.98	0.45	0.98			
Aerobic beginning	14.47	1.00	0.93	23.70	0.27	0.99			
Aerobic for 2 hr	15.48	0.70	0.96	34.67	0.44	0.99			
Aerobic end	NA	NA	NA	30.08	0.39	0.99			

NA: not available.

As shown in Table 2.5, the PHB/PHA ratios also increased, from 0.64 to 0.94. The higher PHB/PHA ratio was again due to the use of acetate as carbon source, thus giving rise to a favorable property of the final polymer and copolymer mixture. It is noted that PHV production is higher under conditions of N limitation than under P limitation, compared Table 2.4 and Table 2.5.

Table 2.5 PHB and PHV production at different C:N weight ratios (%) (Wen et al., 2010)

Running time	C:N = 20			C:N = 60			C:N = 100		
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA
Anaerobic beginning	7.90	4.44	0.64	NA	NA	NA	32.80	4.49	0.88
Anaerobic end	NA	NA	NA	17.07	5.39	0.76	30.49	4.34	0.87
Aerobic beginning	5.49	2.58	0.68	NA	NA	NA	36.10	4.70	0.88
Aerobic for 2 hr	2.93	1.45	0.67	14.94	4.40	0.77	25.52	3.62	0.87
Aerobic end	1.25	0.30	0.80	10.89	2.37	0.82	30.86	2.79	0.92
Running time	C:N = 125			C:N = 180					
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA			
Anaerobic beginning	45.31	3.97	0.92	37.69	4.31	0.89			
Anaerobic end	54.11	4.88	0.92	NA	NA	NA			
Aerobic beginning	NA	NA	NA	40.36	4.54	0.90			
Aerobic for 2 hr	43.34	2.91	0.93	NA	NA	NA			
Aerobic end	40.21	2.48	0.94	39.37	3.92	0.91			

NA: not available.

2.4 Applications of PHAs

PHAs can be used in various ways in domestic, commercial and industrial applications, same as many non-biodegradable petrochemically-derived plastics, either in their pure forms or as specific additives to other oil-derived plastics such as PE owing to their novel features. However, these bio-plastics are far more expensive than petro-chemically based plastics and are therefore very commonly used in a number of specialized applications that conventional plastics cannot suffice or perform satisfactorily (Verlinden et al., 2007).

Over the past decades, PHAs have been widely applied in various fields (Figure 2.12). They have been used in medical application (Zinn et al., 2001), coating

applications (Verlinden et al., 2007), packaging which focused on containers and films (Bucci et al., 2005), disposable items, such as razors, utensils, diapers, feminine hygiene products, cosmetic containers-shampoo bottles and cups (Reddy et al., 2003), and agricultural membrane and geo-films (Chua, 1995). Particularly, PHB is regarded as an environment-friendly and biocompatible plastic with promising applications in medicine, pharmacy and various specialized industries (Philip et al., 2007).

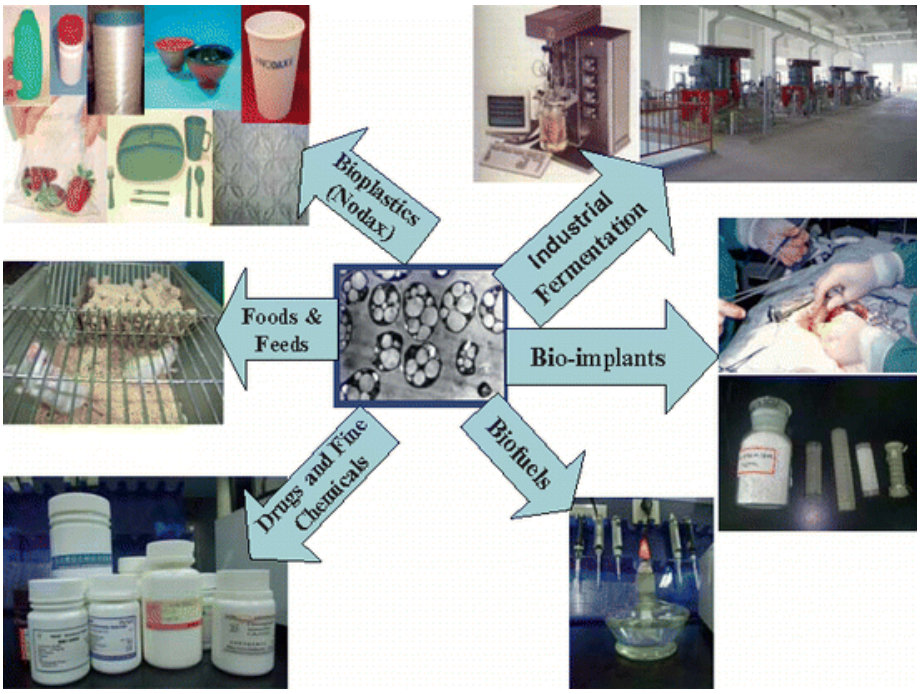


Figure 2.12 Applications of polyhydroxyalkanoates (PHAs) in various fields (Chen, 2009).

Among these categories, the most popular use is medical applications at present, especially applied to develop certain items, such as surgical sutures, nerve cells repair devices, skin repair patches, arm slings, cardiovascular patches, orthopedic

pins and supports, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone-marrow scaffolds, tissue engineered cardiovascular devices and wound dressings (Valappil et al., 2006).

It is shown in the following Table 2.6 and Figure 2.13 for the application of PHAs.

Table 2.6 Application of PHAs

Application Area	Product
Commercial	Packaging films, bags, containers, paper coatings
Agriculture	Biodegradable carriers for long term dosage of drugs, medicines, insecticides, herbicides, fertilizers
Household	Utensils, feminine hygiene products, cosmetics containers, shampoo bottles, cups
Medical area	Surgical pins, staples, swabs, bone and blood vessel replacement, wound dressings

Figure 2.13 The products of PHAs.

The most prominent advantages of PHAs in medical use and pharmaceutical field, e.g. for slow-release capsules in numerous medications. This is because these materials are biocompatible with human tissues, which can be inserted into human body and does not need to be removed again after applications. Furthermore, these materials have lower degradation rate, less inflammatory and they have been proved to be non-toxic to living organisms (Volova et al., 2003).

For such beneficial characteristics of PHAs, they have large potentials to be applied as specialty polymers.

Therefore, it is urgently necessary to substitute conventional plastics with PHAs that degrade in a relatively short period of time when exposed to a suitable and biologically active environment. For example, they are undertaken as drug carrier, drug vector for protein drug delivery (Pouton and Akhtar, 1996), and scaffold material in tissue engineering (Chen and Wu, 2005; Misra et al., 2006; Williams et al., 1999; Zhao et al., 2003).

In the past decades, PHAs became potential candidates for use as drug carriers due to these characteristics (Gould et al., 1987; Korsatkowabnegg and Korsatko, 1990). Unfortunately, only PHB and PHBV have been studied for controlled drug release up to now. The expectation is that a number of other PHAs family members with diverse physic-chemical characteristics and properties will bring more controlled-release or slow-release properties for the drug and pharmaceutical production industry, particularly chiral monomers for these applications are expected to be further exploited in the near future (Chen, 2010a) (Figure 2.14).

However, there are still many limitations and shortcomings. Firstly, to be of practical interest, not every PHAs can be used in medical applications on the special living system and living conditions. The polymeric structure that can

fulfill the specific and complex requirements of one niche application remains a big issue (Vert, 2005). Secondly, PHAs used in contact with blood has to be free of bacterial endotoxins. Thus, the downstream technology of PHA extraction and purification must satisfy rather stringent specific requirements for medical utilization (Sevastianov et al., 2003). Thirdly, they are of limited applications due to the relatively slow biodegradation in the natural environment and high hydraulic stability and non-permeability in sterile tissues (Wang and Bakken, 1998).

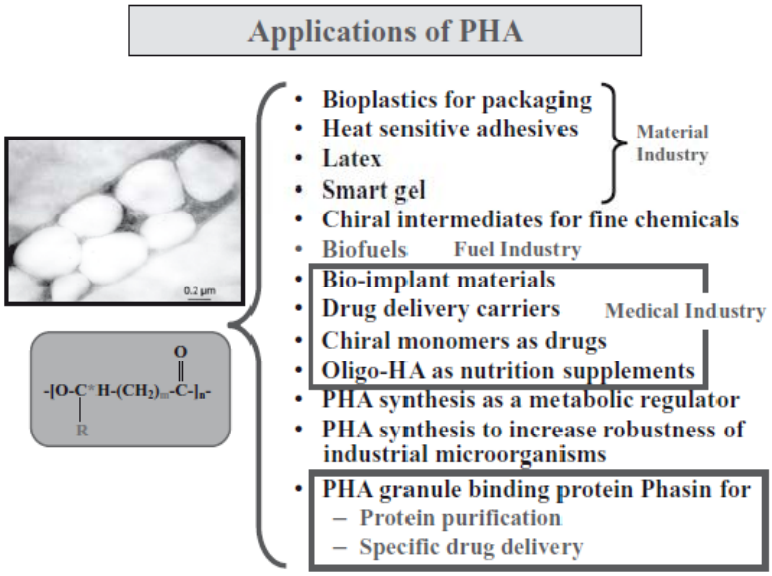


Figure 2.14 PHAs has been developed into an industrial value chain (Chen, 2010a).

Zhang et al. (2009) conducted an experiment to show that microbial PHAs can be used as a new and renewable type of biofuel. It has been roughly estimated by a number of researchers that the production cost of PHA-based biofuel should be around US$1,200 per ton. As the application of PHAs did not require highly

purified PHAs, the production process appeared to be much simpler and of reasonably low costs.

In addition, the extensive range of physical properties and the extended performance of the PHA family can be received by chemical modification (Zinn and Hany, 2005) or blending (Gao et al., 2006; Kunze et al., 2006). For example, copolymerization of PHA monomers combined with common available polymer monomers will generate a lot of new copolymers. There will be wider application of the plastics composed of these new copolymers and provide a broad range of potential end-use. Up to now, we realize that the PHA synthesis enzyme behaves broad substrate specificity and thus a wide variety of monomers can be polymerized. However, this area has not attracted too much attention, possibly due to the high cost of PHA production (Ojumu et al., 2004). Therefore, it is expected that the future trend and development is to focus on the discovery of more efficient and economically feasible fermentation processes for PHA production, downstream isolation, organic extraction and purification, and substantial improvement of PHA material characteristics and properties (Keshavarz and Roy, 2010).

2.5 Microbial growth substrate

One of the most important factors that determine the type of PHA constituents is the variation in concentration and combination of carbon sources in the feed.

For industrial scale production, the cost of microbial growth substrates is a decisive factor. Therefore, appropriately selected carbon sources are essential to meet economic requirements. Use of low-cost substrates and byproducts represent economical and environmental methods (Moita and Lemos, 2012), as the cost of the carbon source contributes significantly to the overall production cost of PHAs (Yamane, 1992; 1993).

Choi and Lee (1999) found that crude and natural carbon substrates, such as cane and beet molasses in sugar refinery, cheese whey in dairy production, plant oils and hydrolysates of starch, cellulose and hemicelluloses in food-processing industries had the low price, so they should be utilized as the excellent substrates for the bacteria. The use of these crude carbon substrates to produce PHAs could lead to significant economical advantages (Quillaguamán et al., 2005).

Novel bacterial and other microbial strains identified in laboratories have been reported regularly, while a number of research groups have struggled to optimize PHA production with inexpensive carbon sources that are needed by all living organisms. But several bacteria can produce PHAs from these inexpensive carbon substrates, in general, the PHA content and productivity are much lower than those received from purified carbon substrates from the previous findings (Ramsay et al., 1995; Yu et al., 1998).

A number of recent research works have demonstrated that the technical

feasibility of producing PHAs in mixed cultures using the specialized growth substrates. It has been demonstrated that the use of mixed cultures with inexpensive substrates (such as industrial waste materials) can substantially reduce the costs of PHA production by as high as 50% (Reis et al., 2003).

2.5.1 Food waste

Rhu et al. (2003) investigated that using food waste to produce PHAs with SBRs. Seed microbes were acclimated with synthetic substrate ahead of the application of the fermented food waste. SBRs were carried out under the condition of limited oxygen and nutrients. As a result, the maximum PHA content reached 51% with P limitation. At last, the authors did an economic analysis. It's suggested that the PHAs produced from the food waste could be a substitute to produce the biodegradable plastic for reducing the use of bags.

A new technology developed by Du and Yu (2002) showed the food wastes were digested in an anaerobic reactor to produce PHAs. The optimum polymer content was 72.6% of dry cell mass.

Wong et al. (2005) found that the accumulation of PHB by two strains of microorganisms grown on several types of food waste. Recently, Din et al. (2012) have produced bioplastic from cafeteria waste. The PHA yield was increased to 68% over cell dried weight.

2.5.2 Renewable resources

I has been observed by a number of research groups that microorganisms are capable of producing PHAs at high production yields from various specially selected carbon sources, which renders these materials to be used as renewable resources. Table 2.7 shows a list of cheap renewable resources, which might also serve as additional carbon or nitrogen sources for microbial growing or PHA accumulating and producing strains.

Table 2.7 PHA-Production from cheap renewable resources for sustainable process development in complex growth and production media (Braunegg et al., 2004)

Carbohydrates	Molasses
	Starch and starch hydrolysates (Maltose)
	Lactose from whey
	Cellulose hydrolysates (e.g., Reject fiber wastes from the paper industry after hydrolysis and ion exchange for heavy metal removal)
Alcohols	Wastes from biodiesel production Methanol plus Glycerol
Fats and oils	Plant and animal wastes
Organic acids	Lactic acid from dairy industry

Pure sucrose is considered to be appropriate carbon sources for lab-scale PHB production, due to its cheap price and availability in the market. Md Din et al. (2006) used renewable resources palm oil mill effluent by mixed-bacterial cultivation. These observations have demonstrated encouraging results, typically

under high carbon fraction along with nutrient-limiting conditions and appropriate temperature ranges. Overall results illustrated high carbon fraction in the culture substrate formulation could induce improvement in the microbial growth and PHAs accumulation in the cultivation.

Alias and Tan (2005) attempted to isolate palm oil-utilizing bacteria from palm oil mill effluent and obtained large quantity of PHAs from palm-oil-utilizing bacteria. (Dionisi et al., 2005a) used olive oil mill effluents as a substrate to produce PHAs.

Starches are also plentiful inexpensive carbon source. Many researchers utilized starch (Rusendi and Sheppard, 1995) and whey (Kim, 2000; Lee, 1997) for PHB production. The results showed oxygen limitation could also increase PHB contents. Furthermore, the use of inexpensive carbon sources in the substrates, such as molasses, starch or whey can substantially contribute to reducing the high production costs of PHB.

However, some of them encountered many difficulties in the process of producing PHAs from starch, as naturally existing microorganisms often display low PHA productivity while grown directly on such substrates as raw starch, and additional downstream processing costs from liquefaction and saccharification processes limit the potential large-scale applications (Chanprateep, 2010).

Cellulose represents an invaluable resource for mankind, with an increasingly growing number of novel applications (Li et al., 2008), including a vast array of derivatives (Gandini, 2011). For example, lingo cellulosic biomass, it's a cost-effective way from microbial fermentation for the commercial production of bio-plastics (Jian, 2007).

2.5.3 Industrial byproducts

A number researchers have studied the possibility of using industrial byproducts as the sole carbon source for economical PHA production (Yu et al., 1999), such as brewery wastes, food-processing wastes, textile dying effluents and ice cream residue (Lee and Gilmore, 2005).

Molasses, from either sugarcane or beet, a common waste from industrial by-product of sugar refinery and production, is one of the cheap carbon sources that is commonly used for PHA production (Castilho et al., 2009). Liu et al. (1998) made a successful experiment and used beet molasses instead of glucose as the substrate to produce PHAs by a recombinant E .coli strain. The final dry cell weight was very high, almost same as glucose as sole carbon source. As in recent years, the price of glucose continued to rise.

According to Bengtsson et al. (2010), a mixed microbial culture that was selected and enriched in glycogen accumulating microorganisms with fermented sugar

cane molasses as substrate high-yield production of PHAs were observed. The produced polymers included five types of monomers, namely 3HB, 3HV, 3-hydroxy-2-methylbutyrate (3H2MB), 3-hydroxy-2-methylvalerate (3H2MV) and the medium chain length monomer 3-hydroxyhexanoate (3HHx).

A three-stage biochemical pathway to produce PHAs from sugar cane molasses included (1) molasses acidogenic fermentation, (2) selection of PHA-accumulating cultures under feast and famine conditions, (3) PHA batch accumulation using the enriched sludge and fermented molasses (Figure 2.15).

The results showed that a microbial culture selected and enriched on fermented molasses demonstrated a slightly lower PHA production yield and accumulation performance. For instance, slow microbial proliferation rates, lower polymer storage yield, specific accumulation rate, higher risks of microbial inhibition by potentially toxic substances in the growth environment, and higher susceptibility to acetate inhibition, than other acetate-selected culture. However, based on the total process costs including the upstream fermentation and downstream purification costs, the pure substrate fee can outbalance the lower polymer yield and production rate of the fermented molasses, so this study is meaningful (Albuquerque et al., 2007).

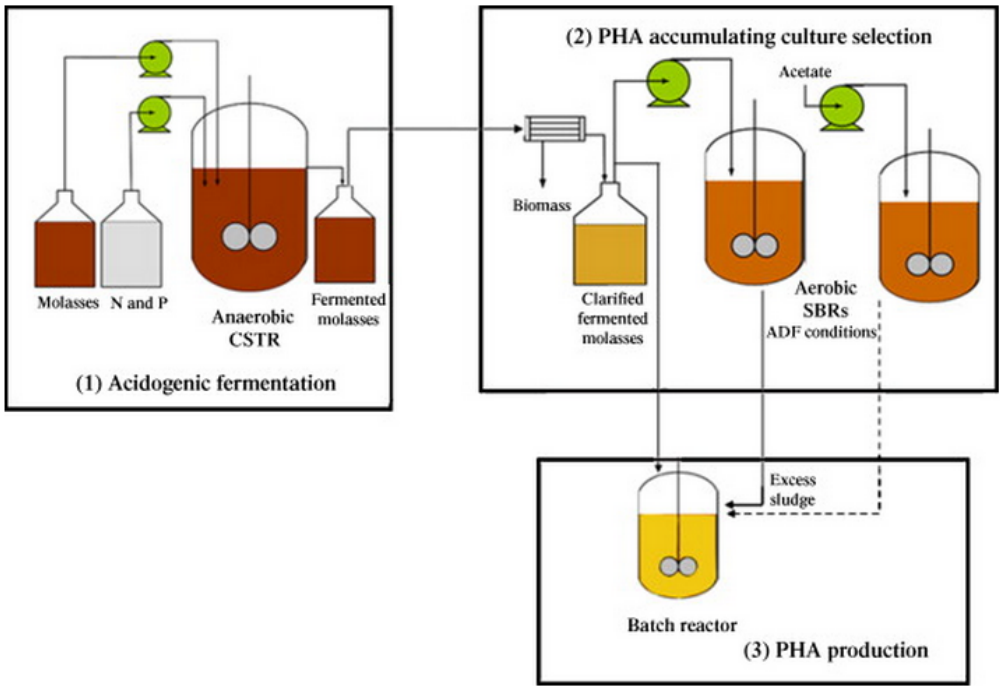


Figure 2.15 Three-step PHA production process from sugar cane molasses by mixed cultures using either fermented molasses (Albuquerque et al., 2007).

It has been reported recently, a 2-stage continuous stirred tank reactor (CSTR) system (under Feast and Famine conditions) was used to effectively select a microbial culture as the effective PHA-storing organisms using fermented molasses as the feedstock in the CSTR or even SBRs (Satoh et al., 2006). Culture enrichment and selection was effectively optimized based on the maximization of a number of selective pressures for PHA storage and could reach a maximum PHA content of 61%. It has been reported by a number of workers that two stages and the feast reactor residual substrate concentration could both present a substantial impact on the efficiency of the microbial enrichment stage. The impact of the feast residual substrate concentration may restrict the selective pressure for PHA storage, but on the other hand, the famine reactor could support

a continuous feeding of substrate if not allowing the microbial culture to attain a substantial growth rate. These successful results opened a whole new perspective to the innovative use of wastewater treatment infrastructure for low-cost PHA production, thus valorizing either excess sludge or wastewaters from municipal sewage treatment facilities (Albuquerque et al., 2010).

Liu et al. (2008) used tomato and other food cannery wastes with a mixed microbial culture to produce PHAs during wastewater treatment. The two-stage PHA production process consisted of SBR under the condition of a specifically designed periodic feast-famine feed pattern to fulfill wastewater treatment, and selection of effective PHA-accumulating microbial culture concurrently. In such a way, PHA-accumulating microbes were enriched under the SBR operating conditions, and PHA content on a cell-weight basis was within the range 7 to 11 weight % in non-filtered sewage, and 2 to 8 weight % in filtered sewage. As a result, a maximum 20 weight % has been reported for PHA content on a cell-weight basis.

Yu et al. (1998) showed the PHA production by *Alcaligenes lallis DSM 1124* utilizing brewery malt waste as carbon substrate to get specific polymer production yield of 70% polymer/cell (g/g). With the use of different types of food wastes as the C source, different PHA copolymers could be produced with distinct polymer properties.

More recently, it has been reported that the waste glycerol, which is a common by-product from the biodiesel industry, has received much interest from many researchers as an inexpensive organic source for use as substrate in PHA production. Guerrero et al. (2012) demonstrated glycerol is a suitable carbon source for EBPR for the first time.

Milk whey has received much attention for its composition as food industrial by-product due to its suitable for the biological process in PHA production. The main constituents of milk whey include lactose, proteins and fats, which are all important in different ways to the fermentation process. Research works need to further utilize milk whey for PHA production nowadays: utilizing activated sludge derived from a dairy wastewater treatment plant as the inoculums. The results of this work showed the production of PHAs from whey with an enriched biomass had the ability of directly using the lactose in a non-sterile fermentation process without pH control (Bosco and Chiampo, 2010).

2.5.4 Fatty acids

Fatty acids are also potential substrates for PHA production. For example, acetate, the most commonly used fatty acids substrate, has been frequently studied by aerobic dynamic feeding (ADF) process, preferentially stored as a homopolymer of PHB (Beun et al., 2000; Serafim et al., 2004).

Recent research works by Lemos et al. (2006) investigated the production of PHAs from such substrates as acetate and propionate by two mixed cultures. During these series of experiments, the independent use of acetic, propionic, butyric and valeric acids under ADF conditions was studied. The resulting polymer composition and yield was also evaluated, showing that the polymer yield and productivity were much higher for acetate than for propionate. The polymer yields on acetate and butyrate were higher than those on propionate and valerate as the sole carbon source. It can be seen that the increase of carbon skeleton length in the molecular structure of the fatty acids results in the declining of the PHA accumulation and production, thus demonstrating the importance of specific choice of substrates.

Zhong et al. (2010) had substantially reduced the production costs by inducing activated sludge bacteria to produce PHAs when the fatty acids were used as carbon source. Moreover, the molar fraction of 3HV in the PHBV copolymer can be adjusted by changing the butyric/valeric acids ratios in the medium.

Generally speaking, fatty acids can be used and polymerized into PHAs by bacteria when there are some growth limiting components, such as nitrogen, phosphate, sulfur, oxygen, and the presence of excess carbon as an energy source.

2.5.5 Volatile fatty acids

Volatile fatty acids (VFAs), as a sub-group of fatty acids, are preferable substrates for PHA production. Various organic compounds will convert to VFAs in anaerobic fermentation that is applied as a pretreatment. VFAs could be utilized as sole carbon and energy source for growth and PHA synthesis.

VFAs are frequently found as intermediate by-products in activated sludge or can be produced from the fermentation of carbohydrates. In the usual fermentation phase, bioreactor operating conditions can be changed in order to attain different VFA compositions and concentrations. Short-chain VFAs are important intermediates in the anaerobic sludge from wastewater, which are the most suitable substrates and an economically feasible alternative source for microbial PHA production (Saito et al., 1995). So it is technically feasible to consider combining a wastewater treatment process with PHA production using activated sludge (Abeling and Seyfried, 1992; Rees et al., 1992; Satoh et al., 1992).

At first, complex biomolecular are hydrolyzed into soluble, biodegradable organics. Then acidogenic and acetogenic bacteria metabolize the hydrolytic products to lower VFAs. Results showed an increase in VFAs production with higher retention times and part of the digested sludge was recirculated. It also produced huge amounts of acetic and butyric acids (Reddy et al., 2003; Sans et al., 1995).

Cai et al. (2009) used different fermentative VFAs as carbon sources to accumulate and synthesize PHAs in sewage-treating activated sludge, in order to reduce the production cost of PHAs and effective disposal of excess sludge simultaneously. PHA accumulation in excess sludge happened feeding by fermentative VFAs with aerobic dynamic feeding process with higher temperature and alkaline condition. The experimental results showed the maximum PHA content accounted for 56.5% of the dry cell. It was a high proportion of PHA content received from using industrial wastes as carbon source and municipal sludge without microbial acclimation as inoculation, which showed that the VFAs generated from excess sludge fermentation were a suitable carbon source for intracellular PHA accumulation by activated sludge. If VFAs concentration was carefully regulated, they could be effective and inevitably choice.

A study by Wang et al. (2010) discovered that the biochemical kinetics of PHA production and VFA consumption by *C. necator* with simultaneous considerations of substrate inhibition, cell growth, maintenance and product. In biosynthesis process, VFAs were observed to have a substantial inhibition on microbial cell growth and deteriorate metabolism. So the results demonstrated initial VFAs concentrations had an effect on the cell activity and maintenance energy required.

Results show that polymers with different monomer composition can be produced by manipulating the feed VFAs profiles, so different carbon substrates will influence monomer composition that affects the physical and mechanical properties of PHAs, therefore, the composition of the VFAs will influence the polymer product finally (Braunegg et al., 1998; Lemos et al., 2006). That means the synthesis of PHAs with controlled functionality and properties is feasible.

As all of the above, VFAs can be converted to PHAs, some suggestions on a strong potential for the valorization of industrial fermentable by-products have good foreground (Lemos et al., 2006).

2.5.6 Glucose

The glucose can be used without demanding poly-p energy, which is a commonly encountered phenomenon which substantially deteriorates the EBPR performance. This study reported the effects of different organic substrates on EBPR, which is a sequencing batch reactor. Experimental data reported show that the EBPR operation efficiency was significantly affected by the increase of the glucose fraction in the feed, as a result of the probable dominance of GAOs (Tasli et al., 1997). This finding is confirmed by Begum and Batista (2012) who point out that a dominance of GAOs over PAOs when EBPR systems are fed with glucose. The possible reason is the GAOs out-compete the PAOs at low pH values; it has been reported that at low pH, GAOs use glycogen as the energy source to

uptake glucose. Consequently, P-removal deteriorated. However, PAOs could accumulate a high content of polyphosphate and obtained higher and faster acetate uptake ability when using acetate substrates, successfully out-competed GAOs (Liu et al., 1997). Therefore, glucose may not be a favorable carbon source to reinforce EBPR operation.

2.5.7 Different carbon sources on EBPR

Hollender et al. (2002) studied the effect of the different carbon sources acetate, acetate/glucose or glucose on the enhanced biological phosphorus removal (EBPR) process under alternating anaerobic – aerobic conditions in one sequencing batch reactor for each carbon source. In the process of experiment, the glucose was consumed completely within the first 30 min of the anaerobic phase while acetate degradation was slow and incomplete. Phosphate released independently of the carbon source during the whole anaerobic phase. 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 PHAs was maximal 17% for acetate and 82% for glucose. PHA storage seems to regulate mainly the phosphate release and uptake because of the strong influence of the carbon source on the PHA concentration and composition.

Primary and waste activated sludge including plenty more sources of waste

organics have been considered as substrates for PHA production, resulting in high treatment and disposal costs (Coats et al., 2007a). Fermented primary sludge is associated with two-phase anaerobic digestion and nutrient removal, so it has also recently shown potential as substrate in PHA production has also been successfully used to accumulate PHA in an un-acclimated activated sludge biomass.

Alcohols are sterile carbon substrates that rarely used, but it could possibly reduce the chance of contamination.

2.5.8 Acid mixtures substrates

For the production of copolymers, co-substrates acting as the precursors for PHAs are often needed, which may be more expensive than the main carbon source and often harmful for cell growth at high concentrations.

A study by Yu et al. (2002) used acetic and propionic acid to product PHAs by Ralstonia eutropha, the results showed acetic acid in acid mixtures increased the fraction (HV) due to reducing the metabolism of propionic acid.

The low PHA productivity and content maybe caused by the improper feeding of propionic acid that brought about toxic effects in the synthesis process. Therefore, a better-controlled propionic acid feeding strategy needs to be developed.

In a word, when the mixture of substrates is present, the overall polymer composition is manifested as the combination of the different substrates used by the culture. The highest polymer yield and the specific polymer storage rate were obtained for acetate, followed by butyrate, propionate and valerate. It's found no matter what microorganisms conducted (pure culture or co-culture), the substrate removal efficiency would decrease as the corresponding increasing carbon chain length of the acids (Lemos et al., 1998; Pijuan et al., 2003; Randall and Liu, 2002).

