The Utilization of Activated Sludge Polyhydroxyalkanoates for the Production of Biodegradable Plastics

Warangkana Punrattanasin

Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Environmental Science and Engineering

Dr. Clifford W. Randall, Chair
Dr. John T. Novak
Dr. Daniel L. Gallagher
Dr. Eugene M. Gregory
Dr. Andrew A. Randall

April 13, 2001
Blacksburg, VA

Keywords: activated sludge; anaerobic-aerobic; biodegradable plastics production; fully aerobic; microaerophilic; nutrient limitation; polyhydroxyalkanoates; polyhydroxybutyrates; polyhydroxyvalerates

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(ABSTRACT)

Sequencing batch reactor (SBR) systems were used for the development of a system and operating procedures for the high production and internal storage of polyhydroxyalkanoates (PHAs) by wastewater treatment (activated sludge) bacterial cultures. The SBRs were operated with microaerophilic/aerobic (MAA), anaerobic/aerobic (AN/AE), and fully aerobic (AE) conditions. It was found that unbalanced growth conditions stimulated massive PHA production in activated sludge biomass. Operating conditions had a significant effect on PHA production and the composition of the accumulated copolymer when either laboratory prepared mixtures of organics or a high acetic acid industrial wastewater were used as the organic substrate mixture. Fully AE conditions with nitrogen (N) and phosphorus (P) limitations were the optimum conditions for PHA production when the laboratory prepared mixtures of organics were used as the substrate. However, fully AE conditions with combinations of N, P, and potassium (K) limitations were better for PHA production when a high acetic acid industrial wastewater was the substrate. Limitation or partial limitation of a single nutrient, either N or P, as used for commercial production utilizing pure cultures, did not promote massive PHA production in activated sludge biomass compared to the simultaneous limitation of two or more nutrients. A maximum cellular PHA accumulation of 70%TSS was obtained under fully AE conditions with multiple alternating periods of growth and N&P limitations. Microaerophilic/aerobic or AN/AE cycling promoted less PHA production compared to fully AE conditions. The relative amounts of the PHA copolymers formed, i.e., polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), were different under different operating conditions, even though the types and amounts of volatile fatty acids (VFAs) in the feed were the same. It was determined that high total phosphorus (TP) content inside the bacterial cells had a significant detrimental impact on PHA production by activated sludge biomass. A two-stage bioprocess was a better approach for obtaining activated sludge PHA accumulation because a growth phase was necessary to grow a bacterial population that contains minimal TP before starting the subsequent PHA accumulation

phase. Seeding sludge obtained from a conventional fully aerobic wastewater treatment system was more suitable than seed obtained from a biological phosphorus removal (BPR) system because bacterial populations from BPR systems tended to convert organic substrates to intracellular carbohydrate content rather than PHA under nutrient limitation conditions. The molecular weights and melting point temperatures of PHAs produced by the mixed culture of activated sludge biomass were comparable to those obtained from pure cultures and have the potential to be used for commercial applications. The results of this study indicate that activated sludge biomass has considerable potential for PHA production for commercial purposes, and likely could do so utilizing wastewater sources of organics. In particular organic rich, nutrient limited wastewaters have potential for efficient PHA production.

ACKNOWLEDGEMENT

I would like to thank The Royal Thai Government for providing me an opportunity to work on their overseas graduate program and financial support during my graduate study at Virginia Tech. I would also like to acknowledge the following sources of research funding and fellowship: Waste Policy Institute (WPI) Scholarship, Edna Bailey Sussman Fund, and Hunnicutt Fellowship. I would also like to thank my committee members, Dr. John Novak, Dr. Eugene Gregory, Dr. Dan Gallagher, and Dr. Andrew Randall, for their assistance throughout the course of this research.

I would like to express my sincere gratitude and appreciation to my advisor, Dr. Clifford Randall, for his tremendous help and support. Without his effort, this research would not have been possible. I also thank Julie Petruska and Jody Smiley for their assistance with laboratory procedures and analytical techniques. I also thank Betty Wingate for her assistance in office related work.

Special thanks to my family for their love and unending support sent across my to encourage me to complete the degree and friends for their friendship to make my stay in Blacksburg more like home.

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INTRODUCTION

It is well understood that synthetic plastics are highly resistant to microbial degradation in landfills. This has become a problem for municipalities worldwide because municipal landfills lose capacity because of the accumulation of synthetic plastics. Imperial Chemicals Industries (ICI), now Monsanto, produces biodegradable plastics from polyhydroxyalkanoates (PHAs) and markets them as "BIOPOL" in Europe. PHAs are an intracellular storage material synthesized by a variety of bacteria, and are materials of interest because they have some properties similar to synthetic plastics. Plastics produced from PHAs have been reported to be truly, fully biodegradable (Page, 1995). In addition, the degradation product of PHA is a common intermediate compound in all higher organisms. Therefore, it is plausible that it is biocompatible to animal tissues and PHA may be use in surgical applications without any toxicity.

The production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate, low polymer production, and the cost of maintaining an axenic, i.e., pure, culture. According to Yamane (1993), higher production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional petroleum-based plastics in the commercial marketplace. Lee (1996) reported that the price of BIOPOL is $16/kg, while the price of synthetic plastics is a lot less expensive, e.g., less than $1/kg for polypropylene. A good candidate for economical PHA production would be a mixed culture that can store high PHA content while growing on an inexpensive substrate.

Satoh, *et al*. (1998) demonstrated the basic feasibility by showing that microaerophilic conditions could be used to increase the percent PHA in activated sludge to as much as 62%TSS, using sodium acetate as the primary organic substrate. If PHAs obtained from activated sludge biomass utilizing wastewaters can be proven to be a suitable raw material for biodegradable plastics, this could be an ideal economic solution for the waste management and wastewater treatment fields. Cultivation strategies to achieve high biomass PHA concentration and high productivity in pure cultures are well defined. However, current knowledge is very limited for PHA production using activated sludge bacterial cultures. The main objective of this research was to explore ways to improve the economics of manufacturing biodegradable plastics by

producing useful by-products (PHAs) from potentially environmentally damaging waste discharges that are expensive to treat. The specific goals of the study included:

1. Develop both an efficient system and suitable operating procedures that would maximize PHA production using the same mixed microbial culture used for activated sludge wastewater treatment, i.e., define optimum operating conditions for maximizing PHA content in the activated sludge biomass.
2. Characterize the composition of the PHAs, i.e., the PHV/PHB ratio, of the copolymers produced by the activated sludge biomass.
3. Investigate parameters affecting the high production of PHA in the mixed culture of activated sludge biomass in order to better design the production process and adequately control its production.
4. Characterize the physical properties of PHAs produced by the activated sludge biomass and compare them with those produced by pure cultures.
5. Test a potential practical application of the developed high PHA production system by utilizing an industrial wastewater as the substrate. Specifically, the high acetic acid industrial wastewater currently discharged and treated by the Celanese Acetate manufacturing plant located at Narrows, Virginia was used as the substrate for PHA production.
6. Perform and present an economic analysis of PHA production utilizing activated sludge biomass and wastewater organics.

References

Lee, S. Y. (1996). Bacterial Polyhydroxyalkanoates. *Biotechnol. and Bioeng*. 49, 1-14.

Page, W. J. (1995). Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics With a Great Future. *Can. J. Microbiol*. 41 (suppl. 1), 1-3.

Satoh, H., Iwamoto, Y., Mino, T., and Matsuo, T. (1998). Activated Sludge as a Possible Source of Biodegradable Plastic. Proceedings, Water Quality International 1998, Book 3 Wastewater Treatment, pp. 304-311. IAWQ 19th Biennial International Conference, Vancouver, BC, Canada, 21-26 June 1998.

Yamane, T. (1993). Yield of Poly-D(-)-3Hydroxybutyrate from Various Carbon Sources: A Theoretical Study. *Biotechnol. and Bioeng*. 41, 165-170.

CHAPTER 1: LITERATURE REVIEW

1. Introduction

It is well known that production and storage of PHAs are integral and essential parts of the excess biological phosphorus removal (EBPR) mechanisms. It is less well known by the wastewater treatment community that bacterial PHAs are used for the manufacture of biodegradable plastics. Primary differences in the two approaches are: EBPR is accomplished using mixed cultures of bacteria, i.e., activated sludge, with the intent of minimizing the amount of phosphorus remaining in solution at the end of the aerobic phase. Plastics production is typically accomplished using pure cultures with the goal of maximizing the amount of stored PHAs at the end of a nutrient limited or oxygen limited phase. Thus, for EBPR the microbial cells are harvested when phosphorus storage is at a maximum, and for plastics production the cells are harvested when PHA storage is at a maximum. Simplistically, the two processes exploit different parts of the same basic biochemical cycle, and this raises the possibility that perhaps wastewaters can be used to produce biodegradable plastics, a marketable by-product. Satoh, *et al*. (1998) demonstrated the basic feasibility by showing that microaerophilic conditions could be used to increase the percent PHA in activated sludge to as much as 62%TSS, using sodium acetate as the primary source of volatile fatty acids (VFAs) in the feed. There are industrial wastewaters such as those from cellulose acetate manufacturing that average as much as 1200 mg/L acetic acid, and would seem to be good substrates for plastics production. Also, fermentation can be used to convert much of municipal sewage to VFAs, and potentially enable sewage as an inexpensive substrate for plastics production.

According to Dawes (1985) there are 4 principal classes of storage polymers in microorganisms: lipids, carbohydrates, polyphosphates, and nitrogen reserve compounds. Each storage polymer will be discussed in detail shortly. For lipid storage, the discussion will be focused mainly on polyhydroxyalkanoates (PHAs). Some organisms can accumulate more than one kind of storage polymers. For example, polyphosphate accumulating organisms (PAOs) that are responsible for phosphorus removal in BPR systems store glycogen, PHA, and polyphosphate. Dawes and Senior (1973) stated that environmental conditions and regulatory mechanisms influence the content of storage polymers accumulated. In addition, the nature of carbon substrate will

determine the type of storage polymers synthesized. For example, when the source of carbon was changed from glucose to acetate, the carbohydrate content of *E. coli* decreased, while the lipid content increased. According to Sasikala and Ramana (1996), Stanier *et al*. (1956), stated that when a carbon substrate is metabolized via acetyl-CoA, i.e., no pyruvate is formed as an intermediate, the flow of carbon is mainly to PHA synthesis. However, if a carbon substrate is metabolized via pyruvate, glycogen storage is predominant. Dawes (1985) stated that the important role of the possession of storage polymers by microorganisms, is that such storage materials provide carbon and energy sources for organisms and permit them to maintain viability longer under nutrient limitation conditions. Organisms that do not possess storage polymers tend to utilize cellular contents, e.g., RNA and protein, during starvation periods.

2. Storage polymers in microorganisms

2.1 Lipids (Polyhydroxyalkanoates, PHAs)

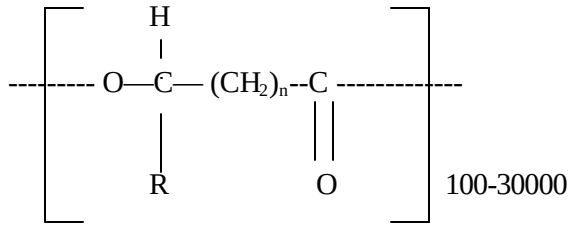
This section will be focused on the production of PHA by microorganisms. Factors affecting the production and its composition will be discussed. Biosynthesis pathways, as well as physical properties, recovery processes, and potential applications of biodegradable plastics produced from PHA will also be included.

Extensive reviews of bacterial PHAs have been performed by Lafferty *et al*. (1988), Anderson and Dawes (1990), Doi (1990), Sasikala and Ramana (1996), Lee (1996), Steinbuchel (1996), and Braunegg *et al*. (1998). PHAs are an intracellular storage material synthesized by a wide variety of bacteria. *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) is a bacterial strain that has been the subject of much of the published research because it can accumulate PHAs up to 80% dry weight (Lee, 1996). Imperial Chemical Industries (ICI), now Zeneca Bio Products, Billingham, England, produces biodegradable plastics from PHA using *R. eutropha,* and markets them as "BIOPOL" in Europe. BIOPOL was recently produced and sold by Monsanto, a company that acquired the business from Zeneca Bio Products in April 1996 (Braunegg *et al*., 1998). PHA is of interest because it possesses thermoplastic characteristics. According to Lafferty *et al*. (1988), PHB has some properties, including tensile strength and flexibility, similar to polyethylene and polystyrene. Plastics produced from PHAs have been reported to be truly

biodegradable in both aerobic and anaerobic environments (Page, 1995), unlike many of the "so-called" biodegradable plastics made synthetically.

PHAs are composed mainly of poly-beta-hydroxybutyric acid (PHB) and poly-beta-hydroxyvaleric acid (PHV), although other forms are possible. More than 80 different forms of PHAs have been detected in bacteria (Lee, 1996). Only two forms of PHAs, i.e., PHB homopolymer and 3HB-3HV copolymer are commercially produced by Zeneca. Figure 1 depicts a general structure of PHAs. Figures 2 and 3 illustrate the structures of the copolymers 3HB-3HV and 3HB-4HB, respectively. Lafferty *et al*. (1988) stated that the accumulation of PHA can be stimulated under unbalanced growth conditions, i.e., when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C:N ratio of the feed substrate is high. Sasikala and Ramana (1996) summarized nutrient limiting conditions that led to PHA accumulation in different microorganisms. In addition to nitrogen, phosphorus, oxygen, and sulfate limitations, limitations of the following compounds also stimulate the accumulation of PHA: iron, magnesium, manganese, potassium, and sodium.

According to Doi (1990), when growth conditions are unbalanced, acetyl-CoA cannot enter the tricarboxylic acid (TCA) cycle to obtain energy for cells due to high concentrations of NADH. According to Dawes (1990), the high concentration of NADH is resulted from the cessation of protein synthesis, a process closely coupled to ATP generation by electron transport chain, during nutrient limitation. The high concentrations of NADH inhibit enzyme citrate synthase, one of the key enzymes of the TCA cycle, leading to an increase in the level of acetyl-CoA. Acetyl-CoA is then used as substrate for PHA biosynthesis by a sequence of three enzymatic reactions (e.g. Figure 4). In addition, high intracellular concentrations of CoA inhibit enzyme 3-ketothiolase, one of the three enzymes of PHA biosynthesis. When the entry of acetyl-CoA to the TCA cycle is not restricted, free CoA is released as the acetyl moiety from citrate synthase activity, i.e., acetyl-CoA is utilized, intracellular CoA concentration increases, and PHA synthesis is inhibited. PHA can serve as a carbon or energy source for microorganisms during starvation periods. In EBPR systems, PHA is formed and stored during the anaerobic period by PAOs when no electron acceptors are available for growth mechanisms. This permits them to sequester most of the available substrate and out-compete organisms that do not have phosphorus



n = 1 R = methyl: polymer = poly(3-hydroxybutyrate)
R = ethyl: polymer = poly(3-hydroxyvalerate)
n = 2 R = hydrogen: polymer = poly(4-hydroxybutyrate)
n = 3 R = hydrogen: polymer = poly(5-hydroxybutyrate)

Figure 1: General Structure of PHA

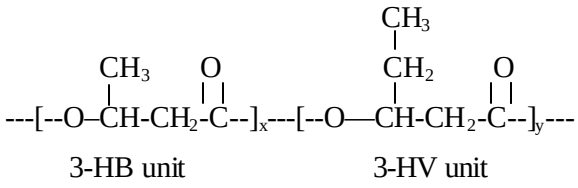


Figure 2: Structure of Copolymer 3HB-3HV

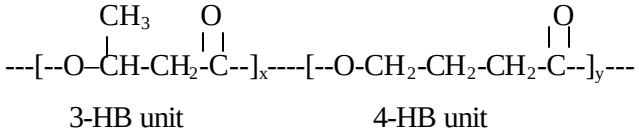


Figure 3: Structure of Copolymer 3HB-4HB

and PHA storage capabilities. According to Lee (1996), there are four different biosynthesis pathways of PHA storage reported to date. They are presented in the following section.

2.1.1 PHA biosynthesis pathways

1) *R. eutropha* PHA biosynthetic pathway

Most of the organisms synthesize PHA using this pathway. The biosynthesis pathways of *R. eutropha*, *Zoogloea ramigera*, and *Azotobacter beijerinckii* are well established (Doi, 1990). Firstly, a substrate is condensed to acetyl-coenzyme A (acetyl-CoA). Two moles of acetyl-CoA are then used to synthesize one mole of PHB. Acetyl-CoA is subjected to a sequence of three enzymatic reactions for PHB synthesis. Figure 4 shows a schematic of PHB biosynthesis by these organisms (Doi, 1990 and Anderson and Dawes, 1990).

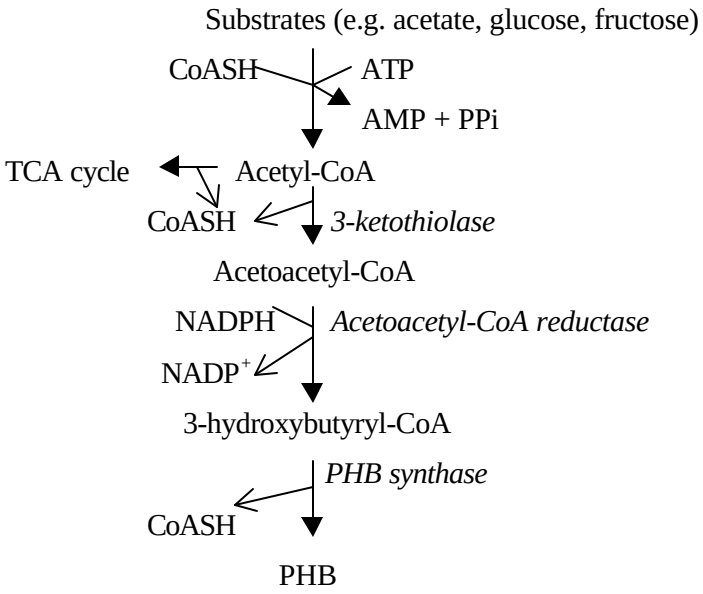


Figure 4: PHA Biosynthesis Pathway of *R. eutropha*, *Zoogloea ramigera*, and *A. beijerinckii*

When propionic acid is used as a sole substrate, PHB-PHV copolymer is formed. Acetyl-CoA is formed by the elimination of carbonyl carbon from propionyl-CoA. Two moles of acetyl-CoA are used to form a HB unit of the copolymer, while a HV unit is formed by the reaction of acetyl-

CoA and propionyl-CoA. Figure 5 shows the biosynthesis pathway of PHB-PHV copolymer by *R. eutropha* (Doi, 1990).

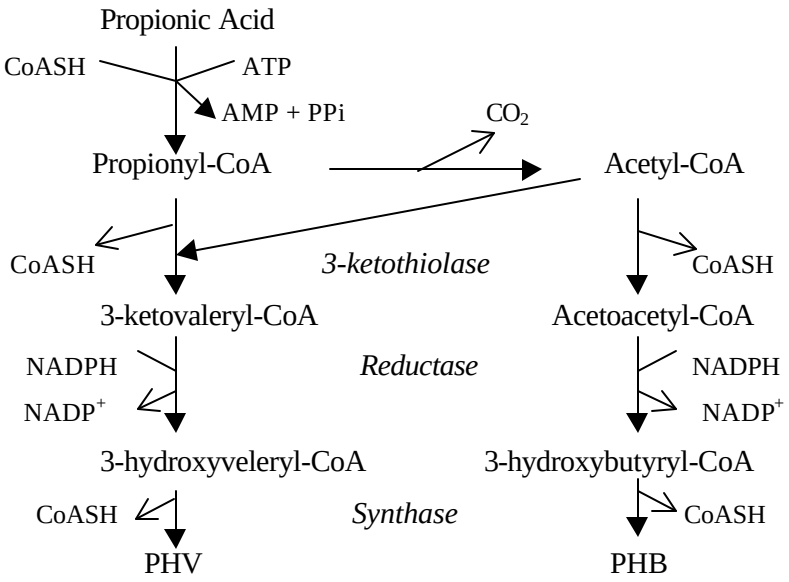


Figure 5: Biosynthesis Pathway of PHB-PHV Copolymer by *R. eutropha*

According to Doi (1990), the degradation of PHA by *R. eutropha* can occur simultaneously with its biosynthesis under nitrogen limitation. This observation is called "a cyclic nature of PHA metabolism". The author reported that the composition of polymer was changed from PHB homopolymer to PHB-49%PHV copolymer when the substrate was changed from butyric acid to pentanoic acid after 96 hours of nitrogen limitation accumulation period, i.e., there was a replacement of PHB by PHB-PHV. Likewise, when *R. eutropha* with a PHV fraction of 56% of its PHA content was fed with butyric acid as a sole substrate under nitrogen limitation, the PHA composition changed markedly, i.e., the fraction of PHV decreased from 56% to 19% after 48 hours. These findings show the simultaneous synthesis and degradation of PHA, i.e., the cyclic nature of PHA metabolism. Figure 6 illustrates cyclic metabolism (Doi, 1990).

2) *Rhodospirillum rubrum* PHA biosynthetic pathway

This pathway is similar to the *R. eutropha* pathway but two enoyl-CoA hydratases are also involved in the second step of catalyzing the conversion of L-3-hydroxybutyryl-CoA to D-3-hydroxybutyryl-CoA via crotonyl-CoA (Anderson and Dawes, 1990; Doi, 1990, Lee, 1996). A

simple schematic of this pathway is shown as: acetate → acetyl CoA → acetoacetyl CoA → L-3-hydroxybutyryl-CoA → crotonyl CoA → D-3-hydroxybutyryl-CoA → PHB.

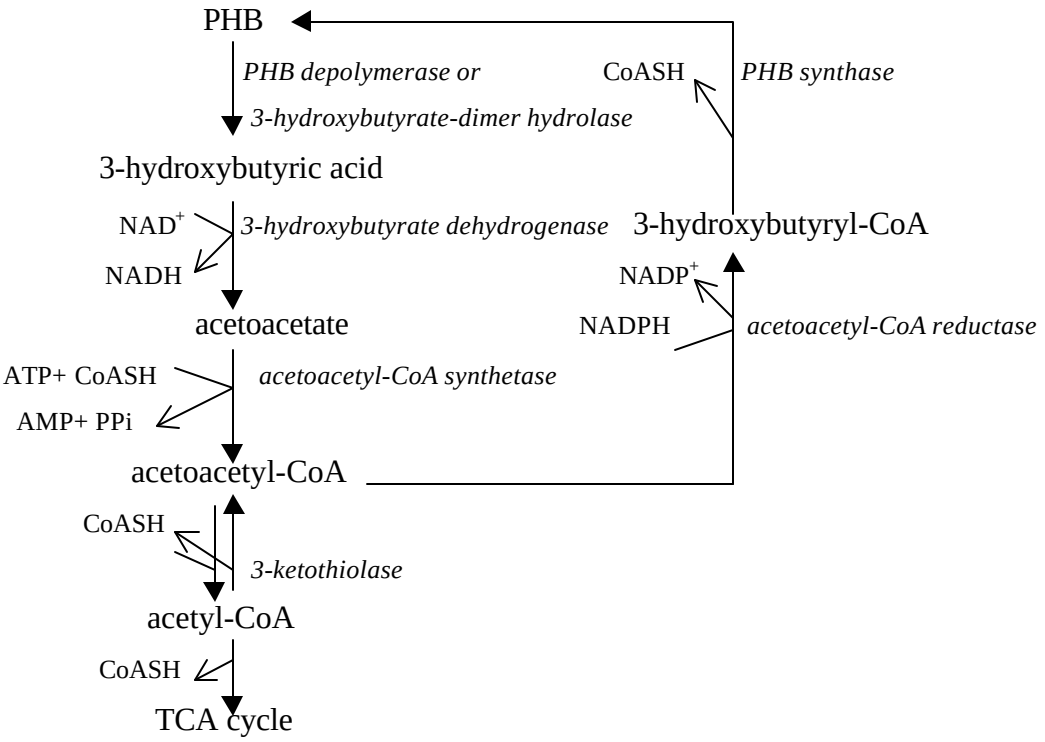


Figure 6: Cyclic Nature of PHA Metabolism

3) *Pseudomonas oleovorans* PHA biosynthetic pathway

This biosynthesis pathway is found in *P. oleovorans* and most pseudomonads from the rRNA homology group I (Lee, 1996). These organisms produce medium-chain-length (MCL) PHAs (from C_6-C_9) from MCL-alkanes, alcohols, or alkanoates. According to Doi (1990), productions of short-chain-length (SCL) PHAs, i.e., PHB homopolymer and PHB-PHV copolymer, could also be produced by these organisms but the productions were less than 1.5%. This PHA biosynthesis involves the cyclic-β-oxidation and thiolytic cleavage of fatty acids, i.e., 3-hydroxyacyl-CoA, and intermediates of the β-oxidation pathways, are used for PHA biosynthesis.

4) *P. aeruginosa* PHA biosynthetic pathway

Most pseudomonads from the rRNA homology group I except *P. oleororans* also produce MCL-PHAs using this pathway. The pathway used in these organisms is called the *P. aeruginosa* PHA biosynthetic pathway. Steinbuchel (1996) stated that MCL-PHAs produced by this pathway are from unrelated substrates, e.g., gluconate or actetate. PHA is synthesized from acetyl-CoA via fatty acid synthetic pathways.

2.1.2 Factors Affecting PHA Synthesis and Its Composition

1) Feed substrate and growth conditions

It is considered to be well known that copolymer composition, i.e., %PHB and %PHV, is primarily influenced by the substrate used (Doi, 1990). The author reported the composition of polymer produced by *R. eutropha* is PHB homopolymer when an even chain length of alkanoic acid is used as a substrate, while PHB-PHV copolymer is produced when an odd chain length of substrate is used. PHB homopolymer is also produced when glucose or fructose is used as a substrate. The concentration of a substrate supplied also affects the amount of polymer produced. For example, when propionate was used as a sole carbon source, the highest PHA content of 56% produced by *R. eutropha* was achieved at the propionate concentration of 14 g/L, while the lowest PHA content of 12% was obtained at the substrate concentration of 2 g/L. Doi *et al*. (1987) studied the copolymer composition produced by *R. eutropha* H16 grown in a medium containing sodium acetate and sodium propionate. The PHV content of the copolymer increased as the propionate concentration in the medium increased. 3HB-3HV copolymer was obtained when propionate was used as a sole carbon. PHA contents produced by *R. eutropha* H16 in this study also varied as the type of substrate and its concentration varied. Holmes *et al*. (1981) of ICI reported that the copolymer of 3HB-3HV was produced by *R. eutropha* using propionic acid and glucose as a carbon source (Doi *et al*. (1990). The mole % of PHV in the copolymer was varied depending on the compositions of the feeding substrate. The PHV content of greater than 95 mole% was obtained when pentanoic and butyric acids were used. Doi *et al.* (1990) stated that the structure and compositions of PHA, as well as its physical and thermal properties can be controlled by composition and concentration of feeding substrates. They ran experiments investigating PHA production by *R. eutropha* using various types of substrate. The copolymer of 3-hydroxybutyrate and 3-hydroxypropionate (3HB-3HP) was obtained when 3-

hydroxypropionic acid, 1,5-pentanediol, 1,7-heptanediols, or 1,9-nonanediol was used as the carbon source. The copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (3HB-4HB) was obtained from 4-hydroxybutyric acid, γ-butyrolactone, 1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol, or 1,12-dodecanediol. The copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate (3HB-3HV) was obtained from propionic or pentanoic acid. In addition, the biodegradability of PHA film (initial weights: 4-8 mg and initial film dimensions: 10X10 mm. in size and 0.03-0.06 mm. thick) was also studied. It was found that the rate of degradation was enhanced when 3HB and 4HB units were present in the copolymer. The presence of 3HV units reduced the degradation rate of copolymer.

Shimizu *et al*. (1994) investigated the PHA production from *R. eutropha* H16 (ATCC 17699) fed with butyric and valeric acids. Optimum conditions for PHB production using butyric acid by this organism were at the concentration of 3 g/L butyric acid and pH of 8.0. PHV or other PHAs was not reported in this study. PHB content of 75 % was obtained under those conditions, while lower PHB contents were achieved when pH was kept at 8 and butyric acid concentrations were 0.03, 0.3, 10 g/L, i.e., PHB contents were 44%, 55%, and 63%, respectively. When butyric acid was kept at 3 g/L, PHB content was lower as pH changed from the optimum value (pH 8). PHB contents of 53% and 58% were obtained at pH values of 6.9 and 7.5, respectively, while no production of PHB was obtained when pH was greater than 8.4. When propionic and butyric were used as substrates, 3HB-3HV copolymers were produced, however, the production of copolymers were lower than that of homopolymer produced by butyric alone. PHA content of 48% and 35% were obtained as the fraction of valeric acid was increased to 40% and 100%, respectively. Steinbuchel and Pieper (1992) studied the production of PHB-PHV copolymer by *R. eutropha* strain R3 under nitrogen limitation. PHA contents were 47%, 35.7%, 29.5%, 21.5% and 43.2% when fructose, gluconate, acetate, succinate and lactate were used as a carbon source, respectively. PHV contents in the copolymer produced from this organism were in the range of 4-7% from all substrates used. When magnesium or sulphur was a limiting condition and fructose was used as a sole substrate, *R. eutropha* strain R3 could accumulate PHA of 45% or 47% with the PHV fraction of 7% or 6%, respectively. When gluconate was used, PHA content of 33% or 38% was obtained with PHV content of 4 or 5% under magnesium or sulphur limiting condition, respectively. PHV was produced by this organism in an absence of propionate or

other precusors for propionyl-CoA. The authors concluded that propionyl-CoA was derived from intermediates of the amino acids, valine and isoleucine. PHV units were then formed by the reaction of propionyl-CoA and acetyl-CoA as discussed above for the biosynthesis pathway of PHB-PHV copolymer by *R. eutropha*. Seeking a less costly substrate, Bourque *et al*. (1992) investigated the production of PHA by 118 methylotrophic microorganisms grown on a cheap substrate like methanol. *Methylobacterium extorquens* was found to accumulate a high PHA content when grown on the mixture of methanol and valerate. PHA content of 60-70% with 20%PHV was produced by this organism. Lee and Yu (1997) operated a two-stage bioprocess for PHA production. The first stage was an anaerobic digester. A mixture of volatile fatty acids produced by the first stage was used by *R. eutropha* for PHA production in a subsequent stage. *R. eutropha* was grown under aerobic and nitrogen-limiting conditions. PHA production of about 34% of cell mass was obtained by *R. eutropha* using digested sludge supernatant. The major component of the sludge PHA was C_4 monomers. The sludge PHA had a melting point of 167°C, 9°C lower than PHB homopolymer. *R. eutropha* consumed approximately 78% of the total organic carbon of the digested sludge supernatant. Acetic acid was the most effective fatty acid used by *R. eutropha* followed by propionic acid, butyric acid, and valeric acid. Biodegradation of the sludge PHA was also studied. Seventy percent of the mass of the sludge PHA was degraded in sludge suspension in 5 weeks and the biodegradability in soil of the sludge PHA was similar to that of PHB, i.e., 22-27% of the mass was degraded in 5 weeks. Bourque *et al*. (1995) investigated the production of PHB by *Methylobacterium extorquens* ATCC 55366 using methanol as a sole carbon and energy source in a fed-batch fermentation system. The production of PHB between 40% and 46% on a dry-weight basis was produced by *M. extorquens*. In addition, biomass production and the growth rate of *M. extorquens* were affected by the mineral composition supplied. The absence of $(NH_4)SO_4$ or $MnSO_4$ and the absence of a combination of $CaCl_2$, $FeSO_4$, $MnSO_4$ and $ZnSO_4$ had a negative impact on biomass production and the specific growth rate of this bacterial strain. High concentration of $(NH_4)SO_4$ were toxic to bacterial growth, while increases in concentrations of $MgSO_4$, $FeSO_4$, and a trace element mixture improved the bacterial growth rate. Stated another way, high-cell-density culture of *M. extorquens* was obtained when a proper combination of mineral medium was supplied. The low concentration of methanol prevented the limitation of oxygen in the culture medium and high molecular mass of PHB was obtained when methanol was fed at a low concentration, i.e., less

than 0.01 g/l. Highest PHB production from this study was obtained under nitrogen starvation conditions. Suzuki *et al*. (1986) reported the maximum PHB production of 66% of dry weight by *Pseudomonas sp. K* using methanol as a sole carbon and energy source. In order to obtain the high content of PHB, a proper medium composition was utilized. In this study, concentrations of phosphate and ammonium were maintained at low levels. Nitrogen deficiency was found to be the most effective way to stimulate the accumulation of PHB. The limitation of dissolved oxygen concentration was found to decrease the rate of biomass growth and PHB production. This finding was contradictory to the results reported by others. According to Lafferty *et al*. (1988), PHB production was stimulated under oxygen limitation by *R. eutropha* and *Azotobacter beijerinckii* (Schuster and Schlegel, 1967 and Nior *et al*., 1972). PHB with a melting point of 176 °C and the average molecular weight of $3.0*10^5$ was obtained from *Pseudomonas sp. K*. Daniel *et al*. (1992) reported the maximum PHB content of 55% by *Pseudomonas 135*. *Pseudomonas 135* was grown in an ammonium-limited fed batch culture using methanol as a sole carbon and energy source. When the cells were cultivated on Mg^{2+} and PO_4^{3-} limited media, the productions of PHB were 42.5% and 34.5%, respectively. PHB produced by this *Psuedomonas* strain had a melting point of 173 °C. The average molecular weights of $3.7*10^5$, $2.5*10^5$ and $3.1*10^5$ were measured in NH_4^+, Mg^{2+} and PO_4^{3-} limited media, respectively.

2) Bacterial strain

Byrom (1992) discussed the industrial production of PHA at ICI. *Ralstonia spp.* was an organism of choice for ICI because it produced an easily extracted PHA with high molecular weight. PHA productions from *Azobacter* and *methylotrophs* were also invetigated. However, PHA with low yield and molecular weight was produced from *methylotrophs* and PHA produced was difficult to extract. *Azobacter* was not an organism of interest because it used carbon substrate for polysaccharide synthesis rather than for PHA production. *R. eutropha* produced 70-80% polymer under phosphate limiting conditions. Copolymer was produced when a mixture of glucose and proprionate was used as the feeding substrate. Byrom (1990) stated that problem experienced using the wild-type of *R. eutropha* was that proprionate was used ineffectively, i.e., only about one third of proprionate was incorporated into the HV unit of the copolymer. The mutant strain, PS-1, was found to utilize proprionate more effectively. A proprionate fraction of 80% or greater was incorporated into the HV unit of the copolymer by the mutant strain. The

fraction of PHV of 0-30% was obtained when the ratio of the two substrates was varied. *Alcaligenes latus* can store PHA up to 80% under normal growth condition. Therefore, one-step PHA production process can be used with this organism (Hrabak, 1992). According to Doi (1990) 2-stage fed batch culture is the most widely used technique to maximize high concentrations of both cell and PHB. First stage or growth phase, optimum nutritional conditions are used to develop a high biomass concentration. Then, a selected nutrient is limited to stimulate PHA production in the second stage or accumulation phase. Yamane *et al.* (1996) studied the production of PHA by *A. latus* using sucrose as a feed substrate. High cell concentration (142 g/L) was obtained in a short culture time (18 hours) and PHB content at the end of the culture time was 50%. They concluded that the innoculum size reduces the culture time. They compared the culture time required for the production of PHB by *R. eutropha* fed with glucose when the same technique (pH-stat fed-batch) were used. The culture time required for *R. eutropha* was longer (30 hours) to obtain high cell concentration (122 g/L), however, higher PHB production (65%) was obtained. The average molecular weight of PHB obtained in this study was $3.16*10^5$. Page (1992) stated that *Azotobacter vineladii* was not considered for commercial production because it produces PHA with low yield and forms capsules. Strain UWD of this organism, however, is of interest because it is a capsule-negative mutant and produces PHA content of approximately 70-80%. Renner *et al*. (1996) studied the production of copolymer by 13 bacteria from the rRNA superfamily III. They reported that different bacteria were able to produce PHAs with different PHV/PHB compositions when growing on the same substrate. Anderson *et al*. (1990) studied the production of copolymer by the genera *Rhodococcus*, *Norcadia*, and *Corynebacterium*. They found that these genera had differences in accumulation and composition when grown on the same single carbon sources.

