İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

COMPARATIVE EVALUATION OF MICROBIAL DIVERSITY AND PHA STORAGE ABILITY OF ACTIVATED SLUDGE UNDER DIFFERENT OPERATING CONDITIONS

Ph.D. Thesis by
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Department : Environmental Engineering

Programme : Environmental Biotechnology

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MAY 2010

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

AKTİF ÇAMURUN PHA DEPOLAMA YETENEĞİNİN VE MİKROBİYAL ÇEŞİTLİLİĞİNİN FARKLI İŞLETME KOŞULLARI ALTINDA KARŞILAŞTIRMALI OLARAK DEĞERLENDİRİLMESİ

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FOREWORD

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ABBREVIATIONS

ADF	**:** Aerobic Dynamic Feeding
ARDRA	: Amplified Ribosomal DNA Restriction Analysis
C/N	: Carbon to Nitrogen
CoA	: Coenzyme A
COD	: Chemical Oxygen Demand
DGGE	: Denaturing Gradient Gel Electrophoresis
DNA	: Deoxyribonucleic Acid
DNF	**:** Delayed Nitrogen Feeding
EBPR	: Enhanced Biological Phosphorus Removal
EMBL	: European Molecular Biology Laboratory
FISH	: Fluorescent In Situ Hybridization
GAO	: Glycogen Accumulating Organism
HAc	: Acetate
HB	: Hydroxybutyrate
HMV	: Hydroxymethylvalerate
HV	: Hydroxyvalerate
lcl-PHA	: long chain length PHA
mcl-PHA	: medium chain length PHA
MLSS	: Mixed Liquor Suspended Solids
MLVSS	: Mixed Liquor Volatile Suspended Solids
N	: Nitrogen
NADH	: Nicotinamide Adenine Denucleotide
NADPH	: Nicotinamide Adenine Denucleotide Phosphate
NH_4-N	: Ammonia Nitrogen
OLR	: Organic Loading Rate
OME	: Olive Mill Effluents
P	: Phosphorus
P(3H2MB)	: Poly-3-Hydroxy-2-Methylbutyrate
P(3H2MV)	: Poly-3-Hydroxy-2-Methylvalerate
P(3HA)	: Poly(3-Hydroxyalkanoate)
P(3HB)	: Poly(3- Hydroxybutyrate)
P(3HB-co-3HV)	: copolymer of 3-Hydroxybutyrate and 3-Hydroxyvalerate
P(3HV)	: Poly(3- Hydroxyvalerate)
PABER	: Polyhydroxyalkanoate Accumulating Bacteria Enhanced Reactor
PAO	: Polyphosphate Accumulating Organism
PCR	: Polymerase Chain Reaction
PHA	**:** Polyhydroxyalkanoate
PHB	**:** Polyhydroxybutyrate
q_p	: Specific polymer storage rate
q_S	: Specific acetate uptake rate
RNA	: Ribonucleic Acid
rRNA	: Ribosomal Ribonucleic Acid
SBR	**:** Sequencing Batch Reactor

scl-PHA	: short chain length PHA
SRT	: Sludge Retention Time
UPGMA	: Unweighted Pair Group Method with Arithmetic Mean
$\mathbf{V_0}$	. initial volume
$\mathbf{V_F}$	: fill volume
VFA	: Volatile Fatty Acids
WWTP	: Wastewater treatment plant
$\mathbf{Y_{P/S}}$	: Yield of polymer on substrate consumed

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COMPARATIVE EVALUATION OF MICROBIAL DIVERSITY AND PHA STORAGE ABILITY OF ACTIVATED SLUDGE UNDER DIFFERENT OPERATING CONDITIONS

SUMMARY

Polyhydroxyalkanoates (PHAs), which are biologically-derived and completely biodegradable polyesters, represents a potentially sustainable substitution to synthetic polymers known as plastics. Currently, high production and recovery costs are the main limitations for the bulk production of bioplastics. PHA production processes based on mixed microbial cultures, such as activated sludge systems, are being investigated as a possible technology to decrease production costs. In activated sludge systems no sterilization is required and bacteria can adapt quite well to the complex and cheap substrates, such as wastewaters. To understand the impact of different enrichment strategies on PHA production, and population dynamics is an obligation because selection of organisms with high storage ability is one of the most critical factors having effect on development on the competitive process for PHA production based on mixed cultures.

In this study, three sequencing batch reactors (SBR) were operated under aerobic dynamic feeding (ADF) conditions for biomass enrichment in order to investigate the effect of nitrogen (N) availability during a SBR cycle, on population dynamics and PHA accumulation ability of selected biomass. Nitrogen was always available in one of the reactors, whereas, in the second SBR, it was depleted completely together with carbon source at the end of feast phase. The third SBR was operated with delayed nitrogen feeding (DNF) strategy which was proposed in this study. In this feeding regime, synthetic wastewater without nitrogen was fed to the SBR and nitrogen source was fed to the reactor following substrate depletion to hinder being substrate and ammonia simultaneously in the reactor.

Changes in polymer storage ability of three biomasses were determined in terms of specific polymer storage rate, yield of polymer on substrate consumed, amount of polymer accumulated, and biomass polymer content. Polymer storage ability of biomasses enriched under ADF conditions were considerably higher when compared to those obtained for inoculum sludge. Substrate was accumulated mainly in the form of Polyhydroxybutyrate (PHB) because acetate was supplied as the sole carbon source. Experimental data showed that nitrogen restraint throughout biomass enrichment stimulated polymer accumulation. Accordingly, polymer content of biomass enriched under dynamic conditions with DNF and also polymer yield and polymer uptake rate obtained for this biomass was higher than those obtained for biomass enriched under dynamic conditions with nitrogen deficiency, and considerably higher than those obtained for biomass enriched under dynamic conditions without nitrogen deficiency.

Community structure of biomass determined clone library construction and subsequent analysis of clone sequences. Changes in bacterial population of

biomasses being enriched under different operating conditions were monitored by denaturing-gradient gel electrophoresis (DGGE) analysis of clone sequences based on 16S ribosomal Ribonucleic acid (rRNA). According to rarefaction analysis, 94% of the species were determined. The bacterial community of the inoculum sludge was consisted of a heterogeneous microbial community, rich in different bacterial species. *Proteobacteria* dominated in the inoculum sludge bacterial clone library with 14.5% belonging to *α-proteobacteria*, and 53% belonging to *β-proteobacteria*. *Proteobacteria* followed by *Verrucomicrobiae* (14.5%), *Bacteroidetes* (13.3%) and *Planctomycetes* (4.8%). Among the members of *β-proteobacteria* class, *Rhodocyclaceae* (42.2%) was the most predominant family represented by clones. Changes in relative abundances of these species during operation of SBRs were monitored by a semi-quantitative method, DGGE. Statistical analysis of results obtained from DGGE indicates that changes in relative abundance of bacterial species in the activated sludge were more significant than changes in number of species during SBR operations.

Although changes in bacterial diversity during operation of three SBRs were different in details, species belonging to *Rhodocyclacea* family in *Betaproteobacteria* phylum and especially *Zoogloea* genus was always predominant in three reactors. Correlation between changes in community structure and PHA storage ability was statistically evaluated. It is concluded that contribution of the species belonging to phyla *Planctomycetes* and *Bacteroidetes* to PHA accumulation were paltry. Relatively lower correlations were obtained for delayed nitrogen feeding process.

Various batch experiments were conducted by feeding different types of substrate and applying various substrate loadings, and carbon to nitrogen (C/N) ratios, in order to investigate responses of biomasses under different conditions. Results obtained from batch experiments showed that concentrations of polymer accumulated by three different sludges increased directly with substrate loading (S/X) and the highest polymer accumulation was obtained for the biomass enriched under delayed nitrogen feeding conditions. The highest sludge polymer content, 47.1% on COD basis, was also obtained for the biomass enriched under these conditions. Relation between polymer storage rate and substrate loading was determined to depend strongly on nitrogen availability during batch test. Substrate loading caused an increase in the specific polymer storage rate during the batch tests where nitrogen does not exist, however it caused a decrease in specific polymer storage rate if nitrogen is available. Yield of polymer on substrate consumed decreased directly with substrate loading for three different sludges. The highest polymer yield (0.71) was obtained during batch tests performed with sludge enriched with delayed nitrogen feeding and the lowest substrate loading applied. Polymer yield increased with substrate concentration if nitrogen concentration kept constant and decreased with substrate concentration if C/N ratio kept constant. Harmony between conditions applied during SBR operation and batch experiments was also determined to be an important factor affecting polymer storage. Substrate was accumulated mainly in the form of hydroxybutyrate (HB) and hydroxyvalerate (HV) when a mixture of acetate and propionate was supplied. Amount of PHV accumulated by the sludge enriched under delayed nitrogen feeding conditions was higher than that accumulated by sludge enriched under nitrogen deficient conditions and noticeably higher than that accumulated by sludge enriched without nitrogen deficiency.

Generally restriction of nitrogen availability during substrate uptake improved polymer storage ability of biomass. Among the three different enrichment strategy, DNF process, which was proposed for the first time in this study, was found to be the most effective one. If this process optimized and combined with other strategies, such as pulsewise feeding control, it can be a stronger alternative to industrial production of PHAs achieved by pure cultures. Ammonia deficient organic wastes can be used as a cheap carbon source in this process for PHA production after a fermentation process.

AKTİF ÇAMURUN PHA DEPOLAMA YETENEĞİNİN VE MİKROBİYAL ÇEŞİTLİLİĞİNİN FARKLI İŞLETME KOŞULLARI ALTINDA KARŞILAŞTIRMALI OLARAK DEĞERLENDİRİLMESİ

ÖZET

Biyolojik olarak üretilen ve biyolojik olarak tümüyle ayrışabilir nitelikte poliesterler olan Polihidroksialkanoatlar (PHA) daha sürdürülebilir olduklarından, plastik dediğimiz sentetik polimerlerin yerini almaya adaydırlar. Biyoloplastiklerin yaygın olarak üretilmelerinin önündeki en büyük engel yüksek üretim maliyetleridir. Aktif çamur gibi karışık mikrobiyal kültürlere dayalı sistemler sterilizasyon gerektirmemeleri ve bakterilerin atıksu gibi karışık ve ucuz besinlere kolayca uyum sağlamaları nedeni ile bu sistemlere dayalı PHA üretimi düşük maliyetli bir olasılık olarak belirmekte ve bu konudaki araştırmalar sürmektedir.

Yüksek depolama özelliğine sahip mikroorganizmaların seçilmesi, rekabet şansı yüksek bir PHA üretim sisteminin geliştirilmesi açısından hayati olduğundan, farklı zenginleştirme stratejilerinin PHA üretimine ve popülasyon dinamiklerine etkisinin anlaşılması bir zorunluluktur.

Bu çalışmada, biyokütle zenginleştirmek amacıyla aerobik dinamik besleme (ADB) koşullarında üç ardışık kesikli reaktör (AKR) işletilmiş ve AKR çevrimi süresince azot varlığının popülasyon dinamiklerine ve seçilen biyokütlenin depolama yeteneğine etkisi araştırılmıştır. Reaktörlerden birinde azot daima mevcutken, diğer reaktöre beslenen sentetik atıksudaki azot konsantrasyonu, bolluk fazının sonunda karbon ile birlikte bitecek şekilde ayarlanmıştır. Üçüncü reaktör ilk defa bu çalışmada önerilen ve gecikmiş azot besleme (GAB) olarak adlandırılan bir yöntemle beslenmiştir. Azot ve karbonun bir arada bulunmalarının engellenmek istendiği bu besleme rejiminde, azot içermeyen bir sentetik atıksu reaktöre beslenmiş ve azot çözeltisi ancak reaktördeki bütün karbon kaynağı tüketildikten sonra sisteme beslenmiştir.

Reaktör işletimleri sırasında biyokütlelerin polimer depolama yeteneğindeki değişimler spesifik polimer depolama hızı, substratın polimere dönüşüm oranı, depolanan polimer miktarı ve biyokütlenin polimer içeriği göz önünde bulundurularak değerlendirilmiştir. ADB koşullarında seçilen biyokütlelerin polimer depolama yeteneklerinin aşı çamurununkine göre oldukça yüksek olduğu tespit edildi. Sisteme beslenen tek karbon kaynağı asetat olduğu için beslenen substrat polihidroksibütirat (PHB) biçiminde depolanmıştır. Deney sonuçları göstermiştir ki, biyokütle zenginleştirilirken, substrat alımı sırasında reaktördeki azotun sınırlandırılması polimer depolanmasını olumlu yönde etkilemiştir. Dolayısıyla GAB'nin uygulandığı dinamik şartlarda zenginleştirilmiş olan biyokütlenin polimer içeriğinin, bu biyokütle için elde edilen dönüşüm oranı ve polimer depolama hızının azot kısıtlamalı olarak zenginleştirilen biyokütle için elde edilen değerlerden daha yüksek, azot kısıtlamasız olarak zenginleştirilen biyokütle için elde edilen değerlerden de çok daha yüksek olduğu tespit edilmiştir.

Biyokütlenin türsel yapısı klon kütüphanesi ve sekans analizi ile belirlenmiştir. Farklı işletme koşullarında zenginleştirilen biyokütlenin bakteriyel popülasyonundaki değişimler ise 16S rRNA üzerine kurulu denaturan gradyent jel elektroforezi (DGJE) analizi yöntemi ile izlenmiştir. Klon kütüphanesinde mevcut türlerin %94'ünün temsil edildiği belirlenmiştir. Aşı çamurunu oluşturan bakteriyel topluluk, farklı türleri içerisinde barındıran zenginlikte ve heterojen bir yapıdadır. *Proteobacteria* aşı çamuru içerisindeki en baskın şubedir. Klon kütüphanesinin %14,5'i *α-proteobacteria* ve %53'ü de *β-proteobacteria* sınıflarından oluşmaktadır. Çamurda tespit edilen bakteriyel türlerin %14,5'inin *Verrucomicrobiae*, %13,3'ünün *Bacteroidetes* ve %4,8'inin de *Planctomycetes* şubelerine ait olduğu belirlenmiştir. Klonların %42,2'sinin en baskın şube olan *β-proteobacteria*'ya bağlı *Rhodocyclaceae* ailesinden oldukları tespit edilmiştir. AKR işletimi sırasında türlerin bağıl çokluğundaki değişimler yarı niceliksel bir yöntem olan DGJE ile belirlenmiştir. DGJE sonuçlarının istatistiksel analizi göstermiştir ki; her bir türün bağıl çokluğundaki değişiklikler önemli olmakla birlikte, tespit edilen türlerin sayısında önemi bir değişiklik gözlenmemiştir. Her bir AKR'deki bakteriyel çeşitlilikte gözlenen değişim farklılık gösterse de *β-proteobacteria*'nın *Rhodocyclaceae* ailesin'e bağlı türler özellikle de *Zoogloae* her üç reaktörde de sürekli baskın olmuştur. Bakteriyel topluluğun yapısındaki değişiklikler ile biyokütlenin depolama yeteneğindeki değişim arasındaki ilgileşim istatistiksel olarak değerlendirilmiştir. *Planctomycetes* ve *Bacteroidetes* şubelerine bağlı türlerin PHA depolamasına katkısının önemsiz olduğu tahmin edilmektedir. Gecikmiş azot beslemenin uygulandığı AKR için elde edilen ilgileşim değerleri diğer iki reaktör için elde edilenlerden daha küçüktür.

Seçilen biyokütlelerin farklı şartlar altındaki tepkilerini gözlemek amacıyla farklı substratların, substrat yüklemelerinin ve karbon/azot (C/N) oranlarının denendiği çeşitli kesikli deneyler gerçekleştirilmiştir. Farklı azot besleme rejimleri ile zenginleştirilmiş üç biyokütle tarafından depolanan polimer konsantrasyonu da artan substrat yüklemesine bağlı olarak artmış fakat en yüksek konsantrasyon, gecikmiş azot besleme ile zenginleştirilmiş biyokütle için elde edilmiştir. Elde edilen en yüksek biyokütle polimer içeriği %47,1 olup bu değer de gecikmiş azot besleme ile zenginleştirilmiş biyokütle için elde edilmiştir. Polimer depolama hızı ile yüklenen substrat arasındaki ilişkinin, kesikli deney sırasında reaktörde azot bulunup bulunmadığına yakından bağlı olduğu tespit edilmiştir. Azotsuz olarak gerçekleştirilen deneylerde artan substrat yüklemeleri spesifik polimer depolama hızında artışa yol açarken, azotla gerçekleştirilen deneylerde bu durum depolama hızında düşüşe yol açmıştır. Substratın polimere dönüşüm oranı her üç biyokütle için de artan substrat yüklemesine bağlı olarak düşmüştür. En yüksek dönüşüm oranı (0,71), gecikmiş azot besleme ile zenginleştirilmiş olan çamur için ve en düşük substrat yüklemesinin uygulandığı deneyde elde edilmiştir. Reaktöre beslenen substrat konsantrasyonunun arttırılması, azot konsantrasyonu sabit tutulması durumunda dönüşüm oranında bir artışa, C/N oranının sabit tutulması durumunda da dönüşüm oranında azalmaya yol açmıştır. AKR işletimi sırasında ve bu reaktörden alınan çamur ile gerçekleştirilen deneyler sırasında uygulanan koşullar arasındaki uyumun polimer depolamasına olumlu yönde etki eden bir etken olduğu belirlenmiştir. Substrat olarak asetat propiyonat karışımının kullanıldığı deneylerde polimer daha çok hidroksibütirat (HB) ve hidroksivalerat (HV) kopolimeri şeklinde depolanmıştır. Aynı şartlar altında gecikmiş azot beslemesi yöntemiyle zenginleştirilmiş biyokütle tarafından depolanan PHV, azot kısıtlı olarak

zenginleştirilen biyokütlenin depoladığından daha fazla, azot kısıtsız olarak zenginleştirilen biyokütlenin depoladığından ise çok daha fazladır.

Genel olarak, ilk defa bu çalışmada önerilen bir proses olan gecikmiş azot besleme ile zenginleştirilmiş biyokütlenin polimer depolama yeteneğinin, azot kısıtlı ve azot kısıtsız şartlarda gerçekleştirilen aerobik dinamik besleme ile zenginleştirilmiş biyokütlelerin depolama yeteneğinden daha üstün olduğu belirlendi. Eğer bu proses optimize edilirse ve çözünmüş oksijen kontrollü besleme gibi yöntemlerle kombine edilirse, saf kültürler kullanılarak gerçekleştirilen endüstriyel PHA üretimi karşısında güçlü bir alternatif olabilir. Nütrient yönünden fakir organik atıklar fermentasyondan sonra bu proseste ucuz karbon kaynağı olarak kullanılabilirler.

1. INTRODUCTION

1.1 Significance of the Subject

Synthetic polymers (known as plastics) have become significant since the 1940s and since then they have been replacing glass, wood and constructional materials. On the other hand plastics also play an important role for many short live applications such as packaging. Exponential growth of the human population has led to the accumulation of huge amount of non-degradable waste materials across our planet. Plastics are recalcitrant to microbial degradation and the increased cost of solid waste disposal is another important environmental problem caused by plastic usage. Plastics occupy high volume fraction in municipal landfills due to their relatively low density. According to Environmental Protection Agency (2000) substitution of synthetic plastics by biodegradable plastics can reduce almost 20% of total waste by volume and 10% by weight. Incineration of these materials is expensive and has also potential hazards. Harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration (Ojumu et al., 2004). Recycling also represents some major disadvantages, as it is difficult to sort the wide variety of plastics and changes in the plastic's material in every recycle step are limiting for the further application range.

The production of petroleum derived plastics depend on availability of fossil fuels, however they are finite source. The world currently consumes approximately 140 million tons of plastics per annum. Processing of these plastics uses approximately 150 million tons of fossil fuels, which are difficult to substitute (Suriyamongkol, et al., 2007).

In recent years there have been a growing public and scientific interest regarding the use and development of biodegradable plastics made from renewable resources, which can undergo complete biodegradation and share similar physical properties with most petroleum derived plastics. Among the candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs) have been drawing much attention, because

of their material properties similar to conventional plastics and complete biodegradability. These microbial polyesters are thermoplastics with biodegradable and biocompatible properties, and the physical properties can be regulated by varying the composition of the copolymers (Doi, Y., 1990). PHAs are synthesized and catabolized by various organisms and do not cause toxic effects in the host. These biopolymers accumulate as storage materials in microbial cells under stress conditions. Actually, the industrial production of PHA is based on pure microbial cultures (wild or genetically modified strains), which may accumulate PHA up to 90% of the cell dry weight (Serafim et al., 2008). Currently, the main limitations for the bulk production of bioplastics are its high production and recovery cost. In recent years, there has been a great interest in investigating potential alternative processes for PHA production aiming at decreasing the polymer production costs. PHA production processes based on mixed microbial cultures are being investigated as a possible technology to decrease production costs, as no sterilization is required and bacteria can adapt quite well to the complex substrate present in low-cost substrate. (Lemos et al., 2008). Many different approaches based on the use of mixed cultures processes have been proposed, but none has yet been implemented at industrial scale. One critical factor on the development of a competitive process for PHA production with mixed cultures is the selection of organisms with high storage capacity. Therefore, it is mandatory to understand the effect of different selection strategies on PHA production, and population dynamics. Aerobic dynamic feeding (ADF) process allows for the selection of an enriched culture with a high and stable capacity of PHA production. In this process sludge is submitted to consecutive periods of external substrate accessibility (feast) and unavailability (famine) under fully aerobic conditions. The process can be economically competitive with PHA production from pure cultures and it has the advantages of being simpler and requiring less investment and operating costs (Serafim et al., 2004). PHA accumulation ability of activated sludge enriched under aerobic dynamic feeding conditions was widely explored by batch experiments carried out under different conditions including also different carbon to nitrogen (C/N) ratios. However, effect of nitrogen deficiency during enrichment period on selection of organisms with high storage capacity was not investigated yet. And still there are very few studies reported about microbial characterization in ADF systems.

This study presents a comparison between polymer accumulation abilities of biomass enriched under ADF conditions operated with different nitrogen regimes and also bacterial populations selected by these operational conditions. The results of this study would contribute to the knowledge on relationship between operating conditions and bacterial community. A novel strategy offered first time in this study, delayed nitrogen feeding, can be a promising enrichment alternative for PHA production by using nitrogen deficient wastewaters as carbon source.

1.2 Aim and Scope

The aim of this study was to investigate effect of nitrogen availability during a sequencing batch reactor (SBR) cycle operated under ADF conditions, for biomass enrichment, on population dynamics and PHA accumulation ability of selected biomass.

In this context, three lab scale SBRs were operated with synthetic wastewater. Nitrogen was always available in one of the reactors, whereas it was depleted completely at the end of feast phase together with carbon source in the second SBR. The third SBR was operated with "delayed nitrogen feeding" strategy which was proposed in this study. In this process accumulation was promoted by limiting growth occupying both internal and external factors. In this feeding regime substrate and ammonia did not exist together in the reactor. Synthetic wastewater without nitrogen was fed to the SBR and nitrogen source was fed to the reactor following substrate depletion.

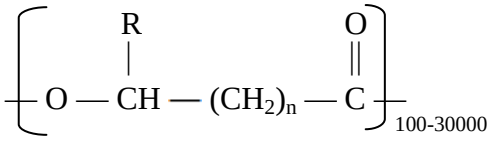
Polymer storage ability of three biomasses enriched under different nitrogen feeding regimes were determined in terms of specific polymer storage rate, yield of polymer on substrate consumed, amount of polymer accumulated, and biomass polymer content. A variety of batch experiments was carried out to investigate responses of biomasses under different conditions. Different substrate types, substrate loadings, and C/N ratios, were applied during batch experiments.

The present work also focused on changes in microbial diversity of three SBR reactors. Changes in community structures of biomasses being enriched under different operating conditions were monitored by a combination of clone library construction and DGGE analysis of clone sequences based on 16S rRNA.

Correlation between changes in community structure and PHA storage ability was statistically evaluated.

2. POLYHYDROXYALKANOATES

PHAs are polyesters of various hydroxyalkanoates that are synthesized by many gram-positive and gram-negative bacteria from at least 75 different genera (Reddy et al., 2003). These polyesters are thermoplastics with biodegradable and biocompatible properties, and the physical properties can be regulated by varying the composition of the copolymers (Doi, 1990). The general structure of polyhydroxyalkanoates was depicted in Figure2.1, which was adapted from Doi, (1990) and Lee, (1996).



n=1	R=	hydrogen	poly (-3-hydroxypropionate)	P(3HP)
		methyl	poly (-3-hydroxybutyrate)	P(3HB)
		ethyl	poly (-3-hydroxyvalerate)	P(3HV)
		propyl	poly (-3-hydroxycaproate)	P(3HC)
		butyl	poly (-3-hydroxyheptanoate)	P(3HH)
		pentyl	poly (-3-hydroxyoctanoate)	P(3HO)
		hexyl	poly (-3-hydroxynonanoate)	P(3HN)
		heptyl	poly (-3-hydroxydecanoate)	P(3HD)
		octyl	poly (-3-hydroxyundecanoate)	P(3HUD)
		nonyl	poly (-3-hydroxydodecanoate)	P(3HDD)
n=2	R=	hydrogen	poly (-4-hydroxybutyrate)	P(4HB)
n=3	R=	hydrogen	poly (-5-hydroxyvalerate)	P(5HV)

Figure 2.1: General structure of polyhydroxyalkanoates.

Poly(3-hydroxybutyrate) [P(3HB)] was first described by Lemoigne (1925) and isolated from Bacillus megaterium. Unlike other biological polymers such as proteins and polysaccharides, this P(3HB) was thermoplastic with a melting temperature around 180ºC. For many bacteria P(3HB) functions either as a carbon and/or energy reserve or as a sink for excess reducing equivalents. P(3HB) exists in the cytoplasmic fluid in the form of granules (Doi, 1990). The first example of microbial copolymers of 3-hydroxyalkanoic acids, poly(3-hydroxyalkanoates) [P(3HA)], was isolated from environmental samples by Wallen and Rohwedder (1974). The copolyesters P(3HA) was present 1.3% of the dry weight of activated sludge and contained four different monomeric units. A controlled-fermentation process was developed by Holmes et al. (1981) and P(3HA) copolyester was produced by feeding bacterial monocultures with a variety of carbon substrates. A copolymer of 3-hydroxybutyrate and 3-

hydroxyvalerate, P(3HB-co-3HV) has been produced commercially by *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) in this process from propionic acid and glucose by Imperial Chemical Industries under the trade name Biopol®.

The products of microbial polyesters, such as films and fibers, can be degraded in soil, sludge or seawater into carbon dioxide and water (Doi, 1990). Under optimum conditions degradation rate is extremely fast (Lee, 1996; Jendrossek, 2001).They can be produced from renewable sources, are recyclable and are considered natural materials. The large diversity of monomers found in PHAs provides a wide spectrum of polymers with varying physical properties (Suriyamongkol et al., 2007). These properties make PHAs good candidates to petrochemical thermoplastics.

Interest in PHAs increased dramatically especially after realization of potential use of these polymers. A considerable effort has being gone by researchers in producing PHA using bacterial monocultures, and mixed cultures as well as eukaryotic cells.

2.1 Physical and Thermal Properties of PHA

The PHAs are non-toxic, biocompatible, biodegradable thermoplastics that can be produced from renewable resources. They have a high degree of polymerization, are highly crystalline, optically active and isotactic (stereochemical regularity in repeating units), peizoelectric and insoluble in water. These features make them highly competitive with polypropylene, the petrochemical-derived plastic (Reddy et al., 2003).

The PHA is typically produced as a polymer of 103 to 104 monomers, which accumulate as inclusions of 0.2-0.5 μm in diameter (Suriyamongkol et al., 2007). They are surrounded by a phospholipid monolayer which is believed to be needed to avoid the contact of PHAs with water (Luengo et al., 2003). The majority of the PHAs are composed of monomers ranging from C3 to C14 carbon atoms with variety of saturated or unsaturated or straight and branched chain containing aliphatic or aromatic side groups (Doi, 1990). PHAs containing up to C5 monomers are classified as short chain length PHAs (scl-PHA). PHAs with C6-C14 and >C14 monomers are classified as medium chain length (mcl-PHA) and long chain length (lcl-PHA) PHAs, respectively (Madison and Huisman, 1999). scl-PHAs have properties close to conventional plastics while the mcl-PHAs are regarded as

elastomers and rubbers (Suriyamongkol et al., 2007). Bacteria synthesize a wide range of PHAs and approximately 150 different constituents of PHAs have been identified (Steinbüchel and Valentin, 1995).

PHB has several useful properties such as moisture resistance, water insolubility, and optical purity, this differentiate PHB from other currently available biodegradable plastics. However, PHB melting point (175ºC) is just slightly lower than its degrading temperature (185ºC), this makes its processing by injection molding difficult (Ojumu, 2004). Doi (1990) reported that the physical and thermal properties of microbial copolyesters can be regulated by varying their molecular structure and copolymer compositions. The P(3HB) homopolymer is a relatively stiff and brittle material. The introduction of hydroxyalkanoate comonomers into a P(3HB) chain greatly improves its mechanical properties. Table 2.1 shows thermal and mechanical properties of P(3HB-co-3HV) copolymers having different compositions. The P(3HB-co-3HV) copolymers becomes more flexible (as demonstrated by the decrease in Young's modulus) and tougher (as demonstrated by the increase in impact strength) as the 3HV content increase (Doi, 1990; Lee, 1996).

Table 2.1: Thermal and mechanical properties of P(3HB-co-3HV) copolymers.

Composition (mol%)		Melting Temperature (ºC)	Glass Transition (ºC)	Heat Distortion Temperature (ºC)	Young's Modulus (GPa)	Tensile Strength (MPa)	Notched Izod Impact Strength (J/m)
3HB	3HV						
100	0	179	10	157	3.5	40	50
97	3	170	8	140	2.9	38	60
91	9	162	6	125	1.9	37	95
86	14	150	4	112	1.5	35	120
80	20	145	-1	99	1.2	32	200
75	25	137	-6	92	0.7	30	400

Data adapted from Doi (1990).

2.2 Practical Applications of PHA

PHA copolymers composed of primarily HB with a fraction of longer chain monomers can be used in a wide range of applications. Many different applications have been described for bioplastics since the first industrial production of Biopol® by ICI Ltd in 1982 (Luengo et al., 2003). Initially, PHAs were used in packaging films mainly in bags, containers, and paper coatings (Madison and Huisman, 1999; Reddy et al., 2003). Similar applications as conventional commodity plastics include the

disposable items, such as razors, diapers, feminine hygiene products, cosmetic containers (Reddy et al., 2003). In addition to potential as a plastic material, PHAs can also be used as chiral precursors for the chemical synthesis of optically active compounds, such as antibiotics, vitamins, aromatics, and pheromones (Holmes, 1985). They are used as carriers for insecticides and herbicides in agricultural applications. Because of their biocompatibility, PHAs are particularly used as biodegradable carriers for long term dosage of drugs, medicines, and hormones. Such compounds are also used as osteosynthetic materials in the stimulation of bone growth owing to their piezoelectric properties, in bone plates, surgical sutures, and blood vessel replacements (Holmes, 1985; Yağmurlu et al, 1999; Reddy et al.,, 2003). These polyesters have been employed also for urological stents, neural- and cardiovascular-tissue engineering, fracture fixation, treatment of narcolepsy and alcohol addiction, cell microencapsulation, support of hypophyseal cells (Luengo et al., 2003).

2.3 Biodegradability of PHA

A remarkable characteristic of microbial polyesters is their biodegradability in microbiologically active environments. Materials made out of PHAs can be degraded in soil, sludge or seawater. PHA is water insoluble and is not affected by moisture, does not degrade under normal conditions of storage, and is stable indefinitely in air (Mergaert et al., 1993; Lee, 1996). However microorganisms, such as bacteria and fungi, colonize on the surface of the polymer and secrete extracellular P(3HB) depolymerases that hydrolyze that environmental P(3HB) and its copolymers into the dimmers and/or monomers in the vicinity of the cells, and the resulting products are absorbed and utilized as nutrients (Doi, 1990).

The effect of different environments on degradation rate of PHAs has been studied by many workers (Doi, 1992; Mergaert et al., 1992, 1993, 1994). Under optimum conditions degradation rate is extremely fast. Lee (1996) showed that P(HB-co-HV) completely degraded after 6, 75, and 350 weeks in anaerobic sewage, soil, and seawater, respectively. PHAs have been reported to degrade in aquatic environments (Lake Lugano, Switzerland) within 254 days even at temperatures not exceeding 6ºC (Jhonstone, 1990). PHAs are compostable over a wide range of temperatures, even at a maximum of around 60ºC with moisture levels at 55% (Reddy et al., 2003).

Boopathy (2000) reported that biodegradation is depend on a number of factors such as microbial activity of the environment, and the exposed surface area, moisture temperature, pH, molecular weight. The nature of monomer units has also been found to affect degradation.

2.4 Polyhydroxyalkanoate Production

Polyhydroxyalkanoates are natural biopolymers that are synthesized and catabolized by various organisms. Since the first finding of P(3HB), in 1926, more than 80 different monomer units have been detected as constituents of PHAs in various bacteria. Today, industrial production of PHAs is possible by using wild and recombinant forms of pure bacterial cultures as well as eukaryotic systems, especially crops. It is important to produce PHA with high productivity and high yield to reduce the overall cost for competing with petroleum derived plastics. Although commercial production has not occurred yet, the interest in the production of PHA by mixed cultures has increased in recent years.

2.4.1 PHA synthesis in pure microbial cultures

A wide variety of microbial species are capable of accumulating PHA. Industrial production processes are based on the use of pure cultures of microorganisms in their wild form, such as *Ralstonia eutropha*, *Alcaligenes latus*, *Burkholderia sacchari*, *Azotobacter vinelandii*, and *Pseudomonas oleovorans* (Doi, 1990; Lemos et al., 2006). More recently, recombinant strains for cost effective PHA production (properties include: rapid growth, high cell density, ability to use several inexpensive substrates, and simple polymer purification) have been developed by cloning the PHA syntheses genes from many microorganisms including *Ralstonia eutropha* and *Escherichia coli* (Dias et al., 2006).

The level of PHA in the cells can be drastically increased from very low percentage to over 80% of cell dry weight when growth is limited by the depletion of an essential nutrient such as nitrogen, oxygen, phosphorus, sulfur, or magnesium. Figure 2.2 shows PHA granules in *A. latus* during the growth and accumulation phases.

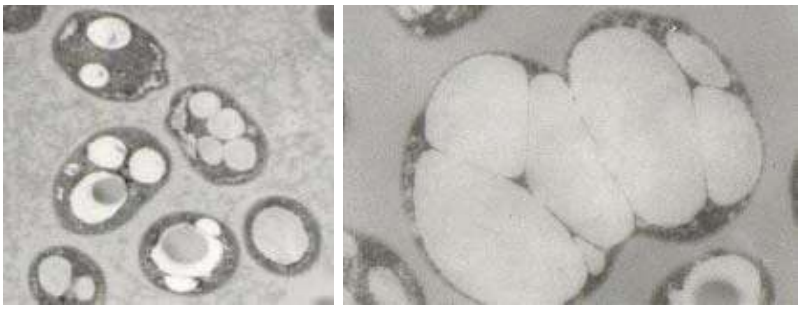


Figure 2.2: PHA granules in *A. latus* during the growth and accumulation phases.

The interest in the use of mixed cultures for the production of polyhydroxyalkanoates has increased in recent years. Mixed cultures selected for PHA production can have a high intracellular storage capacity due to operational conditions that limit their primary metabolism (Dias et al., 2006).

According to Doi (1990), when growth conditions are unbalanced, acetyl-Coenzyme A (-CoA) cannot enter the tricarboxylic acid (TCA) cycle to obtain energy for cells due to high concentrations of Nicotinamide Adenine Denucleotide (NADH). Acetyl-CoA is then used as substrate for PHA biosynthesis by a sequence of three enzymatic reactions. When the entry of acetyl-CoA to the TCA cycle is not restricted, 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.

2.4.1.1 PHA synthesis in pure microbial cultures

More than 300 different microorganisms that synthesize PHA have been isolated (Lee, 1996; Dias et al., 2006). Numerous genes encoding enzymes involved in PHA formation and degradation have been cloned and characterized from a variety of microorganisms. The picture is now clear that nature has evolved several different pathways for PHA formation, each suited to the ecological niche of the PHA-producing microorganism (Reddy et al.,, 2003). In general, the biosynthetic pathway of P(3HB) consists of three enzymatic reactions catalyzed by three different enzymes (Figure2.3) (Madison and Huisman, 1999).