2.6 The Activated Sludge Process

Some microorganisms in activated sludge from municipal sewage treatment plants are known to accumulate PHAs (Chua et al., 2003). The organic materials in activated sludge has the potential to be used as a valuable resource rather than to be disposed of as a waste. As activated sludge is rich in organics, it may be used as a cheap but valuable resource for the production or recovery of PHAs (Morgan-Sagastume et al., 2011). Furthermore, an effective way to decrease the quantity of excess sludge for further treatment compared with pure cultures (Dionisi et al., 2004; Lemos et al., 2006; Satoh et al., 1998; Takabatake et al., 2000). So it has been suggested that PHA synthesis and accumulation in activated sludge may be another promising option for the industrial and low-cost production of PHAs.

As a matter of fact, the capability of PHA production by activated sludge has been found several years before. The often-used activated sludge for PHA production was obtained from anaerobic-oxic biological treatment systems, named as EBPR system (Chua et al., 2003). Many experimental results indicated wherever the batch experiments for PHA production were performed under an anaerobic phase or oxic phase, it obtained activated sludge from the oxic zone of an EBPR system (Chang et al., 2011; Chua et al., 2003; Hu et al., 2005; Kasemsap and Wantawin, 2007; Serafim et al., 2004).

2.6.1 Mixed-culture PHA production

On the other hand, genetically engineered plants containing the bacterial PHA biosynthesis genes are being developed by a number of researchers for the economical production of PHAs. Improvements in fermentation and separation technology will synthesize PHAs more efficiently. Recent discovery and development of bacterial strains or plants will bring the costs down to make PHAs competitive as compared with the conventional plastics (Akaraonye et al., 2010; Lee, 1996). More recently, PHAs have also been discovered in the various bacterial strains isolated from specific selected activated sludge of wastewater treatment plants (Ishihara et al., 1996; Rees et al., 1992; Satoh et al., 1992).

An important advantage of PHA production by mixed microbial cultures in activated sludge, compared to pure cultures is that it allows for an important

reduction in processing costs and it can better adapt to changes in substrates supply and sterilization of the substrate which would have detrimental effects on the substrate quality (Johnson et al., 2010). There is no need for reactor sterilization and some process controls (Salehizadeh and Van Loosdrecht, 2004). Moreover, this procedure could effectively recover renewable carbons from different high-strength industrial wastewater, landfill leacheate and reuse waste sludge from traditional activated sludge systems (Chang et al., 2011). In other words, mixed-culture PHA production can potentially offer the possibility of material recycling by using renewable waste organics (Reis et al., 2011).

So PHA accumulation in mixed cultures is becoming an attractive alternative to established industrial production processes (Lemos et al., 2006; Salehizadeh and Van Loosdrecht, 2004). For example, a number of industrial wastewaters are suitable for mixed culture PHA production. Examples are paper mill wastewater or molasses which are often nutrient deficient. If nutrient supplementation would be required for these streams, this could be an important cost factor. A successful method to enrich PHA producers in a mixed culture is the use of an aerobic dynamic feeding pattern including alternating periods of feast and famine of the carbon sources (Johnson et al., 2010).

It is necessary to find simple polymer recovery processes and applications for PHAs where less purity of the polymer is required (Serafim et al., 2008).

Activated sludge is activated during the process that occurs in treating the sewage and industrial wastewater. This process can remove soluble and insoluble organics from the wastewater and to convert this material into a flocculent microbial suspension. They can settle well in a conventional gravity clarifier and then discharge into the water body.

During the activated sludge process, the soluble carbon is from the wastewater and the dissolved oxygen is from air. After their combination and reaction, they produce to biomass composing material and carbon dioxide gas, which the soluble carbon acted as electron donor and dissolved oxygen acted as electron acceptor. There is another saying that the activated sludge process is a wastewater treatment method in which the carbonaceous organic matter of wastewater provides energy source for the production of new cells for a mixed population of microorganisms in an aquatic aerobic environment.

The overall biochemical reaction leading to organic and nutrients removal in an aeration tank can be described by:

$$\text{Organic matter} + \text{Nutrients} + O_2 \rightarrow CO_2 + H_2O + \text{Biomass} + \text{Energy}$$

Figure 2.16 shown below is the conventional activated sludge process.

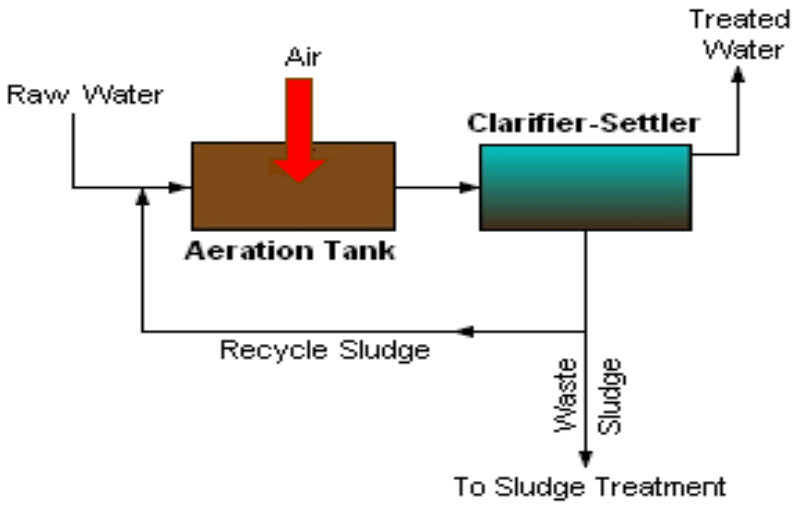


Figure 2.16 The conventional activated sludge process.

The main purposes of wastewater treatment systems are to remove organic pollutants, but it will be very attractive if there is a way to convert the organic pollutants to PHAs, and consequently resulted in the decrease of PHA price.

2.6.2 EBPR process

The anaerobic–aerobic activated sludge process is a modified and improved activated sludge process widely used for the removal of phosphorus from wastewaters. It could accumulate the byproduct PHAs. As shown in Figure 2.17, in this process, activated sludge and influent wastewater are mixed under anaerobic conditions, where no oxygen, nitrate or nitrite. Then the mixture of wastewater and activated sludge is transported to the aeration tank, and the wastewater is treated aerobically. In the subsequent sedimentation tank, treated water and activated sludge are separated gravimetrically. Most of the settled

activated sludge is returned to the top of the reactor and re-used for wastewater treatment again. Some of the activated sludge is withdrawn from the process as excess sludge, because microbial growth causes the production of sludge. Then the microorganisms are exposed to anaerobic and aerobic conditions repeatedly, typically about 40 times in their life. Because wastewater is supplied under anaerobic conditions in which those microorganisms that can take up and accumulate organic substrates, and then grow under aerobic condition, so that have an advantage for survival (Satoh et al., 1999).

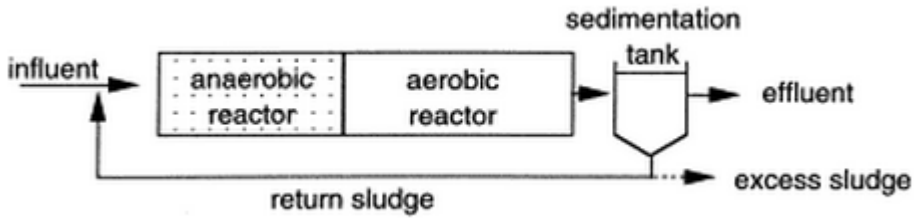


Figure 2.17 Typical schematic of the anaerobic–aerobic activated sludge process (Satoh et al., 1999).

Enhanced biological phosphorus removal (EBPR) is a widely applied method for nutrients removal (Zafiriadis et al., 2011). As we all know, PHAs play a very important role in EBPR process. Synthesis of PHAs by mixed cultures was first found in wastewater treatment plants by EBPR process (Wallen and Rohwedde.Wk, 1974). A culture that can store high PHA concentrations while growing on an inexpensive growth substrate would be a good candidate for PHA production. Activated sludge biomass is one of the suitable cultures for PHA

production.

As widely reported, PAOs and GAOs are common and main groups of bacteria responsible for PHA accumulation under these conditions. PHA synthesis plays an important role in the metabolism about both groups of microorganisms (Serafim et al., 2008). GAOs were presented as more robust than PAOs, easily producing copolymers of PHBV from simple and cheap carbon source, which could be an important advantage for GAOs over PAOs (Dai et al., 2007). However, a research by Bengtsson et al. (2008) contended that rate and efficiency of accumulation of PHAs under anaerobic conditions was limited by the intracellular glycogen stored.

As reported, carbon source is related to the growths of PAOs and GAOs. The prevailing mechanism is that a select group of microbes PAOs in activated sludge under anaerobic conditions can uptake short chain VFAs or their associated salts, sequentially producing PHAs with synchronous phosphorus release to the external medium. PAOs and GAOs use glycogen replaced by polyphosphate as energy source and therefore, do not release or uptake phosphate for metabolic processes (Mino et al., 1998). During starvation periods for PAOs which expend PHAs for reproduction and overload on orthophosphate from their surroundings (You et al., 2011), PHAs can serve as a carbon or energy source, the microbial strains can biosynthesize more PHAs among the growing limited condition.

The main role of polyphosphate under anaerobic conditions has been observed to be as a source of energy both for the reestablishment of the proton motive force, which would be consumed by substrate transport and for substrate storage (Tanaka et al., 1992). It has been reported that the carbon reserves in bio-P bacteria should provide energy for growth and for soluble phosphate accumulation as polyphosphate reserves, thus illustrating that the PHAs is the reduced energy source and its synthesis required substantially reduced powers (Comeau et al., 1986).

The type of carbon source is a main factor influencing EBPR performance (You et al., 2011). Heavy metals in wastewater have great effect on EBPR performance. Wastewater may contain different types of heavy metals that can deteriorate microbial activities due to its source. Some studies on organic matter removal efficiency decreased by certain amounts of different heavy metals has been done by Chua, H. et al. (1999) and Ong et al. (2005a,b).

The EBPR process depends on the ability of some organisms to convert VFAs to PHAs under anaerobic conditions for later use under aerobic conditions (Mino et al., 1998). Under anaerobic condition, the activated sludge takes up carbon and accumulates it as PHAs. Energy for this process comes from the break-down of intracellular polyphosphate, glycogen consumption and substrate degradation in the tricarboxylic acid cycle (TCA) (Martin et al., 2006; Oehmen et al., 2007;

Seviour et al., 2003). Under aerobic condition, PHAs previously accumulated in anaerobic stage is oxidized as the energy source for microorganism growth, synthesizing polyphosphate with ortho-phosphate taking up from the medium (Al-Najjar et al., 2011; Gebremariam et al., 2012; Jeon and Park, 2000).

In recent years, many studies have investigated heavy metals have a great influence on the removal efficiency of carbon, nitrogen and phosphate (Tsai and Wu, 2005; Tsai et al., 2005, 2006; You et al., 2009a, b), especially for the mechanism of phosphate removal which was much more sensitive to heavy metals than those of nitrogen and carbon removals. Therefore, the widespread use of EBPR is hindered by the unstable issues of phosphorus removal performance (Oehmen et al., 2007).

2.6.3 Sequencing Batch Reactor

Sequencing batch reactors are one of the most commonly used technologically-advanced process for mixed culture PHAs accumulation (Albuquerque et al., 2007; Beun et al., 2002; Coats et al., 2011; Dionisi et al., 2005b; Hu et al., 1997; Serafim et al., 2004). They are convenient and compact systems where the full feast and famine cycle may be performed in one single and simple reactor, and the length of each phase may be varied according to different needs (Serafim et al., 2008). A feast phase under 20% of the total cycle length has demonstrated more efficient for selecting for PHA-storing organisms

(Dionisi et al., 2007).

It was first put forward by Majone et al. (1996) the concept of aerobic "feast and famine" process. Under these dynamic conditions, during the feast time, carbon uptake is driven to cell growth and PHA storage. After substrate exhaustion, namely famine regime, the stored polymer previously accumulated is used as energy and carbon sources (Lemos et al., 2006; Majone et al., 1999; Serafim et al., 2004; Serafim et al., 2008).

The different mechanisms for PHA storage between anaerobic/aerobic conditions and the aerobic feast and famine process could be manifested as, in the anaerobic/aerobic process, storage is mainly caused by an external growth limitation due to absence of an electron acceptor (oxygen, nitrate), while in the aerobic feast and famine process, PHA storage occurs owing to an internal growth limitation, since both electron donor and acceptor are present in the feast period (Serafim et al., 2008).

PHA storage by activated sludge can be very important, if the sludge is submitted to feast and famine regime, which is currently named aerobic dynamic feeding. ADF is the most important and often researched PHA storage process, frequently regarded as a superior method to improve the PHA storage capability of activated sludge (Cai et al., 2009).

Serafim et al. (2004) and Johnson et al. (2009) demonstrated that a mixed culture of microbes in an ADF systems showed great potentials for intracellular PHA accumulation reaching very high specific production yields, with polymer productivities on substrate and maximum intracellular PHA contents per unit DCW similar to those reported for pure bacterial cultures. On the other hand, Albuquerque et al. (2011) concluded that by applying a continuous feeding strategy instead of the widely used feast-famine feeding strategy can effectively mitigate the microbial culture process constraints and, at the same time, enable higher rates of substrate uptake and PHA storage. Furthermore, these were consistently maintained so long as the incoming residual substrate concentration was kept reasonably constant. As a result, a considerable improvement in volumetric productivity was achievable due to the use of the aforementioned continuous feeding strategy in the upstream PHA accumulation and production stages.

An innovative research by Dionisi et al. (2004) demonstrated that periodic feeding (feast and famine regime) at a high organic load of a mixture of acetic, propionic and lactic acids had efficient capacity of selecting and enriching a mixed culture to store the copolymer P(HB/HV) at a high rate and yield. The fact is, the storage rate reached the maximum at the beginning and then slightly slower as a substrate excess was present for a long time. The reason was biomass adaptation to the excess substrate caused a progressive increase of growth rate.

The final results showed periodic feeding made it possible to obtain high productivity of a biomass (3.7 g COD $^{-1}$/ day^{-1}) and the storage rates were much higher than typical activated sludge.

The better understanding of F/M process gave rise to the optimal operating conditions in the reactor, leading to a significant PHA storage capacity (Serafim et al., 2004).

In recent years, it is reported that microorganisms in excess activated sludge (EAS) accumulate PHAs as an intermediate metabolic product from the uptake of organic matter in sewage. There are a lot of benefits, it is necessary to combine activated sludge in a wastewater treatment process with PHA production. Additionally, a large number of EAS from a wastewater treatment process would be reduced as plastics. So the plastic prices fall. It is supported by sound reasons.

2.7 Strategy of operational conditions for PHA production

2.7.1 An nutrition imbalance in growth conditions

Many bacteria with nutrition imbalance are capable of making PHAs. Madison and Huisman (1999) noted that PHA accumulation increased when the carbon to nitrogen ratio suddenly increased, due to nutrition imbalance in growth conditions, which led to the cell to change their physiology. This significant discovery makes begin the investigation into the physiological role of PHAs. It

was accepted later that bacteria synthesis and store PHAs when they lack the complete range of nutrients required for cell division but have excess supplies of carbon source (Hu, 2004).

However, Daigger and Grady (1982) found when cells are exposed to a medium with very little limit amounts of nutrient for a long time, the bacteria would modify physiological adaptation as microbial cultures are grown at specific rates less than their maximum.

Nitrogen limitation is a good way for obtaining high PHA contents during the PHA production process (Johnson et al., 2010). Waste streams can be used as a substrate for PHA production. For instance, suitable industrial wastewaters from the food industry or paper industry (Albuquerque et al., 2007; Beccari et al., 2009; Bengtsson et al., 2008; Wong et al., 2005), but which of them have very low nitrogen content and may require supplementation. However, supplementation with nutrients is costly. Therefore, in order to reduce the overall cost for nutrient supplementation, attention will be focused on the wastewaters which are in need of nutrient supplementation.

The continued consumption of excess nitrogen may show growth of non-PHA-storing biomass during famine. In spite of that, biomass growth still appeared restricted. In addition to these, excessive nutrient could have also blocked PHA accumulation because of the increased C-based respiration.

Van Wegen et al. (1998) reported that PHB yield from glucose as carbon substrate could be increased by adding some complex nitrogen sources.

The amount of biomass produced was much less in the cases with N and/or P-limitation. So in these cases PHA yields were higher with nutrient excess (Table 2.8). From this table, it can be seen that nutrient limiting conditions caused higher PHA content and higher PHA yields, compared to the other cases that cell growth was limited in absence of nutrients.

Table 2.8 PHA content and composition, yields and kinetic parameters for the batch experiments (Braunegg et al., 1998)

		Nutrient excess	P-Limitation	N-Limitation	N&P-Limitation
PHA_{max}	% TSS	31.9	48.2	44.0	42.7
PHB:PHV	mol:mol	31:69	39:61	41:59	47:53
Y_{PHB}	C-mol/C-mol	0.08	0.22	0.19	0.27
Y_{PHV}	C-mol/C-mol	0.25	0.44	0.36	0.40
Y_X	C-mol/C-mol	0.31	0.18	0.13	0.26
$-q_{VFA}$	C-mol/C-mol h	0.191	0.086	0.104	0.066
		(0.026)	(0.005)	(0.015)	(0.008)
q_{PHA}	C-mol/C-mol h	0.056	0.064	0.061	0.046
		(0.008)	(0.006)	(0.004)	(0.002)
μ	h^{-1}	0.046	0.015	0.016	0.016
		(0.006)	(0.003)	(0.002)	(0.002)

Standard deviation in brackets.

With nutrient limitation, 43–48% PHAs of TSS was achieved (Figure 2.18). Excess nutrients caused much lower PHA content, the maximum is 32%.

Wen et al. (2010) carried out specifically designed experiments concerning effects of phosphorus and nitrogen limitation on PHA production in activated sludge. The results showed that activated sludge biomass would produce more

PHB than PHV under the nutrient limitation condition, especially under the condition of phosphorus limitation. In addition, the result also illustrated that both phosphorus and nitrogen limitation may cause sludge bulking. P-limitation caused a non-filamentous bulking, while N-limitation resulted in a filamentous bulking.

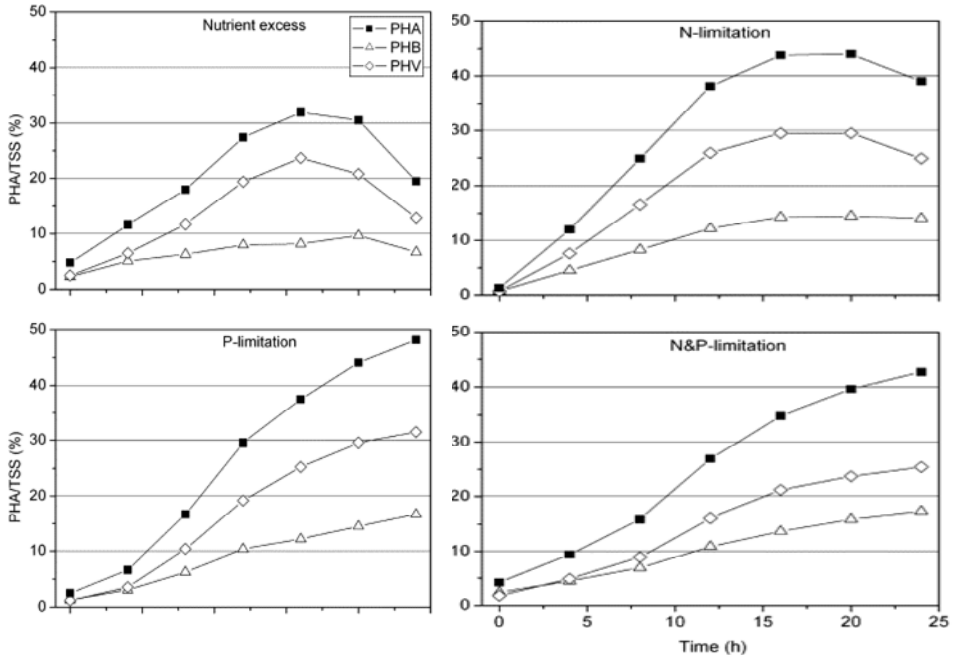


Figure 2.18 PHA content in the sludge during batch experiments with different nutrient conditions (Braunegg et al., 1998).

VFA and NH_4^+-N coinstantaneous consumption during the feast time demonstrates that biomass growth associated with PHA storage. The decrease in DO slowly during the feast time backs up the idea that an increased microbial respiration is related to PHA storage. Less nitrogen consumption in the famine than in the feast has been associated with biomass growth on PHAs

(Albuquerque et al., 2007).

2.7.2 Bacterial strains

PHAs can be produced by many different bacterial cultures, such as natural isolates and recombinant bacteria.

Table 2.9 illustrated the production of various PHAs using natural isolates and recombinant bacteria with different substrates.

In the following Table 2.10, there is an overview of bacterial strains used to produce PHAs, including their corresponding initial carbon sources and produced copolymers.

Table 2.9 Production of PHAs by various bacteria (Reddy et al., 2003)

Microorganism	Carbon source	PHA	PHA content (%w/v)	References
Alcaligenes eutrophus	Gluconate	PHB	46–85	Liebergesell et al. (1994)
	Propionate	PHB	26–36	
	Octanoate	PHB	38–45	
Bacillus megaterium QMB1551	Glucose	PHB	20	Mirtha et al. (1995)
Klebsiella aerogenes recombinants	Molasses	PHB	65	Zhang et al. (1994)
Methylobacterium rhodesianum MB 1267	Fructose/methanol	PHB	30	Ackermann and Babel (1997)
M. extorquens (ATCC55366)	Methanol	PHB	40–46	Borque et al. (1995)
Pseudomonas aeruginosa	Euphorbia and castor oil	PHA	20–30	Eggink et al. (1995)
P. denitrificans	Methanol	P(3HV)	0.02	Yamane et al. (1996)
	Pentanol	P(3HV)	55	
P. oleovorans	Glucanoate	PHB	1.1–5.0	Liebergesell et al. (1994)
	Octanoate	PHB	50–68	
P. putida GPp104	Octanoate	PHB	14–22	Liebergesell et al. (1994)
P. putida	Palm kernel oil	PHA	37	Tan et al. (1997)
	Lauric acid	PHA	25	
	Myristic acid	PHA	28	
	Oleic acid	PHA	19	
P. putida BM01	11-Phenoxyun-decanoic acid	5POHV	15–35	Song and Yoon (1996)
Sphaerotilus natans	Glucose	PHB	40	Takeda et al. (1995)

PHB—polyhydroxybutyric acid), P(3HV)—polyhydroxvaleric acid, 5POHV—poly(3 hydroxy-5-phenylvalerate).

Table 2.10 Overview of bacterial strains used to produce PHAs (Verlinden et al., 2007)

Bacterial strain (s)	Carbon source (s)	Polymer (s) produced	Reference
Aeromonas hydrophila	Lauric acid, oleic acid	mcl-PHAs	(Lee *et al.* 2000; Han *et al.* 2004)
Alcaligenes latus	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	(Wong *et al.* 2004, 2005)
Bacillus cereus	Glucose, ε-caprolactone, sugarbeet molasses	PHB, terpolymer	(Labuzek and Radecka 2001; Yilmaz and Beyatli 2005; Valappil *et al.* 2007)
Bacillus spp.	Nutrient broth, glucose, alkanoates, ε-caprolactone, soy molasses	PHB, PHBV, copolymers	(Katircioglu *et al.* 2003; Shamala *et al.* 2003; Tajima *et al.* 2003; Yilmaz *et al.* 2005; Full *et al.* 2006)
Burkholderia sacchari sp. nov.	Adonitol, arabinose, arabitol, cellobiose, fructose, fucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose, xylitol	PHB, PHBV	(Brämer *et al.* 2001)
Burkholderia cepacia	Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugarbeet molasses	PHB, PHBV	(Keenan *et al.* 2004; Nakas *et al.* 2004; Alias and Tan 2005; Çelik *et al.* 2005)
Caulobacter crescentus	Caulobacter medium, glucose	PHB	(Qi and Rehm 2001)
Escherichia coli mutants	Glucose, glycerol, palm oil, ethanol, sucrose, molasses	(UHMW)PHB	(Mahishi *et al.* 2003; Kahar *et al.* 2005; Park *et al.* 2005a; Nikel *et al.* 2006; Sujatha and Shenbagarathai 2006)
Halomonas boliviensis	Starch hydolysate, maltose, maltotetraose and maltohexaose	PHB	(Quillaguaman *et al.* 2005, 2006)
Legionella pneumophila	Nutrient broth	PHB	(James *et al.* 1999)
Methylocystis sp.	Methane	PHB	(Wendlandt *et al.* 2005)
Microlunatus phosphovorus	Glucose, acetate	PHB	(Akar *et al.* 2006)
Pseudomonas aeruginosa	Glucose, technical oleic acid, waste free fatty acids, waste free frying oil	mcl-PHAs	(Hoffmann and Rehm 2004; Fernández *et al.* 2005)
Pseudomonas oleovorans	Octanoic acid	mcl-PHAs	(Durner *et al.* 2000; Foster *et al.* 2005)
Pseudomonas putida	Glucose, octanoic acid, undecenoic acid	mcl-PHAs	(Tobin and O'Connor 2005; Hartmann *et al.* 2006)
Pseudomonas putida, P. fluorescens, P. jessenii	Glucose, aromatic monomers	aromatic polymers	(Tobin and O'Connor 2005; Ward and O'Connor 2005; Ward *et al.* 2005)
Pseudomonas stutzeri	Glucose, soybean oil, alcohols, alkanoates	mcl-PHAs	(Xu *et al.* 2005)
Rhizobium meliloti, R. viciae, Bradyrhizobium japonicum	Glucose, sucrose, galactose, mannitol, trehalose, xylose, raffinose, maltose, dextrose, lactose, pyruvate, sugar beet molasses, whey	PHB	(Mercan and Beyatli 2005)
Rhodopseudomonas palustris	Acetate, malate, fumarate, succinate, propionate, malonate, gluconate, butyrate, glycerol, citrate	PHB, PHBV	(Mukhopadhyay *et al.* 2005)
Spirulina platensis (cyanobacterium)	Carbon dioxide	PHB	(Jau *et al.* 2005)
Staphylococcus epidermidis	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB	(Wong *et al.* 2004, 2005)
Cupriavidus necator	Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil	PHB, copolymers	(Kim *et al.* 1995; Kichise *et al.* 1999; Taguchi *et al.* 2003; Kahar *et al.* 2004; Khanna and Srivastava 2005a; Volova and Kalacheva 2005; Volova *et al.* 2005)
Cupriavidus necator H16	Hydrogen, carbon dioxide	PHB	(Pohlmann *et al.* 2006)

UHMW: ultra high molecular weight

2.7.3 Factors affecting PHA accumulation

The nutrient conditions, aeration mode, carbon source concentration and initial pH level will more or less affect the PHA yield (Liu et al., 2011).

2.7.3.1 pH

Kasemsap and Wantawin (2007) indicated that when activated sludge of 2%, 6%, and 8% polyphosphate was investigated for batch PHA production by changing the pH values from 6 to 8. The results showed a shorter contact time caused higher productivity, while the PHA content was lower than that in a longer contact period. Increasing the pH from 6 to 8 promoted the PHA production. The reason was excess activated sludge consisting of PAOs and predominant GAOs had a stronger growth ability compared to pure cultures. We can draw from this study that PHA production by ordinary activated sludge is both possible and promising.

2.7.3.2 SRT

Chang et al. (2011) stated that the oxic sludge with 5 days of SRT showed better PHA production performance than anaerobic sludge. In contrast, the anaerobic sludge with 15 days SRT obtained superior PHA production behavior compared to oxic sludge with the same SRT, which suggest that the metabolic behavior of the sludge for PHA production depended significantly on the operating SRT of

the EBPR system, whether they were anaerobic or oxic sludge.

Another research by Coats et al. (2007b) showed the same nutrient limited wastewater was fed to aerobic feast-famine SBRs with different SRTs. At shorter SRT the PHA yield was higher than that at longer SRT, which explained the SBR with shorter SRT was probably stronger nutrient limited than the longer SRT. This confirms that nitrogen limitations get stronger if lower SRTs are used (Johnson et al., 2010).

A study by Jiang et al. (2011) was investigated the temperature and cycle length affected on microbial competition between PHB-producing populations enriched in feast-famine SBRs at different temperatures. The results showed PHB yield at the end of the feast phase was related to the cycle length, independent of the temperature and the microbial community structure in the SBR. The PHB content was associated with the number of cycles per SRT. A longer cycle length caused a higher PHB content at the end of the feast phase with a constant SRT. There may be two reasons: On the one hand, the amount of substrate fed per cycle is increased and biomass concentration at the beginning of the cycle in the reactor decreased by reducing the cycle number per SRT. The substrate to biomass ratio increases, leading to more PHB synthesis accordingly. On the other hand, cells need more PHB to survive with increasing of famine time. The two aspects intensify the selection of microorganisms with a high substrate uptake rate and

high storage capacity. Therefore, the number of cycles per SRT is considered as one of the most important physical selection pressures for enrichment of the bacterial consortium, independent of substrate composition, temperature or PH.