In summary, PHA content and its composition are influenced mainly by the strain of microorganism, the type of substrate employed and its concentration, and environmental growth conditions. The production of PHA by different microorganisms under different growth conditions is summarized in Table 1.

Table 1: PHA Production by Microorganisms

Organism	Substrate, conc.	Limiting condition	PHA (% w/w)	Composition of PHA PHB(%)	PHV(%)	Reference
Azobacter beijerinckii	G, 5g/L	O_2	35	NR	NR	Senior, 1972
	G, 20 g/L		75	NR	NR	
Azotobacter vinelandii UWD	V, 10mM + G, NR V, 20mM + G, NR	NR	94	72	18	Page and Manchak, 1995
	V, 30mM + G, NR		64	78	22	
	V, 10mM		74	75	25	
	V, 20mM		67	84	16	
	V, 30mM		36	79	21	
			29	72	28	
Rhodococcus sp. NCIMB 40126	A, 10 g/L	NH_4^+	29	31	69	Haywood *et al*., 1991
	L, 10 g/L		25	22	78	
	G, 10 g/L		21	25	75	
Methylobacterium extorquens	M, 1 g/L + V, 0.5g/L	None	60-70	80	20	Bourque *et al*., 1992
Methylobacterium extorquens	M, 1.4 g/L	NH_4^+	40-60	100	0	Bourque *et al*., 1995
Rhodococcus sp. ATCC 19070	A, 10 g/L	NH_4^+	4	27	73	Haywood *et al*., 1991
	L, 10 g/L		2	28	72	
	G, 10 g/L		14	9	91	
Rhodococcus ruber NCIMB 40126	G, NR	SO_4^{2-}	16.2	30.3	69.7	Anderson *et al*., 1992
	VA, NR		26.2	18	82	
	G+VA, NR		27.7	21.9	78.1	
Corynebacterium hydrocarboxydans ATCC 21767	A, 10 g/L	NH_4^+	21	50	50	Haywood *et al*., 1991
	L, 10 g/L		2	61	39	
	G, 10 g/L		8	28	72	
Alcaligenes latus	Sucrose, 20 g/L	None	50	100	0	Yamane *et al*., 1996
R. eutropha Strain R3	F, 0.5 g/L	NH_4^+	47	93	7	Steinbuchel and Pieper, 1992
	GL, 0.5 g/L	NH_4^+	35.7	94	6	
	A, 0.5 g/L	NH_4^+	29.5	96	4	
	S, 0.8 g/L	NH_4^+	21.5	93	7	
	L, 0.5 g/L	NH_4^+	43.2	96	4	
	F, NR	Mg^{2+}	45	93	7	
	F, NR	SO_4^{2-}	47	94	6	
	GL,NR	Mg^{2+}	33	95	5	
	GL,NR	SO_4^{2-}	38	96	4	
R. eutropha	Digested sludge supernatant	NH_4^+	34	NR (majority)	NR	Lee and Yu, 1997
R. eutropha H16	A, 22 g/L	NH_4^+	53	100	0	Doi *et al*., 1986
	A,22 g/L+P,10 g/L		51	81	19	
	P, 22 g/L		35	57	43	
R. eutropha H16 (ATCC 17699)	B, 0.03g/L	NH_4^+	44	100	0	Shimizu, *et al*., 1994
	B, 0.3 g/L		55	100	0	
	B, 3 g/L		75	100	0	
	B,10 g/L		63	100	0	
	B,1.8g/L+V,1.2g/L		48	NR	NR	
	V, 3 g/L		38	NR	NR	

Table 1: PHA Production by Microorganisms (Continued)

Organism	Substrate, conc.	Limiting condition	PHA (% w/w)	Composition of PHA PHB(%)	PHV(%)	Reference
R. eutropha H16	A, 20 g/L	NH_4^+	51	100	0	Doi *et al*., 1987
	A, 5 g/L		13	100	0	
	A, 20 g/L + P, 1 g/L		46	98	2	
	A, 20 g/L + P, 2 g/L		52	95	5	
	A, 20 g/L + P, 4 g/L		51	91	9	
	A, 5 g/L + P, 5 g/L		22	79	21	
	A, 5 g/L + P, 10 g/L		38	74	26	
	A, 5 g/L +P,20g/L		45	72	28	
	P, 2 g/L		12	78	22	
	P, 6 g/L		18	76	24	
	P, 10 g/L		28	72	28	
	P, 14 g/L		42	69	31	
	P, 18 g/L		56	73	27	
	P, 22 g/L		31	70	30	
	P, 26 g/L		40	56	44	
	P, 30 g/L		35	55	45	
R. eutropha NCIB 11599	G, 10 g/L+P, 1 g/L G,	NH_4^+	59	85	15	Haywood *et al*., 1989
	10 g/L+V, 1 g/L		60	78	22	
Alcaligenes feacalis NCIB 8156	A, 10 g/L+P, 1 g/L	NH_4^+	14	78	22	Haywood *et al*., 1989
	A, 10 g/L+V, 1 g/L		5	52	48	
Pseudomonas extorquens MP4	M, 10 g/L+P, 1 g/L	NH_4^+	26	95	5	Haywood *et al*., 1989
	M, 10g/L+V, 1 g/L		5	46	54	
Pseudomonas sp. K	M, 1% (v/v)	NH_4^+	52-57	100	0	*Suzuki et al*., 1986
		SO_4^{2-}	48-53	100	0	
		Mg^{2+}	45-50	100	0	
		Fe^{2+}	43-48	100	0	
		Mn^{2+}	50-55	100	0	
		Ca^{2+}	0-10	100	0	
		Zn^{2+}	0-10	100	0	
		Co^{2+}	0-10	100	0	
		Cu^{2+}	0-10	100	0	
		Mo^{6+}	0-10	100	0	
		Na^+	0-10	100	0	
Pseudomonas 135	M, 0.5% (v/v)	NH_4^+	37	100	0	Daniel *et al*., 1992
		Mg^{2+}	42.5	100	0	
		PO_4^{3-}	34.5	100	0	

A = acetate, B = butyric acid, F = fructose, G = glucose, GL = gluconate, M = methanol, L = lactate, P = propionate, S = succinate, V = valerate, VA = valine, NR = not reported

2.1.3 Physical Properties of PHA

PHB has some properties similar to polypropylene with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability (Hrabak, 1992). Booma *et al.* (1994) stated that PHB is an aliphatic homopolymer with a melting point of 179 °C and highly crystalline (80%). It can be degraded at the temperature above its melting point. According to de Koning (1995), Barham *et al*. (1984) reported that the molecular weight of PHB was decreased to approximately half of its original value when it was held at 190°C for 1 hour. PHAs can have physical properties that range from brittle and thermally unstable to soft and tough, depending upon their compositions, i.e., PHV/PHB ratios. The physical properties of PHB, e.g., crystallization, and tensile strength, depend on molecular weight, which is influenced by the strain of microorganism employed, growth conditions, and the purity of the sample obtained. Byrom (1990) summarized two major advantages of the PHB-PHV copolymer over the PHB homopolymer. Firstly, the copolymer has a lower melting point. However HV content in the copolymer does not significantly decrease temperature at which molecular weight degradation occurs. This important feature allows the copolymer to be processed with a larger range of temperature conditions. Secondly, it has a lower flexural modulus or level of crystallinity, which makes it tougher and more flexible. However, a disadvantage of having a low crystallization is that it takes a longer cycle time in the processing step, e.g., the injection-moulding process (Hrabak, 1992). Booma *et al*. (1994) recommended that the PHV content can be varied to obtain composition for the appropriate application. Materials with high PHV content tend to be soft and tough, while materials with low PHV content are hard and brittle. Fractions of PHV in the PHB-PHV copolymer ranging from zero to 30 % have been used by BIOPOL.

The molecular weight of PHA in the range of $2*10^5$ to $3*10^6$ has been reported in the literature (Lee, 1996). High molecular weight of PHB is more useful and desirable for industrial applications. Bourque et al. (1995) stated that the molecular weight of PHB can be reduced during the polymer processing step. In addition, Lafferty et al. (1988) stated that some reduction of PHB molecular weight could occur during the biomass extraction process. Shimizu et al. (1994) found that the average molecular weight of PHB decreased as the concentration of butyric

acid increased but pH had an insignificant effect on the average molecular weight. The highest average molecular weight of $3.3*10^6$ was obtained with a butyric acid concentration of 0.3 g/L, while the average molecular weight of $2*10^6$ was obtained at the optimum conditions for PHA production (butyric acid concentration of 3 g/L at pH 8). Molecular weights that ranged from $1.6*10^6$ to $3.3*10^6$ were reported from this study.

2.1.4 PHA Recovery Processes

In addition to the costs of maintaining pure cultures and the high costs of organic substrates, polymer recovery process is another factor that contributes to the high overall cost of PHA production. In the past 2 decades, several recovery processes have been investigated and studied in order find an economic way to isolate and purify PHA.

According to Doi (1990), Lee (1996), Steinbuchel (1996), and Braunegg (1998), several methods have been used as a recovery process for PHA. These methods include solvent extraction, sodium hypochlorite digestion, and enzymatic digestion. Details of each method as well as their advantages and disadvantages will be discussed and summarized here.

In most cases, bacterial biomass are separated from substrate medium by centrifugation, filtration or flocculation. Then, the biomass are freeze dried (lyophilized). Basically, mild polar compounds, e.g., acetone and alcohols, solubilize non-PHA cellular materials whereas PHA granules remain intact. Non-PHA cellular materials are nucleic acids, lipids, phospholipids, peptidoglycan and proteinaceous materials. On the other hands, chloroform and other chlorinated hydrocarbons solubilize all PHAs. Therefore, both types of solvents are usually applied during recovery process. Finally, evaporation or precipitation with acetone or alcohol can be used to separate the dissolved polymer from the solvent.

1) Solvent extraction

This method is used on a small scale for laboratory experiments as well as on a large scale for commercial production. This method is a widely used method because it is applicable to many PHA producing microorganisms. However, a large amount of solvent is employed because PHA solution is highly viscous. According to Lee (1996), approximately 20 parts of solvent is

employed to extract 1 part of polymer. This requirement makes solvent extraction a costly method. PHAs are soluble in solvents, such as chloroform, methylene chloride or 1,2-dicholoroethane. These 3 solvents can be used to extract PHA from bacterial biomass. In addition, other solvents were also reported to be used to extract PHA, e.g., ethylene carbonate, 1,2-propylene carbonate, mixtures of 1,1,2-trichloroethane with water, and mixtures of chloroform with methanol, ethanol, acetone or hexane.

Doi (1990) described a chloroform extraction method. PHA is extracted with a hot chloroform in a soxhlet apparatus for over 1 hour. Then, PHA extracted is separated from lipids by precipitating with diethyl ether, hexane, methanol, or ethanol. Finally, PHA is redissolved in chloroform and further purified by precipitation with hexane. Ramsay et al. (1994) examined the recovery of PHA from three different chlorinated solvents (chloroform, methylene chloride, and 1,2-dichloroethane). They obtained the best recovery and purity when biomass were pretreated with acetone. The optimum digestion time for all three solvents were 15 minutes. Further digestion resulted in a degradation in the weight molecular weight (MW) of PHA. The degree of recovery when the biomass were pretreated with acetone were 70, 24, and 66% when reflux for 15 minutes with chloroform, methylene chloride, and 1,2-dichloroethane, respectively. Whereas the level of purity of these 3 solvents under these optimum conditions were 96, 95, and 93%, respectively. Temperatures used of these three solvents were 61, 40,and 83°C, respectively. The authors emphasized that extraction conditions have a great impact on the degradation of PHA during the recovery process.

2) Sodium hypochorite digestion

Sodium hypochorite solubilizes non-PHA cellular materials and leaves PHA intact. Then, PHA can be separated from the solution by centrifugation. A severe degradation of polymers during sodium hypochorite digestion is frequently reported. Berger *et al*. (1989) observed 50% reduction in the MW of the polymers when the biomass were digested with sodium hypochorite (Lee, 1996). Because sodium hypochlorite is a strong oxidant, care has to be taken to select for suitable digestion conditions in order to maintain a high molecular weight of the polymers. Ramsay et al. (1990) examined the PHA recovery process from *R. eutropha* using hypochlorite digestion with surfactant pretreatment. Two different surfactants were investigated: Triton X-

100 and sodium dodecyl sulfate (SDS). Improvements in purity and molecular weight can be obtained by pretreating with surfactant prior to the extraction with sodium hypochlorite. They reported that surfactant removed approximately 85% of the total protein and additional protein (10%) was further removed by sodium hypochorite digestion. They also stated that this method resulted in a high MW of extracted PHA and the recovery time was reduced when compared to surfactant-enzymatic treatment or solvent extraction. In addition, a native PHA granule could be maintained during this treatment, which allow PHA to be used for more diverse applications in comparison to solvent extraction method. Table 2 summarizes the results obtained during their study.

Table 2: Results from the study by Ramsay *et al*. (1990)

Pre-treatment	MW	Purity (%)
None	1,200,000	50
Surfactant only		
- SDS (1%)	810,000	87
- Triton X-100 (1%)	800,000	87
Surfactant followed by hypochlorite		
- SDS (1%)	730,000	97
- Triton X-100 (1%)	790,000	98
Hypochlorite only (1-minute treatment)	680,000	87

Ramsay *et al*. (1990) stated that the roles of surfactant are well understood. Generally, surfactant disrupts the phospholipid bilayer of the cell membrane and separates PHA granules from other cell materials. In addition, surfactant denatures or solubilizes proteins, i.e., facilitating the cell disruption (anionic surfactants, SDS, denature protein, whereas non-ionic surfactants, Tritox-100, solubilize protein).

Chen *et al*. (1999) studied the recovery process of PHB from *R. eutropha* using a surfactant-chelate aqueous system. They reported that the amount of surfactant used, the chelate-to-dry biomass ratio, pH, temperature, and treatment time determined the degree of purity and the level of recovery. However, only pH and temperature had an effect on the MW of the extracted PHB. The optimum conditions reported from their study were a surfactant-to-dry mass ratio of 0.12, a chelate-to-dry biomass ratio of 0.08, a pH value of 13, a temperature at 50°C, and a 10-minutes treatment time. A purity and recovery rate of 98.7 and 93.3% were obtained under the optimum

conditions, respectively. The MW of the extracted PHB was 316,000 from an original MW of 402,000.

Holmes and Lim (1990) stated that one disadvantage of using direct solvent extraction is that the high viscosity of the PHA solution prevents removal of residual biomass (Hahn *et al*., 1994). Hahn *et al*. (1994), however, recommended the method called dispersion with hypochlorite and chloroform. They claimed that this method removes most of the non-PHA cellular materials during hypochlorite digestion, which facilitates the separation of PHA from the cells. In addition, digestion with hypochlorite reduced the viscosity of the chloroform phase. Specifically, they investigated optimum conditions for PHA recovery from *R. eutropha* using dispersions of sodium hypochlorite and chloroform. The optimum conditions from their experiments were reported to be 90 minutes digestion time with 30% hypochlorite concentration and a chloroform-to-aqueous phase ratio of 1:1 (v/v). They obtained a degree of recovery of 91% and a level of purity of higher than 97%. The MW reported to be 1,020,000 compared to an original value of 1,272,000. The melting temperature (MT) of the PHA, however, was not affected by the digestion methods. The MT of 176°C was obtained either from hypochlorite digestion or dispersion technique. They recommended that the MW of the extracted PHA could be varied to obtain a desired one for a specific application by varying the concentration of hypochlorite and digestion time during the dispersion. The roles of the solvents used were also summarized. Sodium hypochlorite caused the cells to disrupt, digested non-PHA cellular materials, reduced viscosity of the solvent solution, and acted as bleaching reagent. Chloroform was used to extract and purify PHA, and help protect PHA molecules from hypochlorite. The degradation of MW during the recovery process of PHA from *R. eutropha* using sodium hypochlorite was also observed in the study by Hahn *et al*. (1995). The degradation rate increased as the concentration of sodium hypochlorite increased. However, insignificant degradation of the MW was observed from the recombinant *E. coli*. When dispersion of sodium hypochlorite and chloroform was used, the change in MW and degree of purity were the same for these two microorganisms. When the biomass were treated with sodium hypochlorite, the degree of purity obtained from *R. eutropha* and recombinant *E. coli* were 86 and 93%, respectively. The degree of purity increased grater than 98% when both organisms were treated with the dispersion technique. Roh *et al*. (1995) reported that adding an anti-oxidant, e.g.,

sodium bisulfate, improved the MW of PHB extracted. In addition, the degree of purity could be increased by increasing pH to 12 or adding surfactant.

3) Enzymatic digestion

Due to the high cost of solvent extraction, the enzymatic digestion method was developed by ICI. Steps of this process include thermal treatment (100-150°C) to lyze cells and denature nucleic acids, enzymatic digestion, and washing with anionic surfactant to solubilize non-PHA cellular materials. Finally, concentrated PHA from centrifugation is bleached with hydrogen peroxide. According to Steinbuchel (1996), ICI used a mixture of various enzymes during the enzymatic digestion step. These enzymes are lysozyme, phospholipase, lecithinase, proteinase alcalase and others. These enzymes hydrolyze most of the non-PHA cellular materials but PHA remains intact. Braunegg *et a1*. (1998) reported that ICI used proteolytic enzymes, e.g., trypsin, pepsin, and papain, and mixtures of those enzymes.

Fidler and Dennis (1992) investigated a system to recover PHB granules from *E. coli* by expressing T7 bacteriophage lysozyme gene. The lysozyme penetrates and disrupts the cells, and causes PHB granules to be released. The authors stated that the previous work done by Kalousek *et al*. (1990) did not effectively control the bacteriophage protein to obtain release at the maximum point of PHB accumulation. The system developed by Fidler and Dennis (1992) used a separate plasmid and expressed it at a low level throughout the cell cycle. At the end of the accumulation phase, the cells were harvested and resuspended in the chelating agent, ethylenediaminetetraacetate. This activated the lysozyme to disrupt the cell structure and release PHA granules at the time that PHA accumulation reached the maximum. Triton X-100 was also added to assist the cell disruption. They reported the efficiency of lysis was greater than 99%.

More recently, Rosch *et al*. (1998) studied the release and purification of PHB from *E. coli* by expressing the cloned lysis gene E of bacteriophage PhiX174 from plasmid pSH2 (E-lysis method). This method has been used to open cells and to release gene products (Blasi *et al*., 1990). E-lysis causes cells to open and release PHB through the orifice of the transmembrane tunnel (40-200 mm. in diameter) produced during the process. The driving force to release PHB through the tunnel is the osmotic pressure different of the cytoplasm and the outside medium.

There were two major drawbacks of the E-lysis method: 1) lysis activity was maximum at the exponential growth phase rather than at the maximum PHA accumulation phase and 2) the efficiency of lysis to release PHB was only 50-70%. Therefore, a modified E-lysis was developed to overcome these 2 obstacles. A release of PHB up to 90% was obtained. During the modified E-lysis procedure, $MgSO_4$ was added before the temperature shift, which inhibited cell lysis and production of protein E before PHA accumulation reached the maximum. At the end of the accumulation phase, the cells were centrifuged and resuspended in water or low ionic strength buffer to release the PHB granules. The modified E-lysis also produced a larger hole of the tunnel developed than the normal E-lysis procedure. Then, PHB granule released was isolated by slow speed centrifugation. PHB granules were further purified by flotation buoyant density gradient centrifugation. In addition, an addition of glassmilk or $CaCl_2$ facilitated the aggregation of the PHB granules. They reported that 95% of PHB was precipitated within 10 minutes after $CaCl_2$ addition and 99% of PHB granules were precipitated within 2 minutes after the addition of glassmilk.

2.1.5 Potential Applications of Biodegradable Plastics Produced from PHA

According to Lafferty *et al.* (1988), the possible applications of bacterial PHA is directly connected with their properties such as biological degradability, thermoplastics characteristics, piezoelectric properties, and depolymerization of PHB to monomeric D(-)-3-hydroxybutyric acid. The applications of bacterial PHAs have concentrated on 3 principal area: medical and pharmaceutical, agricultural, and commodity packaging (Holmes, 1985; Huang *et. al*, 1990; Lafferty *et al*., 1988; Lee, 1996). According to Laferrty *et al*. (1988), the most advanced development of bacterial PHAs is in the medical field, especially pharmaceutical applications although they have a considerable potential as consumer goods products.

1) Medical and pharmaceutical applications

The degradation product of P(3HB), D(-)-3-hydroxybutyric acid, is a common intermediate metabolic compound in all higher organisms (Lafferty *et al*., 1988 and Lee, 1996). Therefore, it is plausible that it is biocompatible to animal tissues and P(3HB) can be implanted in animal tissues without any toxic. Some possible applications of bacterial PHAs in the medical and

pharmaceutical applications include: biodegradable carrier for long term dosage of drugs inside the body, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, and stimulation of bone growth and healing by piezoelectric properties. The advantage of using biodegradable plastics during implantation is that it will be biodegraded, i.e., the need for surgical removal is not necessary.

2) Agricultural applications

PHAs are biodegraded in soil. Therefore, the use of PHAs in agriculture is very promising. They can be used as biodegradable carrier for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation. Here again, it is not necessary to remove biodegradable items at the end of the harvesting season.

3) Biodegradable commodity packaging

According to Lafferty *et al*. (1988), PHB homopolymer and PHB-PHV copolymer have some properties, i.e., tensile strength and flexibility, similar to polyethylene and polystyrene. According to the authors, Holmes *et al*. (1981) reported that PHAs can be used in extrusion and moulding processes and blended with synthetic polymer, e.g., chlorinated polyethylene, to make heteropolymers. Also, small additions of PHA improves the property of some conventional polymers, e. g., addition of a small amount of PHA reduces the melt viscosity of acrylonitrile. Tsuchikura (1994) reported that “BIOPOL” with high PHV content is more suitable for extrusion blow moulding and extrusion processes, e.g., made into films, sheets, and fibres, while “BIOPOL” with low PHV content is more suitable for general injection moulding processes. According to Lafferty *et al*. (1988), one particular property of PHB films that make it possible to be used for food packaging is the relatively low oxygen diffusivity. Plastics produced from PHAs have been reported to be biodegraded both in aerobic and anaerobic environments (Page 1995). In summary, possible applications of PHAs for commodity goods include packaging films, bags and containers, disposal items such as razors, utensils, diapers, and feminine products.

2.1.6 Conclusions

The production of biodegradable polyesters on a large scale is limited because of the relative expensive of the substrate required, low polymer production per unit of substrate utilized, and the cost of maintaining axenic cultures. According to Yamane (1993), higher production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional petroleum-base plastics in the commercial marketplace. Also, Byrom (1987) stated that although the fermentation technology and polymer extraction process have been improved, PHA production can not compete with synthetic plastics for large-scale production because of the high cost of fermenting substrate. Lee (1996) reported that the price of BIOPOL is $16/Kg, while the price of synthetic plastics is a lot less expensive, e.g., less than $1/Kg for polypropylene.

A good candidate for PHA production would be a culture that can store high PHA concentrations while growing on an inexpensive growth substrate. Lee (1996) also suggested that the growth rate of a microorganism and its polymer synthesis rate are factors that should be considered when selecting a potential candidate of PHA production.

Polymer recovery process is another factor that contributes to the high cost of PHA production. According to Lee (1996), extraction with solvent is the most commonly used method for the recovery process. Due to the high viscosities of PHA solutions, a large amount of solvent is used, i.e., 20 parts of solvent to extract 1 part of polymers. According to Page (1992), an enzyme-mediated extraction process is used by ICI because it does not significantly damage the molecular mass of PHA extracted. However, it is a costly process.

Because of the high cost of substrate such as glucose and propionic acid, many microorganisms grown on cheap substrates have been investigated in order to reduce substrate expense. Page (1992) studied the production of PHA by *A. vinelandii* UWD using beet molasses and reported that the production cost using beet molasses was one-third of that of glucose.

As noted, Satoh *et al.* (1998) have demonstrated that activated sludge biomass receiving synthetic wastewater contained mainly acetic and propionic acids can be used for this purpose because they obtained PHA accumulations of 62%TSS during their study.

2.2 Carbohydrates

Carbohydrate reserves include glycogen and glycogen-like materials. A wide variety of organisms can accumulate glycogen and glycogen-like materials. Dawes and Senior (1973) and Preiss (1984) did extensive surveys on bacterial species capable of accumulating glycogen and glycogen-like reserves, and their results are summarized in Table 3.

2.2.1 Factors Affecting Carbohydrate Accumulation

Generally, glycogen and glycogen-like materials are accumulated in cells when nitrogen is limited while there is an excess of carbon available in the medium (Dawes, 1985). According to the review by Preiss (1984), in addition to nitrogen limiting conditions, glycogen can be accumulated under sulfur limiting-or phosphorus limiting conditions, or when pH for growth is unfavorable. However, nitrogen limiting condition was reported to be the most stimulating condition for glycogen accumulation in many organisms. Dawes (1985) mentioned adenylate energy charge of a cell as defined by Atkinson (1977). The value of the energy charge indicates the energetic status of the cell and the value in the range of 0.8 to 0.95 should be maintained for optimum cellular growth. Dawes and Senior (1973) suggested that a high adenylate energy charge promotes the synthesis of glycogen, while a low charge value inhibits glycogen synthesis.

$$\text{Adenylate Energy Charge} = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]} \qquad \text{(Dawes and Senior, 1973)}$$

2.2.2 Role of Carbohydrate Reserves

According to reviews by Dawes and Senior (1973) and Preiss (1984), organisms capable of carbohydrate reserves accumulation tend to prolong their viability during starvation periods by utilizing the storage reserve as sources of carbon and energy. In addition to PHA and poly-P, glycogen is believed by some authorities to be an essential part of the metabolism of PAOs in BNR systems (Satoh *et. al,* 1992). In the most recent biochemical model for BNR systems (Mino's model), reducing equivalents (NADH) produced during the degradation of glycogen are

used for PHA biosynthesis during the anaerobic period. It is further believed that the replenishment of glycogen occurs during the aerobic period as PHAs provide a carbon skeleton for its synthesis (Hood and Randall, in press). According to Smolders *et al*. (1994), energy obtained during glycogen degradation is also used for the uptake of organic substrate during the anaerobic period resulting in a decrease in the required energy for substrate uptake by the hydrolysis of poly-P.

Table 3: Microorganisms Capable of Glycogen and Glycogen-Like Reserve Accumulations

Aeromanas formicans	*Aeromanas hydrophila*
Aerobacter aerogenes	*Agrobacterium tumefaciens*
Aphanocapsa 6308	*Arthrobacater crystallopoietes*
Arthrobacter viscosus	*Bacillus cereus*
Bacillus megaterium	*Bacillus stearothermophilus*
Bacterioides fragilis	*Chromatium vinosum*
Chlorobium limicola	*Citrobacter freund*
Clostridium botulinum	*Clostridium pasteurianum*
Enterobacter aerogenes	*Enterobacter cloacae*
Klebsiella pneumoniae	*Escherichia aurescens*
Escherichia coli	*Micrococcus luteus*
Mycobacterium amegmatis	*Mycobacterium phlei*
Mycobacterium smegmatis	*Mycobacterium tuberculosis*
Pasteurella pseudotuberculosis	*Pseudomonas Pseudoflava*
Rhodocyclus purpureus	*Rhodomicrobium vanniellii*
Rhodopseudomonas acidophila	*Rhodopseudomonas capsulatus*
Rhodopseudomonas globiformis	*Rhodopseudomonas gelatinosa*
Rhodopseudomonas palustris	*Rhodopseudomonas sphaeroides*
Rhodopseudomonas viridis	*Rhodospirillum fulvum*
Rhodospirillum molischianum	*Rhodospirillum rubrum*
Rhodospirillum tenue	*Rhodospirillum photometricum*
Salmonella enteritidis	*Salmonella montevideo*
Salmonella typhimurium	*Sarcina lutea*
Serratia liquefaciens	*Serratia marcescens*
Shigella dysenteriae	*Streptococcus mitis*
Streptococcus mutans	*Streptococcus salivarius*
Streptococcus sanguis	*Synechococcus 6031*

2.3 Polyphosphates

Polyphosphates (poly-P) are linear polymers formed by the linkage of orthophosphates by energy-rich phosphoanhydride bonds (Kulaev and Vagabov, 1983). Bacteria poly-P have a chain

length that varies from 2 units (pyrophosphate) to 10^4 units (Dawes, 1985). The roles of polyphosphate include the role of serving as an energy source for organisms and the role of providing a source of phosphorus for some important metabolic processes, i.e., nucleic acid and phospholipid synthesis (Dawes, 1985). The first role of poly-P is, however, dependent on the type of microorganism. For example, *Hydrogemonas* and *Enterobacter aerogenes* do not appear to use poly-P as the energy source, while *Rhodospirillium rubrum*, *Entamoeba hystolytica*, *Propionibacteruim shermanii, Acetobacter xylinum*, and *Bacteriodes symbiosus* appear to possess enzymes for energy production using poly-P. According to Kulaev and Vagabov (1983) the extensive study of poly-P metabolism have been performed only in mycobacterium, corynebacteria, propionic-acid bacteria, streptomycetes, *Aerobacter aerogenes*, and *E. coli.* Enzymes involved in biosynthesis and degradation of polyphosphates have been extensively reviewed by Kulaev and Vagabov (1983) and are summarized below.

2.3.1 Enzymes Involved in Biosynthesis and Degradation of Polyphosphates

1) Polyphosphate:ADP phosphotransferase or polyphosphate kinase

This enzyme was first isolated from *E. coli* and subsequently found in other organisms, e.g., *Corynebacterium sp.* In addition, Dawes (1985) reported that many of aerobic, anaerobic, and facultative bacteria synthesize poly-P using this synthesis route. This enzyme catalyzes the following reaction:

$$ATP + (poly\text{-}P)_n \longleftrightarrow ADP + (poly\text{-}P)_{n+1}$$

2) Polyphosphate: adenosine monophosphate phosphotransferase

This enzyme was detected in mycobacteria and corynebacteria and it catalyzes the following reaction:

$$AMP + (poly\text{-}P)_n \longleftrightarrow ADP + (poly\text{-}P)_{n-1}$$

3) Polyphosphate (metaphosphate)-dependent NAD^+ kinase

Some of the eubacteria, *Acetobacter*, *Archromobacter*, *Brevibacterium*, *Corynebacterium*, and *Micrococcus* have been reported to possess this enzyme. This enzyme catalyzes the following reaction:

$$NAD+ + \text{poly-P (mataphosphate)}_n \longleftrightarrow NADP + \text{poly-P (metaphosphate)}_{n-1}$$

4) Polyphosphate: D-glucose 6-phosphate phosphotransferase

This enzyme was found in a number of mycobacteria, *Norcadia minima*, *Bdellovibrio bacteriovorus*, *Dictyostelium discoideum*, and other fungi and it catalyzes the following reaction:

$$\text{D-Glucose} + (\text{poly-P})_n \longleftrightarrow \text{D-Glucose 6-Phosphate} + (\text{poly-P})_{n-1}$$

5) 1,3-diphosphoglycerate: polyphosphate phosphotransferase

This enzyme was first detected in *N. crassa* and found also in other microorganisms, e.g., *Bdellovibrio bacteriovorus.* This enzyme was also found in fungi and eubacteria. The enzyme catalyzes the following reaction:

$$\text{1,3-Diphosphoglycerate} + (\text{poly-P})_n \longleftrightarrow \text{3-phosphoglycerate} + (\text{poly-P})_{n+1}$$

6) Polyphosphate polyphosphohydrolases or polyphosphate depolymerase

These enzymes cleave the chain of poly-P molecules into smaller fractions. The energy released from the cleavage may be utilized for transport the fragmented molecules through the membrane. Following is the reaction catalyzed by these enzymes:

$$(\text{poly-P})_n + \text{water} \longleftrightarrow (\text{poly-P})_{n-x} + (\text{poly-P})_x$$

7) Polyphosphate phosphohydrolases or polyphosphatases

These enzymes cleave the terminal phosphate from the poly-P molecule, i.e., hydrolyzing poly-P to orthophosphate. The cleavage of poly-P by these enzymes is similar to enzymatic degradation processes of proteins and polysaccharides. These enzymes are detected in many organisms, e.g., *Norcadia erythropolia*, *Brevibacterium sp*., *Corynebacterium sp*., *Streptomycetes levoris*, bacteria of the genera *Bacillus* and *Micrococcus*. Following is the reaction catalyzed by these enzymes:

$$(\text{poly-P})_n + \text{water} \longleftrightarrow (\text{poly-P})_{n-1} + P_i$$

For BNR systems, poly-P plays an essential part for the metabolism of PAOs. ATP produced by the degradation of poly-P is used for the uptake and storage of organic substrates during anaerobic conditions. As a result of poly-P degradation, orthophosphate is released into the

medium as observed during the typical anaerobic P release in BNR systems. The ability of the PAOs to utilize poly-P during anaerobic period gives them competitive advantage over other bacteria in activated sludge systems. van Groenestijn *et al*. (1987) investigated the production of ATP from poly-P in *Acinetobacter* strain 210A. They reported that ATP produced by this organism is due to a combined reaction of polyphosphate: AMP phosphotransferase and adenylate kinase. The activities of the enzymes polyphosphate glucokinase and polyphosphate dependent NAD kinase were not detected in this organism. Mino *et al*. (1985a, 1985b) studied the fraction of intracellular polyphosphates from BPR sludge using a modification of the Schmidt-Thannhauser-Schneider (STS) method. High and low molecular weight polyphosphates can be separately determined by the STS method. Low molecular weight poly-P was degraded during the anaerobic period, while it was synthesized during the aerobic period. They suggested that low molecular weight poly-P was used as an energy source during the anaerobic period, while high molecular weight poly-P serves as a source of phosphorus for cell growth. They also suggested that high molecular weight poly-P can provide phosphorus for other reactions, e.g., the formation of low molecular weight poly-P during aerobic periods. Varma and Peck (1983) studied functions of short- and long-chain poly-P in anaerobic bacteria. They obtained the samples from the sediment of a lake. Short-chain poly-P, e.g. tripolyphosphate (PPPi) and tetrapolyphosphate (PPPPi) promoted the growth of these anaerobic bacteria, i.e., there were increases in absorbances, protein contents and direct counts of the cultures. They suggested that the polyphosphates serve as an energy source for bacterial growth. However, there were differences in growth when the cultures were grown on PPPi and PPPPi. The authors suggested different enzymes are involved in the conversions of different short-chain polyphosphates to pyrophosphate.