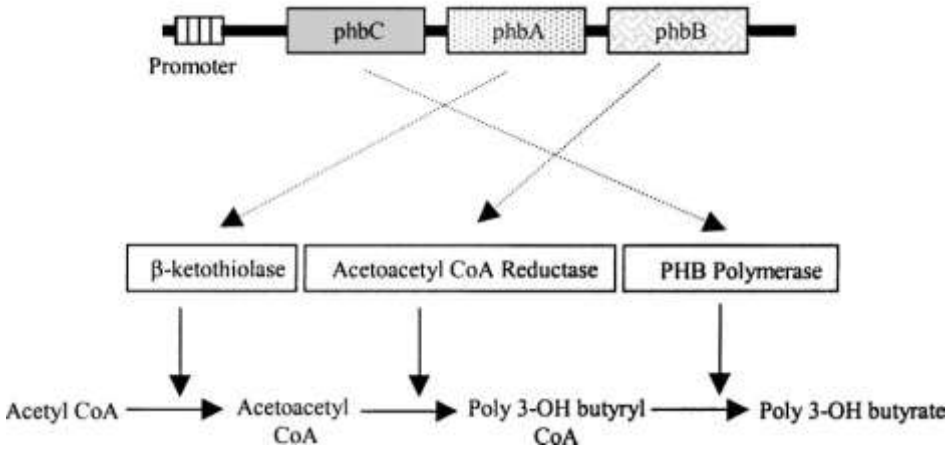


Figure 2.3: Biosynthetic pathway of poly(3-hydroxybutyrate).

The first reaction consists of the condensation of two acetyl-CoA molecules into acetoacetyl-CoA by β-ketoacylCoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an Nicotinamide Adenine Denucleotide Phosphate (NADPH) dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Lastly, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by P(3HB) polymerase, encoded by phbC (Huisman et al., 1999). Most of the organisms synthesize PHA by using this pathway. The pathway and regulation of P(3HB) synthesis have been studied extensively in *R. eutropha*, *Zoogloea ramigera*, and *Azotobacter beijerinckii* (Doi, 1990).

2.4.1.2 PHA Production by Recombinant Bacteria

Most natural PHA producers take a long time to grow during fermentation and extraction of polymers from their cells is difficult. On the other hand, although *E. coli* does not naturally produce PHA, this bacterium is considered to be appropriate host for generating higher yields of biopolymer because of its fast growth and the ease with which it can be lysed (Li et al., 2007). In recent years, a combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHA production in microorganism (Suriyamongkol et al., 2007). pha genes first introduced into *E. coli* by Slater et al. (1988). Since E. coli can utilize various carbon sources, including glucose, sucrose, lactose, and xylose, a further cost reduction in PHA is possible by using cheap substrates such as molasses and whey (Lee et al., 1994). However PHB accumulation level was not as high as what could be obtained with the natural producers of the biopolymer. One of the major obstacles

in producing PHB in recombinant organism is associated with the instability of introduced pha genes. Loss of plasmid due to metabolic load often limits high yield of biopolymer (Madison and Huisman, 1999).

2.4.2 PHA production by mixed cultures

In recent years, there has been a great interest in investigating potential alternative processes for PHA production aiming at decreasing the polymer production costs. Those include the use of low value substrates, as waste feedstocks and microbial mixed cultures. The combination of these two factors allows saving energy (no sterilization is required), reduces fermentation equipment costs (less expensive materials for reactor construction) and minimizes the need for control equipment (less control is required) (Dias et al., 2008).

In general, mixed cultures are microbial populations of unknown composition, which are able to perform specific intracellular and extracellular reactions, and are selected by the operational conditions imposed on the biological system (Dias et al., 2006). The microorganisms involved experience rapidly changing conditions of availability of nutrients and can adapt continuously to change in substrate. Microorganisms which are able to quickly store available substrate and consume the storage to achieve a more balanced growth have strong competitive advantage over microorganisms without the capacity of substrate storage (Van Loosdrecht et al., 1997). PHA has an important role as carbon, energy and reducing power storage material in various microorganisms encountered in activated sludge systems and especially is known to play an important role in mixed cultures both anaerobic/aerobic and aerobic dynamic feeding processing, where electron donor and availability are separated (Satoh et al., 1998).

As stated by many researchers (Lee, and Choi 1999; Satoh et al, 1998; Chua and Yu 1999; Takabatake et al., 2000, 2002) PHA production from waste can provide double benefits because environmentally polluting waste is converted into environmentally friendly biodegradable polymer. In an economical point of view, the cost of substrate that contributes most significantly, to the overall production cost of PHAs, can be decreased if waste product/steam is used as substrate. This makes it possible to produce PHA more economically, and at the same time to treat wastewater without extra disposal cost.

The most important constrain for producing PHA by activated sludge is relatively less PHA content of the sludge. Activated sludge accumulates PHA to around 20% of cell dry weight under anaerobic conditions. This ratio is very low when compared to PHA content of pure cultures which are about 80%.

However there is a considerable effort going to increase the PHA content of the sludge. The PHA content of activated sludge was increased to 62% in a microaerophilic-aerobic sludge process (Satoh et al., 1998; Takabatake, et al., 2002), but the PHA production was not stable. Serafim et al., (2004) showed that the PHB content of activated sludge submitted to aerobic dynamic conditions can reach 65% of cell dry weight using a pulse substrate feeding strategy. This process can be economically competitive with PHA production from pure cultures and it has the advantages of being simpler and requiring less investment and operating cost (Dias, et al., 2006).

2.4.2.1 Anaerobic-aerobic process

The anaerobic-aerobic processing, following its invention in the middle of 1970s, has being widely used for the removal of phosphorus (P) from wastewater. Synthesis of PHA by mixed cultures was first observed in wastewater treatment plants (WWTP) designed for enhanced biological phosphorus removal (EBPR) (Wallen and Rohwedder, 1974). In such an activated sludge process, microorganisms are circulated through anaerobic and aerobic phases, where organic substrates are available to cells only during the anaerobic period (Barnard, 1975).

In this process, some bacteria assimilate volatile fatty acids (VFA) under anaerobic conditions and store them as polyhydroxyalkanoates (PHA) (Nicholls and Osborne, 1979; Wentzel et al., 1985; Comeau et al., 1986). During anaerobic phase, microorganisms use intracellular polyP as an energy source to synthesize PHA, and release orthophosphate generated from polyP degradation. In the aerobic period, microorganisms with stored PHA are able to use these as carbon and energy source to grow and to assimilate phosphate to synthesize polyP (Mino et al., 1987; Wentzel et al., 1985; Comeau et al., 1986; Seviour et al., 2003).

The main groups of bacteria responsible for PHA accumulation selected under these conditions are polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs). PAOs are probably the most widely recognized for

producing storage polymers (PHA, glycogen and polyphosphates). The whole competitive advantage for these organisms is based on their capacity to utilize the energy stored as poly-P to store exogenous substrate in the form of PHA when there is no electron acceptor (oxygen or nitrate) available for energy generation (Salehizadeh and Van Loosdrecht, 2004). GAOs, which were recognized as competitors of PAOs, effectively rely on substrates which can be fermented (e.g., glucose), and they store the fermentation products inside the cell rather than excreting them. These organisms can also use internal stored glycogen for fermentation to PHB. The energy released in the glycolysis process is subsequently used to accumulate fermentation products (e.g., acetate) in the form of PHB. PAOs and GAOs proliferate in systems where the substrate is present regularly while an electron acceptor is absent (Cech and Hartman, 1993). Both groups of microorganisms can take up acetate (as a model substrate for metabolic studies) and activate it to acetyl-CoA. Acetyl-CoA is then consumed for the synthesis of PHB by condensation to acetoacetyl-CoA, reduction to hydroxybutyryl-CoA and finally polymerization to PHB (Figure 2.4) (Salehizadeh and Van Loosdrecht, 2004).

Two different metabolic models have been proposed by Comeau et al. (1986) and Mino et al. (1987) to explain the interaction between phosphorus release under anaerobic condition and uptake and storage of short-chain fatty acids. Source of electrons for formation of the PHA was the main difference between models. Comeau et al. (1986) proposed the oxidation of substrate in the TCA cycle, while Mino et al. (1998) were indicating that the conversion of glycogen to acetyl-CoA delivered the essential reduction of power for forming the PHA.

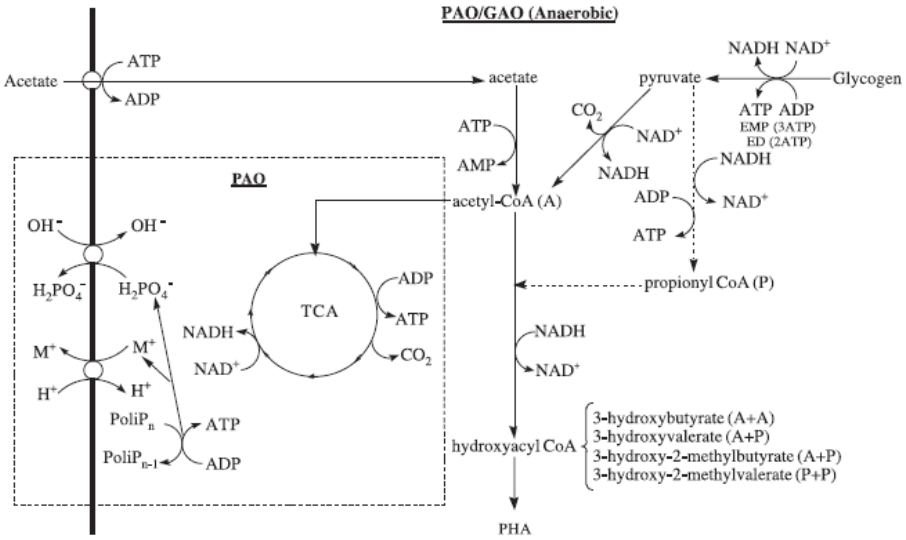


Figure 2.4: PHA production metabolism in PAO/GAO system.

The amount of PHA accumulated by these groups of microorganisms is generally less than 20% (Satoh et al. 1996). A PHA content between 30 and 57% was achieved using a reactor designated as polyhydroxyalkanoate accumulating bacteria enhanced reactor (PABER), however PHA content in this anaerobic-aerobic process was not stable (Takabatake et al. 2000).

2.4.2.2 Microaerophilic-aerobic process

Satoh et al., (1998) proposed a novel strategy for PHA accumulation. Activated sludge acclimatized under microaerophilic-aerobic conditions accumulated PHA of 60% or more. In the microaerophilic-aerobic reactor, microorganisms are contacted with the organic substrates in the existence of a limited amount of oxygen.

In such conditions, microorganisms can take up organic substrates by getting energy through oxidative degradation of some part of the organic substrates. If supply of oxygen is sufficient, the microorganisms may be able to get enough energy for assimilative activities such as the production of protein, glycogen, and other cellular components simultaneously with taking up organic substrates. But if the supply of oxygen is adequately controlled, we may be able to suppress such assimilative activity while letting microorganisms accumulate PHA. The following aerobic conditions where excess oxygen is supplied allow microorganisms grow with the consumption of PHA. Production of PHA requires less energy when compared to production of glycogen does. In the subsequent microaerophilic-aerobic conditions,

the microorganisms that are dominant will be able to take up organic substrates and accumulate them as PHA under microaerophilic conditions while getting energy by oxidative consumption of part of the organic substrates. They will not have the ability to utilize energy reserve materials such as polyphosphate or glycogen for anaerobic substrate uptake, since they do not have reason to have it.

Although a maximum content of PHA achieved in this process (62%) was higher than that achieved in anaerobic-aerobic one, relatively less studies carried out to investigate these systems (Takabatake et al., 2000; Punrattanasin et al., 2006) because of instability of PHA production in this process.

2.4.2.3 Aerobic Dynamic Feeding

Sludge with significant PHA storage capacity was also observed in aerobic WWTP, where selectors for bulking control were introduced. The concept of aerobic "feast and famine" process was first proposed by Majone et al. (1996). This process configuration originates periods of excess of carbon (in the selector reactor) alternated with substrate limitation (main reactor) favoring the selection of floc-formers with enhanced PHA storage capacity (Majone et al. 1996). In order to understand the mechanisms responsible for the enhanced PHA storage capacity of the enriched mixed culture present in these systems, conditions of carbon excess (feast) and limitation (famine) were simulated in lab-scale reactors. The enhanced capacity of the culture to store PHA under these conditions was confirmed (Majone et al. 1996).

According to Salehizadeh and Van Loosdrecht (2004), among the mentioned systems for industrial production of PHAs, the feast and famine approach is the most promising because of high PHA accumulation. This approach promotes the conversion of the carbon substrate to PHA and not to glycogen or other intracellular material. Fundamentals, metabolism, microbiology and operation parameters of this process will be discussed in subsequent chapter.

2.4.3 PHA Production in transgenic plants

Starch is one of the most abundant biopolymers, which is sold at 20 cents/kg. With an aim to produce PHA as cheap as starch, several researchers have been investigating the possibility of producing P(3HB) in transgenic plant (Lee, 1996).

Since P-ketothiolase, the first enzyme of PHA synthesis, is present in the cytoplasm of higher plants, only the reductase and the PHA synthase are required to synthesize PHA in plant cells (Poirier et al., 1992). Another driving force in exploring PHA production in plant systems was relying of these systems only on water, soil nutrients, atmospheric CO_2, and sunlight. Following a considerable effort, the genetically engineered genes of *A. eutrophus* were successfully targeted to the plant plastids, and the enzymes were active in the plastids. The hybrid expressing the A. eutrophus PHA synthesis enzymes accumulated P(3HB) up to 10 mg/g fresh weight, representing 14% of dry weight (Nawrath et al., 1994). Although these results show that production of PHA by transgenic plant may become economical, there are still barriers to increasing PHA production in plants.

Barriers were generally associated with expression of transgenes and metabolic load on plant growth (Suriyamongkol et al., 2007). Bohmert et al. (2002) demonstrated that constitutive expression of phaA was detrimental to plant growth as early as during the transformation step. Preventing the expression of phaA during transformation/regeneration procedure by using inducible promoter helped the generation of transformants in some plants. Competition between several metabolic pathways for acetyl-CoA was another important obstacle encountered. Specific enzyme inhibitors were used to suppress these anabolic pathways in order to increase the availability of acetyl-CoA for PHB production (Suzuki et al., 2002). Increasing the availability of acetyl-CoA in plant cells might be another strategy to improve polymer yield in plants (Suriyamongkol et al., 2007).

2.5 Polymer Recovery

PHA polyester can be extracted from bacterial cells with a suitable organic solvent such as chloroform, methylene chloride, 1,2-dichloroethane, propylene carbonate, tetrahydrofuran methyl cyanide, or ethyl cyanide (Doi, 1990; Dias et al., 2006). The recovery methods developed for pure cultures are based on two different principles: the polymer solubility in appropriate solvents, and disruption of the cell membrane. Although large effort has been devoted to understanding and optimization of PHA production by mixed cultures, the same cannot be said regarding the downstream process. Currently, a low-cost, highly efficient, and environmentally friendly, PHA recovery process is not generally accepted or implemented. Recently, supercritical

CO_2 extraction and non-PHA selective cell mass dissolution by protons, with PHA crystallization are two novel methods proposed as environmentally friendly and cost effective. These methods may also be applied to the recovery of PHA produced by mixed cultures (Dias et al., 2006).

In the production of PHA in crops, the extraction and purification processes yet another challenge. Unlike extractions of bacteria there are other useful byproducts that can also be extracted from harvested crops. Any extraction process from plant tissue should accommodate extraction of such compounds in unmodified form. Two new methods for PHA extraction from crops have been also described which were based on wet and dry milling methods used in corn industry (Suriyamongkol et al., 2007).

2.6 Economics of PHA Production

The development of PHB was begun by ICI in 1975 as a response to the increase in oil prices. ICI, Zeneca Bio Product started making Biopol® as early as in 1982 from Ralstonia eutropha, which can store PHA up to 80% of its cell dry weight. Commercialization of PHA has being continued to grow up since the production of Biopol®. Biopol has being marketed by Berlin Packaging Corp. USA, especially for production of bottles for cosmetics. Bioscience Ltd., Finland has been producing material from PHA for medical applications. BioVentures Alberta Inc., Canada has been producing PHA from recombinant E. coli. Metabolix (Cambridge, MA) is another company producing P(3HB-co-3HV) by R. eutropha. Currently,European consumption of biodegradable polymers is roughly estimated at 50,000 ton/year which accounts for less than 1% of the world annual production of polymers (>100 million tons). Commercial applications and wider use of PHA is prevented mainly by their high production cost ($4-6/kg) compared with the petroleum-derived plastics (Akaraonye et al., 2010).

Suriyamongkol et al. (2007) stated that production of PHA in agricultural crops is likely to be economically viable if it can be produced as a byproduct with some other plant constituents such as oil or starch. The main factor affecting production cost of PHA by pure bacterial cultures is stated by Yaoping (2007), as the high cost of the substrate and of the equipment required for aseptic operation. With the aim of commercializing PHA, a substantial effort has been devoted to reducing the

production cost through the development of bacterial strains and more efficient fermentation/recovery processes. However the price of the substrate and its sterilization still has the largest influence on the cost of production of PHA. Because sterilization is not required in PHA production by using mixed cultures, they have recently started to attract a lot of attention. Using mixed bacterial culture instead of pure cultures can be a cheaper alternative for PHA production. It has the potential to produce large amounts of PHAs with seemingly lower costs due to lower sterility, equipment and control requirements and the ability to utilize a wide range of cheap substrates including industrial and agricultural wastes (Reis et al., 2003; Rhu et al., 2003). In recent years, the use of organic wastes such as food wastes, and olive mill effluents is being studied as an alternative substrate for PHA production (Yu et al., 1999; Dionisi et al., 2005a). PHA production from wastes can provide double benefits since environmentally polluting waste is converted into environmentally friendly biodegradable polymer. The use of mixed cultures and waste materials can substantially decrease the cost of PHA and increase their market potential (Satoh et al., 1998). Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production carried out by Gurieff and Lant (2007) showed that if cheap, reliable sources of renewable energy were made available through the help of legislation and subsidies, the current technology PHA process would become both financially and environmentally attractive.

3. ADF PROCESS for PHA PRODUCTION

Feed regime of activated sludge processes can be highly dynamic, especially when equalization facilities are not employed. The biomass subjected to successive periods of external substrate availability and no external substrate availability experiences an unbalanced growth. Under dynamic conditions, growth of biomass and storage of polymer occur simultaneously when there is an excess of external substrate. When all the external substrate is consumed, stored polymer can be used as carbon and energy source. Dynamic conditions add a selection pressure that favors microorganism with an ability to establish internal carbon reservoirs. According to Dias et al. (2006) PHA storage by activated sludge under fully aerobic conditions can be a particularly important process, if the sludge is submitted to consecutive periods of external substrate accessibility (feast) and unavailability (famine). This process is currently known as ADF or ‘‘feast and famine’’. These conditions allow for the selection of an enriched culture with a high and stable capacity of PHA production.

3.1 Fundamentals

In the presence of an external substrate, microorganisms have the option to use the substrate for growth or for accumulation of intracellular reserves. Accumulation occurs when growth is limited by external factors such as a lack of nutrients (for example, phosphorus or nitrogen) or internal factors such as an insufficient amount of Ribonucleic acid (RNA) or enzymes required for growth. The previous mechanism is the one applied during industrial production of PHA by *R. eutropha*. Cells start to accumulate P(3HB) after they encounter phosphate limitation after about 60 hours of growth. The later mechanism is similar to the one observed during industrial production of PHA by recombinant *E. coli*. Synthesis of P(3HB) by recombinant E. coli does not require limitation of a specific nutrient, but is dependent on the amount of acetyl-CoA available.

According to Dias et al. (2006), the latter mechanism is the most accepted explanation for the storage phenomena in the feast and famine process, where

substrate availability alternates periodically. Starvation by substrate for a certain period of time can cause a decrease in the amount of intracellular components RNA and enzymes needed for growth. Following this period of substrate limitation, cells faced with an excess of substrate can take it up rapidly. However, the growth rate does not increase at a rate that corresponds with the substrate uptake rate. This difference is related to the fraction of substrate stored or used for cell maintenance. After starvation, storage occurs preferentially instead of cell growth because the amount of enzymes required for storage are lower than the RNA and enzymes needed for growth at maximum rate (Daiger, and Grady 1982). During the famine period, the stored substrate is used for cell growth and cell maintenance. Operation of a reactor under feast and famine conditions can selectively enrich a mixed population with a high storage capacity.

Higher PHA production yields obtained during batch experiments carried out without nitrogen proofs that external factors also have a considerable effect on polymer storage by mixed microbial cultures enriched under dynamic conditions.

3.2 Metabolism

Currently, there is no experimental evidence regarding the metabolism performed by PHA-producing bacteria in mixed cultures. However, it is assumed that PHA metabolism in mixed cultures is similar to that reported for pure cultures using the same carbon substrate (Serafim et al., 2008) (Figure3.1).

In activated sludge submitted to aerobic dynamic feeding, considering acetate as the carbon substrate, the acetyl- CoA (two carbons) produced is partially channeled to the TCA for growth and NAD(P)H production, and partially used for PHA production (Figure 3.1). For polyhydroxybutyrate synthesis, the main polymer formed from acetate, two units of acetyl-CoA condense to produce acetoacetyl-CoA, which is reduced to hydroxybutyryl-CoA at the expense of NAD(P)H, and finally gives the hydroxybutyrate monomer (HB, four carbons). PHB is metabolized when no external substrate is available (Dias et al., 2006).

This monomer is the direct precursor for the synthesis of P(3HB). Propionate gives rise to three different precursors. If two molecules of propionyl-CoA condense, they will originate 3-hydroxy-2-methylvaleryl-CoA, the precursor for poly-3-hydroxy-2-

methylvalerate [P(3H2MV)]. If acetyl-CoA is present or formed from the breakdown of propionyl-CoA (in the case of mixture of substrates), the junction of both molecules, acetyl and propionyl-CoA can produce P(3HV) or poly-3-hydroxy-2-methylbutyrate [P(3H2MB)]. Butyrate can be used for the production of P(3HB) and valerate to P(3HV) directly but also used through β-oxidation. These four polymers are the most frequent scl-PHA (Serafim et al., 2008).

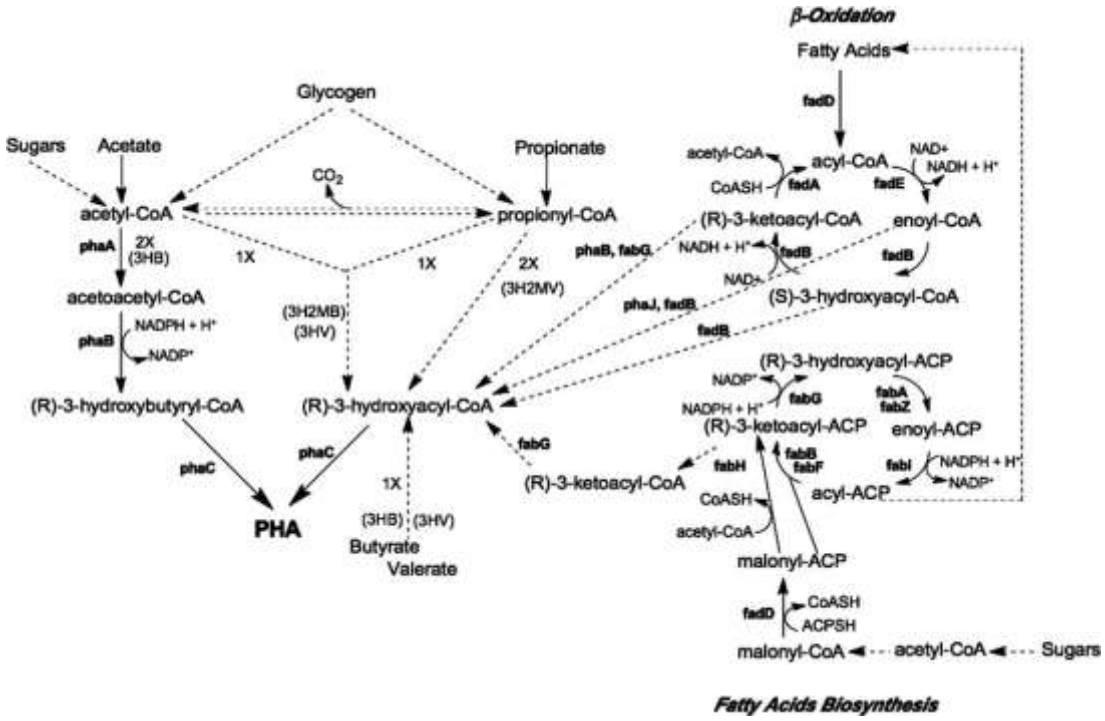


Figure 3.1: Metabolic pathways for polyhydroxyalkanoates synthesis (abcD-gene responsible for the synthesis of the enzyme involved in the step).

For scl-PHA, the substrates used are mainly short-chain VFAs such as acetic, propionic, butyric and valeric acids, or/and sugars, like glucose. The specificity of PHA synthase, the enzyme involved on the polymerization of the precursors, determines the type of PHA produced, either scl-PHA or medium-chain length polyhydroxyalkanoates, mcl-PHA (Rehm, 2003). Break down of longer chain fatty acids by β-oxidation reactions produces two-carbon chain precursor, by acetyl-CoA, for an even number of carbons, or to acetyl-CoA and propionyl-CoA, for an odd number of carbons. These precursors follow the general metabolism described above, while precursors with a higher number of can yield the corresponding hydroxyacyl-CoA (Dias, et al., 2006; Serafim et al., 2008).

In general, the global yield (which accounts for the substrate used for cell growth, polymer synthesis, and maintenance) for acetate, butyrate, and valerate is close to

quantitative, indicating that almost all the carbon was used for these three processes. For propionate, the global yield is usually less than 1.0 g substrate/g substrate, due to the production of CO_2 from decarboxylation that is not accounted for in respiration measurements. For the production of HV (2C+3C; the main monomer produced), HB (2C+2C), or HMB (2C+3C), there is always the need to convert at least one propionyl-CoA (3C) into acetyl-CoA (2C) (Dias et al., 2006).

3.3 Microbiology

Currently, more than 300 different microorganisms that synthesize PHA have been isolated (Lee, 1996; Dias et al., 2006). Numerous studies have been conducted to identify the bacterial diversity of anaerobic-aerobic systems in both full-scale and lab-scale reactors through the use of molecular techniques. The main driving force in exploring population structure of anaerobic-aerobic activated sludge was to establish a relationship between population and EBPR performance. It was widely accepted that communities of these systems are phylogenetically very diverse. The *Rhodocyclus* group of *Betaproteobacteria* was represented to a greater extend in activated sludge systems performing EBPR (Crocetti, et al., 2000; Dionisi, et al., 2002; Levantesi, et al., 2002; Kong, et al., 2002; Seviour et al., 2003; Jeon, et al., 2003; Wong et al., 2005; Oehmen et al, 2007). However little information is available about the microorganisms responsible for PHA accumulation under ADF.

The evolution of bacterial community through reactor operating period was studied Dionisi et al. (2005b) by applying denaturing-gradient gel electrophoresis (DGGE). The operating conditions of the SBR were shown to select for a restricted microbial population which appeared quite different in terms of composition with respect to the initial microbial cenosis in the activated sludge used as inoculum. A partial identification of the selected population was obtained by the sequencing of the major bands of the DGGE profile. On the basis of the sequencing of the major bands in the DGGE profiles, four main genera were identified: a *Methylobacteriaceae bacterium, Flavobacterium sp, Candidatus Meganema perideroedes*, and *Thauera sp*. Former two had been isolated from activated sludge before (van der Gast et al., 2004; Liu et al., 2005). *Candidatus Meganema perideroedes* has been previously described as filamentous bacteria occasionally causing bulking problems in activated sludge treatment plants (Thomsen et al., 2002) and it has been shown to be present and able

to store PHAs in an aerobic reactor intermittently fed with acetate (Levantesi et al., 2004). The genus *Thauera* had been isolated from activated sludge (Valle et al., 2004), which is known to be a member of *Rhodocyclus* group within *Betaproteobacteria*.

In another study, Dionisi et al. (2006) after confirming the speciation of the population for PHA accumulation by DGGE, constructed a clone library from the total deoxyribonucleic acid (DNA) extracted from the SBR sludge. The screening of the clones was performed using amplified ribosomal DNA restriction analysis (ARDRA). Each of the 14 different operational taxonomic units (OTU) was sequenced. The most abundant taxonomic group obtained was the *Betaproteobacteria*, in which at least two different species of *Thauera*, two species of *Alcaligenes*, *Comamonas* sp., *Achromobacter* sp., and *Pseudomonas* sp. were present. *Thauera* sp. was the most abundant organism in the sludge, but neither *Thauera* sp. nor *Achromobacter* sp. have been shown to produce PHA. Many strain belonging to *Alcaligenes* genus were isolated from activated sludge (Dias and Bhat, 1964), and many of them were actually described as PHA over producers (Doi et al., 1990; Yu et al., 1999). The genus *Comamonas* has also been evaluated for PHA production, and *Comamonas acidovorans* found application in the production of PHAs with a high 4-hydoxybutyrate monomer content (Saito and Doi, 1994). Bacteria from the *Gammaproteobacteria* group were detected, namely the genera *Kluyvera*, *Pseudomonas*, and *Acinetobacter*, although *Kluyvera* has also not yet been shown to produce PHA. Finally, *Xanthobacter* sp., belonging to the *Alphaproteobacteria*, and *Curtobacterium* sp. were also identified, where the latter has not yet been associated with PHA accumulation. Further investigation regarding the aforementioned microbial groups is necessary to certify their status as PHA producers.

A different approach was employed by Serafim et al., (2006). Fluorescent in situ hybridization (FISH) applied in combination with Nile Blue staining showed that the microbial group able to store the higher amount of PHA belonged to the *Azoarcus* genus within *Betaproteobacteria*. Other two main morphotypes identified were belong to the *Alphaproteobacteria* class.

The selected populations in two reactors fed with acetate and with propionate presented a similar microbial composition, despite both systems representing

different metabolisms that resulted in diverse polymer compositions, kinetic and stoichiometric parameters.

Majone et al. (2006) monitored the dynamics of the bacterial speciation, taking place in the SBR operated under feast and famine conditions, by means of DGGE analyses. Stabilization in the microbial speciation was observed to attain in 18 days. Bacterial community observed in this study were similar to that observed by Dionisi et al. (2005b). The main genera identified were *Thauera, Candidatus Meganema perideroedes* and *Flavobacterium*.

The feast and famine process was also applied to pure cultures like *Amaricoccus kaplicensis* and *Paracoccus pantothrophus*. Both cultures showed a similar behavior to that of the mixed cultures submitted to ADF: PHA storage and growth while the substrate was available, and PHA consumption during famine conditions. The abundance of these strains in a mixed-culture process has not yet been evaluated (Dias et al., 2006).

A comprehensive study was carried out by Lemos et al., (2008) to identify PHA storing bacteria by applying reverse transcriptase–polymerase chain reaction on micromanipulated cells and four genera, *Amaricoccus, Azoarcus, Thauera* and *Paraccoccus* were detected. FISH was also applied in this study to confirm results. The use of acetate or propionate as acarbon substrate and diverse operating conditions on selected microbial populations was also investigated in the mentioned study.

3.4 Process Operation

3.4.1 Substrates

Selection of a suitable substrate is an important factor for optimizing the PHA production and affects the PHA content, composition and the polymer properties. Over 40% of total operating expense of PHA production is related to the raw materials, and more than 70% of this cost is attributed to the carbon source. Therefore, a more cost-effective process should include the selection of cheap substrates (e.g., whey, molasses, palm oil mill effluents, starch, xylose, malt, and soy wastes) which can be effectively used by the microorganisms to synthesize PHA at

high productivities, and that the resulting PHA polymer properties are suitable for a wide range of industrial applications (Dias et al., 2006).

Unlike pure cultures, carbohydrates are not directly stored as PHA by mixed cultures, they are preferentially accumulated as glycogen (Karahan et al., 2006). Accordingly PHA production from sugar-enriched raw materials requires a previous anaerobic fermentation step for their transformation into VFA. This is the reason why the majority of the studies related to PHA production by mixed cultures are based on the use of organic acids.

Acetate, which is transformed into a homopolymer of PHB, is one of the most well-studied substrates for PHA production by mixed cultures submitted to feast and famine conditions. Copolymers of poly(3HB-co-3HV) can be synthesized by bacteria from higher-chain VFA, such as propionate, butyrate, and valerate.

When propionate was fed as the sole substrate, both a homopolymer of HV (Lemos et al., 2006) and a copolymer of poly(3HB-co-3HV) or terpolymer (HB/HV/HMV) (Dionisi et al., 2004a) were observed to be produced. The difference in polymer composition observed in both studies is probably due to differences in the microbial population structure. Similar storage yields were obtained in both studies (0.30 g PHA/g substrate and 0.35 g PHA/g substrate). The HV fraction in polymer produced from propionate as the sole carbon source by pure culture of *R. eutropha* was much lower (45%) (Doi et al., 1987) than that produced by activated sludge (83%) (Lemos et al., 2006).

Butyrate and valerate have also been used in the feast and famine process. Butyrate, was converted into a homopolymer P(3HB) (Lemos et al., 2006). The polymer yield (0.44 g PHA/g substrate) was lower than the value obtained by the same culture with acetate (0.52 g PHA/g substrate), but higher than feeding with propionate (0.30 g PHA/g substrate). Valerate was stored by this culture as a terpolymer of P(HB/HV/HMV) with a yield of 0.37 g PHA/g substrate. The synthesis of PHA from propionate requires a decarboxylation step, which may also be the case for valerate. This likely explains the lower polymer yield from these two substrates (Dias et al., 2006).

Glutamic acid was also fed as the only carbon source to mixed cultures cultivated under dynamic conditions (Dionisi et al., 2004b). Storage of PHB occurred with a

low yield (0.10 g COD PHB/g COD substrate) simultaneously to biomass growth. Interestingly, neither other PHAs nor other possible storage polymers, like polyglutamic acid, were detected.

Mixtures of substrates are generally used to obtain copolymers with different monomer compositions, aiming at the tailored synthesis of PHA with given target mechanical properties (Dias et al., 2006). In a feast and famine process, a mixture of 50% of propionate and acetate was transformed in a copolymer poly(3HB-co-3HV) with molar fractions close to 1/1 (Lemos et al., 2006). Interestingly, the quantity of HV per mole of carbon consumed was higher when acetate and propionate were supplied simultaneously than when only propionate was fed. Indeed, when both substrates are present, the acetyl-CoA units required for HV synthesis can be produced directly from acetate, leaving more propionyl-CoA available for hydroxyvaleryl-CoA synthesis.

Relative concentration of valeric and butyric acids were studied by Chua et al. (1999). The incorporation of valeric acid in the substrate caused increase of the molar fraction of 3HV in the polymer and lower overall polymer yields. When a mixture of acetate, propionate, butyrate, and valerate was fed to a batch reactor, the former two were observed to be removed completely. However only a small fraction of butyrate and valerate was consumed (Beccari et al., 1998). A copolymer of poly(HB-co-HV) was produced in this study with a storage yield of 0.39 g PHA/g substrate. Mixtures of acetate and glucose were studied by Carta et al. (2001) Glycogen and PHB were stored simultaneously by the sludge with yields of 0.74 g glycogen/ g substrate and 0.51 g PHB /g substrate, respectively.

The production of PHA by mixed cultures under feast and famine conditions was also studied with real wastewater. Studies involving carbohydrate-based wastes include a prefermentation step in order to produce VFA.

Olive oil mill effluents (OME) were converted by Dionisi et al. (2005a) in a continuous anaerobic reactor to a mixture of organic acids (acetic, butyric, propionic, isobutyric, and valeric), which were then fed to a batch reactor inoculated with an enrichment of PHA-accumulating bacteria. The storage yield (1 g PHA /g VFA on COD a basis) was much higher than the one obtained with synthetic substrate containing acetic, lactic, and propionic acids (0.39 g PHA /g VFA on a COD basis). This was explained by the conversion of organic compounds other than VFA, which

were also present in the fermented OME. The polymer content obtained was 54%. The non-fermented OME (containing only acetic and lactic acids as VFA) was also used, but the total amount of PHA produced was three times lower than that obtained from the fermented OME.

Other real wastes that have been used for the production of PHA include fermented food waste (Rhu et al., 2003), sugar cane molasses (Albuquerque et al., 2007), paper mill wastewater (Bengtsson et al., 2008), and alkaline excess sludge (Mengmeng et al., 2009).

3.4.2 Reactor operational strategies

The majority of the studies related to the production of PHA by the mixed cultures submitted to feast and famine conditions were carried out in SBR, operated with cycles of feeding, reaction, settling, and draw. The length of the total cycle varied from 2 to 12 h, however the substrate feeding period was always very short (Dias et al., 2006). Serafim et al., (2004) fed substrate to the SBR by pulses and controlled the addition on-line by the rise in oxygen concentration in the reactor. In this case, the length of the famine phase was fixed (10 h), while that of the feast phase varied freely according to the time required to consume all of the carbon supplied. Pulse-wise addition of substrate was also carried out in batch reactors inoculated with biomass taken from SBRs enriched in PHA-accumulating bacteria to supply more than one pulse of substrate in order to obtain higher PHA content and to avoid substrate inhibition.