2.7.3.3 Oxygen Supply

Many studies proved that one of the favored conditions for PHA accumulation by bacteria is oxygen limitation or low oxidation-reduction potentials. Satoh et al. (1998) investigated that microaerophilic-aerobic process accumulated more PHAs than the original anaerobic-aerobic process, as a small amount of oxygen was entered to the reactor. Therefore, it is believed that the dissolved oxygen plays a very important role in the PHA production from activated sludge in SBR.

It is also reported that oxygen plays a key role for the regulation of metabolic pathway. The polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) in activated sludge are very sensitive to the variation of dissolve oxygen (DO) concentration, which could result in changes of microbial community or metabolic pathway.

As suggested in Satoh et al. (1998), while acetate as sole carbon source, the presence of excess oxygen during the period of +100mv ORP (i.e. more pure oxygen was supplied), leading to the balanced growth conditions, and acetyl-CoA was submitted to the TCA cycle for energy generation and

intermediates synthesis. As a result, the concentration of free CoASH became higher. The key enzyme for PHA synthesis, 3-ketothiolase, was inhibited by high concentration of free CoASH, and brought on the inhibition of PHA synthesis.

Oxygen also regulates the 3-Hydroxyvalerate (3HV) molar fraction in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymer. There may be two possible explanations. Firstly, different DO concentration became a key factor which caused the selected growth of bacteria in the EAS, and then resulted in different metabolic pathways for the accumulation of PHAs with different 3HV mole fraction. Secondly, some unknown mechanisms would be affected by the DO concentration when using acetate as sole carbon substrate during the process of 3HV unit synthesis in PHBV copolymer. Some studies even indicated that the production of polymer was inhibited by valeric acid. Similar results were reported by Braunegg et al. (1998) that aerobic feeding and dynamically unstable operating conditions acted as physical selection pressures that triggered off a strong ability of microorganisms to establish intracellular carbon reserves. Activated sludge in sewage treatment works under normal aerobic conditions demonstrated substantial PHA storage capacity, especially if the sludge is under conditions with alternating organic surge loadings (feast-famine conditions).

2.7.3.4 Metal ions

In a study by You et al. (2011) showed that certain heavy metals, such as lead,

had a significant impact on phosphate uptake mechanism in activated sludge of PAOs. A preliminary hypothesis suggesting that PAOs microbial community could effectively hydrolyze polyphosphate and metabolize external carbon sources to produce PHB as a form of energy storage when volatile fatty acids such as acetic acid was present in excess. Another hypothesis suggested that the divalent lead ion (Pb^{2+}) could have interrupted microbial metabolism of PAOs and hence adversely affected the energy for phosphorus uptake in aerobic sludge.

3 METHODOLODY

The entire work was designed as a five-stage project as shown in the following flow diagram (Figure 3.1). Activated sludge from a conventional municipal sewage treatment works was obtained and conducted for the PHA accumulation by using selected sole carbon sources in a laboratory-scale activated-sludge simulator system. In the first stage, the optimal aeration time settling time were determined, based on the quality of the sludge and treated effluent. In the second stage, with the fixed aeration and settling time for the activated-sludge simulator system, the optimal carbon-phosphorus (C:P) ratio was determined by observing the intracellular PHA accumulation rates.

In the third stage, investigation was directed at evaluation of the optimal values of carbon-nitrogen (C:N) ratio on PHA production yields under the other pre-determined optimal conditions. After repeated operation of the activated sludge simulator system through these three stages of works, the activated sludge was considered to have been selected with a microbial consortium that is best suited for efficient PHA accumulation under the predetermined operating conditions. The process operating parameters and the carbon-nutrient ratio were designed to exert a selection pressure on the microbial consortium in the activated sludge thus selecting the intended micro-ecosystem.

In the fourth stage of the study, the pilot-scale SBR system was built for PHA accumulation based on optimal condition determined from the laboratory-scale system. The selected activated sludge was used to seed the system. The PHA production yields and COD removal efficiency were studied on a long-term basis for its stability and reliability.

In the final stage, the composition of co-polymeric materials, namely PHBV, produced by activated sludge bacteria was controlled by regulating the concentration of butyric acid (C4) and valeric acid (C5) ratio in the medium under the condition of pilot-scale system. The organic feed to PHBV conversion mechanism were studied and modeled as a mathematical relation. This stage of operation was designed to illustrate that the composition of the co-polymers, the physical, thermal and mechanic properties, could be controlled by manipulating the influent organic compositions in the medium.

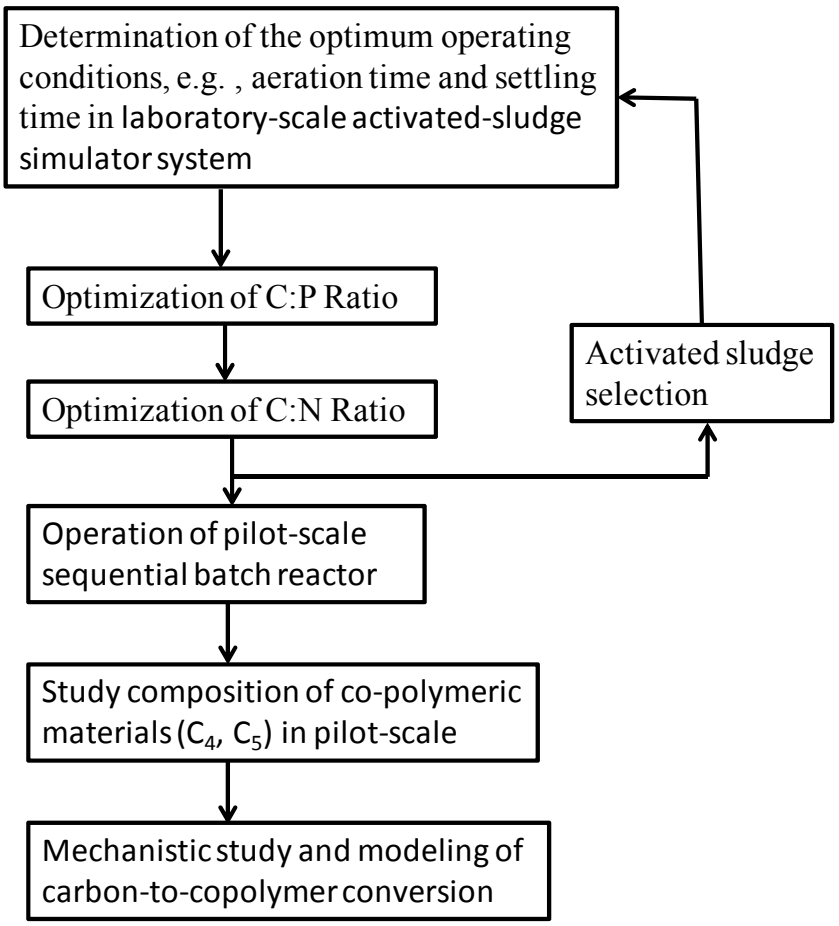


Figure 3.1 The flow diagram for the design of experimental framework.

3.1 Experimental design

In this study, several factors affecting the overall economics of PHA production were investigated. In order to examine how these factors contribute to the process efficiency, main focus of the research works was the design of laboratory-scale activated-sludge simulator system and pilot-scale sequential batch reactor (SBR) system to determine the optimum operating conditions of activated sludge process for increasing the PHA production yield.

The experiments were carried out in five stages to investigate and optimize the production of biodegradable plastics by activated sludge bacteria. In the first stage of the experiments, a laboratory-scale biological reactor, namely a SBR system, was fabricated and set up for PHA production. The optimal aeration and setting time should be adjusted.

Secondly, when aeration and setting time were fixed for the activated-sludge simulator system, different C:P ratio that affected the PHA yield (delP) were tested，then the optimal C:P ratio was accordingly determined.

In the third stage, investigation was directed at evaluation of the optimal values of carbon-nitrogen (C:N) ratio on PHA production yields under the other pre-determined optimal conditions. It was designed to investigate the effect of different values of C:N ratio on the process operating efficiency, PHA formation yield, specific rates of substrate utilization, PHA productivity, biomass growth and concentration during wastewater treatment process under the condition of optimal aeration, settling time and C:P ratio that have been determined in the previous stages.

In the fourth stage of the study, the pilot-scale SBR system was built for PHA accumulation based on optimal condition determined from the laboratory-scale system, and the PHA production yields and COD removal efficiency were studied on a long-term basis, compared them in PHA production and COD removal

efficiency.

The final stage of experiment was to study composition of co-polymeric materials produced by activated sludge bacteria by regulating the concentration of butyric acid (C_4) and valeric acid (C5) ratio in the medium under the condition of pilot-scale system. Moreover, the physical, thermal and mechanic properties of different co-polymers were also investigated.

3.1.1 Acclimation phase

The activated sludge collected and applied for PHA production was harvested from laboratory-scale conventional SBR process acclimatized with industrial wastewater (from HK Amoy Food Ltd). The activated sludge from the industrial wastewater treatment plant was initially enriched in a synthetic wastewater media using glucose as carbon source. The performance and stability of the acclimation SBR was evaluated regularly before the commencement of the batch tests under different operating conditions.

It is necessary to understand the importance of concentration and operation parameters which can enhance the PHA production capability. This helped in the selection for the suitable wastewater for sludge acclimatization.

After the acclimation period, the SBR achieved operation stability, and the sludge retention time (SRT) of 15 days was maintained. The acclimation was

operated at uncontrolled room temperature in the range of 27–31°C.

Nitrogen and phosphorus, according to the weight ratio of C:N: P=150: 5: 1, was supplied at this stage for cell growth. After enrichment, the activated sludge was inoculated into the SBR. The carbon-nitrogen-phosphorus weight ratio at the acclimation phase before P limitation experiment was controlled at 150:5:1 with COD 312 mg/L.

3.1.2 Reactor set up and operations

The lab-scale sequential batch activated sludge reactors (SBRs) were used as the wastewater treatment process, the first stage of the proposed PHA production system (Figure 3.2).

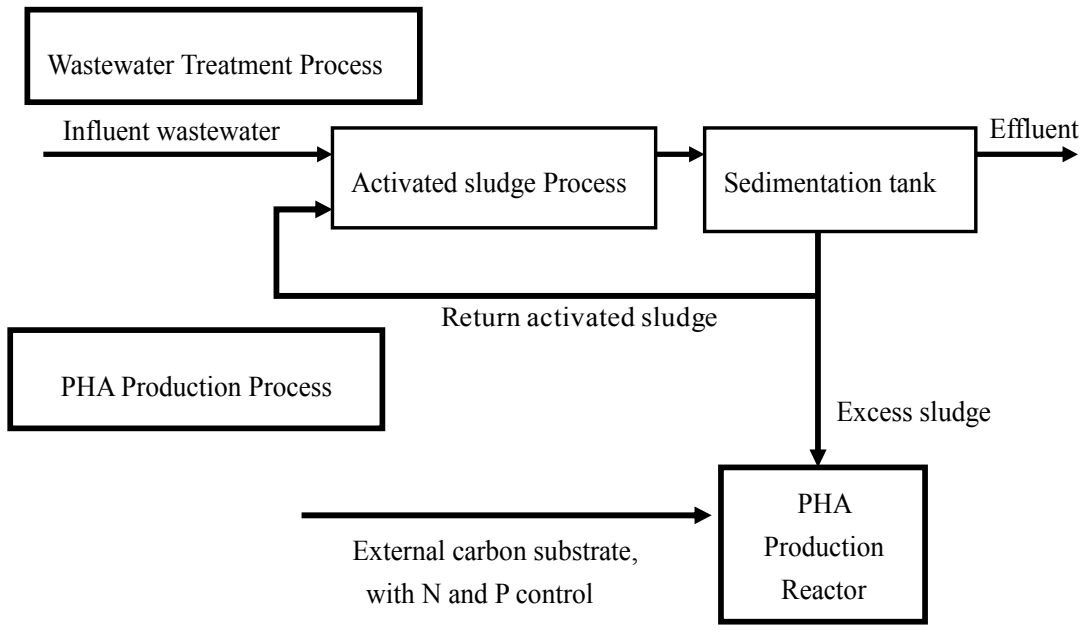


Figure 3.2 PHA production system by using activated sludge treating wastewater.

For the Experimental Design Reactor Set-up, the figure is shown below. The SBR system equipped with peristaltic pumps for influent feeding and effluent withdrawing, air compressor for aeration and timers for automatically controlling.

Figure 3.3 Biological reactors of 10 liters effective volume.

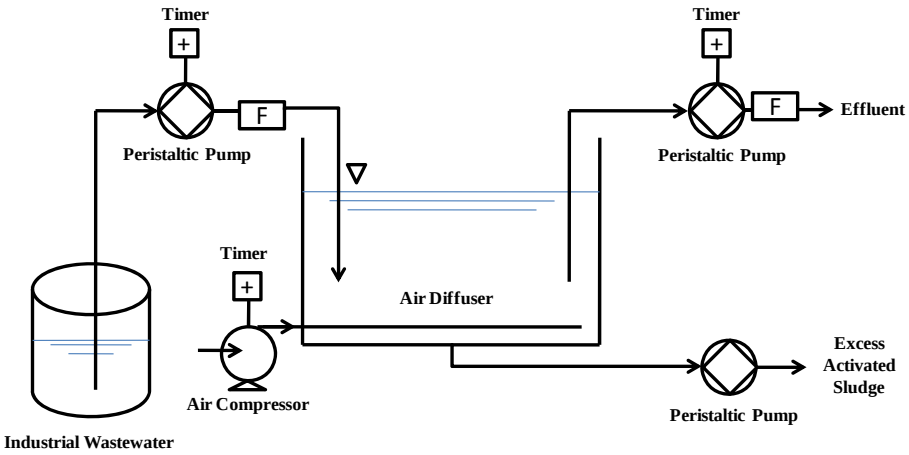


Figure 3.4 Schematic diagram of the wastewater treatment process.

The industrial wastewater was collected from HK Amoy food-processing industry in Tai Po Industrial Estate. The wastewater quality fluctuated, with

narrow ranges as shown below:

COD=290–320 mg /L

Total organic carbon (TOC) = 380–460mg /L

Phosphate (PO4-P) =1.7–2.9 mg /L

Activated sludge was collected from an industrial wastewater treatment plant. The activated sludge was first thickened by settling for at least 2 hours. Then, 1 litre of the settled and thickened activated sludge was transferred and cultivated in a laboratory-scale SBR of 10-L effective volume without pH control under aerobic conditions. The inoculated reactor started off with an initial MLSS of 3,000 mg/L.

The batch loading rate or F/M ratio was set at around 0.24 mg BOD/mg MLSS-d and the average organic reduction efficiency was relatively stable at around 82-89%. The reactor was fed with the industrial wastewater of an average COD of 312 mg/L and BOD of 180 mg/L, including glucose. The wastewater was supplemented with NH4CI at 10.40 mg/L to result in a C:N ratio of 30.

Nitrogen and phosphorus were added in the form of NH_4Cl and KH_2PO4 (10.40 mg and 0.98 mg per L of wastewater, respectively). The wastewater was also supplemented with trace mineral, and a growth factor as described in

Table 3.1.

Table 3.1 Composition of mineral supplements of the synthetic culture medium with macro and micro nutrients

Nutrient Supplements	Concentration (mg/L)
KH_2P04	0.9800
$MgS0_4.7H_2O$	0.0190
$FeCl_3$	0.0285
$CaCl_2$	0.0038
$Al_2(S04)_3$. $l8H_20$	0.0020
$CoCl_2.6H_2O$	0.0076
Thiamine hydrogen chloride	0.0076
$NaSi0_3.5H_20$	0.0038
H_3B0_3	0.0038
$(NH_4)_6Mo_20_{24}.4H_20$	0.0190
$CuS0_4.5H_20$	0.0190
$ZnS0_4.7H_20$	0.0190
$MnCl_2.2H_20$	0.0025

As optimization of operational conditions in activated sludge process is essential for PHA production enhancement as well as for satisfactory effluent quality. Sludge retention time was investigated and controlled in this study because they are important and easily manipulated in activated sludge process. The hydraulic retention time in the selector was adjusted to be just long enough for almost complete uptake of the incoming COD. The total removal of soluble COD for the

process was gradually stabling at an average level of 85%.

After the reactor was operated for a period of time long enough to attain stable conditions, 300 ml of activated sludge samples were taken periodically in the aeration period from the reactor for PHA content and biomass concentration analysis. Because the effective volume of the reactor was only 10 L, the sample volume had to be controlled in order not to upset the process stability. Also, in order not to affect the COD removal performance of wastewater treatment system, no duplication of sample was taken.

In each operating cycle, liquid- and solid-phase samples were taken regularly (once in every 2 weeks) at the end of the aerobic aeration period to analyze the COD, pH, MLSS and DO, as a consistent monitoring of the process performance stability, reliability, effluent quality and sludge quality.

3.2 Operation of SBR

One cycle of the SBR operation consisted of five stages, namely fill, aeration, settle, decant and idle, with an effective working volume of 10 L. Firstly, the optimal aeration and settle time, and the hydraulic retention time (HRT) were determined. Subsequently a HRT of 8 h were maintained in an air-conditioned room with temperature between 20°C (winter) to 28°C (summer).

3.2.1 Optimum aeration and settling time

The aeration and settling time is important for achieving the optimum PHA accumulation. The effect of the aeration time (1, 1.5, 2, 2.5, 3 and 3.5h) on PHA accumulation was studied under continuous aeration, by determining the trend of COD depletion, while the settling time was optimized by investigating the profile of supernatant SS levels. The optimum aeration and settling times were at the instants when the COD and SS profiles reached a relatively stable level, which had a fluctuation of less than 10%.

3.2.2 SBR parameters

SBR was operated with 4 cycles per day (**Figure 3.5**).

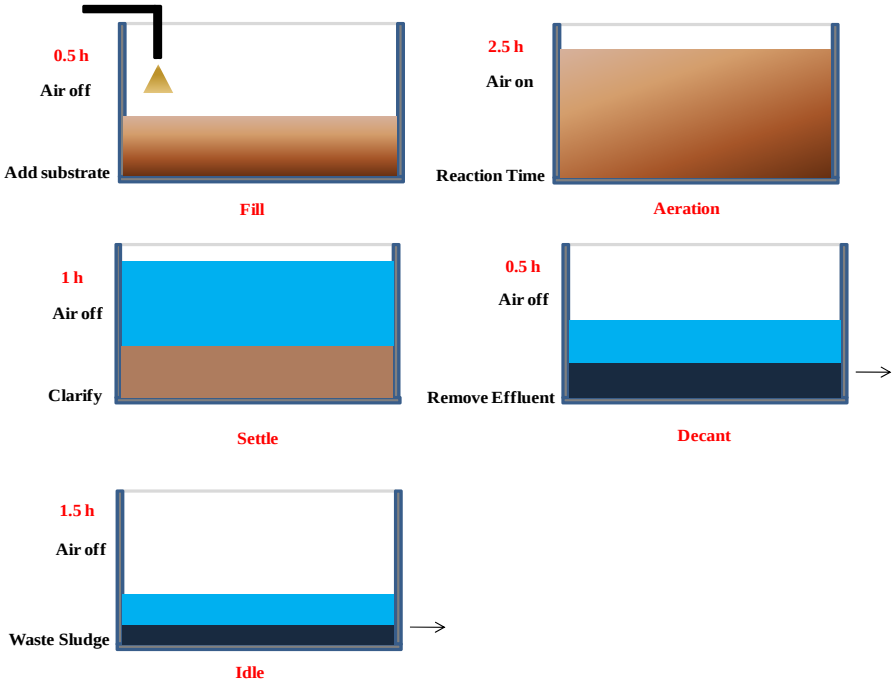


Figure 3.5 Operation sequence for one cycle of SBR process.

Table 3.2 Description of the operational steps for the laboratory-scale SBR

Operational step	Aeration status	The length of time	Flow rate	Description
Fill	Air off	0.5 hours	10L/h	This is the time for adding in substrate medium to the reactor. One litre of substrate is fed each time.
Aeration	Air on	2.5 hours		Air compressors and diffusers were activated by timer control in order to provide sufficient oxygen for bacterial growth and mixing for the activated sludge liquor.
Settle	Air off	1 hours		This period was to allow biomass separation to occur. A clear supernatant would be obtained for discharge towards the end of the stage.
Decant	Air off	0.5 hours	10L/h	The peristaltic pump was started to remove one litre clarified treated water supernatant from the reactor.
Idle	Air off	1.5 hours		This period was to provide time for the reactor to complete its cycle time before switching to another cycle.

Table 3.3 Designed Operating Conditions of the SBR

Effective Volume	10 L
HRT	8 hours
SRT	15 days
Temperature	20℃-28℃
Influent COD	~312 mg/L

The stirring rate was maintained at 500 rpm using a mechanical agitator. The reactors were operated at room temperature in the range of 20 – 28℃.

3.2.3 Optimum C:P Ratio

The optimum carbon-phosphorus (C:P) ratio was evaluated by changing the C:P ratio from 150 to 400 at increment interval of 50. The PHA accumulation at different C:P ratios were sampled and measured.

The C:P ratio was altered by keeping the COD level at around 310 mg/L and changing the KH_2PO_4 concentration. The subsequent PHA productivity was determined by taking samples of the reactor mixed liquor, separating the biomass, and conducting an organic solvent extraction of PHA for quantitative analysis.

3.2.4 Effect of C:N Ratio on PHA production

After the reactor operated under stable conditions with the C:N ratio of 30, the supplied nitrogen was reduced to result in C:N ratio of 45, 60, 75, 90, 105 and 120, making it different degrees of nutrient deficiency. Since long-time nitrogen

deficiency would have adverse influence on microbial growth and the organic treatment performance, an intermittent nitrogen feeding was required after every 4 cycles to enhance cell growth to each C:N ratio above 30. In order to control intermittent nitrogen feeding, an additional time-controlled peristaltic pump was installed into the system and a refrigerated storage tank for synthetic medium with C:N ratio of 30 was prepared. In a nitrogen feeding cycle, an influent with C:N ratio of 30 was fed into the reactor. Activated sludge samples for each C:N ratios were periodically taken from the reactor during the aeration period in a randomly selected operation cycle for analysis.

3.3 Pilot-scale SBR operation

The pilot-scale SBR system was built for PHA accumulation based on optimum condition determined from the laboratory-scale system. It had an average working volume of 600 L, equipped with mechanical foam disrupters which allowed for intensive aeration and kept an average DO concentration of 2.5 mg/L throughout the process operation.

The SBR system operating cycle could also be divided into Five Stages: Fill, React, Settle, Decant and Idle. The cycle length of 6 h was 0.5 hr, 2.5 hr, 1 hr, 0.5 hr and 1.5 hr, respectively.

Table 3.4 Description of the operating parameters for the pilot-scale SBR

Operational step	Aeration status	Duration of stage	Flow rate
Fill	Air off	0.5 hours	600 L/h
Aeration	Air on	2.5 hours	
Settle	Air off	1 hours	
Decant	Air off	0.5 hours	600 L/h
Idle	Air off	1.5 hours	

Figure 3.6 in below shows a scheme of the PHA production by activated sludge in the pilot-scale work.

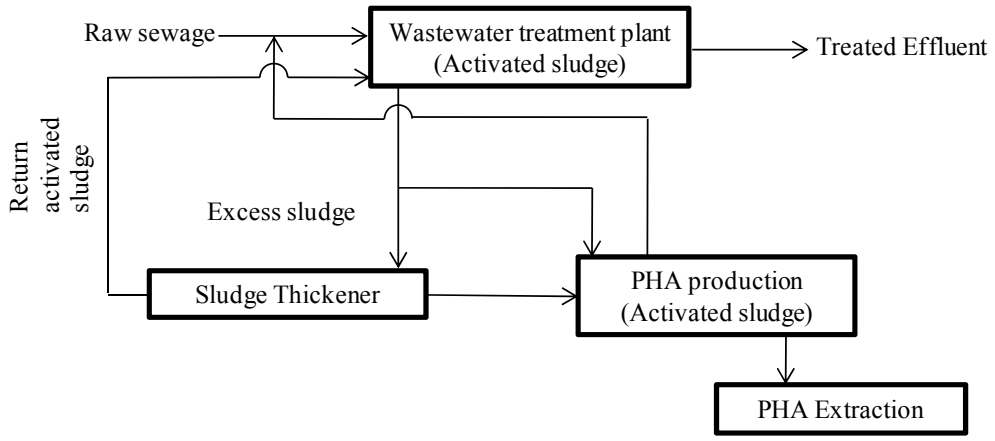


Figure 3.6 The process flow diagram for pilot-scale SBR.

The essential process operating parameters were measured on a daily basis during the operation of the pilot-scale work.

Table 3.5 The parameters measured on a daily basis

pH	Measured by using a digital pH meter (Orion model EA940)
DO	Measured by an on-line DO monitoring system (YSI Model N055)
MLSS	Standard (American Public Health Association 2005) 2540D
BOD5	Standard (American Public Health Association 2005) 5560C
COD	Standard (American Public Health Association 2005) 5220D

Figure 3.7 On-site pilot-scale SBR at Wuxi Sewage treatment works.

The schematic diagram of the SBR is given in Figure 3.8.

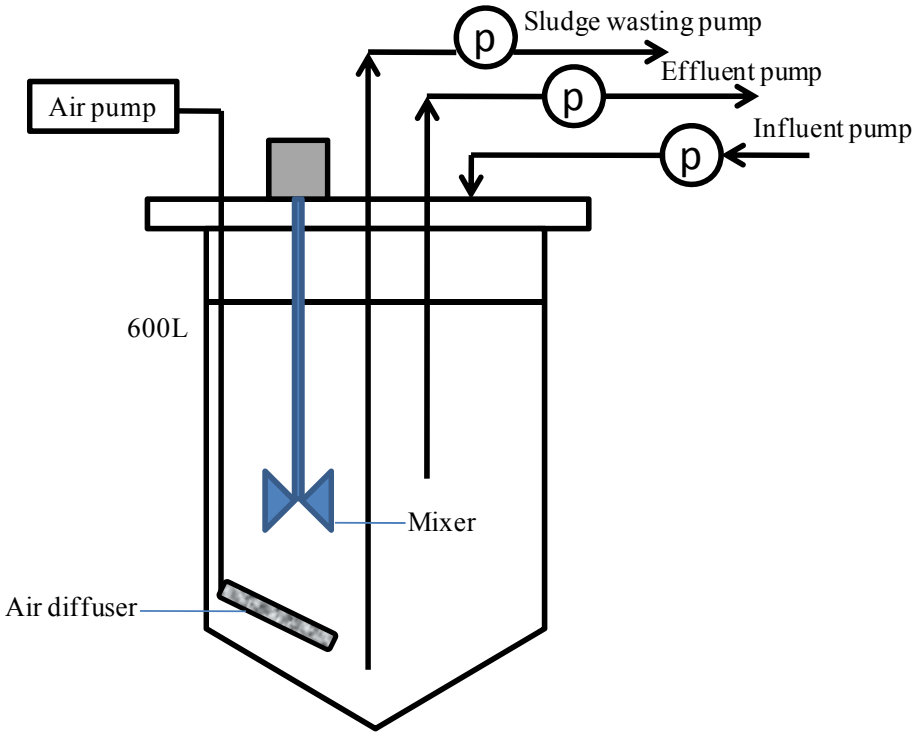


Figure 3.8 Schematic diagram of the SBR.

3.3.1 The SBR system operating cycle and essential parameters

At the start-up step, the return activated sludge (RAS) of the original wastewater treatment plant was use to cultivate the pilot-scale process. The reactor was inoculated with activated sludge taken from a municipal sewage treatment works in Wuxi treating mixed sewage of domestic and industrial origins. The sludge in the pilot-scale SBR system was fed with synthetic wastewater, with a composition that included KH_2PO_4, urea, $FeCl_3$, CHOCOOH, glucose, and NH_4Cl. The DO concentration was controlled at 2.5 ± 0.3 mg/L. The SBR system was acclimatized at 15 days of sludge retention time (SRT). Influent and

effluent water qualities of the SBR system were analyzed twice per week.

3.3.2 Setting C:N ratio by external dosing

The return activated sludge of the plant was taken to seed the reactor and was cultured with the aforementioned conditions. The effluent of the SBR system was analyzed. The COD and the SS of the effluent were determined to judge whether the system was under stable status. After the system was stable, the C:N ratio was set by external dosing. The optimum C:N ratio from laboratory-scale studies would be set at the beginning of reaction by using the dosing system. Once the C:N ratio was fixed, the system would run for several days to attain another stable status. The sludge samples were taken several times when the system was stable.

3.3.3 Effects of in-coming organic composition on co-polymeric composition

Control of co-polymeric composition was studied by carrying out a series of pilot-scale SBR system operations with varying in-coming carbon compositions. The nitrogen-free medium contained butyric acid (C_4) and valeric acid (C_5) as carbon sources, and supplementary trace minerals and a growth factor as described in Table 3.1 were added. The C4 to C5 weight ratios in the medium were separately adjusted to 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100 (g/g) and

the resulting co-polymers produced were sampled, extracted, purified and analyzed.

3.4 Analytical techniques

The PHA content (%) was defined as the percentage of the ratio of PHA concentration to dry cell weight (DCW). Process performance in the fermenter and PHA accumulation tests were evaluated by measuring substrate uptake, PHA production, nutrient consumption, and solids levels.