2.4 Nitrogen Reserves

Cyanophycin and phycocyanin are two types of nitrogen reserves reported in cyanobacteria and they are utilized under nitrogen limiting conditions. Generally, microorganisms accumulate insignificant amount of nitrogen reserves. The accumulation of nitrogen reserves will not be discussed in great detail here.

3. References

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiological Reviews.* 54 (4), 450-472.

Anderson, A. J., Haywood, G. W., Williams, D. R., and Dawes, E.A. (1990). The Production of Polyhydroxyalkanoates from Unrelated Carbon Sources. In: *Novel Biodegradable Microbial Polymers*, E. A. Dawes (ed.), Kluwer Academic Publishers, The Netherlands, 119-129.

Anderson, A. J., Williams, D. R., Taidi, B., Dawes, E.A., and Ewing, D. F. (1992). Studies on Copolyester synthesis by *Rhodococcus ruber* and Factors Influencing the Molecular Mass of polyhydroxybutyrate accumulated by *Methylobacterium extorquens* and *Alcaligenes eutrophus*. *FEMS Microbiology Reviews.* 130, 93-102.

Booma, M., Selke, S. E., and Giacin, J. R. (1994). Degradable Plastics. *Journal of Elastomers and Plastics*. 26, 104-142.

Bourque, Ouellette, B., Andre, G., and Groleau, D. (1992). Production of Poly-β-hydroxybutyrate from Methanol: Characterization of a New Isolate of *Methylobacterium extorquens*. *Appl. Microbiol. Biotechnol*. 37, 7-12.

Bourque, D., Pomerleau, Y., and Groleau, D. (1995). High-Cell-Density Production of Poly-β-hydroxybutyrate (PHB) from Methanol by *Methylobacterium extorquens*: Production of High-Molecular-Mass PHB. *Appl. Microbiol. Biotechnol*. 44, 367-376.

Brandl, H., Bachofen, B., Mayer, J., and Wintermantel, E. (1995). Degradation and Applications of Polyhydroxyalkanoates. *Can. J. Microbiol*. 41(suppl. 1), 143-153.

Braunegg, G., Lefebvre, G., and Genser, K. F. (1998). Polyalkanoates, Biopolyester From Renewable Resources: Physiological and Engineering Aspects. *Journal of Biotechnology*. 65, 127-161.

Byrom, D. (1987). Polymer Synthesis by Microorganisms: Technology and Economics. *TIBTECH.* 5, 246-250.

Byrom, D. (1992). Production of Poly-β-hydroxybutyrate: Poly-β-hydroxyvalerate Copolymers. *FEMS Microbiology Reviews.* 103, 247-250.

Byrom, D. (1990). Industrial production of Copolymer from *Alcaligenes eutrophus*. In: *Novel*

Biodegradable Microbial Polymers, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 113-117.

Chen, Y., Chen, J., Yu, C., Du, G., and Lun, S. (1999). Recovery of poly-3-hydroxybutyrate from *Alcaligenes eutrophus* by surfactant-chelate aqueous system. *Process Biochemistry*. 34 (2), 153-157.

Daniel, M., Choi, J. H., Kim, J. H., and Lebeault, J. M. (1992). Effect of Nutrient Deficiency on Accumulation and Relative Molecular Weight of Poly-β-hydroxybutyric acid by Methylotrophic Bacterium, *Pseudomonas* 135. *Appl. Microbiol. Biotechnol*. 37,702-706.

Dawes, E. A. (1985). Starvation, Survival and Energy Reserves. In: *Bacteria in Their Natural Environment*, Fletcher, M. and Floodgate, G. D. (eds.), Academic Press, Florida, 43-79.

Dawes, E. A. (1990). Novel Microbial Polymers: An Introductory Overview. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 3-16.

Dawes, E. A. and Senior, P. J. (1973). The Role and Regulation of Energy Reserve Polymers in Micro-organisms. *Advances in Microbial Physiology*. 10, 135-266.

de Koning, G. (1995). Physical Properties of Bacterial Poly((R)-3-hydroxyalkanoates). *Can. J. Microbiol*. 41(suppl. 1), 303-309.

Doi, Y., Kunioka, M., Nakamura, Y., and Soga, K. (1986). Nuclear Magnetic Resonance Studies on Poly(β-hydroxybutyrate) and a Copolymer of β-hydroxybutyrate and β-hydroxyvalerate Isolated from *Alcaligenes eutrophus* H16. *Macromolecules*, 19, 2860-2864.

Doi, Y., Kunioka, M., Nakamura, Y., and Soga, K. (1987). Biosynthesis of Copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-Lebeled Acetate and Propionate. *Macromolecules*, 20, 2988-2991.

Doi, Y. (1990). *Microbial Polyesters*. VCH Publishers, Inc. New York, USA.

Doi, Y., Segawa, A., Nakamura, S., and Kunioka, M. (1990). Production of biodegradable Copolymers by *Alcaligenes eutrophus*. In: Novel Biodegradable Microbial Polymers, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 37-48.

Fidler, S. and Dennis, D. (1992). Polyhydroxyalkanoate Production in Recombinant *Escherichia coli*. *FEMS Microbiology Reviews.* 103, 231-236.

Hahn, S. K., Chang, Y. K., Kim, B. S., and Chang, H. N. (1994). Optimization of Microbial Poly(3-hydroxybutyrate) Recovery Using Dispersions of Sodium Hypochlorite Solution and Chloroform. *Biotechnology and Bioengineering*. 44, 250-262.

Haywood, G. W., Anderson, A. J., and Dawes, E. A. (1989). A Survey of the Accumulation of Novel Polyhydroxyalkanoates by Bacteria. *Biotechnology Letters*. 11(7), 471-476.

Haywood, G. W., Anderson, A. J., Williams, D. R., and Dawes, E. A. (1991). Accumulation of a Poly (hydroxyalkanoate) Copolymer Containing Primarily 3-Hydroxyvarate from Simple Carbohydrate substrates by *Rhodococcus sp*. NCIMB 40126. *International Journal of Biological Macromolecules*. 13, 83-88.

Holmes, P. A. (1985). Application of PHB- a Microbially Produced Biodegradable Thermoplastics. *Phys. Technol*. 16, 32-36.

Hood, C. R. and Randall, A. A. (in press). A Biochemical Hypothesis Explaining the Response of Enhanced Biological Phosphorus Removal Biomass to Organic Substrates, *Water Research*.

Hrabak, O. (1992). Industrial Production of Poly-β-hydroxybutyrate. *FEMS Microbiology Reviews.* 103, 251-256.

Huang, J., Shetty, A. S., and Wang, M. (1990). Biodegradable Plastics: A Review. *Advances in Polymer Technology*, 10(1), 23-30.

Kulaev, I. S. and Vagabov, V. M. (1983). Polyphosphate Metabolism in Microorganisms. *Adv. Microbial Physiol*. 24, 83-171.

Lafferty, R. M., Korsatko, B., and Korsatko, W. (1988). Microbial Production of Poly-β-hydroxybutyric Acid. In: *Biotechnology*, Rehm, H. J. and Reed, G. (eds.), VCH Publishers, New York, 135-176.

Lee, S. Y. (1996). Review Bacterial Polyhydroxyalkanoates. *Biotechnol. and Bioeng*. 49, 1-14.

Lee, S. and Yu, J. (1997). Production of Biodegradable Thermoplastics from Municipal Sludge by a Two-Stage Bioprocess. *Resources, Conservation and Recycling*. 19, 151-164.

Madigan, M. T., Martinko, J. M. and Parker, J., 1997. *Brock Biology of Microorganisms.* Princeton Hall, New Jersey.

Mino, T., Kawakami, T., and Matsuo, T. (1985a). Location of Phosphorus in Activated Sludge and Fraction of Intracellular Polyphosphate in Biological Phosphorus Removal Process. *Wat. Sci. Technol*. 17(2/3), 93-106.

Mino, T., Kawakami, T., and Matsuo, T. (1985b). Behaviour of Intracellular Polyphosphate in Biological Phosphorus Removal Process. *Wat. Sci. Technol*. 17(11/12), 11-21.

Page, W. J. (1992). Production of Polyhydroxyalkanoates by *Azotobacter vinelandii* Strain UWD in Beet Molasses Culture, *FEMS Microbiology Reviews.* 103, 149-158.

Page, W. J. (1995). Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics With a Great Future. *Can. J. Microbiol*. 41(suppl. 1), 1-3.

Page, W. J., and Manchak, J. (1995). The Role of β-oxidation of Short-Chain Alkanoates in Polyhydroxyalkanoate Copolymer Synthesis in *Azotobacter vinelandii* UWD. *Can. J. Microbiol*. 41 (suppl. 1), 106-115.

Preiss, J. (1984). Bacterial Glycogen Synthesis and Its regulation. *Ann. Rev. Microbiol*. 38, 419-458.

Ramsay, J. A., Berger, E., Voyer, C., Chavarie, C., and Ramsay, B. A. (1990). Extraction of Poly-3-hydroxybutyrate Using Chlorinated Solvents. *Biotechnol. Techniq*. 8(8), 589-594.

Ramsay, J. A., Berger, E., Ramsay, B. A., and Chavarie, C. (1994). Recovery of Poly-3-hydroxyalkanoic Acid Granules by a Surfactant-Hypochlorite Treatment. *Biotechnol. Techniq*. 9(10), 709-712.

Renner, G., Haage, G., and Braunegg, G. (1996). Production of Short-Side-Chain Polyhydroxyalkanoates by Various Bacteria from the rRNA Superfamily III. *Appl. Microbiol. Biotechnol*. 46, 268-272.

Resch, S., Gruber, K., Wanner, G., Slater, S., Dennnis, D., and Lubitz, W. (1998). Aqueous Release and Purification of Poly(β-Hydroxybutyrate) from *Escherichia coli. Journal of Biotechnology*. 65, 173-182.

Roh, K. S., Yeom, S. H., and Yoo, Y. J. (1995). The Effects of Sodium Bisulfate in Extraction of PHB by Hypochlorite. *Biotechnology Techniques*. 4(4), 221-226.

Sasikala, CH. and Ramana, CH. V. (1996). Biodegradable Polyesters. In: *Advances in Applied Microbiology, Volume 42*, Neidleman, S. L. and Laskin, A. I. (eds.), Academic Press, California, 97-218.

Satoh, H., Mino, T., and Matsuo, T. (1992). Uptake of Organic Substrates and Accumulation of Polyhydroxyalkanoates Liked with Glycolysis of Intracellular Carbohydrates under Anaerobic Conditions in the Biological Excess Phosphorus Removal Processes. *Wat. Sci. Tech*. 26, 5-6, 933-942.

Satoh, H., Iwamoto, Y., Mino, T., and Matsuo, T. (1998). Activated Sludge as a Possible Source of Biodegradable Plastic. Proceedings, Water Quality International 1998, Book 3 Wastewater Treatment, pp. 304-311. IAWQ 19th Biennial International Conference, Vancouver, BC, Canada, 21-26 June 1998.

Senior, P. J., Beech, G. A., Ritchie, G. A. F., and Dawes, E. A. (1972). The Role of Oxygen Limitation in the Formation of Poly-β-Hydroxybutyrate During batch and Continuous Culture of *Azotobacter beijerinckii*. *The Biochemistry Journal.* 128, 1193-1201.

Shimizu, H., Tamura, S., Ishihara, Y., Shioya, S., and Suga, K. (1994). Control of Molecular Weight Distribution and Mole Fraction in Poly(-D(-)-3-hydroxyalkanoates) (PHA) Production by *Alcaligenes eutrophus.* In: *Biodegradable Plastics and Polymers*, Doi, Y. and Fukuda, K. (eds.), Elsevier Science B.V., New York, 365-372.

Smolders, G. J. F., van der Meij, J, and van Loosdrecht, M. C. M. (1994). Model of the Anaerobic Metabolism of the Biological Phosphorus Removal Process: Stoichiometry and pH Influence. *Biotechnology and Bioengineering*. 43, 461-470.

Steinbuchel, A. and Pieper, U. (1992). Production of a Copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric Acid from Single Unrelated Carbon Sources by a Mutant of *Alcaligenes eutrophus*. *Appl. Microbiol. Biotechnol*. 37, 1-6.

Steinbuchel, A. (1996). PHB and Other Polyhydroxyalkanoic Acids. In: *Biotechnology*, Rehm, H. J. and Reed, G. (eds.), VCH, New York, 403-464.

Suzuki, T., Yamane, T., and Shimizu, S. (1986). Mass Production of Poly-β-hydroxybutyric acid by Fully Automatic Fed-Batch Culture of Methylotroph. *Appl. Microbiol. Biotechnol.* 23, 322-329.

Tsuchikura, K. (1994). 'BIOPOL' Properties and Processing. In: *Biodegradable Plastics and Polymers*, Doi, Y. and Fukuda, K. (eds.), Elsevier Science B.V., New York, 362-364.

van Groenestijn, J. W., Deinema, M. H., and Zehnder, A. J. B. (1987). ATP Production from Polyphosphate in *Acinetobacter* Strain 210A. *Arch Microbiol*. 148, 14-19.

Varma, A. K. and Peck, H. D. (1983). Utilization of Short and Long-Chain Polyphosphate as Energy Sources for the Anaerobic Growth of Bacteria. *FEMS Microbiol. Lett*. 16, 281-285.

Yamane, T. (1993). Yield of Poly-D(-)-3Hydroxybutyrate from Various Carbon Sources: A Theoretical Study. *Biotechnology and Bioengineering*. 41(1), 165-170.

Yamane, T., Fukunaga, M., and Lee, Y. W. (1996). Increase PHB productivity by High-Cell-Density Fed-batch Culture of *Alcaligenes latus*, a Growth-Associated PHB Producer. *Biotechnol. and Bioeng*. 50, 197-202.

CHAPTER 2
PRODUCTION OF POLYHYDROXYALKANOATES FOR BIODEGRADABLE PLASTICS USING ACTIVATED SLUDGE BIOMASS: SYSTEM DEVELOPMENT

Warangkana Punrattanasin[1], Clifford W. Randall[1], and Andrew A. Randall[2]

[1]*Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.*
[2]*Department of Civil and Environmental Engineering, University of Central Florida, Orlando, FL 32816-2450, U.S.A.*

Part of the manuscript was submitted to 1st World Congress of the International Association of Water Quality, Paris, 3-7 July, 2000

ABSTRACT

Sequencing batch reactor (SBR) systems were used for the development of a system and operating procedures for the high production and storage of polyhydroxyalkanoates (PHAs) by wastewater treatment (activated sludge) bacterial cultures. The systems were maintained at a nominal hydraulic retention time (HRT) of 10 hours, and a temperature of 20°C. The solids retention time (SRT) was maintained at 10 days during the first experiment, but a two-stage bioprocess approach that had a growth phase and a separate PHA accumulation phase was used for subsequent experiments. After the initial experiment, the system was fed a 660 mg/L mixture of acetate and propionate as the sole sources of organic carbon. Whereas the initial experiment with microaerophilic (MAA)/aerobic (AE) cycling was unsuccessful, the two-stage bioprocess approach proved to be a successful strategy for PHA production by activated sludge biomass. Unbalanced growth conditions stimulated massive production of PHA. Operating conditions had a significant effect on PHA production and the composition of the accumulated copolymer. Fully AE conditions with N&P limitations were the optimum conditions for PHA production using activated sludge biomass. Maximum cellular PHA accumulation of 70%TSS was obtained under fully AE conditions with multiple periods of simultaneous nitrogen (N) and phosphorus (P) limitations. Surprisingly, the relative amounts of the PHA copolymers formed, i.e., polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) were different under different operating conditions, even though the types and amounts of volatile fatty acids (VFAs) in the feed were the same. The results indicate that activated sludge biomass has considerable potential

for PHA production for commercial purposes, and likely could do so utilizing wastewater sources of organics.

KEYWORDS

Biodegradable plastics production; activated sludge; microaerophilic; anaerobic-aerobic; fully aerobic; nutrient limitation; biopolymer storage; polyhydroxyalkanoates; polyhydroxybutyrates; polyhydroxyvalerates

INTRODUCTION

It is well known that production and storage of PHAs are integral and essential parts of the excess biological phosphorus removal (EBPR) mechanisms. It is less well known by the wastewater treatment community that PHA production by bacteria also is used for the manufacture of biodegradable plastics. Primary differences in the two approaches are: EBPR is accomplished using mixed cultures of bacteria, i.e., activated sludge, with the intent of minimizing the amount of phosphorus remaining in solution at the end of the aerobic phase. Plastics production is typically accomplished using pure cultures with the goal of maximizing the amount of stored PHAs at the end of a nutrient limited or oxygen limited phase. Thus, for EBPR the microbial cells are harvested when phosphorus storage is at a maximum, and for plastics production the cells are harvested when PHA storage is at a maximum. Simplistically, the two processes exploit different parts of the same basic biochemical cycle, and this raises the possibility that perhaps wastewaters can be used to produce biodegradable plastics, i.e., a marketable by-product. Satoh, et al. (1998) demonstrated the basic feasibility by showing that microaerophilic conditions could be used to increase the percent PHA in activated sludge to as much as 62%TSS, using sodium acetate as the primary source of VFAs in the feed.

Extensive reviews of bacterial PHAs have been performed by Lafferty *et al*. (1988), Anderson and Dawes (1990), Doi (1990), Sasikala and Ramana (1996), Lee (1996), Steinbuchel (1996), and Braunegg *et al*. (1998). PHAs are an intracellular storage material synthesized by a wide variety of bacteria. They are composed mainly of poly-beta-hydroxybutyric acid (PHB) and poly-beta-hydroxyvaleric acid (PHV), although other forms are possible. Lafferty et al., (1988) stated that the accumulation of PHA can be stimulated under unbalanced growth conditions, i.e.,

when nutrients such as N, P or sulfate become limiting, when oxygen concentration is low, or when the carbon:nitrogen (C:N) ratio of the feed substrate is high. According to Doi (1990), when growth conditions are unbalanced, acetyl-CoA cannot enter the tricarboxylic acid (TCA) cycle to obtain energy for cells due to high concentrations of NADH. The high concentrations of NADH inhibit the enzyme citrate synthase leading to an increase in the level of acetyl-CoA. Acetyl-CoA is then used for PHA biosynthesis by a sequence of enzymatic reactions. PHA can serve as a carbon or energy source for microorganisms during starvation periods. In EBPR systems, PHA is formed and stored during the anaerobic period by phosphorus accumulating organisms (PAOs) when no electron acceptors are available for growth mechanisms. This permits them to sequester most of the substrate and out-compete organisms that do not have phosphorus and PHA storage capabilities.

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) is a bacterial strain that has been the subject of much of the biodegradable plastics published research because it can accumulate PHAs up to 80% dry weight (Lee, 1995). Imperial Chemical Industries (ICI), now Monsanto, produces biodegradable plastics from PHA using *R. eutropha*, and markets them as "BIOPOL" in Europe. Plastics produced from PHAs have been reported to be truly biodegradable (Page, 1995), unlike many of the "so-called" biodegradable plastics made synthetically. They are biodegraded in both aerobic and anaerobic environments. However, PHAs can have physical properties that range from brittle and thermally unstable to soft and tough, depending upon their PHV/PHB ratios. Byrom (1990) summarized the two major advantages of the copolymer (PHB-PHV) over the homopolymer (PHB). Firstly, the copolymer has a lower melting point, which reduces thermal degradation. Secondly, it has a lower flexural modulus, which makes it tougher and more flexible. It is well known that copolymer composition, i.e., %PHB and %PHV, is primarily influenced by the substrate used (Doi, 1990). Doi et al. (1987) studied the copolymer composition produced by *R. eutropha* H16 grown in a medium containing sodium acetate and sodium propionate. The PHV content of the copolymer increased as the propionate concentration in the medium increased.

Hood and Randall (in press) proposed two hypotheses to explain the observed variation in PHA production and EBPR when different types and mixtures of substrates are present. These

hypotheses were based on knowledge of biochemical pathways and a balance of reducing equivalents. The hypotheses were that either PHB resulted in greater P uptake than PHV, or that VFAs that contribute towards consumption of reducing equivalents during biotransformation to PHAs result in greater PHA quantity. Either appeared to explain differences in EBPR performance with different VFAs. For example, they predict much better performance with acetic acid than with propionic acid, which is contrary to results reported by Manoharan (1988), but consistent with results reported by Abu-ghararah and Randall (1989) and others. Subsequent work by Liu *et al*. (2000) showed that PHB resulted in greater P uptake than PHV, explaining differences between VFAs in EBPR. Unpublished results also show strong PHA formation with combinations of acetic and propionic acids.

According to Yamane (1993), higher production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional, petroleum-base plastics in the commercial marketplace. A good candidate for PHA production would be a culture that can store high PHA concentrations while growing on an inexpensive growth substrate. As noted, Satoh et al. (1998) have demonstrated that activated sludge biomass can be used for this purpose because they obtained PHA accumulations of 62%TSS during their study. There are industrial wastewaters such as those from cellulose acetate manufacturing that average as much as 1200 mg/L acetic acid, and would seem to be good substrates for plastics production. Also, fermentation can be used to convert much of municipal sewage to VFAs, and potentially render sewage as an inexpensive substrate for plastics production.

Cultivation strategies to achieve high biomass PHA concentration and high productivity in pure cultures are well defined. However, the knowledge is rather limited for PHA production using activated sludge bacterial cultures. The objectives of this study were to develop both an efficient system and suitable operating procedures that would maximize PHA production in an activated sludge system using acetate and propionate as the primary organic substrates. A goal was to define optimum operating conditions for maximizing the %PHA in the activated sludge biomass, and to characterize the composition of the PHAs, i.e., the PHV/PHB ratio, of the copolymers stored in the cells.

MATERIALS AND METHODS

Experimental design

Three variable parameters were selected for study, i.e., oxygen, N, and P. Three types of oxygen operating conditions were investigated, i.e., the absence of oxygen (anaerobic, AN), oxygen limitation (MAA), and without oxygen limitation (fully aerobic, AE). Two sets of conditions were selected for the N and P experiments, i.e., limitation of either N or P, or limitation of both N and P. Initially a full factorial design of 12 experiments was planned, but the preliminary results showed that oxygen limitation (both MAA/AE cycling and AN/AE cycling) did not promote massive production of PHA when compared with fully aerobic experiments. Therefore, a partial factorial design was used to reduce the number of experiments by focusing on investigating the effects of nutrient limitation under fully aerobic conditions.

Regarding the PHA harvesting and the economics of PHA production, bacterial biomass should be maintained at a high concentration during the peak production of PHA in order to maximize its productivity. However, there were large decreases in the total biomass during the nutrient limiting conditions for both AN/AE and MAA/AE experiments. It is not surprising to observe the rate of decline of the biomass during the nutrient limitation phase because N&P are essential nutrients required for cellular growth by all living organisms. According to Preusting *et al*. (1993), PHA productivity can be further increased by increasing biomass concentration and or PHA content of the biomass. They were successful in increasing the PHA productivity of *Psedumonas oleovorans* by increasing the cell concentration, which was accomplished by increasing the limiting nutrient (ammonium) in the feed solution from 16.7 to 116.6 mM. The cell density of *P. oleovorans* was increased from 2-3 g/L to 11.6 g/L and the PHA productivity was increased from 0.17 to 0.58 gPHA/L/hour. Therefore, partial limitations of nutrients were also investigated (Experiments 4 and 10) to determine if the partial addition of nutrients will help maintain the high concentration of the biomass as well as stimulate the massive production of PHA. The complete set of experimental conditions investigated in this study are listed below:

- Experiment 1: Effects of MAA/AE cycling without nutrient limitation
- Experiment 2: Effects of MAA/AE cycling with combined N and P limitations
- Experiment 3: Effects of AN/AE cycling with combined N and P limitations

- Experiment 4: Effects of MAA/AE cycling with partial N limitation
- Experiment 5: Effects of MAA/AE cycling with P limitation
- Experiment 6: Effects of AN/AE cycling with P limitation
- Experiment 7: Effects of fully aerobic (AE) conditions with P limitation
- Experiment 8: Effects of fully AE conditions with N&P limitations
- Experiment 9: Effects of fully AE conditions with N limitation
- Experiment 10: Effects of fully AE conditions with partial N&P limitations

Reactor set up and operations

A 6-hour cycle SBR system with a working volume of 4 liters (L) was used for all experiments, and was maintained at a constant temperature of 20°C. The cycle times were electronically controlled using timers, with an overall HRT of 10 hours. The cycle periods were: a 30 minute (m) nitrogen purging and influent supply period; a 1 hour (hr) oxygen limited period; a 3 hr aeration period; and a 1.5 hr settling period with effluent discharge during the last 15 m. When the reactor was operated under fully aerobic conditions, the aeration period of the reactor cycle was 4 hr. The volume in the reactor after discharge was 1.6 L, resulting in a discharge volume of 2.4 L per cycle. The SRT was maintained at 10 days during the first experiment. In subsequent experiments, a two-stage bioprocess approach was used, similar to what is used for commercial PHA production (Doi, 1990). That is, optimum nutritional conditions were used to develop a high biomass concentration (growth phase) using MAA/AE, AN/AE, or fully AE cycling. During the growth phase, the SRT was maintained at 10 days to ensure that the biomass in each experiment before starting the next cultivation phase was similar in terms of biomass concentration and microbial population, i.e., the biomass were allowed to adjust and grow on the same feed components for about 2-3 SRTs before the PHA accumulation phase. Then, one or more nutrients were limited to stimulate PHA production (accumulation phase). No biomass was wasted from the SBR reactor during the PHA accumulation phase to maximize biomass concentration in the reactor, except that wasted with the supernatant drawoff. In summary, during this cultivation approach the two phases were operated in the same SBR reactor. The operating conditions of the experiment are summarized in Table 1. The initial synthetic wastewater composition (Experiment 1) was the same as used by Satoh *et al*. (1998), i.e., the influent feed contained near equal amounts of acetic (204 mg/LCOD) and propionic (226

mg/LCOD) acids, plus bactopeptone (173 mg/LCOD) and yeast extract (64 mg/L). In subsequent experiments, the feed components were modified, i.e., the system was fed with a 660 mg/LCOD mixture of the equal amounts of acetate and propionate as the sole sources of organic carbon. However, the influent COD concentrations, which entered the SBR systems were below the expected value of 660 mg/LCOD. The average of the influent COD concentration, as well as standard deviation and coefficient of variation, of each experiment is summarized in Table 2. The reduction in the influent COD observed could result from biological activity in feeding tanks. Nutrient and micronutrient concentrations used in both Experiment 1 and subsequent experiments are listed in Table 3. When the systems were not operated under nutrient limitations, the concentrations of $(NH_4)_2SO_4$ and KH_2PO_4 before dilution were approximately 33 mg/L NH_4-N and 17 mg/L PO_4-P, respectively. Activated sludge from the Blacksburg-VPI Sanitation Authority nitrification-denitrification wastewater treatment plant or from the fully aerobic Peppers Ferry Regional wastewater treatment plant (Radford, VA) was used to innoculate the system.

Table 1: Operating Conditions of the SBR reactors

Effective Volume	4 L
HRT	12 hours
SRT	10 days
Temperature	20°C
Influent COD	~660 mg/L
Air Supply Rate During MAA Conditions	0-10 ml/min

Table2: Influent COD Concentrations of the Experiments

	Average Influent COD (mg/L)	Standard Deviation	Coefficient of Variation
Experiment 2	585	15.5	2.7
Experiment 3	571	17	3
Experiment 4	613	10.4	1.7
Experiment 5	606	9.3	1.5
Experiment 6	604	9.9	1.6
Experiment 7	599	15.8	2.6
Experiment 8	600	13	2.2
Experiment 9	601	20	3.3
Experiment 10	597	18.8	3.2

Note: the coefficient of variation is defined as: (100*standard deviation of the sample set)/ mean value.

Table 3: Nutrients and Micronutrients Concentrations

Nutrients	Conc. (mg/L) Before dilution	Conc. (mg/L) after dilution	Micronutrients	Conc. (μg/L) Before dilution	Conc. (μg/L) after dilution
$CaCl_2$	28.3	17	$FeSO_4.7H_2O$	183	110
$MgSO_4$	148	89	H_3BO_3	18.3	11
KCl	117	70	$CuCl_2.2H_2O$	3.7	2.2
			KI	22	13.2
			$MnSO_4.H_2O$	14.7	8.8
			$NaMoO_4.2H_2O$	7.3	4.4
			$ZnCl_2$	14.7	8.8
			$CoCl_2.6H_2O$	18.3	11

Monitoring and analytical methods

Each experiment was monitored for mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solid (MLVSS), PHA, PHB, PHV, chemical oxygen demand (COD), anions (nitrite, nitrate, phosphate, and sulfate), and cations (ammonia-N, potassium, magnesium, and calcium). MLSS, MLVSS, and COD were analyzed according to Standard Methods for the Examination of Water and Wastewater, 19th Edition, 1995, using sections 2540D, 2540E, and 5220C, respectively. Anions were analyzed by a Dionex 2010I ion chromatography (IC) with an IONPAC AS4A-SC column and electrochemical conductivity detector (Dionex Corp., Sunnyvale, CA). Cations were analyzed by Dionex 120 ion chromatography (Dionex Corp., Sunnyvale, CA). PHA, PHB, and PHV were measured by methanolysis-GC method described by Hart (1994), with some modifications. Firstly, the mixed liquor suspended solid (MLSS) was centrifuged at 10,000 rpm for at least 10 minutes. The volume of MLSS needed should yield at least 50 mg for solids. Solids were dried for 24 hours at 100°C. Solids were weighed as they were placed into a 5 mL high pressure Wheaton "V-vial". At least 5 PHA external standards were used to make a PHA standard curve for each analysis run. Benzoic acid was used as an internal standard. Benzoic acid solution was prepared by mixing 50 mg of benzoic acid into 100 mL of 3% sulfuric acid in methanol solution (v/v). 2 mL of the benzoic acid solution was added into each vial. Then, 2 mL of chloroform was added. The vials were then sealed by tightening the cap and incubated in an oven at 100 °C for 3.5 hours. The vials were allowed to cool and 1 mL of distilled water was added into each vial. The vials were shaken for 10 minutes to allow the organic and inorganic layers to mix properly. Upon the completion of digestion, 1 μL of the chloroform phase (bottom layer) was injected into a GC with a 3 meter 2% Reoplex 400 Chromosorb GAW column attached to an FID detector. The oven temperature was 130 degrees

C, while injector and detector temperatures were 160 and 200 degrees C, respectively. The air flow rate was 428 mL/min, while hydrogen and nitrogen gas flow rates were 30 and 29 mL/min, respectively. Chloroform appeared as a peak during the first minute of run time. PHB then appeared as a peak at about 3.5 minutes, PHV appeared at about 5 minutes, and benzoic acid appeared at 6.5 minutes.

A minimum of 2 analyses were made for each sample to represent each data point of PHA measured. Error bars representing analytical variability based on plus and minus one standard deviation of the sample mean were performed to represent the degree of variability of the data as shown in all figures in the results and discussion section.

RESULTS AND DISCUSSION

Experiment 1: Effects of MAA/AE cycling without nutrient limitation

Experiment 1 failed to achieve the PHA accumulation success reported by Satoh *et al*. (1998). After approximately 50 days of operation, activated sludge PHA content under MAA/AE cycling conditions was less than 10%TSS. Also, the biomass in this reactor gradually decreased from 2000 to less than 900 mg/L due to poor sludge settleability, which resulted in excessive biomass loss during supernatant discharge. Filamentous bacteria were observed to be dominant when sludge was examined microscopically. Figure 1 shows the effects of MAA/AE conditions on PHA storage and biomass production, i.e., a steady decline of both. The results were inconsistent with the study by Satoh *et al*. (1998) wherein they reported PHA storage of 62%TSS using a MAA/AE reactor without nutrient limitation. Next, sludge was taken from the MAA/AE reactor for batch experiments under MAA conditions with air supplied at two different rates (1 and 3 ml/min). PHA production in the 46 hr periods of the batch experiments never reached 10%TSS, as shown in the two graphs of Figure 2. This could have resulted from the inability to adequately maintain the optimum air flow rate during the MAA phase. Satoh *et al*. (1998) stated that less PHA production was observed when the air flow rates were greater or lower than the optimum one (1.2 ml/min). The continuous one-stage methodology was discontinued after this experiment, and the 2-stage bioprocess approach was applied for the rest of the experiments.