Biomass concentration used for PHA accumulation and selection of microbial cultures with a high storage capacity is essential for productivity of the mixed-culture processes. Both can be achieved by a PHA production process, based on continuous culture selection followed by batch PHA accumulation. Dionisi et al. (2004a) proposed a three step process for “side stream” PHA production: acidogenic fermentation to transform a high-concentration biodegradable waste at high rate into a mixture of acetate and other carboxylic acids; SBR operation under feast and famine conditions to enrich and produce sludge with a high storage capacity; batch reactor which is fed with a high substrate concentration in order to increase the polymer content in the biomass, and where the excess sludge produced in the second

step is introduced. This three stage process was employed in different studies (Dionisi et al., 2005b, 2006; Albuquerque et al., 2007; Bengtsson et al., 2008).

3.4.3 Operational parameters

Dias et al. (2006) stated that the polymer yield and productivity, for the same kind of substrate, depend on the reactor operating conditions such as the substrate concentration, organic loading rate (OLR), carbon to nitrogen ratio (C/N), sludge retention time (SRT), pH, oxygen concentration, and temperature.

3.4.3.1 Substrate concentration

Serafim et al. (2004) evaluated the impact of initial acetate concentration on PHA production. The amount of polymer produced was observed to be determined by the quantity of substrate supplied. A linear relationship was observed between the amount of polymer produced and the substrate consumed, in the range of 0.90-2.70 g/L of acetate by Serafim et al. (2004) and 0.18- 1.44 g/L of acetate by Beun et al. (2002).

3.4.3.2 Organic loading rate

The effect of the organic loading rate in a SBR operated under aerobic dynamic feeding was evaluated by Dionisi et al.(2006) between 7.5 and 27.6 g substrate /L/h). The maximum polymer concentration and storage rate observed for 17.6 g substrate/L/h and decreased sharply for higher OLR values.

3.4.3.3 Sludge retention time (SRT)

Beun et al. (2000) stated that the specific acetate uptake rate in the feast period decreased with increasing SRT, but it was not linearly dependent on the SRT. In systems with biomass growth only, a linear relation between substrate uptake and biomass growth exist. However in dynamically fed systems, in which biomass growth and PHB production occur simultaneously, more complicated relations exist because the anabolic rate depends on the anabolic enzymes level in the cell. Biomass growth during acetate uptake is no longer related to the growth rate in the system dictated by the SRT. PHB yield per substrate and specific productivity observed by these authors were almost constant between the SRT values of 3.8 and 19.8. Dionisi

et al.(2002) also obtained a relatively constant storage yield in a SRT range of 0.37-3 d.

Chua et al. (2003) proposed that sludge acclimatization with a short SRT may be preferable for PHA production purpose. This is because the sludge yield under a shorter SRT is higher than that under a longer SRT. Therefore, activated sludge process operated with a short SRT can supply sufficient amount of sludge for PHA production compared to that with a long SRT.

3.4.3.4 Carbon to nitrogen ratio (C/N)

The PHA storage in the feast and famine process occurs when growth is limited. Although growth is believed to be limited mostly by internal factors, ammonia limitation was also used to control the fraction of substrate driven through cell growth (Serafim et al., 2004; Lemos et al., 2006). In these studies, ammonia was exhausted in the beginning of the SBR cycle together with substrate. It was shown in batch experiments that, in the range of 0-40 mg N/L, the growth yield increased proportionally with ammonia concentration while the storage yield decreased. When no ammonia was supplied, the storage yield (0.83 Cmmol HB/Cmmol HAc) was more than double that obtained for 40 mg N/L (0.37 Cmmol HB/Cmmol HAc). The highest amount of PHB stored was reached for 10 mg N/L (38.6%) and the lowest for 40 mg N/L (25.4%). Bengtsson et al. (2008) studied production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater by performing batch tests with varying nutrient levels. They reported that limiting nitrogen and/or phosphorus produced higher amount of polymer and also polymer yield.

On the other hand, Dionisi et al. (2005b) compared one batch reactor fed with an excess of ammonia with another reactor fed without a nitrogen source and observed no significant impact on the efficiency of PHA storage. The difference observed in both studies can be attributed to differences in the method of selecting the PHA-accumulating bacteria. In the studies of Serafim et al.(2004) and Lemos et al.,(2006) ammonia was only available at the beginning of the feast phase, whereas in the studies of Dionisi et al.(2001, 2004a, 2005b) and Beun et al.(2000, 2002) ammonia was always present along the feast and famine cycles. By evaluating the results obtained in the mentioned studies it may be concluded that ammonia is an important

parameter to be controlled in the ‘‘feast’’ and ‘‘famine’’ processes for PHB production. However effect ammonia limitation during SBR operation on PHA accumulation in batch reactor needed to be proofed experimentally.

3.4.3.5 pH

The effect of pH on the PHA content was studied by Chua et al.,(2003). They found that, through controlling the pH at 6 or 7, the PHA content (less than 5%) was lower than at pH 8 or 9 (25–32%). Serafim at al. (2004) also observed that PHB content and polymer storage yield were higher at pH 8 than at pH 7, and increased sharply when pH was not controlled. When pH was not controlled, it was reported to fluctuate between 8 and 9.5 by tending to increase during feast phase and to decrease slightly following the substrate exhaustion. It is more advantageous to have a non-pH-controlled system from the point of view of the operation simplicity, as well as PHA productivity.

3.4.3.6 Temperature

The effect of temperature on PHA production was evaluated by (Krishna, C., and van Loosdrecht, M.C.M., 1999). A decrease in the yield of PHB on acetate from 0.43 to 0.072 g PHA /g substrate and a decrease in the specific productivity from 0.12 to 0.060 g PHA /g cell dry weight /h, was observed when temperature increased from 15 to 35ºC. Increased temperature also led to a slightly lower biomass formation in feast phase. At the same time however the CO_2 production strongly increased because the PHB accumulation decreased and the contribution of maintenance processes increased. Low temperatures (between 15 and 20ºC) allow for a less costly process and favors polymer produced in the cells.

4. MATERIALS and METHODS

4.1 Origin of the Seed

The enrichment reactors were inoculated with sludge obtained from a full scale SBR treating wastewater of a paper mill producing cardboard from recycled fibers (Halkalı Kağıt Karton Sanayi ve Tic. A.Ş.).

4.2 Experimental Design and Operation of Enrichment Reactors

Three SBRs having working volume of 3L were utilized to enrich activated sludge under aerobic dynamic feeding conditions. One was fed by sufficient amount of nitrogen (COD/N/P: 100/12/2) through operating period, hereinafter called as "SBR N+". One was fed by nitrogen deficient synthetic wastewater (COD/N/P: 100/2/2), hereinafter called as "SBR N-". The other reactor was fed by nitrogen deficient synthetic wastewater, however ammonia was supplied to the reactor separately and 1 hour after the supplement of synthetic wastewater. The SBR operated under ADF conditions with delayed nitrogen feeding was hereinafter called as "SBR N_D-". Two of the SBRs (N+ and N-) were operated simultaneously (Figure 4.1), however SBR N_D- was started to be operate after former two SBRs operation finish.

For all SBRs, a fill volume, V_F, of 1.5 L was selected, leaving remaining 1.5 L for the initial volume, V_0, and corresponding to a V_0/V_F ratio of 1/1. Timers controlled the stirring, pumps for air, medium feed and removal, and biomass removal. The SBR operation was designed for four cycles a day. The SBR cycles consisted of 5 h of reaction including feast and famine phases, 30 min settling and 30 min withdrawing upper half of the volume, which was replaced with fresh medium at the beginning of the next cycle. Filling was achieved in 3 minutes. A defined volume of biomass was removed to keep a SRT of 8 days. Theoretically activated sludge systems were considered to run at steady state conditions after three sludge ages of operation. Accordingly, SBRs can be considered to reach steady state conditions in one month. Although activated sludge concentrations and MLVSS/MLSS ratios were

almost constant in three reactors after one month of operation, SBRs were operated for an additional one month before batch experiments. The reactors were operated without pH control. The temperature was kept at 20±2°C. Nitrification was hindered by using thiourea. SBR N+ and SBR N- were operated for 82 days and SBR N_D- was operated for 80 days.

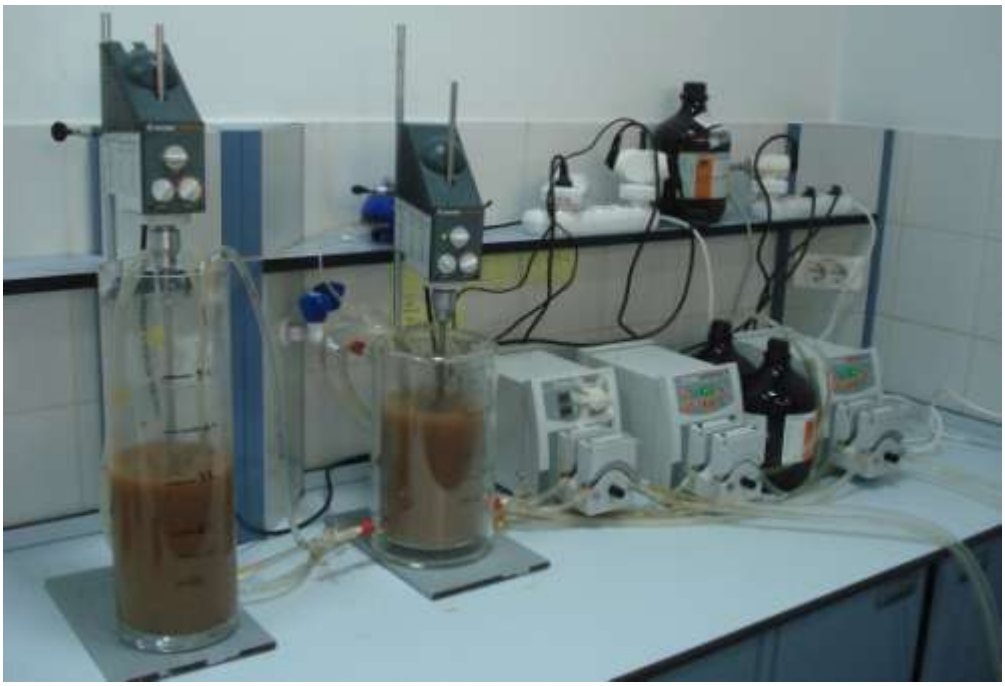


Figure 4.1: A view of SBR N+ and SBR N-.

4.3 Characteristics of Synthetic Wastewater

The SBRs were fed by synthetic wastewater of which COD concentration was 300 mg/ L. Sodium acetate was used as the sole carbon source. The SBRs were fed with a mixture of an organic and three inorganic nutrient solutions (sol C for carbon, sol N for ammonia, sol P for phosphorus, and sol M for micro nutrients). Nutrient solutions were pumped separately into the reactor to avoid any biological activity in the feeding, which could be resulted in reduction in the effluent COD. Stock solutions of C, N, P, and M were prepared with distilled water, diluted with tap water to desired concentrations in feeding bottles, and then supplied to the reactors by timer controlled pumps. Compositions of stock solutions and corresponding concentrations of the compounds in influent were given in Table 4.1.

Table 4.1: Compositions of macro and micro compounds.

Solution	Component	Compound	Stock (g/L)	Influent (mg/L)
Sol C	$CH_3COONa.3H_2O$	COD	24	300
Sol N	NH_4Cl	N	24	36[a], 6[b]
Sol P	KH_2PO_4	P	12	3
	K_2HPO_4	P	12	3
Sol M	$MgSO_4.7H_2O$	Mg	0.74	4.44
	$FeSO_4.7H_2O$	Fe	0.10	0.60
	$ZnSO_4.H_2O$	Zn	0.09	0.55
	$CaCl_2.2H_2O$	Ca	0.36	2.16
	$MnSO_4.H_2O$	Mn	0.07	0.40

a: SBR N+; b: SBR N- and SBR N_D-

4.4 Batch Experiments

Aerobic batch tests were performed with either the enriched biomass from both SBRs or the activated sludge used as inoculum. Batch experiments with enriched biomass were carried out after about two months of SBR operation. Activated sludge either enriched biomass which was withdrawn at the end of cycle or inoculum sludge, put in a smaller rector, washed, and diluted to selected volume by tap water. Sol P and Sol M nutrient solutions were prepared and appended to ensure similar concentrations of phosphorus and micro nutrients in the influent of enrichment reactors. Then Sol C and Sol N were speared to initialize the experiments. As summarized in Table 4.2, sludges taken from three enrichment reactors and inoculum sludge were exposed to different organic loads, organic acids, and C/N ratios during batch experiments. When acetate and propionate mixture was used as carbon source, their ratio was 1/1 on COD basis.

Table 4.2: Summary of conditions applied during batch experiments.

Batch	Origin of sludge	Organic load (g COD S / g COD X)	VFA	C/N	Duration (h)	Ammonia spearing time (h)
$N+_0$	Inoculum	0.1	Acetate	100/12	2	0
$N-_0$	Inoculum	0.1	Acetate	100/2	2	0
N_D-_0	Inoculum	0.1	Acetate	100/0	2	0
$N+_1$	SBR N+	0.1	Acetate	100/12	5	0
$N+_2$	SBR N+	0.2	Acetate	100/12	5	0
$N+_3$	SBR N+	0.1	Acetate + Propionate	100/12	5	0
$N+_4$	SBR N+	0.4	Acetate	100/12	5	0
$N+_5$	SBR N+	0.8	Acetate	100/12	5	0
$N+_6$	SBR N+	0.4	Acetate	100/0	5	0
$N-_1$	SBR N-	0.1	Acetate	100/2	5	0
$N-_2$	SBR N-	0.2	Acetate	100/2	5	0
$N-_3$	SBR N-	0.1	Acetate + Propionate	100/2	5	0
$N-_4$	SBR N-	0.4	Acetate	100/2	5	0
$N-_5$	SBR N-	0.8	Acetate	100/2	5	0
$N-_6$	SBR N-	0.4	Acetate	100/0	5	0
N_D-_1	SBR N_D-	0.1	Acetate	100/2	5	1
N_D-_2	SBR N_D-	0.2	Acetate	100/2	5	1
N_D-_3	SBR N_D-	0.1	Acetate + Propionate	100/2	5	1
N_D-_4	SBR N_D-	0.4	Acetate	100/2	5	3
N_D-_5	SBR N_D-	0.8	Acetate	100/2	8	4
N_D-_6	SBR N_D-	0.1	Acetate	100/2	3	0

4.5 Sampling

Samples were collected on days 1, 5, 7, 12, 15, 19, 26, 33, 40, 62, 72, and 82 from SBR N+ and SBR N-, and on days 1, 9, 14, 23, 27, 35, 45, 51, 57, 66, 74, and 80 from SBR N_D- to monitor evaluation of SBR performances. For COD, ammonia nitrogen (NH_4-N), PHA, and pH measurements, every sampling day, six, six, and eight samples were collected from SBR N+, SBR N-, and SBR N_D-, respectively. Sampling minutes were varied during operation. At the beginning of operating period samples were taken from SBR N+ and SBR N- in minutes 0, 15, 30, 60, 120, and 300, and from SBR ND- in minutes 0, 15, 30, 60, 90, 120, 180, and 300. Depending on increasing rates of substrate uptake and polymer storage during operation, samples were taken more frequently in beginning of cycles in the following sampling days. Therefore samples were taken from SBR N+ and SBR N- in minutes 0, 10, 15, 30, 60, and 300 and from SBR N_D- in minutes 0, 5, 15, 30, 60, 75, 90, and 300, at the end of operating periods. One sample for MLVSS measurement and one for the subsequent microbiological analysis were collected from all SBRs in every sampling day. The performance of enrichment reactors was further investigated by measuring the concentration profiles throughout a representative cycle during the steady state

operation. During detailed cycle measurements samples collected from enrichment reactors more frequently.

Performance of batch experiments was monitored through measurements of acetate (HAc), COD, NH_4-N, PHA, and pH. During batch experiments samples were taken more frequently. Sample collection during batch experiments was summarized in Table 4.3.

Table 4.3: Summary of sample collection points during batch experiments.

Batch	Sampling minute
N+0, N-0, N_D-0	0, 5, 15, 30, 60, 75, 90, 120
N+1, N+2, N+3, N-1, N-2, N-3	0, 3, 7, 11, 15, 20, 30, 60, 120, 180, 240, 300
N+4, N-4	0, 5, 15, 30, 45, 60, 75, 90, 120, 180, 300
N+5	0, 5, 15, 30, 60, 90, 120, 180, 300
N-5	0, 5, 15, 30, 60, 90, 120, 180, 240, 300
N+6, N-6	0, 5, 15, 30, 45, 60, 75, 90, 120, 180, 240, 300
N_D-1	0, 3, 7, 11, 15, 30, 60, 62, 65, 68, 75, 90, 120, 300
N_D-2	0, 3, 7, 15, 30, 45, 60, 62, 65, 68, 75, 90, 120, 300
N_D-3	0, 3, 7, 11, 15, 30, 60, 62, 65, 68, 75, 90, 300
N_D-4	0, 10, 30, 60, 70, 80, 90, 120, 180, 185, 195, 210, 240, 270
N_D-5	0, 5, 15, 30, 60, 90, 120, 150, 180, 210, 240, 245, 270, 300
N_D-6	0, 3, 7, 11, 15, 30, 60, 90, 120, 180

4.6 Chemical Analysis

Biomass samples for PHA measurement were taken into two 10 mL centrifuge tubes containing 2 drops of formaldehyde then centrifuged and washed by phosphate buffer solution. The pellet obtained after second centrifugation was lyophilized. Extraction, hydrolization, and esterification steps were proceed in a mixture of hydrochloric acid, 1-propanol, and dichloroethane at 100°C as described by Beun et al., (2000). The mixture was washed by water to remove free acids and centrifuged to separate phases. The organic phase was analyzed by gas chromotograph (Agilent 6890N). Benzoic acid was used as an internal standard throughout the procedure.

Samples taken for COD, and NH_4-N analyses were filtered through 0.45 μm syringe filters. COD, MLSS, and MLVSS, were measured according to Standard Methods (APHA,1995). Ammonia concentration was determined via a spectrophotometer, using the direct Nesslerization method (Greenberg et al., 1992). Acetate was analyzed by gas chromotograph (Agilent 6890N). For pH measurements, a Thermo Orion 720 A+ model pH meter was used.

4.7 Microbiological Analysis

4.7.1 DNA extraction and PCR amplification of 16S rRNA genes

Genomic DNA was extracted from 1 ml aliquots of the stored samples using the FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions. Amplification of 16S rDNA from the extracted DNA was performed with primers given in Table 1. Amplification was done in a 50 µL reaction volume containing 25 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate 1.5 mM MgCl2, 5 µL of 10 x Taq buffer and 4 U of Taq DNA polymerase (Fermentas, Latvia). PCR amplification was performed in a Techne TC-412 thermal cycler (Barloworld Scientific Ltd. UK) with an initial denaturation at 94ºC for 1 min, annealing for 1 min and extension at 72 ºC for 2 min and final extension at 72ºC for 10 min. PCR products were visualized by staining with ethidium bromide after agarose gel electrophoresis (Thermo-Scientific Ltd., U.K.) and the images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France).

4.7.2 Denaturating Gradient Gel Electrophoresis

PCR primers for DGGE analysis were given in Table 4.4. PCR products were run on a 10% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0) over a 30-70% denaturing gradient (100% denaturant is 7 M urea and 40% (v/v) formamide). To aid the conversion and normalization of gels, a marker was added on the outside of the gel as well as after every four samples. Electrophoresis was performed using the D-Code system (Bio-Rad Laboratories, Ltd., UK). Gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after stained with SybrGold (1:10000 diluted; Molecular Probes Inc., UK) according to the supplier's instructions.

Table 4.4: Primers used in PCR applications

Primer	Target	Experimental Stage	Position[a]	Reference
Bact341f_GC[b] Bact534r	Bacterial 16S rDNA	DGGE	341-357 534-518	Muyzer et al., 1993
Bact8f Bact1541r		Cloning	8-27 1541-1522	Lane, 1991
Bact342f		Sequencing	342-361	Edwards et al., 1988
M13f M13r	B-galactosidase	Clone screening	-	Schrenk et al., 2003

[a] Escherichia coli numbering
[b] 5´-GC clamp on Bact341f (GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGG)

Images were analyzed by using the Bionumerics 5.0 software (Applied Maths, Kortrijk, Belgium). Similarities between tracks were calculated by using the Dice coefficient (SD) (unweighted data based on band presence or absence) and semi-quantitative clustering. For analysis using Dice coefficient a band position tolerance of 0.7% was applied. This was the minimum tolerance at which all marker lanes clustered at 100%.

For semi-quantitative analysis DGGE bands were scored as present (score = 1) or absent (score = 0), and we also scored bands for relative density using a 1–5 semi quantitative scale (1 = least dense, 5 = most dense, 0 = band absent). DGGE data were analyzed by principal component analysis (PCA) in order to correlate the first component (PC1) with the other variables.

4.7.3 Cloning, sequencing, and phylogenetic analyzes of 16S rRNA gene fragments

PCR amplified 16S rRNA gene sequences from the inoculum sludge was cloned. PCR products from the cloned sequences and the other samples were compared using DGGE to relate the bands in DGGE profiles with the cloned DNA.

PCR primers used for cloning, clone screening and sequencing were given in Table 1. PCR products were cloned with a TOPO TA cloning kit (Invitrogen Ltd., Paisley, United Kingdom) according to manufacturer's instructions. PCR amplified vector inserts of the correct size were purified by ethanol precipitation and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). 800 bp of bacterial sequence data were generated.

The sequences were analyzed in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html) and checked for reading errors with the alignment programs of the ARB package (Ludwig et al., 2004). Homology searches of the sequences in DNA databases were performed with FASTA provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html). 16S rRNA gene sequences showing 97% similarity or higher was considered to belong to the same phylotype. None of the sequences showed low (below 70%) relatedness with known bacterial phylogenetic groups. Sequences showing similarities lower than 97% were placed within tentative taxa according to their closest relative in DNA databases. The sequences were submitted to the EMBL database under accession numbers given in Table 2. Sequences representing distinct phylotypes and their closest relatives were aligned by using the MEGA software (Tamura et al., 2007). Distance analyses of aligned base positions were also performed by using the MEGA software and trees were generated from distance matrices using the neighbour-joining method. Rarefaction curves and coverage values were obtained using the distribution of clone types present in the clone libraries (Röling and Head, 2005). Statistical analyses were performed using the software MINITAB 15 (Minitab Ltd., England)

4.8 Calculations

Total PHA accumulation through a cycle was calculated by subtracting the amount of PHA at the beginning of the cycle from that at the end of feast phase (Δ PHA, mg COD/L). The first derivative at time zero were calculated by adjusting a linear function to the experimental data to determine the specific PHA storage rate (q_P, in Cmmol PHA/Cmmol X.h), and the specific HAc uptake rate ($-q_S$, in Cmmol HAc/Cmmol X.h). The yield of PHB ($Y_{P/S}$ in Cmmol HB/Cmmol HAc) on substrate consumed was calculated by dividing the amount of PHB formed by the amount of acetate consumed.

5. EXPERIMENTAL RESULTS

5.1 Performances of Enrichment Reactors throughout Operating Periods

5.1.1 Enrichment reactor operated under ADF conditions without nitrogen limitation (SBR N+)

Overall COD removal efficiency of SBR N+ was 75.5% in the first cycle, which increased to 95% in the following 10 days of operation and then remained constant to the end of operation. Remaining COD was probably arisen from microbial end products. Especially during the earlier days of operation, COD consumption rate continued to increase. After two weeks of operation, almost all COD was observed to be consumed in first 15 minutes. Accordingly, first 15 minutes was considered as feast phase, while the remaining 285 minutes was being considered as famine phase.

The biomass concentration in the reactor was 2430 mg/L after 40 days of operation and then observed to be almost stable. MLVSS/MLSS ratio was less than 0.5 in the beginning of operating period and increased gradually depending on sludge removal. The ratio was about 0.78 at the end of operation, which was almost constant after 40^{th} day.

SBR N+ was operated under ADF conditions without nitrogen limitation. C/N ratio of synthetic wastewater was 100/12. This ratio, which supplies more nitrogen than needed for microbiological growth, kept nitrogen concentration in the reactor always higher than 21 mg/L during operating period. During a cycle, 14±1 mg/L of NH_4-N was observed to be consumed.

PHA content of inoculum sludge was about 8.5% of biomass on COD basis. The polymer was consisted of 3 fractions which were PHB, PHV, and 3H2MV. The major fraction was PHV with about 6% at the beginning of operation, while PHB and 3H2MV contents were only 1.15% and 1.3% respectively. Because acetate was the only carbon source entering the reactor, only biomass PHB content increased through operating period. Sludge 3H2MV content remained nearly constant during 82 days of operation. Microorganisms neither accumulate PHV nor 3H2MV, but existing PHV

was depleted. Sludge PHV content was decreased from 6% to about 1% in one month then remained nearly constant. Amount of sludge polymer content was nearly the same after one month of operation; however PHB was the major component with about 6%. During the subsequent days of operation, PHB accumulation performance of the sludge was observed to increase. PHB accumulated by biomass, which was about 6% of sludge dry weight in 33rd day, increased to about 12.14% during the following operation. Total polymer content of the sludge was about 14.24% as mg COD/L at the end of operating period, and 85% of the polymer were PHB. Profile of polymer fractions during the operation of SBR N+ were depicted in Figure 5.1.

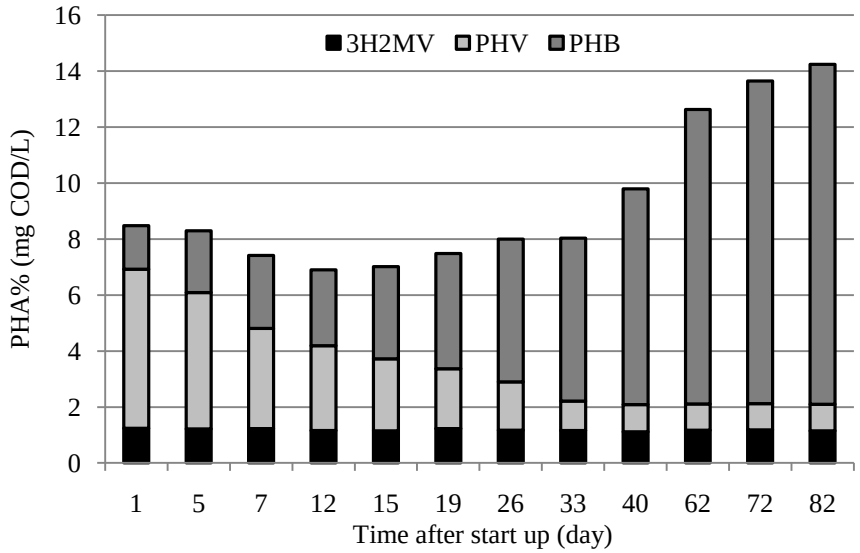


Figure 5.1: Profile of polymer fractions during the operation of SBR N+.

During a cycle, when carbon source was depleted completely, PHB content of the sludge reached its maximum. Peaks in the Figure 5.2 represent these maximums and also indicate borders between feast and famine phases. Both amount of polymer accumulated through a cycle and storage rate increased through SBR operation. Accumulated PHA concentration through a cycle, which was 37 mg COD/L at the beginning, increased to 145 in 2 months and then remained nearly unchanged.

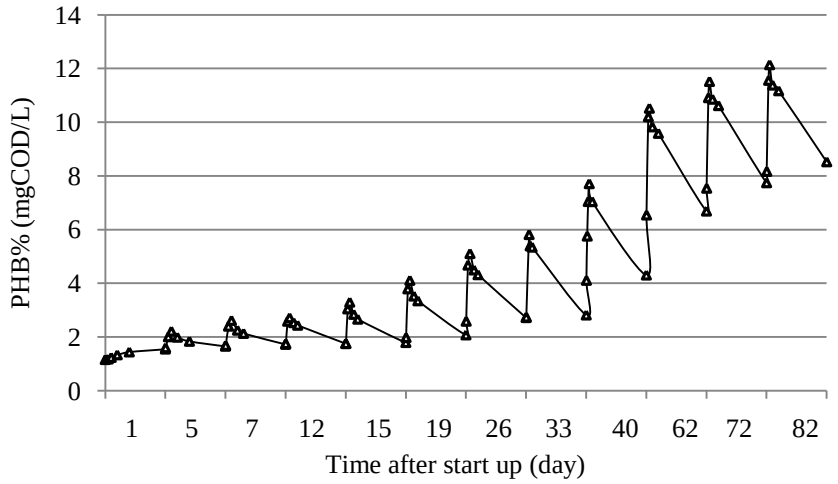


Figure 5.2: PHB content of biomass throughout operation of SBR N+.

The transformations occurring in intracellular and extracellular compounds during a cycle of SBR N+ at steady state operation were shown in Figure5.3. Almost all acetate, which was 300 mg/L as COD in the beginning of cycle, was taken up in 15 minutes. When acetate was consumed, PHA concentration reached its maximum by increasing from 310 to 455 mg/L as COD. Acetate and ammonia consumption and PHB production occurred with a slightly decreasing rate through almost all of the "feast" period. After depletion of acetate in the reactor, microorganisms started to consume produced PHA with a decreasing rate. Consumption rate of intracellular polymer was very slow during the last three hours of operation when compared to beginning of the famine phase. Although ammonia was continued to be consume during the whole cycle, consumption rate decreased sharply following substrate depletion. Ammonia uptake was insignificant especially during the last three hours of operation. At the end of the cycle ammonia concentration was about 21 mg/L.

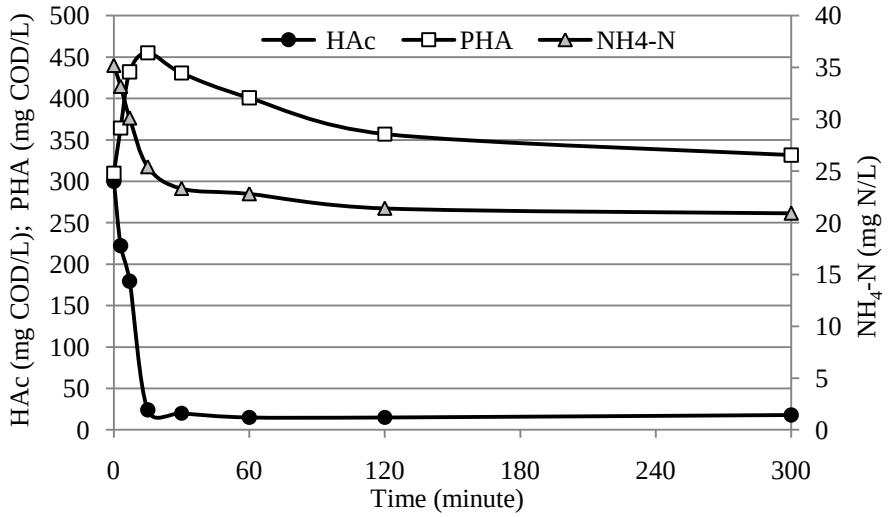


Figure 5.3: The transformations occurring in intracellular and extracellular compounds during a cycle of SBR N+.

The pH increased at the beginning of cycle, and decreased slightly following the substrate exhaustion. Profile of pH was depicted in Figure 5.4. It was fluctuated between 8.65 and 9.

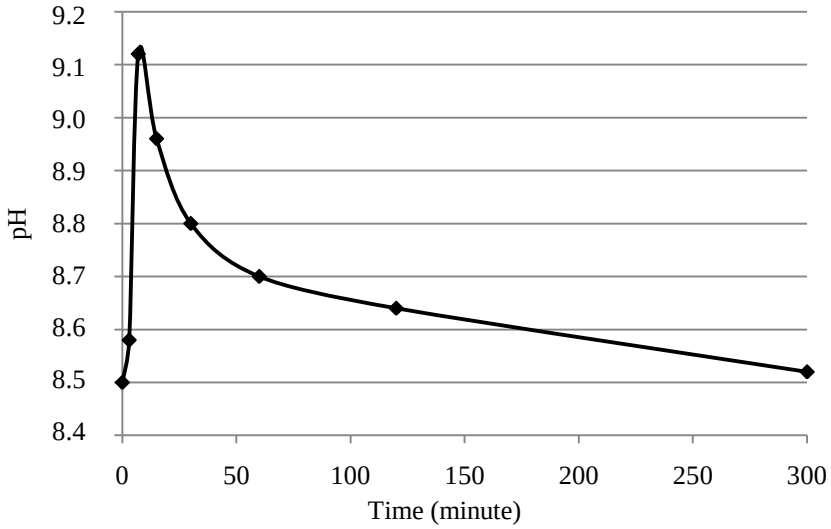


Figure 5.4: Profile of pH during a cycle of SBR N+.

Evaluation of the specific PHA storage rate, the specific HAc uptake rate, the yield of PHA on substrate consumed, and the amount of PHA accumulated during a cycle throughout the operation of SBR N+ was summarized in Table. The specific polymer storage rate increased from 0.01 to 0.18 during the first 40 days of operation and then remained almost stable. The specific HAc uptake rate increased from 0.08 to 0.43 through operating period, it was also almost stable after 40^{th} day. The amount of

polymer accumulated during a cycle increased from 51.6 mg/L to 135.9 on COD basis. The amount of polymer produced per substrate consumed was almost stable after 62 days of operation, which increased from 0.17 to 0.43 throughout operation.

Table 5.1: Performance of SBR N+ throughout operation.

Time after start up (day)	$-q_S$	q_P	$Y_{P/S}$	Δ PHA
5	0.08	0.01	0.17	51.6
7	0.10	0.02	0.19	55.2
12	0.18	0.05	0.26	83.1
15	0.24	0.07	0.28	90.8
19	0.29	0.10	0.30	97.9
26	0.33	0.11	0.35	113.0
33	0.30	0.11	0.37	114.3
40	0.46	0.18	0.38	122.1
62	0.43	0.19	0.43	132.7
72	0.43	0.18	0.44	135.9
82	0.43	0.18	0.43	135.9

q_S in Cmmol HAc/Cmmol X.h; q_P in Cmmol PHA/Cmmol X.h; $Y_{P/S}$ in Cmmol PHA/Cmmol HAc; Δ PHA in mg COD/L.

5.1.2 Enrichment reactor operated under ADF conditions with nitrogen limitation (SBR N-)

Overall COD removal efficiency of the SBR N- was 77.1% in the first cycle, which increased to 92.4% in the first 5 days, then slightly to 95% in 33 days and then remained almost constant to the end of operation. After 33 days of operation, almost all COD supplied to the reactor was observed to be consumed in first 15 minutes. Accordingly, first 15 minutes was considered as feast phase, while the remaining 285 minutes was being considered as famine phase. Remaining COD concentration at the end of cycle was 16 mg/L, which was probably arisen from microbial end products.

The biomass in the reactor was 2310 mg/L after 40 days of operation and then stayed almost constant. MLVSS/MLSS ratio was about 0.73 at the end of operation, which was almost stable after 40^{th} day.

SBR N- was operated under ADF conditions with nitrogen limitation. Nitrogen concentration of synthetic wastewater was 6 mg/L, which was lower than nitrogen needed for microbiological growth. However, during the first cycle, complete utilization of nitrogen took 2 hours. Nitrogen uptake rate continued to increase slightly during operation. After 33 days, almost all nitrogen was observed to be consumed in 15 minutes, which means, nitrogen also became unavailable for

microorganisms during the remaining 285 minutes of operation, besides COD. During the subsequent days of operation, changes in nitrogen consumption rate and duration of nitrogen deficient phase did not changed significantly.

Among the 3 different fractions of polymer in inoculum sludge, PHV was the major one with 5.74%. Total polymer content was about 8.3% as mg COD/L. PHB and 3H2MV contents were about 1.2% and 1.3% respectively. Sludge 3H2MV content did not change during operating period. Sludge PHV content decreased slightly to 0.93% in 2 months and then did not changed. PHB content of biomass increased continuously during operation of SBR N- depending on acetate feeding. After 82 days of operation PHB content of the biomass was 18.63% as mg COD/L, which was covering 90% of total polymer content. Total polymer concentration accumulated through a cycle was increased from 58.6 mg/L to 185.5 on COD basis during the operation of SBR N-. Profiles of sludge polymer contents during the SBR operation and sludge PHB content during the cycles of SBR were shown in Figure 5.5 and Figure 5.6, respectively.