Samples taken from the reactor for analysis of residual carbon concentration (COD) (Standard Code: 5220), total kjeldahl nitrogen (TKN) (Standard Code: 4500-Norg), dissolved oxygen (DO), pH and DCW (Standard Code: 2540D).

DO was measured by DO meter (YSI Model No. 55). The pH was monitored by pH meter (ORION Model EA 940). The TKN was determined using a Tecator Autoanalyzer (KJEL TEC AUTO 1030 ANALYZER). The activated sludge sample was first centrifuged and only the supernatant was taken for TKN analysis. The cell concentration was determined by measuring DCW. The dry cell mass was performed by mass liquor suspended solid (MLSS).

1. Mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solid (MLVSS) and COD involved in each experiment are analyzed by Standard Methods for the Examination of Water and Wastewater;

2. Solvent extraction method is adopted for PHA recovery process;

3. PHA,PHB and PHV are measured by Gas Chromatography (GC) method;

3.4.1 The extraction of PHAs from activated sludge (laboratory-scale)

The activated sludge from the SBR was centrifuged at 3,000 rpm for 15 minutes. The PHA content of the washed and dried biomass was determined by extraction. A chloroform-based method was used to extract PHAs from biomass samples, with methanol as precipitation agent.

The three separate phases were obtained. The upper phase was the hypochlorite solution, the middle phase contained non-PHA cell material and undisrupted cells, and the bottom phase was the chloroform layer including 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) and filtered out the products by simple filtration. The sample was put on an aluminum foil and then dried by evaporation for 24 hours to a constant weight. The weight of the extracted polymers were measured to determine the productivity.

Glass transition temperature (Tg), melting temperature (Tm), and melting enthalpy (ΔHm) were analyzed by differential scanning calorimetry (DSC, TA

Instruments Q1000).

3.4.2 Gas Chromatography method

The determination of PHAs was performed by gas chromatography. One liter of activate sludge from SBR 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 I ml of esterification solution, which is consisted of 3ml 98% H_2S04 and 0.29 g benzoic acid dissolved in 97 ml methanol as an internal standard. Sodium 3-hydroxybutyrate was used as the standard for the quantification of 3HB. 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 uL of lower organic phase was injected by split injection into a G.C. Nitrogen was the carrier gas, at a flow rate of 10 ml/min.

The detector over and injector temperatures were 250°C, 180°C and 120°C, respectively. Initial temperature setting of column was 60°C for 4 min, then

increased in 12°C/min to 180°C and maintained for 6 min.

PHA purity and its composition could be determined by this GC method, and the quantity of PHAs 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 PHAs in the samples could be identified and estimated. The procedure was repeated for the standard PHB and P (3HB-co-3HV).

3.4.3 The extraction of PHAs from activated sludge (pilot-scale)

Before analysis the PHAs accumulated in the excess activated sludge, it's should be extracted. There is little different from laboratory-scale. From the experiment site to the laboratory, 0.5 vol. % of formaldehyde solution was added to the sludge sample due to inhibiting the activities of the bacterial cells.

50 ml of EAS is centrifuged under 3000 rpm for 20 min. The supernatant was sucked off and the sample was then frozen in a freezer overnight in order to make sure no liquor exists. The frozen sample was processed in a free dryer to lyophilize over 24 hours to remove the H_2O by sublimation. The DL-3-Hydroxybutyric acid (HB) sodium salt was dissolved in acidified methanol and was used as the standard. It's weighed 20 mg of lyophilized sample into Pyrex test tubes (volume, 15 ml) with Teflon-lined caps. Then added 2 ml

esterification solution, which contains 3 ml concentrated H_2SO4 and 2.9 g benzoic acid in every 100 ml, and 2 ml chloroform. Esterfication reaction at 100℃ was processed using water bath for 3.5 hours. After cooling, 1.9 ml denser chloroform phase was transferred in another Pyrex tube (volume, 10 ml) containing 0.5 ml of distilled water. This re-extraction step was necessary, because it greatly improved the reliability of the method by removing acids and particulate debris which caused premature degradation of the GC column. After 5 min vigorous shaking and 3 min centrifugation at 3000 rpm, the PHA methyl ester was extracted in the chloroform phase at the bottom of the tube.

3.4.4 Determination of melting temperature

Melting point of the extracted polymer was determined by using an Electrothermal 9100 digital mp apparatus. The temperature increase rate program was set at 10°C/min from room temperature to 200°C.

4 RESULTS & DISCUSSION

4.1 Optimal aeration and settling time

The aeration time is an important parameter for the operation and optimization of the SBR process, because it governs the reaction time, the availability of dissolved oxygen and the completeness of biochemical reactions. If the aeration time is insufficient, there will not be enough time and oxygen supply for the microbes in the activated sludge to decompose the soluble and suspended organic matters in the wastewater, which in turn adversely affects the treatment efficiency and discharge effluent quality. On the other hand, if the aeration time is too long, it will increase energy consumption, trigger off the sludge to enter into an endogenous respiration phase and cause a shift in microbial species balance in the ecosystem, and again adversely affecting the quality of the sludge and the final treated effluent.

The curve shown in Figure 4.1 depicts the trend of decrease of the residual COD in the culture liquor in the SBR simulator. It shows that the residual COD level in the system was a prime factor determining the stability of the activated sludge under different duration of aeration time. In the total operating time of 3.5 hours of aeration times (with 30-minute interval for sampling), the COD decreased from around 312.5mg/L to 31.7 mg/L, equivalent to around 90 % of organic

removal efficiency, in around 2.5 h and remained more or less stable after that. By stable, it was defined as a fluctuation of COD level of less than 10 % of the average COD values. This treatment efficiency surpassed that reported in the literature for comparable systems (Chua et al., 1999; Dionisi et al., 2004) both in terms of COD removal and aeration time. Therefore, the aeration time of 2.5 h, with the COD attaining a low tolerant or persistent level was fixed as the optimum operating parameter for subsequent sludge selection and PHA accumulation.

The settling time of the SBR operation was similarly selected with a total settling time of 1.5 hours and 15-minute intervals for sampling. The sampled supernatant MLSS decreased from the initial 2,500 mg/L to a stabilized level of 50 mg/L after a settling of 1 hour. MLSS level remained stable with less than 10 % fluctuation for the remaining observation. Therefore, the settling of 1 hour was taken as the optimum operating parameter for subsequent sludge selection and PHA accumulation.

The optimized aeration time and settling time, together with the preselected feeding time, decanting time and idling time, formed the physical selection pressures to attain a microbial consortium that was dominated by species that are hyper-accumulator of PHAs as their carbon reserves.

Table 4.1 Residual COD profile at different aeration times

Aeration Time (h)	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**
COD (mg/L)	313±15.6	68.9±10.4	42.5±7.1	33.5±5.7	31.9±4.6	31.7±1.6

Data are means ± SD (n=5)

Table 4.2 Supernatant SS profile at different settling times

Settling Time (h)	**0.25**	**0.50**	**0.75**	**1.00**	**1.25**	**1.5**
SS (mg/L)	2500±100	1101±80.3	302.8±50.3	53.7±12.3	53.1±13.5	50.3±10.3

Data are means ± SD (n=5); SS means MLSS because the samples were collected from a homogeneous reactor liquor

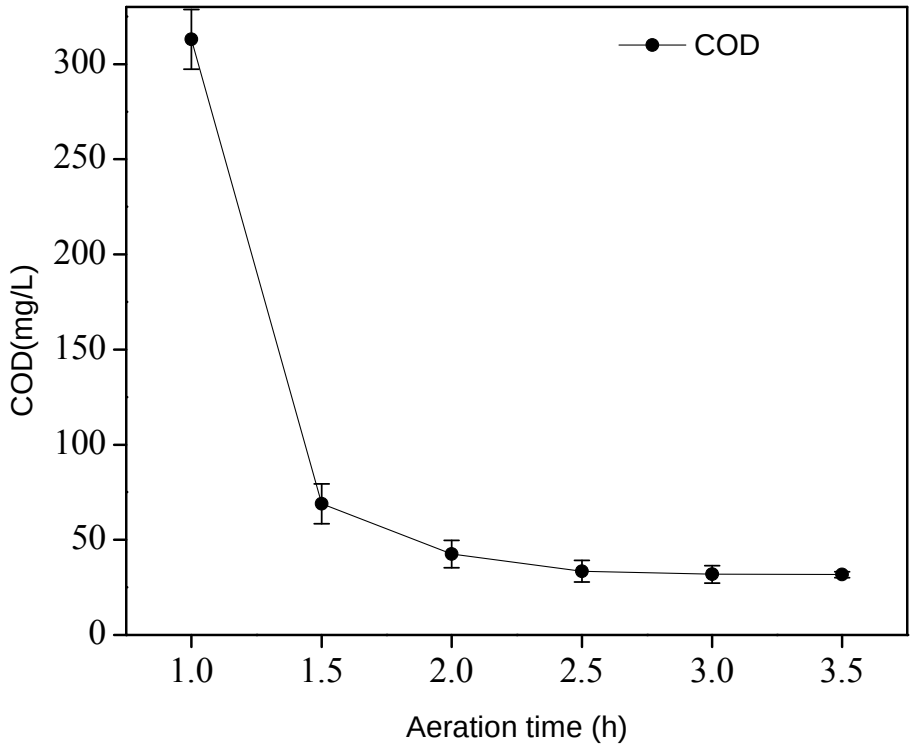


Figure 4.1 Residual COD profile at different aeration times.

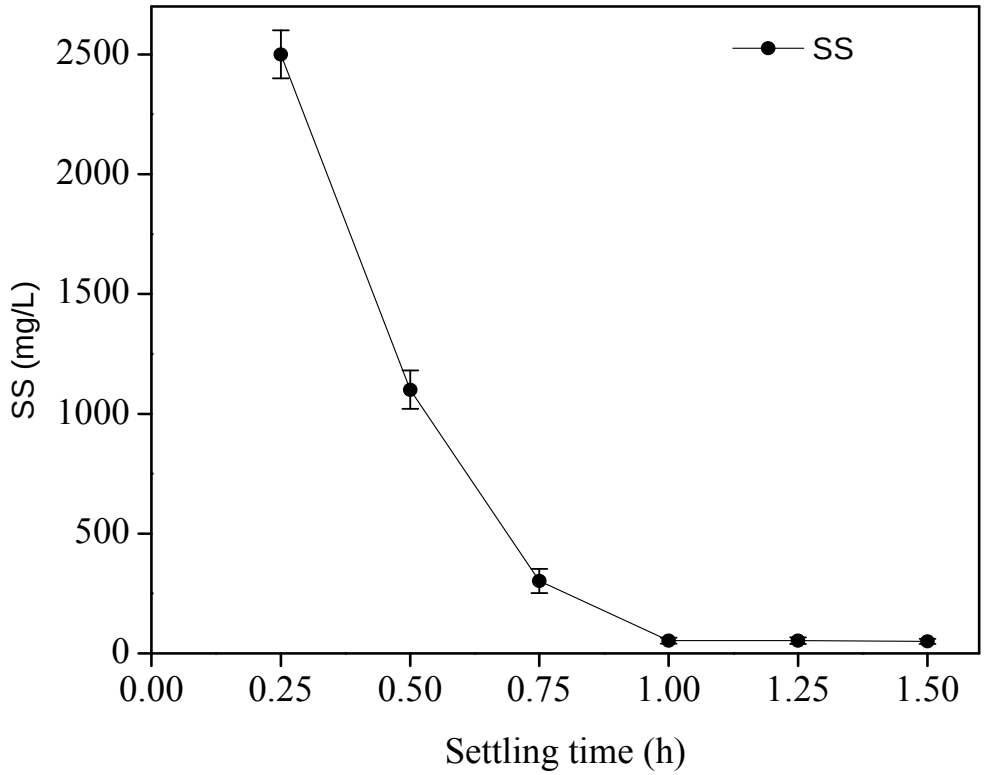


Figure 4.2 Supernatant SS profile at different settling times.

Physical selection for activated sludge

The above results showed that an activated sludge process may be optimized under physical operating conditions, namely 2.5h aeration time for active soluble and suspended organic degradation, nitrification conversions and 1h settling time for water-biomass separation and denitrification activities. If the aeration time was allowed to proceed longer than 2.5 h, the various species of microorganisms in the sludge will go into the endogenous respiration period due to the lack of degradable organic substances and hence deteriorated the sludge quality. Furthermore, the intracellular PHAs, accumulated as the carbon reserves, will be consumed for energy regeneration and cellular metabolic subsistence. As a result, over aeration will cause reduction in sludge biomass and PHA production yield, which was consistent with that observed by Lee and Choi (1999).

These results showed that both the operating parameters, namely the aeration and settling times, are effective physical pressures for selecting the intended activated sludge. With the influent COD of 313 mg/L (equivalent BOD_5 of 150 mg/L) and MLSS of 2,500 mg/L, when the aeration time was optimized as 2.5 hr, the resulting process organic loading rate or F/M ratio (BOD_5/MLSS-HRT) was 0.24 mg/mg-d. This compared well with the published literature that the F/M ratio of a typical activated process should fall within the range of 0.1-0.5 mg/mg-d (Zafiriadis et al., 2011; Renou et al., 2008). This could further showed that the

physical selection pressure could effectively establish an activated sludge that was stable, robust and ready for further selection for PHA accumulation.

Concurrently, the initial TKN of 10.9 mg/L in the reactor liquor, which included organic nitrogen in the forms of proteins, polypeptides and amino acids, and ammonia nitrogen, took slightly more than 2.5 hours to degrade to a stable and low level of 2.1 mg/L in the nitrification process. In the initial phase of the aeration time, the TKN were contributed mainly by Org-N, while NH3-N took over as the dominating species as aeration proceeded. These nitrification processes were mediated by *Nitrosomonas spp*. and *Nitrobacters spp.*. and a number of other saprophytic bacteria which were not the PHA accumulators in the activated sludge (Gebremariam et al., 2012; Krishna and van Loosdrecht, 1999). While TKN was degraded, nitrite nitrogen and nitrate nitrogen were being formed (Figure 4.3). Again 2.5 hour of aeration and reaction time was sufficient for the near completion of the entire nitrification process, which was indicated by the leveling out of the four curves in the graph. When the activated sludge simulator system entered into the settle phase, the microbial consortium also entered into the denitrification phase under the anoxic condition with low dissolved oxygen and ORP levels. The process of conversion of nitrite nitrogen to gaseous nitrogen (N_2), during the optimized settling time of 1 hour, was mediated by *Pseudomonas spp*., which are known to be PHA accumulators (Yu et al., 1999; Gebremariam et al., 2012; Krishna and van

Loosdrecht, 1999).

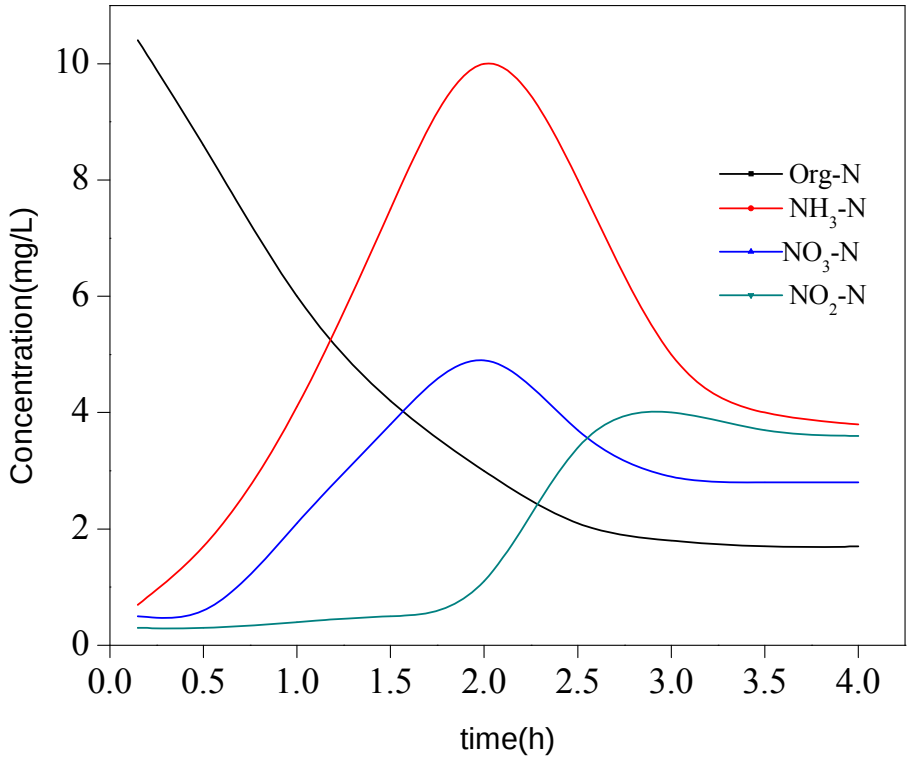


Figure 4.3 Inter-conversion of different forms of nitrogen during aeration phase.

If aeration and settling time could be well controlled, it would get optimal production rate of PHAs. The aeration time (2.5h) and setting time (1h) in each cycle was used subsequently in both the sludge selection in the laboratory simulator and the pilot-scale system.

4.2 Optimal C:P ratio

Figure 4.4 shows the variation in PHA yield over the aeration time under different C:P ratios. The PHA yield increased from around 0.080 g to 0.115 g. The results showed that the highest PHA yield of 0.122 g was obtained with C:P

ratio of 300. Then the PHA yield decreased with further increment of C:P ratio, which was attributed to the severe phosphorus deficiency affected the normal growth of the microbes. Increasing C:P ratios led to the fastest PHA accumulation showing that phosphorus deficiency was a sensitive biochemical or nutrient factor that triggered off the accumulation of intracellular food reserves. These results were consistent with those in classical observations reported in the literature (Fleit, 1995; Comeau et al., 1986). The typical peak of PHA production was observed under the physical operating and selection conditions at the aeration time of 2.5 h. These results also illustrated that PHAs was readily and spontaneously accumulated with the C:P ratio of 300 under the conditions of aeration time of 2.5 h and settling time of 1 h.

Table 4.3 PHA accumulation under different C:P ratios

C:P ration	**150**	**200**	**250**	**300**	**350**	**400**
del P (g)	0.0801±0.001	0.0962±0.002	0.119±0.003	0.122±0.002	0.118±0.002	0.115±0.001

Data are means ± SD (n=5)

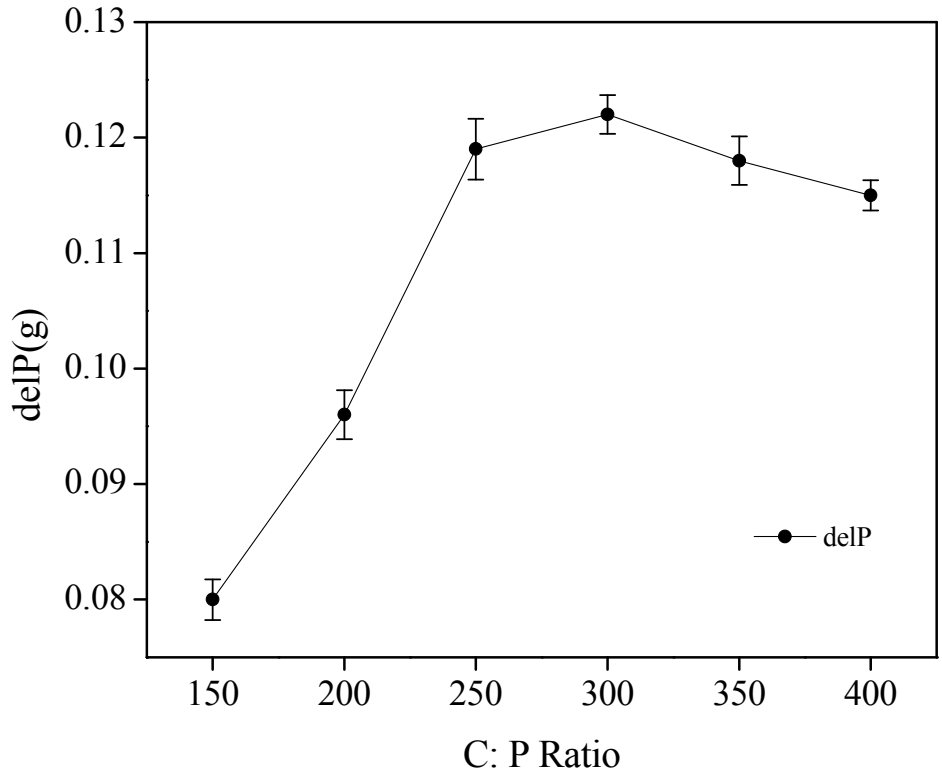


Figure 4.4 PHA accumulation under different C:P ratios.

4.3 Optimal C:N ratio

4.3.1 Performance of SBR under different C:N ratios

After obtaining the optimal aeration, settling time and C:P ratio, the biological reactor was operated in aerobic dynamic feeding with industrial wastewater collected from a local food-processing manufactory (HK Amoy Food Ltd.), the organic concentration measured in terms of COD was 312 mg/L. The nitrogen concentration of the wastewater was controlled to meet an intended C:N ratio of 30. As the reactor was operating under stable conditions, in order to evaluate its impact on PHA production, the nitrogen concentration in the wastewater was

reduced, resulting in C:N ratio of 45, 60, 75, 90, 105 and 120, which led to different degrees of nitrogen deficiency for PHA accumulation. The purpose of varying C:N ration was designed to create an unfavorable inhibiting environment for the growth of the microbial cells in the reactor liquor. The SBR was first observed to have stabilized at C:N ratio of 30, and was then operated under the higher C:N ratios. After the SBR system was operated in a stable condition under each C:N ratio, samples of the reactor liquor were periodically collected for analysis during the 2.5-hour reaction time in operation cycles randomly. In each run, the SBR was operated for at least 2 months, in order to attain the stable conditions.

4.3.1.1 Production of PHAs under C:N ratio of 30

After attaining the steady state conditions, samples of mixed liquor were collected for every 15 minutes during the 2.5 hours reaction time in a randomly selected operation cycle. Steady-state conditions were defined as that all measurable parameters fluctuated within a 10 % range over time, which was consistent with normal practices (Gurieff and Lant, 2007; Satoh et al., 1999). Analysis of the parameters included residual organic and nutrient concentrations, cell growth and PHA polymeric accumulation. The values of these parameters are shown in Table 4.4. The concentration of carbon, measured as residual COD, in the mixed liquor of the reactor decreased with time from 328.5mg/L to

35.8mg/L, with 89.1% COD removal efficiency. These observations surpassed that reported in the literature for similar activated sludge systems operated with similar conditions (Seviour e al., 2003; Reddy and Mohan, 2012b). In the meantime, the concentration of nitrogen, measured as residual TKN, in the mixed liquor of the reactor decreased from 10.9 mg/L to 2.1 mg/L. The pH of the reactor liquor was not controlled and fluctuated between 6.49 and 6.91, which was taken to be the normal operating range for activated sludge treating municipal sewage (Ciggin et al., 2011).

In Figure 4.5 Carbon and nitrogen consumption at C:N ratio of 30 revealed that both the residual COD and TKN maintained a consistent depletion rate throughout the 2.5 hours reaction time. However, as the residual COD and TKN decreased, the overall biomass increased from 23.342g to 25.255g, obtaining 1.913 g dry cell weight, at the same time, the polymer content increased from 0.107 g to 0.227 g, accumulating 0.120 g of net intracellular polymers as food reserves (Figure 4.6). These results indicated that the C:N ratio was ideal for microbial proliferation and biomass buildup, but was not initiating substantial PHA accumulation.

This indicated that the activated sludge microorganisms have the ability to take up some soluble and suspended organic carbon provided for growth and metabolic maintenance, and the smaller remainder, into bacterial reserve materials, PHAs.

Table 4.4 Laboratory - scale SBR performance under C:N ratio of 30

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	328.5	10.9	6.68	23.342	0.107
0.25	278.3	9.1	6.53		
0.50	190.2	7.5	6.60	23.841	0.149
0.75	160.7	6.3	6.91		
1.00	120.8	5.4	6.63	24.356	0.201
1.25	95.2	4.1	6.90		
1.50	55.9	2.9	6.54	25.014	0.210
1.75	41.8	2.6	6.49		
2.00	38.7	2.2	6.53	25.180	0.219
2.25	36.5	2.1	6.47		
2.50	35.8	2.1	6.41	25.255	0.227

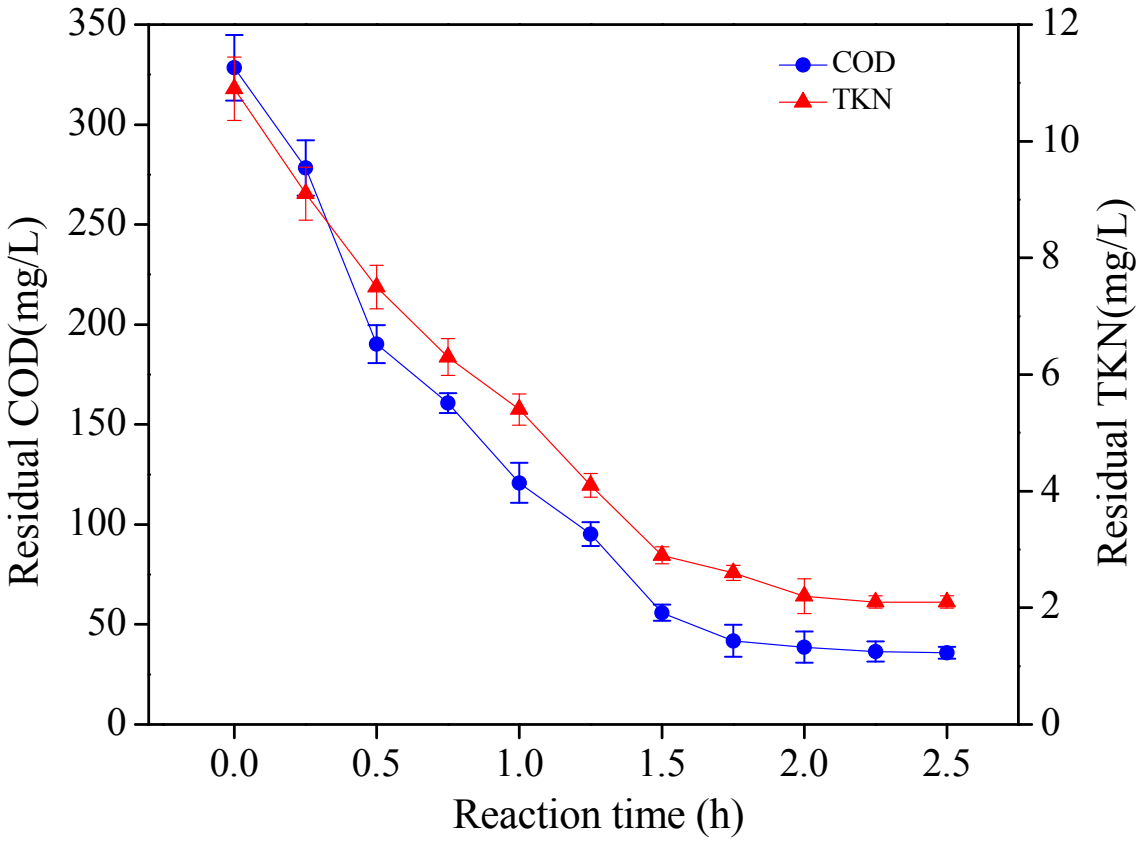


Figure 4.5 Carbon and nitrogen consumption at C:N ratio of 30.

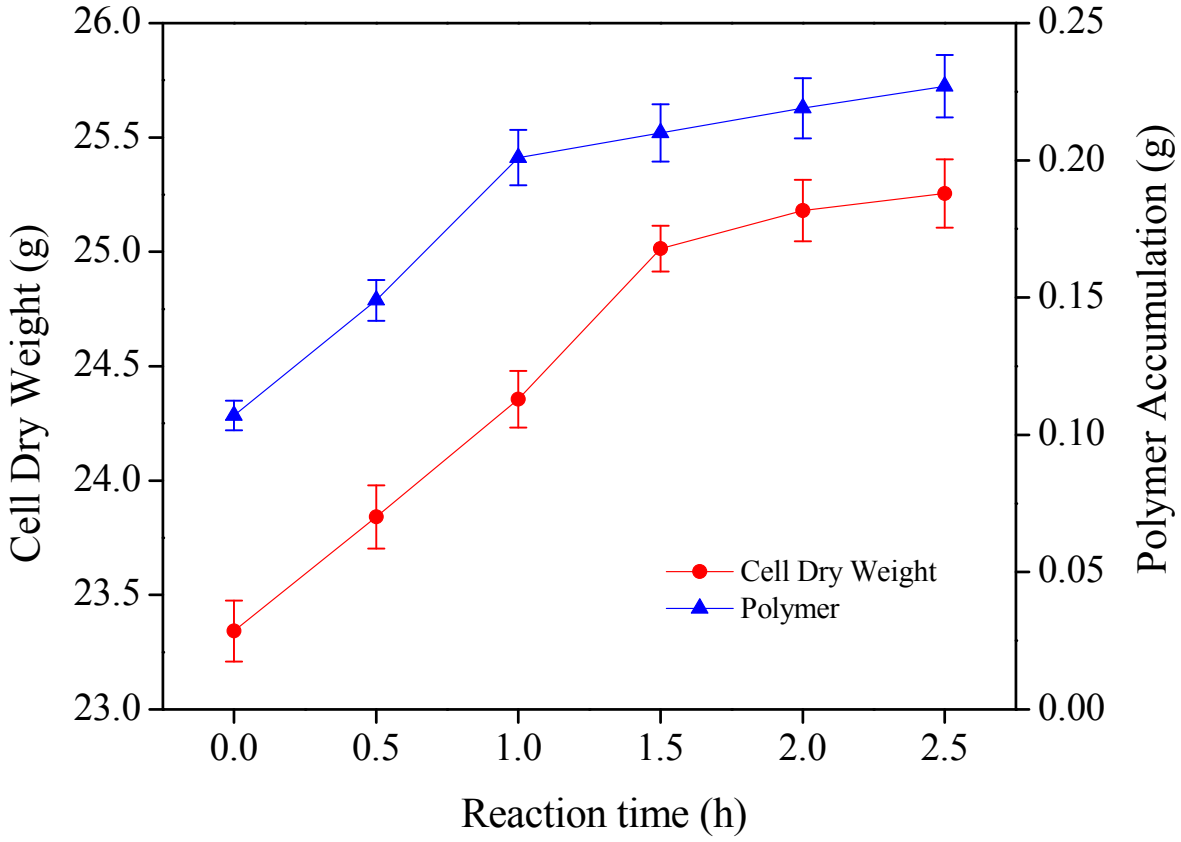


Figure 4.6 Growth and polymer accumulation under C:N ratio of 30.