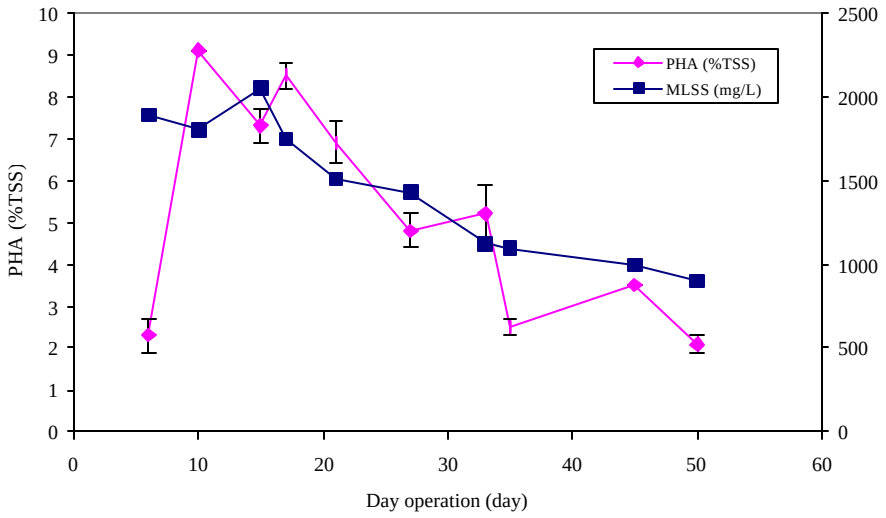


Figure 1: The Effect of MAA/AE Cycling on Biomass and PHA Accumulation Changes

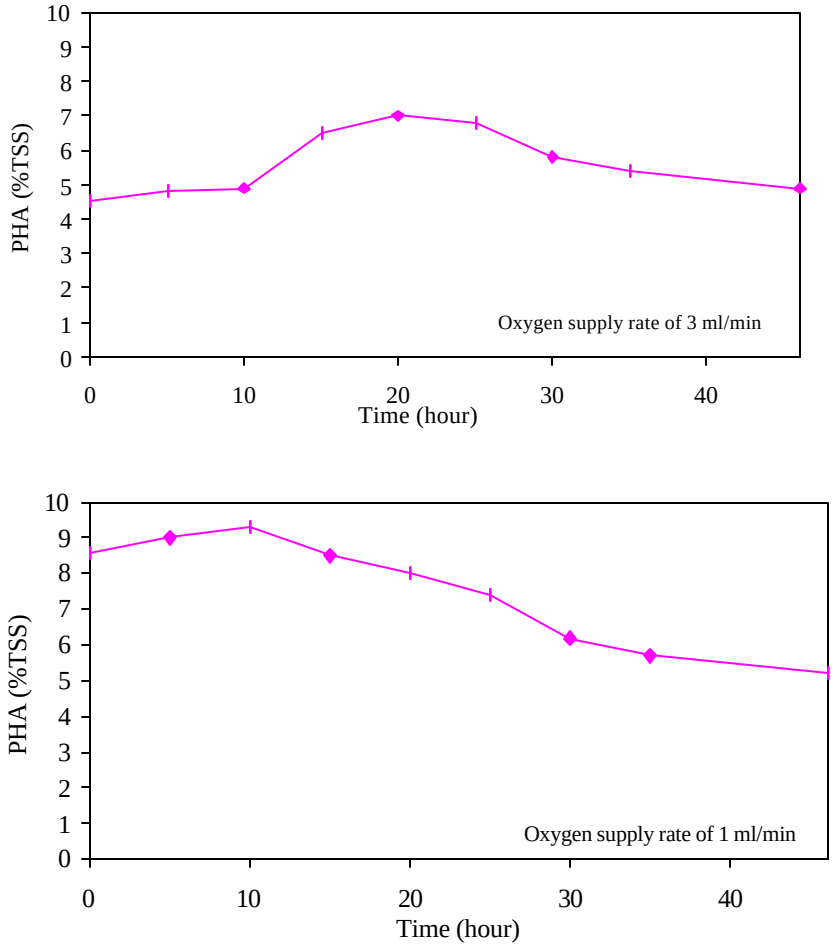


Figure 2: The Effects of Aeration Rate on PHA Accumulation Changes During MAA/AE Cycle

Experiment 2: Effects of MAA/AE cycling with combined N and P limitations

First, the reactor was operated at a 10 day SRT for 2-3 SRTs without N&P limitations to develop a robust activated sludge. Then the MAA/AE reactor was operated for approximately 70 days without addition of N&P. Figure 3 shows the profiles of PHA and biomass concentration of the MAA/AE reactor with N&P limitations. As shown in the figure, PHA contents increased rapidly the first 2 days after N&P were eliminated from the feed. The PHA accumulation continued to increase and reached the maximum content of 48%TSS, 20 days after N&P were eliminated. The production of PHA was observed during both MAA and aerobic phases. This observation is different from what is observed in typical BNR systems, i.e., PHA production in a BNR system is observed during the anaerobic phase where most of the COD is removed from solution, while PHA consumption occurs in the aerobic phase when it is used as a source of carbon and energy for biomass growth and polyphosphate storage. After PHA content reached the maximum % value of 48%TSS, it then decreased simultaneously with the biomass concentration. In contrast to typical BNR systems, when PHA production was maximized using MAA/AE cycling with N&P limitation, most of the COD was consumed during the aerobic period, i.e., COD consumption during the MAA period was negligible. Then, during PHA decrease the biomass did not remove COD from solution, as shown in Figure 4. Reduction of soluble COD was not observed after 26 days without N&P addition, and COD during the aerobic period was higher than the influent concentration. This is not surprising since N&P are essential nutrients required for cellular growth by all living organisms. According to Lafferty *et al*. (1988), Anderson and Dawes (1990), and Doi (1990), PHA production varies from species to species and is influenced by growth conditions. Experiment 2 showed that the activated sludge mixed culture was producing massive amounts of PHA under the combination of MAA/AE cycling with N&P limitations, however, high PHA contents could not be maintained for long periods when N&P were absence from the feed. Economical biodegradable plastics production would require that PHA be harvested at its maximum productivity (mg/L/day). PHA concentration (mg/L) and PHA productivity (mg/L/day) were calculated for the MAA/AE cycling experiment. The maximum PHA production and PHA productivity in this experiment were 972 mg/L and 37.4 mg/L/day, respectively. The results also suggest that the commercially developed PHA production strategy of first developing a culture without nutritional limitations, and then subjecting it to one or more limitations for a short period before harvesting, is most likely to be a

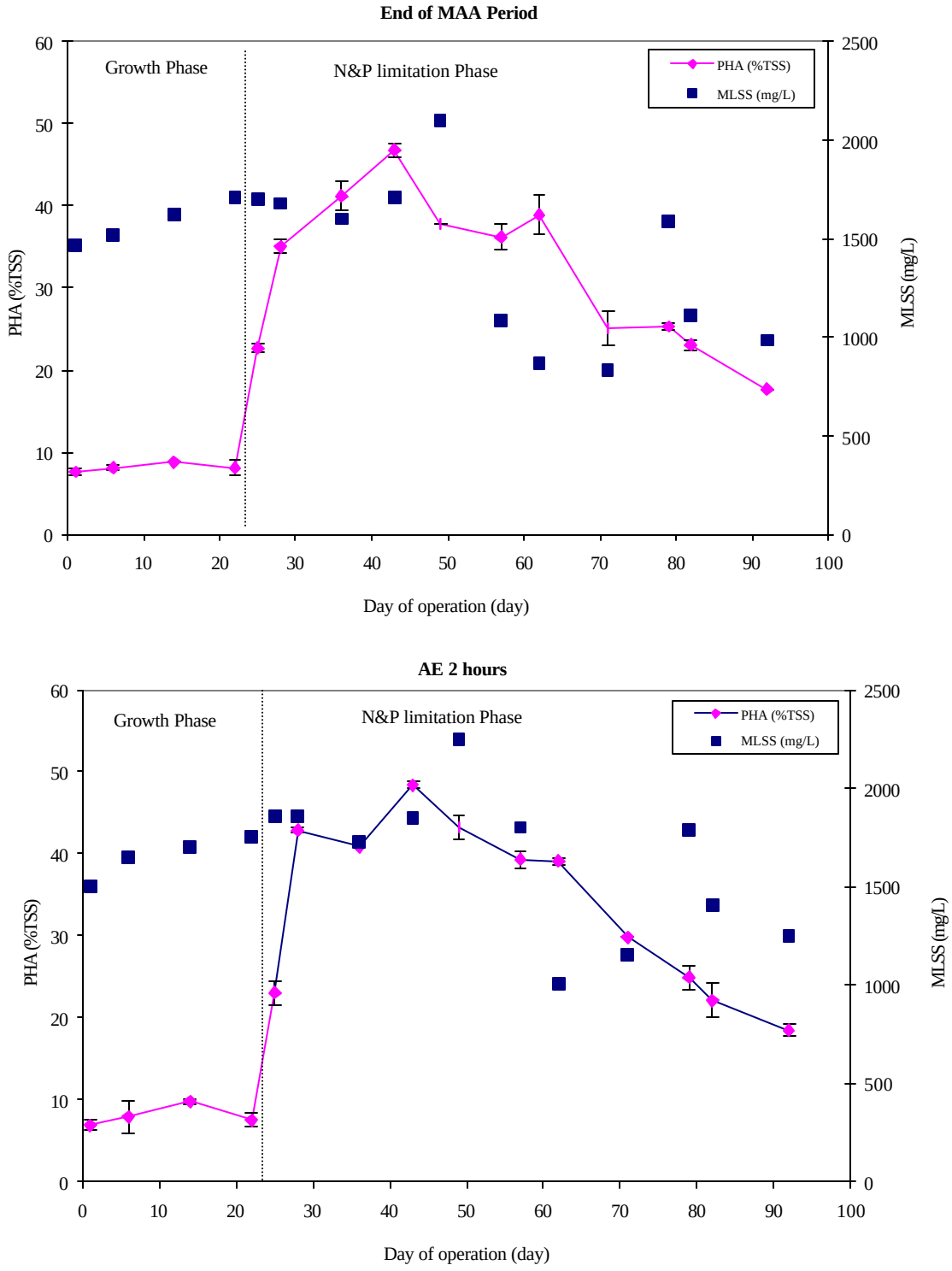


Figure 3: Experiment 2 – MAA/AE Cycling: Changes in PHA and MLSS

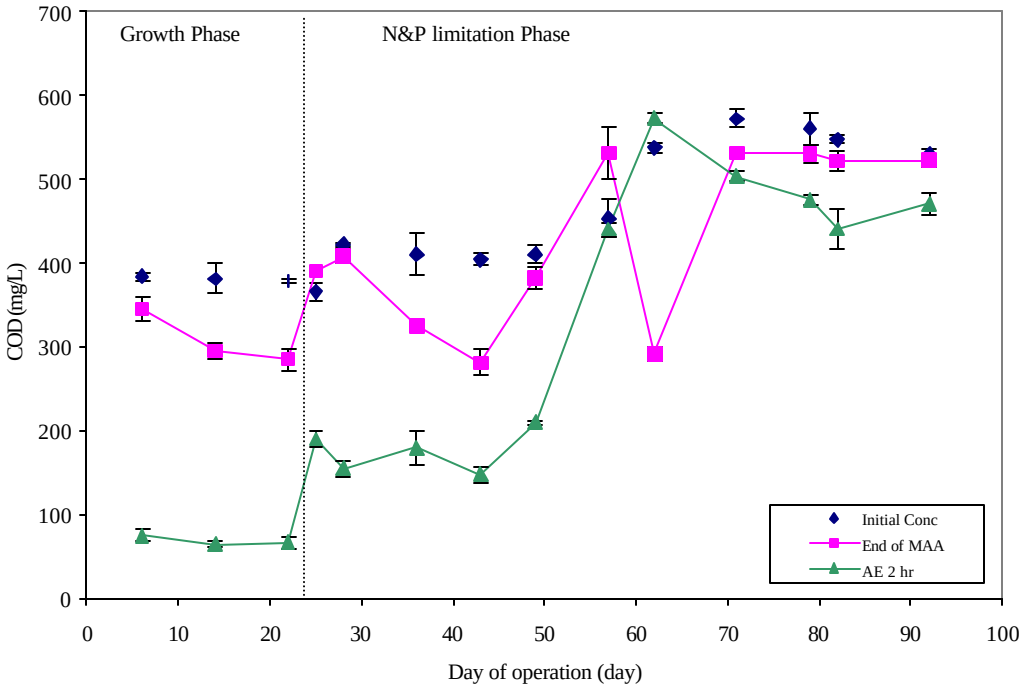


Figure 4: COD Profiles of Experiment 2 – MAA/AE Cycling

successful strategy for PHA production utilizing activated sludge.

Experiment 3: Effects of AN/AE cycling with combined N and P limitations

Experiment 3 was designed to investigate the effects of N&P limitation during typical EBPR operation, i.e., separate from MAA effects but using anaerobic/aerobic cycling. An anaerobic/aerobic (AN/AE) EBPR reactor was operated for approximately 70 days. During the first 24 days of the acclimation period, the activated sludge PHA content was approximately 10-12%TSS. Then, N&P were eliminated from the feed. Figure 5 shows PHA and biomass concentration profiles from the AN/AE reactor with N&P limitations. PHA production was observed during both the anaerobic and aerobic phases. The same pattern of PHA accumulation was observed in Experiments 2 and 3, i.e., after reaching maximum accumulation of PHA, both %PHA and biomass concentration declined. The maximum PHA content of 47%TSS was obtained 29 days after N&P removal in this experiment, then, the PHA content and the biomass concentration began to decrease. The maximum PHA accumulation obtained using AN/AE cycling with N&P limitations was slightly lower than that obtained using the combination of MAA/AE cycling and N&P limitations. The maximum PHA concentration and PHA productivity in this experiment were 1494 mg/L and 78.6 mg/L/day, respectively which was greater than MAA/AE productivity. COD profiles of the reactor are shown in Figure 6. The reduction of COD was observed throughout the course of this experiment even during the decline of PHA production. However, its reduction was negligible during the anaerobic period, just as observed during the MAA period of Experiment 2.

Experiment 4: Effects of MAA/AE cycling with partial N limitation

This experiment was performed in an effort to further increase the cell growth in the reactor. A low level of ammonia N was added to the feed along with adequate P. An MAA/AE SBR was first operated with adequate nutrients to obtain a sufficient amount of biomass in the system, i.e., 1800-2000 mg/L TSS. Then, the influent ammonia-N concentration was reduced from 20 mg/L to approximately 2 mg/L N, while the influent P concentration was maintained at 10 mg/L. Figure 7 shows the PHA and biomass concentration profiles across the reactor. After the N was reduced, the PHA production rapidly increased to approximately 30-36 %TSS, and remained nearly constant at this level throughout the experimental period. Maximum PHA content of

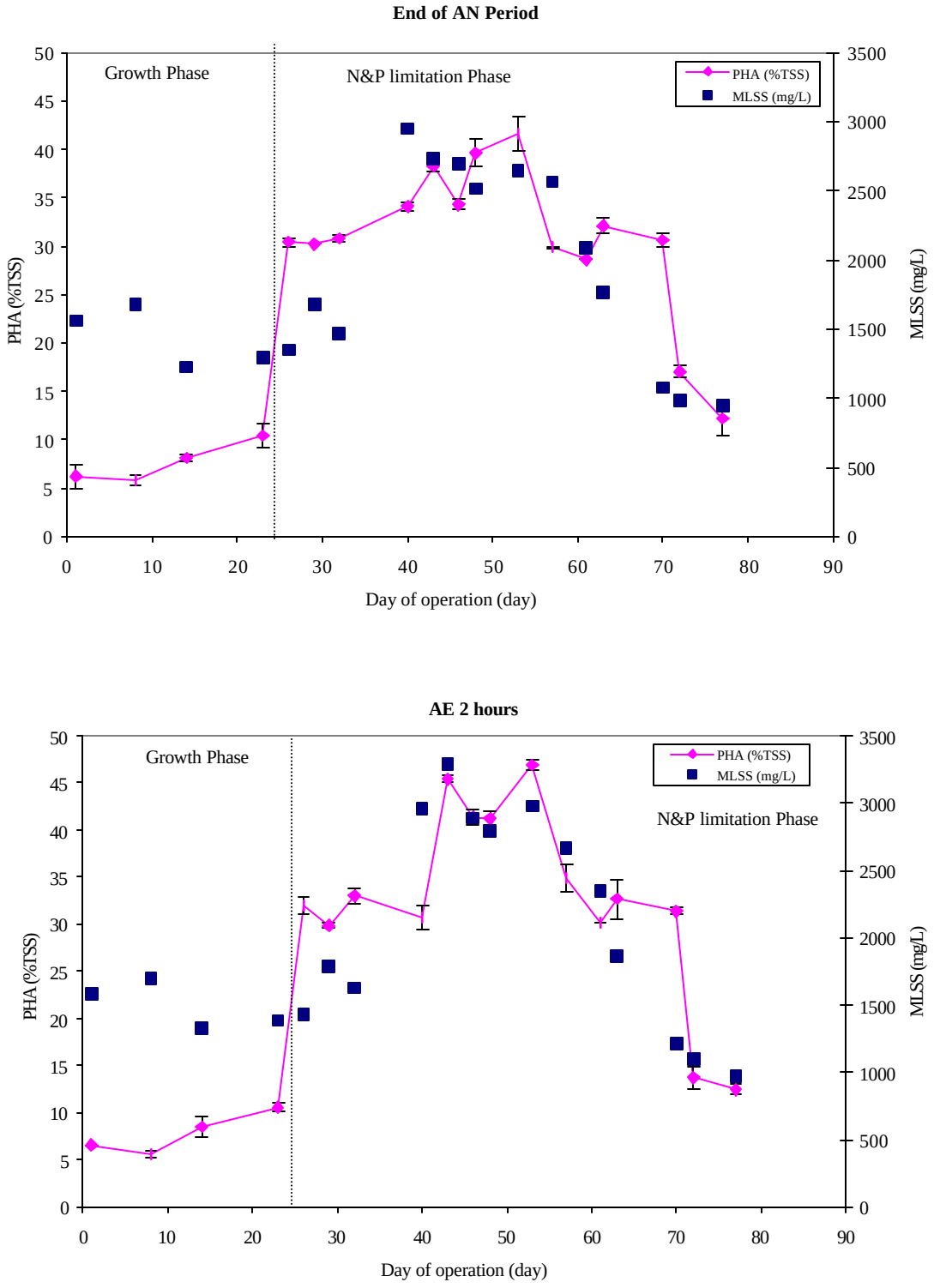


Figure 5: Experiment 3 – AN/AE Cycling: Changes in PHA and MLSS

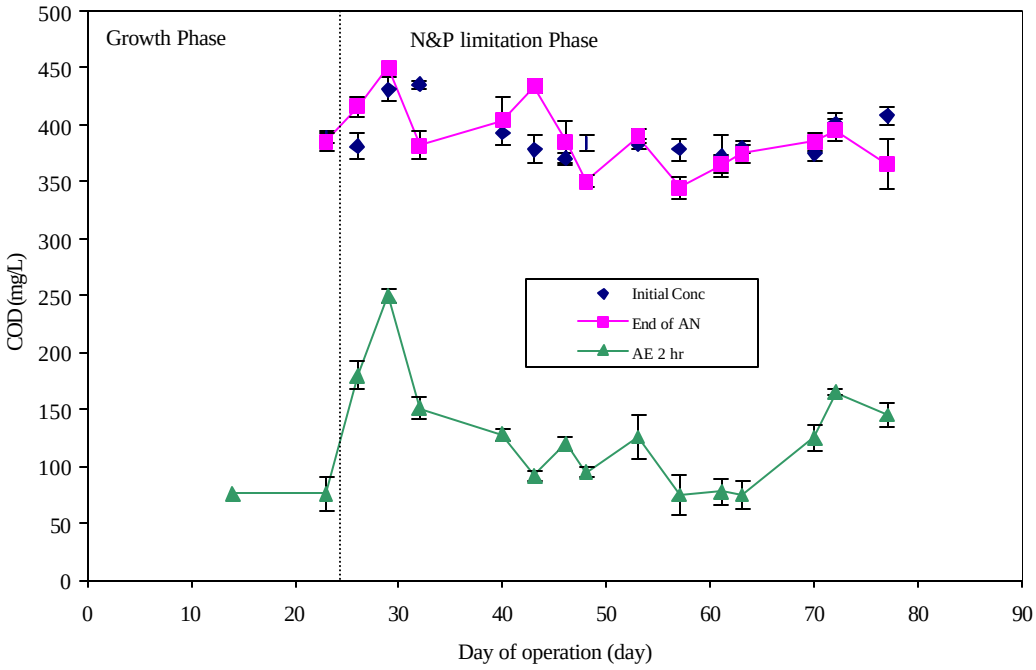


Figure 6: COD Profiles of Experiment 3 – AN/AE Cycling

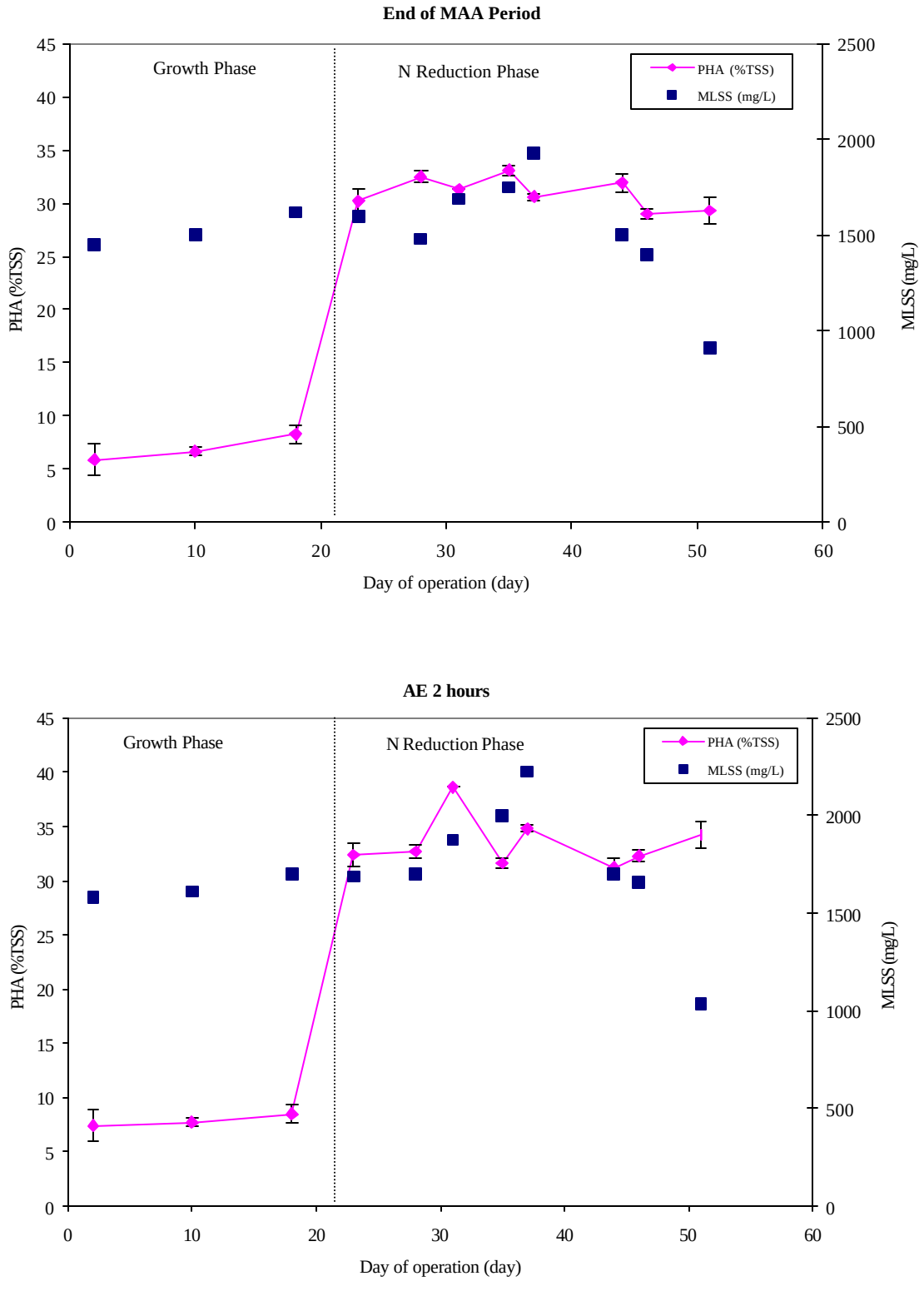


Figure 7: Experiment 4 – MAA/AE Cycling: Changes in PHA and MLSS

39%TSS was obtained 11 days after N was reduced. The MLSS concentration gradually increased, then declined beginning 17 days after N was reduced. PHA accumulation was less than that obtained during Experiments 2 and 3. The low concentration of added nitrogen was not sufficient to further increase the concentration of activated sludge biomass and the maximum PHA content achieved was lower than those obtained in the previous experiments. The maximum PHA concentration and PHA productivity in this experiment were 774 mg/L and 45.5 mg/L/day, respectively. The results clearly showed that operating conditions and nutrient limiting conditions have a significant effect on the production of PHA. MAA/AE cycling with N&P limitations and AN/AE cycling with N&P limitations stimulated the accumulation of PHA better than MAA/AE cycling with partial N limitation. The utilization of COD in this experiment was similar to that of Experiment 3, i.e., COD reduction during the MAA period was negligible but overall reduction was observed throughout the course of experiment.

Experiment 5: Effects of MAA/AE cycling with P limitation

After the biomass growth phase, the MLSS concentration during this experiment was in the range of 1800-1950 mg/L. Then the MAA/AE reactor was operated for approximately 60 days without addition of P. Figure 8 shows the profiles of PHA and biomass concentration in the reactor. As shown in the figure, PHA content gradually increased and reached the maximum content of approximately 62%, 30 days after P elimination. The production of PHA was observed during both the MAA and aerobic periods as observed in all experiments. The biomass concentration gradually decreased after P was eliminated. The maximum PHA concentration and PHA productivity rate in this experiment were 755 mg/L and 58.1 mg/L/day, respectively. COD consumption was observed throughout the course of the experiment although PHA contents were declining at the end of the experimental period. Also, as observed in the previous experiments, COD reduction during the MAA period was negligible.

Experiment 6: Effects of AN/AE cycling with P limitation

Experiment 6 was run in parallel with Experiment 5. They both were fed with the same influent compositions. Figure 9 shows the profiles of PHA and biomass concentration during Experiment 6. As shown in the figures, PHA content gradually increased as observed in Experiment 5 and

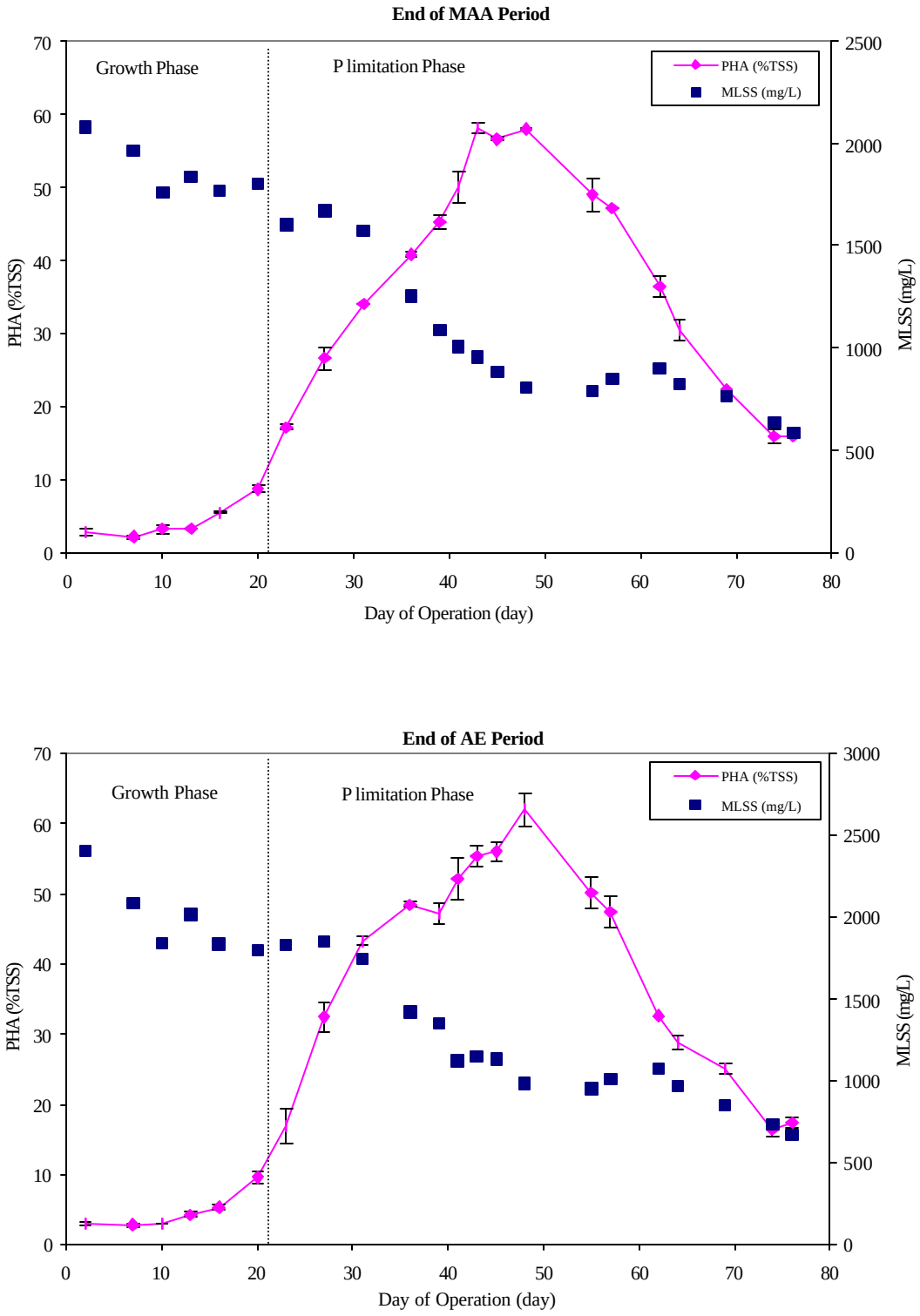


Figure 8: Experiment 5 – MAA/AE Cycling: Changes in PHA and MLSS

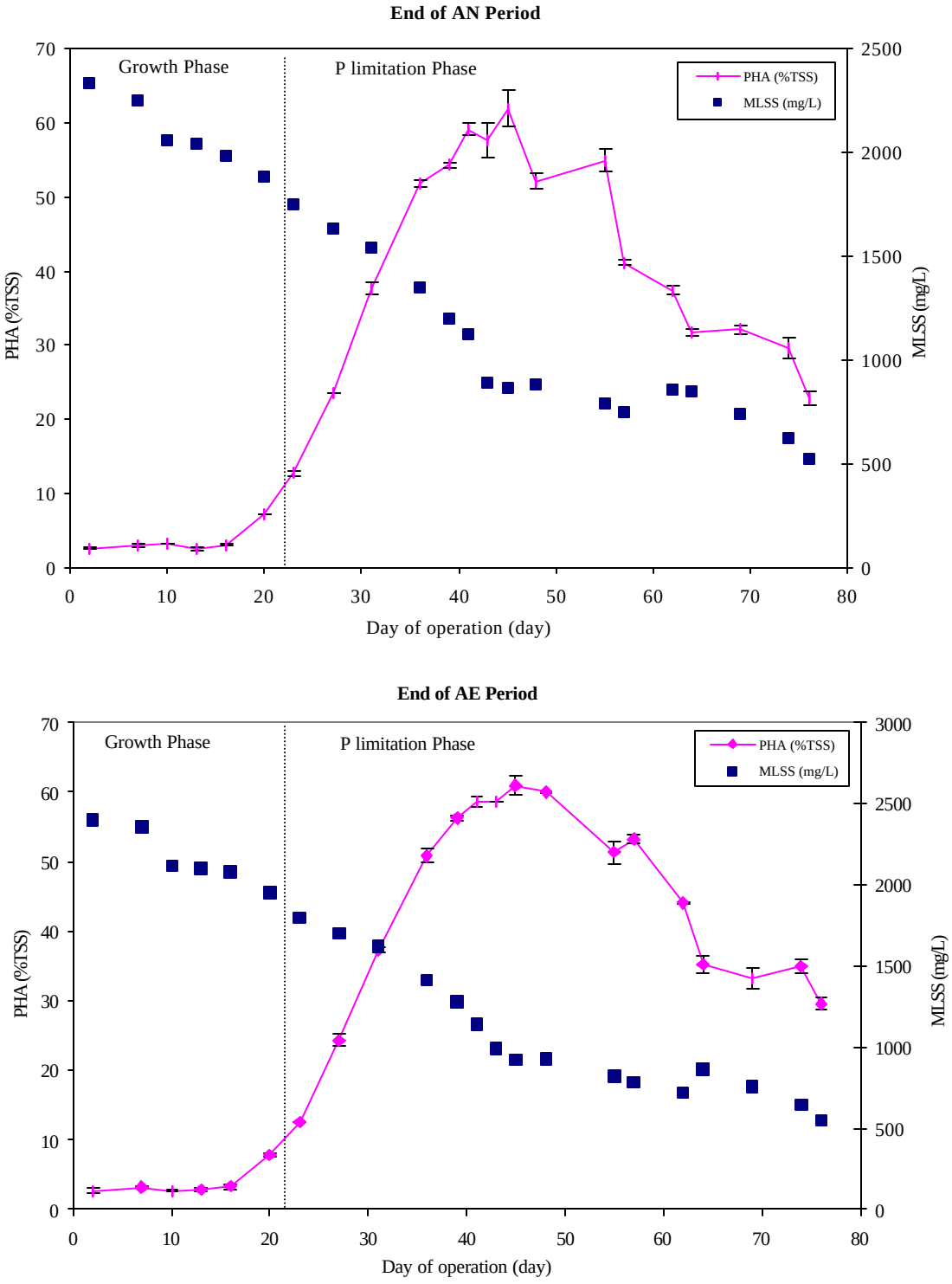


Figure 9: Experiment 6 – AN/AE Cycling: Changes in PHA and MLSS

reached the maximum content of approximately 62% on day 45, 27 days after P elimination, i.e., PHA reached the maximum a bit faster than in Experiment 5. PHA production was observed during both anaerobic and aerobic phases as observed in all experiments. The same pattern of the biomass was observed in Experiments 5 and 6, i.e., a gradual decrease after P was eliminated. The maximum PHA concentration and PHA productivity in this experiment were 720 mg/L and 34.3 mg/L/day, respectively. The maximum production and productivity in experiments 5 and 6 were lower than those obtained in the previous four experiments due to the lower biomass production throughout the course of the experiments. The changes of COD during Experiment 6 were similar to those of Experiment 5. However, COD consumption in this experiment was somewhat better than Experiment 5, i.e., lower COD concentrations were measured in the effluent.

Experiment 7: Effects of fully AE conditions with P limitation

The system was operated under fully AE conditions with a growth phase of 24 days. The biomass concentration before the PHA accumulation phase was similar to that obtained during the preceding experiment, i.e., 1820 mg/L. Figure 10 shows the profiles of PHA and biomass concentration in the reactor. As shown in Figure 10, PHA content increased rapidly the first 2 days after P was eliminated from the feed. The PHA accumulation continued to increase and reached the maximum content of 53%TSS, 24 days after P was eliminated. The biomass in the reactor gradually decreased throughout the course of the experiment, i.e., from 1820 mg/L to less than 800 mg/L. The maximum PHA concentration and PHA productivity in this experiment were 757 mg/L and 379 mg/L/day, respectively. PHA productivity in this experiment was significantly higher than what was obtained during Experiments 1-6 because maximum PHA production was accomplished with a short production time, i.e., 2 days after P was eliminated from the feed. However, the maximum %PHA produced at that particular time was low, i.e., 26%TSS. Regarding the PHA purification process, it is more economical to harvest and purify PHA when the biomass contains high PHA concentrations. According to Choi and Lee (1999), a low PHA content in bacterial cells results in a larger amount of digesting agents required to separate PHA from other intracellular materials, a higher cost of waste disposal, and an increase in equipment-related costs. Details of PHA economic analysis was discussed by Punrattanasin *et*

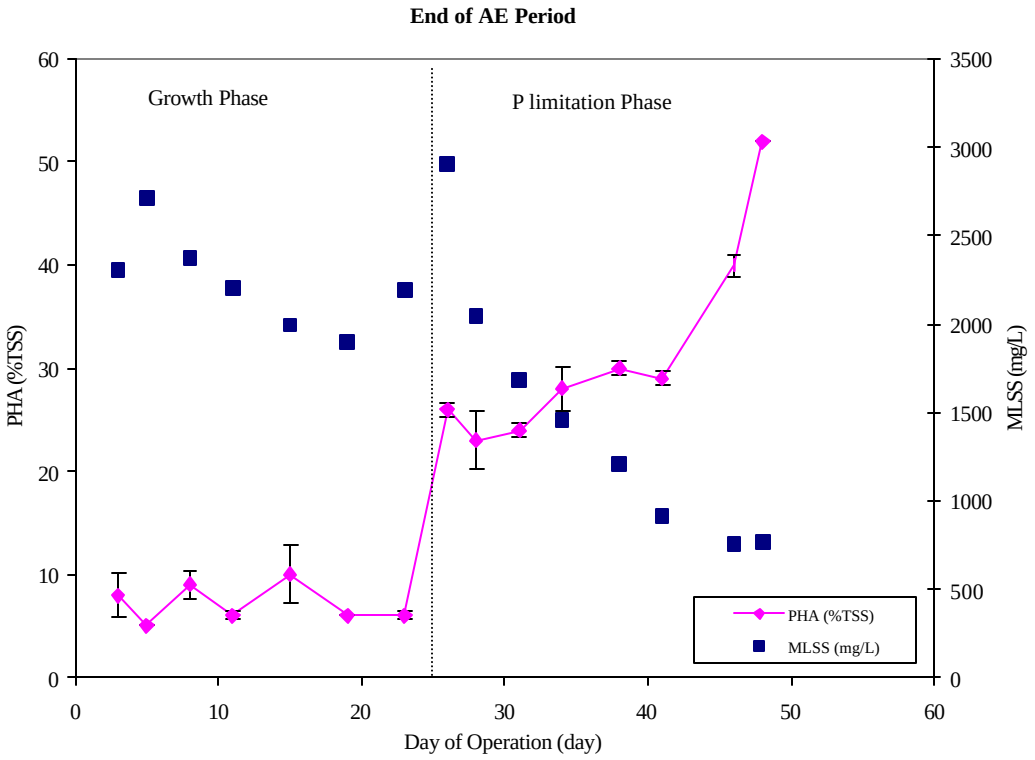


Figure 10: Experiment 7 – Fully AE: Changes in PHA and MLSS

al. (2001). COD consumption was observed throughout the course of the experiment although the biomass concentration continuously declined throughout the experiment.