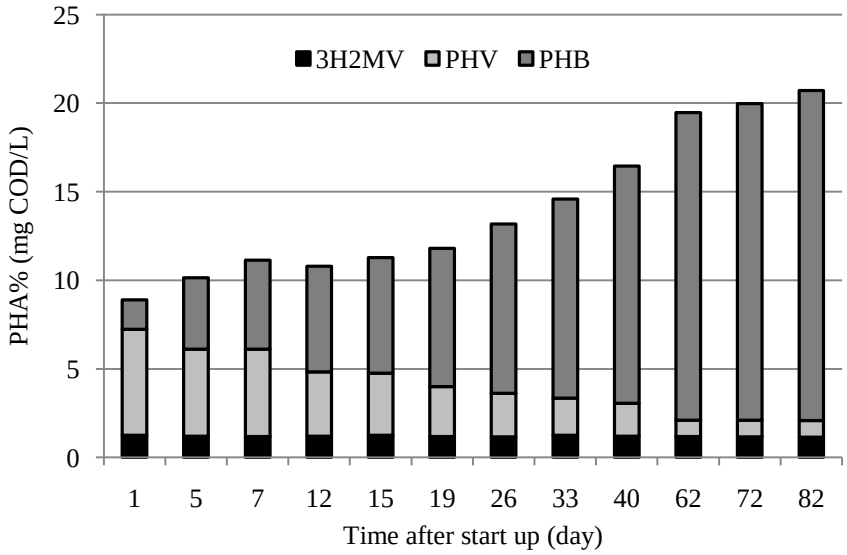


Figure 5.5: Profile of polymer fractions during the operation of SBR N-.

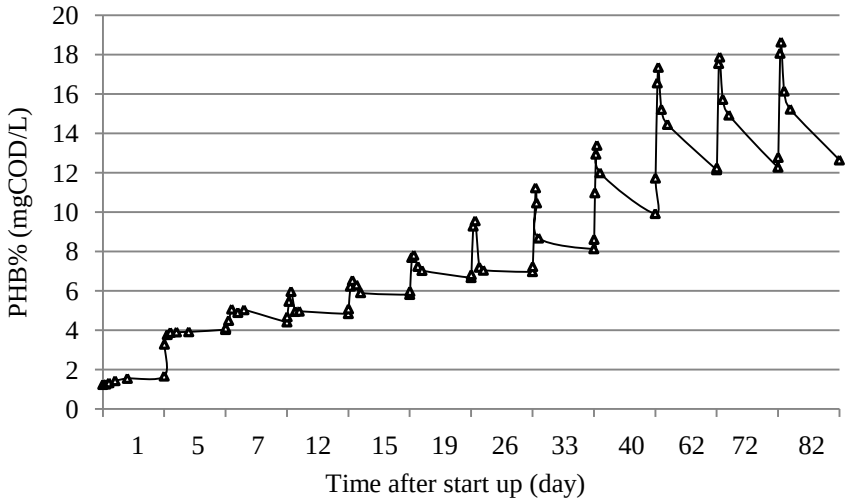


Figure 5.6: PHB content of biomass throughout operation of SBR N-.

Figure 5.7 depicts the transformations occurring in intracellular and extracellular compounds during a cycle of SBR N- at steady state conditions. Almost all acetate was taken up in 15 minutes. When acetate was consumed, PHA concentration reached its maximum by increasing from 375 to 575 mg/L as COD. Acetate consumption and PHB production occurred with a slightly decreasing rate through almost all of the "feast" period. Produced PHB was started to be consumed following depletion of acetate in the reactor. Ammonia was consumed completely in 15 minutes with a constant rate.

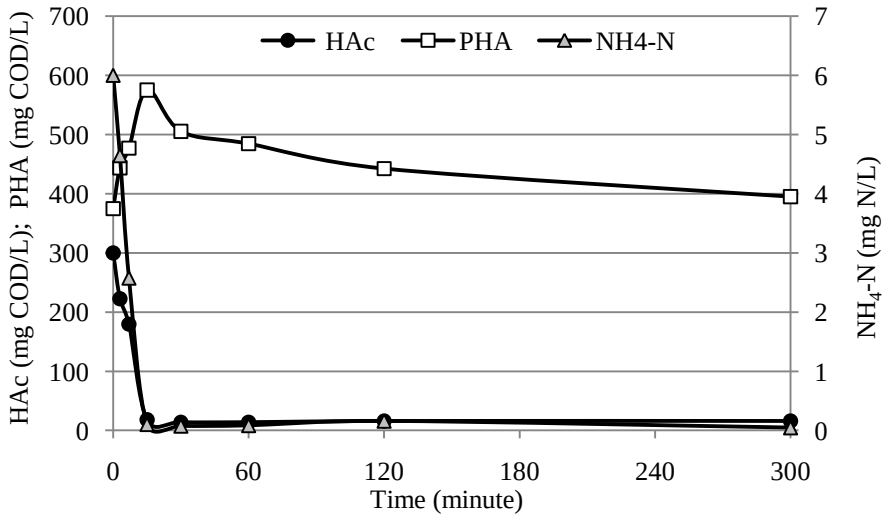


Figure 5.7: The transformations occurring in intracellular and extracellular compounds during a cycle of SBR N-

Changing pH values during a cycle of SBR N- was depicted in Figure 5.8. The pH increased at the beginning of cycle, and decreased slightly following the substrate exhaustion. It was fluctuated between 8.5 and 9.

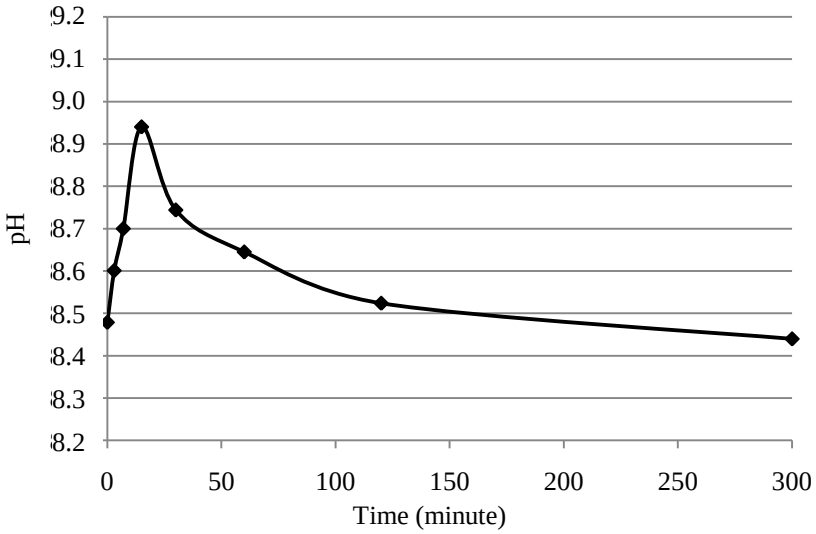


Figure 5.8: Profile of pH during a cycle of SBR N-.

The specific polymer storage rate, the specific HAc uptake rate, the yield of PHA on substrate consumed, and the amount of polymer accumulated during a cycle were calculated for the operating period of SBR N- and the results were summarized in Table. The specific polymer storage rate increased from 0.01 to 0.28 while the specific substrate uptake rate increased from 0.08 to 0.49. The yield of polymer on substrate consumed increased from 0.22 to 0.58 throughout operation. Corresponding amount of polymer accumulated during a cycle at the end of operation was 185.5 mg COD/L.

Table 5.2: Performance of SBR N- throughout operation.

Time after start up (day)	$-q_S$	q_P	$Y_{P/S}$	Δ PHA
5	0.08	0.01	0.22	58.7
7	0.09	0.02	0.26	82.00
12	0.15	0.04	0.31	100.0
15	0.18	0.06	0.35	112.6
19	0.22	0.09	0.38	119.3
26	0.31	0.14	0.42	132.9
33	0.32	0.14	0.45	144.1
40	0.50	0.23	0.48	155.9
62	0.48	0.26	0.56	179.4
72	0.49	0.29	0.58	186.2
82	0.49	0.28	0.58	185.5

q_S in Cmmol HAc/Cmmol X.h; q_P in Cmmol PHA/Cmmol X.h; $Y_{P/S}$ in Cmmol PHA/Cmmol HAc; Δ PHA in mg COD/L.

5.1.3 Enrichment reactor operated under ADF conditions with delayed nitrogen feeding (SBR N_D-)

Overall COD removal efficiency of the SBR N_D- was 91.7% in the first cycle, which increased to about 98% in the first 9 days and then remained constant. After one month of operation COD consumed in first 30 minutes was about 96% of overall COD. The amount of COD consumed after 30^{th} minute was insignificant. However this first 30 minutes can not be considered as feast period because of nitrogen deficiency.

The biomass concentration in the reactor was 2560 mg/L after one month of operation and then stayed almost constant. MLVSS/MLSS ratio was about 0.44 at the beginning of operation and increased gradually depending on sludge withdrawal from the reactor. The ratio was about 0.74 after one month and then slightly increased to the 0.77 during the following days of operation.

SBR N_D- was operated under ADF conditions with delayed nitrogen feeding. Nitrogen concentration supplied to the reactor was arranged to provide a C/N ratio of 100/2. However this nitrogen supplied to the reactor one hour after COD supplement. During the first cycle, only 40% of nitrogen was utilized completely. After 1 week of operation 95% of overall nitrogen was observed to be consumed throughout cycle. After 45 days of operation, nitrogen was available only for 15 minutes in the reactor, following its spiking.

PHA content of inoculum sludge was about 5.2% of biomass as mg COD/L. Half of the total polymer content was covered by PHB, while PHV and 3H2MV contents

were 1.83% and 1.45% respectively. Sludge PHV and 3H2MVcontent remained nearly constant during operation. Because acetate was the only carbon source entering the reactor, only biomass PHB content increased through operation period. Profile of sludge PHB content during the cycles of SBR N_D-was shown in Figure 5.9. After 80 days of operation PHB content of the biomass increased from 2.6% to 25.7%.

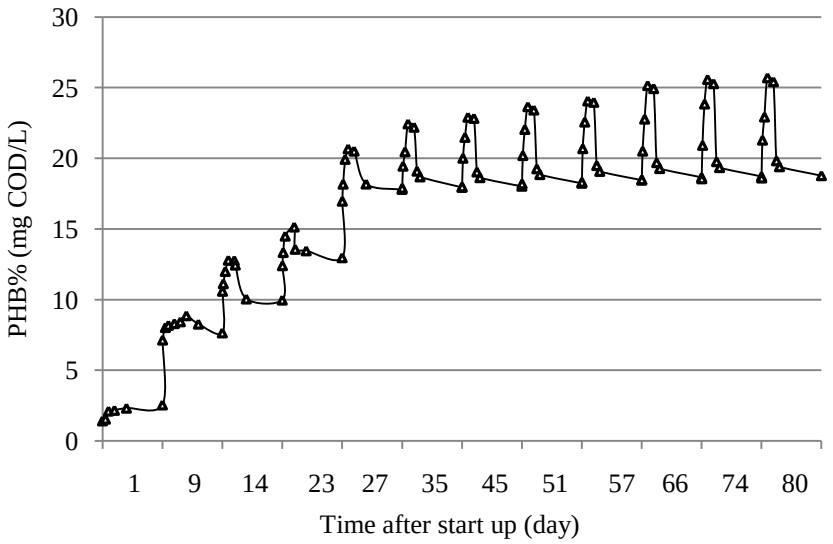


Figure 5.9: PHB content of biomass throughout operation of SBR N_D-.

At the end of operation, PHB covered 90.3% of total polymer content. Profile of sludge polymer fractions during the operation of SBR N_D- were shown in Figure 5.10. Concentration of PHA accumulated through a cycle was increased from 68.9 mg/L to 257.4 during operating period. PHA accumulation ability has especially improved during the first month, and then continued to increase with a declined rate.

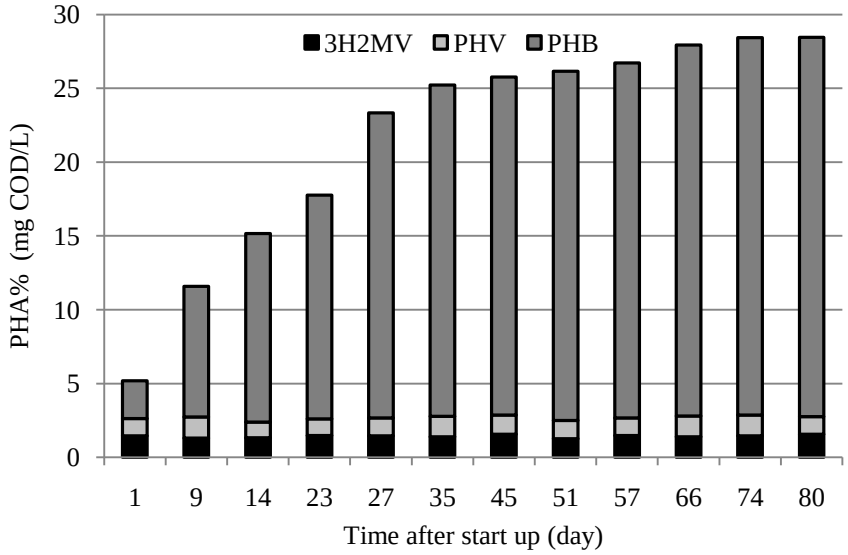


Figure 5.10: Profile of polymer fractions during the operation of SBR N_D-.

Figure 5.11 demonstrates the transformations of intracellular and extracellular compounds during a cycle of SBR N_D- at steady state operation. Almost all acetate was consumed in 30 minutes. PHA concentration increased from 653 to 887 mg/L as COD and reached its maximum when acetate was consumed completely. The PHA was started to be depleted with a slow rate after acetate depletion. Consumption rate of PHA was increased sharply following ammonia addition to the reactor. Ammonia was consumed almost completely in 15 minutes with a slightly decreasing rate.

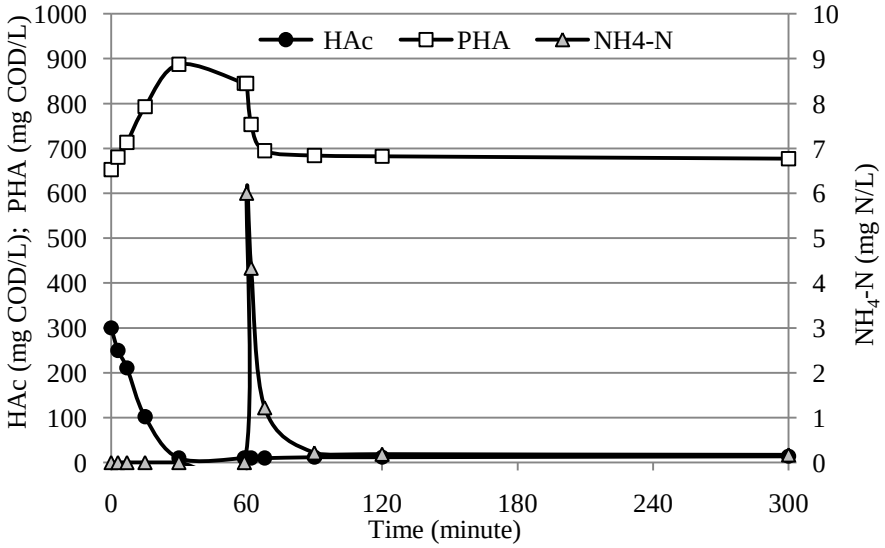


Figure 5.11: The transformations occurring in intracellular and extracellular compounds during a cycle of SBR N_D-.

The pH increased at the beginning of cycle from 8.5 to 9, and decreased slightly following the substrate exhaustion to 8.5 again. Figure 5.12 shows changing pH values during a cycle of SBR N_D-.

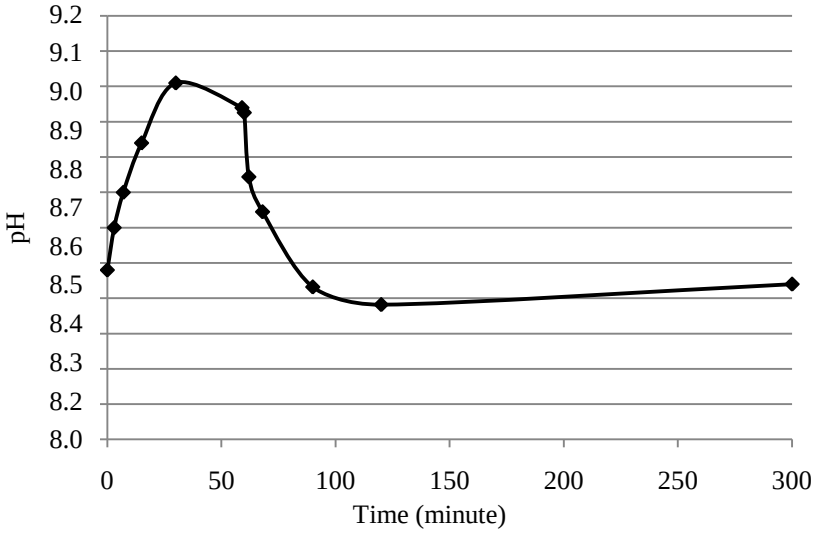


Figure 5.12: Profile of pH during a cycle of SBR N_D-.

The specific polymer storage rate, the specific HAc uptake rate, the yield of PHA on substrate consumed, and the amount of polymer accumulated during a cycle were calculated for the operating period of SBR N_D-and the results were given in Table 5.3. The specific polymer storage rate increased from 0.03 to 0.26 during the operation. The specific substrate uptake rate increased from 0.12 to 0.41. The amount of polymer accumulated during a cycle increased from 85.6 mg COD/L to 224.8 throughout operation of N_D-, corresponding to a polymer yield of 0.68 at the end of operation.

Table 5.3: Performance of SBR N_D- throughout operation.

Time after start up (day)	$-q_S$	q_P	$Y_{P/S}$	Δ PHA
9	0.12	0.03	0.27	85.6
14	0.27	0.08	0.27	87.8
23	0.32	0.10	0.32	106.9
27	0.33	0.13	0.39	127.7
35	0.37	0.16	0.42	138.5
45	0.38	0.20	0.50	163.6
51	0.39	0.23	0.58	191.3
57	0.39	0.23	0.57	187.8
66	0.40	0.22	0.63	207.4
74	0.40	0.25	0.68	225.0
80	0.41	0.26	0.68	224.8

q_S in Cmmol HAc/Cmmol X.h; q_P in Cmmol PHA/Cmmol X.h; $Y_{P/S}$ in Cmmol PHA/Cmmol HAc; Δ PHA in mg COD/L.

5.2 Changes in Bacterial Diversity throughout Operating Periods of Enrichment Reactors

The bacterial community of the inoculum sludge was investigated by construction of 16S rRNA clone library and subsequent sequencing analysis. The bacterial community changes were monitored by DGGE analyses of PCR amplified 16S rRNA genes from the samples of inoculum sludge and those taken from the reactor operations. Number of clones screened was 83 and number of different phylotypes detected was 26. The coverage of the clone library (94%) and rarefaction analysis (Figure 5.13) revealed that the major part of the microbial diversity was recovered. Rarefaction curve was close to reach an asymptote predicting that most of the probable unique phylotypes were analyzed (Parkes et al., 2005).

Figure 5.13 shows phylogenetic relationships of clones. Accession numbers and similarity of the clones to their closest relative were also depicted in the figure. Considering all the sequences, members of the *Proteobacteria* dominated in the inoculum sludge bacterial clone library with 14.5% belonging to *α-proteobacteria*, and 53% belonging to *β-proteobacteria*. *Proteobacteria* followed by *Verrucomicrobiae* (14.5%), *Bacteroidetes* (13.3%) and *Planctomycetes* (4.8%).

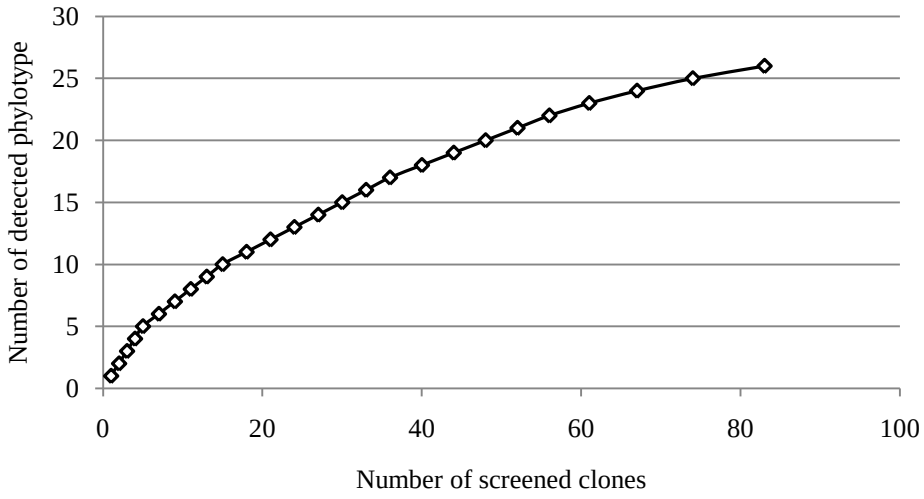


Figure 5.13: Rarefaction analysis of clone library.

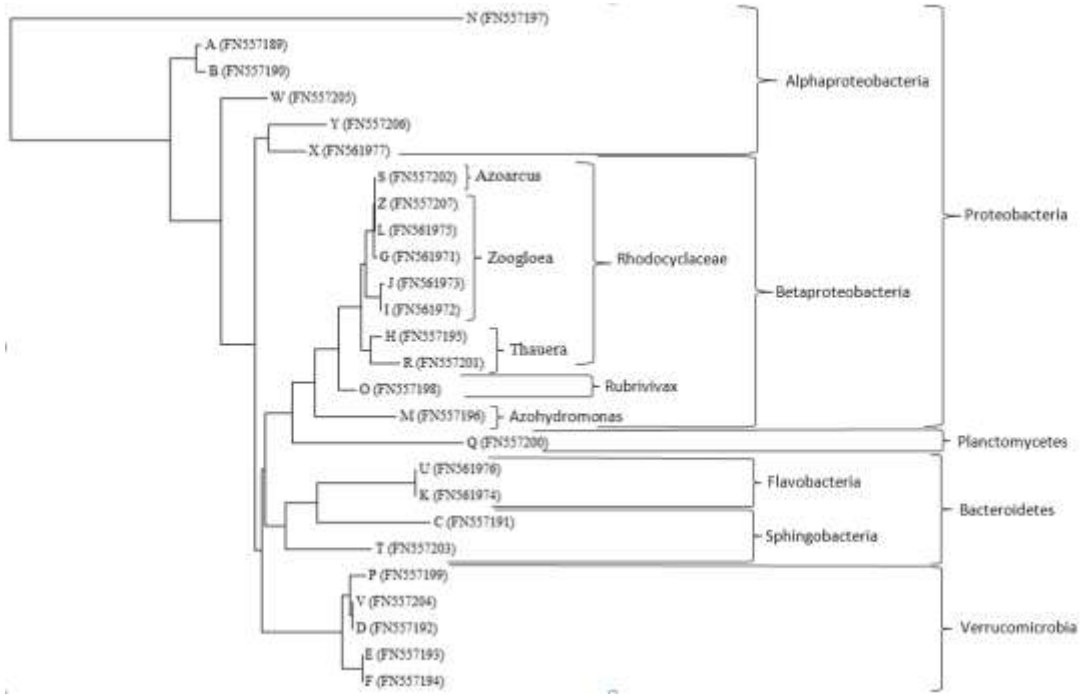


Figure 5.14: Phylogenetic relationships of the clones.

Among the members of *β-proteobacteria* class, *Rhodocyclaceae* (42.2%) was the most predominant family represented by clones. Other members of this class such as *Rubrivivax* (10.8%), and *Alcaligenaceae* (1.2%), belonged to *Burkholderiales* order. Among the members of *Rhodocyclaceae*, *Zoogloea* was the most predominant genus with 19.3%, and followed by *Thauera* (15.7%), and *Azoarcus* (6%).

Table 5.4: Phylogenetic affiliation of the clones.

Clone no.	Accession no	Phylogenetic relationship	
		Species	% Similarity
A	FN557189	*Brevundimonas sp. E8*	85
B	FN557190	*Citromicrobium sp. JL-354*	82.2
C	FN557191	*Terrimonas lutea*	93.3
D	FN557192	*Roseibacillus ishigakijimensis*	84.9
E	FN557193	*Roseibacillus persicicus*	87
F	FN557194	*Haloferula harenae*	86.8
G	FN561971	*Zoogloea caeni*	99.2
H	FN557195	*Thauera sp. PIV-1*	94.4
I	FN561972	*Zoogloea oryzae*	97.5
J	FN561973	*Zoogloea oryzae*	99.7
K	FN561974	*Flavobacterium sp. AKB-2008-JO15*	98.1
L	FN561975	*Zoogloea oryzae*	97.9
M	FN557196	*Azohydromonas australica*	94.2
N	FN557197	*Caulobacter sp. K31*	73.1
O	FN557198	*Rubrivivax sp. SNRB4-8*	96.2
P	FN557199	*Verrucomicrobium sp. GD*	88.7
Q	FN557200	*Planctomyces maris*	85.8
R	FN557201	*Thauera sp. MZ1T*	92.1
S	FN557202	*Azoarcus sp. 22Lin*	96.4
T	FN557203	*Sphingobacterium sp. P-7*	80.9
U	FN561976	*Flavobacterium sp. AKB-2008-JO15*	98.1
V	FN557204	*Luteolibacter pohnpeiensis*	84.1
W	FN557205	*Azospirillum sp.*	88.1
X	FN561977	*Hyphomicrobium sp. Ellin112*	97
Y	FN557206	*Phaeospirillum chandramohanii*	88.8
Z	FN557207	*Zoogloea sp. EMB 108*	95

5.2.1 Bacterial diversity of SBR N+

DGGE protocol allows direct identification of the presence and relative abundance of different bacterial species and profiling of populations in a complex microbial community in both a qualitative and a semi-quantitative way (Muyzer et al., 1993). The dynamics of the bacterial speciation, taking place in the SBR, was monitored by means of DGGE analyses. The number of PCR products or bands migrating in each DGGE lane represents the number of 16S rRNA gene sequence similarity groups or operational taxonomic units, which usually were called species for simplicity (Hughes et al., 2001). The initial inoculum showed the presence of a heterogeneous microbial community, rich in different bacterial species, with some dominant, whose members were represented by stronger bands. Diverse distribution of the major bands, disappearance and appearance of some bands, throughout operation indicate changes in bacterial community during operation of SBR N+. Similarities between

DGGE lanes were calculated by using whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and UPGMA clustering and shown in Figure 5.15. Bacterial community in the reactors changed continuously. Similarity based on Pearson correlation between sample taken from the reactor at the 82nd day and inoculum sludge was 53.3%.

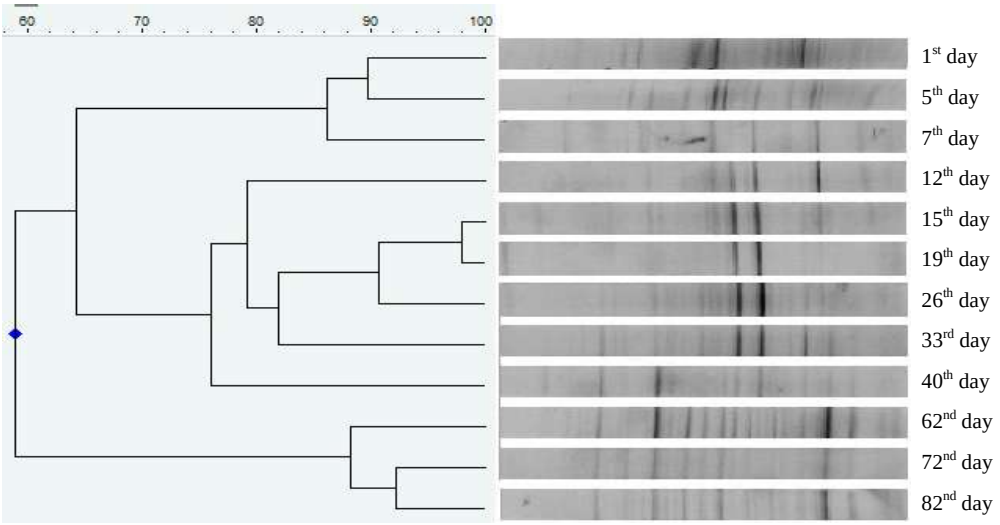


Figure 5.15: Similarities between bacterial communities sampled from SBR N+.

Changes in relative intensities of the DGGE bands belonging to different subclasses encountered throughout operation of SBR N+ were shown in Figure 5.16.

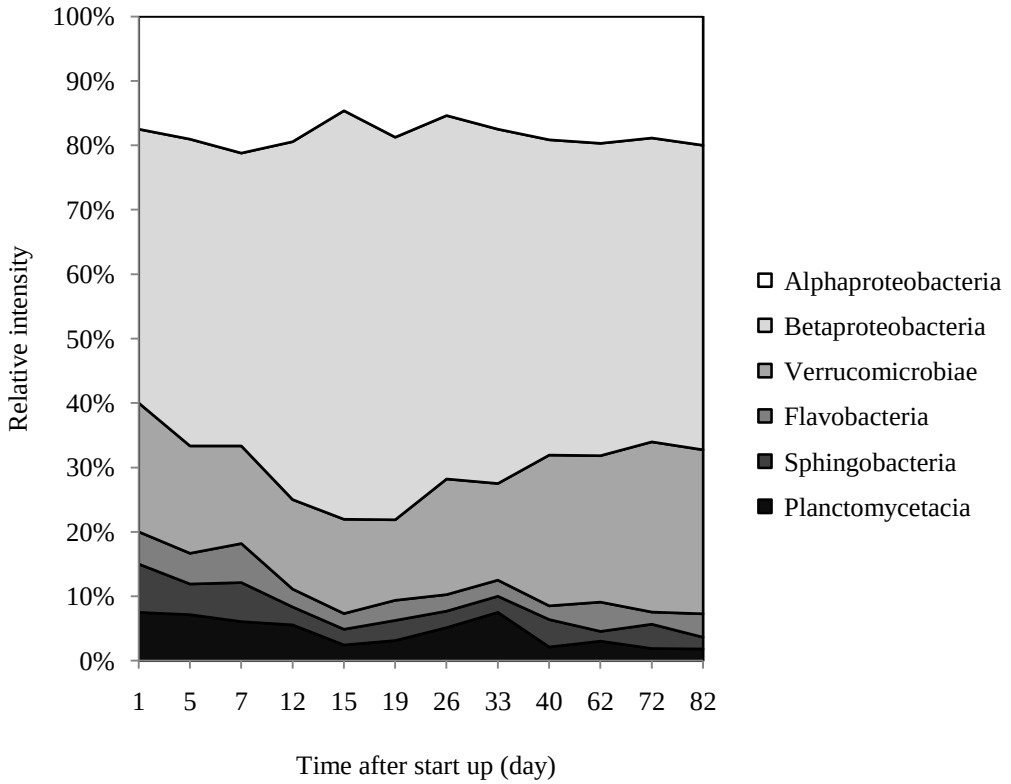


Figure 5.16: Changes in relative intensities of classes in SBR N+.

Relative intensities of the bands belonging to beta subclass of *Proteobacteria* were observed to fluctuate between 42.5% and 63.4% during the operation of SBR N+. Among the members of *β-proteobacteria* class, *Rhodocyclaceae* was the most predominant family. Relative abundance of *Rhodocyclaceae* family of β-proteobacteria increased from 32.5% to 58.5% in two weeks and then slightly decreased to 41.8%. This family covered by members of three genus; *Zoogloea, Thauera,* and *Azoarcus*. Relative intensities of the bands belonging to *Zoogloea* increased from 15% to 21.8% through operation by fluctuating in a wide range (15%-41.4%). Relative intensities of the bands belonging to *Thauera* decreased from 12.5% to 10.9%, and that belonging to *Azoarcus* increased from 5% to 9.1% and.

Relative intensities of the bands belonging to alpha subclasses of *proteobacteria* increased from 17.5% to 20%. Relative abundance of *Planctomycetes* decreased slightly from 7.5% to 1.8%. Relative intensity of *Bacteroidetes* phylum which was covered by members of *Flavobacteria* and *Sphingobacteria* was slightly decreased from 12.5% to 5.4% throughout operation of SBR N+. Throughout operation, relative abundance of the members of *Verrucomicrobia* was observed to increase from 20% to 25.5% after a slight decrease at the beginning of operation.

5.2.2 Bacterial diversity of SBR N-

DGGE profile of the bacterial community structure associated with the samples collected from the SBR N- at different days was depicted in Figure 5.17. The activated sludge here used as SBR inoculum showed the presence of a heterogeneous microbial community, rich in different bacterial species, with some evidence for the presence of a dominant, restricted microbial population whose members are represented by the major bands. During the 26 days of SBR run the bacterial population whose members were numerically more represented than others in the initial inoculum disappeared, and a new bacterial cenose was selected. Similarity between inoculum sludge and the one taken from the reactor at 26th day was 64%. Within two weeks the number of species in the population increased again. The least similarity based on Pearson correlation between bacterial populations (46.4%) was observed for days 12 and 82. Similarity between samples taken first and last day was 59.2%.

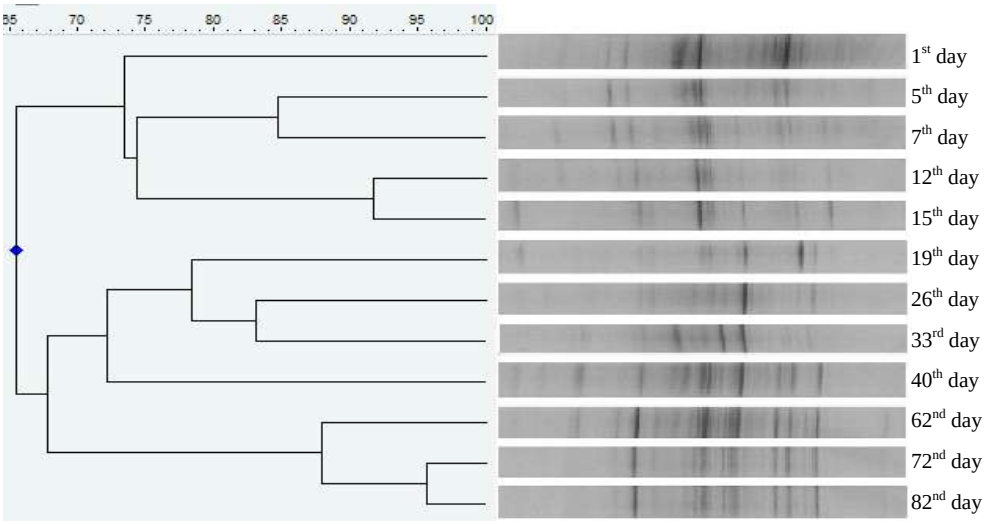


Figure 5.17: Similarities between bacterial communities sampled from SBR N-.

Figure 5.18 shows the changes in relative intensities of the DGGE bands belonging to different subclasses encountered throughout operation of SBR N-.

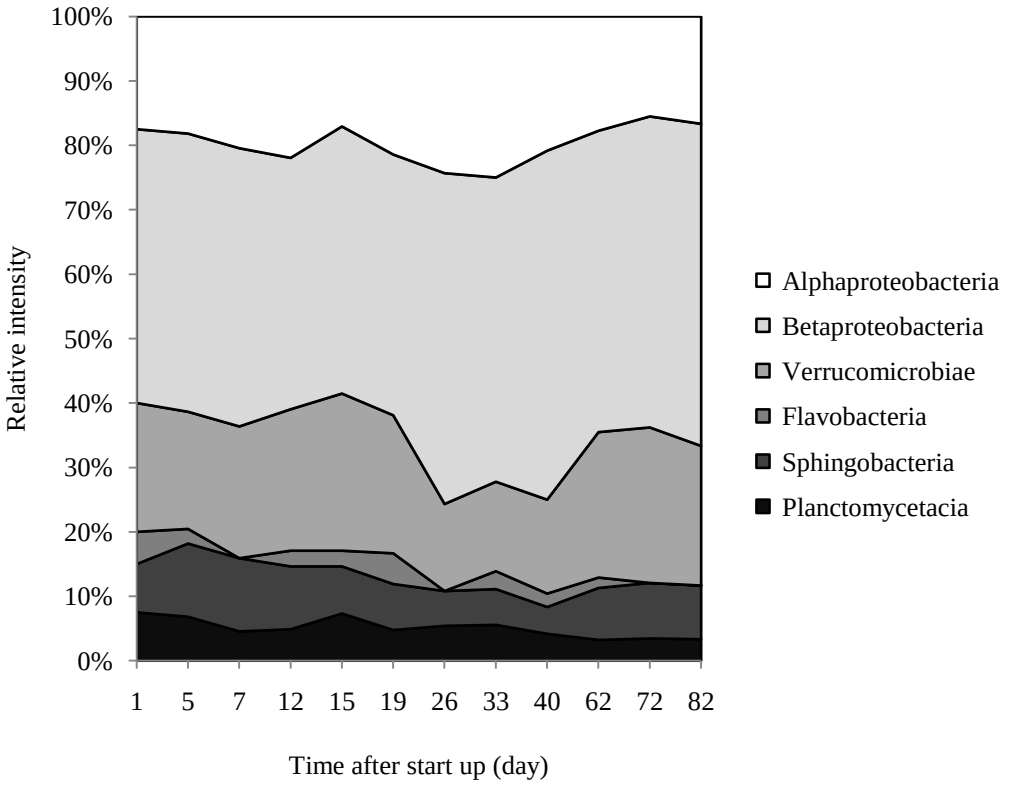


Figure 5.18: Changes in relative intensities of classes in SBR N-.