4.3.1.2 Production of PHAs under C:N ratio of 45

After the experiment under C:N ratio of 30 was completed, ammonium chloride concentration was proportionately reduced to result in C:N ratio of 45. The SBR was operated for about two weeks until stable conditions were achieved.

It was found that when C:N ratio was increased to 45, similar observations were obtained as performance in the case under C:N ratio of 30. The residual COD and TKN decreased from time while the overall biomass and polymer accumulation increased. The residual COD reduced over time from 305.7 mg/L to 38.5 mg/L,

with 84.7% COD removal efficiency. During the same time, the residual TKN decreased from 6.8 mg/L to 2.0 mg/L (Figure 4.7). The pH fluctuated between 6.49 and 6.84. However, as the residual COD and TKN decreased, the net cell growth decreased to 1.700 g and the polymer accumulation increased to 0.163 g. These showed that the slight nitrogen deficiency hampered cell growth and encouraged PHA accumulation. These observations agreed with that reported by others (Coats et al., 2007b).

The performance of each parameter is shown in Table 4.5. The growth and polymer accumulation are shown in Figure 4.8.

Table 4.5 Laboratory - scale SBR performance under C:N ratio of 45

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	305.7	6.8	6.60	22.643	0.112
0.25	279.2	6.1	6.64		
0.50	245.3	5.4	6.81	22.929	0.114
0.75	180.4	4.2	6.73		
1.00	110.2	3.5	6.62	23.851	0.186
1.25	80.9	2.4	6.50		
1.50	60.1	2.3	6.72	24.128	0.240
1.75	54.6	2.1	6.84		
2.00	48.0	2.1	6.45	24.250	0.261
2.25	40.1	2.0	6.71		
2.50	38.5	2.0	6.49	24.343	0.275

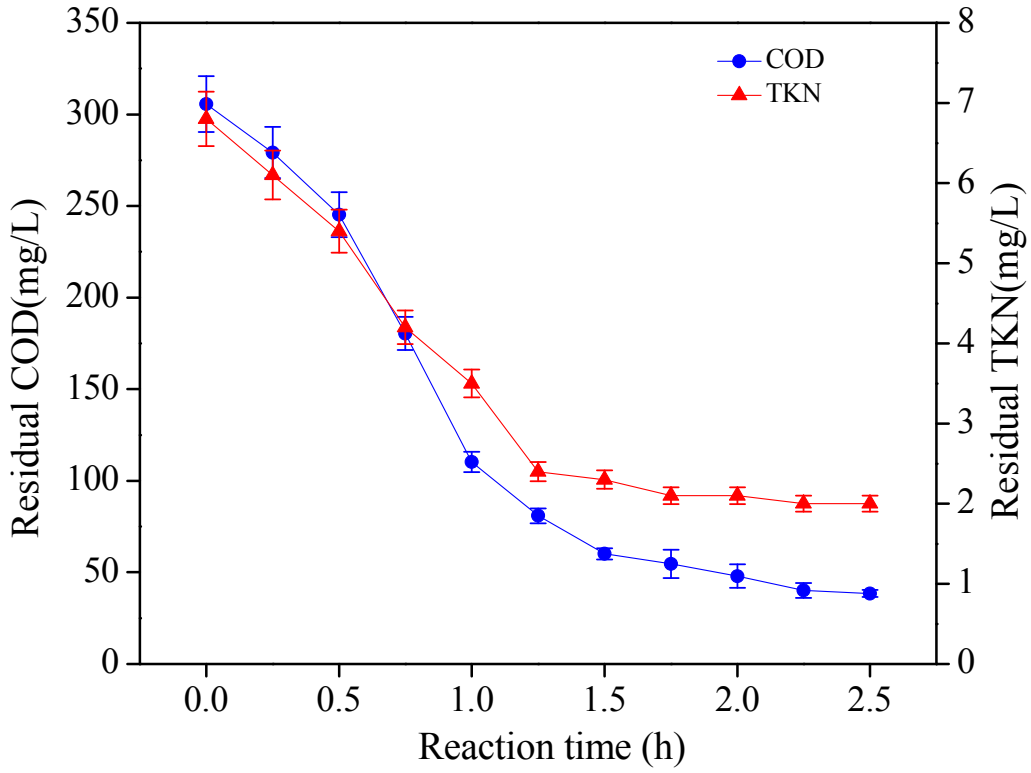


Figure 4.7 Carbon and nitrogen consumption at C:N ratio of 45.

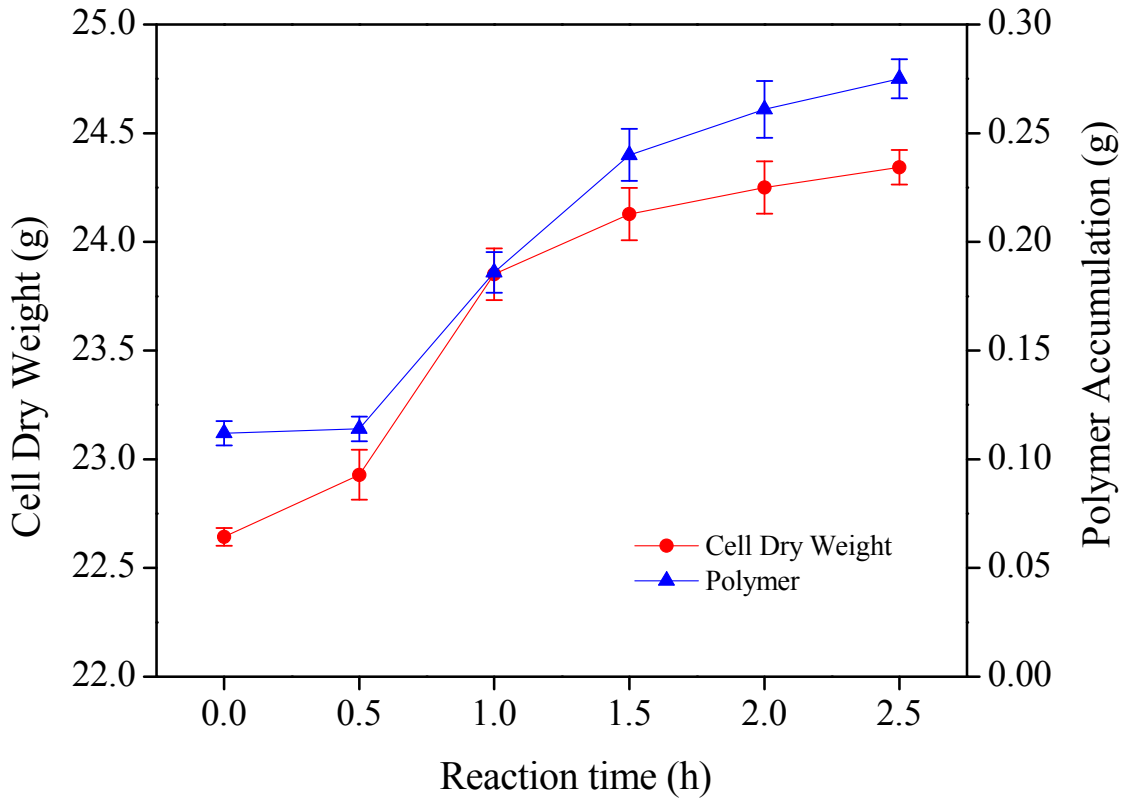


Figure 4.8 Growth and polymer accumulation under C:N ratio of 45.

4.3.1.3 Production of PHAs under C:N ratio of 60

In this operation, ammonium chloride content was further reduced to create a C:N ratio of 60. For this process, nine days was required for the reactor to attain a steady state. Similar trends were observed as the C:N ratios of 30 and 45. The parameters are shown in Table 4.6. It was found that the residual COD reduced over time from 307.3 mg/L to 47.3 mg/L, with 84.60% COD removal efficiency, and the residual TKN decreased from 5.1 mg/L to 1.9 mg/L (Figure 4.9). As the residual COD and TKN decreased, the overall biomass increased from 22.107 g to 23.618 g, obtaining 1.511 g net cell growth. The polymer content increased from

0.142g to 0.350 g, accumulating 0.208 g net intracellular polymers (Figure 4.10). These variations were consistent with the those observed with C:N ratios of 30 and 45.

Table 4.6 Laboratory - scale SBR performance under C:N ratio of 60

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	307.3	5.1	6.58	22.107	0.142
0.25	280.7	4.5	6.74		
0.50	240.4	3.9	6.85	22.306	0.165
0.75	170.2	3.6	6.49		
1.00	114.6	3.2	6.67	22.911	0.237
1.25	82.9	2.9	6.54		
1.50	60.4	2.4	6.71	23.310	0.316
1.75	52.6	2.1	6.79		
2.00	50.1	2.0	6.71	23.491	0.338
2.25	48.0	1.9	6.59		
2.50	47.3	1.9	6.62	23.618	0.350

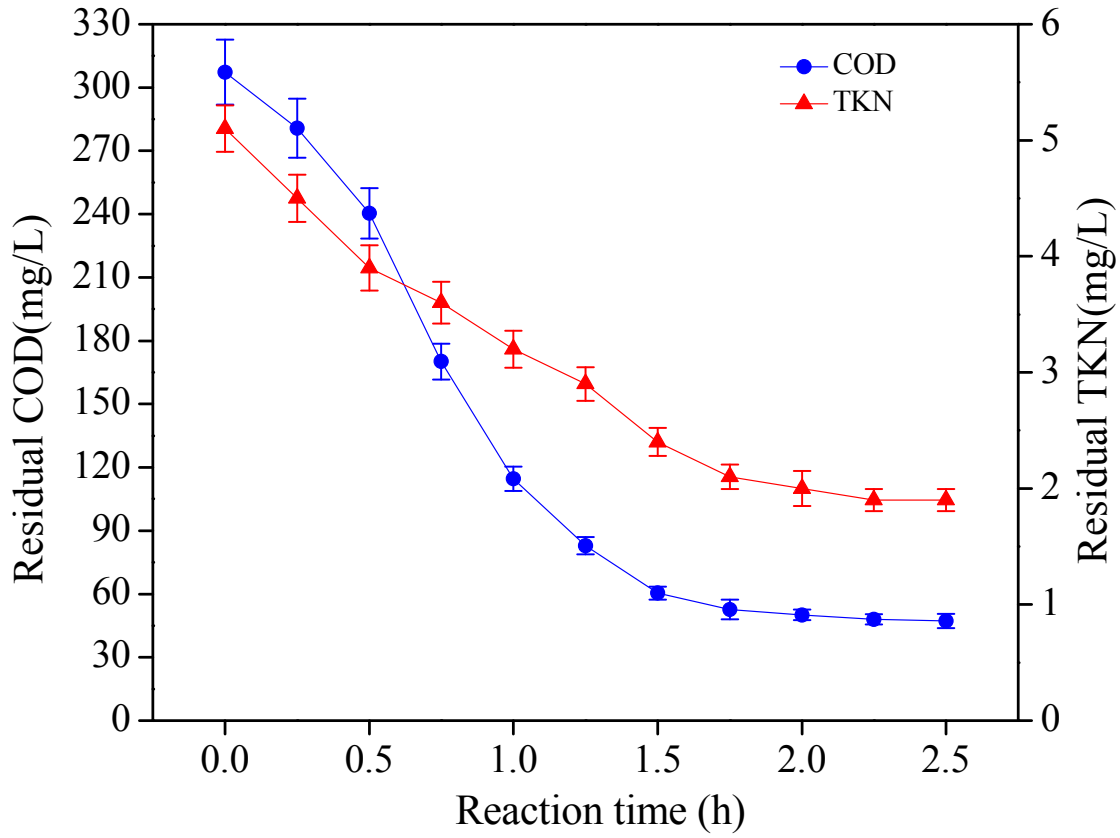


Figure 4.9 carbon and nitrogen consumption at C:N ratio of 60.

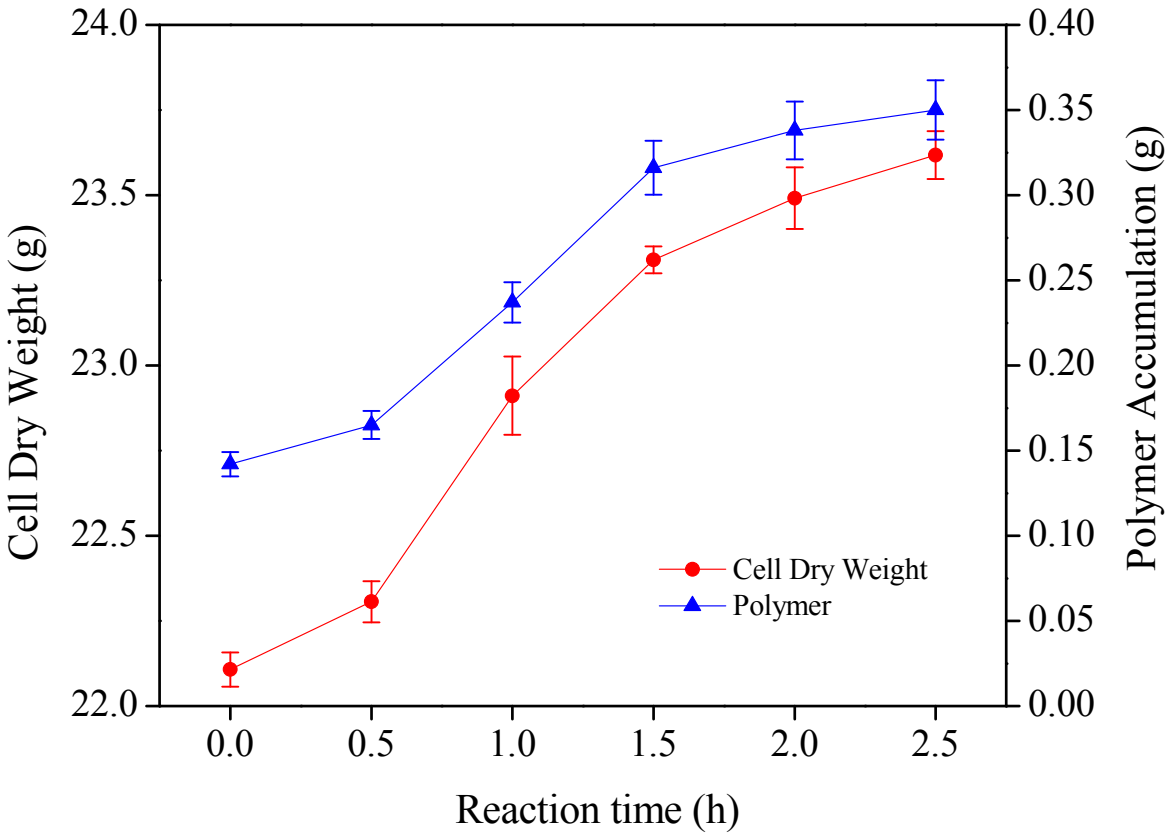


Figure 4.10 Growth and polymer accumulation under C:N ratio of 60.

4.3.1.4 Production of PHAs under C:N ratio of 75

Under C:N ratio of 75, as shown in Table 4.7, the residual COD and TKN reduced over time as previous C:N ratios (Figure 4.11), with 83.5% COD removal efficiency. On the contrary, the overall cell growth increased from 21.804 g to 22.288 g, resulting in 1.204 g net cell growth. The polymer content increased from 0.165 g to 0.386 g, accumulating 0.221 g net intracellular polymers (Figure 4.12).

Table 4.7 Laboratory - scale SBR performance under C:N ratio of 75

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	304.2	4.0	6.77	21.084	0.165
0.25	287.4	3.6	6.59		
0.50	223.3	3.5	6.64	21.189	0.203
0.75	160.9	3.3	6.81		
1.00	126.2	3.0	6.80	21.507	0.274
1.25	111.4	2.7	6.49		
1.50	81.8	2.4	6.79	22.046	0.312
1.75	60.7	2.1	6.63		
2.00	56.3	1.9	6.67	22.160	0.361
2.25	53.2	1.8	6.72		
2.50	50.2	1.8	6.84	22.288	0.386

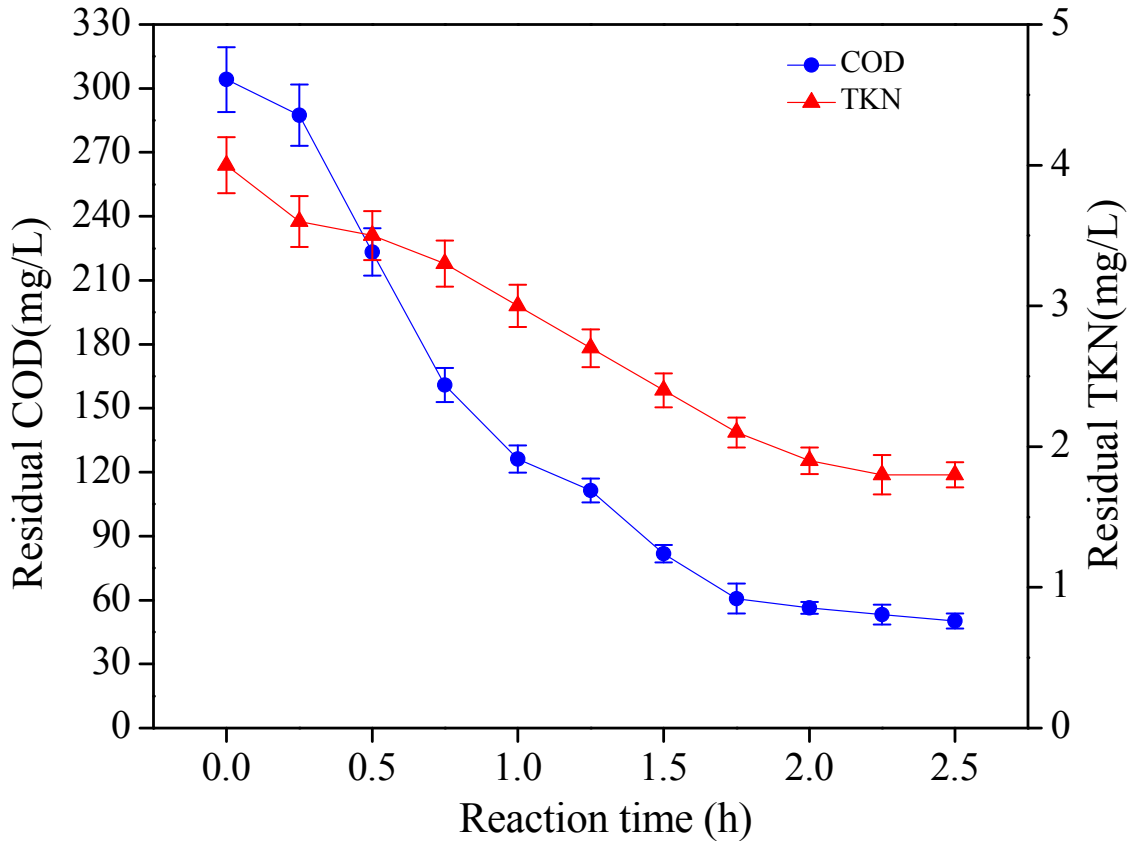


Figure 4.11 Carbon and nitrogen consumptions at C:N ratio of 75.

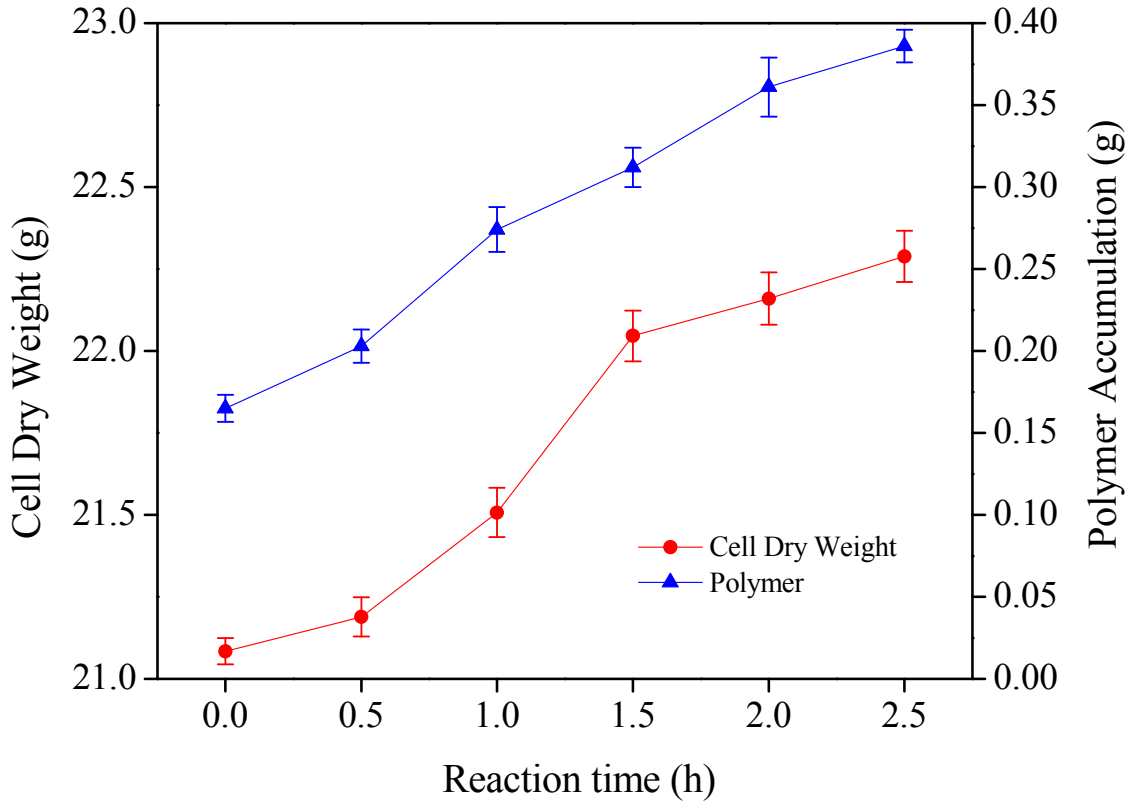


Figure 4.12 Growth and polymer accumulation under C:N ratio of 75.

4.3.1.5 Production of PHAs under C:N ratio of 90

When the C:N ratio was increased to 90, the residual COD reduced over time from 303.9 mg/L to 51.4 mg/L during the reaction time, with 83.1% COD removal efficiency, the residual TKN decreased from 3.4 mg/L to 1.2 mg/L. The measurable liquor characteristics in the reactor are summarized in Table 4.8.

With this more pronounced nitrogen deficiency, the residual TKN was near depleted in the first 15 minutes, and the reactor almost entered into nitrogen-deficient condition throughout the remaining react time (Figure 4.13). This observation was somewhat different from that of the previous operations

with C:N ratio of 30, 45, 60 and 75, showing the residual TKN concentration in the reactor liquor maintaining at a relatively consistent depletion rate. The values of pH was still fairly stable, maintaining in the range of 6.51 to 6.91. The net cell growth of 0.965 g DCW was lower than previous lower C:N ratios. On the contrary, the polymer content increased from 0.197 g to 0.447 g, accumulating 0.250 g intracellular polymers, which was observably more than that when C:N ratio was 30, 45, 60 and 75. The rate of polymer accumulation increased significantly after 0.5h, which was when the reaction came into severe nitrogen deficiency (Figure 4.14).

Table 4.8 Laboratory - scale SBR performance under C:N ratio of 90

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	303.9	3.4	6.87	20.776	0.197
0.25	290.4	1.8	6.79		
0.50	213.8	1.6	6.74	20.779	0.217
0.75	150.9	1.5	6.51		
1.00	136.4	1.6	6.81	20.876	0.336
1.25	110.6	1.5	6.73		
1.50	55.1	1.4	6.94	21.305	0.404
1.75	60.4	1.2	6.91		
2.00	55.6	1.3	6.74	21.596	0.431
2.25	52.9	1.2	6.70		
2.50	51.4	1.2	6.80	21.741	0.447

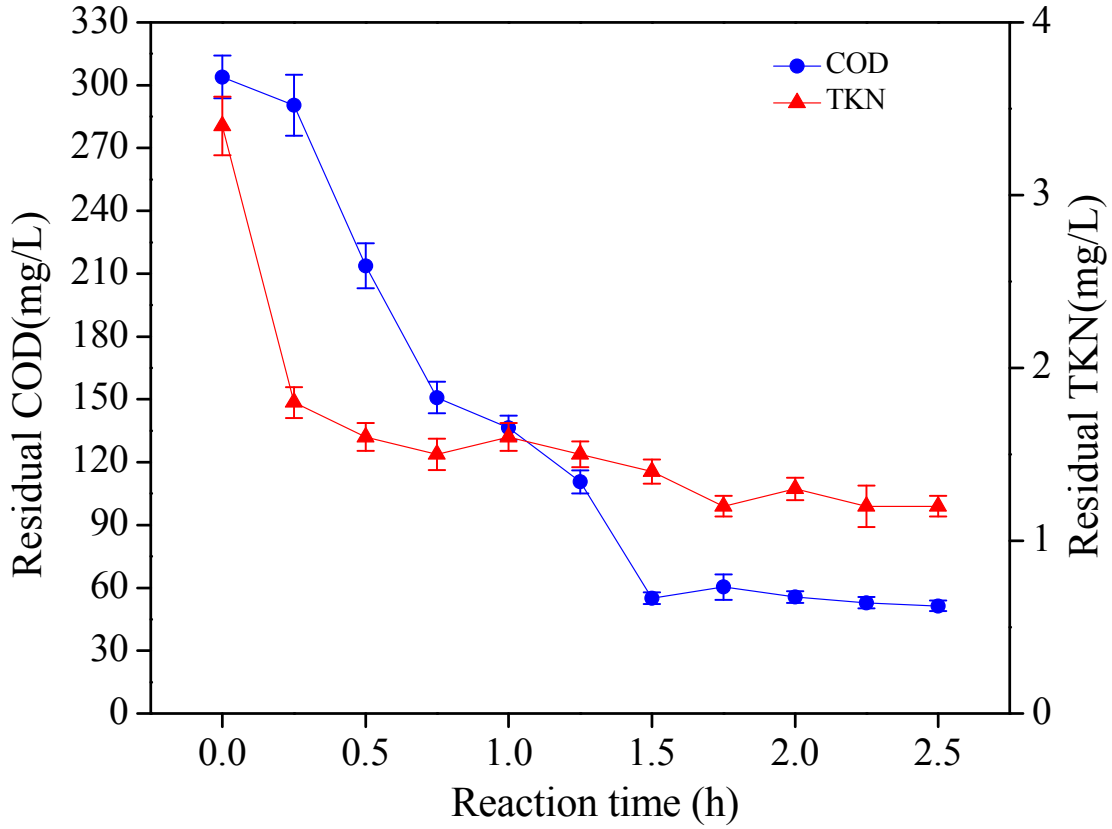


Figure 4.13 Carbon and nitrogen consumption at C:N ratio of 90.