Experiment 8: Effects of fully AE conditions with N&P limitations

The biomass concentration before the PHA accumulation phase was 2260 mg/L. Figure 11 shows the profiles of PHA and biomass concentration during Experiment 8. The PHA contents increased rapidly the first 2 days after N&P were eliminated from the feed. The PHA accumulation continued to increase and reached the maximum content of 57%TSS, 4 days after N&P were eliminated. The biomass in this reactor increased during the first 4 days of PHA accumulation phase, then gradually declined. The maximum PHA concentration and PHA productivity in this experiment were 1988 mg/L and 497 mg/L/day, respectively, the best PHA production and productivity obtained thus far. The corresponding PHA content on the day of maximum productivity was 54%TSS. High COD removal of greater than 85% was observed during the first 4 days after N&P were eliminated. However, low COD removals of less than 60% were observed thereafter.

Experiment 9: Effects of fully AE conditions with N limitation

During Experiment 4 (MAA/AE cycling with partial N limitation), nitrogen was not totally eliminated from the feed in order to obtain some cell growth with hopes of reducing the rate of decline of biomass solids during the nutrient limitation phase. However, the low concentration of added nitrogen was insufficient to maintain the high biomass concentration for a long period of time. Nitrogen concentration was totally eliminated from the feed in this experiment to determine the maximum PHA content produced under fully AE conditions with N limitation. Figure 12 shows the profiles of PHA and biomass concentration of this experiment. PHA content gradually increased and reached the maximum content of 42%TSS, 8 days after N was eliminated. The biomass in this reactor increased during the first 6 days of the PHA accumulation phase, then gradually declined. The maximum PHA concentration and PHA productivity in this experiment were 1250 mg/L and 156.3 mg/L/day, respectively. The maximum PHA content, production, and productivity in the experiment were higher than those obtained during Experiment 4. High COD removal of greater than 90% was observed during the

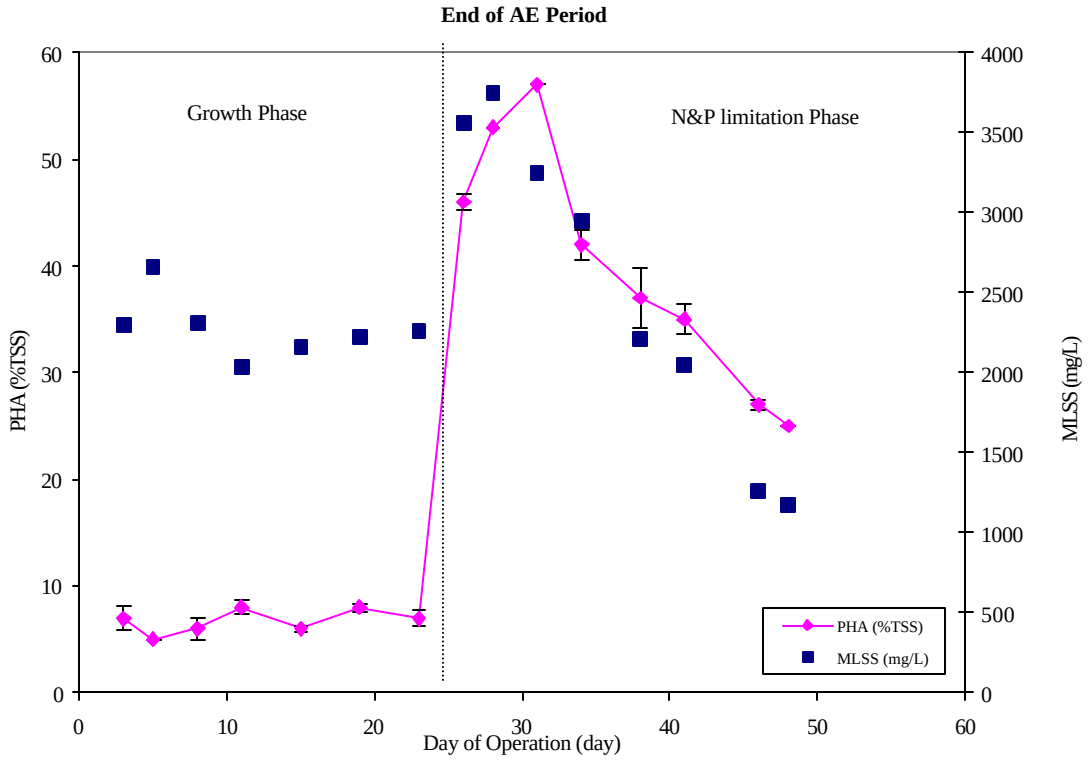


Figure 11: Experiment 8 – Fully AE: Changes in PHA and MLSS

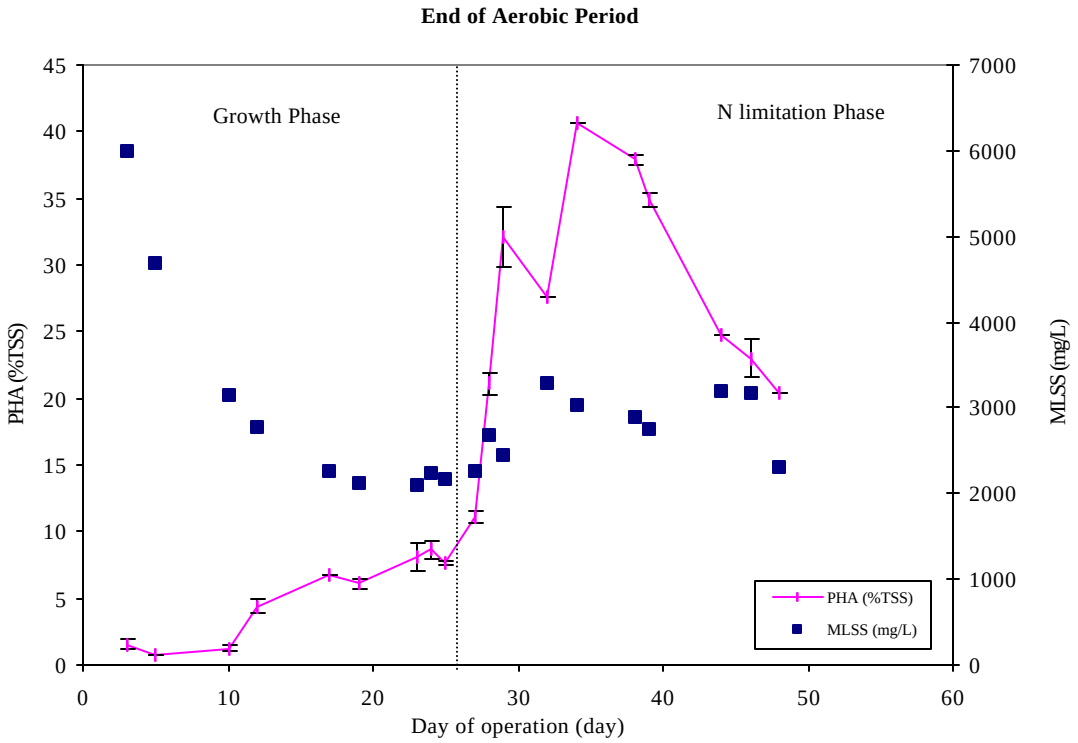


Figure 12: Experiment 9 – Fully AE: Changes in PHA and MLSS

first 3 days after N was eliminated. However, low COD removal of less than 60% was observed thereafter.

Experiment 10: Effects of fully AE conditions with partial N&P limitations

Operating conditions of Experiment 8, i.e., fully AE with total N&P limitations, were the best conditions for PHA production using activated sludge biomass investigated thus far. In this experiment, an attempt was made to increase the biomass concentration further by partial limitations of the essential nutrients (N&P) in order to obtain a higher PHA productivity. The system was first operated under the normal growth phase and the utilization of N&P were monitored. Then, N&P were partially added in the amount equivalent to 15% of that utilized during the normal growth phase. Figure 13 shows the profiles of PHA and biomass concentrations of the experiment. Higher biomass concentration was obtained in comparison to those obtained in the previous experiments, except from Experiment 8. However, a longer PHA production time was required to achieved the maximum %PHA produced, i.e., PHA content of approximately 43%TSS was not obtained until 13 days after N&P were partially eliminated. A longer PHA production time resulted in a lower PHA productivity of this experiment. The maximum PHA concentration and PHA productivity in this experiment were 1011 mg/L and 77.8 mg/L/day, respectively.

Reproducibility of PHA production by a fully AE system with N&P limitations

According to the results obtained from Experiments 1-10, the operating conditions of Experiment 8, fully AE with N&P limitations, were the best conditions for PHA production using the mixed cultures of activated sludge biomass. In order to extend the results of the laboratory experiments to full-scale production of PHA, the reproducibility of PHA production is an issue of concern. In addition, other factors affecting the production and storage of PHA in activated sludge biomass need to be more completely defined.

Two systems (Systems 1 and 2) were set up and operated under the same conditions as used in Experiment 8. Both of the systems were first operated under normal growth phase conditions. Biomass (MLSS) concentrations before the PHA accumulation phase of Systems 1 and 2 were 3204 and 2738 mg/L, respectively. Figure 14 shows the profiles of PHA and biomass

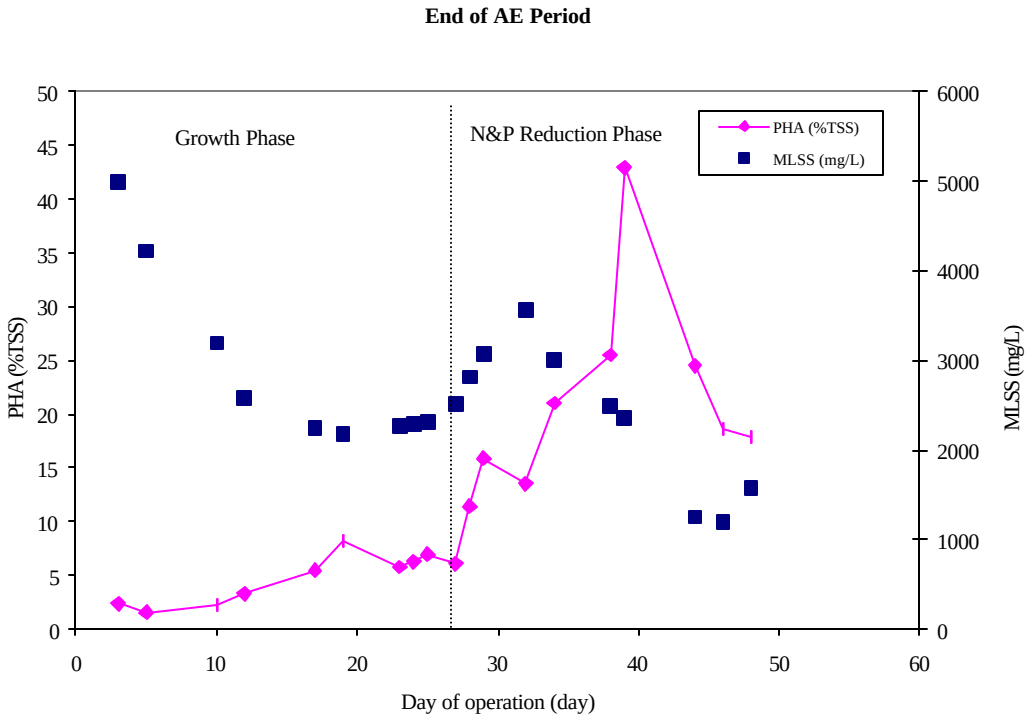


Figure 13: Experiment 10 – Fully AE: Changes in PHA and MLSS

concentrations of these 2 systems. The maximum PHA content produced by Systems 1 and 2 were 46%TSS (7 days after N&P limitations) and 54%TSS (9 days after N&P limitations), respectively. PHA increased and reached the maximum content and then gradually declined. The systems were returned to operate under normal growth phase for approximately 5 days, then nutrients were limited a second time. During the second N&P limitations, the maximum PHA content of systems 1 and 2 were 70%TSS (5 days after N&P limitations) and 67%TSS (5 days after N&P limitations), respectively. The maximum PHA concentration and PHA productivity of System 1 were 2265 mg/L and 453 mg/L/day, respectively, while those of System 2 were 1919 mg/L and 384 mg/L/day, respectively. The results during this phase of study indicated that it is important to enrich the population of PHA producing bacteria by operating the system under two consecutive periods of normal growth and nutrient limitation to achieve high %PHA accumulation. In addition, the production time was also reduced by operating the systems under two consecutive periods of N&P limitations. However, it was not successful when an attempt was made to further increase %PHA accumulation by operating the system under N&P limitations a third consecutive time. Sludge bulking occurred throughout the course of the third N&P limitation period. A lot of biomass solids were lost in the effluent discharge. Maximum PHA content during the third limitation period was less than 30%TSS.

Copolymer composition

Surprisingly, the PHV/PHB ratios of the copolymers produced by the activated sludge mixed culture were different under the different operating conditions of Experiments 2 to 10, even though the organic substrates used were the same. This was surprising because during commercial production of PHAs the substrates are changed when PHAs of different properties are desired. Also, the compositional change with experimental conditions was not predicted by the hypothesis proposed by Hood and Randall (in press) and Liu *et al*. (2000), even though it appears to adequately explain different EBPR performances with different VFAs and VFA combinations. This difference is probably due to the fact that PHA carbon could be recycled multiple times in these continuous experiments unlike the batch experiment of Liu *et al*. (2000). For example it is well know that acetic acid results in 3HB but in multiple cycle experiments or because of glycogen carbon 3HV is also formed to varying degrees, e.g., Pereira *et al*. (1996). The PHV/PHA ratios of the experiments are summarized in Tables 4 and5.

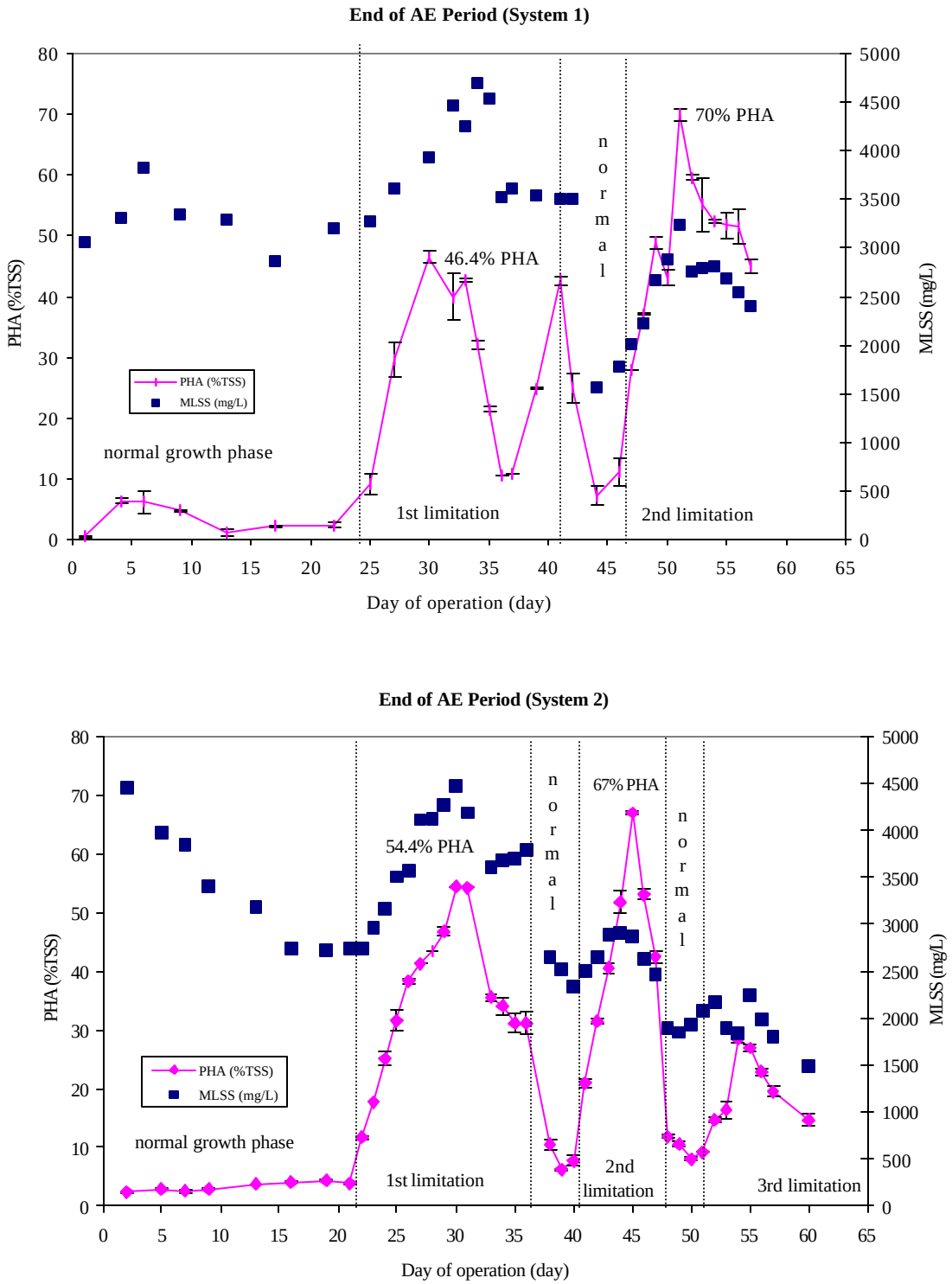


Figure 14: The Production of PHA and MLSS Concentration During Reproducibility Phase

It is considered to be well known that copolymer composition, i.e., %PHB and %PHV, is primarily influenced by the substrate used (Doi, 1990). Doi *et al.* (1987) studied the copolymer composition produced by *R. eutropha* H16 grown in a medium containing sodium acetate and sodium propionate. The PHV content of the copolymer increased as the propionate concentration in the medium increased. They found that copolymer was obtained even when propionate was used as the sole organic carbon compound. During the study reported herein, the same organic substrates were used during Experiments 2-10, yet the PHV/PHA ratio of the copolymer changed in accordance with operating conditions.

It is possible that when the bacteria responsible for PHA accumulation are subjected to multiple nutrient limitations, they synthesize PHA using different biosynthesis pathways. Perhaps propionate is used less effectively when there is more than one limiting condition. This could result in a lesser fraction of the available propionate being incorporated into the HV unit of the copolymer produced under MAA/AE cycling or AN/AE cycling, relative to fully AE operation. Also, the PHV/PHA ratios obtained when N&P were partially limited were higher than those obtained when N&P were totally eliminated from the feed. Renner *et al*. (1996) studied the production of copolymer by 13 bacteria from the rRNA superfamily III. They reported that different bacteria were able to produce PHAs with different PHV/PHB compositions when growing on the same substrate. Anderson *et al*. (1990) studied the production of copolymer by the genera *Rhodococcus*, *Nocardia*, and *Corynebacterium*. They found that these genera had differences in PHA accumulation and composition when grown on the same single carbon compounds. Therefore, it is possible that the different operating conditions in this study selected for different groups of PHA producing bacteria, and the different microbial populations were responsible for both the differences in PHA production and the observed differences in the %PHB and %PHV of the resulting copolymer.

According to Doi (1990), PHA production and degradation could occur simultaneously under nutrient limiting conditions, which is known as cyclic metabolism of PHA. Holmes (1985) and Lafferty *et* al. (1988) stated that PHA is degraded more rapidly in an anaerobic environment than in an aerobic one. In addition, PHB homopolymer was found to be degraded faster than PHB-PHV copolymer. Therefore, it is possible that the degradation rate of PHA under AN/AE

cycling or MAA/AE cycling is somewhat more rapid than that under fully AE conditions or under multiple limiting conditions. The rapid rate of PHA degradation under these conditions could result in more available acetyl-CoA that can be used to form more HB units in the polymer chains as observed in higher PHB fractions in the copolymer produced under MAA/AE cycling and AN/AE cycling.

PHA productivity and PHA yield

The production of PHA, biomass concentration, and COD removal of Experiments 2-10 and the experiments during confirmation phase are summarized in Tables 4 and 5, respectively. Choi and Lee (1999) reported that the best PHA productivity reported to date was obtained from the study by Wang and Lee (1997). They obtained PHA productivity of 4.94 gPHB/L/hr from *A. latus*. PHA productivity obtained in this study was significantly lower than that reported by Wang and Lee (1997) and other studies using pure cultures due to a lower biomass concentration in the system. Concentrations of biomass and substrate used in commercial production are significantly higher than the concentrations of the mixed culture of activated sludge that exist at a wastewater treatment plant, and of organic substrate in wastewaters. For example, the cell concentration of *A. latus* was 76 g/L and 5-20 g/L of sucrose was applied in the study by Wang and Lee (1997), while the concentration of the biomass in an activated sludge wastewater treatment system is almost always lower than 5,000 mg/L and the organic matter in domestic wastewater is usually lower than 500 mg/LCOD. An ideal organism for PHA production would be a culture that can store high PHA content and grow rapidly on an inexpensive substrate. Pure cultures of selected bacteria are best suited for PHA production, but the cost of maintaining pure culture conditions and providing the suitable substrate has increased the cost of biodegradable plastics to the point that it is not competitive with synthetic plastics, in spite of the environmental benefits. These experiments have shown, however, that it is possible and potentially economical to develop a PHA production system for biodegradable plastics using activated sludge biomass and wastewater as a raw material source, such as in a side-stream process coupled with a main-stream wastewater treatment system.

According to Choi and Lee (1999), the cost of the carbon substrate is a major factor in the overall PHA production costs, i.e., 38% of the total operating cost based on the production scale

of 100,000 ton/year. The yields of PHA per unit substrates in this study are summarized in Tables 4 and 5. The yield of PHA per unit substrate reported in the study by Wang and Lee (1997) was 0.42 gPHA/g sucrose or equivalent to 0.37 gPHB/gCOD (1 gram of sucrose is equal to 1.123 gram of COD). PHA yields per unit COD during this study was similar to those obtained with *A. latus*: 0.39 gPHA/gCOD during Experiment 8 vs. 0.37 gPHA/gCOD from *A. latus*. These experiments demonstrated that activated sludge biomass used the VFA substrates as efficient as the pure cultures and substrates used during commercial production. Perhaps the cost of PHA production could be further reduced if a higher strength wastewater could be used and the activated sludge culture could be further concentrated, such as with a membrane separation system, or concentrated biomass from the secondary clarifier underflow could be used to feed the side-stream PHA production system.

Table 4: Summary of PHA Production, Biomass Concentration, and COD removal of Experiments 2-10

	Exp.2	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**	**Exp. 7**	**Exp. 8**	**Exp. 9**	**Exp. 10**
Max. PHA (%TSS)	48	47	39	62	62	53	57	42	43
Corresponding MLSS (mg/L)	1848	2980	1875	985	870	720	3250	3000	2353
Days to Accumulate Max. %PHA (day)	20	29	11	30	27	24	7	8	13
Max. PHA Concentration (mg/L)	972	1494	774	755	720	757	1988	1250	1011
Corresponding PHA (%TSS)	43	45	35	43	56	26	53	42	43
Days to Accumulate Max. Production (day)	26	19	17	13	21	2	4	8	13
PHA Productivity (mg/L/day)	37	79	46	58	34	379	497	156	78
PHV/PHA on day of Max. Production (%)	12-13	38	29-30	50-53	27-44	65-66	45	36-37	55-56
COD Removal on day of Max. Production (%)	52	77	92	92	91	90	86	27	96
Carbohydrate Content on day of Max %PHA (%TSS)	NM	17.8	12.9	24.6	20.9	22.2	12.6	10	17.2
Yield of PHA on Substrates (mgPHA/mgCOD)	0.05	0.07	0.04	0.04	0.03	0.27	0.39	0.34	0.05

Note: NM = not measured

Table 5: Summary of PHA Production, Biomass Concentration, and COD removal of the Experiments during the Reproducibility Phase

	System 1		System 2	
	First limitation	Second limitation	First limitation	Second limitation
Max. PHA (%TSS)	46	70	54	67
Corresponding MLSS (mg/L)	3934	3238	4469	2865
Days to Accumulate Max. %PHA (day)	7	5	9	5
Max. PHA Concentration (mg/L)	1827	2265	2431	1919
Corresponding PHA (%TSS)	46	70	54	67
Days to Accumulate Max. Production (day)	7	5	9	5
PHA Productivity (mg/L/day)	261	453	270	384
PHV/PHA on day of Max. Production (%)	43	50	36	45
COD Removal on day of Max. Production (%)	82	91	92	93
Yield of PHA on Substrates (mgPHA/mgCOD)	0.23	0.34	0.25	0.28

SUMMARY AND CONCLUSIONS

An objective of this study was to develop a high PHA production system using the mixed culture of activated sludge biomass that exists in wastewater treatment plants. Some approaches used for commercial production using pure cultures could be applied to PHA production using activated sludge biomass. The two-stage bioprocess approach proved to be the most successful strategy for PHA production, and fully aerobic conditions were both better and simpler for PHA production. However, modifications were required to maximize the production of PHA using activated sludge biomass. A combination of N and P limitations stimulated the highest PHA production by activated sludge biomass in comparison to a single limitation (N or P) typically used for pure culture commercial production. Nonetheless, the feasibility of the approach was demonstrated. Activated sludge biomass cultures have the potential to be used for the production of PHA for biodegradable plastics, with the possibility that wastewaters could be used as a low cost raw material. This would reduce the production cost of PHA, reduce the environmental problems of disposal of synthetic plastics in landfills, simultaneously treat the wastewater, and reduce the amount of excess sludge sent for ultimate disposal. In addition, the cost of developing and maintaining axenic cultures would be eliminated. Development of this technology could

result in major economic and environmental impact by contributing to shifts in the types of plastics used for many consumer oriented applications, and altering the economics and objectives of wastewater treatment. Based upon the results of the study, the following conclusions were made:

1. Operating conditions have a significant effect on PHA production and the composition of the accumulated copolymer. Fully AE conditions with N&P limitations were the optimum conditions for PHA production using activated sludge biomass. The PHA production results obtained by Satoh et al. (1998) using MAA/AE cycle without nutrient limitation could not be reproduced. AN/AE cycling as used for a BNR system did not promote a massive production of PHA. PHV/PHA ratios of the copolymers produced were different under different operating conditions.
2. The combination of N&P limitations promotes the maximum PHA production. This finding is different from commercial production wherein only one nutrient limitation has been applied for high PHA production, i.e., N or P limitation depending on the type of microorganism employed.
3. The 2-stage bioprocess is a better approach for obtaining activated sludge PHA accumulation than the continuous SBR process.
4. Partial limitation of either N or N&P did not improve PHA productivity compared to full limitation. Low PHA accumulation was obtained and a longer PHA production time was required to achieve the maximum quantity under partial nutrient limitation.
5. Two consecutive cycles of N&P limitations under fully AE conditions resulted in the maximum amount of %PHA accumulation and reduced the required production time. It appears that it is necessary to enrich the PHA accumulating bacteria by this approach before optimum results are obtained.
6. Activated sludge can be used as a source of organisms that overproduce PHA, and that produce PHA with different PHV/PHA ratios. Mixed cultures of activated sludge could be used to produce polymers for specific applications.
7. The yield of PHA per unit substrate as measured by COD obtained in this study was similar to that obtained during current commercial production. This demonstrates that the activated

sludge biomass under the conditions of these experiments converted the substrate to PHA as efficient as the pure cultures used in commercial production.

8. It is recommended that activated sludge PHA production systems that utilize wastewater as the production substrate be coupled as a side-stream process with a main-stream wastewater treatment system for practical production.

REFERENCES

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiological Reviews.* 54 (4), 450-472.

Anderson, A. J., Haywood, G. W., Williams, D. R., and Dawes, E.A. (1990). The Production of Polyhydroxyalkanoates from Unrelated Carbon Sources. In: *Novel Biodegradable Microbial Polymers*, E. A. Dawes (ed.), Kluwer Academic Publishers, The Netherlands, 119-129.

Braunegg, G., Lefebvre, G., and Genser, K. (1998). Polyhydroxyalkanoates, Biopolyesters From Renewable Resources: Physiological and Engineering Aspects. *Journal of Biotechnology*. 65, 127-161.

Byrom, D. (1990). Industrial production of Copolymer from *Alcaligenes eutrophus*. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 113-117.

Choi, J and Lee, S. Y. (1999). Factors Affecting the Economics of Polyhydroxyalkanoates Production by Bacterial Fermentation. *Appl. Microbiol. Biotechnol*. 51, 13-21.

Dawes, E. A. (1990). Novel Microbial Polymers: An Introductory Overview. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 3-16.

Dawes, E. A. and Senior, P. J. (1973). The Role and Regulation of Energy Reserve Polymers in Micro-organisms. *Advances in Microbial Physiology*. 10, 135-266.

Doi, Y. (1990). *Microbial Polyesters*. VCH Publishers, Inc. New York, USA.

Doi, Y., Kunioka, M., Nakamura, Y, and Sago K. (1987). Biosynthesis of Copolyesters in *Alcaligenes eutrophus* H16 from $_{13}$C-Labeled Acetate and Propionate. *Macromolecules.* 20, 2988-2991.

Doi, Y., Segawa, A., Nakamura, S., and Kunioka, M. (1990). Production of biodegradable Copolymers by *Alcaligenes eutrophus*. In: Novel Biodegradable Microbial Polymers, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 37-48.

Huang, J., Shetty, A. S., and Wang, M. (1990). Biodegradable Plastics: A Review. *Advances in Polymer Technology*, 10(1), 23-30.

Haywood, G. W., Anderson, A. J., and Dawes, E. A. (1989). A Survey of the Accumulation of Novel Polyhydroxyalkanoates by Bacteria. *Biotechnology Letters*. 11(7), 471-476.

Hart, V. S. (1994). An Examination of Biological Phosphorus Removal Using Bacteria Counting and Poly-beta-hydroxybutyrate Analysis in Batch and Continuous Flow System. Ms.Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Hood, C. R. and Randall, A. A. (in press). A Biochemical Hypothesis Explaining the Response of Enhanced Biological Phosphorus Removal Biomass to Organic Substrates, *Water Research*.

Holmes, P. A., (1985). Applications of PHB– A Microbially Produced Biodegradable Thermoplastic. *Physics in Technology,* 16: 32-36.

Hrabak, O. (1992). Industrial Production of Poly-β-hydroxybutyrate. *FEMS Microbiology Reviews.* 103, 251-256.

Lafferty, R. M., Korsatko, B., and Korsatko, W. (1988). Microbial Production of Poly-β-hydroxybutyric Acid. In: *Biotechnology*, Rehm, H. J. and Reed, G. (eds.), VCH Publishers, New York, USA, 135-176.

Lee, S. Y. (1996). Bacterial Polyhydroxyalkanoates. *Biotechnology and Bioengineering*. 49, 1-14.

Lee, S. and Yu, J. (1997). Production of Biodegradable Thermoplastics from Municipal Sludge by a Two-Stage bioprocess. *Resources, Conservation and Recycling*. 19, 151-164.

Liu, Y. H., Geiger, C. and Randall, A.A. (2000). The Role of PHA Form in Determining the Response of Enhanced Biological Phosphorus Removal Biomass to Volatile Fatty Acids, Proceedings, WEFTEC 2000, 73rd Annual Conference & Exposition on Water Quality and Wastewater Treatment, Anaheim, CA, USA, 14-18 October 2000.

Madigan, M. T., Martinko, J. M. and Parker, J., 1997. *Brock Biology of Microorganisms.* Princeton Hall, New Jersey.

Page, W. J. (1995). Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics With a Great Future. *Can. J. Microbiol*. 41(suppl. 1), 1-3.

Pereira, H., Lemos, P. C., and Reis, M. A. M. (1996). Model for Carbon Metabolism in Biological Phosphorus Removal Processes Based on *In Vivo* 13C-NMR Labeling Experiments. *Water Research*. 30 (9), 2128-2138.

Preusting, H., Hazenberg, W., and Witholt, B. (1993). Continuous Production of Poly(3-hydroxyalkanoates) by Pseudomonas oleovorans in a High-Cell-Density, Two-Liquid-Phase Chemostat. *Enzyme Microb. Technol*. 15, 311-316.

Punrattanasin, W., Randall, C. W., and Randall, A. A. (2001). Production of Polyhydroxyalkanoates for Biodegradable Plastics Using Activated Sludge Biomass: Production using High Acetic Acid Industrial Wastewater and Economic Analysis. Ph.D. Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Renner, G., Haage, G., and Braunegg, G. (1996). Production of Short-Side-Chain Polyhydroxyalkanoates by Various Bacteria from the rRNA Superfamily III. *Appl. Microbiol. Biotechnol*. 46, 268-272.

Sasikala, CH. and Ramana, CH. V. (1996). Biodegradable Polyesters. In: *Advances in Applied Microbiology, Volume 42*, Neidleman, S. L. and Laskin, A. I. (eds.), Academic Press, California, 97-218.

Satoh, H., Iwamoto, Y., Mino, T., and Matsuo, T. (1998). Activated Sludge as a Possible Source of Biodegradable Plastic. Proceedings, Water Quality International 1998, Book 3 Wastewater Treatment, pp. 304-311. IAWQ 19th Biennial International Conference, Vancouver, BC, Canada, 21-26 June 1998.