During the operation of SBR N-, relative intensities of the bands belonging to beta subclass of *Proteobacteria* increased from 42.5% to 50%. Relative abundance of the members belonging to *Rhodocyclaceae* family of *β-proteobacteria*, which was also the most predominant family in the beginning of operation, increased almost continuously and covered 43.3% of the community in SBR N- at the end of the operation. Relative abundances of *Zoogloea* and *Thauera* this family were observed to increase during operation. Relative intensities of the bands belonging to *Zoogloea* and *Thauera* increased from 15% to 26.7% and 12.5% to 13.3% through operating period of SBR N-, respectively. However a slight decrease (1.6%) was observed in relative intensity of the band belonging to member of *Azoarcus*.

Relative intensities of the bands belonging to alpha subclasses of proteobacteria fluctuated between 15.5% and 25%. Relative abundance of *Planctomycetes* decreased slightly from 7.5% to 3.3%. Relative intensity of *Bacteroidetes* phylum which was covered by members of *Flavobacteria* and *Sphingobacteria* decreased from 12.5% to 8.3% throughout operation of SBR N-, following a slight increase in the beginning of operation. Relative abundance of the members of *Verrucomicrobia* fluctuated between 13.5% and 24.49%.

5.2.3 Bacterial diversity of SBR N_D-

Figure 5.19 shows DGGE profile of the bacterial community structure associated with the samples collected from the SBR N_D- at different days. During the operation of SBR run a considerable shift in the species composition of the predominant microbial population occurred with respect to the initial activated sludge. Similarity between samples taken from first and last day was 61.1%.

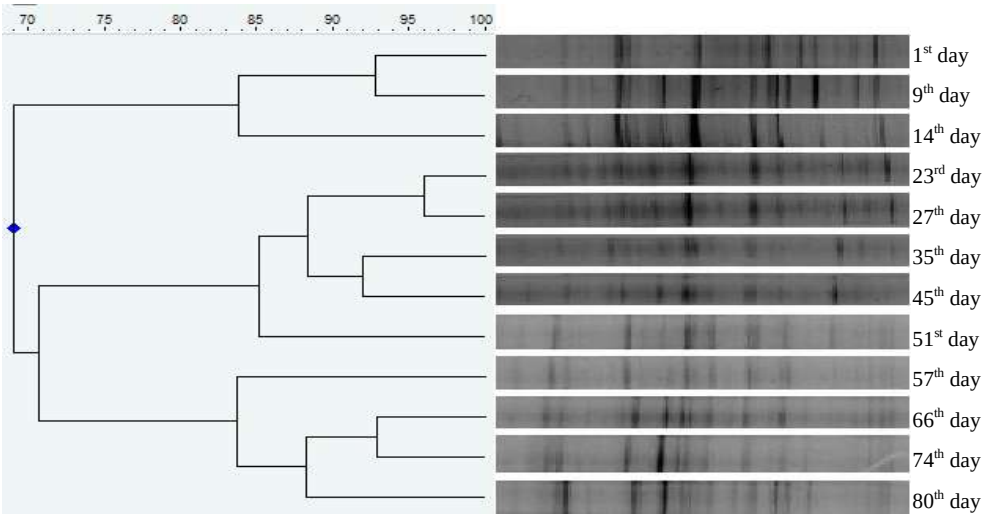


Figure 5.19: Similarities between bacterial communities sampled from SBR N-.

Changes in relative abundances of subclasses encountered throughout operation of SBR N_D- were represented in Figure 5.20.

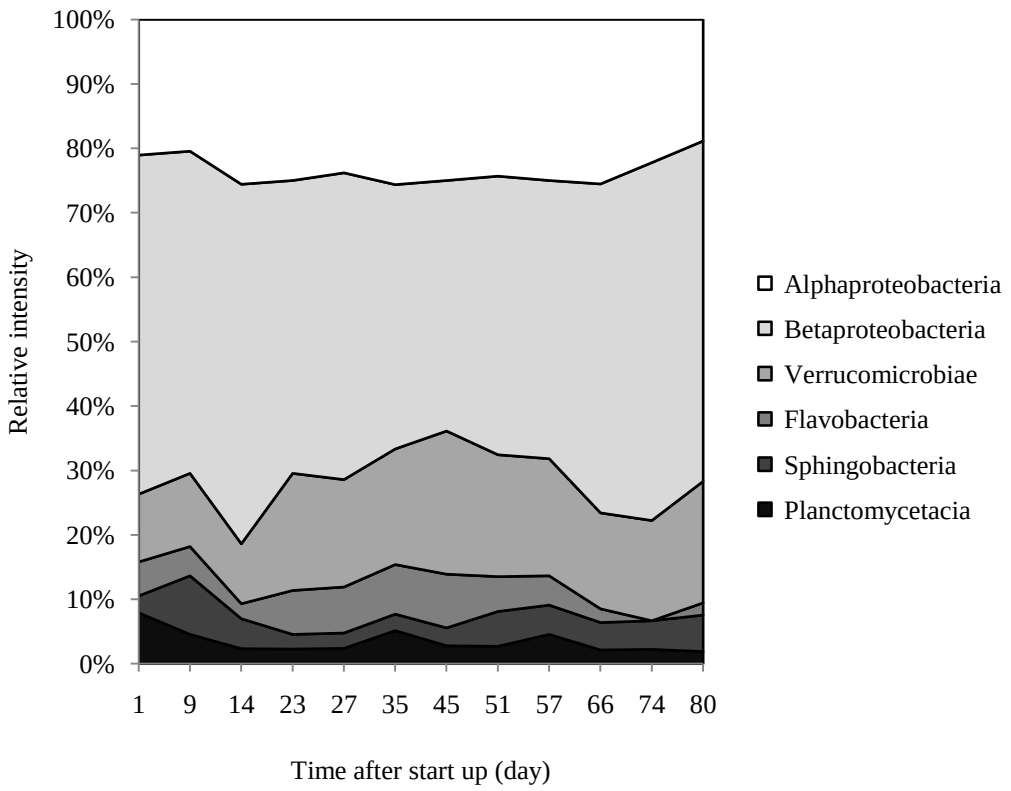


Figure 5.20: Changes in relative intensities of classes in SBR N-.

Relative abundances of the members belonging to beta subclass of *Proteobacteria* were covering more than half the community in the beginning of operation. Relative abundance of this class decreased slightly to the 38.9% in 45 days and then increased to 52.8% slightly. Relative intensities of the DGGE bands belonging to members of *Rhodocyclaceae* family in β-proteobacteria were increased from 39.5% to 45.3%. This family covered by members of three genus; *Zoogloea*, *Thauera*, and *Azoarcus*. Relative intensities of the bands belonging to *Zoogloea* and *Azoarcus* increased from 18.4% to 22.6% and 5.3% to 7.6 through operating period of SBR N-, respectively. However, slight decreases were also observed in relative intensities of some bands belonging to members of *Zoogloea*. Relative intensity of the bands belonging to *Thauera* fluctuated between 7.7% and 15.8%.

Relative intensities of the bands belonging to alpha subclasses of *proteobacteria* were observed to fluctuate between 18.9% and 25.6%. Relative intensity of the band belonging to *Planctomycetes* was 1.9% at the end of operating period, which was 7.9% in inoculum sludge. Relative intensity of *Bacteroidetes* phylum fluctuated in a wide range (4.4% - 13.6%) throughout operation of SBR N_D-, but difference between initial and final abundance of this class was insignificant (0.4%). Relative abundance

of the members of *Verrucomicrobia* increased from 10.5% to 18.9%, which fluctuated between 9.3% and 22.2% during operation.

5.3 Batch Experiments

5.3.1 Batch experiments carried out with inoculum sludge

Three batch experiments were carried out with inoculum sludge to investigate the response of biomass before enrichment. Specific substrate loading for three batch experiments carried out with inoculum sludge was 0.1 g COD S/g COD X, which was identical to those in SBR operations. Acetate was used as carbon sole source. The C/N ratios of synthetic wastewaters used in two of the batch tests were 100/12 and 100/2, which are identical to SBR N+ and SBR N-. The third experiment was carried out with a synthetic wastewater including no nitrogen. Polymer content of inoculum sludge was about 3.9% as mg COD/L at the beginning.

5.3.1.1 Batch $N+_0$

Batch $N+_0$ was carried out with inoculum sludge, applying substrate loading of 0.1 g COD S/g COD X, and C/N ratio of 100/12. Profiles of acetate and PHA concentrations during batch experiment $N+_0$ was shown in Figure 5.21. PHA content of biomass increased from 3.9% to 4.3% as mg COD/L, corresponding to an increase in PHA concentration from 128.4 mg/L to 138.3 mg/L on COD basis. Acetate uptake rate dropped sharply after 75 minute. Remaining acetate concentration at the end of experiment was 93.2 mg COD/L. The yield of polymer on substrate consumed was 0.05. Specific rates for polymer storage and HAc uptake were calculated as 0.004 Cmmol PHA/Cmmol X.h and 0.066 Cmmol HAc/Cmmol X.h, respectively.

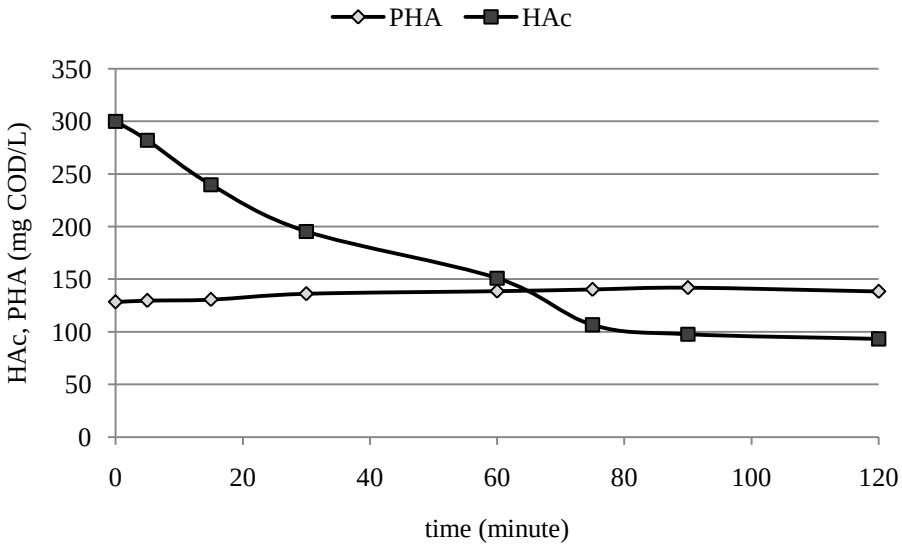


Figure 5.21: Profiles of acetate and PHA during batch experiment $N+_0$.

5.3.1.2 Batch $N-_0$

Substrate loading of 0.1 g COD S/g COD X, and C/N ratio of 100/2 was applied during batch experiment $N-_0$. Only 45% of supplied substrate was consumed during 2 hours. Profiles of acetate and PHA concentrations during the experiment were shown in Figure 5.22. PHA content of biomass increased from 3.9% to 4.4% as mg COD/L. PHA concentration increased from 127.4 to 144.3 as mg COD/L, and the polymer yield calculated as 0.12. Specific rates for polymer storage and HAc uptake were 0.007 Cmmol PHA/Cmmol X.h and 0.063 Cmmol HAc/Cmmol X.h, respectively.

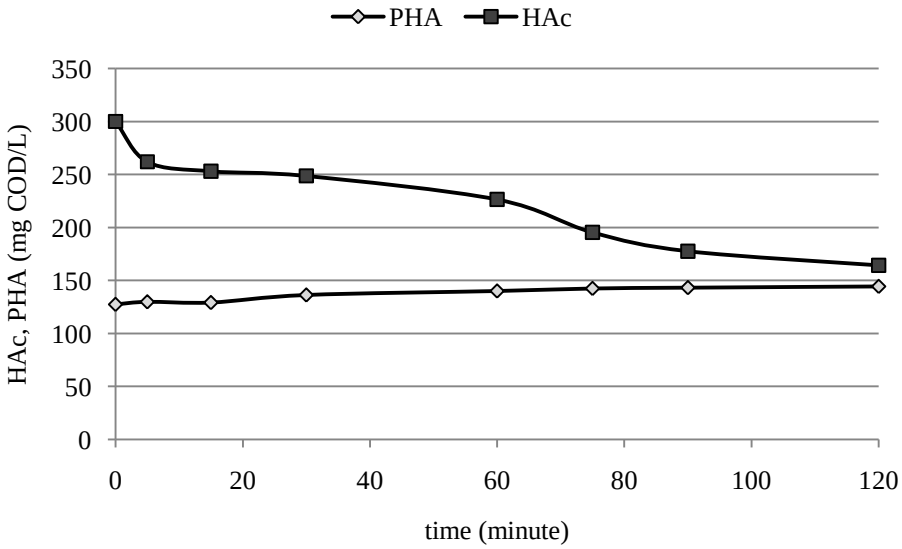


Figure 5.22: Profiles of acetate and PHA during batch experiment $N-_0$.

5.3.1.3 Batch N_{D-0}

Batch N_{D-0} was carried out with inoculum sludge and a synthetic wastewater without nitrogen, applying substrate loading of 0.1 g COD S/g COD X. Figure 5.23 shows profiles of acetate and PHA concentrations during the experiment. The PHA concentration increased from 128.5 mg COD/L to 161.4 mg COD/L, while acetate concentration decreased from 300 mg COD/L to 186.5. Corresponding yield of polymer on substrate consumed was 0.29. Polymer content of biomass increased from 4.0% to 5.0% on COD basis. Specific polymer storage rate was calculated as 0.024 Cmmol PHA/Cmmol X.h, and specific acetate uptake rate was calculated as 0.064 Cmmol HAc/Cmmol X.h.

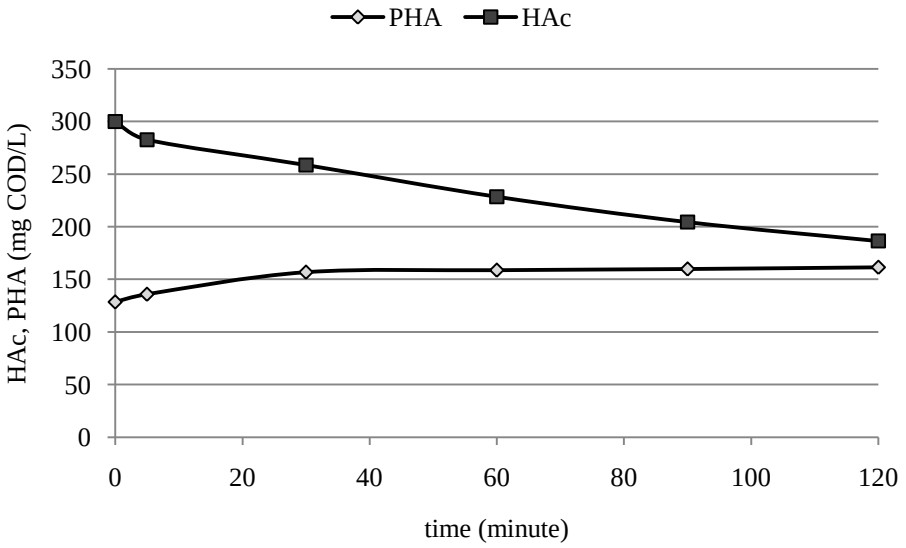


Figure 5.23: Profiles of acetate and PHA during batch experiment N_{D-0}.

5.3.2 Batch experiments carried out with biomass enriched in SBR N+

The response of biomass after enrichment under ADF conditions without nitrogen deficiency was investigated by six batch experiments. Propionate-acetate mixture was used as carbon source during an experiment. One experiment was carried out by applying a synthetic wastewater without nitrogen. Four experiments were carried out by applying synthetic wastewater in different organic loadings.

5.3.2.1 Batch N_{+1}

Batch $N+_1$ was carried out by applying substrate loading of 0.1 g COD S/g COD X, and C/N ratio of 100/12, which were similar to those in SBR N+ and batch $N+_0$. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment $N+_1$

were shown in Figure 5.24. In 15 minutes, almost all acetate was consumed and NH_4-N concentration decreased from 36 mg/L to 25.4 mg/L. Ammonia uptake rate decreased sharply following acetate exhaustion. Remaining NH_4-N concentration at the end of experiment was 19.4 mg/L. PHA content of biomass increased from 10.4% to 14.8% as mg COD/L, corresponding to an increase in PHA concentration from 349.6 mg/L to 496.6 mg/L on COD basis. The polymer accumulated in the form of PHB and concentrations of PHV and 3H2MV did not change during the batch experiment. When biomass polymer content reached its maximum, the polymer composition was 85% of HB, 10% of HV, and 5% of HMV on molar basis. The yield of polymer on substrate consumed was 0.43. Specific rates for polymer storage and HAc uptake were calculated as 0.28 Cmmol PHA/Cmmol X.h and 0.79 Cmmol HAc/Cmmol X.h, respectively. The maximum specific growth rate and the growth yield were determined as 0.13 h^{-1} and 0.37, respectively.

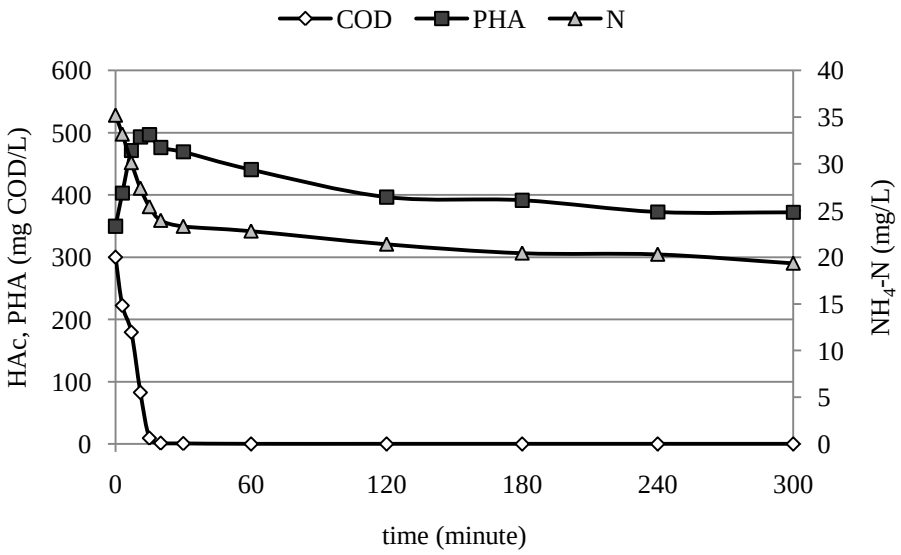


Figure 5.24: Profiles of acetate, PHA, and NH_4-N during batch $N+_1$.

5.3.2.2 Batch N_{+2}

Batch $N+_2$ was carried out by applying C/N ratio of 100/12, and substrate loading of 0.2 g COD S/g COD X, which was two times of those in SBR N+, batch $N+_0$, and batch $N+_1$. Consumption of acetate took 30 minutes and 14 mg/L of NH_4-N was taken up during 30 minutes. Then ammonia uptake rate decreased sharply following acetate exhaustion. Concentration of NH_4-N taken up in the subsequent 270 minutes was 8 mg/L. PHA content of biomass increased from 10.8% to 19.1% as mg COD/L, corresponding to an increase in PHA concentration from 362.7 mg/L to 642.0 mg/L

on COD basis. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment $N+_2$ were shown in Figure 5.25.

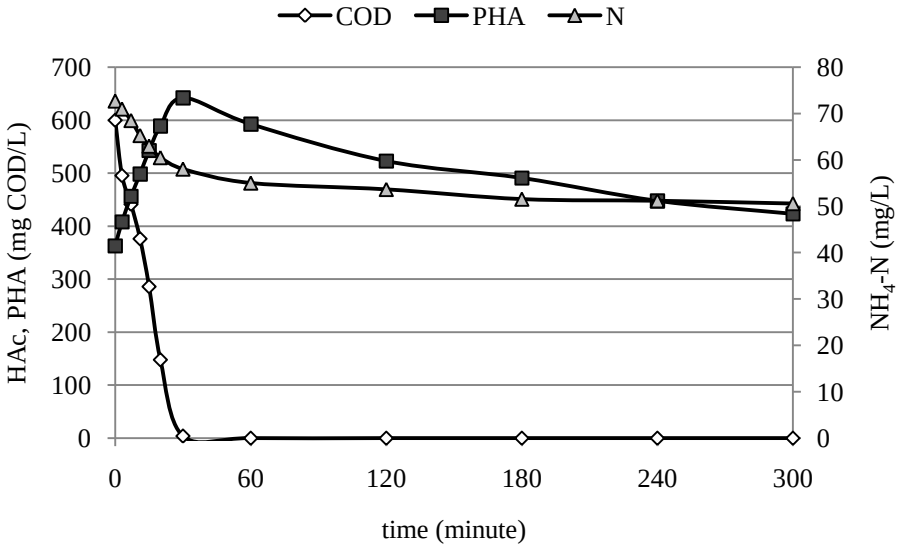


Figure 5.25: Profiles of acetate, PHA, and NH_4-N during batch $N+_2$.

The polymer accumulated mostly in the form of PHB however a small increase in biomass PHV content was also observed (0.3% on COD basis) during the batch experiment. The polymer was composed of 88% of HB, 8% of HV, and 4% of HMV on molar basis. The yield of polymer on substrate consumed was 0.42. Specific rates for polymer storage and HAc uptake were calculated as 0.24 Cmmol PHA/Cmmol X.h and 0.92 Cmmol HAc/Cmmol X.h, respectively. The growth yield was calculated as 0.28 and the maximum specific growth rate was 0.10 h^{-1}.

5.3.2.3 Batch N_{+3}

Batch $N+_3$ was carried out by applying substrate loading of 0.1 g COD S/g COD X, and C/N ratio of 100/12, which were similar to those in SBR N+, batch $N+_0$, and batch $N+_1$. However a propionate-acetate mixture was used as carbon source (50% of HAc, 50% of HPr on COD basis). Profiles of acetate, propionate, PHB, PHV and NH_4-N concentrations during batch experiment $N+_3$ were shown in Figure. Acetate and propionate were consumed simultaneously in 20 minutes until acetate depletion. Propionate concentration in the reactor was 61.4 mg/L on COD basis when acetate depleted and complete depletion of propionate took 240 minutes. Rate of propionate uptake was decreased sharply following acetate exhaustion. Concentration of NH_4-N taken up during the experiment was 11 mg/L. PHA content of biomass increased from 9.9% to 12.1% as mg COD/L, corresponding to an increase in PHA

concentration from 331.4 mg/L to 407.1 mg/L on COD basis. PHB and PHV accumulation occurred simultaneously until acetate depletion, and then PHB assimilation and PHV accumulation took place simultaneously. However rate of PHV accumulation decreased sharply after acetate depletion. Concentrations of PHB and PHV increased from 232.9 mg/L to 257.5 mg/L and from 59.7 mg/L to 113.9 mg/L on COD basis, respectively. When acetate was depleted completely the polymer composition was 71% of HB, 23% of HV, and 6% of HMV on molar basis.

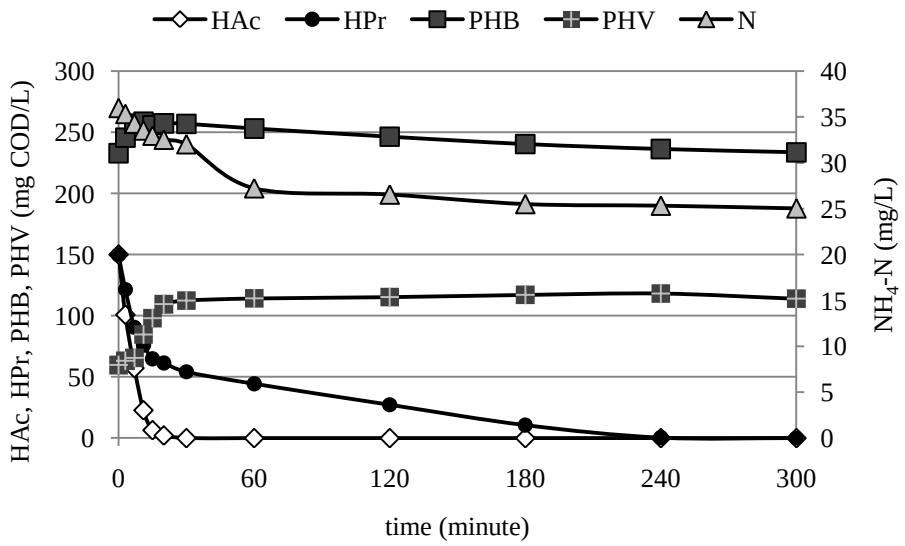


Figure 5.26: Profiles of acetate, propionate, PHB, PHV and NH_4-N during batch $N+_3$.

The yield of PHA on VFA consumed was 0.29. Specific rates for polymer storage and substrate uptake were calculated as 0.09 Cmmol PHA/Cmmol X.h and 0.39 Cmmol VFA/Cmmol X.h, respectively. The maximum specific growth rate and the growth yield were calculated as 0.035 h^{-1} and 0.6, respectively.

5.3.2.4 Batch N_{+4}

Batch $N+_4$ was carried out by applying C/N ratio of 100/12, and substrate loading of 0.4 g COD S/g COD X, which was four times of those in SBR N+, batch $N+_0$, and batch $N+_1$. Acetate was depleted in 60 minutes. Concentration of NH_4-N taken up during test was 31 mg/L of which 70% was taken up in 60 minutes. PHA content of biomass increased from 10.3% to 24.9% as mg COD/L, corresponding to an increase in PHA concentration from 345.8 mg/L to 836.8 mg/L on COD basis. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment $N+_4$ were shown in Figure 5.27.

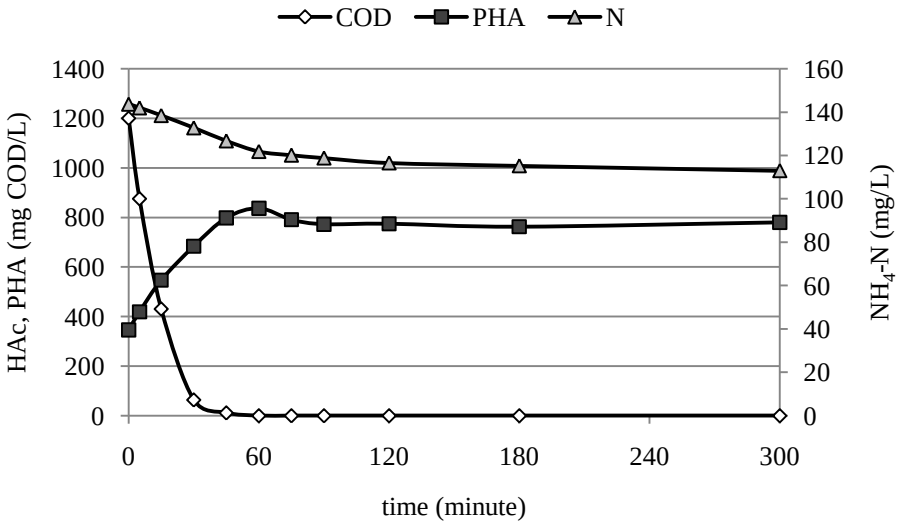


Figure 5.27: Profiles of acetate, PHA, and NH_4-N during batch $N+_4$.

The polymer accumulated mostly in the form of PHB however biomass PHV content was also observed to increase from 1.6% to 3.4% on COD basis during the batch experiment. Only 13% of the PHV was accumulated before acetate depletion. The polymer was composed of 88% of HB, 7% of HV, and 5% of HMV on molar basis. The yield of polymer on substrate consumed was 0.36. The specific polymer storage rate and the specific HAc uptake rate were calculated as 0.23 Cmmol PHA/Cmmol X.h and 1.12 Cmmol HAc/Cmmol X.h, respectively. The growth yield was calculated as 0.21 and the maximum specific growth rate was calculated as 0.074 h^{-1}.

5.3.2.5 Batch N_{+5}

Batch $N+_5$ was carried out by applying C/N ratio of 100/12. Applied substrate loading and corresponding acetate concentration supplied during the experiment were 0.8 g COD S/g COD X and 2400 mg COD/L, respectively. In 180 minutes, acetate was depleted completely and 55 mg/L of NH_4-N was taken up, which 89% of total NH_4-N removal. PHA concentration increased to 1286.4 mg/L on COD basis, corresponding to an increase in PHA content of biomass from 10.3% to 38.3% as mg COD/L. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment $N+_5$ were shown in Figure 5.28.

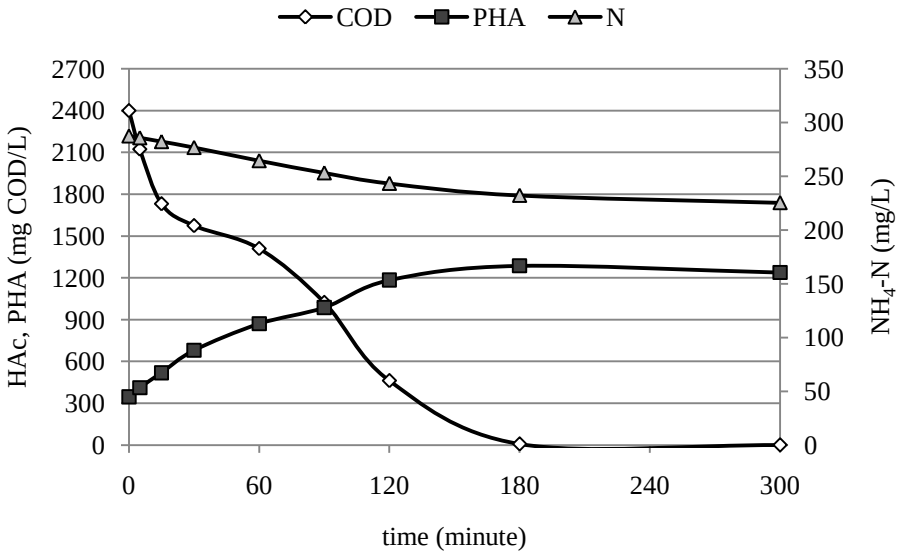


Figure 5.28: Profiles of acetate, PHA, and NH_4-N during batch $N+_5$.

Biomass PHV content increased from 1.7% to 2.1% on COD basis. The polymer accumulated mostly in the form of PHB during the experiment. The polymer was composed of 92% of HB, 5% of HV, and 3% of HMV on molar basis. The polymer yield and the specific polymer storage rate were 0.35 Cmmol/Cmmol and 0.21 Cmmol PHA/Cmmol X.h, respectively. The specific HAc uptake rate was calculated as 0.99 Cmmol HAc/Cmmol X.h. The growth yield was 0.26 and the maximum specific growth rate was calculated as 0.062 h^{-1}.

5.3.2.6 Batch N_{+6}

Batch $N+_6$ was carried out by applying substrate loading of 0.4 g COD S/g COD X, which was identical to that in batch $N+_4$. No ammonia was supplied during the batch experiment. Supplied acetate, which was 1200 mg COD/L, was depleted in 120 minutes. PHA content of biomass increased from 10.7% to 27.0% as mg COD/L, corresponding to an increase in PHA concentration from 357.4 mg/L to 907.4 mg/L on COD basis. Profiles of acetate and PHA concentrations during the batch experiment were shown in Figure 5.29.

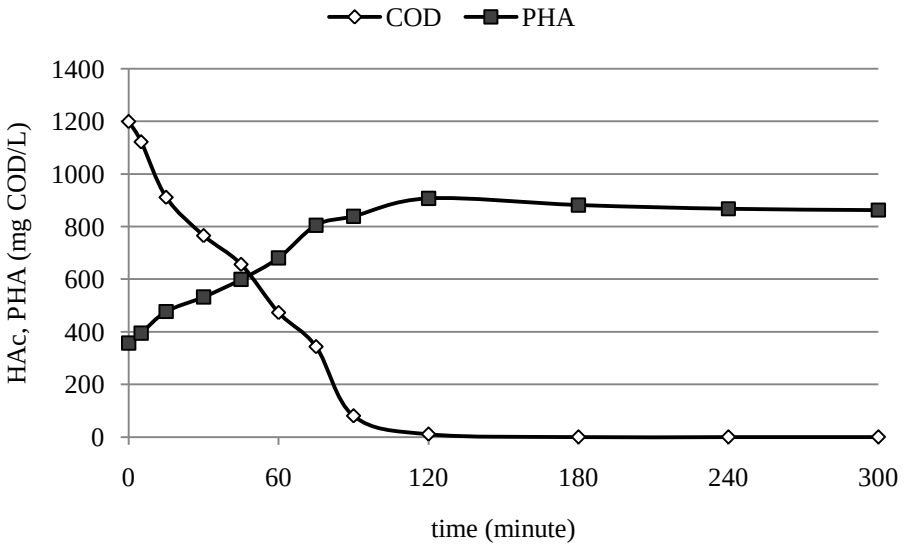


Figure 5.29: Profiles of acetate and PHA during batch $N+_6$.

The polymer accumulated mostly in the form of PHB however biomass PHV content was also observed to increase from 1.7% to 3.5% on COD basis during the batch experiment. The polymer was composed of 88% of HB, 7% of HV, and 5% of HMV on molar basis. The yield of polymer on substrate consumed was 0.40. The specific polymer storage rate and the specific HAc uptake rate were calculated as 0.12 Cmmol PHA/Cmmol X.h and 0.28 Cmmol HAc/Cmmol X.h, respectively.

5.3.3 Batch experiments carried out with biomass enriched in SBR N-

The response of biomass enriched under nitrogen deficient dynamic conditions was investigated by six batch experiments. Conditions of batch experiments were identical to those in batch experiments carried out with biomass enriched in SBR N+. However C/N ratio applied during the batch tests carried out with sludge N- was identical to that in SBR N-, which was 100/2. Only exception was batch $N-_6$, in which no ammonia was supplied.

5.3.3.1 Batch $N-_1$

Batch $N-_1$ was carried out by applying substrate loading of 0.1 g COD S/g COD X, which was similar to that in SBR N- and batch $N-_0$. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment $N-_1$ were shown in Figure 5.30. In 15 minutes, almost all acetate and NH_4-N were consumed. PHA content of biomass increased from 12.9% to 19.2% as mg COD/L, corresponding to an increase in PHA concentration from 411.7 mg/L to 611.3 mg/L on COD basis. The polymer

accumulated in the form of PHB and concentrations of PHV and 3H2MV did not change during the batch experiment. When biomass polymer content reached its maximum, the polymer composition was 89% of HB, 7% of HV, and 4% of HMV on molar basis. The yield of polymer on substrate consumed was 0.61. Specific rates for polymer storage and HAc uptake were calculated as 0.38 Cmmol PHA/Cmmol X.h and 0.54 Cmmol HAc/Cmmol X.h, respectively. The maximum specific growth rate and the growth yield were calculated as 0.084 h^{-1} and, 0.23 respectively.