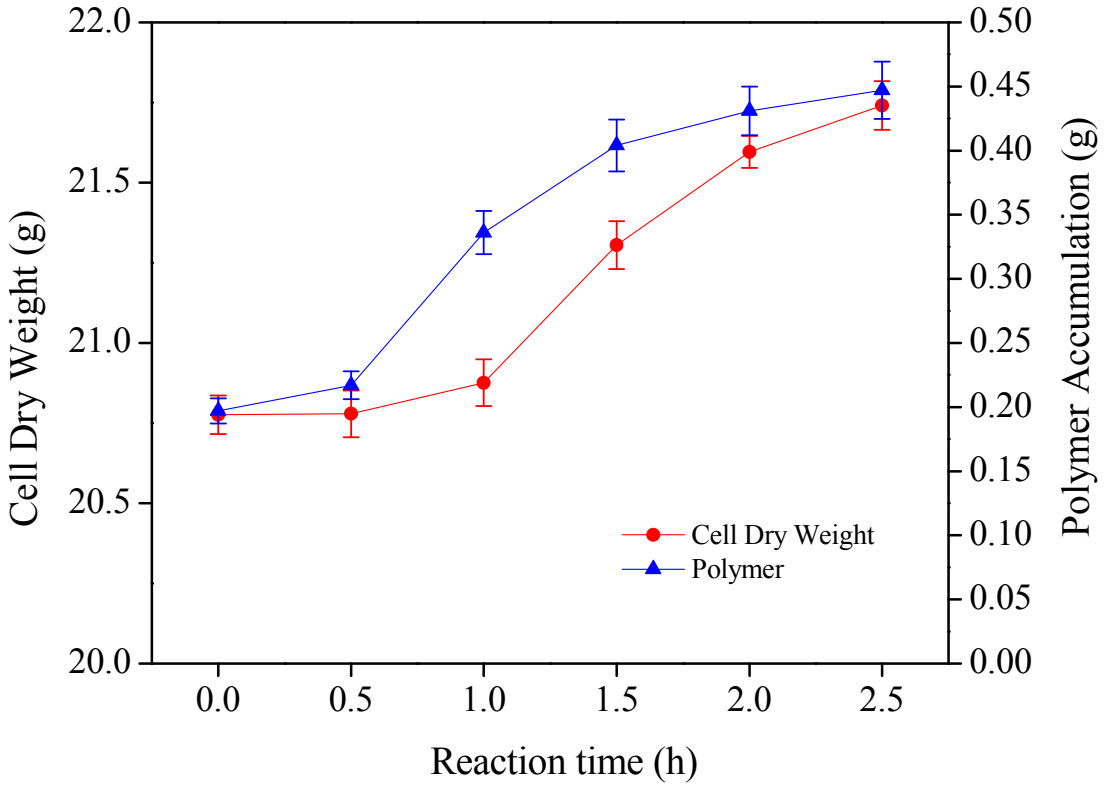


Figure 4.14 Growth and polymer accumulation under C:N ratio of 90.

4.3.1.6 Production of PHAs under C:N ratio of 105

When the C:N ratio was further adjusted to 105:1, the SBR was operated around a week to a steady state. The various water and sludge quality parameters of this reactor are shown in Table 4.9.

The residual COD reduced from 304.5 mg/L to 53.9 mg/L during the 2.5 h reaction time, with 82.3% COD removal efficiency (Figure 4.15). The residual TKN depleted very fast at the first 15mins as the C:N ratio of 90. But the overall biomass and polymer accumulation were on the increasing trends. The net cell growth decreased to 0.812 g. The polymer content increased from 0.394 g to 0.181

g, accumulating 0.213 g net intracellular polymers (Figure 4.16), which was less than that when the C:N ratio of 90, showing that C:N ratio of 90 was possibly that optimal condition for PHA accumulation. The pH in the liquor reactor was not obviously affected and fluctuated between 6.50 and 6.93.

Table 4.9 Laboratory -scale SBR performance under C:N ratio of 105

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	304.5	2.9	6.79	20.803	0.181
0.25	290.2	1.8	6.65		
0.50	208.5	1.6	6.74	21.301	0.204
0.75	162.4	1.5	6.59		
1.00	135.8	1.4	6.62	21.527	0.326
1.25	124.1	1.2	6.50		
1.50	80.2	1.3	6.91	21.565	0.360
1.75	75.6	0.9	6.93		
2.00	63.7	0.9	6.78	21.598	0.380
2.25	58.0	0.9	6.56		
2.50	53.9	0.9	6.74	21.615	0.394

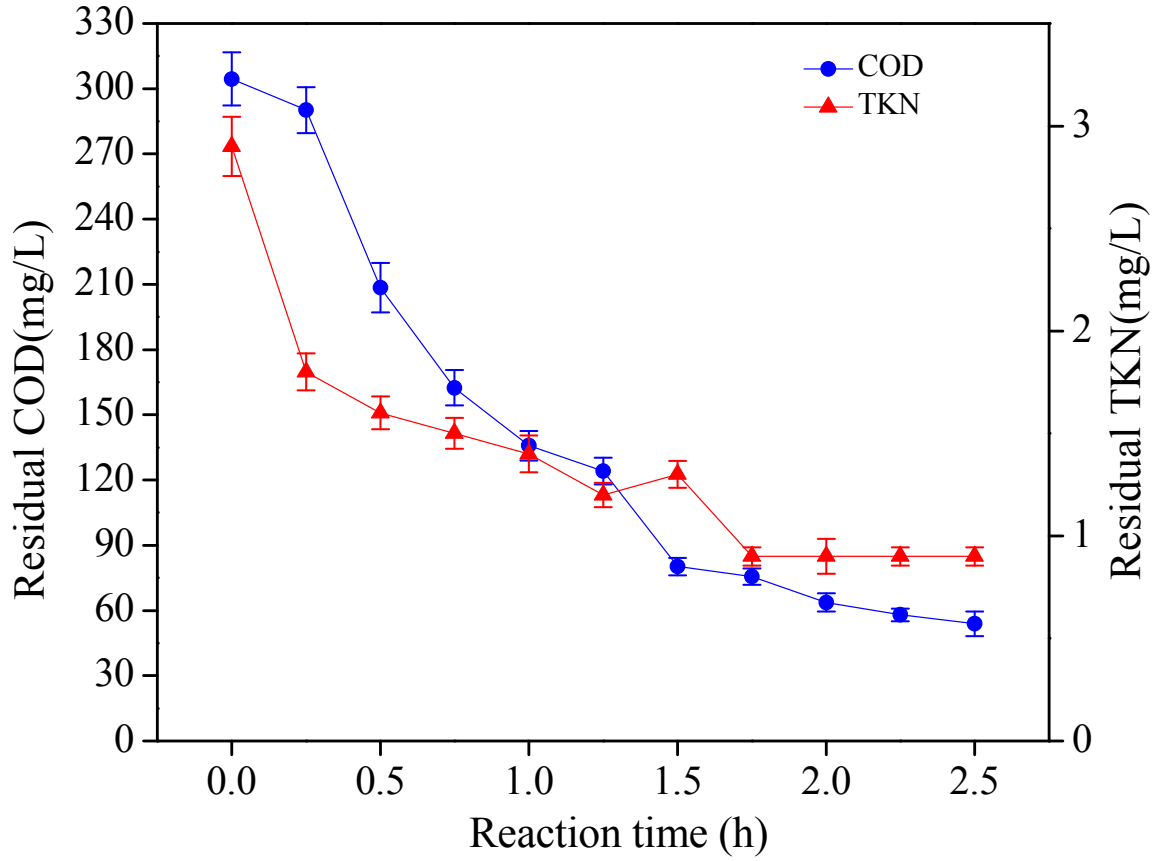


Figure 4.15 Carbon and nitrogen consumption at C:N ratio of 105.

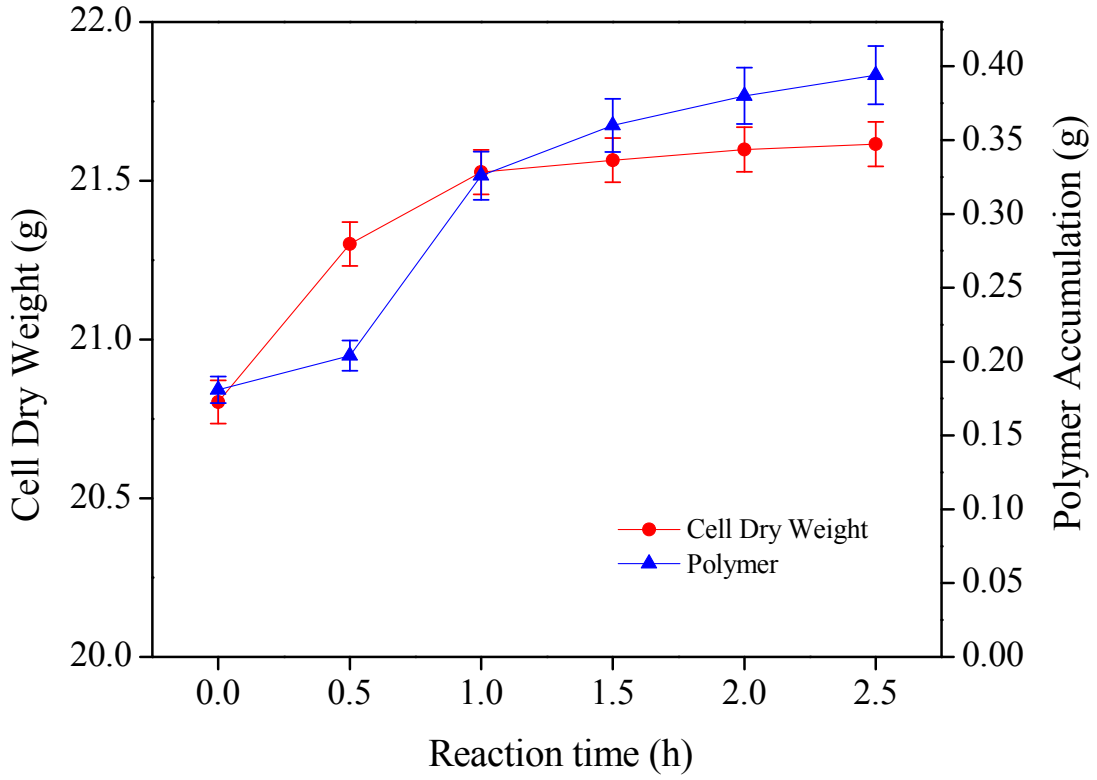


Figure 4.16 Growth and polymer accumulation under C:N ratio of 105.

4.3.1.7 Production of PHAs under C:N ratio of 120

Under C:N ratio of 120, the trends of residual COD, TKN and pH were found similar as the C:N ratio of 60 and 75. The various parameters of this reactor are shown in Table 4.10.

The residual COD reduced from 303.4 mg/L to 54.6 mg/L during the 2.5 h reaction time, with 82 % COD removal efficiency (Figure 4.17). The residual TKN decreased from 2.5 mg/L to 0.7 mg/L. However, the cell growth increased from 20.879 g to 21.580 g, with a smaller 0.0.701 g net cell growth as compared to previous operations. The polymer content increased from 0.173 g to 0.377 g,

attaining 0.204 g intracellular polymers (Figure 4.18). With these experimental data, it was observed that the net cell growth and polymer accumulation were less than that when C:N ratio was 90 and 105, further confirming that 90 was the optimum C:N ratio.

Table 4.10 Laboratory -scale SBR performance under C:N ratio of 120

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	303.4	2.5	6.80	20.879	0.173
0.25	270.6	2.3	6.56		
0.50	230.1	1.8	6.67	21.208	0.184
0.75	160.9	1.8	6.58		
1.00	124.8	1.3	6.91	21.246	0.286
1.25	108.3	0.9	6.94		
1.50	98.9	0.8	6.54	21.501	0.340
1.75	61.7	0.8	6.70		
2.00	58.0	0.7	6.52	21.558	0.359
2.25	56.1	0.7	6.65		
2.50	54.6	0.7	6.71	21.580	0.377

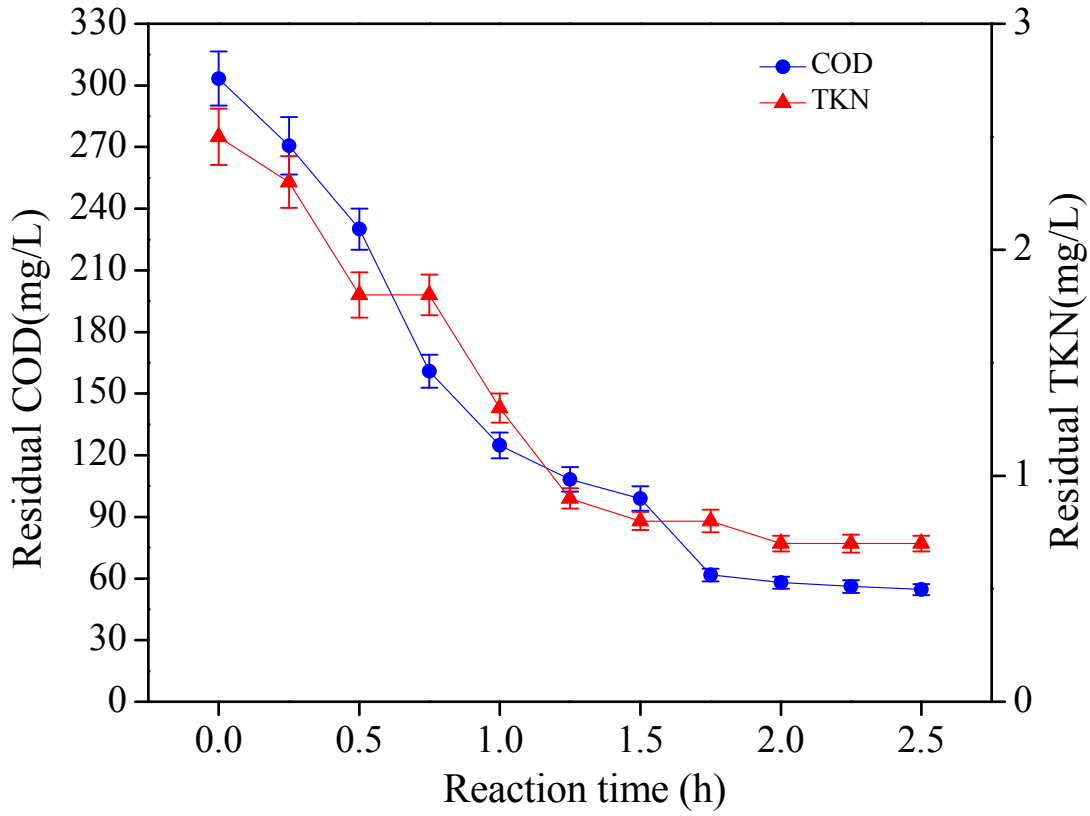


Figure 4.17 Carbon and nitrogen consumption at C:N ratio of 120.

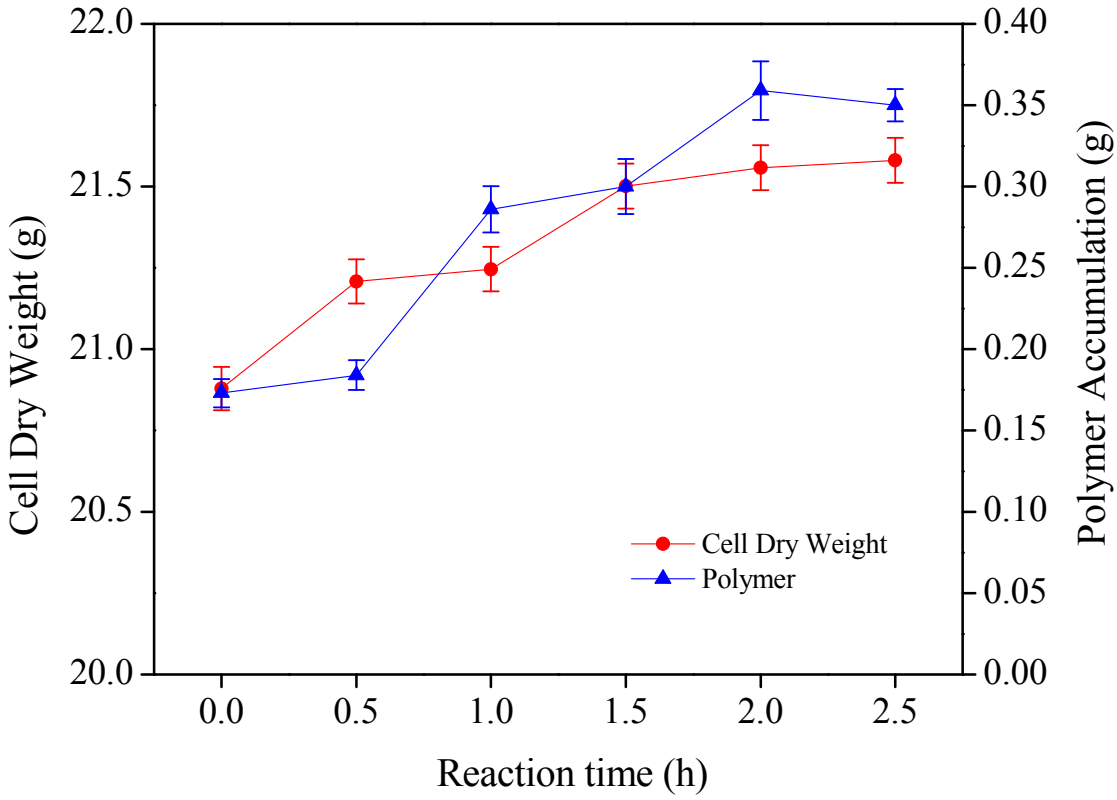


Figure 4.18 Growth and polymer accumulation under C:N ratio of 120.

4.3.2 Optimization of Polymer Production

The net cell growth, polymer accumulation, COD consumption, polymer productivity and three dimensionless yield factors under various C:N ratios are all shown in Table 4.11.

The consumption of COD (delS), cell growth (delX), polymer accumulation (del p) during the 2.5 hour reaction time under various C:N ratios are shown in Figure 4.19. showing that the net cell growth, del X, decreased from 1.913 g to 0.701 g as C:N ratio increased, especially pronounced when the C:N ration was further increased to 105 and 120, the net cell growth decreased to between 0.812 and

0.701g, but the polymer accumulation remained almost unchanged, even reduced to 0.213 g and 0.204 g. These phenomena were attributed to the nitrogen deficiency hampering the normal cell division and growth in the activated sludge consortium. This was agreeable with that reported in the literature that normal C:N ratio required for microbial growth is around the range of 20-25 (Chinwetkitvanich et al., 2004; Chua and Yu, 1999a). These further showed that C:N ratio, together with the previous C:P, aeration and settling times, were effective nutrient and physical selection pressure that produce a stable, robust and PHA accumulating sludge. This also validated that the novel selection procedure developed in this work was effective for the intended purposes.

As observable from

Figure 4.20, when the C:N ratio increases from 30 to 120, the specific polymer yield (Yp/x) increases from 0.063 to 0.291 g polymer/g cell weight. However, the specific growth yield (Yx/s) decreases from 0.654 to 0.282 g cell weight/g COD consumed. This indicated that the nitrogen-deficient condition affected the normal growth and cytoplasmic synthesis of biomass in the activated sludge. The increased C:N ratio caused an increased in specific polymer yield or intracellular polymeric granule fraction, which represent that the unfavorable nitrogen-deficient condition resulted in the microorganisms in the activated sludge to accumulate more intracellular carbon reserve in the form of storage

polymers.

The specific polymer yield (Yp/x), SPY, which indicated the PHA accumulation efficiency in microbial cells, is an important parameter in the microbial production of PHAs. It can be seen from Figure 4.19 that SPY could reach a highest value of 0.291g polymer/g cell weight, which means that 29.1 weight % of the activated sludge was composed of the polymers. If this portion was extracted for use as biodegradable plastics, the treatment and disposal of the excess sludge fee could be reduced by a corresponding percentage, which represented a substantial contribution towards cost reduction in sewage work operation.

In line with the general objective in the study to reduce the cost of producing PHAs, the optimal operating condition was considered that less carbon source was consumed while more polymers were accumulated in the sludge. Large Y p/s represented that more PHAs accumulated in the cell and less carbon or raw carbon-based material consumed. The dimensionless parameter, Y p/s, can thus be used as the most important and effective indicator for process efficiency.

Figure 4.20 also shows that Y p/s reached a maximum value of 0.099 g polymer/ g COD consumed under C:N ratio of 90, When C:N ratio continuously increased from 105 to 120, Y p/s decreased slightly instead.

Table 4.11 polymer productivity under different C:N ratios

C:N Ratio	delX (g)	del P (g)	delS (g)	Yx/s (g/g)	Yp/x (g/g)	Yp/s (g/g)	COD Removal Efficiency(%)
30	1.913	0.120	2.927	0.654	0.063	0.041	89.10%
45	1.700	0.163	2.672	0.636	0.096	0.061	87.40%
60	1.511	0.208	2.600	0.581	0.138	0.080	84.60%
75	1.204	0.221	2.540	0.474	0.184	0.087	83.50%
90	0.965	0.250	2.525	0.382	0.259	0.099	83.10%
105	0.812	0.213	2.506	0.324	0.262	0.085	82.30%
120	0.701	0.204	2.488	0.282	0.291	0.082	82.00%

del X= net cell growth as measured in dry cell weight during 2.5 hour reaction time
del P= net accumulation of intracellular polymers during 2.5 hour reaction time

del S=net consumption of COD during 2.5 hour reaction time

The $Y_{X/S}$ (g DCW / g COD consumed) is the specific growth yield. (SGY)

The $Y_{P/X}$ (g polymer / g DCW) is the specific polymer yield. (SPY)F

The $Y_{P/S}$ (g polymer /g COD consumed) is the overall polymer production yield. (PPY)

The relation among three of them are shown below.

$Y_{P/S} = Y_{X/S}\ Y_{P/X}$

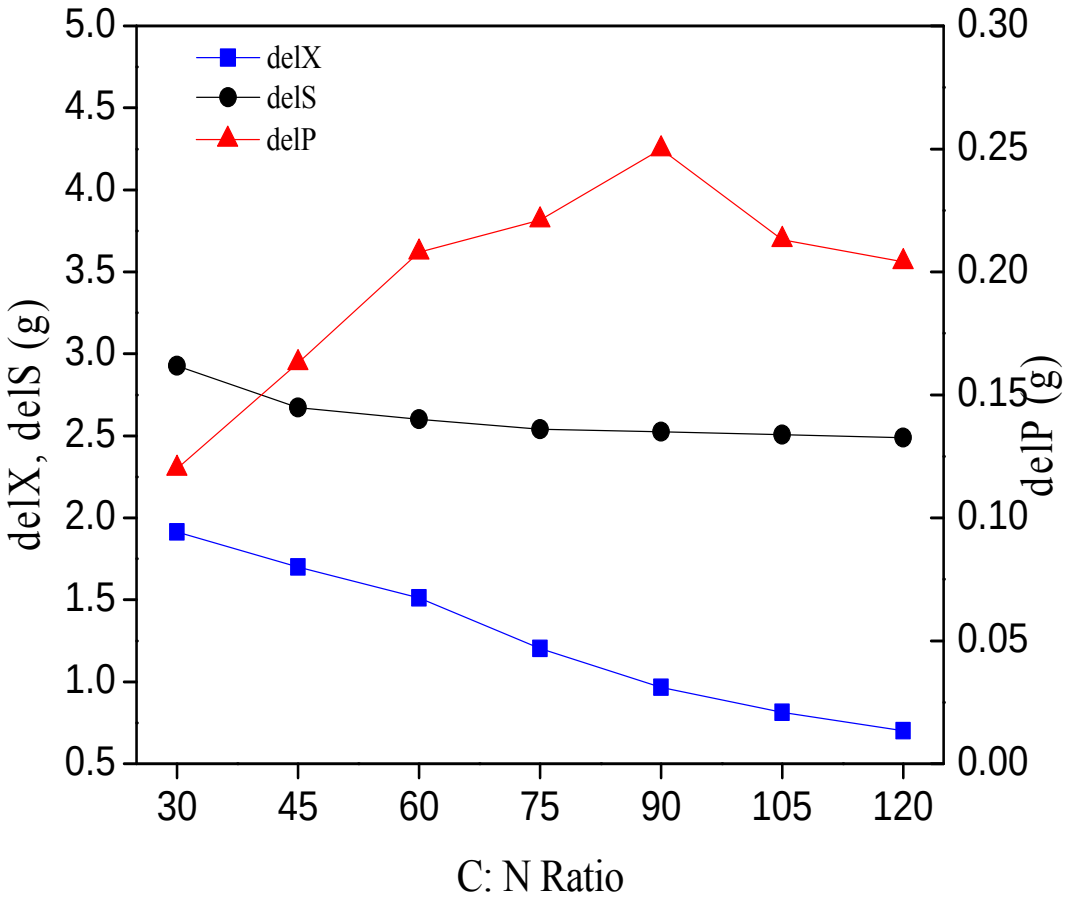


Figure 4.19 Polymer productivity under different C:N ratios. (del X represented the absolute increase in DCW, del S represented the decrease in substrate COD, del P represented the absolute accumulation of PHAs.)

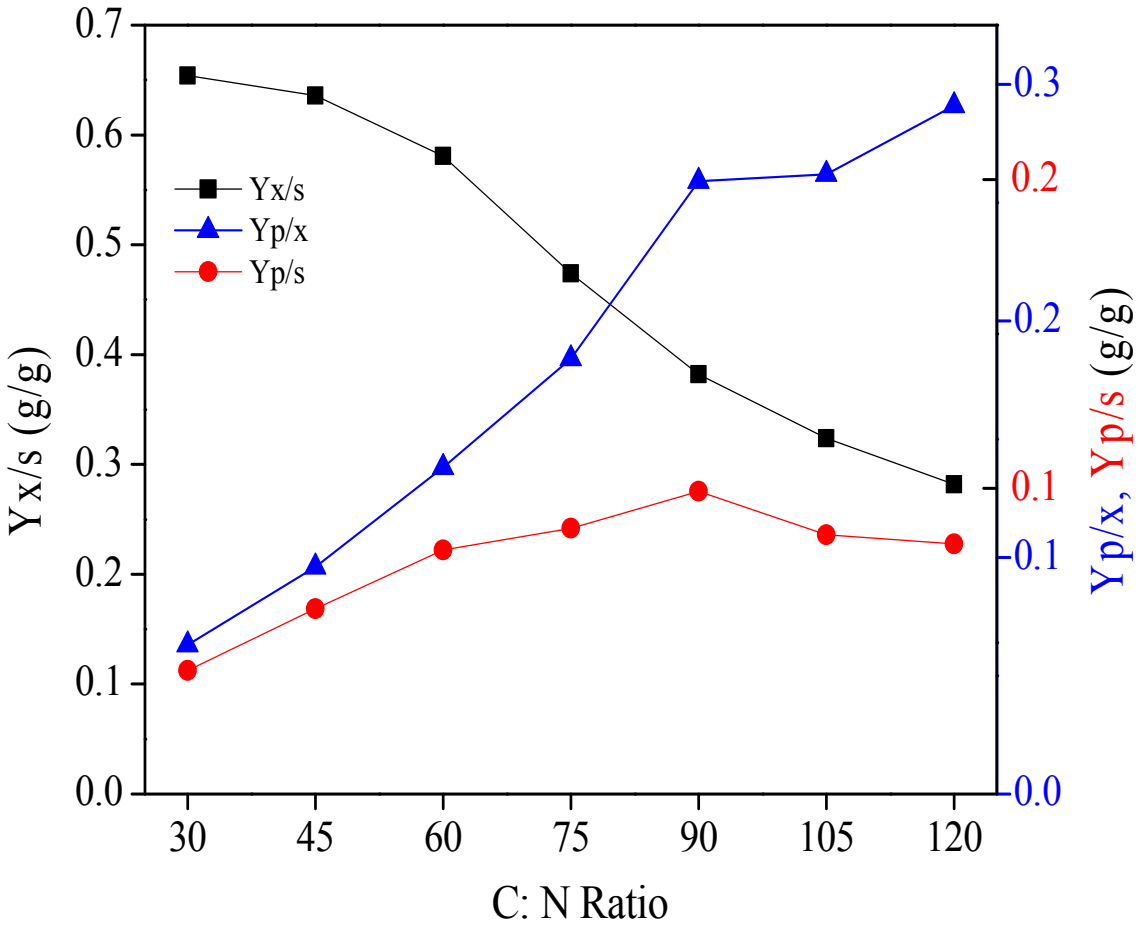


Figure 4.20 Polymer yields under different C:N ratios. (The three dimensionless yield factors were determined for subsequent mathematical modeling for engineering design, operation and optimization of the SBR process.)

The overall polymer production yield, Yp/s, was an essential indicator of cost in producing PHAs on economic aspects. When Y p/s was larger, the profit of production was proportionately higher. Therefore, when considering the productivity of PHAs of unit COD consumed from the incoming substrate, the C:N ratio of 90 will be the optimal point. The overall polymer production achieves 0.099 g polymer/g COD consumed at such a C:N ratio. After reaching the maximum PHA yield, the process performance then gradually decreased over time. The reason for this could be the consumption of intracellular PHAs as a source of carbon and energy after glucose depletion in the reactor liquor. Therefore, the various dimensionless parameters developed in this work, namely Yp/s, Y p/x and Y x/s, can be used as the process predictive model for the design, optimization, operation and control. These novel model parameters will be validated in subsequent pilot-scale opeartion, and will be a valuable tool for full-scale industrial opearations.