Yamane, T. (1993). Yield of Poly-D(-)-3Hydroxybutyrate from Various Carbon Sources: A Theoretical Study. *Biotechnology and Bioengineering*. 41, 165-170.

Wang, F. and Lee, S. Y. (1997). Poly(3-hydroxybutyrates) Production with High Polymer Content by Fed-Batch Culture of *Alcaligenes latus* Under Nitrogen Limitation. *Appl. Environ. Microbiol.* 63, 3703-3706.

CHAPTER 3
PRODUCTION OF POLYHYDROXYALKANOATES FOR BIODEGRADABLE PLASTICS USING ACTIVATED SLUDGE BIOMASS: DETERMINATION OF CONTROL PARAMETERS AND PHYSICAL PROPERTIES

Warangkana Punrattanasin[1], Clifford W. Randall[1], and Andrew A. Randall[2]

[1]*Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.*
[2]*Department of Civil and Environmental Engineering, University of Central Florida, Orlando, FL 32816-2450, U.S.A.*

ABSTRACT

Sequencing batch reactor (SBR) systems were operated using 2 different approaches, i.e., one-stage and two-stage bioprocesses, to investigate parameters affecting the high production of polyhydroxyalkanoates (PHA) suitable for biodegradable plastics production by mixed cultures of activated sludge biomass. The primary purpose was to determine parameters that can be used to control high PHA production and assist with the design and operation of a system that optimizes PHA productivity. A secondary objective was to determine the physical properties of PHA extracted from the biomass for comparison with PHA commercially produced using pure cultures. The previously investigated two-stage bioprocess (Punrattanasin *et al*., 2001) was a successful strategy for PHA production because the growth phase minimizes the total phosphorus (TP) content of the bacterial cells before starting the subsequent nutrient-limited PHA accumulation phase. It was clearly shown that the presence of significant concentrations of phosphorus in the cells during nutrient limitation periods will reduce the production of PHA. Therefore, it is important to monitor TP during the growth phase, and the subsequent PHA accumulation phase should be started when TP content is at a minimum. Further, the PHA production approach used not only has an impact on PHA production, but also on the PHA composition, i.e., PHV/PHA ratio. It also was determined that the source of the seeding sludge used in the PHA production system should be carefully selected. Seeding sludge obtained from a conventional fully aerobic wastewater treatment system was more suitable than sludge obtained from a biological phosphorus removal (BPR) system because the bacterial population from the BPR system tended to convert organic substrates to intracellular carbohydrates rather than to PHA under nutrient limiting conditions. It was demonstrated that PHA stored in activated sludge

biomass can be extracted and purified using the chloroform extraction method, and a high purity extract can be obtained. Molecular weight and melting point temperature determinations of the PHA obtained from the mixed cultures of activated sludge biomass compared favorably with those obtained from pure cultures and have the potential to be used for commercial applications.

KEYWORDS

Activated sludge biomass; polyhydroxyalkanoates production; polyhydroxybutyrates; polyhydroxyvalerates; one-stage bioprocess; two-stage bioprocess; biodegradable plastics; physical properties.

INTRODUCTION

In recent years, attempts have been made to develop a process for high PHA production using mixed cultures of activated sludge biomass. Punrattanasin *et al*. (2001) have shown that mixed cultures of activated sludge biomass have considerable potential for PHA production for commercial purposes, and likely could do so using wastewaters as a low cost substrate. They demonstrated that operating conditions have a great impact on PHA production and its composition. They obtained PHA contents up to 70%TSS with a PHA yield of 0.39 mgPHA/mgCOD using a mixture of acetic and propionic acids fed under fully aerobic conditions with multiple alternating periods of simultaneous nitrogen and phosphorus limitations. There were significant similarities between the optimum conditions determined in their study compared to those used for commercial PHA production using pure cultures. These were: 1) the two-stage bioprocess approach widely used for commercial production proved to be the most successful strategy for PHA production using activated sludge biomass. 2) fully aerobic (AE) conditions were both better and simpler for PHA production, compared to microaerophilic/aerobic (MAA) or anaerobic/aerobic (AN/AE) cycling conditions. However, a combination of nitrogen and phosphorus limitations stimulated the highest PHA production by activated sludge biomass in comparison to a single limitation (nitrogen or phosphorus) typically used for pure culture commercial production.

Several studies are reported in the literature wherein the carbon to nitrogen (C:N) ratio of the feed substrate was used as a control parameter to stimulate high PHA accumulation in activated

sludge biomass. Chua *et al*. (1997) induced PHA accumulation in activated sludge biomass by controlling the C:N ratios of the feed substrates. They studied 4 different C:N ratios (24:1, 48:1, 96:1, and 144:1). Maximum specific polymer yield of 0.374 g polymer/g cell (or 37.4% PHA) was obtained with the highest C:N ratio of 144:1. The maximum PHA production yield of 0.0939 g polymer/ g TOC was obtained using the C:N ratio of 96:1. Yu *et al*. (1999) studied the production of PHA from *A. latus* and activated sludge biomass using industrial food wastes, i.e., malt and soya wastes, as organic substrates. High C:N ratios were used to stimulate PHA production by *A. latus* and microorganisms from activated sludge. PHA contents produced by *A. latus* using malt and soya wastes were 70.1% and 32.6%, respectively. When activated sludge biomass was used, PHA contents of 43.3% were obtained from malt waste. The C:N ratio of the feed substrate used for *A. latus* was lower than 20:1, while that used for the activated sludge biomass was not specified. Ma *et al*. (2000) also used the C:N ratio approach to induce PHA production in activated sludge biomass. Glucose at the concentration of 2500 mg/L was used as a source of organic substrate in their study. Polymer production increased from 0.061 (or 6.1%PHA) to 0.274 (or 27.4%PHA) g polymer/g cell as the C:N ratio increased from 24:1 to 144:1, while the specific growth yield decreased from 0.642 to 0.305 g cell/g COD removed as the C:N increased. They suggested that prolonged nitrogen limitation had an adverse effect on biomass production. They controlled the nitrogen non-feeding cycle (NNFC) to nitrogen feeding cycle (NFC) ratio of the SBR systems to achieve higher PHA biomass concentrations. A maximum PHA production rate of 0.111 g polymer/g COD consumed was obtained using a C:N ratio of 96:1 and a NNFC:NFC ratio of 3:1. Biomass concentration was further increased by operating the system with low NNFC:NFC ratios, i.e., 2:1 and 1:1. However, higher PHA production was not obtained because the PHA content of the cells was low compared to the previous results.

A PHA production system using activated sludge biomass is a relatively new development, and experience with it is limited. A C:N ratio can be used to stimulate PHA production in activated sludge biomass, but the maximum PHA contents achieved are rather low, i.e., less than 45% of TSS as reported by Chua *et al*. (1997), Yu *et al*. (1999), and Ma *et al*. (2000). Details regarding parameters controlling the high PHA production in the activated sludge biomass also are rather limited. The purpose of this research was to investigate parameters affecting the production of

PHA in mixed cultures of activated sludge biomass to improve the design of the production process, more adequately control PHA production, and improve the potential usefulness of the system for commercial purposes. Also, an extraction and purification process was evaluated, and the physical properties of the PHA produced by the activated sludge biomass were determined and compared with those produced by pure cultures as reported in the literature.

MATERIALS AND METHODS

Reactor setup

Activated sludge SBR reactors with a working volume of 4 liters were used for all experiments. The reactors were maintained in a 20°C constant temperature room and operated with hydraulic retention times (HRT) of 10 hours. The reactor cycle included a 30 minute (m) nitrogen purging and influent supply period; a 1 hour (hr) oxygen limited period; a 3 hr aeration period; and a 1.5 hr settling period with effluent discharge during the last 15 m. When the reactor was operated under fully aerobic conditions, the aeration period of the reactor cycle was 4 hr. The one-stage bioprocess approach was utilized during the first phase of the study, i.e., nutrient limitation was introduced upon the start up of the reactors and no non-limited growth phase was incorporated. The results obtained were unsatisfactory, so the two-stage bioprocess approach was used for all subsequent experiments. The two-stage bioprocess included optimum nutritional conditions during the initial (growth) phase, which was followed by a nutrient limited PHA accumulation phase. The SRT was maintained at 10 days during the growth phase but no biomass was wasted from the reactors during the PHA accumulation phase except the quantity inadvertently wasted with the supernatant drawoff. Activated sludge from either the fully aerobic Peppers Ferry Regional wastewater treatment plant (Radford, VA) or from a biological phosphorus removal (BPR) pilot plant located at Virginia Tech was used to innoculate the reactors.

Analytical methods

PHA, PHB, and PHV were measured by the methanolysis-GC method described by Hart (1994), with some modifications. Details of the PHA measurement are summarized by Punrattanasin *et al*. (2001). Intracellular carbohydrate content was measured using the Phenol method as outlined in Manual of Methods for General Bacteriology (American Society for Microbiology, 1981).

MLSS, MLVSS, and COD were analyzed according to Standard Methods for the Examination of Water and Wastewater, 19th Edition, 1995, using sections 2540D, 2540E, and 5220C, respectively. TP was measured by persulfate digestion method (section 4500-P B.5) followed by ascorbic acid method (section 4500-P E). Anions including nitrite, nitrate, phosphate, and sulfate were analyzed by a Dionex 2010I ion chromatograph (IC) with an IONPAC AS4A-SC column and electrochemical conductivity detector (Dionex Corp., Sunnyvale, CA). Cations including ammonia-nitrogen, magnesium, potassium, and calcium were analyzed by a Dionex 120 ion chromatograph (Dionex Corp., Sunnyvale, CA).

Chloroform extraction was used to purify PHA from non-PHA cellular materials. The extraction process was obtained by personal communication with Satoh (1998). Firstly, activated sludge biomass was dried under vacuum condition using a freeze-drying unit. Freeze dried sludge pellets were extracted for PHA using hot chloroform in a soxhlet extractor. Approximately 1-2 grams of dried sludge pellets and 100 mL of chloroform were used per batch of extraction. Sludge pellets were extracted for approximately 4-6 hours. Then, the chloroform mixture was condensed to approximately 5 mL. PHA was extracted from the chloroform mixture by adding 100 mL of methanol. Pure PHA was obtained as the resulting white precipitate and was collected via filtration. Precipitate was blow dried to remove the remaining solvent and the residue was used for molecular weight and boiling point temperature determinations. Molecular weight was determined using a Water model 2690 gel permeation chromatography (GPC) equipped with a differential reflective index detector and Viscotek differential viscometer. Approximately 3 mg/mL of the sample was eluted by chloroform at a flow rate of 1 mL/min. The GPC was operated at 30°C and polystyrene was used as the standard. A 100 μL of a PHA solution was injected for each analysis. Boiling point temperature was measured using a differential scanning calorimeter (DSC). Approximately 10 mg of the purified PHA sample was encapsulated in an aluminum pan and heated at the rate of 20°C/min from 30 to 195°C. The purity of the PHA extracted was determined by measuring PHA content and total protein of the precipitate. PHA content was determined using the methanolysis-GC method and total protein was measured using the bicinchoninic acid protein kit (Sigma Procedure No. TORO-562, Sigma Chemicals, St. Louis, MO).

RESULTS AND DISCUSSION

Effects of TP inside bacterial cells on PHA production

Punrattanasin *et al*. (2001) demonstrated that the 2-stage bioprocess approach could be used to stimulate PHA production up to 70%TSS by mixed cultures of activated sludge biomass under fully aerobic conditions with multiple periods of simultaneous nitrogen and phosphorus limitations. In the study by Punrattanasin *et al*. (2001), the activated sludge biomass was obtained from a conventional fully aerobic wastewater treatment plant. From an economical point of view, PHA production cost could be minimized if the production time could be reduced. A possible way to reduce PHA production time would be by eliminating the growth phase of the 2-stage PHA production system, i.e., reduce it to a one-stage bioprocess. A series of experiments were conducted to assess the feasibility of this approach.

Phase 1: One-stage PHA production system

Two SBR reactors were operated under the same conditions as used by Punrattanasin *et al*. (2001), i.e., fully AE with P limitation and fully AE with simultaneous N&P limitations. However, a normal growth phase was not included as part of the cultivation approach. Limitation of either P or N&P were introduced upon start up of the production systems. As can be seen from Figures 1 and 2, PHA production increased rather slowly. The maximum PHA produced under fully AE with N&P limitations was 33%TSS compared to 57%TSS obtained by Punrattanasin *et al*., 2001 when the system was operated using the 2-stage bioprocess. Maximum PHA production under P limitation was low (22%TSS). In summary, the performance of the one stage PHA production system was significantly lower than that of the 2-stage bioprocess system used by Punrattanasin *et al*. (2001). It was speculated that the activated sludge bacteria from the full-scale plant might have some phosphorus stored inside the cells, with the result that they still have a source of utilizable P, and the systems were never operated under true N&P or P limitation conditions in these experiments. To support this hypothesis, the systems were restarted and the TP contents inside the bacterial cells were monitored along with other parameters.

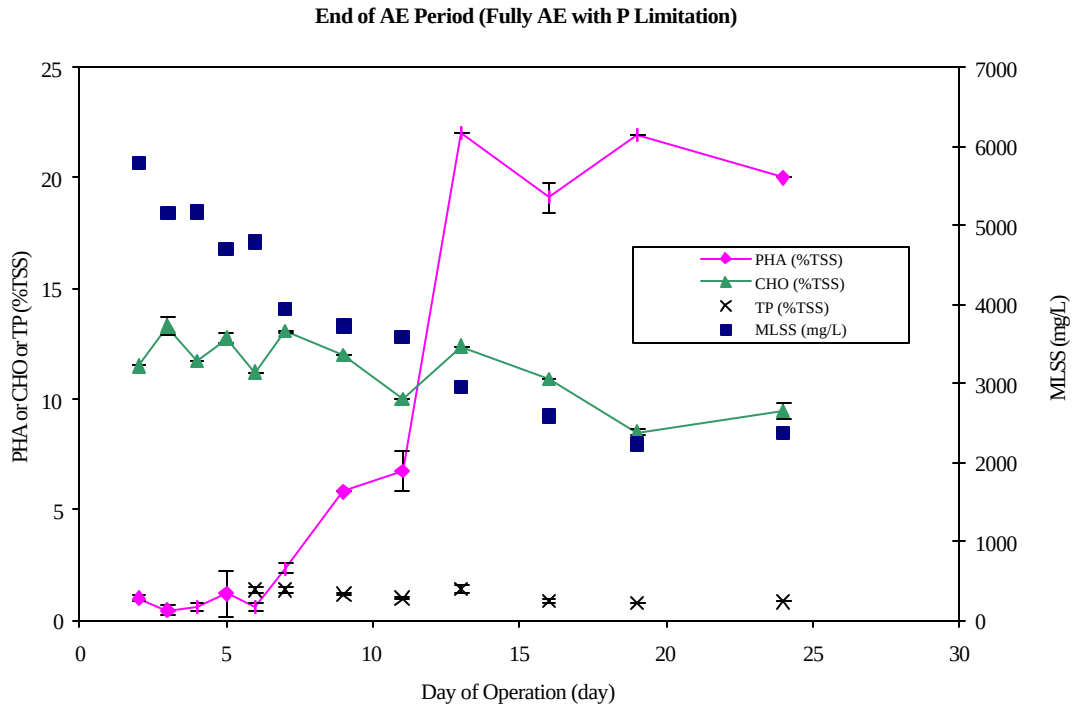


Figure 1: Changes in PHA, MLSS, Carbohydrate, and TP: One-Stage Production System

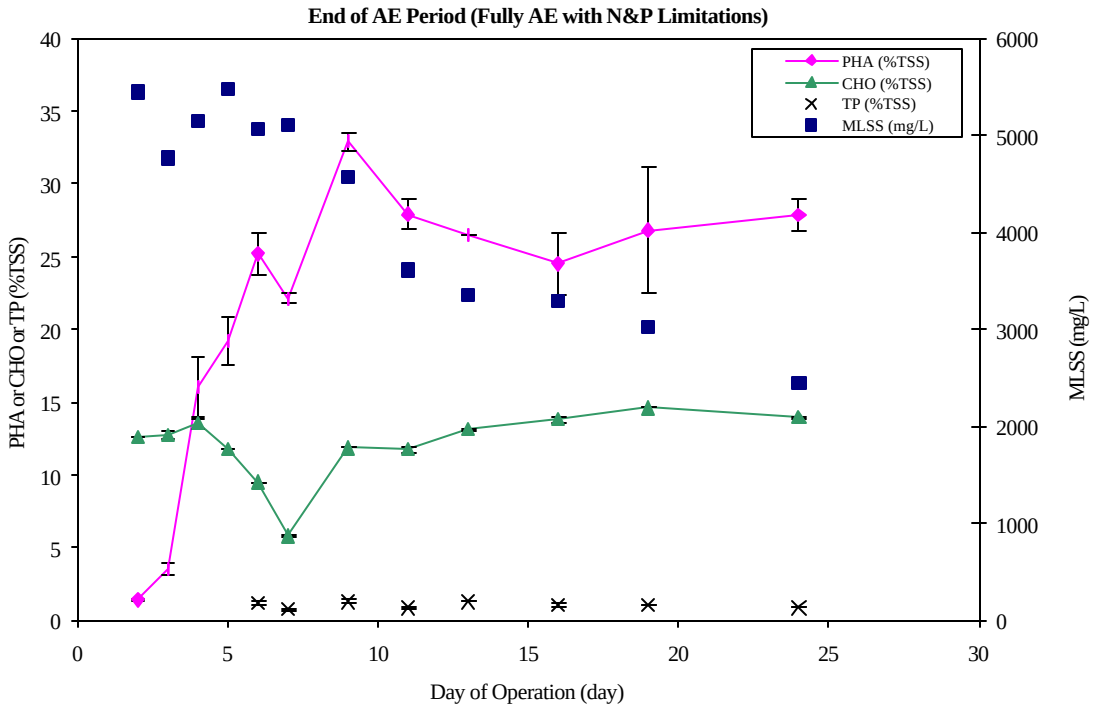


Figure 2: Changes in PHA, MLSS, Carbohydrate, and TP: One-Stage Production System

Phase 2: The correlation of TP content and PHA production

Two SBRs reactors were operated to investigate the potential correlation of the TP content inside the bacterial cells with PHA production after phosphorus inputs had been eliminated. The first system (System 1) was operated under fully AE conditions while utilizing a growth phase, as in the 2-stage bioprocess, to minimize the TP content of the bacterial cells before starting the nutrient-limited PHA accumulation phase. The seeding sludge for System 1 was obtained from the conventional fully aerobic wastewater treatment plant used for the Phase 1 study. However, System 2 was operated under anaerobic/aerobic (AN/AE) conditions with N&P limitations. The seeding sludge for System 2 was obtained from the last aerobic reactor of a BPR pilot plant, where TP content inside the bacterial cells would be at a maximum. The system was operated without a growth phase to enable investigation of PHA production when the biomass contains large amounts of TP inside the cells at the beginning of the nutrient limiting period.

The growth phase of System 1 was similar to that used by Punrattanasin *et al*. (2001). The TP contents of the cells during the growth phase ranged from 1.9 to 2.7%TSS, except for a single value of 4.7%TSS obtained on 9/15/00. A maximum PHA content of 46%TSS, which coincided with the lowest TP content of 0.6%TSS, was obtained 7 days after N&P additions were eliminated. Maximum PHA production was 1827 mg/L as compared to 1988 mg/L reported by Punrattanasin *et al*. (2001). The yield of PHA per unit substrate was 0.24 mgPHA/ mgCOD, as compared to 0.39 mgPHA/mgCOD reported by Punrattanasin *et al*. (2001). The cellular TP content corresponded well with PHA production, i. e., %PHA increased as %TP decreased as shown in Figure 3. The PHA concentration reached the maximum content and then declined. Next, the system was operated under normal growth phase conditions for a time, then nutrients were limited a second time. During the second N&P limitation period, the PHA content reached a maximum of 70%TSS, and the maximum occurred when the TP content was at the minimum value of 0.9%TSS. These values were observed 5 days after N&P additions were eliminated. Maximum PHA production and productivity were 2265 mg/L and 453 mg/L/day, respectively, and the yield of PHA per unit substrates was 0.34 mgPHA/ mgCOD. PHA production, biomass concentration, TP, and cellular carbohydrate contents during the System 1 experiments are shown in Figure 4.

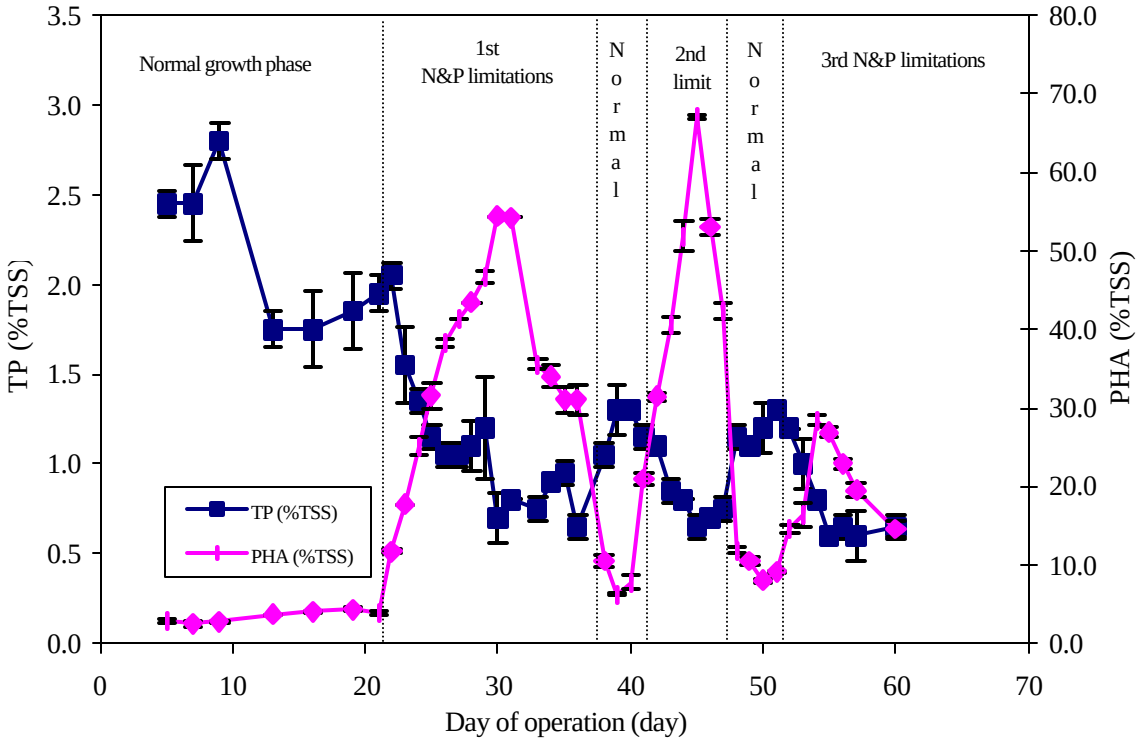


Figure 3: Profiles of TP and PHA under Fully AE with Multiple N&P Limitations

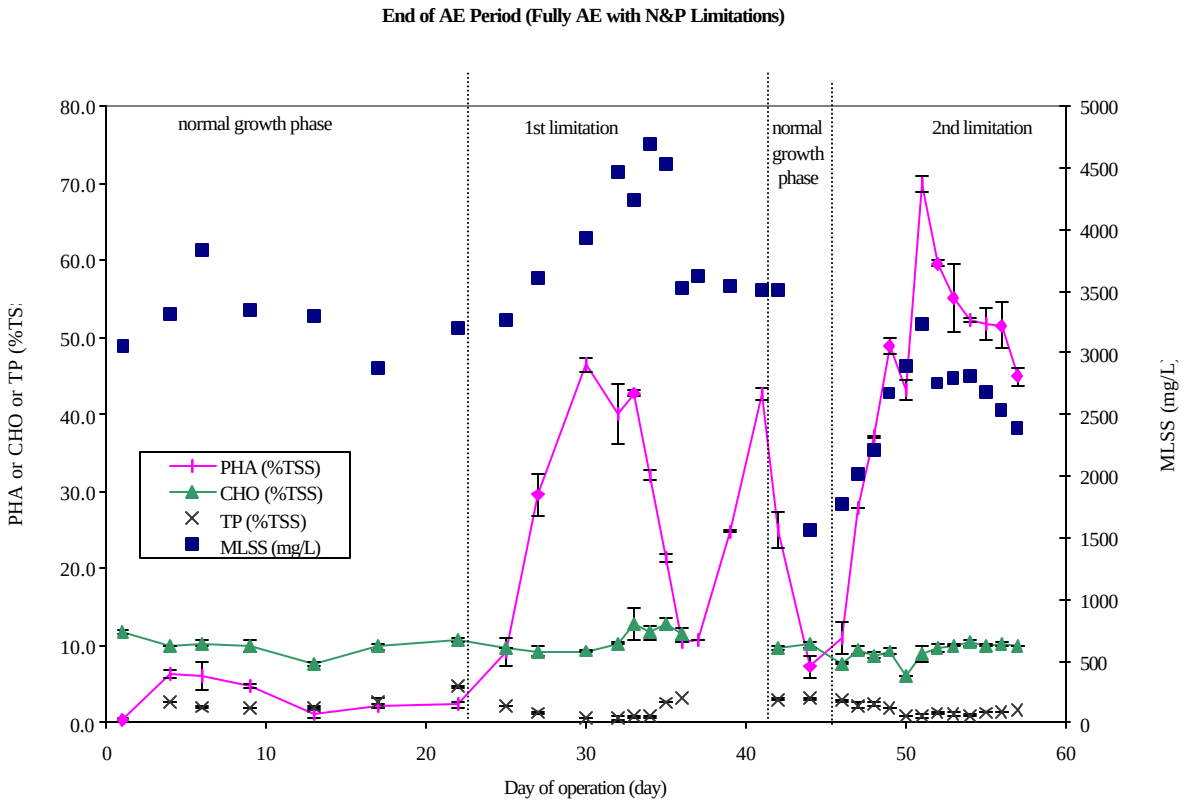


Figure 4: The Production of PHA, MLSS, Carbohydrate, and TP: Conventional Fully AE Seed

In System 2, the maximum PHA content obtained was 25%TSS. Additionally, a longer production time (16 days) was required to reach the maximum PHA value after N&P were eliminated from the feed. The PHA content reached the maximum, then gradually declined. The TP cellular concentrations during the limitation phase were in the range of 1.0 to 1.9 %TSS. The yield of PHA per unit substrate was low, i.e., 0.03 mgPHA/ mgCOD. The system was then returned to fully aerobic growth phase operation because high %PHA was not achieved under AN/AE conditions. During the first N&P limitations after fully aerobic conditions were implemented, a maximum PHA content of 22%TSS and a TP content of 1.0%TSS were obtained 9 days after N&P limitations began. The yield of PHA per unit substrate was 0.09 mgPHA/mgCOD. Then, the system was operated under normal growth phase conditions for a period, and nutrients were limited a second time under fully AE conditions. During the second N&P limitation period, a maximum PHA content of 30%TSS and a TP content of 0.8%TSS were observed 8 days after N&P limitations began. The yield of PHA per unit substrate was 0.10 mgPHA/ mgCOD. The system was returned to operation under normal growth phase conditions again, then N&P additions were eliminated a third time. This time the maximum PHA content was 34%TSS and the TP content was 0.6%TSS 6 days after N&P addition was stopped. The yield of PHA per unit substrate was 0.15 mgPHA/mgCOD. Figure 5 illustrates the trends for PHA production, biomass concentration, TP content, and carbohydrate content of the biomass in System 2 during this series of manipulations. As can be seen from Figure 5, PHA production time was reduced, while PHA content and yield of PHA per unit substrate were increased when the system were subjected to multiple N&P limitations. However, the desired high PHA content was not achieved when the starting biomass was obtained from the BPR system.

In summary, it appears to be important to acclimate the biomass before starting the PHA accumulation phase because activated sludge obtained from a wastewater treatment plant usually contains TP inside the bacterial cells. Even though the activated sludge biomass may be obtained from a conventional fully aerobic system, the biomass still could contain high TP concentrations if there was an insufficient supply of oxygen in the aeration tanks, resulting in anaerobic conditions inside the activated sludge biomass flocs. Therefore, phosphorus release and uptake could occur in the aeration tanks of the system, and result in unintentional BPR performance. This phenomenon is usually observed during summer months where biomass

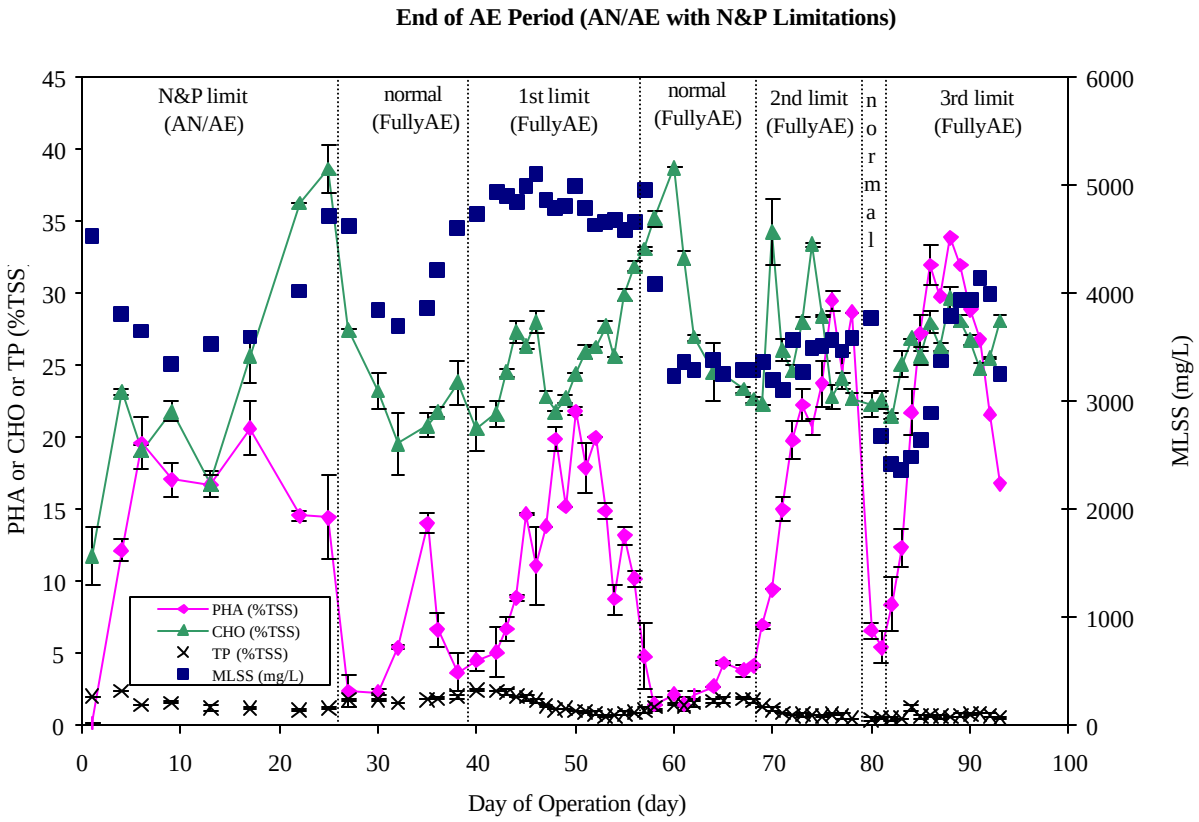


Figure 5: The Production of PHA, MLSS, Carbohydrate, and TP: BPR Seed

growth is rapid and oxygen consumption is high. Therefore, a growth phase is required to grow a bacterial population that contains minimal TP before starting the subsequent PHA accumulation phase. Stated another way, the 2-stage bioprocess is more suitable than the 1-stage process for PHA production. It is suggested that the TP content inside the bacterial cells should be monitored under growth phase conditions. The subsequent PHA accumulation phase should be started once TP content is at a minimum concentration. High PHA production could not be achieved when the system was originally seeded with the BPR sludge. It is possible that bacterial populations from BPR systems are more suited for carbohydrate production when they are subjected to nutrient limiting conditions. For example, significantly higher cellular carbohydrate contents were detected in System 2 during Phase 2 of the study. According to Lee and Choi (1999), some bacteria can survive in a BPR system even though they do not accumulate TP and use it as an energy source in the anaerobic phase. These bacteria obtain energy under the anaerobic phase by the glycolysis of intracellular carbohydrates such as glycogen. The prevalence of these bacteria could result in the deterioration of BPR processes. These bacterial populations are usually referred to as glycogen accumulating organisms (GAOs). Therefore, nutrient limiting conditions should be avoided in BPR systems in order to prevent the prevalent of GAOs. The effects of carbohydrate content on PHA production will be discussed in detail in the next section.

Phase 3: Effect of a long growth phase on PHA production

Two SBRs systems were operated under the same conditions as used in Phase 1. However, the PHA accumulation phase was delayed due to the installation of data acquisition systems in the laboratories. For commercial PHA production, a long operation of growth phase has been recommended to increase the biomass concentration before starting the PHA accumulation phase. According to Preusting *et al*. (1993), two possible approaches can be used to improve the economics of PHA production: 1) increase the %PHA accumulated, and 2) increase the biomass concentration of the PHA producing bacteria. Therefore, they suggest that long growth phase periods of operation could be used to achieve high PHA production by increasing the biomass concentration in the PHA production system.

During this phase of study, the systems were operated with a long growth phase of 35 days, in comparison to 23 days used by Punrattanasin *et al*. (2001). The maximum PHA content of 36%TSS was obtained 6 days after N&P were eliminated and the biomass concentration was 2488 mg/L. The maximum PHA content and biomass concentration obtained by Punrattanasin *et al*. (2001) were 57%TSS and 3250 mg/L, respectively. Yield of PHA per unit substrates in this study was 0.23 mgPHA/mgCOD. The PHA content of the system operated with a long growth phase under P limitation was 10% lower throughout the course of the 40-day experiment. According to Kim *et al*. (1994), when the culture of *R. eutropha* NCIMB 11599 was maintained under growth phase for a longer period of time to achieve a higher cell concentration (90 g/L) before starting N limitation, high PHA production was not achieved. The author stated that the culture was unstable but the cause of this failure was unknown.

Effects of intracellular carbohydrate content on PHA production

The production of intracellular carbohydrate was an issue of interest because a wide variety of microorganisms can accumulate intracellular carbohydrates under nutrient limitating conditions. Intracellular carbohydrates include glycogen and glycogen-like materials. According to Dawes (1985), intracellular carbohydrates are accumulated when nitrogen is limited while there is an excess of carbon available in the medium. In addition to nitrogen limiting conditions, carbohydrates can be accumulated under sulfur limiting or phosphorus limiting conditions, or when pH for growth is unfavorable (Preiss, 1984). However, nitrogen-limiting condition was reported to be the most stimulating condition for glycogen accumulation in many organisms. The microorganisms of choice for PHA production would be ones that can convert carbon substrate to PHA, but not to other intracellular storage materials. Byrom (1992) discussed the industrial production of PHA by ICI. *Alcaligenes spp.* was an organism of choice for ICI because it produced high PHA content, and the PHA produced was easy to extract and had high molecular weight. PHA productions by *Azobacter* spp. also was investigated. However, *Azobacter* was not an organism of interest because it used carbon substrate for polysaccharide synthesis rather than for PHA production.