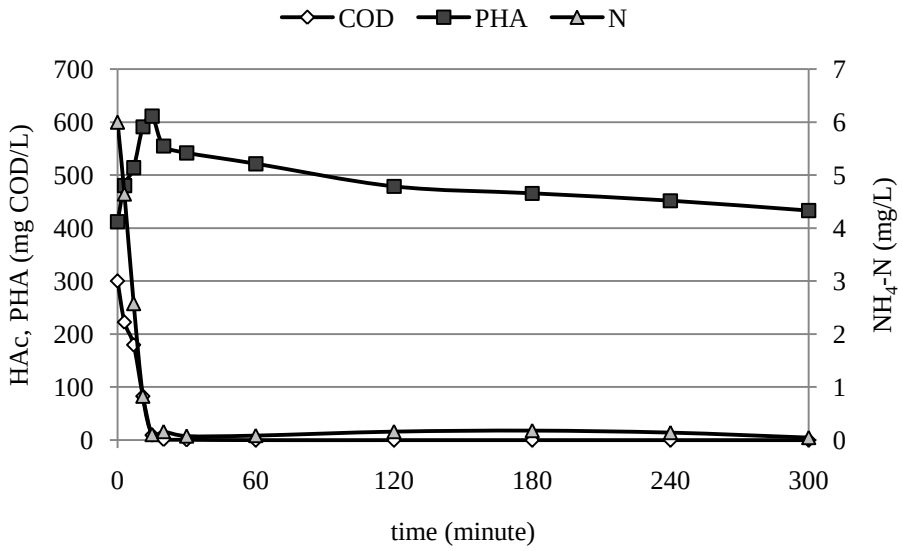


Figure 5.30: Profiles of acetate, PHA, and NH_4-N during batch N_{-1}

5.3.3.2 Batch N_{-2}

Supplied substrate concentration and corresponding substrate loading during batch N_{-2} were 600 mg COD/L, and 0.2 g COD S/g COD X, respectively, which were two times of those in SBR N-, batch N_{-0}, and batch N_{-1}. Acetate and ammonia were depleted almost completely in 30 and 60 minutes, respectively. Ammonia uptake rate decreased sharply following acetate exhaustion. PHA content of biomass increased from 13.2% to 22.7% as mg COD/L, corresponding to an increase of 302.6 mg/L in PHA concentration on COD basis. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N_{-2} were shown in Figure 5.31.

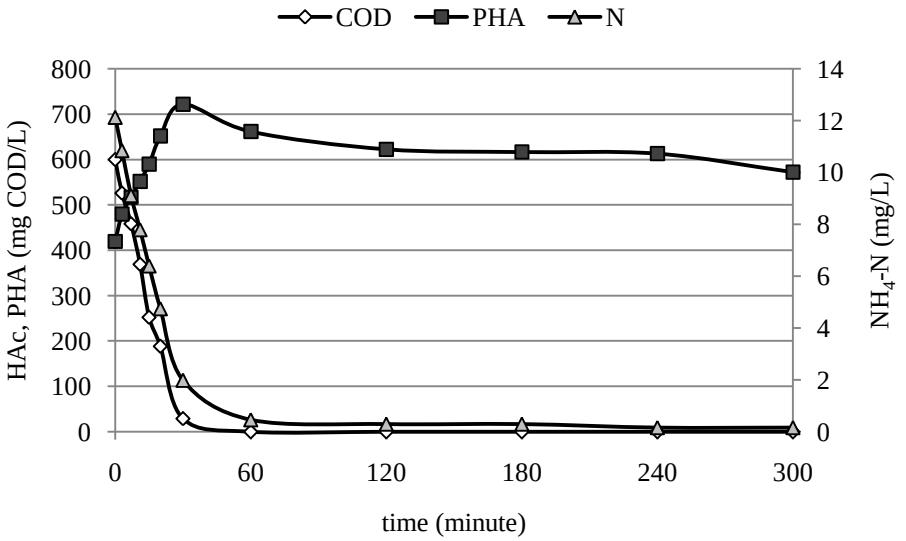


Figure 5.31: Profiles of acetate, PHA, and NH_4-N during batch N_{-2}.

The polymer accumulated mostly in the form of PHB however a small increase in biomass PHV content was also observed (0.2% on COD basis) during the batch experiment. The polymer was composed of 91% of HB, 6% of HV, and 3% of HMV on molar basis. The yield of polymer on substrate consumed was 0.47. Specific rates for polymer storage and HAc uptake were calculated as 0.33 Cmmol PHA/Cmmol X.h and 0.46 Cmmol HAc/Cmmol X.h, respectively. The maximum specific growth rate and the growth yield were calculated as 0.072 h^{-1} and 0.20, respectively.

5.3.3.3 Batch N_{-3}

Batch N_{-3} was carried out by applying substrate loading of 0.1 g COD S/g COD X, and C/N ratio of 100/2, which were similar to those in SBR N-, batch N_{-0}, and batch N_{-1}. However a propionate-acetate mixture was used as carbon source (50% of HAc, 50% of HPr on COD basis). Figure 5.32 shows profiles of acetate, propionate, PHB, PHV and NH_4-N concentrations during batch experiment N_{-3}. Acetate and propionate were consumed simultaneously in 30 minutes until acetate depletion. Propionate concentration in the reactor was 44.7 mg/L on COD basis when acetate depleted and complete depletion of propionate and NH_4-N took 120 minutes. PHA content of biomass increased from 11.8% to 16.8% as mg COD/L, corresponding to an increase of 157.9 mg COD/L in PHA concentration. PHB and PHV accumulation occurred simultaneously until acetate depletion, and then PHB assimilation and PHV accumulation took place simultaneously until propionate depletion. Concentrations of PHB and PHV increased from 321.1 mg/L to 368.6 mg/L and from 55.6 mg/L to

182.2 mg/L on COD basis, respectively. When acetate was depleted completely the polymer composition was 71% of HB, 24% of HV, and 5% of HMV on molar basis. When all propionate was taken up, polymer was consisted of 69% of HB, 27% of HV, and 4% of HMV on molar basis.

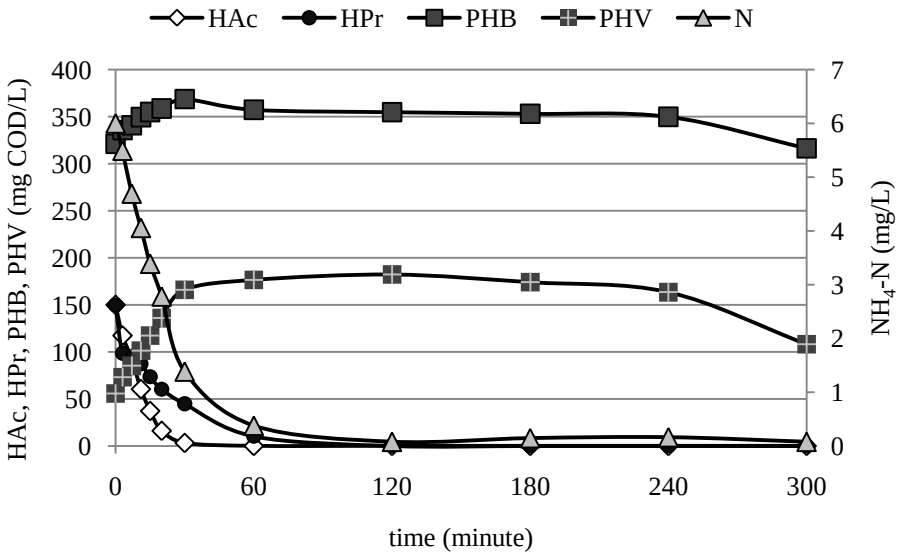


Figure 5.32: Profiles of acetate, propionate, PHB, PHV and NH_4-N during batch N-$_3$.

The yield of PHA on VFA consumed was 0.50. Specific rates for polymer storage and substrate uptake were calculated as 0.17 Cmmol PHA/Cmmol X.h and 0.39 Cmmol VFA/Cmmol X.h, respectively. The maximum specific growth rate and the growth yield were 0.033 h^{-1} and 0.25, respectively.

5.3.3.4 Batch N$_{-4}$

Batch N-$_4$ was carried out by applying substrate loading of 0.4 g COD S/g COD X, which was four times of that in SBR N-. Applied C/N ratio was identical to that in SBR N-. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N-$_4$ were shown in Figure 5.33. Acetate was depleted in 60 minutes. Concentration of NH_4-N taken up during test was 21 mg/L of which 69% was taken up in 60 minutes. Ammonia uptake rate decreased smoothly following the substrate depletion. PHA content of biomass increased from 12.9% to 28.8% as mg COD/L, corresponding to an increase in PHA concentration from 411.4 mg/L to 917.3 mg/L on COD basis.

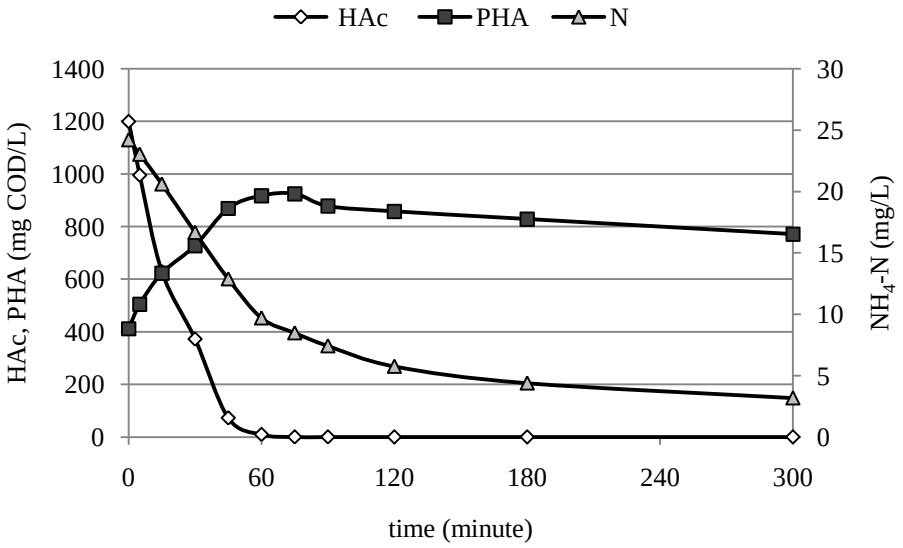


Figure 5.33: Profiles of acetate, PHA, and NH_4-N during batch N_{-4}.

The polymer accumulated mostly in the form of PHB however a small increase was also observed in biomass PHV content (0.37% on COD basis) during the batch experiment. The polymer was composed of 93% of HB, 5% of HV, and 2% of HMV on molar basis. The yield of polymer on substrate consumed was 0.37. The specific polymer storage rate and the specific HAc uptake rate were calculated as 0.31 Cmmol PHA/Cmmol X.h and 0.79 Cmmol HAc/Cmmol X.h, respectively. The growth yield was calculated as 0.14 and the maximum specific growth rate was calculated as 0.052 h^{-1}.

5.3.3.5 Batch N_{-5}

The batch test was carried out by applying substrate loading of 0.8 g COD S/g COD X, which was eight times of that in SBR N-.Corresponding acetate concentration supplied during the experiment was 2400 mg COD/L. In 120 minutes, acetate was depleted completely and 28.6 mg/L of NH_4-N was taken up. Following substrate depletion, ammonia uptake rate decreased slightly and 9.5 mg/L of NH_4-N was consumed during the subsequent 180 minutes. PHA concentration increased from 422.5 mg/L to 1378.5 mg/L on COD basis, corresponding to an increase in PHA content of biomass from 13.3% to 43.3% as mg COD/L. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N_{-5} were shown in Figure 5.34.

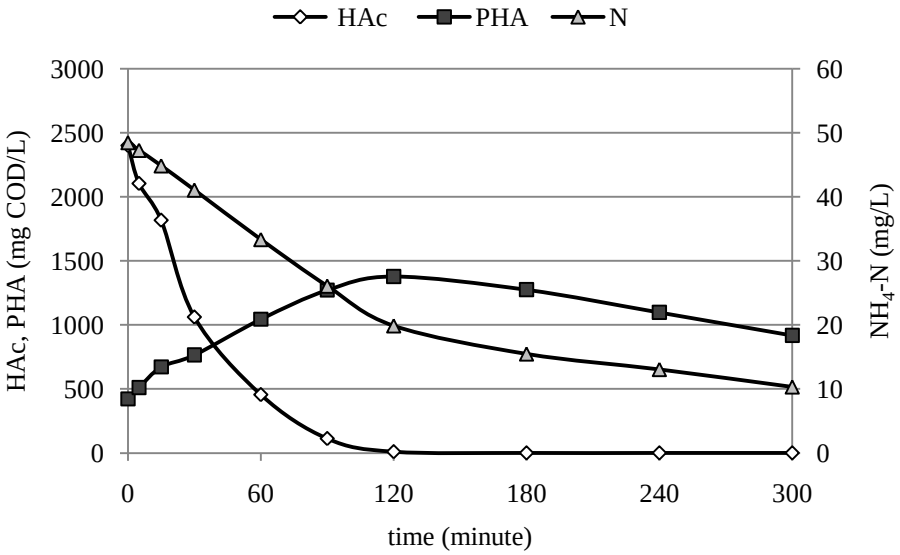


Figure 5.34: Profiles of acetate, PHA, and NH_4-N during batch N_{-5}.

The polymer accumulated mostly in the form of PHB during the experiment however, biomass PHV content also increased from 1.8% to 2.3% on COD basis. The polymer was composed of 94% of HB, 4% of HV, and 2% of HMV on molar basis. The specific polymer storage rate and the polymer yield were 0.30 Cmmol PHA/Cmmol X.h and 0.35, respectively. The specific HAc uptake rate was calculated as 1.12 Cmmol HAc/Cmmol X.h. The growth yield was 0.14 and the maximum specific growth rate was calculated as 0.05 h^{-1}.

5.3.3.6 Batch N_{-6}

Batch N_{-6} was carried out by applying substrate loading of 0.4 g COD S/g COD X, which was identical to that in batch N_{-4}. No ammonia was supplied during the batch experiment. Profiles of acetate and PHA concentrations during the batch experiment were shown in Figure 5.35. Supplied acetate, which was 1200 mg COD/L, was depleted in 90 minutes. PHA content of biomass increased from 13.6% to 31.3% as mg COD/L, corresponding to an increase in PHA concentration from 434.3 mg/L to 998.3 mg/L on COD basis.

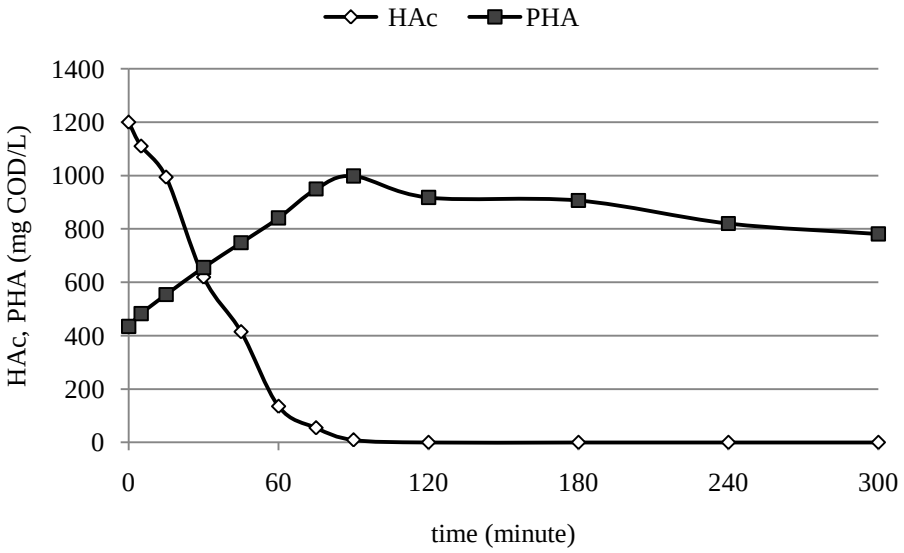


Figure 5.35: Profiles of acetate and PHA during batch N_{-6}.

The polymer accumulated mostly in the form of PHB during the batch experiment. The polymer was composed of 93% of HB, 5% of HV, and 2% of HMV on molar basis. The polymer yield was calculated as 0.43. The specific polymer storage rate and the specific HAc uptake rate were calculated as 0.16 Cmmol PHA/Cmmol X.h and 0.34 Cmmol HAc/Cmmol X.h, respectively.

5.3.4 Batch experiments carried out with biomass enriched in SBR N_D-

The response of biomass after enrichment under ADF conditions with delayed nitrogen feeding was investigated by six batch experiments. Propionate-acetate mixture was used as carbon source during an experiment. One experiment was carried out by supplying nitrogen together with carbon source at the beginning of experiment. Four experiments were carried out by applying synthetic wastewater in different organic loadings.

5.3.4.1 Batch N_{D-1}

Batch N_D-$_1$ was carried out by applying substrate loading of 0.1 g COD S/g COD X, which was similar to that in SBR N_D- and batch N_D-$_0$. The test started with addition synthetic wastewater having 300 mg COD /L of acetate and no nitrogen. Required ammonia to satisfy a C/N ratio of 100/2 was added 1 hour later. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N_D-$_1$ were shown in Figure 5.36. In 30 minutes, almost all acetate was consumed, and NH_4-N was consumed in 15 minutes following its addition. PHA content of biomass increased from 22.0% to

29.0% as mg COD/L, corresponding to an increase in PHA concentration from 741.9 mg/L to 976.8 mg/L on COD basis. The polymer accumulated in the form of PHB and concentrations of PHV and 3H2MV did not change during the batch experiment. When biomass polymer content reached its maximum, the polymer composition was 90% of HB, 6% of HV, and 4% of HMV on molar basis. The yield of polymer on substrate consumed was 0.71. Specific rates for polymer storage and HAc uptake were calculated as 0.15 Cmmol PHA/Cmmol X.h and 0.30 Cmmol HAc/Cmmol X.h, respectively.

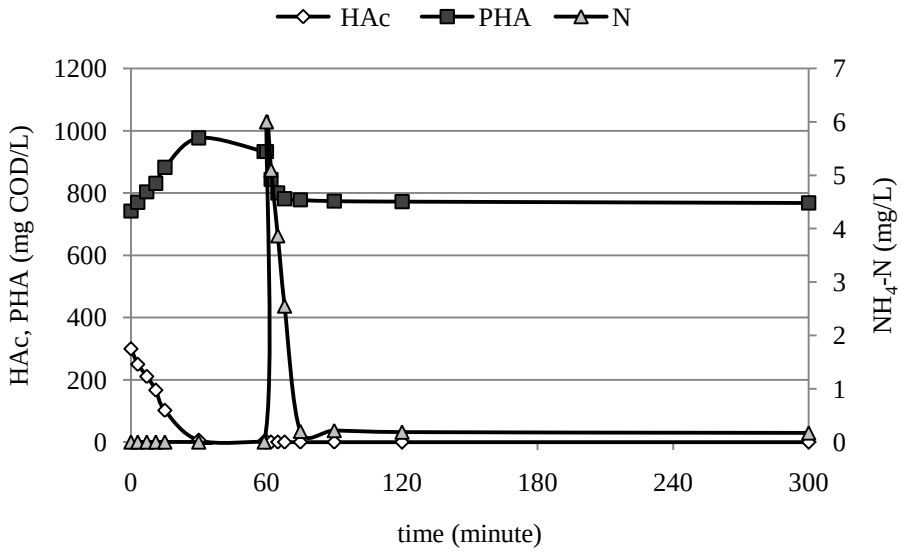


Figure 5.36: Profiles of acetate, PHA, and NH_4-N during batch N_{D-1}.

5.3.4.2 Batch N_{D-2}

Supplied substrate concentration and corresponding substrate loading during batch N_{D-2} were 600 mg COD/L, and 0.2 g COD S/g COD X, respectively, which were two times of those in SBR N_{D-}, batch N_{D-0}, and batch N_{D-1}. Acetate was depleted almost completely in 45 minutes. Ammonia was added 1 hour after start up and depleted completely in 240 minutes. PHA content of biomass increased from 22.5% to 35.7% as mg COD/L, corresponding to an increase in PHA concentration from 759.3 mg/L to 1201.6 mg/L on COD basis. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N_{D-2} were shown in Figure 5.37.

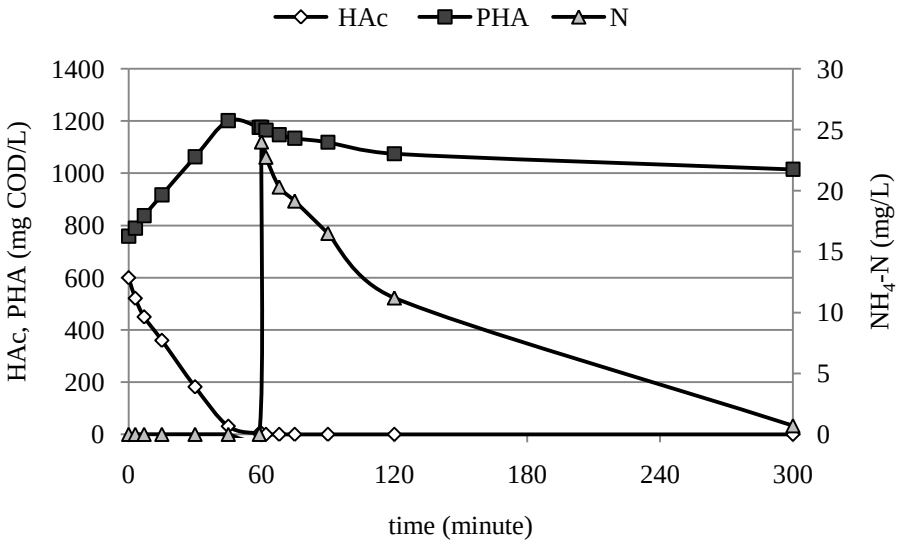


Figure 5.37: Profiles of acetate, PHA, and NH_4-N during batch N_{D-2}.

The polymer accumulated mostly in the form of PHB however a small increase in biomass PHV content was also observed (0.8% on COD basis) during the batch experiment. PHV uptake was observed to continue after acetate depletion and ammonia addition. The polymer was composed of 94% of HB, 4% of HV, and 2% of HMV on molar basis. The yield of polymer on substrate consumed was 0.69. Specific rates for polymer storage and HAc uptake were calculated as 0.16 Cmmol PHA/Cmmol X.h and 0.47 Cmmol HAc/Cmmol X.h, respectively.

5.3.4.3 Batch N_{D-3}

Batch N_{D-3} was carried out by applying substrate loading of 0.1 g COD S/g COD X, which was similar to that in SBR N_D- and batch N_{D-1}. Only difference was the carbon source supplied, which consisted of a propionate-acetate mixture (50% of HAc, 50% of HPr on COD basis). The test started with addition synthetic wastewater having 300 mg COD /L and no nitrogen. Required ammonia to satisfy a C/N ratio of 100/2 was added 1 hour later. Figure 5.38 shows profiles of acetate, propionate, PHB, PHV and NH_4-N concentrations during batch experiment N_{D-3}. Acetate and propionate were consumed simultaneously in 30 minutes until acetate depletion. Half of the propionate supplied was consumed when acetate depleted, and propionate uptake rate decreased after acetate depletion. Remaining propionate after 60 minutes, which was 26 mg COD/L, was observed to be consumed in two minutes following ammonia addition. Supplied ammonia was consumed almost completely in 30 minutes following its addition. Total PHA content of biomass increased from 22.3%

to 27.3% as mg COD/L, corresponding to an increase of in PHA concentration from 807.7 mg/L to 988.4 mg/L on COD basis. PHB and PHV accumulation occurred simultaneously until acetate depletion, and then PHB assimilation and PHV accumulation took place simultaneously until propionate depletion. Increase in concentration of PHB was 78 mg/L on COD basis when acetate depleted. Increase in concentration of PHV was 101.8 mg/L when acetate was depleted and was 137.8 mg/L when propionate was depleted on COD basis. When all substrate was taken up, polymer was consisted of 80% of HB, 17% of HV, and 3% of HMV on molar basis.

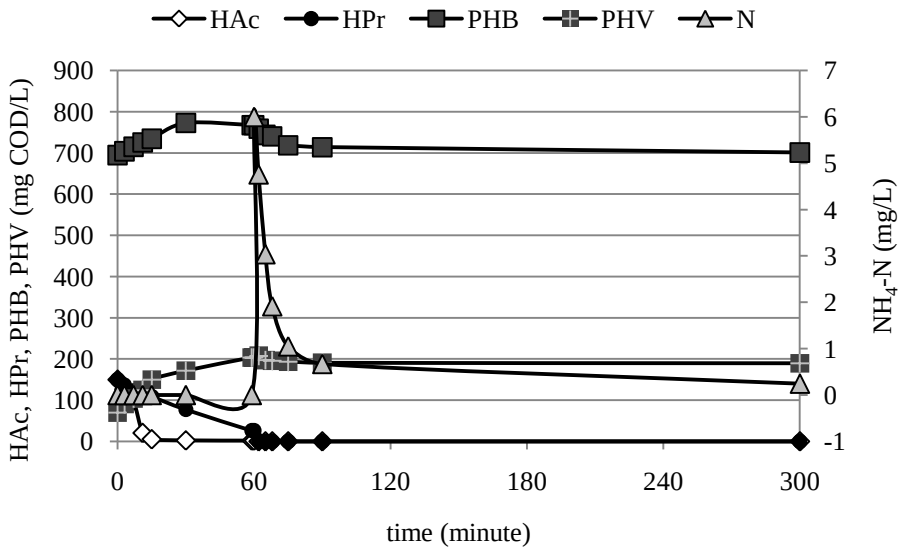


Figure 5.38: Profiles of acetate, propionate, PHB, PHV and NH_4-N during batch N_{D-3}.

The yield of PHA on VFA consumed was 0.74. Specific rates for polymer storage and substrate uptake were calculated as 0.14 Cmmol PHA/Cmmol X.h and 0.17 Cmmol VFA/Cmmol X.h, respectively.

5.3.4.4 Batch N_{D-4}

Batch N_D-4 was carried out by applying substrate loading of 0.4 g COD S/g COD X, which was four times of that in SBR N_D-. Applied C/N ratio was identical to that in SBR N_D-. The test started with addition synthetic wastewater having 1200 mg COD /L and no nitrogen. Required ammonia to satisfy a C/N ratio of 100/2 was added 3 hours later. Profiles of acetate, PHA, and NH_4-N concentrations during the batch experiment were shown in Figure 5.39. Acetate was depleted in 120 minutes. Concentration of NH_4-N taken up during test was 17.7 mg/L. PHA content of

biomass increased to 41.2% as mg COD/L, corresponding to an increase in PHA concentration from 734.8 mg/L to 1388.9 mg/L on COD basis.

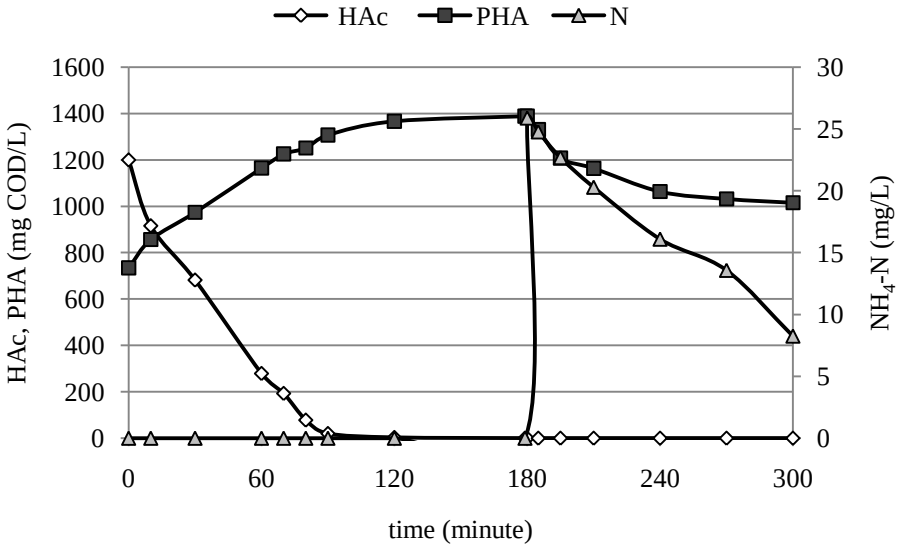


Figure 5.39: Profiles of acetate, PHA, and NH_4-N during batch $N_{D-4.}$

The polymer accumulated mostly in the form of PHB however a small increase was also observed in biomass PHV content (0.6% on COD basis) during the batch experiment. The polymer was composed of 94% of HB, 4% of HV, and 2% of HMV on molar basis. The yield of polymer on substrate consumed was 0.48. The specific polymer storage rate and the specific HAc uptake rate were calculated as 0.191 Cmmol PHA/Cmmol X.h and 0.5 Cmmol HAc/Cmmol X.h, respectively.

5.3.4.5 Batch N_{D-5}

The batch test was carried out by applying substrate loading of 0.8 g COD S/g COD X, which was eight times of that in SBR N_D-. The test started with addition synthetic wastewater having 2400 mg COD /L and no nitrogen. Required ammonia to satisfy a C/N ratio of 100/2 was added 4 hours later. In 210 minutes, acetate was depleted almost completely. 124 mg/L of NH_4-N was taken up during the experiment following its addition. PHA concentration increased from 782.6 mg/L to 1586.6 mg/L on COD basis, corresponding to an increase in PHA content of biomass from 23.2% to 47.1% as mg COD/L. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N_D-$_5$ were shown in Figure 5.40.

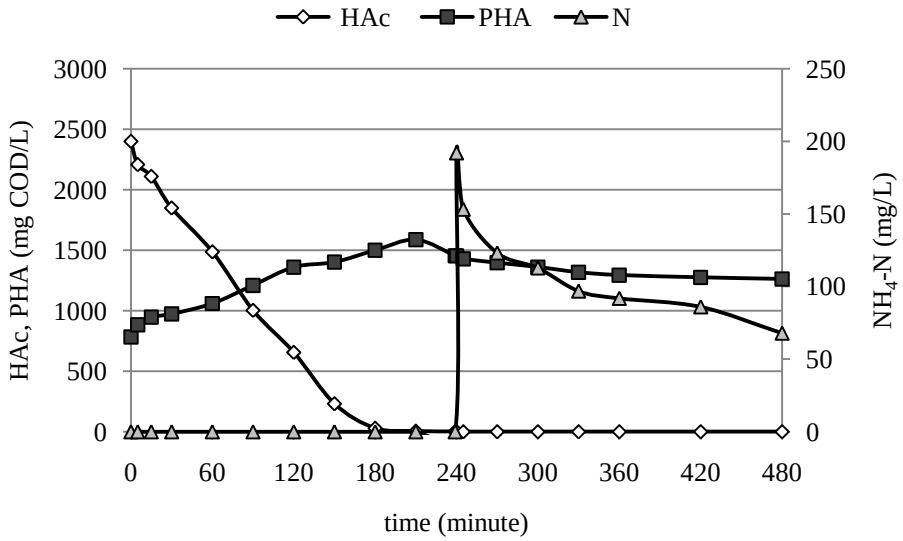


Figure 5.40: Profiles of acetate, PHA, and NH_4-N during batch N_{D-5}.

The polymer accumulated mostly in the form of PHB during the experiment however, biomass PHV content also increased from 1.8% to 2.7% on COD basis. The polymer was composed of 94% of HB, 4% of HV, and 2% of HMV on molar basis. The specific polymer storage rate and the polymer yield were 0.32 Cmmol PHA/Cmmol X.h and 0.3, respectively. The specific HAc uptake rate was calculated as 0.68 Cmmol HAc/Cmmol X.h.

5.3.4.6 Batch N_{D-6}

The batch test N_D-$_6$ was carried out to understand the response of biomass enriched under dynamic conditions with delayed nitrogen feeding to wastewater including nitrogen. Substrate loading of 0.1 g COD S/g COD X, and C/N ratio of 100/2, which were similar to those in SBR N_D- and batch N_D-$_1$ were applied in the test. However, similar to N-$_1$, required ammonia to satisfy a C/N ratio of 100/2 was added together with acetate at the beginning of the test. Profiles of acetate, PHA, and NH_4-N concentrations during batch experiment N_D-$_6$ were shown in Figure 5.41. In 30 minutes, almost all acetate and NH_4-N were consumed. PHA content of biomass increased from 22.3% to 24.3% as mg COD/L, corresponding to an increase in PHA concentration from 749.6 mg/L to 819.9 mg/L on COD basis. The polymer accumulated in the form of PHB and concentrations of PHV and 3H2MV did not change during the batch experiment. When biomass polymer content reached its maximum, the polymer composition was 91% of HB, 6% of HV, and 3% of HMV on molar basis. The yield of polymer on substrate consumed was 0.22. Specific rates for

polymer storage and HAc uptake were calculated as 0.05 Cmmol PHA/Cmmol X.h and 0.44 Cmmol HAc/Cmmol X.h, respectively. The maximum specific growth rate and the growth yield were calculated as 0.039 h^{-1} and, 0.23 respectively.

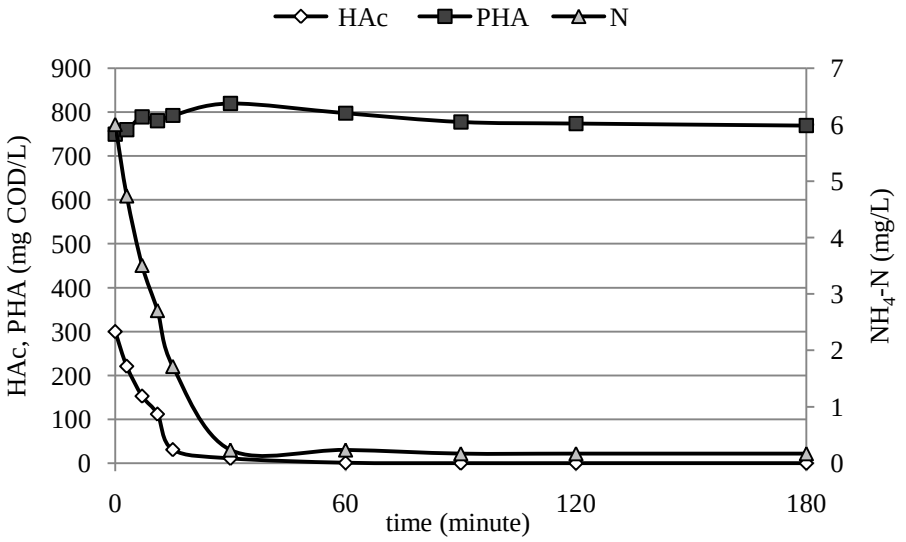


Figure 5.41: Profiles of acetate, PHA, and NH_4-N during batch $N_{D\text{-}6}$.

6. DISCUSSIONS

6.1 Polymer Accumulation by Inoculum Sludge

Four types of fresh activated sludges from municipal, pulp-paper, starch and dairy wastewater treatment plants were studied previously for PHA production by Yan, et al., (2006) and the pulp-paper activated sludge was reported to show the highest potential for PHA production. However batch experiments carried out with inoculum sludge in this study clarified that polymer accumulation by the inoculum sludge was of no great concern. The highest increase in biomass PHA content achieved by inoculum sludge was observed during batch N_D-0, which was 1% on COD basis. Substrate uptake rates were also noticeably lower. Probably, operating conditions applied in wastewater treatment plant (lower organic loading rate and considerably higher sludge age) adversely affected activity of sludge and also its polymer storage ability.

6.1.1 Effect of different C/N ratios on polymer accumulation by inoculum sludge

Obtained max polymer content of sludge, amount of PHA accumulated, the specific acetate uptake rate, the specific polymer storage rate, and the yield of polymer on substrate consumed in batch experiments carried out with inoculum sludge were compared for different C/N ratios in Table 6.1. Similar substrate uptake rates were obtained for all experiments. However polymer storage ability of inoculum sludge in case of amount, rate, and yield were observed to increase with decreasing ammonia concentration. Highest polymer yield, polymer storage rate, and polymer accumulation was observed for batch test performed with synthetic wastewater with no ammonia. Obtained polymer yield in this batch test was comparable with those obtained in the literature and also with those obtained in this study for enriched sludge. However, because of low acetate uptake rate, amount of polymer stored by inoculum sludge at the end of the test was very low. Supplied acetate, which was 300 mg COD/L, could not be utilized completely in 120 minutes, during the batch tests carried out with inoculum sludge.

Table 6.1: Effect of C/N ratio on polymer accumulation by inoculum sludge.

C/N	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
100/12	4.36	13.5	0.066	0.004	0.04
100/2	4.45	17.4	0.063	0.007	0.10
100/0	4.96	32.9	0.064	0.024	0.26

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.2 Effect of Biomass Enrichment on Polymer Accumulation

Substrate loadings and C/N ratios applied in batch experiments carried out with inoculum sludge ($N+_0$, $N-_0$, N_D-_0) were also applied in three batch experiment carried out with biomass enriched under different conditions ($N+_1$, $N-_1$, N_D-_1). The comparison of the results obtained from batch experiments performed before and after enrichment served to the purpose of understanding effect of enrichment of biomass under dynamic conditions on polymer accumulation.

6.2.1 Enrichment without nitrogen deficiency

Initial biomass, acetate and ammonia concentrations, and experimental conditions were same for batch $N+_0$ and $N+_1$. Only difference between both tests performed with C/N ratio of 100/12 was the origin of biomass. Batch $N+_0$ was carried out with inoculum sludge and batch $N+_1$ was carried out with sludge enriched under aerobic dynamic conditions without nitrogen deficiency. PHA concentrations in the reactors during both tests were compared in Figure 6.1. Amount of polymer accumulated by enriched sludge was noticeably higher than that accumulated by inoculum sludge. Polymer concentration in the reactor increased 13.5 mg COD/L during the batch test carried out with inoculum sludge, while 147 mg COD/L of increase obtained for enriched sludge under same conditions. The polymer content of sludge, amount of PHA accumulated, the specific acetate uptake rate, the specific polymer storage rate, and the yield of polymer on substrate consumed for biomass enriched under dynamic conditions were compared with corresponding values obtained for inoculum sludge (Table 6.2). A remarkable disparity between values obtained for two sludges indicate that polymer storage ability of activated sludge was highly improved under aerobic dynamic feeding conditions.