The nitrogen-deficient conditions when the C:N ratio in the industrial wastewater was above 30 did not significantly affect the efficiency of organic reduction, indicating that the PHA accumulating process did not significantly affect the sewage treatment process. As additional nitrogen was intermittently fed in the reactor liquor, microbial growth and organic reduction efficiency were not significantly affected in the SBR because of different degrees of nitrogen deficiency had a relatively mild adverse effect on the health of the microbial

ecosystem, which was consistent with previous findings (Chua et al., 1999; Morgan Sagastume et al., 2010). As a result, the COD removal efficiency kept above 82% for various C:N ratios throughout the entire study. These experimental results were a contrast, however, to the widely accepted viewpoint in the field of conventional environmental engineering that the C:N ratio in activated sludge processes have to be kept about 20 in order to enable normal microbial cell synthesis (Wang et al., 2007). One possible explanation for these inherent contradictions is that the sporadic nitrogen deficiency was considered mild and did not cause a slow-down in microbial growth and did not have adverse effect on the organic treatment performance by the process. Therefore, it was concluded that it was better for intermittent nitrogen feeding procedure to optimize the polymer production without significantly affecting the normal treatment performance of the activated sludge process.

Hong et al. (2009) made an attempt to optimize some operating conditions and nutritional factors for the enhancement of PHA production in activated sludge by response surface methodology. A yield of 49.5% of DCW was achieved under optimum conditions of NH_3–N 4.8 mg /L, C:N ratio of 60 and initial pH 9.0, which was not as good as that obtained in this work. This is because the operating conditions were not as easy to control and maintain as those selected in this work.

4.3.3 Filamentous organism as the dominating species in the sludge

Microscopic investigations of the sampled activated sludge demonstrated that filamentous bacteria were the dominating PHA accumulating species in the biomass (Figure 4.21). Large PHA granules (in the range of 0.5-1.0 microns in diameter, readily observable as shaded patches in the micrograph at the low resolutions) could be observed in the filaments after Sudan black or Nile blue staining. The filaments dominated the biomass to a highest extent when the C:N ratio was set at the optimal level of 90. These filamentous species were likely dominated by *Nocardia spp*., as indicated by the typical morphological characteristics of having obvious right angled filamentous branching (Wong, 2005). Furthermore, another genus of rod cells were also observed in the PHA accumulating bacteria, which were likely *Alcaligene spp.* (Figure 4.22). These typical rod cells were seen to be accumulating intracellular granular food reserves, which were also observed as dark patches in the bacterial cells in the micrograph. Braunegg et al. (1998) found that there were also two morphotypes of floc-forming PHA accumulating bacteria that were evident in the biomass through his research findings, however they were not identified. These filaments are morphologically different from the *Nocardia spp*. observed in this work in such a distinctive way that they are not branched, as shown in the Figure 4.23. However, these filaments were not observable in the sludge selected in this work.

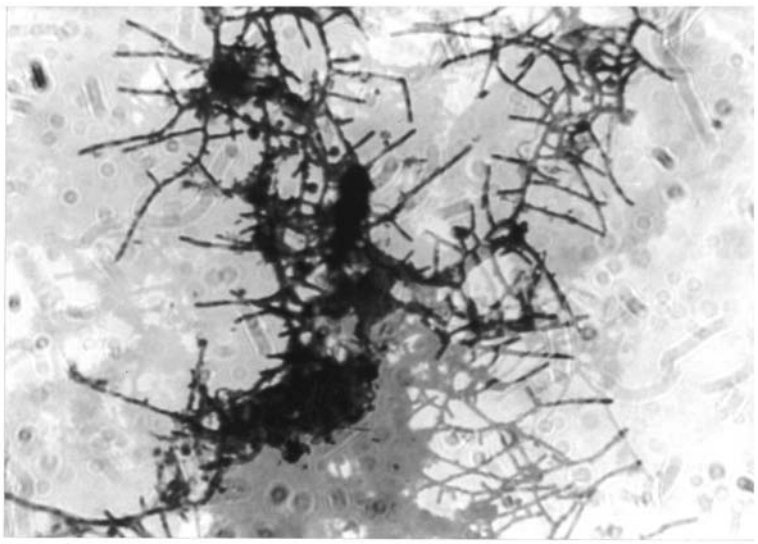


Figure 4.21 Branched filaments (*Nocardia spp.*) as the dominant genus in the PHA accumulating activated sludge (x 1,000 magnification).

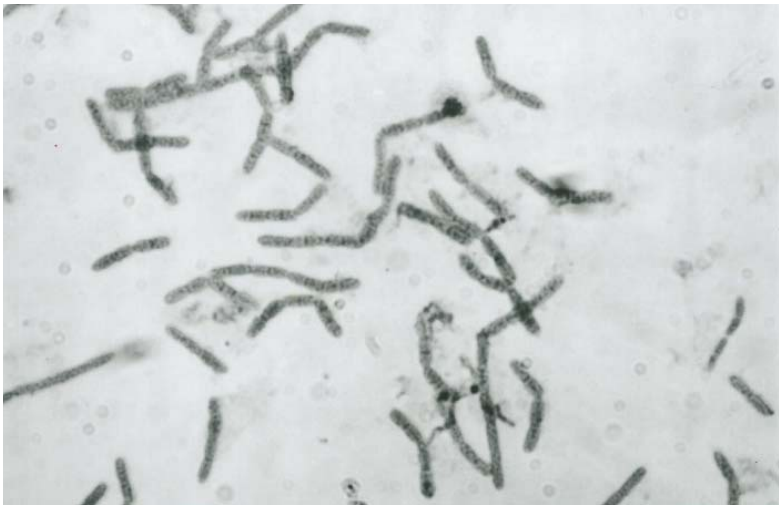


Figure 4.22 Rod cells (*Alcaligene spp.*) as the second dominating PHA accumulating activated sludge (x 1,000 magnification).

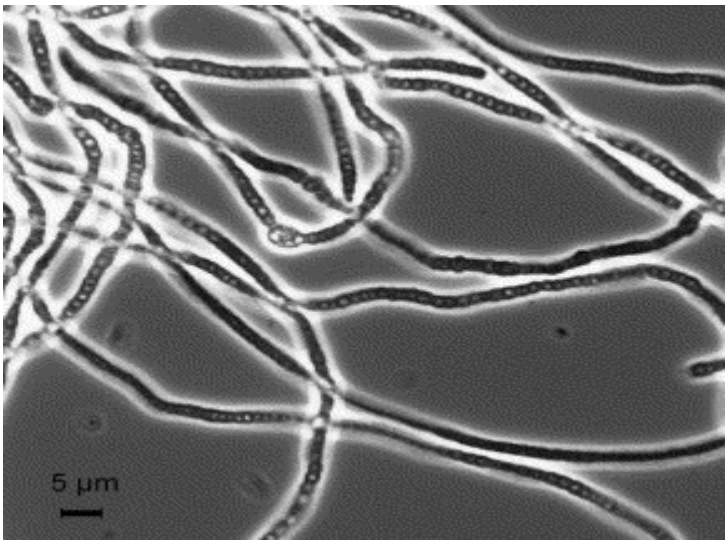


Figure 4.23 Phase contrast micrograph showing the PHA-accumulating filamentous organism dominating the sludge (Braunegg et al., 1998).

4.3.4 Process optimization and sludge selection

The prime novelty and main difference between this work and previous studies reported in the literature lied upon the optimized process operating conditions and the characteristics of the selected sludge.

The novelty and differences were expressed in six ways as follows.

(1) Different Sludge Retention Time (SRT)

The aeration and settling time optimized in the first stage of this work served as physical selection pressures while C:P and C:N ratios optimized in the second and third stages of this work served as biochemical or nutrient selection pressure on the activated sludge for higher PHA accumulation. A research reported by Chua et al.(2003) investigated the decreasing SRT from 10d to 3d could lead to

an increase in PHA production from 20% to 30% of sludge dry weight. However, the specific reasons were not explicitly elucidated. Nevertheless, two possible causes were proposed. Firstly, the short SRT select the cell group with big nutrient-uptake capacity. Secondly, the short SRT resulted in low MLSS concentration which could render every microbial cell taking up more carbon source. As a result, the results from this work surpassed that from Ma (2000) having a higher production yields. The SRT could influent the biomass PHA-storage capacity and sludge selection since short SRTs tend to lead to higher growth rates and less substrate storage, which agreed with that reported by Dias et al. (2006).

According to Dionisi et al. (2007), findings were quite on the contrary, they discovered a strong influence of the cycle length on microbial decomposition of organics. The reason was the higher organic loading rate may be related to the different effect of cycle length on microbial decomposition. As the amount of substrate fed per cycle had relation to the cycle length, the substrate concentration with longer cycle, was significantly higher. As a result, bacteria may be inhibited or selected by the substrate concentration tolerance.

In order to reach high biomass productivities and yields in a potential commercial PHA production process, lower SRTs are favorable because younger and hence more active sludge prevails. It was found that 1 day SRT was

feasible for enriching a PHB producing culture (Dionisi et al., 2001; Dionisi et al., 2006). However, the nitrogen-limited culture at 0.5 d SRT caused some problems as a result of substantial biofilm formation and ran the risk of losing most of its PHB storage capacity over time. The enriched culture then showed a very low PHB storage capacity and production yield. During an operation under 0.5 d SRT, the feast period was longer than the famine period which resulted in the selective pressure for PHB producers distinctly not strong enough to obtain a stable PHB producing culture with a high storage capacity (Johnson et al., 2010).

A conclusion was draw from these observations that low SRTs are favorable for biomass proliferation and PHA production. However, if the SRT was too low, it became unfavorable for PHA storage. In longer SBR operating cycles, cultures have to store a higher amount of PHB or PHAs in order to grow throughout the longer famine phase. Towards the end of the first three phases of this work, the activated sludge in the laboratory-scale process simulator was considered well selected for optimal accumulation of PHAs.

(2) pH

Activated sludge is known to be a complex and dynamic micro ecosystem, in which different microorganisms have different adaptation range of pH values. If exceeding the range, especially when the influent pH values change suddenly and significantly, the microbial metabolism and product would change, inhibiting the

microbial activities, and even causing death of the sludge. Thus the operation of the reactor and hence the sewage treatment efficiency will be affected.

Sludge displayed same PHA production capability when acclimatized under pH range between 7 and 8 in normal sequencing batch reactors reported in the literature (You et al, 2009a). In this work however, the pH of the reactor liquor was not controlled and fluctuated naturally between 6.41 and 6.94. The stability in pH values in the reactor liquor was attributed to the effective control by the novel sludge selection procedure developed in this work. The physical selection, namely aeration and settling times and hence SRT, and the biochemical or nutrient selection, namely C:P and C:N ratios, resulted in a robust microbial consortium in the activated sludge. This selected microbial ecosystem, was capable of maintaining inherently a relatively stable pH range in the liquor, thus resulted in optimum PHA accumulation.

These findings were somewhat different from that reported by others. pH values, as reported by others, affected greatly the PHA accumulation behavior of activated sludge with batch experiments. For example, Liu et al. (2011) found at an initial pH value of 7.0, the maximum PHB content was 67.0%. The results indicated that the pH value was an important parameter for PHB production, and the PHB production could be significantly improved by controlling the pH value.

In the experiments carried out in this work, an initial pH value was around

6.4-7.0. If the initial pH values were adjusted, it would cause a change in the growth condition of the microorganisms and possibly led to an increase in the polymer storage yield. However, from the perspective of process economics, this was not encouraging because pH adjustment on an industrial scale would have translated into substantial operating costs.

Some researchers reported that a low PHB content was obtained at a pH value of 6.0, but the yield was increased when pH value kept at 8 or 9 (Chua et al., 2003; Johnson et al., 2009). However, Fleit (1995) received the lowest PHB content at an initial pH value of 5.0, which was likely owing to the presence of undissociated acetic acid. But the PHB content at an initial pH value of 5.5 was much higher than that at 5.0. So the PHB accumulation behavior seemed very sensitive when the initial pH value was increased from 5.0 to 5.5.

(3) Operating parameters rendering the enrichment of PHA-storing organisms in SBR

A long famine (starving) time and short feasting (organic surge load) time, achieved through aeration and settling control of resulting F/M ratio, demonstrated efficiency in ensuring the selection ability of PHA-storing organisms with high-strength (high COD levels) substrates under the condition of aerobic dynamic feeding system. The famine phase should be operated as a long aeration and settling time to make sure that PHA-storing microorganisms could

use the stored carbon for growth. On the contrary, the other microorganisms were starved and underwent a relatively lower growth rate. The time period of the feasting phase (achieved as an organic surge load in actual activated sludge process operation) kept up much shorter operation than the famine stage to insure an appropriate selection pressure. For this aspect, changing the operating parameters seems to be a very important consideration for optimizing PHA accumulation. In order to convert the famine-feast time length ratio and effectively caused a different PHA-storage yield, it is generally recognized that the higher the famine-feast ratio, the more PHA-storage yields should result(Chua et al., 1999; Yu et al., 1998). In addition, to the famine-feast ratio, the maximum substrate concentration during the feast phase and sludge age show important influence. The substrate concentration in the initial phase of the feasting stage is directly correlated with the SBR simulator operating cycle length and it has to be high enough to facilitate PHA storage but low enough to refrain from biomass inhibition. Reduction of PHA-storing capacity was observed in a biomass that was exposed to a long feasting operation, which agreed with that observed by others (Morgan-Sagastume et al., 2010).

(4) Dominant microbial species

The modification of the complex microbial species in the ecosystem of the sludge caused the different yield in the PHA accumulation, leading to changes in

microbial metabolic and biochemical pathways from carbohydrates and fatty acids to polymer in different microbial species (Hu et al., 1997). The microbial community structure has a close connection with the process operating conditions in PHA accumulation from activated sludge, which served as selectors for microbial consortium species.

It was observed in this work that the selected activated sludge in the first three stages was dominated by branched filamentous species, possibly associated with the *Nocardia spp. (Norcadia amarae*). However, the relatively high PHA accumulation yield attained suggested that the selected activated sludge also constituted hyper-accumulating species for PHAs, namely *Alcaligenes spp*. (possibly *Alcaligenes eutrophus and Alcaligenes lactus*) and the denitrifying *Pseudomonas spp*. (possibly *Pseudomonas aerugenosa*). These are the hyper-accumulating species for PHAs as long as genetically engineered species are not used (Wang et al., 2010; 2007). However, rigorous microbial identification was beyond the scope of this work.

(5) Concerted effects of C:P and C:N ratios

The sequential optimization of C:P and C:N ratios was never done before according to the published literature. C:P and C:N ratios were reported to be separately optimized, but either one was usually carried out while the other parameter was not in its optimum state. The concerted effects of the C:P and

C:N ratios as two important nutrient selection pressure were the prime attribute for the resulting robust selected activated sludge.

The accumulation of PHAs can be stimulated under unfavorable and unbalanced growth conditions, when nutrients such as N and P, or possibly potassium and sulfate as micro and macro nutrients become limiting (Braunegg et al., 2004; Chua et al., 1999). Furthermore, when dissolved oxygen concentration is low, or when the ratio of C:N in the feed substrate is high, PHA accumulation is widely recognized to be substantially promoted.

There are some possible reasons from the in depth investigation into the metabolic pathway, which were beyond the scope of this work. From the perspective of process design and operation, the increasing C:N ratio formed the inhibition for the cell growth, so the PHA accumulation was enhanced and accumulation and conversion pathway was accelerated. As the C:N ratio increases from 30 to 120, the PHAs accumulated intracellularly in the cells of specific species increased almost linearly and proportionately. The high C:N ratios allowed the cells to accumulate more PHAs as food reserves.

When looked upon from another perspective, the increasing C:P and C:N ratio also inhibited the growth of the microbial cells, hence the specific growth yield was decreasing. When the C:N ratio was fixed at a certain level, the absolute cell quantities and the PHA amount in every single cell reached a certain level, which

was the so-called optimal or dynamic equilibrium point. At this level, the overall polymer production yield attained the peak value. If the C:N and C:P values continued to increase, the specific polymer yield also increased. However, the overall polymer production yield was decreasing. In other words, the polymer amount reached a high fraction in each cell, but from the perspective of the consumed carbon source, it was not the most economically feasible method. These results added on to the earlier findings by Hu (2004).

(6) Aeration rate

Aeration rate was also widely accepted as one of the important factors affecting the SBR process (Majone et al., 1999; Liu 2009). Under the condition of anoxic or anaerobic ambient due to insufficient aeration, microbial metabolism is suppressed due to lack of dissolved oxygen. If there is excessive aeration, severe turbulence would cause the activated sludge flocs to rupture, turbidity of effluent increases, which leads to flocculation and increase energy consumption in the plant operation. Therefore, good control and optimization of aeration rate is generally considered as one of the most important parameter for process operation. The aeration time and settling time in this work were profoundly controlled to result in different SRT and F/M ratio, which in turn, affected the PHA accumulation rates and yields. These selection pressures, in conjunction with the nutrient selection pressures, resulted in a novel sludge selection

procedure that has never been reported in published literature.

Effects of substrate load and nutrient concentration on PHA accumulation

It was discovered in this work that nitrogen deficient conditions with profoundly controlled nitrogen-feeding patterns favored PHA accumulation. This was achieved by controlling the carbon: nitrogen (C:N) ratio by adjusting the nitrogen (ammonium chloride) concentration and feeding pattern while keeping the carbon concentration constant in the reactor. The PHA yield was substantially affected by the nutritional conditions in the form of N and P concentrations. Higher substrate loads, lower nitrogen and phosphorous concentrations, and well control feeding pattern caused higher PHA accumulations. These accumulation yields surpassed that of previous works (Reddy and Mohan, 2012a; Wong et al., 2005; Satoh et al., 1992).

Yao et al. (1999) also reported that the PHA accumulation was strongly associated with nitrogen limitation. This finding is confirmed by Din et al. (2006), Bernat et al. (2008) and Johnson et al. (2009) who pointed out that high yield in PHB accumulation could be achieved by limiting the nitrogen or phosphorous sources. As limiting ammonia availability during batch experiments gave rise to higher polymer production by suppressing cell growth (Basak et al., 2011). However, none of these workers worked on concurrent effects of N and P.

In this work, given glucose and food-processing effluent as the carbon sources, PHA concentration and content changed over time in the presence and absence to different degrees of nitrogen and phosphorous was surveyed under different physical operating conditions. As the accumulation proceeded, the PHA yield gradually increased and then decreased along with time. The above results indicated that in the absence or deficiency of the nitrogen and phosphorous, glucose or any other carbon sources could be transformed into PHA by the microorganisms in the sludge, while some carbon source was consumed for microbial metabolism. This confirmed that the nitrogen and phosphorous were two important parameters, with appropriate control over aeration time for complete biological reaction, in the production of PHA by the activated sludge.

4.4 Pilot-scale SBR system

The pilot-scale sequencing batch reactor (SBR) system was designed and built for PHA accumulation, and was operated with the physical and carbon:nutrient parameters that were similar to that established in the previous laboratory-scale system. In general, the pilot-scale system was observed to have displayed cost-effective and process stability in PHA accumulation.

The 600-L pilot-scale SBR reactor was initially seeded with the enriched activated sludge that was previously selected by the aeration, settling and carbon:nutrient parameters in the laboratory-scale system. As it has been well

recognized that the most important consideration to realize feasible PHA applications is the production cost. Along this line, in order to accelerate the accumulation rates of PHAs and increase the production yield, the C:N ratio was set at the previously optimized level of 90 to create an appropriate nutrient-deficient and somewhat growth-inhibitory environment in order to exert the required pressure on the selected microbial consortium in the sludge.

The C:N ratio is achieved by external addition of carbon source in the incoming wastewater into the pilot-scale system. The carbon source used in this study (both laboratory-scale and pilot-scale) was glucose, which was considered as the cheapest and the most spontaneously metabolized carbon source.

After the system was set up, seeded, started up and acclimatized for a period of three weeks, the average influent and effluent water qualities as well as a number of essential operational conditions, generally achieved steady state conditions, are shown in Table 4.12.

The data show that the sludge used in the operation had high carbon and nitrogen concurrent removal efficiency. The system was thought to be stable after 20 days of operation, which was considered to be fast in acclimatization to the new ambient (Yu et al., 2006).

Table 4.12 Influent and effluent water qualities of the pilot-scale SBR system under steady state conditions

Water quality parameters	Influent (mg/L)	Effluent (mg/L)	Removal Efficiency (%)	Operational parameters
Total COD	390±57	76±22.1	80	
Soluble COD	250±49	21±14	82	
Total P	3.9±0.6	1.25±0.5	68	
PO_4^{-3}-P	1.3±0.8	0.4±0.2	69	
Total nitrogen	4.8±0.9	0.72±0.3	85	
NH_4^+-N	2.4±0.3	0.14±0.03	94	
NOx-N	0.2±0.1	0.39±0.12		
MLSS (mg/L)				2700±223
MLVSS (mg/)				2500±219

4.4.1 Production of PHAs under the optimal C:N ratio of 90

When stable operation was attained in the pilot-scale SBR under C:N ratio of 90, samples of the mixed liquor were periodically collected (with 15-min time intervals) for analysis during the 2.5-h aeration/reaction time in a randomly selected operation cycle. The essential parameters analyzed included residual COD and TKN concentrations, overall cell growth and polymer accumulation. The values of these parameters were shown in Table 4.13.

The residual COD decreased with time from an initial level of 386.1mg/L to the final stabilized level of 76.1 mg/L, with an average of 80.29 % COD removal efficiency. In the meantime, the residual TKN decreased substantially from the

initial 4.3 mg/L to a low residual level of 1.5 mg/L (Figure 4.24). The pH of the reactor liquor was not controlled and fluctuated between lower and upper limits of 6.39 and 6.82, respectively.

Figure 4.24 also reveals that both the residual COD and TKN maintained a consistent depletion rate throughout the 2.5 hours reaction time. However, as the residual COD and TKN decreased, the cell growth was observed as indicated by an increased of cumulative cell biomass from 1589.237g to 1661.591g, obtaining 72.354 g of net cell growth. At the same time, the intracellular polymer content increased from 10.8 g to 27.912 g, accumulating a total of 17.112 g of net intracellular polymers (Figure 4.25). Eventually, the dimensionless yield factor, namely Yp/x, was calculated by taking the absolute increase in PHA accumulation divided by the absolute increase in dry cell weight. Similar calculation procedures are adopted for the rest of the data processing.

It was found that when the residual COD and TKN declined, the overall biomass and polymer accumulation ascended gradually and consistently. These were attributed to direct carbon and nutrient conversion to biomass and intracellular food reserves. This showed a trend that was more or less similar to that observed in the laboratory-scale results, indicating that the process scale-up, seeding and acclimatization were carried out appropriately.

Table 4.13 Pilot -scale SBR performance under the optimal C:N ratio of 90

Reaction time (h)	Residual COD (mg/L)	Residual TKN (mg/L)	pH	Dry Cell Weight (g)	Polymer Accumulation (g)
0.00	386.1	4.3	6.71	1589.237	10.800
0.25	360.4	4.0	6.82		
0.50	334.5	3.5	6.53	1604.017	13.604
0.75	290.9	3.3	6.50		
1.00	268.5	3.0	6.39	1627.445	17.231
1.25	215.9	2.7	6.63		
1.50	135.8	2.4	6.54	1645.652	22.189
1.75	90.3	2.0	6.78		
2.00	85.4	1.8	6.59	1657.238	25.984
2.25	78.6	1.6	6.65		
2.50	76.1	1.5	6.72	1661.591	27.912

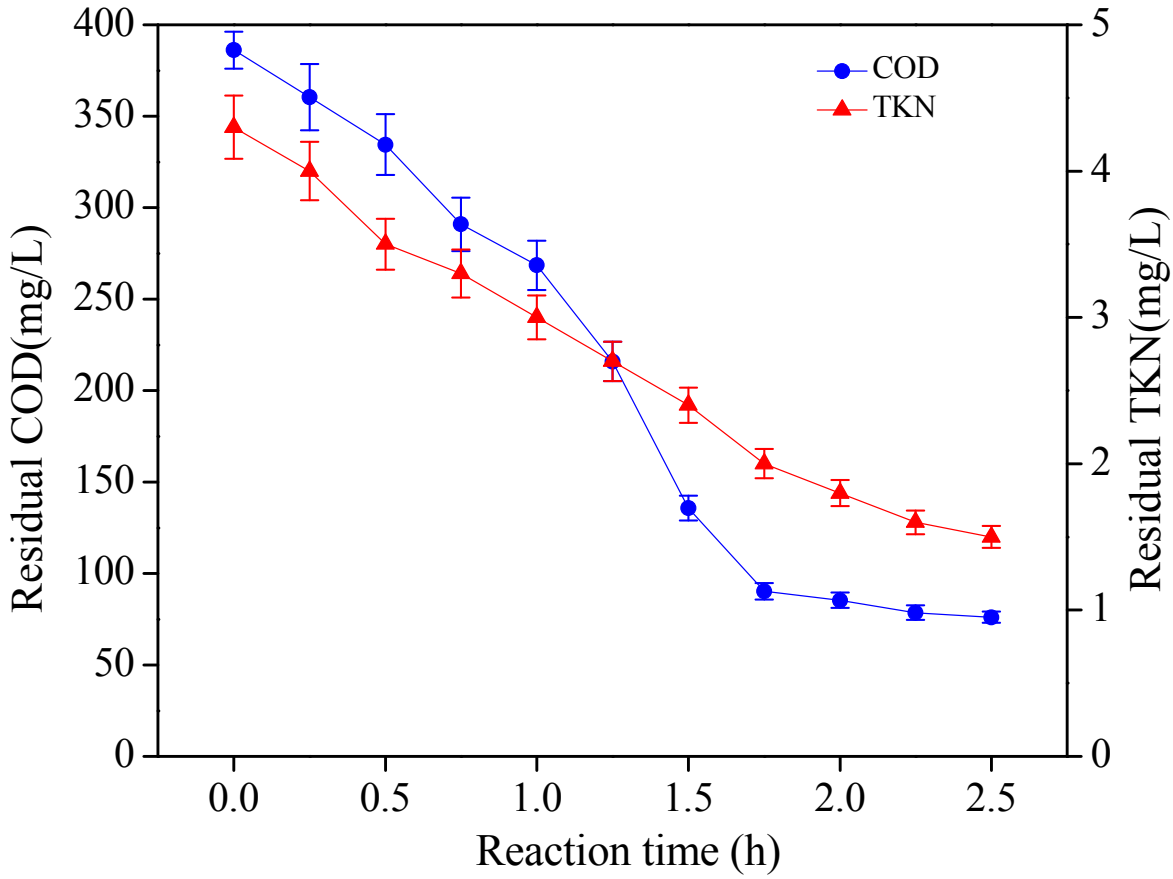


Figure 4.24 Carbon and nitrogen consumption at the optimal C:N ratio of 90 in pilot-scale.

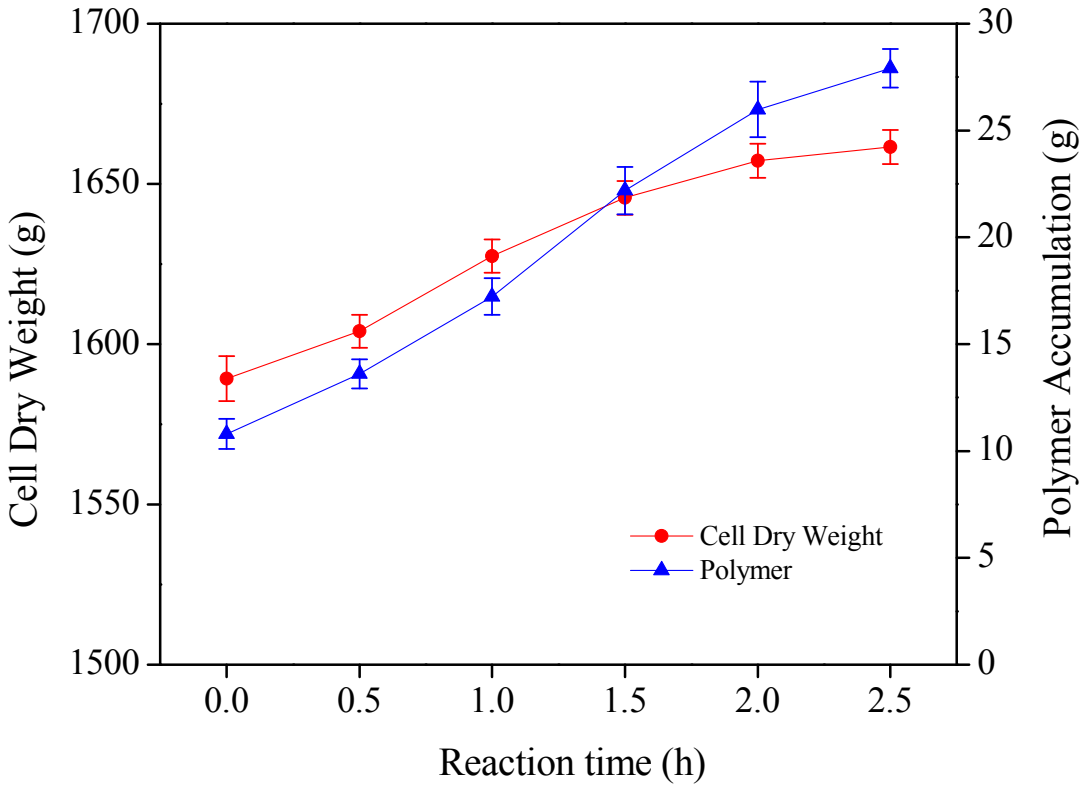


Figure 4.25 Growth and polymer accumulation under the optimal C:N ratio of 90 in pilot-scale.