Intracellular carbohydrates were measured in all 3 phases of this study. As shown in Figures 1 to 3, the carbohydrate contents were in the range of 6-15%TSS when the seeded sludge were

obtained from the fully AE wastewater treatment system, even when the systems were operated using the 1-stage bioprocess approach. The lowest intracellular carbohydrate production was observed in the best PHA production system, i.e., fully AE conditions with multiple, simultaneous N&P limitations. As shown in Figure 3, carbohydrate contents in the range of 9-13%TSS were observed during the first N&P limitation period. When PHA content was further increased by subjecting the system to the second N&P limitation period, the carbohydrate content decreased to the range of 6-10%TSS. In contrast, when the seeded sludge was obtained from the BPR system (System 2 of Phase 2), intracellular carbohydrate production was high during the limitation phase, and ranged from 20-38%TSS. When System 2 was subsequently operated under fully AE conditions, the high intracellular carbohydrate concentrations were still maintained as shown in Figure 4. It clearly illustrates that the source of the biomass used for PHA production has a great impact upon production. Therefore, it is important to select the proper source of seeding sludge for a PHA production system using mixed cultures of activated sludge biomass.

As discussed by Punrattanasin *et al*. (2001), operating conditions had a significant impact on the PHA production and its composition. Intracellular carbohydrate contents were also different under different operating conditions. Table 1 summaries the productions of intracellular carbohydrates under different operating conditions. The best PHA production system would be the one that promotes the conversion of organic substrates to PHA, not to intracellular carbohydrates. The Table 1 results show that both AN/AE and MAA/AE cycling produced PHA concentrations as high as 62%TSS, while fully AE conditions with N&P limitations achieved a PHA content of 57%TSS during the first limitation period. When an additional experiment was run, the fully AE conditions with N&P limitations achieved a PHA concentration of 46%TSS during the first period of nutrient limitation, but it increased to 70%TSS during the second period of nutrient limitation. Also note that the average carbohydrate content of the AN/AE experiments was 19.3%TSS, while the carbohydrate content of the MAA/AE experiments averaged 18.8%TSS. By contrast, the fully AE conditions with N&P limitations had carbohydrate concentrations that averaged only 12%TSS, and the carbohydrate content of the fully AE sludge after the second N&P limitation period was only 8.9%. The results indicate that

fully AE conditions with multiple N&P limitations are the best operating conditions for a high PHA production system using mixed cultures of activated sludge biomass.

Copolymer composition

According to Punrattanasin *et al*. (2001), polymer compositions, i.e., the PHV/PHA ratios of the copolymers produced were different under different operating conditions. In this study, copolymers produced were also different under different cultivation approaches, i.e., PHV/PHA ratios obtained from the 1-stage bioprocess were different from those of the 2-stage bioprocess. Table 2 summarizes the PHV/PHA ratios of copolymer produced from this study.

Table 1: Summary of Intracellular PHA & Carbohydrate Contents Under Different Operating Conditions

Operating condition	Maximum PHA achieved (%TSS)	Carbohydrate content on day of max. PHA (%TSS)
AN/AE cycling w/ N&P limitations	47	17.8
MAA/AE cycling w/ partial N limitation	39	12.9
MAA/AE cycling w/ P limitation	62	24.6
AN/AE cycling w/ P limitation	62	20.9
Fully AE w/ P limitation	53	22.2
Fully AE w/ N&P limitations	57	12.6
Fully AE w/ N limitation	42	10
Fully AE w/ partial N&P limitations	43	17.2
Fully AE w/ multiple N&P limitations		
- first limitation	46	9.3
- second limitation	70	8.9
AN/AE cycling w/ multiple N&P limitations		
- first limitation under AN/AE cycling	21	25.6
- first limitation under fully AE	22	24.4
- second limitation under fully AE	30	21.3
- third limitation under fully AE	34	32.2

Table 2: PHV/PHA Ratios of the Copolymers Produced in Fully Aerobic Reactors

	PHV/PHA Ratio (%)
Phase 1: 1-stage PHA production system	
- Fully AE with N&P limitations	51
- Fully AE with P limitation	77
Phase 2: 2-stage PHA production system	
- Fully AE with N&P limitations (first limitation)	43
- Second limitation	50
Phase 3: 2-stage PHA production system (long growth phase)	
- Fully AE with N&P limitations	44
- Fully AE with P limitation	68-74

Purification process using chloroform extraction and physical properties determinations using GPC and DSC

Solvent extraction is commonly used on a small scale for laboratory experiments as well as on a large scale for commercial productions. It is a widely used method because it is applicable to many PHA producing microorganisms. However, a large amount of solvents must be employed due to a highly viscous PHA solution. According to Lee (1996), approximately 20 parts of solvent is employed to extract 1 part of polymer. This requirement makes solvent extraction a costly method. PHAs are soluble in solvents, e.g., chloroform, methylene chloride or 1,2-dicholoroethane. These 3 solvents can be used to extract PHA from bacterial biomass. In addition, other solvents have been used to extract PHA, e.g., ethylene carbonate, 1,2-propylene carbonate, mixtures of 1,1,2-trichloroethane with water, and mixtures of chloroform with methanol, ethanol, acetone or hexane.

In this study, chloroform extraction was used to purify PHA from non-PHA cellular materials. PHA was extracted using a hot chloroform solution in a soxhlet apparatus for 4-6 hours. Then, the extracted PHA was separated from lipids by precipitating with methanol, resulting in a white precipitate of the purified PHA, which was collected via filtration. The degree of purity was then determined by measuring %PHA using a methanolyis-GC method (Punrattnasin *et al*., 2001) and by determining the protein content of the purified samples. The PHA concentrations of the purified samples were in the range of 93.7% to 103.4%, while the protein concentrations were approximately 1% or less. Measured %PHA of greater than 100% in this study logically resulted from the variability of the analytical procedure (methanolyis-GC method). The variability of the methanolyis-GC method will be discussed shortly. The results showed that chloroform extraction separated PHA from non-PHA cellular materials very well. This finding is consistent with Ramsay *et al*. (1994). They examined the purity and recovery of PHA from three different chlorinated solvents: chloroform, methylene chloride, and 1,2-dichloroethane. They obtained the best recovery and purity when biomass was pretreated with acetone. The optimum digestion time of 15 minutes was reported for the recovery process for all three solvents. Further digestion resulted in a degradation of the molecular weight (MW) of the PHA. The degrees of purity of PHAs extracted with these 3 solvents under optimum conditions were 96, 95, and 93%,

respectively. Table 3 summarizes %PHA of the purified samples after chloroform extraction of this study. The degrees of purity of purified PHA were 93% or higher.

The degree of recovery in this study was approximately 40-60%, e.g., when 2 gram biomass samples that contained 50% PHA were extracted, approximately 400-600 mg of purified PHA was obtained at the end of the purification process. The low degree of recovery was due to the difficulty of separating the purified PHA from the filter paper, i.e, part of the purified PHA attached to the filter paper. Therefore, the true degree of recovery was calculated by subtracting the mass of the biomass that remained after chloroform extraction from the original mass used at the beginning of the extraction process. The true degree of recovery ranged from 82-89%. It is recommended that a better separation process be developed and employed to obtain a higher degree of PHA recovery.

Table 3: %PHA of purified samples

	Original heat dry sample			Purified sample		
	PHB(%TSS)	PHV(%TSS)	PHA(%TSS)	PHB(%TSS)	PHV(%TSS)	PHA(%TSS)
AE (AN/AE w/ P limitation) 10/18/99	23.8	29.5	53.3	37.1	64.7	101.8
AE (AN/AE w/ P limitation) 10/18/99	25.6	26.5	52.1	38.5	64.9	103.4
MAA (MAA/AE w/ P limit) 10/23/99-10/26/99	38.2	27.3	65.5	42.3	58.4	100.7
MAA (MAA/AE w/ P limit) 10/23/99-10/26/99	34.9	28.3	63.2	41.9	57.8	99.7
AE (fully AE w/ multiple N&P limitations 10/17/00	29.4	23.1	52.5	52.4	42.2	94.6
AE (fully AE w/ multiple N&P limitations 10/17/00	28.2	23.8	52.0	52.2	45.1	93.7
AE (fully AE w/ multiple N&P limitations 10/17/00	29.2	23.7	52.9	54.3	42.5	96.8

Another fraction of the purified PHA was used for physical properties determinations using gel permeation chromatography (GPC) and differential scanning calorimeter (DSC). The MW of the sample from the MAA/AE with P limitation and the fully AE with multiple N&P limitation reactors were 323,000 and 300,000, respectively. When the sample from the fully AE with

multiple N&P limitation reactor was pretreated with acetone (2 grams of lyophilized biomass was mixed with 20 mL of acetone for 20 minutes, then the biomass was recovered by filtration) prior to the chloroform extraction, the high MW of 1,051,000 was obtained. Acetone removed most of the non-PHA cellular material making PHA easy to extract. During the extraction process, isolated PHA immediately dissolved in chloroform and therefore protected the polymer from degradation. A MW of greater than 250,000 has been reported to be acceptable for industrial applications. Table 4 summaries molecular weights of PHA produced by pure cultures. The melting point temperature of the sample from the MAA/AE with P limitation reactor was 75°C. The PHV fraction of this particular sample was in the range of 42-45%. The melting point temperature of a commercial standard containing 24% PHV (Sigma Chemicals, St. Louis, MO) was also measured. Its melting point temperature was approximately 110°C. According to the literature, PHB homopolymer (100% PHB) has a melting point temperature of approximately 179°C. Byrom (1990) summarized two major advantages of the PHB-PHV copolymer over the PHB homopolymer. Firstly, the copolymer has a lower melting point. However the HV content in the copolymer does not significantly decrease the temperature at which molecular weight degradation occurs. This important feature allows the copolymer to be processed over a larger range of temperature conditions. Secondly, it has a lower flexural modulus or level of crystallinity, which makes it tougher and more flexible.

Measure of analytical variability of PHA measurement (methanolysis-GC method)

Variability of PHA measured could occur because of the analytical method used (methanolysis-GC method). During the study, a minimum of 2 analyses were made for each sample to represent each data point of PHA measured. Error bars representing analytical variability based on plus and minus one standard deviation of the sample mean were performed to represent the degree of variability of the data as shown in all figures.

To better quantify the analytical variability, two PHA samples from the fully aerobic with N&P limitations reactor were collected and analyzed. Each sample was separated into 7 small fractions. The PHA content of each fraction was determined and the sample mean, standard deviation, and coefficient of variation of each sample were calculated and are summarized in

Table 5. The coefficient of variation is defined as: (100*standard deviation of the sample set)/ mean value.

Table 4: Molecular Weights of PHA Produced by Pure Cultures

Microorganism	Type of PHA	Carbon Source	MW * 10^{-5}
Alcaligenes eutrophus	PHB	Fructose	7.37
	PHB	Butyric acid	4.32
	PHB	Glucose	9.0-20
	PHB	Glucose	21-23
	P(3HB-co-3HV)	Pentanoic acid	2.54
Bacillus megaterium	PHB	Glucose	1.66
Zoogloea ramigera	PHB	Glucose	5.42
Protomonas extorquens	P(3HB-co-3HV)	Propanol	4.37
Azotobacter vinelandii	PHB	Glucose and fish peptone	17-28
	PHB	Beet molasses	10-45
Methylobacterium extorquens	PHB	Methanol	2.0-6.0
	PHB	Methanol	1.7-6.2
	PHB	Methanol and ethanol	3.2
	PHB	Methanol and glycerol	2.6-3.9
	PHB	Methanol and fructose	11.1-11.3
	PHB	Sodium succinate	7.2-16.6
	PHB	Sodium succinate	9.0-17.0
Pseudomonas 135	PHB	Methanol	2.6-3.7
Pseudomonas cepacia	PHB	Glucose, xylose, and lactose	2.2-8.7

Table 5: Measure of Analytical Variability of PHA Measurement

Sample 1 (12/8/00)	PHA (%TSS)	Sample 2 (12/9/00)	PHA (%TSS)
Fraction 1	36.2	Fraction 1	32.4
Fraction 2	35.2	Fraction 2	34.5
Fraction 3	35.1	Fraction 3	35.3
Fraction 4	36.9	Fraction 4	34.8
Fraction 5	34.4	Fraction 5	37.3
Fraction 6	35.2	Fraction 6	34.6
Fraction 7	36.1	Fraction 7	35.1
Sample Mean	35.6	Sample Mean	34.9
Standard Deviation	0.85	Standard Deviation	1.44
Coefficient of Variation	2.39	Coefficient of Variation	4.13

Measurement of Total Variability in PHA Production

The biological nature of the mixed culture of activated sludge biomass as well as sampling effects would also cause variability. The total variability in PHA production determinations was measured through 3 independent replicates from the fully AE with N&P limitation reactors.

Figure 6 illustrates PHA production over time for those 3 reactors. Maximum %PHA produced from each reactor, sample mean, standard deviation, and coefficient of variation are summarized in Table 6.

Table 6: Measurement of Total Variability in PHA Production Determinations

	Max PHA Produced (%TSS) (first limitation)	Max PHA Produced (%TSS) (second limitation)
Reactor 1	57	-
Reactor 2	46.4	70
Reactor 3	54.4	67
Sample Mean	52.6	68.5
Standard Deviation	5.5	2.1
Coefficient of Variation	10.5	3.1

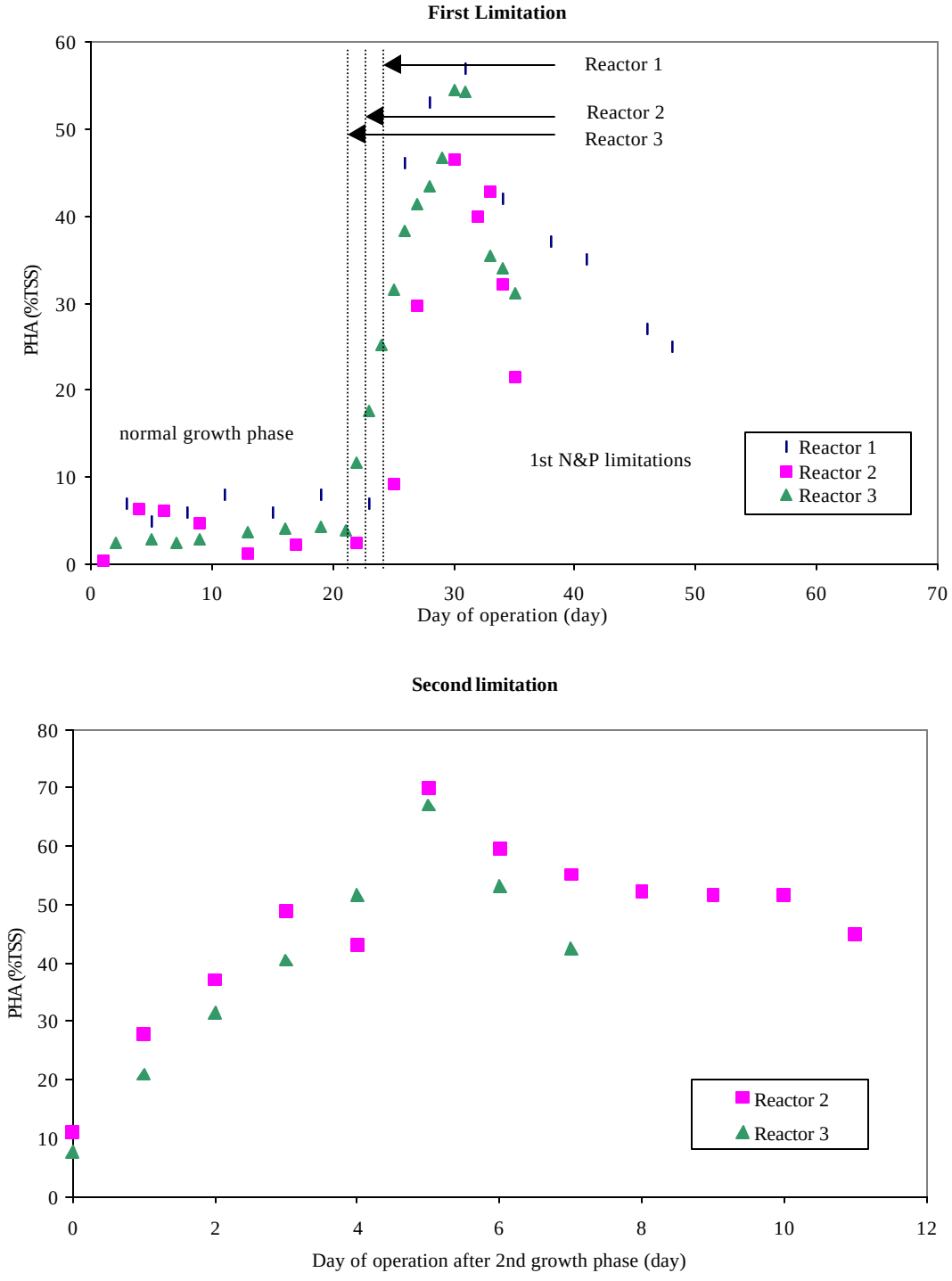


Figure 6: Measurement of Total Variability in PHA Production

SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate parameters that affect high PHA production by mixed cultures of activated sludge biomass, and identify those that could be used to control the production process. The results have shown that the TP content inside the bacterial cells at the beginning of nutrient limitation and the production of intracellular carbohydrates are the most important parameters affecting high PHA production by activated sludge biomass. The results also show that the physical properties of PHA produced by activated sludge biomass during this study are comparable to those obtained from commercial production by pure cultures. Several conclusions can be drawn from this study.

1. The TP content of the bacterial cells during the PHA accumulation phase had a significant impact on PHA production by activated sludge biomass. The two-stage bioprocess was a better approach for obtaining high activated sludge PHA accumulation because a growth phase was necessary to grow a bacterial population that contains minimal TP before starting the subsequent PHA accumulation phase. However, an overlong growth phase may have caused an adverse effect on PHA production.
2. It is recommended that cellular TP be monitored during the growth phase so that the subsequent PHA accumulation phase can be started when the TP content is at a minimum.
3. Seeding sludge obtained for a conventional fully aerobic activated sludge wastewater treatment system was more suitable for PHA production than seed obtained from a BPR system, because bacterial population from the BPR system tended to convert organic substrates to intracellular carbohydrate content rather than PHA under nutrient limitation conditions.
4. The PHA production approach used (1-stage versus 2-stage bioprocess) not only has an impact on PHA production, but also on its composition, i.e., PHV/PHA ratio.
5. PHA of high purity can be obtained from activated sludge biomass with high % recovery using the chloroform extraction method.
6. PHA produced by activated sludge biomass tends to have a high molecular weight and a low melting point temperature, and is highly suitable for biodegradable plastics production.

REFERENCES

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiological Reviews.* 54 (4), 450-472.

Braunegg, G., Lefebvre, G., and Genser, K. (1998). Polyhydroxyalkanoates, Biopolyesters From Renewable Resources: Physiological and Engineering Aspects. *Journal of Biotechnology*. 65, 127-161.

Byrom, D. (1990). Industrial production of Copolymer from *Alcaligenes eutrophus*. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 113-117.

Byrom, D. (1992). Production of Poly-β-hydroxybutyrate: Poly-β-hydroxyvalerate Copolymers. *FEMS Microbiology Reviews.* 103, 247-250.

Choi, J and Lee, S. Y. (1999). Factors Affecting the Economics of Polyhydroxyalkanoates Production by Bacterial Fermentation. *Appl. Microbiol. Biotechnol*. 51, 13-21.

Chua, H., Yu, P. H. F., and Ho, L. Y. (1997). Coupling of Wastewater Treatment with Storage Polymer Production. *Appl. Biochem. and Biotech*. 63, 627-653.

Dawes, E. A. (1985). Starvation, Survival and Energy Reserves. In: *Bacteria in Their Natural Environment*, Fletcher, M. and Floodgate, G. D. (eds.), Academic Press, Florida, 43-79.

Dawes, E. A. (1990). Novel Microbial Polymers: An Introductory Overview. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 3-16.

Dawes, E. A. and Senior, P. J. (1973). The Role and Regulation of Energy Reserve Polymers in Micro-organisms. *Advances in Microbial Physiology*. 10, 135-266.

Doi, Y. (1990). *Microbial Polyesters*. VCH Publishers, Inc. New York, USA.

Huang, J., Shetty, A. S., and Wang, M. (1990). Biodegradable Plastics: A Review. *Advances in Polymer Technology*, 10(1), 23-30.

Hart, V. S. (1994). An Examination of Biological Phosphorus Removal Using Bacterial Counting and Poly-beta-hydroxybutyrate Analysis in Batch and Continuous Flow Systems. Ms.Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Kim, B. S., Lee, S. C., Lee, S. Y., Chang, H. N., Chang, Y. K., and Woo, S. I. (1994). Production of poly(3-hydroxybutyric acid) by *R. eutropha* with glucose concentration control. *Biotechnology and Bioengineering*, 43, 892-898.

Lafferty, R. M., Korsatko, B., and Korsatko, W. (1988). Microbial Production of Poly-β-hydroxybutyric Acid. In: *Biotechnology*, Rehm, H. J. and Reed, G. (eds.), VCH Publishers, New York, USA, 135-176.

Lee, S. Y. (1996). Bacterial Polyhydroxyalkanoates. *Biotechnology and Bioengineering*. 49, 1-14.

Lee, S. and Yu, J. (1997). Production of Biodegradable Thermoplastics from Municipal Sludge by a Two-Stage bioprocess. *Resources, Conservation and Recycling*. 19, 151-164.

Lee, S. Y. and Choi, J. (1999). Production and Degradation of Polyhydroxyalkanoates in Waste Environment. Waste Management. 19, 133-139.

Lemos, P. C., Vianna, C., Salgueiro, E. N., Ramos, A. M., Crespo, J. P. S. G., and Reis, M. A. M. (1998). Effect of Carbon Source on the Formation of Polyhydroxyalkanoates (PHA) by a Phosphate-Accumulating Mixed Culture. *Enzyme and Microbial Technology*. 22, 662-671.

Ma, C. K., Chua, H., Yu, P. H. F., and Hong, K. (2000). Optimal Production of Polyhydroxyalkanoates in Activated Sludge Biomass. *Appl. Biochem. and Biotech.* 84-6, 981-989.

Madigan, M. T., Martinko, J. M. and Parker, J. (1997). *Brock Biology of Microorganisms.* Princeton Hall, New Jersey.

Page, W. J. (1995). Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics With a Great Future. *Can. J. Microbiol*. 41(suppl. 1), 1-3.

Preiss, J. (1984). Bacterial Glycogen Synthesis and Its regulation. *Ann. Rev. Microbiol*. 38, 419-458.

Preusting, H., Hazenberg, W., and Witholt, B. (1993). Continuous Production of Poly(3-hydroxyalkanoates) by Pseudomonas oleovorans in a High-Cell-Density, Two-Liquid-Phase Chemostat. *Enzyme Microb. Technol*. 15, 311-316.

Punrattanasin, W., Randall, C. W., and Randall, A. A. (2001). Production of Polyhydroxyalkanoates for Biodegradable Plastics Using Activated Sludge Biomass:

System Development. Ph.D. Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Ramsay, J. A., Berger, E., Voyer, R., Chavarie, C., and Ramsay, B. A. (1994). Extraction of poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology Techniques*. 8, 589-594.

Satoh, H. (1998). Department of Urban and Environmental Engineering, University of Tokyo. 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Yu, P. H. F., Chua, H., Huang A. L., Lo W. H., and Ho, K. P. (1999). Transformation of Industrial Food Wastes Into Polyhydroxyalkanotes. *Water Science and Technology*. 40 (1), 365-370.

MANUSCRIPT 3
PRODUCTION OF POLYHYDROXYALKANOATES FOR BIODEGRADABLE PLASTICS USING ACTIVATED SLUDGE BIOMASS: UTILIZATION OF AN INDUSTRIAL WASTEWATER AND ECONOMIC ANALYSIS

Warangkana Punrattanasin[1], Clifford W. Randall[1], and Andrew A. Randall[2]

[1]*Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.*
[2]*Department of Civil and Environmental Engineering, University of Central Florida, Orlando, FL 32816-2450, U.S.A.*

ABSTRACT

Laboratory scale sequencing batch reactors were set up to investigate high PHA production using the high acetic acid industrial wastewater currently discharged and treated by the Celanese Acetate Corporation plant located at Narrows, VA. The Celanese wastewater was composed mainly of acetic acid with a total chemical oxygen demand of approximately 1800 mg/L. The purpose of the study was to determine if it is possible to feed this wastewater to a culture of wastewater bacteria under nutrient limited conditions and produce PHAs of sufficient quality and quantity for use as the raw material for the production of biodegradable plastics. Optimum cultivation strategy was investigated during the early phase of the study. It was found that the one-stage bioprocess is a better approach than the two-stage bioprocess for PHA production using Celanese wastewater as the substrate. Bioprocess operating conditions were found to have a significant effect on PHA production using Celanese wastewater. Fully aerobic (AE) reactor conditions with the original mixture of wastewater containing minimal concentrations of nitrogen, phosphorus, and potassium were determined to be better conditions for PHA production compared to fully AE with either N&P limitations or P limitation. The maximum PHA content, biomass concentration, and PHA productivity were 40%TSS, 5766 mg/L, and 577 mg/L/day, respectively. Punrattanasin *et al*. (2001a) found that the enrichment of PHA producing bacteria by operating under alternating periods of growth and N&P limitation conditions was an effective way to achieve high PHA production when the substrate was a mixture of acetic and propionic acid. However, it was not successful when the Celanese wastewater was the substrate. Also, the PHV/PHA ratios of the copolymers produced could not be increased significantly by operating the system under P limitation as reported by

Punrattanasin *et al.* (2001a). It is believed the negative results were because of the high acetic acid concentrations in the wastewater. According to the review of PHA economics, important factors affecting the economics of PHA production include cellular PHA content, PHA productivity, PHA yield per unit substrate, and substrate cost. In order to optimize the economics of PHA production using Celanese wastewater, it is important that the cellular PHA content produced be increased. It is recommended that the utilization of Celanese wastewater as a substrate for pure cultures, i.e., *R. eutropha, A. latus* or recombinant *E. coli* be investigated. It also is recommended that fermented domestic sewage be investigated as a potential substrate for PHA production using activated sludge biomass.

KEYWORDS

Biodegradable plastics production; activated sludge; one-stage bioprocess; two-stage bioprocess; high acetic acid industrial wastewater; nutrient limitation; polyhydroxyalkanoates; polyhydroxybutyrates; polyhydroxyvalerates

INTRODUCTION

A laboratory scale high PHA production system using activated sludge mixed microbial cultures was successfully developed in the Virginia Tech Environmental Engineering Laboratories utilizing a mixture of acetic and propionic acids as a substrate (Punrattanasin *et al*. 2001a & b). The main objective of this study was to test the high PHA production system using an industrial wastewater as the substrate. Specifically, the high acetic acid industrial wastewater currently discharged and treated by the Celanese Acetate plant located at Narrows, Virginia was used as an inexpensive substrate for PHA production. This Celanese plant currently manufactures cellulose acetate fibers, producing them from wood fibers and acetic acid. The manufacturing process results in the discharge of a very strong wastewater that is both an environmental and an economical liability. This wastewater is very expensive to treat to the extent required before discharge into the environment. Also, it is known that most of the current "so-called" biodegradable plastics on the market are not genuinely biodegradable, but simply fragment into smaller, non-biodegradable pieces. If the utilization of the Celanese wastewater for the production of biodegradable plastics is feasible, then the treatment liability could be turned into

an asset, not only for Celanese Acetate, but for the environment in general as truly biodegradable plastics replace the dominant poorly-biodegradable forms.

The Celanese wastewater utilized was composed dominantly of acetic acid with a total chemical oxygen demand (COD) of approximately 1800 mg/L. Other compounds in the wastewater, listed in order of magnitude, included isopropyl alcohol, acetone, isopropyl acetate, methyl ethyl ketone, and mesityl oxide. The concentrations of ammonia-nitrogen, phosphorus, and potassium were minimal, i.e., NH_4-N = 0.7 mg/L, PO_4-P not detectable, K^+ = 0.2 mg/L. The purpose of this study was to determine if it is possible to utilize this wastewater as a substrate for activated sludge biomass under nutrient limited conditions and produce PHAs of sufficient quality and quantity for use as the raw material for the production of biodegradable plastics. Finally, an economic analysis of PHA production was developed, discussed and summarized.

MATERIALS AND METHODS

Reactor set up and operations

Sequencing batch reactor (SBR) laboratory systems were set up and maintained at a constant temperature of 20°C. Operating parameters of the SBR were the same as used in Punrattanasin *et al.* (2001a & b). The systems were operated under fully aerobic (AE) conditions for all experiments during this study. During the early phases of the study, different cultivation approaches were used to stimulate PHA production in order to determine the optimum cultivation technique for the Celanese wastewater. System 1 was operated using a two-stage bioprocess approach, similar to what is used for commercial PHA production. That is, optimum nutritional conditions were used to develop a high biomass concentration (growth phase). Then, nutrients were limited to stimulate PHA production (accumulation phase). System 2 was operated using a one-stage bioprocess approach, i.e., the system was operated without a growth phase. During the PHA accumulation phase, both systems were fed with the mixture of wastewater containing minimal concentrations of nitrogen (N), phosphorus (P), and potassium (K). After the proper cultivation approach for Celanese wastewater was determined, the effect of K limitation on PHA production was separated from the effects of N and P, i.e., the wastewater was supplemented with potassium chloride and the system was operated under N&P limitations

(System 3). Punrattansin *at al.* (2001a) found that the composition of PHA, i.e., the PHV/PHA ratios were different under different operating conditions, and that P limiting conditions produced the PHA with the highest PHV/PHA ratio. The Celanese wastewater consisted mainly of acetic acid, therefore PHB was expected to be the majority fraction of the copolymer produced. Polymer with higher PHV fraction is known to be more desirable for industrial applications because the polymer has a greater toughness and is more flexible (Byrom, 1990). Therefore, System 4 was designed to investigate the production of PHA and its composition under P limitation conditions.

Wastewater Characteristics

The wastewater stream with minimal amounts of N, P, and K^+ was sampled and analyzed. The wastewater contained approximately 1200 mg/L acetic acid, 400 mg/L isopropyl alcohol, 100 mg/L acetone, 40 mg/L methyl ethyl ketone, and approximately 10-20 mg/L methyl cyanide. The concentrations of ammonia-nitrogen, phosphorus, and potassium were minimal, i.e., NH_4-N = 0.7 mg/L, PO_4-P not detectable, K^+ = 0.2 mg/L.

Monitoring and analytical methods

The following parameters were measured during the study: mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solid (MLVSS), total PHA, PHB, PHV, carbohydrates, total phosphorus in the biomass (TP), COD, and ammonia-nitrogen. MLSS, MLVSS, COD, and TP were analyzed according to Standard Methods for the Examination of Water and Wastewater, 19th Edition, 1995, using sections 2540D, 2540E, 5220C, and 4500-P B.5 persulfate digestion method followed by 4500-P E. ascorbic acid method, respectively. Ammonia-nitrogen was analyzed by a Dionex 120 ion chromatography (Dionex Corp., Sunnyvale, CA). PHA, PHB, and PHV was measured by the methanolysis-GC method described by Hart (1994), with some modifications. Details of the PHA measurement are summarized by Punrattanasin *et al*. (2001a). Intracellular carbohydrate content was measured using the Phenol method as outlined in Manual of Methods for General Bacteriology (American Society for Microbiology, 1981).

RESULTS AND DISCUSSION

Phase 1: Cultivation strategy for PHA production using high acetic acid industrial wastewater

This phase of the study was designed to investigate the proper cultivation approach for PHA production using Celanese wastewater. Two SBR systems were set up and operated under fully AE conditions. The Celanese wastewater fed into these 2 systems during the PHA accumulation phase contained minimum concentrations of N, P, and K. System 1 was operated using the two-stage bioprocess approach, while System 2 was operated using the one-stage bioprocess approach.

The systems were unstable the first two weeks of the operation because of problems. In addition, dissolved oxygen (DO) could not be maintained at high concentrations resulting in a low DO filamentous bulking problem. Therefore, the systems' overall hydraulic retention times were increased from 10 to 20 hours and defoamer was added to the systems to control the foaming problem.