Dionisi et al. (2005b) enriched activated sludge under feast and famine conditions without any nitrogen limitation and compared polymer production rates of both

sludges. They have reported much higher storage rates for enriched sludge (0.278 g COD/g COD.h) than that for non-enriched activated sludge from municipal wastewater treatment plants (0.012-0.044 g COD/g COD.h). Majone et al. (2006) has reported 2 times higher PHA productivity and 20 times higher polymer uptake rates for enriched sludge under dynamic conditions than activated sludge. In this study more than 70 times higher polymer storage rate was obtained for enriched sludge under aerobic dynamic feeding conditions.

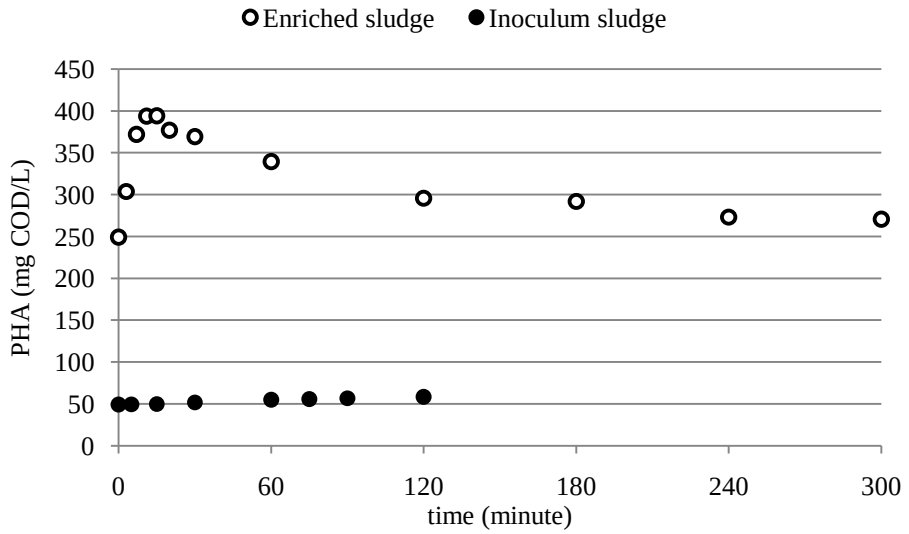


Figure 6.1: Comparison of PHA profiles obtained during Batch $N+_0$ and Batch $N+_1$.

Table 6.2: Effect of biomass enrichment without nitrogen deficiency on polymer accumulation.

	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
Inoculum sludge	4.36	13.5	0.066	0.004	0.04
Enriched sludge	14.80	147	0.790	0.283	0.44

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.2.2 Enrichment with nitrogen deficiency

Substrate loading (0.1 g COD S/g COD X), and C/N ratio (100/2) employed during batch $N-_0$, were repeated for batch $N-_1$, which was carried out with biomass enriched under aerobic dynamic conditions with nitrogen deficiency. Fluctuations of PHA concentration during the batch tests carried out with inoculum and enriched sludge were shown in Figure 6.2. Significant differences between the amounts of polymer accumulated by both sludges clarified that enrichment of activated sludge under nitrogen deficient conditions made a great influence on PHA storage. The polymer content of sludge, amount of PHA accumulated, the specific acetate uptake rate, the

specific polymer storage rate, and the yield of polymer on substrate consumed for enriched biomass were compared with corresponding values obtained for inoculum sludge (Table 6.3). Increase in polymer concentration during the batch test carried out with inoculum sludge was 17.4 mg COD/L for C/N ratio of 100/2. Obtained increase under same conditions for enriched sludge was 199.5 mg COD/L. Remarkable differences were also observed for kinetic and stoichiometric values obtained for two sludges. The specific polymer storage rate of enriched sludge was more than 50 times higher than that of inoculum sludge. There are two studies in the literature, in which nitrogen limitation was also applied besides dynamic feeding during biomass enrichment (Serafim et al., 2004; Lemos et al., 2006). However, comparisons could not be found in these studies between polymer accumulation capabilities inoculum and enriched sludge.

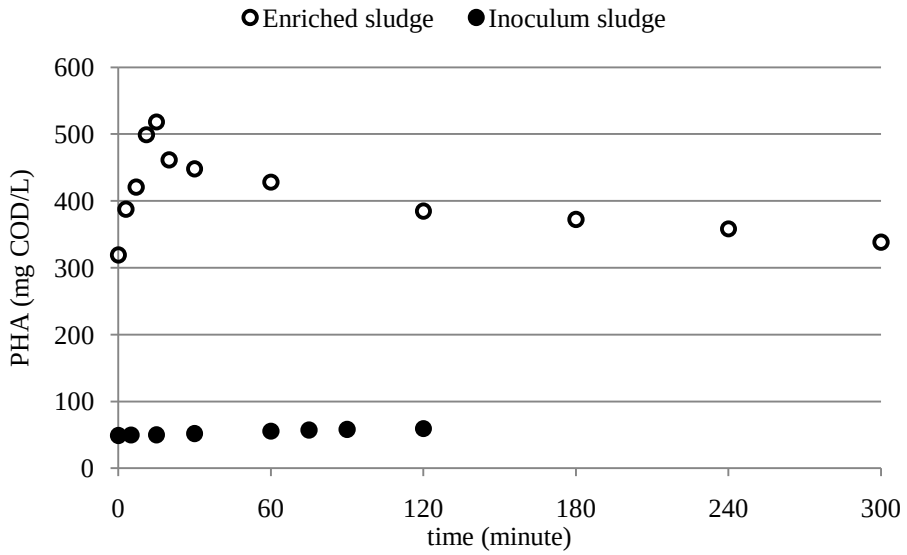


Figure 6.2: Comparison of PHA profiles obtained during Batch $N\text{-}_0$ and Batch $N\text{-}_1$.

Table 6.3: Effect of biomass enrichment with nitrogen deficiency on polymer accumulation.

	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
Inoculum sludge	4.45	17.4	0.063	0.007	0.10
Enriched sludge	19.19	199.5	0.487	0.383	0.61

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.2.3 Enrichment with delayed nitrogen feeding

Batch experiments $N_D\text{-}_0$ and $N_D\text{-}_1$ were both initialized by adding a synthetic wastewater with no ammonia to the reactors. Identical conditions and substrate loading were employed for both experiments. The batch $N_D\text{-}_0$ was performed with

inoculum sludge while N_D-$_1$ was performed with activated sludge enriched under aerobic dynamic conditions with delayed nitrogen feeding. Profiles of PHA concentrations in the reactors during both tests (Figure 6.3) shows that amount of polymer accumulated by enriched sludge was remarkably higher than that accumulated by inoculum sludge. Table 6.4 indicates that not only amount of polymer accumulated but also rate and yield of polymer storage were also improved significantly during the enrichment of biomass. Although best results were obtained for batch N_D-$_0$ among the tests carried out with inoculum sludge, substrate could not be utilized completely in 120 minutes in this test, and polymer yield was also considerably low. However, enriched biomass utilized acetate completely in 30 minutes and accumulated 71% of the substrate as PHA under same conditions.

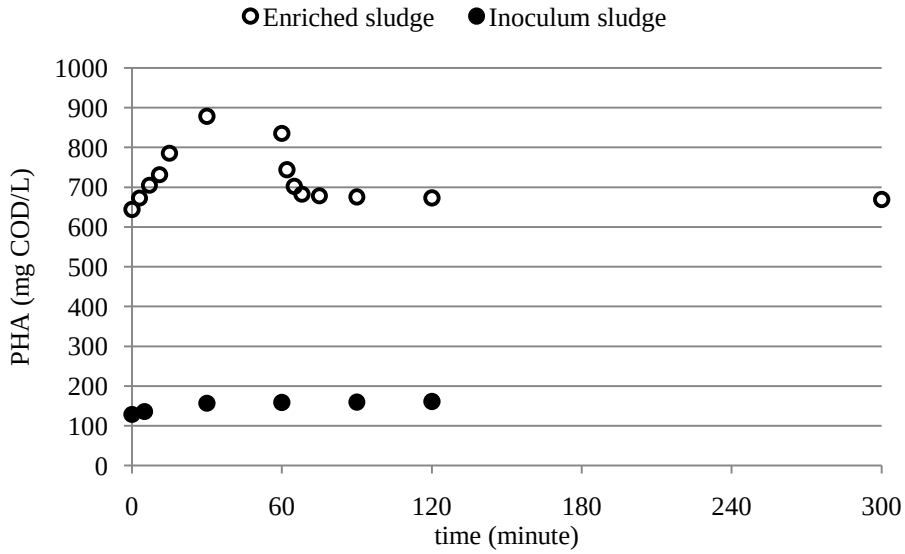


Figure 6.3: Comparison of PHA profiles obtained during Batch N-$_0$ and Batch N_D-$_1$.

Table 6.4: Effect of biomass enrichment with delayed nitrogen feeding on polymer accumulation.

	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
Inoculum sludge	4.96	32.9	0.064	0.024	0.26
Enriched sludge	29	234.9	0.226	0.140	0.71

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.2.4 Comparison of enrichment strategies

Comparison between polymer contents of enriched biomass obtained during batch experiments carried out with different C/N ratios and corresponding values obtained for inoculum sludge were shown in Figure 6.4. The lowest improvement in case of polymer content was observed for enrichment without nitrogen deficiency. Obtained

polymer content during batch experiment performed with C/N ratio of 100/12 was 3.4 times higher after 82 days of enrichment under aerobic dynamic conditions without nitrogen deficiency. Enrichment of biomass under nitrogen deficient conditions produced better results. During batch experiment performed with C/N ratio of 100/2, obtained polymer content of biomass was 4.3 times higher after enrichment under nitrogen deficient conditions. The polymer content of sludge enriched under delayed nitrogen feeding conditions reached 29% during batch test which was 5.8 times higher than corresponding value obtained for inoculum sludge.

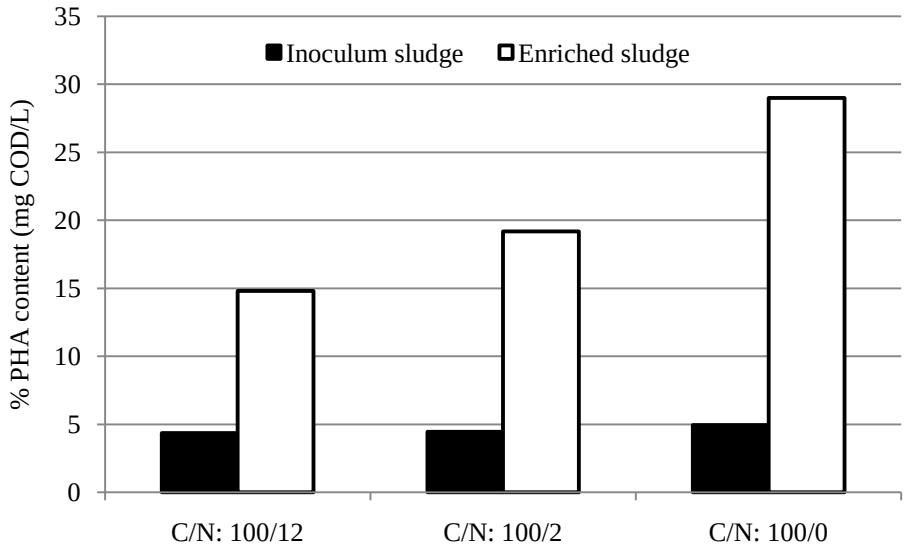


Figure 6.4: Comparison between polymer contents obtained during batch experiments carried out with different C/N ratios.

Acetate uptake rate of biomass observed to increase during enrichment under aerobic dynamic conditions. The highest increase was obtained for biomass enriched without nitrogen deficiency. The specific acetate uptake rate of biomass obtained for enriched biomass was 12 times higher than that obtained for inoculum sludge. The lowest increase was observed for biomass enriched with delayed nitrogen feeding. The specific acetate uptake rate of biomass enriched in SBR N_D- was 3.5 times higher than that obtained for inoculum sludge. Comparison between specific acetate uptake rates was given in Figure 6.5.

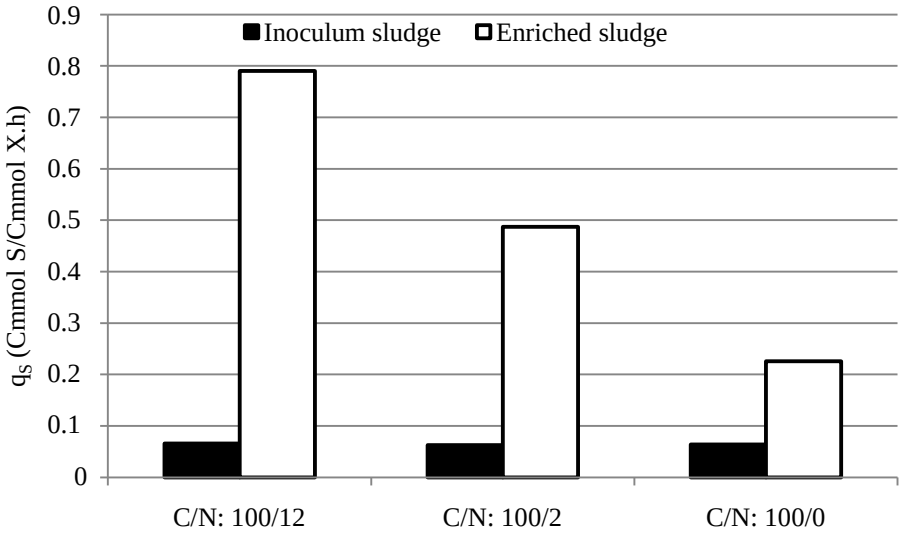


Figure 6.5: Comparison between specific acetate uptake rates obtained during batch experiments carried out with different C/N ratios.

Figure 6.6 shows comparison between specific polymer storage rates of enriched biomass obtained during batch experiments carried out with different C/N ratios and corresponding values obtained for inoculum sludge. The highest polymer uptake rate was observed for biomass enriched under nitrogen deficient conditions, however the highest increase in corresponding value during enrichment was observed for nitrogen sufficient conditions. The specific polymer storage rate of biomass improved 70.8 times under dynamic conditions without nitrogen deficiency. During batch experiments performed with C/N ratio of 100/0, obtained polymer uptake rate was only 3.5 times higher after enrichment under dynamic conditions with delayed nitrogen feeding.

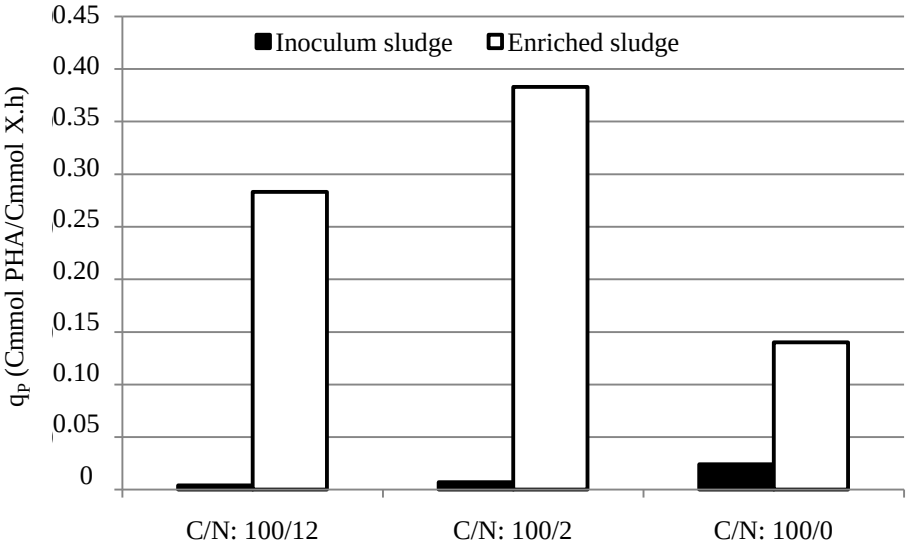


Figure 6.6: Comparison between specific polymer storage rates obtained during batch experiments carried out with different C/N ratios.

Comparison between yields of polymer on substrate consumed obtained during batch experiments carried out with different C/N ratios and corresponding values obtained for inoculum sludge were shown in Figure 6.7. As expected, higher polymer yields were obtained when ammonia deficiency becomes a growth limiting factor during batch experiments performed with both inoculum and enriched sludge. The polymer yield obtained during batch experiment carried out with C/N ratio of 100/12 was observed to increase 10.9 times during enrichment under dynamic conditions without nitrogen deficiency. Corresponding value increased 6.1 and 2.7 times under nitrogen deficient conditions and dynamic conditions with delayed nitrogen feeding, respectively.

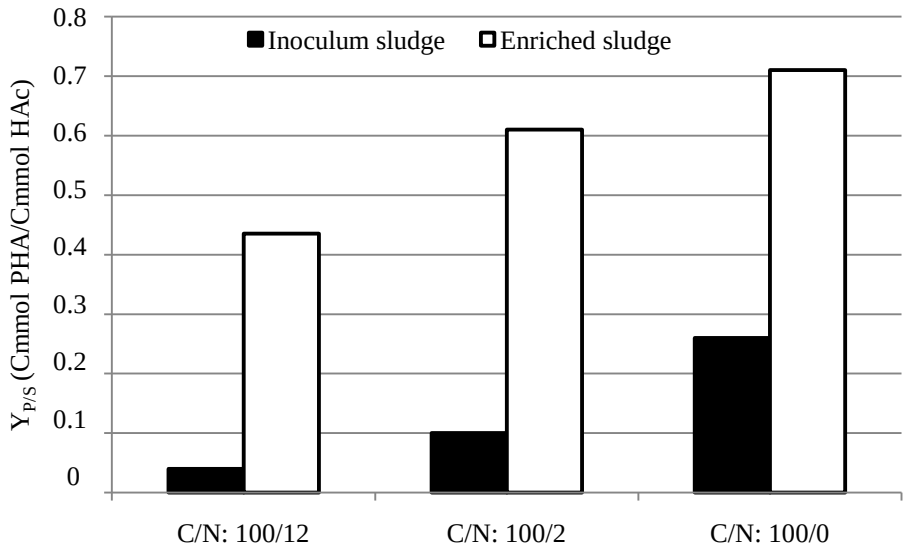


Figure 6.7: Comparison between polymer yields obtained during batch experiments carried out with different C/N ratios.

Polymer storage ability of inoculum sludge, which was considerably poor, has been observed to improve significantly under aerobic dynamic feeding conditions. Besides dynamic feeding, suppressing growth by applying nitrogen deficiency during SBR operation and batch experiments also contributed polymer production. As stated by Serafim et al. (2004), the fraction of substrate used for growth depends, among other factors, on the availability of the growth nutrients in the medium. Yield of polymer on substrate consumed decreased directly with ammonia concentration in the reactor during acetate uptake. Highest polymer production and polymer yield were obtained for delayed nitrogen feeding, in which nitrogen is not available in the reactor during acetate uptake.

6.3 Effect of Substrate Loading on Polymer Accumulation

Biomasses enriched under aerobic dynamic feeding conditions with different nitrogen regimes were used in batch experiments carried out with different substrate loadings to understand effect of substrate in higher concentrations on polymer accumulation. Applied C/N ratio during batch experiments were similar to that applied during SBR operation for every biomass. Substrate loadings during batch experiments were 0.1, 0.2, 0.4, and 0.8 g COD S/g COD X.

6.3.1 Effect of substrate loading on polymer accumulation by sludge N+

Different batch experiments were carried out varying acetate concentration between 300 and 2400 mg COD/L and holding C/N ratio constant. The specific substrate uptake rate observed to increase with substrate loading in the range of 0.1-0.8 g COD S/g COD X, almost linearly dependent on substrate loading. Comparison of polymer accumulated by sludge N+ during batch experiments carried out with different substrate loadings were shown in Figure 6.8. The concentration of polymer produced increased directly with substrate loading. The higher sludge PHA content (38.3%) was obtained for 0.8 g COD S/g COD X of substrate loading (Table 6.5). However the specific polymer storage rate slightly decreased (from 0.28 to 0.21 Cmmol PHA/Cmmol X.h) within the range of substrate loading used. The polymer storage yield on substrate consumed also decreased with increasing substrate loading. Obtained polymer yield for substrate loading of 0.1 g COD S/g COD X was 0.44, while that obtained for 0.8 g COD S/g COD X was 0.35.

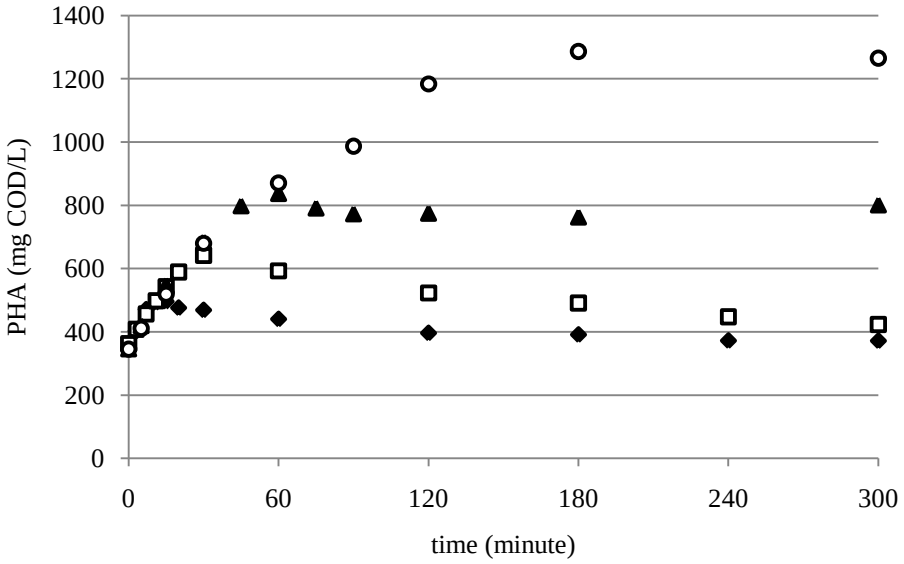


Figure 6.8: Comparison of polymer accumulation by sludge N+ during batch tests performed with different substrate loadings, ◆, 0.1; □, 0.2; ▲, 0.4; ○, 0.8 g COD S/g COD X.

Table 6.5: Effect of substrate loading on polymer accumulation by sludge N+.

S/X	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
0.1	14.8	147.0	0.79	0.28	0.44
0.2	19.1	279.3	0.92	0.24	0.42
0.4	24.9	491.0	1.16	0.23	0.36
0.8	38.3	941.2	1.32	0.21	0.35

S/X in g COD S/g COD X, PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.3.2 Effect of substrate loading on polymer accumulation by sludge N-

Comparison of polymer accumulated by sludge N- during batch experiments carried out with different substrate loadings were shown in Figure 6.9. The concentration of polymer produced increased directly with substrate loading. The lowest sludge PHA content (19.2%) was obtained for 0.1 g COD S/g COD X of substrate loading and the highest one (43.3%) was obtained for 0.8 g COD S/g COD X of substrate loading (Table 6.6). The specific substrate uptake rate also observed to increase with substrate loading. However the specific polymer storage rate slightly decreased (from 0.38 to 0.30 Cmmol PHA/Cmmol X.h) within the range of substrate loading used. The polymer storage yield on substrate consumed also decreased with increasing substrate loading within the range of substrate loading used. The highest (0.61) polymer yield was reached for 0.1 g COD S/g COD X and the lowest for 0.8 g COD S/g COD X (0.36).

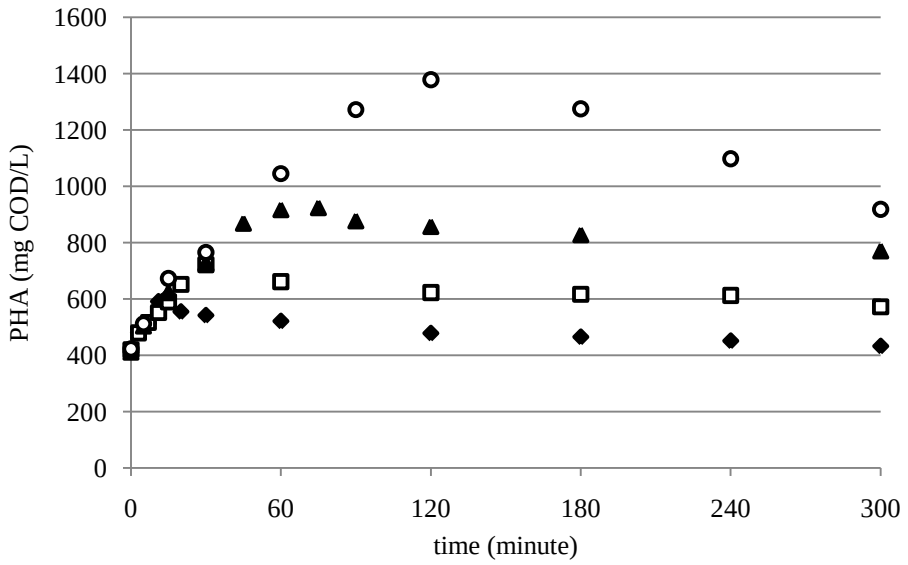


Figure 6.9: Comparison of polymer accumulation by sludge N- during batch tests performed with different substrate loadings, ◆, 0.1; □, 0.2; ▲, 0.4; ○, 0.8 g COD S/g COD X.

Table 6.6: Effect of substrate loading on polymer accumulation by sludge N-.

S/X	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
0.1	19.2	199.5	0.49	0.38	0.61
0.2	22.7	302.3	0.54	0.33	0.47
0.4	28.8	505.9	0.77	0.31	0.38
0.8	43.3	956.0	1.12	0.30	0.36

S/X in g COD S/g COD X, PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.3.3 Effect of substrate loading on polymer accumulation by sludge N_D-

Figure 6.10 shows comparison of polymer accumulated by sludge N_D- during batch experiments carried out with different substrate loadings. Increasing substrate loading caused highest amount of polymer to be accumulated by sludge N_D-. The lowest sludge PHA content (29.0%) was obtained for 0.1 g COD S/g COD X of substrate loading and the highest one (47.1%) was obtained for 0.8 g COD S/g COD X of substrate loading (Table 6.7). The specific substrate uptake rate and the specific polymer storage rate also observed to increase within the range of substrate loading used. The highest specific substrate uptake rate (0.68 Cmmol HAc/Cmmol X.h) and the highest specific polymer storage rate (0.32 Cmmol PHA/Cmmol X.h) were obtained for 0.8 g COD S/g COD X of substrate loading (Table). However polymer storage yield on substrate consumed decreased with increasing substrate loading within the range of substrate loading used. The highest (0.71) polymer yield was obtained for 0.1 g COD S/g COD X and the lowest for 0.8 g COD S/g COD X (0.30).

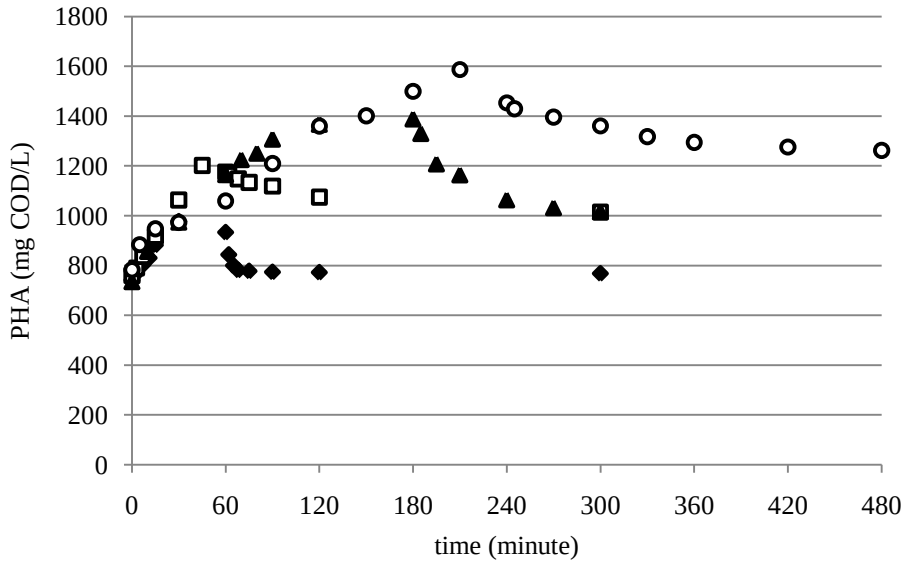


Figure 6.10: Comparison of polymer accumulation by sludge N_D- during batch tests performed with different substrate loadings, ◆, 0.1; ◻, 0.2; ▲, 0.4; ○, 0.8 g COD S/g COD X.

Table 6.7: Effect of substrate loading on polymer accumulation by sludge N_D-.

S/X	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
0.1	29.0	234.9	0.30	0.15	0.71
0.2	35.7	442.3	0.47	0.16	0.69
0.4	41.2	654.1	0.51	0.19	0.48
0.8	47.1	804.0	0.68	0.32	0.30

S/X in g COD S/g COD X, PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.3.4 Overall evaluation of batch experiments carried out with different substrate loadings

In general, the concentration of polymer produced increased directly with substrate loading for three different sludges enriched under different conditions. However sludge polymer contents obtained for sludge N_D- were higher than sludge N- and sludge N+. The lowest polymer content (14.8%) was obtained for sludge N+ and for substrate loading of 0.1 g COD S/g COD X, while the highest (47.1%) one was obtained for sludge N_D- and for substrate loading of 0.8 g COD S/g COD X. The highest PHA concentration (1586.6 mg COD/L) during experiments was also obtained for sludge N_D- and for substrate loading of 0.8 g COD S/g COD X. The range of polymer contents were in the range of values (31.9% - 48.2%) obtained by Bengtsson et al. (2008) for fermented paper mill wastewater, of those (10% - 44%) obtained by Majone et al. (2006) for a mixture of acetic, propionic and lactic acid, of those (8.7% - 32.3%) obtained by Dionisi et al. (2006) for a mixture of acetic

propionic and lactic acid, and of those (17.5% - 67.2%) obtained by Serafim et al. (2004) for acetate as sole carbon source.

The specific acetate uptake rate observed to increase directly with substrate loading for batch experiments carried out with three different sludges. However higher uptake rates were observed for sludge N+. Dionisi et al. (2005b) also reported faster substrate removal with nitrogen. The lowest acetate uptake rate (0.30 Cmmol HAc/Cmmol X.h) was obtained for batch experiment carried out with sludge N_D- and for substrate loading of 0.1 g COD S/g COD X, while the highest (1.32 Cmmol HAc/Cmmol X.h) one was obtained for sludge N+ and for substrate loading of 0.8 g COD S/g COD X. Serafim et al. (2004) also obtained increasing substrate uptake rates for higher substrate loading, when C/N ratio was kept constant like in this study. They obtained 0.83 and 0.94 Cmmol HAc/Cmmol X.h of substrate uptake rates for 0.25 and 0.35 g COD S/g COD X of substrate loading. The range of acetate uptake rates (0.30 - 1.32 Cmmol HAc/Cmmol X.h) in this study fell in the same range of values (0.50 – 0.90 Cmmol HAc/Cmmol X.h) obtained by Dionisi et al. (2001) and of those (0.45 - 1.55 Cmmol HAc/Cmmol X.h) obtained by Serafim et al. (2004).

In general, lower polymer storage rates were observed during batch tests carried out with sludge N+, where higher ammonia concentrations were supplied when compared to those carried out by sludge N-. The specific polymer storage rates observed to decrease with increasing substrate loading for batch tests performed with sludge N+ and sludge N-. However increasing substrate loadings caused an increase in the specific polymer storage rate during the batch tests carried out with sludge N_D-. Because, for every sludge, C/N ratios were kept constant and identical to those applied during SBR operations, relatively higher ammonia concentrations entered the reactors during batch tests performed with higher substrate loadings. Decrease in specific storage rate probably caused by higher ammonia concentrations rather than higher substrate loading. The same phenomenon was also observed by Serafim et al. (2004). Increasing polymer storage rate with increasing substrate loading in batch experiments carried out with sludge N_D-, where ammonia was not available during polymer accumulation, also supporting this idea. The lowest polymer storage rate (0.15 Cmmol PHA/Cmmol X.h) was obtained for batch experiment carried out with sludge N_D- and for substrate loading of 0.1 g COD S/g COD X, while the highest

(0.38 Cmmol HAc/Cmmol X.h) one was obtained for sludge N- applying same substrate loading. The range of specific polymer storage rates was in the range of values (0.20 - 0.37 Cmmol HAc/Cmmol X.h) obtained by Albuquerque et al. (2008) and was slightly lower than those (0.25 – 0.75 Cmmol HAc/Cmmol X.h) obtained by Serafim et al (2004).

Yield of polymer on substrate consumed decreased directly with substrate loading for three different sludges. During the batch experiments, duration required for substrate consumption increased with substrate loadings as expected. In feast and famine process accumulation is considered to occur when growth is limited by insufficient amount of RNA or enzymes. Probably increasing duration for substrate availability caused an increase in the amount of intracellular components (RNA and enzymes), and polymer yield decreased depending on increase in the fraction of substrate used for growth. Difference between higher (0.44) and lower (0.35) polymer yield obtained was small for batch experiments performed without nitrogen limitation. On the other hand polymer yield decreased considerably with substrate loading where nitrogen was deficient or was not exist. Both the lowest (0.30) and the highest (0.71) polymer yields were obtained during batch tests performed with sludge N_D- and for substrate loading of 0.8 and 0.1 g COD S/g COD X, respectively. The range of polymer yields were in the range of values (0.37 - 0.62 X.h) obtained by Albuquerque et al. (2008), and those (0.25 – 0.75 Cmmol HAc/Cmmol X.h) obtained by Serafim et al (2004). Serafim et al. (2006) and Dionisi et al. (2006) obtained polymer yields of 0.58 and 0.49 when acetate was used as sole carbon source, respectively. Dionisi et al. (2006) observed that polymer storage yield decreased sharply to the range of 0.05 – 0.16 with increasing organic loading rate. Serafim et al. (2004) investigated optimum operating conditions for dynamically fed aerobic systems and obtained polymer yields in a wide range (0.37 - 0.79). Similar to findings in this study, they also observed that polymer yield increased with substrate concentration if nitrogen concentration kept constant and decreased with substrate concentration if C/N ratio kept constant.

Results clearly indicate that both specific substrate uptake rate and amount of polymer accumulated by biomass increased with substrate loading for all conditions. An inverse relation was observed between polymer yield and substrate loading for all

conditions and relation between polymer storage rate and substrate loading was determined to depend strongly on nitrogen availability.

6.4 Effect of C/N Ratio on Polymer Accumulation

Because applied C/N ratio during batch experiments carried out with different substrate loadings were similar to that applied during SBR operation for every biomass, 3 different C/N ratios (100/12, 100/2, and 100/0) were used for 3 different sludge (sludge N+, sludge N-, and sludge N_D-). Accordingly it could not be understood clearly that whether polymer production obtained for biomass enriched with delayed nitrogen feeding arise from enrichment conditions or C/N ratio applied during batch experiments. To understand the impact of initial C/N ratio on polymer production of sludge N_D-, batch experiments were carried out with two different initial C/N ratios (100/2, 100/0) and changes in polymer concentrations during the tests were shown in Figure 6.11. Comparison of polymer accumulation during batch tests N_D-$_1$ and N_D-$_6$ clearly indicates that applied nitrogen regime strongly impacts on polymer accumulation during also batch experiment. Amount of polymer accumulated by biomass enriched with delayed nitrogen feeding was 234.9 mg COD/L when initial C/N ratio was 100/0. However same sludge accumulated only 70.3 mg COD/L when initial C/N ratio was 100/2. Results given Table 6.8 indicates that not only amount of polymer but also polymer yield and specific polymer storage rate were decreased about 3 times when biomass fed by a wastewater having ammonia. The specific substrate uptake rate of sludge N_D-, as expected, increased with initial ammonia concentration. Serafim et al. (2004) observed a considerable decrease in polymer yield (from 0.83 to 0.58) when they shifted C/N ratio from 100/0 to 100/2. However differences in polymer storage rate and amount of polymer obtained for two experiments in the mentioned study was smaller when compared to those obtained in this study.