4.4.2 Comparison of PHA production in laboratory-scale and pilot-scale operations

In this section, compariosn between laboratory-scale and pilot-scale studies was made in order to assess the technical and economic feasibility of large scale production of PHAs. This was also designed to validate the dimensionless parameters, namely the three yield factors, as effective tools for process design and scale-up. The values of comparison of the essential parameters were shown in Table 4.14, based on the same operational parameters and definitions.

When the optimal C:N ratio of 90 was maintained, it can be observed from the results shown in Figure 4.26, that the specific growth yield (Yx/s) achieved in the pilot-scale system was higher than that in the laboratory-scale operations, which indicated the bacterial consortium in the pilot-scale SBR system had a stronger capacity of growth. These observations were somewhat different from the usual believes that a process operated under well-controlled laboratory conditions should produce better performance results (Lee and Gilmore, 2005; Sarafim et al., 2004). The better process performance, as measured in terms of the dimensionless yield parameters, namely the specific growth yield ($Y_{x/s}$), could be attributed to the improved stability of the selected sludge in the larger scale process, and hence a more robust microbial ecosystem, in tolerating environmental fluctuations such as organic surge loads and hydraulic surge loads.

However, in terms of other dimensionless yield parameters, there were contrary observations. The specific polymer yield, Yp/x was 0.237 g polymer/g cell weight in pilot-scale, which was less than the 0.259 g polymer/g cell weight observed in laboratory-scale operation under similar operational condition. The specific polymer yield was an important parameter that showed the intracelluar accumulating PHA rate of microorganism in mixed activated sludge. Such results illustrated that when the incoming feed was supplemented with the same amount of carbon substrate thus giving rise to a nitrogen-deficient ambient would result in an increased storage of PHAs in laboratory-scale compared to the pilot-scale

operation.

The Y p/s was 0.092 g polymer / g COD consumed in pilot-scale, which was also slightly less than the 0.099 g polymer/g COD consumed in laboratory-scale operation. One possible explanation for the observed better performance in lab-scale system is that daily wastewater influent quality was more stable compared to that in the pilot-scale system, resulting in lower Yp/s in the on-site pilot-scale system. The quality and quantity of the influent wastewater were better controlled under laboratory conditions. Even so, 0.092 g polymer / g COD consumed of Y p/s was also in an acceptable range and can still be considered as pratical in future full-scale application. Furthermore, the dimensionless yield factors, although deviated slightly in the pilot-scale system, were still consistent with those observed in the laboratory operations. These shows that these three novel dimensionless yield factors were well validated in the pilot-scale operation, and hence can be used as an effective tool and predictive models for process scale-up, design, operation and optimisation.

The COD removal efficiency, averaged at 80.29%, was found to be 2.81% lower in the pilot-scale system compared to the previous laboratory-scale operations. However, the designed pilot-scale SBR system was observed to be stable and reliable for long-term applications under fluctuating ambient, climate and other site conditions.

Table 4.14 Comparison between laboratory-scale and pilot-scale SBR performance under the optimal C:N ratio of 90

Model	delX (g)	del P (g)	delS (g)	Yx/s (g/g)	Yp/x (g/g)	Yp/s (g/g)	COD Removal Efficiency(%)
Laboratory-scale	0.965	0.250	2.525	0.382 ±0.012	0.259 ±0.011	0.099 ±0.003	83.10%
Pilot-scale	72.354	17.112	186	0.389 ±0.016	0.237 ±0.010	0.092 ±0.046	80.29 %

The $Y_{X/S}$ (g DCW / g COD consumed) is the specific growth yield. (SGY)

The $Y_{P/X}$ (g polymer / g DCW) is the specific polymer yield. (SPY)

The $Y_{P/S}$ (g polymer /g COD consumed) is the overall polymer production yield. (PPY)

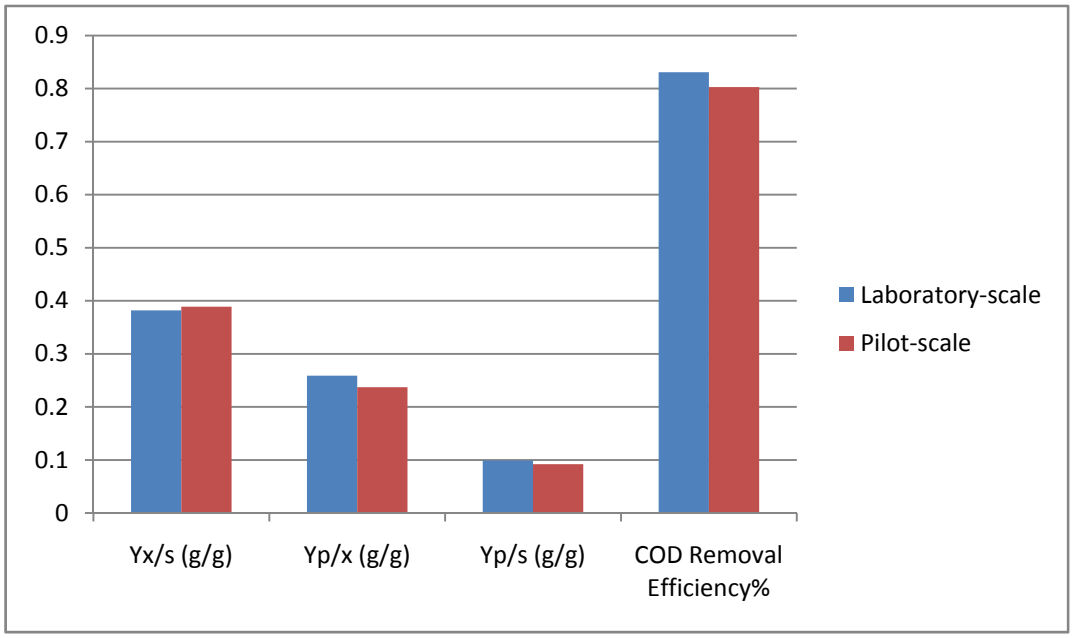


Figure 4.26 Comparison between laboratory-scale and pilot-scale SBR performance under the optimal C:N ratio of 90.

The pilot-scale process, while enjoyed the benefits of process stability, performed slightly inferior compared to the laboratory-scale process, due to a number of physical factors. The four possible reasons are (1) the transference loss due to physical size of equipment; (2) the different SRT which was inevitable due to different de-sludging operations; (3) the somewhat unsteady state in the pilot-scale SBR system due to site conditions, and (4) the different microorganisms in the consortium due to climatic changes. These are consistent with that observed by others (Tsai et al., 2006; Saito et al., 1995).

The possible attributes that contributed to this aspect are analyzed as follows.

(1) The geographical distance from the experiment site to the laboratory

The pilot-scale plant was located in Wuxi city, Jiangsu province. It took some time to transport the seed culture from the laboratory (in Hong Kong) to the site. Within the travelling time, the samples, which contain enriched biomass and residual ingredients, might have gone through biochemical reactions and endogenous respiration.

(2) The variation of C:N ratio

The biggest difference between the laboratory-scale study and pilot-scale study was the variation of the influent wastewater and other site conditions. In particular, the COD and TKN concentration of the influent changed greatly, with

an average percentage of fluctuation of around 20 %, due to the mixed municipal and industrial effluents from the city of Wuxi. Though the C:N ratio may be set on certain ratio through adding to external carbon sources and nutrient solution, sometimes the actual exact ratio might not be the preset one on the large-scale operations. For example, the C:N ratio at 90 actually varied from 80 to 101 (which was again around 20 % fluctuation). The difference between these two values is 21, which is enough to affect production yield quite substantially.

(3) Mineral ions

Activated sludge from two different wastewater treatment plants which include different metal ions and trace element. The mineral compositions also changed greatly from time to time on site, which was almost impossible to be artificially adjusted. The PHA degradation and synthesis rates decreased with increasing copper concentration, irrespective of the SRTs, which was agreeable with that reported by others (Tsai and Chen, 2011). This is another inherent factor that affected the performance of the pilot-scale system.

Under the effect of Ca^{2+} and Mg^{2+}, the PHB accumulation rate inside some bacterial strains was reduced. The heavy metallic ions, Cu^{2+} inhibited the growth and proliferation of the bacterial strains. Mn^{2+} was found to enhance the PHB accumulation in *S. natans*. The Cr^{3+} and Ni^{2+} in low concentrations will also accumulate together with the PHB production. Mn^{2+} (low conc.), Cr^{3+} (high conc.)

and Ni^{2+} (high conc.) could enhance the PHB accumulation in *E. Coil* (Lo, 2004). These reported observations agreed with that observed in the pilot-scale processes, which was subject to the heavy-metal effects present in the in-coming municipal-industrial mixed sewage.

(4) Temperature

The results of the work by Krishna and Van Loosdrecht (1999) showed the accumulation of storage polymers was strongly related to temperature, with more PHB formation at lower temperatures. This finding was further confirmed by Chinwetkitvanich et al. (2004) who pointed out that PHA production was greater in the 10°C system than in the 20 and 30°C systems under phosphorus limitation conditions, suggesting that the biochemical reactions were generally exothermic reactions that were hindered by higher temperatures.

In the laboratory-scale and pilot-scale SBR systems, which had no temperature control, were both subject to temperature variations. So the mixed liquor temperature in pilot-scale reactor fluctuated more drastically due to the site and climatic conditions, resulted in different and generally lower PHA yields.

4.5 Effect of C_4 to C_5 weight ratio on co-polymeric composition in pilot-scale system

From Table 4.15, when butyric acid was used as sole carbon source in the

in-coming feed, there was only P(3HB) homopolymer produced as intracellular food reserves. The 3-hydroxyvalerate (3HV) mole fraction in the PHBV reached a maximum of 48% (by weight) when valeric acid was used as sole carbon source. So, polymers and co-polymers produced by butyric acids were rich in HB monomeric units. The higher HV content in the polymeric formulation can be achieved by adding a higher concentration of valeric acids to the reactor.

However, as valeric acid in the medium was increased, both SPY and PPY were adversely affected. The specific polymer yield (Yp/x) decreased from 0.42 to 0.05 g polymer/g cell weight (Table 4.15). Besides, it also resulted in a decline in polymer production yield (Yp/s) from 0.51 to 0.07 g polymer/g COD consumed, showing that valeric acid was a less favorable carbon source for food reserve accumulation.

According to Ishihara et al. (1996) who found, in their survey, that the mole fraction of HV units in copolymers (0–40%) was linearly related to the mole fraction of valeric acid in the medium, which showed general agreement with our pilot-scale results.

The increase in valeric acid content also exhibited an inhibitory effect on polymer production, giving rise to a decline in polymer/ g COD consumed, was an indication of fatty acid with 5 carbons is less spontaneously metabolized that that with 4 carbons. The wide range of polymer production yields between 0.51

and 0.07 (g/g), could be ascribed to the complex microbial species in the ecosystem of the activated sludge, resulting in widely varied metabolic pathways from fatty acid to polymer in different microbial species. This was in general agreement with our earlier deduction that the enriched and selected sludge should be dominated by a number of hyper accumulators of PHAs, including *Nocardia spp*, *Alkaligene spp.* and *Pseudomonas spp.*, thus giving rise to the complexity in microbial consortium and variation in polymeric production yields. It is also believed that the variation of valeric acid concentration in medium caused the changes in the balance of microbial species, and hence effecting metabolic pathways in activated sludge, which in turn, caused the wide ranging overall polymer production yields. These results were in general agreement with that reported by Morgan-Sagastume et al. (2011).

Table 4.15 Copolymer accumulation by activated sludge under different carbon sources ratios of butyric and valeric acids

Carbon Conc. (g/L)		C4: C5 (g/g)	Y p/x (g/g)	Y p/s (g/g)	HV Fraction mol %	Tm (℃)
C4	C5					
3.20	0.00	100:0	0.42	0.51	0	178.9
2.56	0.64	80:20	0.37	0.23	13	146.5
1.92	1.28	60:40	0.31	0.17	28	138.2
1.28	1.92	40:60	0.28	0.12	36	126.9
0.64	2.56	20:80	0.07	0.09	46	112.4
0.00	3.20	0:100	0.05	0.07	48	98.5

Yp/x Specific copolyester yield was calculated as the mass of copolyesters accumulated during the nutrient-deficient stage divided by the cell mass.

Yp/s Copolyester production yield was calculated as the mass of copolyesters accumulated during the nutrient-deficient stage divided by the COD consumed.

It has been proposed by other researchers on the biochemical mechanistic pathways (Reddy et al., 2003). The mole fraction of 3HV in the accumulated copolymer increased proportionately with the valeric acid concentration in in-coming medium. This may be due to the fact that when butyric acid was used as the sole carbon source, propionyl-coA (precursor of 3HV unit) was not produced and as a result, only 3HB units were formed, but when valeric acid was used as the sole carbon source, both acetyl-coA and propionyl-coA were produced and available for forming 3HB and 3HV units. However, mechanistic pathways are beyond the scope of this study.

Figure 4.27 illustrates a more or less linear relationship between 3HV mole fraction in the copolymer and the valeric acid concentration. The maximum value of 3HV mole fraction in copolymer PHBV was restricted to 48 mol%.

This linear relationship between 3HV mole fraction in copolymer and the valeric acid concentration may be correlated through a linear expression, as developed as follows. If the 3HV mole fraction was represented by Y and the valeric acid concentration in medium (Wt %) was represented by X, then a mathematical model may be constructed as Equation (1), which applies as follows.

$$Y=0.4957x+3.7143 \qquad \text{R-Square} = 0.9475 \qquad (1)$$

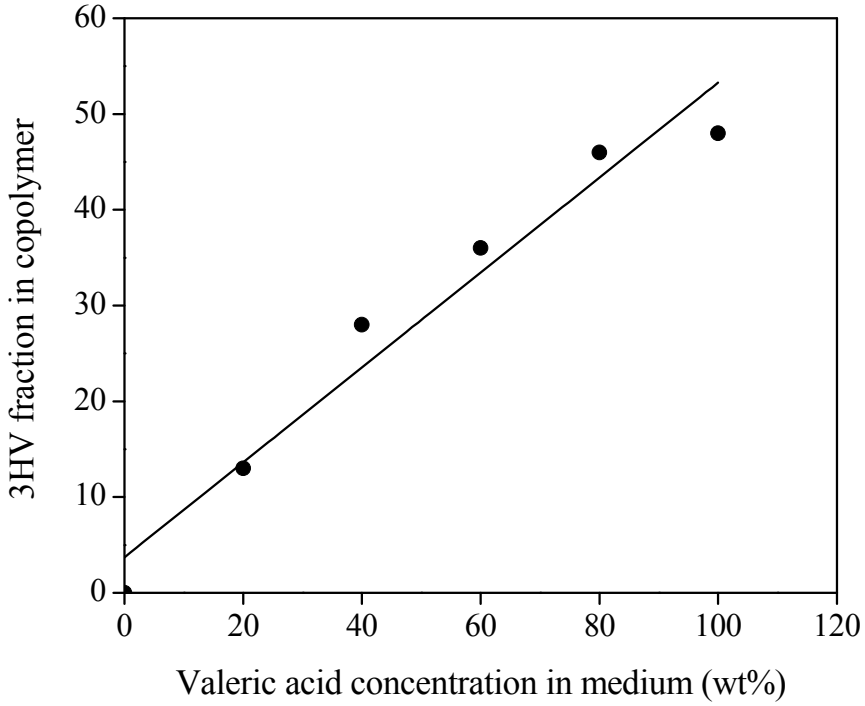


Figure 4.27 Relationship between 3HV fraction in copolymer and valeric acid concentration in the medium (wt %).

These results showed that the 3HV mole fraction of the PHBV copolymer accumulated in activated sludge could be controlled by adjusting the valeric acid concentration in the medium. The linear fit of the Y-X correlation also indicated that the copolymer composition in actual production could be predicted from the influent medium composition, hence enabling effective control of co-polymeric composition by the manipulation of the in-coming feed compositions.

As discussed previously, the maximum value of 3HV mole fraction in copolymer PHBV was restricted to 48 mol% due to relatively rapid metabolism of propionyl-coA to succinyl-coA and then to acetyl-coA for producing energy in *TCA* cycle, instead of entering into the HV monomer synthesis (Chua and Yu,

1999b).

4.5.1 Thermal properties of the Co-polymer

Melting temperature (Tm) is one of the important thermal properties for characterizing PHA polymers. As shown in Figure 4.28, when the 3HV units in the co-polymer increased from 0.0 to 48.0 mol%, the melting temperature (Tm) decreased fairly drastically from 178.9 to 98.5°C. The copolymers with maximum and minimum values of Tm were obtained when butyric acid and valeric acid were respectively used as the sole carbon sources in the medium. So an increase of 3HV monomeric units in the PHBV gave rise to a nearly proportionate decrease in polymer melting temperature, as seen from the mathematical model that follows. If the 3HV mole fraction in the copolymer was represented by X1, then Equation (2) applies.

$$Tm = -1.4475X_1 + 174.8206 \quad \text{R-Square} = 0.9357 \qquad (2)$$

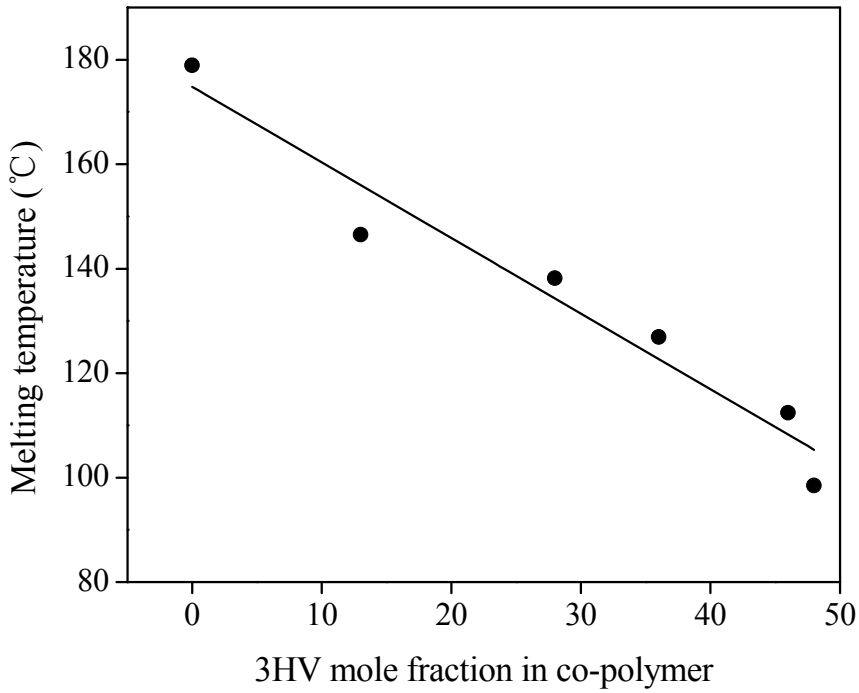


Figure 4.28 Relationship between melting temperature and 3HV fraction in co-polymer.

The experimental results were well coincided with the previous published thermal properties of polymers and copolymers of PHAs. Therefore, the mechanical and thermal properties of copolymer varied with 3HB to 3HV ratio, such as compressive, tensile, shear and flexural strengths. This mathematical model also allows prediction of final co-polymeric thermal, physical and mechanical properties with varying in-coming carbon compositions.

Wider range of properties renders more widespread industrial and commercial applications of the co-polymeric PHAs. Lower melting point could give rise to a loss of stiffness as well as poorer thermal resistance during the process, but it has obtained a great improvement in impact strength. These pilot-scale experimental

results revealed that specific high-value property materials for special usages may be obtained from activated sludge by adjusting the valeric acid concentration in the medium, which agreed with that published by Hu et al. (1997). It may also be deduced further that other fatty acids, or even other organics, in the in-coming formulation may further produce a wider range of possibly useful co-polymeric properties,

Typical samples of the acclimated co-polymeric materials extracted from the pilot-scale activated sludge microorganisms are shown in Figure 4.29. The sample with high HB ratio (Fig. 4.27(a)) showed lower tensile strength and elasticity, hence giving a readily breakable texture. The sample with higher HV ratio (Fig. 4.27(c)) showed lower brittleness and hardness, hence giving a flexible and stretchable texture. These variations in physical, mechanical and thermal properties render these novel materials very useful in various domestic, commercial and industrial applications.

a) The copolymer with higher 3HB mole fraction was more brittle

b) The copolymer with intermediate 3HB mole fraction had an improvement in impact strength

c) The copolymer with higher 3HV mole fraction was more flexible

Figure 4.29 Different textures of polymers extracted from activated sludge.

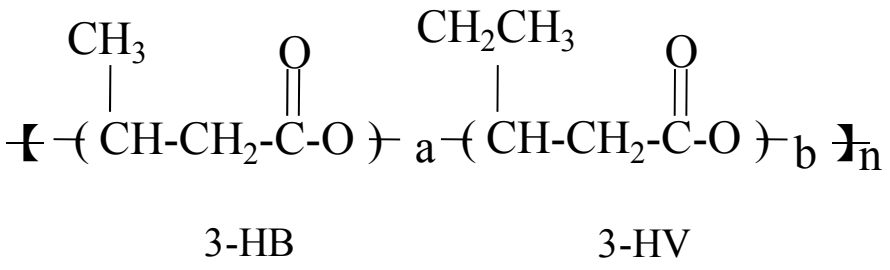


Figure 4.30 Structural formula of extracted polymers.

4.5.2 Further Discussion

These pilot-scale results also indicated that the 3HV mole fraction of the PHBV copolymer accumulated in activated sludge, in terms of co-polymeric formulation, production efficiency and accumulation yield, could be controlled by adjusting the valeric acid and overall carbon concentrations in the medium. The established and validated mathematical models are effective and useful in process design and product quality prediction.

Cell growth and proliferation rate were observed to be affected by the changing proportion of VFAs in the in-coming feed. Higher proportions of the type of VFAs, namely butyrates, involved in HB production were fairly accurately predicted by the model. The most likely explanation for this could be attributed to the difference in substrates used for biomass enrichment and species selection, hence activating the microbial group that are most suited for the accumulation of specific co-polymers. Generally, even-number carbon sources obtained a superior

PHA production capability and cell growth rates than odd-numbers, which was similar to that published by others (Chang et al., 2012).

Lemos et al. (2006) presented a then controversial argument that the co-polymer composition, and consequently the co-polymer properties, could be manipulated in any desired way by varying the volatile fatty acid feed composition, which is now deem promising as validated by our pilot-scale results. So the carbon sources play the very important role in governing with metabolic pathway to be the prevailing one and hence the production and the composition of the final PHA formulation (Brandl et al., 1988; Huisman et al., 1989). It means physical properties could be regulated by varying the molecular structure and composition of copolymers (Doi, 1995), which can now be quite accurately predicted by our established mathematical models.

Hu et al., (1997) investigated that the variation of valeric acid concentration in medium lead to the changes in the shift and eventual balance of microbial species, as a result, the prevailing metabolic pathways in activated sludge during the 48-h incubation which finally resulted in the wide ranging Yp/s values. Therefore, the mathematical expressions and the dimensionless yield factors together form an effective model for process design, scale-up, operation, optimization and control in PHA production as well as for predicting the final co-polymeric composition, physical, mechanical and thermal properties.

5 CONCLUSION

The main focus of this work was to conduct an in-depth investigation on the PHA accumulation in both the laboratory- and pilot-scale activated sludge sewage treatment systems.

A novel and effective sludge selection procedure was developed, using a series of physical pressures such as aeration time and settling time, and nutrient pressures such as C:P and C:N ratios, to established a stable and efficient PHA-accumulating sludge in an laboratory-scale SBR simulator system.

The laboratory-scale activated sludge process was scaled up 100-fold into an on-site pilot-scale SBR system and three novel dimensionless yields parameters were validated and proved to be effective model for process scale-up and design. The works have never been done by others and are substantial contributions towards better understandings of the process mechanisms of the PHA accumulation system.

The substantial results and useful observations from this work were organized into five main stages.

Firstly, in the operation of the SBR system, the optimal aeration time of 2.5 h and the optimal settling time of 1.0 h were determined, based on the quality of the

sludge and treated effluent in the laboratory-scale activated sludge simulator system. These resulted in an organic loading rate or F/M ratio of 0.24 mg/mg-d, which was established as the physical selection pressure for the intended activated sludge.

Secondly, with the fixed aeration and settling time that have previously been optimized for the activated-sludge simulator system, the optimal carbon-phosphorus (C:P) ratio was fixed at 300, determined by observing the PHA accumulation rates in the biomass.

In the third stage, in-depth investigation was directed at evaluating the optimal values of carbon-nitrogen (C:N) ratio on PHA production yields under the other pre-determined optimal conditions. Results showed that as the C:N ratio increased from 30 to 120, specific polymeric yield increased to a maximum of 0.291 g polymer/g dry cell weight, while specific growth yield decreased with increasing C:N ratio. The highest overall polymer production yield of 0.099 g polymer/g COD consumed was achieved under the C:N ratio of 90. Therefore, a severe nitrogen deficiency triggered the intracellular accumulation of PHAs as a food reserve in the activated-sludge microbial community. Sporadic adjustments of the C:N ratio did not significantly affect the COD removal efficiency, which maintained at around 80%. Therefore, C:P and C:N ratios (the nutrient selection pressures), together with the previous physical selection pressure, had

successfully selected an activated sludge that was stable, robust and was a hyper-accumulator for PHAs. The novel physical and nutrient selecting procedure, gave rise to an activated sludge with the three novel dimensionless parameters, namely $Y_{x/s}$, $Y_{p/x}$ and $Y_{p/s}$, showing very promising results.

After these three stages of works, the activated sludge was considered to have been selected with an effective microbial consortium that is best suited for efficient PHA accumulation under the predetermined operating conditions. A specific polymeric yield as high as 0.30 g polymer/g dry cell weight indicated that the activated sludge is dominated by hyper accumulators of PHAs such as the branched filamentous *Nocardia spp.*, and other rod cells *Alkaligenes spp.* and *Psudomonas spp.*.

In the fourth stage, the pilot-scale SBR system was built for PHA accumulation based on optimal condition determined from the laboratory-scale system. The previously selected activated sludge was used to seed the system. The PHA production yields and COD removal efficiency were studied on a long-term basis. The results showed that the specific growth yield (Yx/s) from the pilot-scale SBR system was higher than that in the laboratory-scale simulator. The specific polymer yield (Yp/x) was 0.237 g polymer/g dry cell weight in pilot-scale, which was less than the 0.259 g polymer/g cell weight observed in the laboratory-scale simulator system. The overall polymer production yield (Yp/s) was 0.092 g

polymer / g COD consumed in pilot-scale, which was less than the 0.099 g polymer/g COD consumed in laboratory-scale simulator under the C:N ratio of 90. The average COD removal efficiency of 83.10% in the laboratory-scale simulator was found to be 2.81% higher than that in the pilot-scale system. While having slight variations, the dimensionless parameters namely $Y_{x/s}$, $Y_{p/x}$ and $Y_{p/s}$, remained fairly stable, showing that these models were established as useful tools for design, scale-up, and operation of PHA-accumulating activated sludge processes.

In the final stage, the composition of the macro-molecular co-polymeric materials, namely PHBV, produced by activated sludge bacteria was controlled by regulating the concentration of butyric acid (C4) and valeric acid (C5) ratio in the medium under the condition of pilot-scale system. The organic feed to PHBV conversion mechanism were studied and modeled. When butyric acid was used as sole carbon source, there was only PHB homo-polymer produced instead of PHBV co-polymers. On the other hand, the highest 3HV mole fraction 48% in the co-polymer accumulated was when valeric acid was used as sole carbon source. The melting temperature of the PHAs produced by activated sludge decreased from 178.9℃ to 98.5℃, with an increase in the 3HV fraction, indicated that 3HV unit act as defects in the PHBV crystal lattice. Therefore, the composition of the co-polymers, the physical, thermal and mechanic properties, could be controlled by manipulating the influent organic compositions in the medium. The carbon

source to PHBV copolymers conversion mechanisms and relations were elucidated.

With these results and observations obtained in the five stages of works, it can be concluded that PHAs, including copolymers of PHBVs, with industrially applicable properties can be produced at substantially reduced costs using a specifically selected activated sludge, through a novel procedure, from sewage treatment works. The PHA accumulation process was scaled up in a pilot scale system and the conversion process mechanism was elucidated. The novel use of dimensionless parameters, namely Yp/s, Y p/x and Y x/s, served as the process predictive model for the design, optimization, operation and control. These model parameters were validated in pilot-scale opeartion, and were valuable tools for full-scale industrial opearations. This is a major step ahead towards environment-friendly plastics production and efficient sewage sludge disposal. Furthermore, this established method may serve as an environment-friendly means to convert sewage sludge as a waste into PHAs as a valuable product.

6 RECOMMENDATION FOR FURTHER STUDIES

In future research work, it is recommended that the specific microbial genera and species be identified and the associated physiological characteristics studied in detail in order to effectively enrich the activated sludge for improved PHA production yield.

It is also recommended that the waste-to-PHA conversion process can be mathematically modeled in order to better understand the biochemical mechanisms and process design.

Finally, various cheaper carbon sources, such as mixed fatty acids from excess activated sludge and sewage, molasses and fiber from industrial wastes should be considered and investigated for their effects on PHA production efficiency and the associated co-polymeric composition, physical, mechanical and thermal properties.

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