System 1 was operated under growth phase conditions for approximately 22 days before the subsequent nutrient limited PHA accumulation phase was started. The wastewater was supplemented with ammonium chloride, potassium phosphate, and potassium chloride during the growth phase. The ammonium-N and orthophosphate concentrations in the wastewater were in the range of 35-40 mg/L NH_4-N and 10-15 mg/L PO_4-P, respectively. The profiles of biomass concentration, PHA, TP, and carbohydrate contents measured for System 1 are shown in Figure 1. A maximum PHA content of 18%TSS was obtained 2 days after the limitation phase began. The biomass concentration gradually decreased during the accumulation phase, and a biomass concentration of 856 mg/L was observed at the end of the study. Figure 2 illustrates the profiles of biomass concentration, and the PHA, TP, and carbohydrate biomass contents of System 2. A maximum PHA content of 40%TSS, and an MLSS of 5766 mg/L were obtained 4 days after the experiment began, and coincided with the lowest TP content of 0.8%TSS. Maximum PHA production and productivity of System 2 were 2309 mg/L and 577 mg/L/day, respectively. Yield of PHA per unit substrate was 0.5 mgPHA/mgCOD. The stored PHA reached the maximum content and gradually declined thereafter. System 2 was then returned to growth operation, i.e., the nutrients N, P and K were added to the wastewater. Then, the system was operated under nutrient limitations for a second time. Sludge bulking occurred 3 days after the second period of N,P and K limitations began. A lot of biomass solids were lost in the effluent discharge during this period. A maximum PHA content of 12%TSS was obtained 3 days after the second

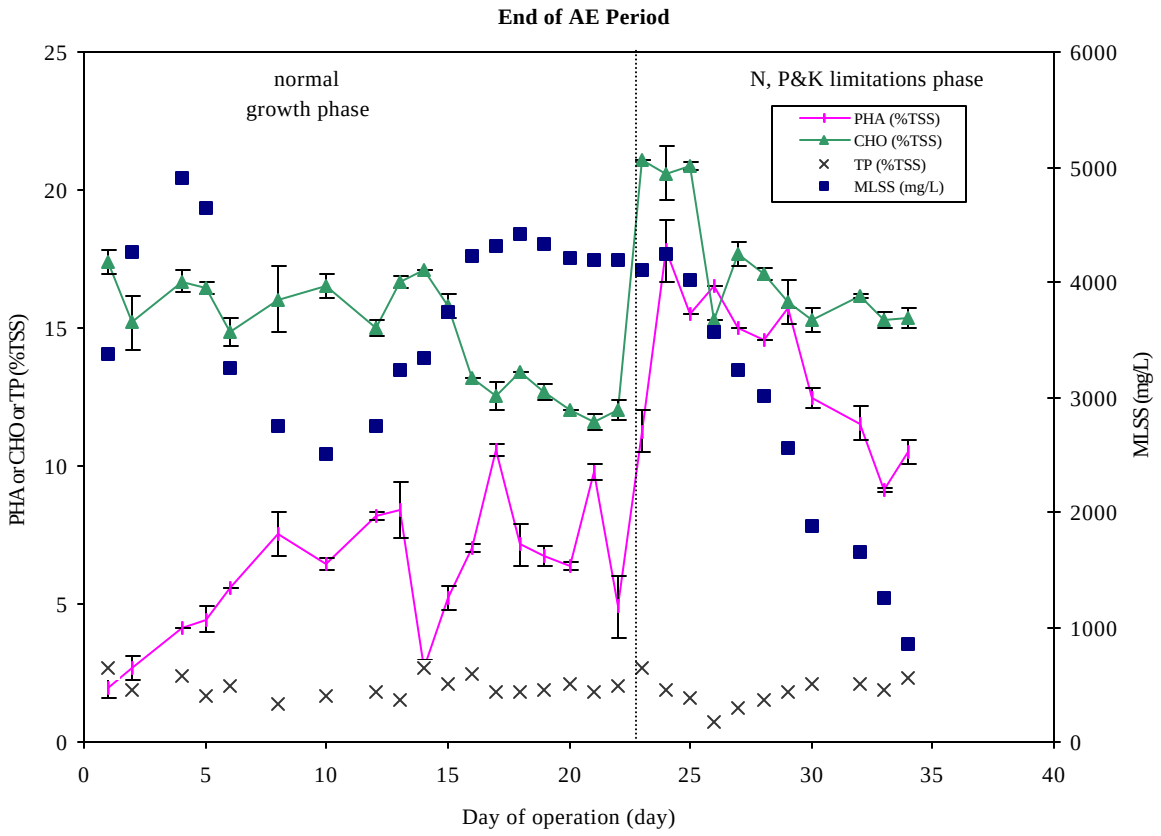


Figure 1: Changes in PHA, MLSS, Carbohydrate, and TP of System 1: Two-Stage Production with N, P&K Limitations

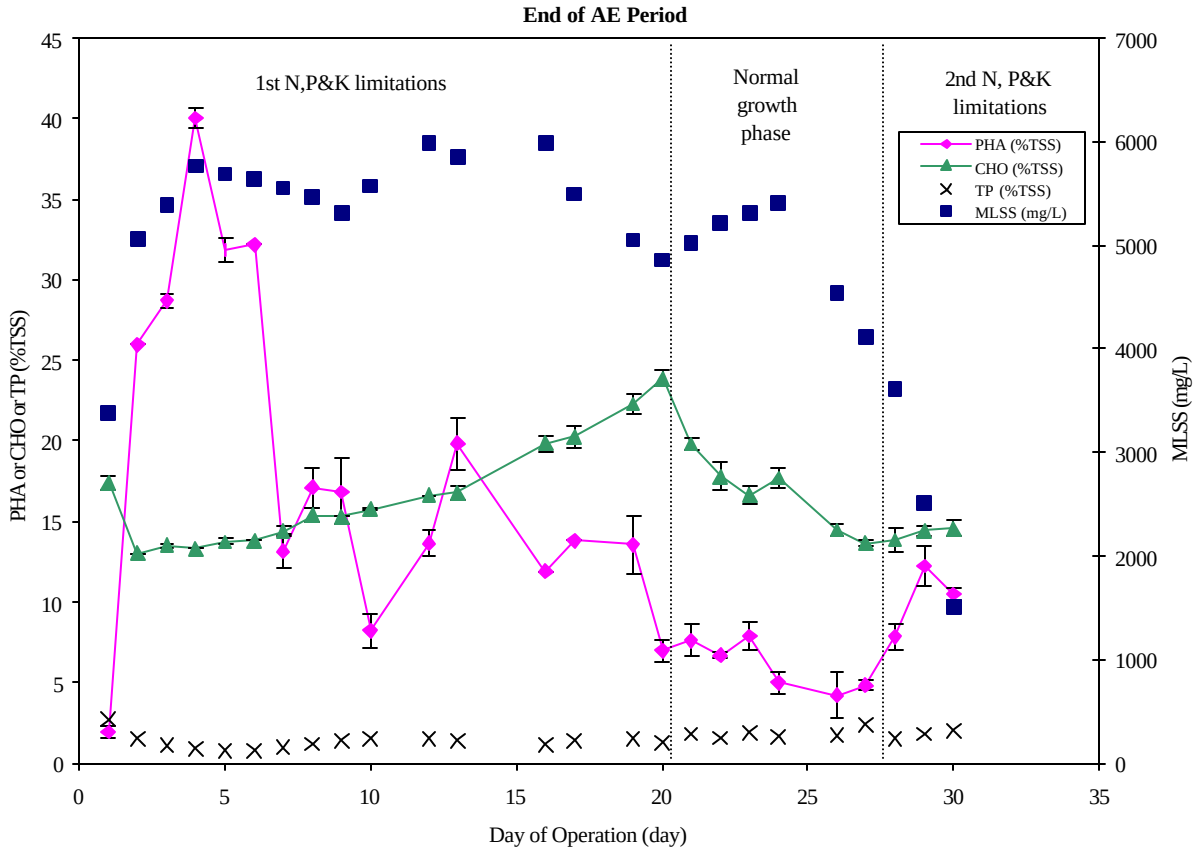


Figure 2: Changes in PHA, MLSS, Carbohydrate, and TP of System 2: One-Stage Production with N, P&K Limitations

limitation period began. The results obtained during this phase of the study are summarized and compared with those obtained from the study using the laboratory prepared mixtures of organics (fully AE with multiple N&P limitations) in Table 1.

In summary, a growth phase was not beneficial for PHA production using the Celanese wastewater, i.e., the one-stage approach was most applicable. Therefore, the PHA production time using the Celanese wastewater would be less than that for the acetic acid-propionic acid mixture, and the PHA production cost also would be less. In this study, we have shown that high PHA productivity can be achieved with the Celanese wastewater due to the high biomass concentration achieved using it. The MLSS of System 2 was 5766 mg/L, resulting in PHA production of 2309 mg/L and PHA productivity of 577 mg/L/day, which are higher than those obtained using the laboratory prepared mixtures of organics. According to Preusting *et al*. (1992), two possible strategies can be used to improve the economics of PHA productions: 1) increase the biomass %PHA content, and 2) increase the biomass concentration of the PHA producing bacteria. In this study, we have shown that high PHA productivity was achieved by maintaining high biomass concentrations.

Table 1: Production of PHA, biomass concentration, carbohydrate, and TP contents

	System 1	System 2	Fully AE+ multiple N&P limitations
Max PHA Content (%TSS)	18	40	70
Production Time (day)	2	4	5
Corresponding MLSS (mg/L)	4250	5766	3238
PHA Production (mg/L)	757	2306	2267
PHA Productivity (mg/L/day)	379	577	453
PHV/PHA (%)	1.8	2.1	50
Yield of PHA per unit COD	0.25	0.5	0.34
TP content (%TSS)	2.0	0.8	0.9
Carbohydrate content (%TSS)	15-21	13.3	8.9

Phase 2: PHA production under N&P limitations

This phase of the study was designed to separate the effects of potassium limitation from the effects of simultaneous N&P limitations. Potassium chloride was added to the wastewater obtained from the Celanese Plant. Applying the results of the Phase 1 study, the one-stage bioprocess approach was used for this phase of the study and for a subsequent experiment. Figure 3 illustrates the production of PHA, biomass concentration, TP, and carbohydrate

contents of the system. A maximum PHA content of 29%TSS was obtained 4 days after N&P limitations began. PHA production and productivity were 1460 mg/L and 365 mg/L/day, respectively. The PHV/PHA ratio of the system ranged from 0.4 to 1.4%, and the carbohydrate content on the day of maximum PHA content was 14.2%. The PHA reached the maximum content and gradually declined thereafter. The system was then returned to growth phase operation, after which the system was operated under nutrient limitations for a second time. The maximum PHA content achieved during the second limitation period was low, i.e., 10 %TSS. The carbohydrate contents of the biomass during the second limitation period were in the range of 19 to 25%TSS. The biomass concentration gradually decreased after the start of the second period of nutrient limitation.

Phase 3: PHA production under P limitation

This phase of the study was designed to investigate if the PHV fraction of the produced PHA could be increased by operating the system under P limitation conditions. It was shown by Punrattanasin *et al*. (2001a) that the PHV fraction was different under different operating conditions when the substrate was a mixture of acetic and propionic acids. For example, PHV fractions of 45% and 65-66% were obtained under fully AE with N&P limitation conditions, and fully AE conditions with P limitation, respectively. The Celanese wastewater was supplemented with potassium phosphate and orthophosphate concentration of the wastewater was in the range of 10-15 mg/L PO_4-P. Figure 4 shows the profiles of PHA, biomass concentration, TP, and carbohydrate contents of the system during the experiment. A maximum PHA content of 27 %TSS was obtained 3 days after P limitation began. PHA production and productivity were 1176 mg/L and 392 mg/L/day, respectively. The PHV/PHA ratios of the system ranged from 0.8 to 2.1%, i.e., a little higher than those obtained with simultaneous N&P limitations. The carbohydrate concentration on the day of maximum PHA content was 14.0%TSS. PHA reached the maximum content and gradually declined thereafter. The system was returned to growth operation for a period of time and then subjected to nutrient limitations for a second time. The maximum PHA content achieved during the second limitation was low, i.e., 3 %TSS. The carbohydrate contents during the second limitation period were in the range of 18 to 35%TSS. The biomass concentration gradually decreased during this period, as observed in the N&P-limitations experiment.

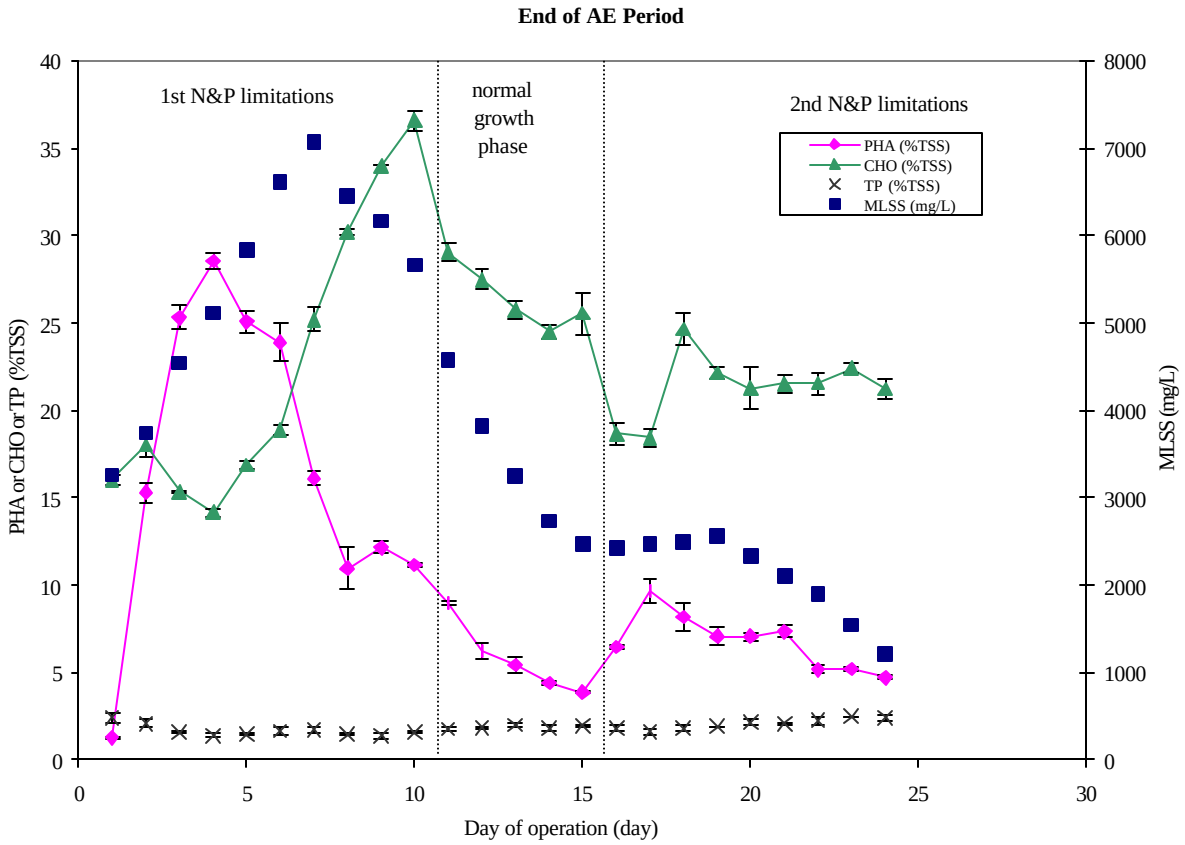


Figure 3: Changes in PHA, MLSS, Carbohydrate, and TP of One-Stage Production with N&P Limitations

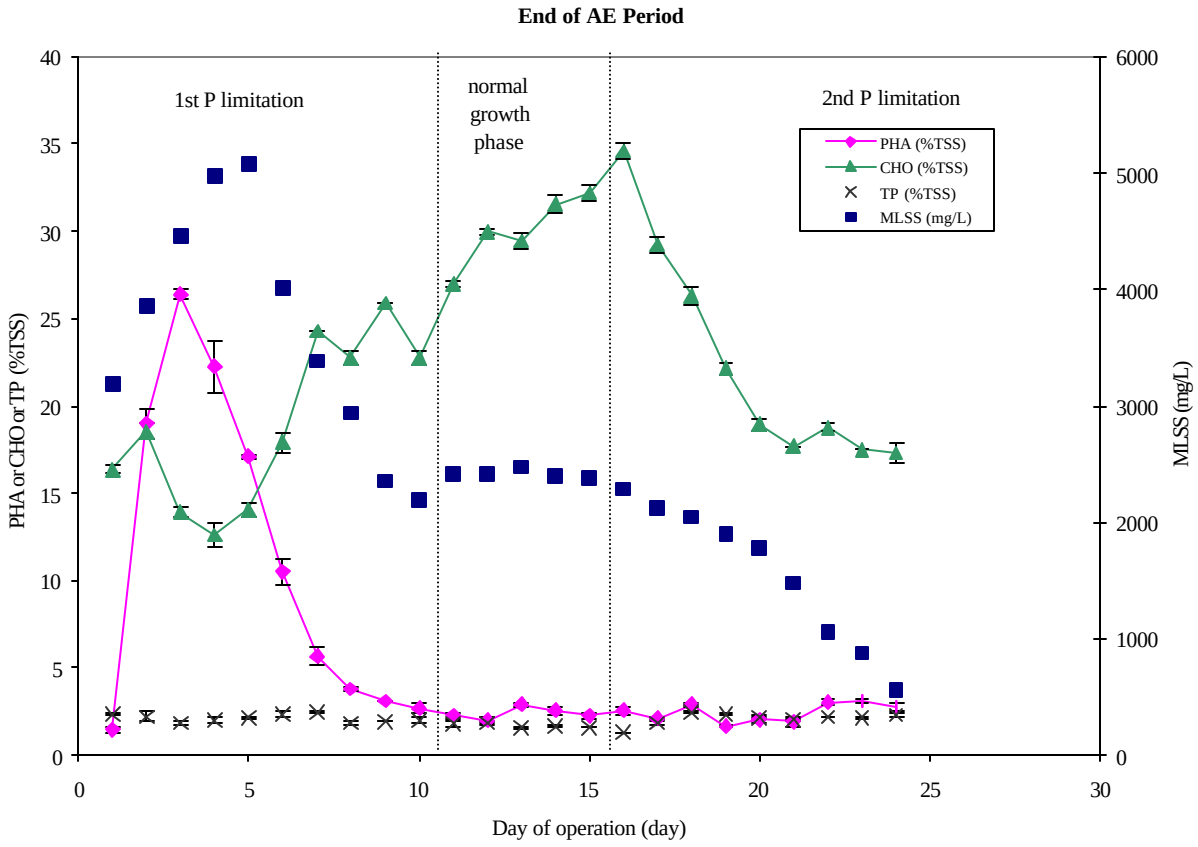


Figure 4: Changes in PHA, MLSS, Carbohydrate, and TP of One-Stage Production with P Limitation

The results during the latter phases of the study showed that high PHA content and productivity could not be achieved under either N&P or P limitation. The PHA content and productivity were significantly lower than those obtained from the original mixture of wastewater (N, P, and K limitations). In addition, the operating condition did not have a significant effect on PHA composition, i. e., the PHV/PHA ratio. This result was probably observed because the wastewater was composed mainly of acetic acid. Therefore, it probably would be necessary to supply a cosubstrate, e.g., propionic acid, to the Celanese wastewater in order to produce a larger fraction of PHV in the copolymer produced.

In summary, it was demonstrated during the experiments that there is considerable potential for the utilization of Celanese wastewater for the production of large quantities of PHAs by the activated sludge biomass that already exists in the on-site wastewater treatment system. Also, it was determined that, unlike when the substrate was a mixture of acetic and propionic acids, the use of a growth phase and multiple periods of growth and nutrient limitation did not enhance PHA production when using the Celanese wastewater as a substrate. Instead, the system was prone to bulking problems. A lot of biomass solids were lost in the effluent discharge, resulting in failure of the system. The maximum PHA content during the second period of nutrient limitations was too small for commercial production. During the second period of nutrient limitation during the N&P- and P-limitation experiments, most of the organic carbon substrates were converted to carbohydrates rather than to PHA. Nonetheless, the basic feasibility of the approach was demonstrated and further work is warranted to more fully define the optimum methods that should be used for commercial application.

Economic analysis

It has been known for a considerable time that PHAs are attractive biopolymers for biodegradable plastics production because they have similar physical and mechanical properties to synthetic plastics. According to Hrabak (1992), PHB has some properties similar to polypropylene with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability. However, the production of biodegradable polyesters on a large scale is limited because of the relative expense of the substrate required, low polymer production, and the cost of maintaining an axenic culture. According to Yamane (1993), higher

production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional petroleum-base plastics in the commercial marketplace. Lee (1996) reported that the price of BIOPOL is $16/Kg, while the price of synthetic plastics is a lot less expensive, e.g., less than $1/Kg for polypropylene. According to van Wegen *et al*. (1998), the price difference between PHA and conventional petroleum-base plastics for a plastic bag, which requires 6.6 gram of polymer, and a shampoo bottle, which requires 60 grams of polymer, were 9 and 84 cents, respectively. A good candidate for PHA production would be a culture that can store high PHA concentrations while growing on an inexpensive growth substrate. *R eutropha*, *A. latus*, *Azotobacter vinelandii*, methylotrophs, and recombinant *Escherichia coli* are reported to be suitable bacterial strains for PHA production because they produce high PHA concentration and productivity. Because of the high cost of substrate such as glucose and propionic acid, many microorganisms grown on cheap substrates have been investigated in order to reduce substrate expense. Page (1992) studied the production of PHA by *A. vinelandii* UWD using beet molasses and reported that the production cost using beet molasses was one-third of that of glucose.

The polymer recovery process is another factor that contributes to the high cost of PHA production. According to Lee (1996), extraction with solvent is the most commonly used method for the recovery process. Due to the high viscosities of PHA solutions, a large amount of solvent has to be used, i.e., 20 parts of solvent to extract 1 part of polymer. According to Page (1992), an enzyme-mediated extraction process is used by ICI because it does not significantly damage the molecular mass of the PHA extracted. However, it is a costly process.

Recently, more extensive PHA economic analyses were performed to estimate PHA production cost and identify approaches to possibly reduce its production cost (Choi and Lee (1997), van Wegen *et al*. (1998), Lee and Choi (1998), and Choi and Lee (1999)). Choi and Lee (1997) used the software BioPro Designer® to estimate the approximate price of PHB produced by *R. eutropha*, *A. latus*, *Methylobacterium oraganophilum*, and recombinant *E. coli* at the commercial scale. The simulated data were obtained from published information in the literature. Two different recovery processes were investigated and analyzed, i.e., surfactant-hypochlorite digestion (assumed recovery efficiency of 95%) and dispersion of chloroform and hypochlorite

(assumed recovery efficiency of 90%). The targeted PHB production in their study was approximately 3000 tonnes/ year. Fixed capital cost in their simulation included total plant direct cost, total plant indirect cost, and other costs, e.g., contractor's fee and contingency. Annual operating cost included direct fixed capital-dependent items, labor-related items, administration and overhead expense, raw materials, other consumables, e.g., membrane and filter cloths, utilities, and waste treatment/ disposal. The cost of raw materials accounted for approximately 39.5% of the total cost. The cost of carbon substrate was approximately 70-80% of the total raw material cost. The operating cost of the process utilizing the dispersion recovery method was higher than the cost of the surfactant-hypochlorite digestion method for all production processes for 4 different bacterial strains. The lowest PHB production cost of $5.58/kg PHB was obtained with *R. eutropha* using glucose as a carbon substrate.

Recombinant *E. coli* has been reported to be able to utilize inexpensive substrates. Based on the simulations of Choi and Lee (1997), PHA production costs could be reduced to $5.00, $4.47, $4.79, and $4.61/kg PHB if hydrolyzed corn starch, whey, cane molasses, and hemicellulose hydrolysate, respectively, were used as a carbon source for recombinant *E. coli.* When the simulations were scaled up to the production of one million tonnes/ year, the PHB production costs for *R. eutropha* and recombinant *E. coli* using glucose were reduced to $4.75 and $5.01/kg PHB, respectively. However, as the production scale increased, the fraction of raw material cost to the total cost also increased. The raw material cost increased up to approximately 48% as the production scale increased from 3000 to one million tonnes per year when glucose was used as substrate. For recombinant *E. coli*, if hydrolyzed cornstarch was used instead of glucose, PHA production would be reduced from $5.01 to $3.84/kg PHB at the production scale of one million tonnes per year. Based on the large-scale PHB production, the PHB production cost would be in the range of $3-4/kg PHB. For the manufacturing of shampoo bottles that require 50 grams of polymer per bottle, the cost difference of the bottle made from PHB and polypropylene would be only 15 cents.

van Wegen *et al*. (1998) also estimated PHA production cost utilizing recombinant *E. coli.* They stated advantages for using *E. coli* for PHA production over *R. eutropha*. The advantages include: 1) *E. coli* has a higher growth rate and PHA productivity, 2) PHA produced by *E. coli* is

easier to extract, 3) a nutrient limitation phase is not required making the production phase simple. According to van Wegen *et al*. (1998) recombinant *E. coli* accumulated high PHA contents in complex media, e.g., Luria-Bertani broth or glucose supplemented with corn steep liquor, casein hydrolysate, and yeast extract. The recovery process used in this study was homogenization and centrifugation, i.e., the process combines homogenization, disc-stack centrifugation, and sodium hypochlorite treatment. The authors stated that this recovery process resulted in high PHA recovery and purity, and was suitable for large-scale production. The economic analysis in this study was based on the targeted production of 4300 tonnes/year. The approximate price of PHB was estimated to be $6.08/kg. The substrate cost was found to contribute significantly to the total cost. They stated that if the substrate was free, PHA production cost would be only $3.21/kg, i.e., 53% of the cost for PHA produced using glucose-based substrates. Sensitivity analysis was also carried out and it was found that important factors affecting PHB production costs were PHA content, the recovery process, and PHA yield per unit substrate.

Choi and Lee (1999) investigated the effects of PHA productivity, PHA content, PHA yield, the cost of carbon substrate, and the PHA recovery process on the economics of PHA production by *R. eutropha*, *A. latus*, *Methylobacterium oraganophilum*, and recombinant *E. coli*. PHA production used in their simulations was 100,000 tonnes/year and surfactant/hypochlorite digestion was used as a recovery process. The efficiency of the recovery process was assumed to be 95%. The lowest production cost of $2.6/ kgPHB was obtained from *A. latus*. The low production cost resulted from the high PHA content (88%PHB) and high productivity (4.94 gPHB/L/hr) of *A. latus*. They found that PHA productivity only affected equipment-related costs, i.e., equipment-related costs decreased as the productivity increased. When the productivity was increased from 1.98 to 3.2 g/L/hr, PHB production cost decreased from $5.37 to $4.91/kgPHB. PHA content was found to have multiple effects on the production process , i.e., it affects the equipment-related costs, the efficiency of the recovery process on both recovery yield and degree of purity, and the PHA yield per unit substrate. In addition, less digestion agent is needed and lower amounts of waste are generated as PHA content increases. Lee and Choi (1998) reported a recovery cost of $4.8/kg PHB for a PHB content of 50%, and a much cheaper recovery cost of $0.92/kg PHB for PHB content of 88%. The recovery process using

surfactant-hypochlorite digestion was less costly than PHA recovery using solvent extraction, e.g., chloroform, because the process requires less solvent and less waste disposal. Pure PHA could be obtained from both methods. An enzymatic digestion method developed by Zeneca for BIOPOL production is rather costly and complicated. Choi and Lee (1998) reported that PHA produced from recombinant *E. coli* could be effectively separated from non-PHA cellular materials by a simple and less expensive method, i.e., digestion with 0.2M NaOH for 2 hours. PHB with a degree of purity of 98.5% was obtained. They stated that if this simple recovery process was used, PHA production cost would be 75% of the surfactant-hypochlorite digestion method.

Lee and Choi (1998) reported that the substrate cost could contribute up to 38% of the total operating cost for PHA production using recombinant *E. coli* (PHB content of 77% and PHA productivity of 3.2g/L/hr). If hydrolyzed corn starch is used instead of glucose with recombinant *E. coli*, PHA production cost would be reduced from $4.91 to $3.72/ kg PHB. According to Choi and Lee (1999), other factors affecting the economics of PHA production include oxygen requirement and scale-up of the system. As the production scale increases, PHA production cost decreases. However, the cost fraction of the substrate increases as the production scale increases.

A sensitivity analysis was also carried out by Choi and Lee (1999). They found that PHA content has a significant effect on PHA production process economics. PHA productivity has an effect only on the equipment-related cost. PHA yield per unit substrate only has an effect to the substrate cost, i.e., substrate cost decreases as the PHA yield increases. PHA yield per unit substrate did not affect direct-fixed-capital-dependent items, labor-dependent cost, and utility costs. The production scale did not affect the PHA yield but did affect production costs, i.e., PHA production cost is higher for smaller-scale production.

According to the review of PHA economic analysis, several factors have an impact on PHA production cost. The important factors include PHA content, PHA productivity, substrate cost, and PHA yield per unit substrate. According to the study by Punrattansin *et al*. (2001a & b), the mixed culture of activated sludge biomass could be used for PHA production. High PHA content (70%TSS) and high PHA yield per unit substrate were obtained during their study. In

addition, PHA composition, i.e., the PHV/PHA ratio, obtained during their study is desirable for industrial applications. With these results, the cost of the cosubstrate, e.g., propionic acid, would be reduced. According to Choi and Lee (1999), the cost of propionic acid is rather high, i.e., approximately $1/kg versus $0.493/kg of glucose. Also, the PHA productivity achieved with activated sludge was rather low in comparison to the productivity obtained using pure cultures. To optimize the economics of PHA production using activated sludge biomass, it is recommended that activated sludge PHA production systems that utilize wastewater as the production substrate be coupled as a side-stream process with a main-stream wastewater treatment system. When the Celanese wastewater and the activated sludge mixed microbial cultures that existed in the wastewater treatment plant were used, the PHA content achieved was lower than when the acetic-propionic mixture was used, i.e., the maximum PHA content, obtained under N, P, and K limitations, was 40%TSS. According to the economic analysis review, the PHA content affects the PHA production cost in multiple ways, i.e., equipment-related costs, the efficiency of the recovery process, and the PHA yield per unit substrate. One possible way to improve the economics of PHA production using Celanese wastewater is to investigate the utilization of the Celanese wastewater as a source of substrate for pure cultures, e.g., *R. eutropha*, *A. latus* or recombinant *E. coli.* A study by Yu *et al*. (1999) showed that malt waste could be used for the production of PHA by *A. latus*. Higher PHA content was obtained from *A. latus* compared to activated sludge biomass, i.e., PHA contents were 70% and 43.3%, respectively. Lee *et al*. (1997) investigated PHA production by recombinant *E. coli* using concentrated whey solution and PHA content up to 80% was obtained. A similar experiment with the Celanese wastewater would seem to be worthwhile.

SUMMARY AND CONCLUSIONS

1. The 1-stage bioprocess is a better approach for PHA production using the Celanese wastewater than the 2-stage bioprocess using a growth cycle.
2. Operating conditions have a significant effect on PHA production when the Celanese wastewater is the substrate. Fully AE production conditions utilizing the original mixture of wastewater with minimal concentrations of N, P, and K, were more optimum for PHA production than either N&P limitations or only P limitation.

3. The PHV/PHA ratios of the copolymers produced could not be significantly increased by operating the system under P limitation conditions. This contrasted with the results obtained with the acetic-propionic mixture as reported by Punrattanasin *et al*. (2001a & b). This limitation probably occurred because of the high acetic acid concentration in the Celanese wastewater and a lack of cosubstrate transformable to propionyl-CoA.
4. Important factors affecting the economics of PHA production include cellular PHA content, PHA productivity, PHA yield per unit substrate, and substrate cost.
5. It is probable that the economics of PHA production using the Celanese wastewater could likely be improved if pure cultures were used, rather than activated sludge biomass. It is recommended that the utilization of Celanese wastewater for PHA production be investigated using pure cultures of bacterial that are known to be efficient PHA producers.

REFERENCES

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiological Reviews.* 54 (4), 450-472. 119-129.

Braunegg, G., Lefebvre, G., and Genser, K. (1998). Polyhydroxyalkanoates, Biopolyesters From Renewable Resources: Physiological and Engineering Aspects. *Journal of Biotechnology*. 65, 127-161.

Byrom, D. (1990). Industrial production of Copolymer from *Alcaligenes eutrophus*. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 113-117.

Choi, J and Lee, S. Y. (1997). Process Analysis and Economic Evaluation for Poly(3-hydroxybutyrates) Production by Fermentation. *Bioprocess Engineering*. 17, 335-342.

Choi, J and Lee, S. Y. (1999). Factors Affecting the Economics of Polyhydroxyalkanoates Production by Bacterial Fermentation. *Appl. Microbiol. Biotechnol*. 51, 13-21.

Dawes, E. A. (1990). Novel Microbial Polymers: An Introductory Overview. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 3-16.

Dawes, E. A. and Senior, P. J. (1973). The Role and Regulation of Energy Reserve Polymers in Micro-organisms. *Advances in Microbial Physiology*. 10, 135-266.

Doi, Y. (1990). *Microbial Polyesters*. VCH Publishers, Inc. New York, USA.

Doi, Y., Kunioka, M., Nakamura, Y, and Sago K. (1987). Biosynthesis of Copolyesters in *Alcaligenes eutrophus* H16 from $_{13}$C-Labeled Acetate and Propionate. *Macromolecules.* 20, 2988-2991.

Doi, Y., Segawa, A., Nakamura, S., and Kunioka, M. (1990). Production of biodegradable Copolymers by *Alcaligenes eutrophus*. In: Novel Biodegradable Microbial Polymers, Dawes, E. A. (ed.), Kluwer Academic Publishers, The Netherlands, 37-48.

Huang, J., Shetty, A. S., and Wang, M. (1990). Biodegradable Plastics: A Review. *Advances in Polymer Technology*, 10(1), 23-30.

Hart, V. S. (1994). An Examination of Biological Phosphorus Removal Using Bacterial Counting and Poly-beta-hydroxybutyrate Analysis in Batch and Continuous Flow System. Ms.Thesis. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Holmes, P. A., (1985). Applications of PHB– A Microbially Produced Biodegradable Thermoplastic. *Physics in Technology,* 16: 32-36.

Hrabak, O. (1992). Industrial Production of Poly-β-hydroxybutyrate. *FEMS Microbiology Reviews.* 103, 251-256.

Lafferty, R. M., Korsatko, B., and Korsatko, W. (1988). Microbial Production of Poly-β-hydroxybutyric Acid. In: *Biotechnology*, Rehm, H. J. and Reed, G. (eds.), VCH Publishers, New York, USA, 135-176.

Lee, S. Y. (1996). Bacterial Polyhydroxyalkanoates. *Biotechnol. and Bioeng.* 49, 1-14.

Lee, S. Y., Middelberg, A. P. J., and Lee, Y. K. Poly(3-hydroxybutyrates) Production from Whey using Recombinant *Escherichia coli. Biotechnology Letter*. 19, 1033-1035.

Lee, S. Y. and Yu, J. (1997). Production of Biodegradable Thermoplastics from Municipal Sludge by a Two-Stage bioprocess. *Resources, Conservation and Recycling*. 19, 151-164.

Lee, S. Y. and Yu, J. (1998). Effect of Fermentation Performance on the Economics of Poly(3-hydroxybutyrates) Production by *Alcaligenes latus. Polymer Degrad. Stabil*. 59, 387-393.

Madigan, M. T., Martinko, J. M. and Parker, J., 1997. *Brock Biology of Microorganisms.* Princeton Hall, New Jersey.

Page, W. J. (1992). Production of Polyhydroxyalkanoates by *Azotobacter vinelandii* Strain UWD in Beet Molasses Culture, *FEMS Microbiology Reviews.* 103, 149-158.

Page, W. J. (1995). Bacterial Polyhydroxyalkanoates, Natural Biodegradable Plastics With a Great Future. *Can. J. Microbiol*. 41(suppl. 1), 1-3.

Preusting, H., Hazenberg, W., and Witholt, B. (1993). Continuous Production of Poly(3-hydroxyalkanoates) by Pseudomonas oleovorans in a High-Cell-Density, Two-Liquid-Phase Chemostat. *Enzyme Microb. Technol*. 15, 311-316.

Punrattanasin, W., Randall, C. W., and Randall, A. A. (2001a). Production of Polyhydroxyalkanoates for Biodegradable Plastics Using Activated Sludge Biomass: System Development. Ph.D. Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Punrattanasin, W., Randall, C. W., and Randall, A. A. (2001b). Production of Polyhydroxyalkanoates for Biodegradable Plastics Using Activated Sludge Biomass: Determination of Control Parameter and Physical Properties. Ph.D. Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Ramsay, J. A., Berger, E., Voyer, R., Chavarie, C., and Ramsay, B. A. (1994). Extraction of poly-3-hydroxybutyrate using chlorinated solvents. *Biotechnology Techniques*. 8, 589-594.

Wang, F. and Lee, S. Y. (1997). Poly(3-hydroxybutyrates) Production with High Polymer Content by Fed-Batch Culture of *Alcaligenes latus* Under Nitrogen Limitation. *Appl. Environ. Microbiol.* 63, 3703-3706.

Yamane, T. (1993). Yield of Poly-D(-)-3Hydroxybutyrate from Various Carbon Sources: A Theoretical Study. *Biotechnology and Bioengineering*. 41, 165-170.

Yu, P. H. F., Chua, H., Huang A. L., Lo W. H., and Ho, K. P. (1999). Transformation of Industrial Food Wastes Into Polyhydroxyalkanotes. *Water Science and Technology*. 40 (1), 365-370.