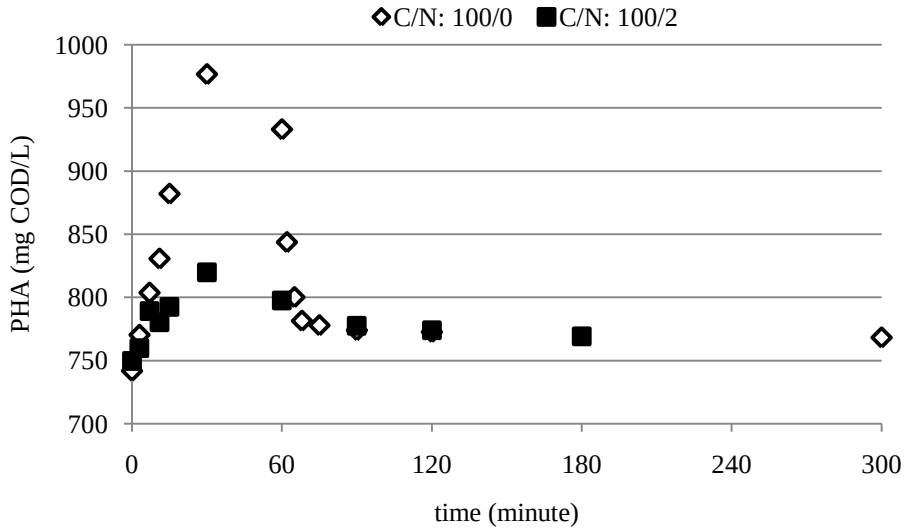


Figure 6.11: Comparison of polymer accumulation of sludge N_D- during batch tests carried out with different C/N ratios.

Table 6.8: Polymer storage performance of sludge ND- during batch tests carried out with different C/N ratios.

C/N	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
100/0	29	234.9	0.226	0.140	0.71
100/2	24.3	70.3	0.443	0.055	0.22

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

6.5 Effect of Sludge Origin on Polymer Accumulation

Another comparison was carried out between the results of batch experiments of $N\text{-}_1$ and $N_D\text{-}_6$ to understand response of different sludges to same substrate loading and C/N ratio. Both experiments were performed with substrate loading of 0.1 g COD S/g COD X and C/N ratio of 100/2. The sludge used in batch $N\text{-}_1$ enriched under dynamic conditions with nitrogen deficiency and that used in batch $N_D\text{-}_6$ enriched under dynamic conditions and not only with nitrogen deficiency but also with delayed feeding of nitrogen. Polymer accumulation during batch the tests were shown in Figure 6.12. Polymer concentration increased from 411.7 mg COD/L to 611.3 mg COD/L during the batch test performed with sludge N-, while that increased from 749.6 mg COD/L to 819.9 mg COD /L during batch test carried out with sludge N_D-. Although the polymer content of sludge N_D- was higher than that of sludge N-, this was caused by differences between initial polymer contents of the sludges. Surprisingly, as can be noticed in Table 6.9, sludge N- accumulated more polymer than sludge N_D-. Obtained specific substrate uptake rates for both sludges

were close to each other. However specific polymer storage rate and polymer yield, similar to amount of polymer accumulated, obtained for sludge N- were higher than those obtained for sludge N_D-.

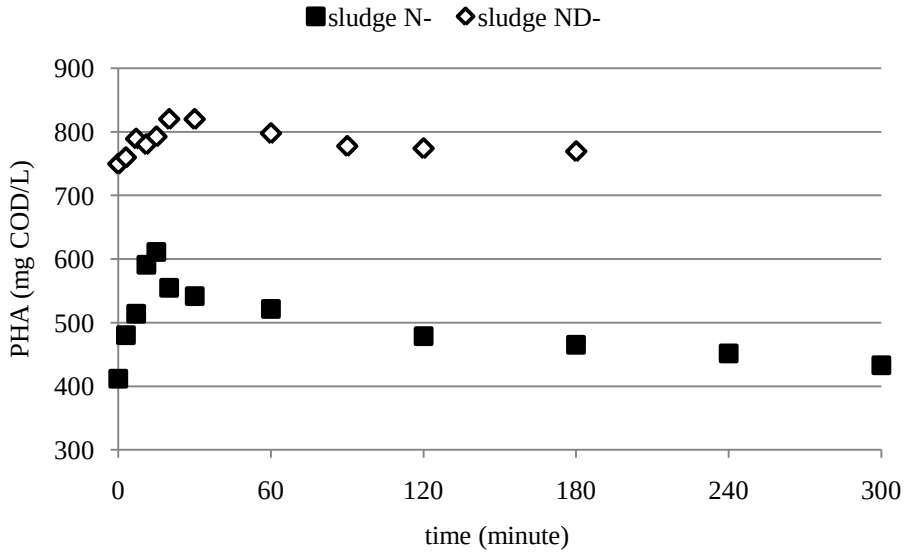


Figure 6.12: Comparison of polymer accumulation of sludges N- and N_D- during batch tests carried out with C/N ratio of 100/2.

Table 6.9: Polymer storage performance of sludge N- and ND- during batch tests carried out with C/N ratio of 100/2.

Sludge origin	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
SBR N-	19.2	199.5	0.487	0.383	0.61
SBR N_D-	24.3	70.3	0.443	0.055	0.22

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

In this study, sludge enriched with delayed nitrogen feeding generally showed better polymer accumulation properties when compared to other sludges. However, comparison of the results of batch $N\text{-}_1$ and batch $N_D\text{-}_6$ showed interestingly that neither enrichment strategy nor conditions during batch experiment enough by itself to obtain high polymer accumulation. Harmony between SBR operation and batch experiments may be considered as a third factor affecting polymer storage. Dionisi et al. (2005b) compared one batch reactor fed with an excess of ammonia with another reactor fed without a nitrogen source and observed no significant impact on the efficiency of PHA storage because biomass in the mentioned study was selected without nitrogen limitation. However Serafim et al. (2004) and Lemos et al. (2006) obtained better results for batch test carried out with lower ammonia concentrations since ammonia was only available at the beginning of the feast phase, during SBR

operation in these studies. Accordingly higher polymer accumulation, polymer yield, and polymer storage rate obtained for sludge N- than those obtained for sludge N_D- does not indicate inferiority of delayed nitrogen feeding but importance of sludge history. To proof this idea one more comparison was done between batch experiments $N+_6$, $N-_6$, and N_D-_4.

The three batch experiments were performed with substrate loading of 0.4 g COD S/g COD X and initiated with wastewater without nitrogen. Impact of relatively higher substrate loading on biomass enriched under dynamic feeding conditions without nitrogen deficiency, with nitrogen deficiency and with delayed nitrogen feeding was investigated and results were given in Table 6.10.

The lowest sludge PHA content (549.9 mg COD/L) was obtained for sludge N+ and the highest one (654.1 mg COD/L) was obtained for sludge N_D-. The highest specific substrate uptake rate, specific polymer storage rate, and polymer yield also observed for the sludge enriched under aerobic dynamic conditions with delayed nitrogen feeding and lowest ones for sludge enriched without nitrogen limitation. Results proved importance of sludge history on polymer storage as well as superiority of delayed nitrogen feeding during sludge enrichment.

Table 6.10: Polymer storage performances of sludges N+, N- and N_D- during batch tests carried out with wastewater without nitrogen.

Sludge origin	PHA content (%)	Δ PHA	$-q_S$	q_P	$Y_{P/S}$
SBR N+	27.0	549.9	0.277	0.120	0.41
SBR N-	31.3	564.0	0.339	0.160	0.43
SBR N_D-	41.2	654.1	0.506	0.193	0.48

PHA content %, and Δ PHA in mg COD/L, q_S in Cmmol HAc/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol HAc.

Effect of external factors, such as nitrogen limitation, on polymer production was shown by batch experiments previously (Serafim et al. 2004; Lemos et al. 2006). Serafim et al. (2004) obtained an extremely high polymer yield (0.83) during a batch test where no ammonia was supplied. They stated that, in the feast and famine process, growth and polymer storage occurred simultaneously, but in the absence of ammonia acetate was almost completely used in polymer production. Results given in this study clearly indicate that similarity between conditions, such as C/N ratio, applied during SBR operation and batch experiments improves polymer production. The delayed nitrogen feeding, which was proposed first time in this study, can be an

effective alternative to other enrichment processes because of its similarity to the most productive batch experiment reported.

6.6 Effect of Substrate Shift on Polymer Production

Three batch experiments were carried out by supplying an acetate propionate mixture (1/1 on COD basis) to understand effect of substrate shift on polymer accumulation by biomass enriched under different conditions. Batch experiments were performed with substrate loading of 0.1 g COD S/gCOD X and with C/N ratio similar to ones applied during enrichment period for every sludge and the results were compared with those obtained for single substrate under same conditions.

A terpolymer of HB:HV:HMV formation (Serafim et al., 2006) or a homopolymer of HB:HV formation (Serafim et al., 2006; Dionisi et al., 2004a) was reported when acetate and propionate mixture was used. Similarly, substrate accumulated mainly in the form of HB and HV in this study when a mixture of substrate was supplied. A slight increase also observed in the concentration of 3H2MV. The comparison of the results obtained from batch experiments performed with single and mixed substrate for different sludges was shown in Table 6.11. When acetate/propionate mixture was used as carbon source, obtained overall substrate uptake rates were lower. Similar to finding of Dionisi et al. (2004a) and Serafim et al. (2006) propionate uptake rates were considerably lower than acetate uptake rates for all sludges. The uptake rates obtained for sludge N_D- was lower than those obtained for sludge N+ and N-. This was probably caused by absence of ammonia rather than sludge history. During the batch experiments carried out with all sludges higher amount of polymer content, higher polymer accumulation, and higher specific productivity were obtained for acetate as sole carbon source. Serafim et al. (2006) obtained a polymer content of 32.5% with acetate as sole carbon source and biomass acclimatized to acetate. On the other hand they obtained considerably lower polymer content (15.6%) when they fed same sludge with propionate. Dionisi et al. (2004a) reported storage yield of 0.49 for acetate and 0.36 for acetate/propionate mixture. Yields of PHA on substrate consumed in this study for sludge N+ and N- were higher when acetate used as substrate. Surprisingly, yield of PHA on substrate consumed by sludge N_D- was similar to one when acetate/propionate mixture was used as carbon source. Amount of PHV accumulated by sludge N_D- was higher than that accumulated by sludge N-

and noticeably higher than that accumulated by sludge N+. Results indicate that sludge enriched under dynamic conditions with delayed nitrogen feeding capable of accumulate more PHV.

Table 6.11: Comparison of polymer storage performance obtained from batch tests carried out with single and mixed substrate for different sludges.

	Acetate			Acetate/Propionate		
Sludge	N+	N-	N_D-	N+	N-	N_D-
PHA content (%)	14.8	19.2	29.0	12.1	18.0	27.3
Δ PHA	147.0	199.5	234.9	79.5	140.5	180.7
Δ PHB				24.3	47.5	78.0
ΔPHV				58.5	126.6	132.1
$-q_S$	0.791	0.487	0.297	0.389	0.386	0.167
$-q_{HAc}$				0.293	0.204	0.127
$-q_{HPr}$				0.096	0.088	0.041
q_P	0.283	0.383	0.150	0.085	0.174	0.140
$Y_{P/S}$	0.44	0.61	0.71	0.29	0.50	0.71

PHA content %, Δ PHA, Δ PHB, and Δ PHV in mg COD/L, $-q_S$, in Cmmol S/Cmmol X.h, $-q_{HAc}$ in Cmmol HAc/Cmmol X.h and $-q_{HPr}$ in Cmmol HPr/Cmmol X.h, q_P in Cmmol PHA/Cmmol X.h, $Y_{P/S}$ in Cmmol PHA/Cmmol S.

Obtained polymer compositions in batch experiments carried out with different sludges and different carbon sources were compared in Figure 6.13. In general, higher HV, and slightly higher 3H2MV fractions were observed when acetate/propionate mixture was used as carbon source instead of acetate. Although sludge N_D- accumulated more PHV than other two sludges, HV contents of polymer obtained for sludge N_D- (16.5%) was lower than sludge N+ (25.5%) and sludge N- (26.6%), due to higher initial polymer content of sludge N_D-.

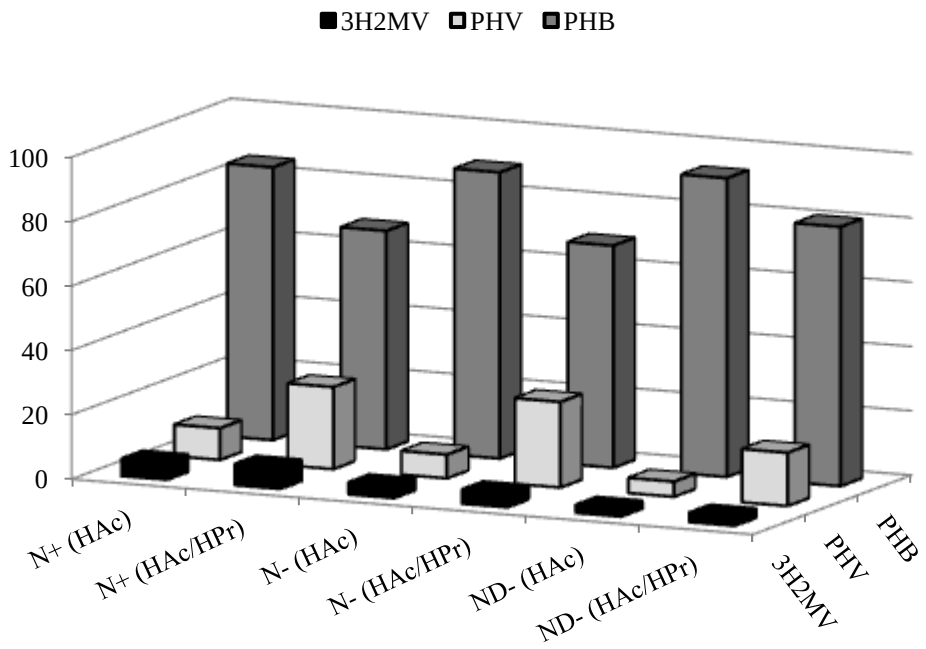


Figure 6.13: Polymer compositions obtained in batch experiments carried out with different sludges and different carbon sources.

6.7 Evaluation of Changes in Bacterial Communities

There is very limited information in the literature about microbiology of ADF systems. Dionisi et al. (2005b) presented changes in community structure by DGGE and select four predominant bands at the end of operation for sequencing. Dionisi et al. (2006) applied same procedure to investigate microbial structure however they select 14 bands for sequencing. Majone et al. (2006) monitored dynamics of bacterial speciation by means of DGGE analysis, and sequenced 3 dominant bands and found similar results with Dionisi et al. (2005b). Serafim et al. (2006) applied a different approach to investigate microbial community in ADF reactor. They applied FISH in combination with Nile Blue staining to show microbial groups able to store PHA, however the probes selected were generally the ones used for EBPR systems. Only in a recent study (Lemos et al., 2008) a detailed information obtained about bacteria selected under aerobic dynamic feeding conditions.

Although it is considered as prone to biases, DGGE protocol allows direct identification of the presence and relative abundance of different bacterial species and profiling of populations in a complex microbial community in both a qualitative and a semi-quantitative way (Muyzer et al., 1993). Traditionally, relationships

between DGGE profiles and environmental variables have been suggested by tracking the appearance and disappearance of bands and linking these to other changes occurring in the ecosystem (Riemann et al., 2000).

The main drawback of DGGE method is involvement of extraction of nucleic acids and subsequent PCR, which may both cause some biases. Although different intensities of DGGE bands are not be interpreted as quantitative measures of the abundance of each species to each other, DGGE profiles have been analyzed successfully using a variety of statistical approaches (Fromin et al., 2002). Although correlation between microbial community and operating conditions (Ma et al. 2006) or geochemistry of the environment (Fry et al., 2006; Parkers et al., 2005) was investigated before, this statistical approach have not been applied to ADF reactors to investigate correlation between bacterial diversity and PHA production. In this study, according to rarefaction analysis, 94% of the species were determined and changes in relative abundance of these species were monitored by a semi-quantitative method (DGGE) throughout operation of three SBR. Correlation between relative abundance of the species encountered and polymer accumulation (polymer yield, polymer uptake rate, and amount of polymer accumulated) were calculated. During the examining of correlation between bacterial structure and polymer accumulation, it must be keep in mind that DGGE is a semi-quantitative method and open to some biases. Because of information about community structure of biomass selected under ADF conditions is very limited in the literature, results obtained by combination of DGGE, cloning, and sequencing were also preferred t o be evaluated statistically. Confirmation of the obtained results by RNA based methods may produce more definite results.

6.7.1 Changes in bacterial community of SBR N+

Changes in bacterial communities were determined by using the Dice coefficient (unweighted data based on band presence or absence) and band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and UPGMA clustering. Calculated results showing similarities between bacterial communities of SBR N+ sampled in different days were given in Table 6.12 and Table 6.13. As can be seen easily, calculation based on densitometric curves produced lower similarities when compared to that based on unweighted data. Results indicate that changes in relative abundance of bacterial species in the

activated sludge were more significant than changes in number of species during operation. However disappearance and appearance of some bands were also observed throughout operation. Bands belonging to members of two genus in *Alphaproteobacteria* (*Caulobacter* and *Phaeospirillum*), members of *Burkholderiales* in *Betaproteobacteria*, and members of two genus in *Verrucomicrobiae* (*Rubrivivax* and *Luteolibacter*) were observed to disappear during operation of SBR N+ and they appeared again. Furthermore members of *Rubrivivax* became one of the dominant genus at the end of operating period. However bands belonging to members of *Hyphomicrobium* genus in *Alphaproteobacteria* and members of two genus, a *Flavobacterium* and a *Sphingobacterium*, in *Bacteroidetes* were disappeared permanently.

Table 6.12: Similarities between bacterial communities of SBR N+ based on band presence or absence.

Days	1	5	7	12	15	19	26	33	40	62	72	82
1	100											
5	98.0	100										
7	91.7	93.6	100									
12	88.4	91.3	93.0	100								
15	91.7	93.6	90.9	97.7	100							
19	89.4	87.0	89.4	90.5	93.0	100						
26	91.7	89.4	86.4	93.0	95.5	93.0	100					
33	87.0	84.4	81	87.8	90.5	87.8	95.2	100				
40	87.0	84.4	76.2	82.9	85.7	87.8	90.5	95.0	100			
62	93.9	91.7	84.4	90.9	93.3	90.9	97.8	93.0	93.0	100		
72	93.9	91.7	84.4	90.9	93.3	90.9	97.8	93.0	93.0	100	100	
82	93.9	91.7	84.4	90.9	93.3	90.9	97.8	93.0	93.0	100	100	100

>90
80.1-90
70.1-80
60.1-70
≤60

Table 6.13: Similarities between bacterial communities of SBR N+ based on Pearson correlation.

Days	1	5	7	12	15	19	26	33	40	62	72	82
1	100											
5	89.7	100										
7	86.7	85.8	100									
12	72.9	70.0	80.6	100								
15	62.6	68.2	71.1	81.7	100							
19	64.4	65.1	71.5	82.2	98	100						
26	57.6	58.7	63.2	77.4	89.9	91.6	100					
33	58.2	55.4	60.9	75.8	78.9	81.9	85	100				
40	56.5	60.8	59.8	67.7	78.3	77.9	75.8	80.7	100			
62	55.0	59.1	52.0	58.1	62.6	62.4	64.3	66.7	73.7	100		
72	54.4	58.3	52.4	51.5	59.8	59.6	59.5	62.1	76.0	88.1	100	
82	53.3	58.5	52.5	46.4	54.7	54.4	54.3	56.9	69.9	88.5	92.3	100

>90
80.1-90
70.1-80
60.1-70
≤60

Pearson correlation obtained noticeably lower similarities between sludge samples taken from the reactor in different days, which indicates significant changes in community structure. The lowest similarity was obtained between days 12 and 82 for SBR N+ which was 46.4%. Similarity between sludge samples taken from inoculum and from the reactor in last day was 53.3%. In general, abundances of species belonging to *Alphaproteobacteria*, *Betaproteobacteria*, and *Verrucomicrobiae* slightly increased and members belonging to *Flavobacteria*, *Sphingobacteria*, and *Planctomycetacia* decreased slightly. Among the six dominant bands appeared at the end of operation, one was belonging to *Brevundimonas* genus in *Alphaproteobacteria*, three were belonging to three different genus in *Betaproteobacteria* (*Thauera*, *Zoogloea*, and *Azoarcus*), and two were belonging to two different genus in *Verrucomicrobiae* (*Roseibacillus* and *Verrucomicrobium*). *Brevundimonas* was previously determined to have the capacity to accumulate PHA with yields ranging from 64% to 72% of the dry cell weight (Silva et al., 2007). Even though until now the ability to store PHA by the microorganisms belonging to genus *Thauera* and *Azoarcus* has not been studied, their involvement in the process cannot be excluded. Different species related to *Thauera* genus were previously found to be predominant by Dionisi et al. (2005b, 2006) and Majone et al. (2006) in dynamically fed aerobic reactors. Importance of *Azoarcus* species in PHA accumulation in ADF was also reported by Serafim et al. (2006). In a dynamically fed reactor, where acetate was used as carbon source, *Thauera* and *Azoarcus* cells observed by Lemos et al. (2008) to compose more than 60% of the biomass, of which PHA content was

previously reported as 36.9% by Serafim et al. (2004). According to van Niekerk et al. (1987) feast and famine conditions stimulated development of population mainly composed of bacteria belonging to *Zoogloea* genus. *Thauera* and *Zoogloea* were also reported as PHA accumulating organisms encountered in full scale anaerobic-aerobic wastewater treatment plants (Oshiki et al., 2008). *Zoogloea ramigera* has been studied extensively and the pathway and regulation of PHB synthesis in this bacteria described by Doi (1990). Until now, *Verrucomicrobiae* has not been associated to PHA production. It is reported as a minor group in phosphate removing activated sludge (Liu et al., 2005).

6.7.2 Relationship between polymer accumulation and bacterial structure in SBR N+

Principal Component Analysis was applied to simplify DGGE data for subsequent statistical analysis. Correlations between these PCA results and parameters characterizing both reactor performances were calculated to understand whether relationship between community structure and system performance is significant or not. High r values over 0.78 (Table 6.14) indicated that there was a strong relationship between system performance and microbial diversity in SBR N+. Subsequent correlation between relative intensity of DGGE bands representing certain species and parameters representing reactor performance such as polymer storage rate, polymer storage yield, and amount of polymer accumulated were also calculated to describe degree of relation between microbial species and polymer storage. This statistical approach had been applied before by Ma et al. (2006) to investigate correlation between dissolved oxygen concentration and microbial community in a reactor and by other researches (Fry et al., 2006; Parkers et al., 2005) to investigate relationship between microbial community and geochemistry of the environment.

Statistically significant results ($p<0.05$) were presented in Table. All phyla and classes encountered were included by statistical analysis. Because alpha and beta subclasses of proteobacteria covered nearly 70% of the microbial community, some dominant sub branches of these classes were also included by statistical calculations. Considering statistical analysis we can say that, in general, the abundance of species belonging to *Proteobacteria* and *Verrucomicrobia* phyla positively affected polymer storage performance and no correlation observed for *Planctomycetes* and

Bacteroidetes phyla. However this general picture is not enough to understand relationship between system performance and bacterial structure because different species even in the same genus were observed to correlate differently with PHA accumulation. Abundance of microorganisms belonging to *Hyphomicrobium* in *Alphaproteobacteria* was observed to correlate negatively with polymer production however that of *Caulobacteraceae* in the same class correlate positively with polymer production. Interestingly, only two of five species belonging to *Zoogloea*, which was the most abundant genus, were correlated with polymer accumulation ability of sludge N+ and none of the species belonging to *Thauera* were observed to correlate with polymer accumulation. A closest relative of the most well known PHA producer (*Alcaligenes latus*), *Azohydromonas* (clone M) in *Betaproteobacteria* correlated well with PHA accumulation. Although no correlation was observed between *Bacteroidetes* and PHA accumulation two species belonging to *Flavobacterium* and *Sphingobacterium* were observed to correlate negatively with PHA accumulation. Nearly all species belonging to *Verrucomicrobiae* correlated with PHA accumulation in SBR N+.

Table 6.14: Relationship between polymer accumulation and bacterial species in SBR N+.

	q_P	$Y_{P/S}$	Δ PHA
PCA	0.781	0.864	0.796
clone A	0.867	0.874	0.841
clone N	0.635		
Caulobacteraceae	0.841	0.807	0.771
clone X	-0.9	-0.93	-0.933
Alphaproteobacteria	0.694	0.621	
Thauera			
clone G		0.628	0.638
clone I	0.622		
Zoogloae			0.625
Rhodocycloceae	0.753	0.751	0.756
clone M	0.794	0.786	0.771
Betaproteobacteria	0.729	0.734	0.729
Planctomycetes			
clone U	-0.697	-0.767	-0.81
Flavobacterium			
clone T	-0.697	-0.767	-0.81
Sphingobacterium			
Bacteroidetes			
clone D	0.759	0.647	0.622
clone E	0.733	0.699	0.714
clone F	0.728	0.754	0.721
clone V	0.947	0.917	0.891
Verrucomicrobiae	0.829	0.775	0.742

6.7.3 Changes in bacterial community of SBR N-

Changes in bacterial communities of SBR N- determined by using the Dice coefficient and Pearson correlation coefficients were given in Table 6.15 and Table 6.16, respectively. Similar to results obtained for SBR N+, calculation based on densitometric curves produced lower similarities when compared to that based on unweighted data, which indicates that changes in number of species during operation less than changes in relative abundance of bacterial species in the activated sludge. However bands belonging to members of *Hyphomicrobium* and *Citromicrobium* genus in *Alphaproteobacteria* and two bands belonging to members of *Flavobacterium* were disappeared at the beginning of operation and did not appeared again. Interestingly *Flavobacterium* was reported to be predominant in different mixed cultures developed under feast and famine conditions (Dionisi et al., 2005b;

Majone et al., 2006). Disappearance and appearance of some bands were also observed throughout operation.

Table 6.15: Similarities between bacterial communities of SBR N- based on band presence or absence.

Days	1	5	7	12	15	19	26	33	40	62	72	82
1	100											
5	93.9	100										
7	93.9	91.3	100									
12	93.9	87	95.7	100								
15	91.7	84.4	93.3	97.8	100							
19	93.9	87.0	91.3	95.7	97.8	100						
26	87.0	88.4	88.4	88.4	85.7	83.7	100					
33	87.0	83.7	83.7	83.7	81.0	79.1	90.0	100				
40	87.0	88.4	83.7	83.7	81.0	83.7	90.0	90.0	100			
62	93.9	91.3	91.3	91.3	88.9	91.3	93.0	83.7	93.0	100		
72	91.7	88.9	93.3	93.3	90.9	88.9	95.2	85.7	90.5	97.8	100	
82	91.7	88.9	93.3	93.3	90.9	88.9	95.2	85.7	90.5	97.8	100	100

>90
80.1-90
70.1-80
60.1-70
≤60

Table 6.16: Similarities between bacterial communities of SBR N- based on Pearson correlation.

Days	1	5	7	12	15	19	26	33	40	62	72	82
1	100											
5	74.4	100										
7	72.9	84.8	100									
12	74.4	73.8	83	100								
15	71.9	66.2	74.7	91.8	100							
19	68.8	65.0	65.7	75.9	79.2	100						
26	64.4	59.3	60.5	65.0	64.1	82.7	100					
33	62.8	61.6	63.2	61.9	60	74.2	83.1	100				
40	56.1	56.7	64.6	61.3	61.5	65.8	72.9	77.6	100			
62	63.1	62.4	68.3	65.8	68.9	69.7	67.5	65.5	67.9	100		
72	62.6	67.0	72.5	69.5	70.8	70.0	68.6	63.2	67.8	89.2	100	
82	59.2	63.3	73.2	72.8	74	69.2	71.9	62.4	70.6	86.8	95.6	100

>90
80.1-90
70.1-80
60.1-70
≤60

Noticeably lower similarities calculated by Pearson correlation between sludge samples indicates significant changes in community structure during operation. Similarity between sludge samples taken from inoculum and from the reactor in last day was 59.2%.

In general, similar to SBR N+, abundances of species belonging to *Betaproteobacteria*, and *Verrucomicrobiae* slightly increased and members belonging to *Flavobacteria*, and *Planctomycetacia* decreased slightly. Different from

SBR N+, abundances of species belonging to *Alphaproteobacteria* decreased and *Sphingobacteria* increased slightly. Among the seven dominant bands appeared at the end of operation, one was belonging to *Caulobacter* genus in *Alphaproteobacteria*, which was a close relative of a PHA producer, Brevundimonas.

Four dominant bands were belonging to *Rhodocyclaceae* family in *Betaproteobacteria*, two of them to *Thauera* and other two to *Zoogloea*. Two dominant bands belonging to two different genus in *Verrucomicrobiae* determined at the end of SBR N+ operation were also dominant in SBR N-.

6.7.4 Relationship between polymer accumulation and bacterial structure in SBR N-

Correlations between PCA results and parameters characterizing reactor performances (Table 6.17) indicated that there was a strong relationship between system performance and microbial diversity in SBR N-. Subsequent correlation between relative intensity of DGGE bands representing certain species and parameters representing reactor performance shows that, in general, the abundance of species belonging to Alpha and Beta subclasses of *Proteobacteria* positively affected polymer storage performance and no correlation observed for *Planctomycetes*, *Bacteroidetes*, and *Verrucomicrobiae* phyla.

Abundance of microorganisms belonging to *Hyphomicrobium* (clone X) and *Citromicrobium* (clone B) genus in *Alphaproteobacteria* were observed to correlate negatively with polymer production however abundance of microorganisms belonging to *Azospirillum* (clone W) in the same class correlated positively with polymer production. *Azospirillum* genus has been previously reported to produce high levels of PHB under suboptimal growth conditions (Kadouri et al. 2002). Other PHB producer group in *Alphaproteobacteria*, *Caulobacteraceae*, correlated with only amount of polymer accumulated, however none of the group in this family observed to correlate with neither amount of polymer nor polymer yield and polymer storage rate. One of two species in *Thauera* genus (clone R) correlated with polymer production however no correlation was observed between this genus and polymer production.

Three of five species belonging to *Zoogloea* were correlated with polymer accumulation ability of sludge N-. *Zoogloea* genus in *Rhodocyclaceae* family and

this family itself also observed to correlate with polymer production. *Azohydromonas* (clone M) in *Betaproteobacteria* correlated well with PHA accumulation, which was a closer relative of a PHA producer, *Alcaligenes Latus*. One species belonging to *Sphingobacterium* (clone T) were observed to correlate with PHA accumulation. Interestingly, opposite to that observed for SBR N+, none of the species belonging to *Verrucomicrobiae* correlated with PHA accumulation in SBR N-.

Table 6.17: Relationship between polymer accumulation and bacterial species in SBR N-.

	q_P	$Y_{P/S}$	Δ PHA
PCA	0.883	0.848	0.857
Caulobacteraceae			0.664
clone W	0.751	0.671	0.726
clone B		-0.601	-0.637
clone X	-0.872	-0.862	-0.913
Alphaproteobacteria	0.678	0.617	0.728
clone R	0.899	0.682	0.769
Thauera			
clone G	0.775	0.645	0.718
clone I	0.613		0.667
clone L	0.702	0.723	0.686
Zoogloae	0.905	0.698	0.872
Rhodocycloceae	0.864	0.659	0.817
clone M	0.842	0.729	0.814
clone O	0.686		
Betaproteobacteria	0.89	0.684	0.808
Planctomycetes			
Flavobacterium			
clone T	0.647	0.666	0.722
Sphingobacterium			
Bacteroidetes			
Verrucomicrobiae			

6.7.5 Changes in bacterial community of SBR N_D-

Changes in bacterial communities of SBR N_D- determined by using the Dice coefficient (SD) and Pearson correlation coefficients were given in Table 6.18 and Table 6.19, respectively. Similar to results obtained for SBR N+ and SBR N-, calculation based on densitometric curves produced lower similarities when compared to that based on unweighted data, which indicates that changes in number of species during operation less than changes in relative abundance of bacterial species in the activated sludge. Interestingly, although the greatest improvement in

PHA accumulation ability of biomass occurred in sludge N_D-, changes in bacterial diversity was lesser in SBR N_D- when compared to other two reactor. Similarity based on Dice correlation between sludge samples taken from inoculum and from the reactor in last day was 93.3%, which indicates a slight difference in number of species. Bands belonging to members of *Citromicrobium* genus in *Alphaproteobacteria* and band belonging to members of *Flavobacterium* in *Bacteroidetes* were disappeared during operation and did not appeared again. Bands belonging to members of *Roseibacillus* genus in *Verrucomicrobiae*, which were not detectable in the beginning, appeared and became one of the predominant bands. Similarity based on Pearson correlation between sludge samples taken from inoculum and from the reactor in last day was 61.1%, which indicates an important difference in relative abundance of bacterial species. In general relative abundances of species belonging to *Planctomycetes*, *Flavobacteria* and *Alphaproteobacteria* decreased and those belonging to *Sphingobacteria* and *Verrucomicrobiae* increased during operation. Although relative abundance of the species belonging to *Betaproteobacteria* decreased in the beginning of operation, it increased again and reached the point that observed in inoculum sludge. Among the five dominant bands appeared at the end of operation, four were belonging to *Betaproteobacteria* (2 *Thauera*, 1 *Zoogloea*, and 1 *Azoarcus*). Other dominant band was belonging to members of *Roseibacillus* genus in *Verrucomicrobiae*.

Table 6.18: Similarities between bacterial communities of SBR N_D- based on band presence or absence.

Days	1	9	14	23	27	35	45	51	57	66	74	80
1	100											
9	97.9	100										
14	95.7	97.9	100									
23	91.7	93.9	91.7	100								
27	91.7	93.9	91.7	100	100							
35	91.7	93.9	91.7	100	100	100						
45	91.7	93.9	91.7	100	100	100	100					
51	93.9	96	93.9	98.0	98.0	98.0	98.0	100				
57	95.8	98	95.8	96.0	96.0	96.0	96.0	98.0	100			
66	93.3	91.3	93.3	89.4	89.4	89.4	89.4	91.7	93.6	100		
74	90.9	88.9	90.9	87.0	87.0	87.0	87.0	89.4	91.3	97.7	100	
80	93.3	91.3	88.9	89.4	89.4	89.4	89.4	91.7	93.6	95.5	97.7	100

>90
80.1-90
70.1-80
60.1-70
≤60

Table 6.19: Similarities between bacterial communities of SBR N_D- based on Pearson correlation.

Days	1	9	14	23	27	35	45	51	57	66	74	80
1	100											
9	92.8	100										
14	82.6	84.9	100									
23	81.6	82.1	76.3	100								
27	75.4	76.6	70.9	96.0	100							
35	67.1	66.9	63.2	87.1	90.7	100						
45	62.2	63	58.8	86.2	89.7	92.0	100					
51	71.3	70.2	67.7	82.2	85.5	83.7	89.1	100				
57	73.6	75.2	69.4	80.4	81.3	76.8	81.8	82.8	100			
66	65.4	67.7	63.9	70.4	71.1	66.7	70.4	72.6	88.5	100		
74	66.4	71.3	68.0	71.2	71.9	65.6	63.7	66.7	83.4	92.9	100	
80	61.1	65.8	62.1	65.8	66.6	60.9	61.2	65.2	79.3	86.6	89.9	100

>90
80.1-90
70.1-80
60.1-70
≤60

6.7.6 Relationship between polymer accumulation and bacterial structure in SBR N_D-

Opposite to SBR N+ and SBR N-, no correlation was observed between PCA results and parameters characterizing reactor performances for SBR N_D-(Table 6.20). Probably fluctuations in relative abundance of the species and almost continuous increase in polymer production hindered correlation. Studying microbiology of delayed nitrogen feeding in a longer period may produce better results for correlation between microbial structure and polymer accumulation. Although no correlation was observed in general, some species or subgroups correlated with polymer accumulation in SBR N_D-. Correlation between relative intensity of DGGE bands representing certain species and parameters representing reactor performance shows that, among the phyla encountered, only *Verrucomicrobiae* correlated with polymer accumulation. *Caulobacteraceae* in *Alphaproteobacteria* was the only family correlating.

Two of the species in *Zoogloea* and one species belonging to *Rubrivivax* in *Betaproteobacteria* were correlated with polymer accumulation. *Rubrivivax*, a photosynthetic bacterium, reported to use the available electron to produce hydrogen and also intracellular PHB (Li and Fang, 2008). Opposite to that observed for SBR N+, and SBR N-, *Azohydromonas* in *Betaproteobacteria* (clone M) was observed to correlate negatively with polymer accumulation. It was a closer relative of *Alcaligenes Latus*, well known PHA producer. Groethe (1999) reported that

Alcaligenes Latus growth on substrate and accumulate polymer simultaneously but in the absence of ammonia no PHA was produced. Because carbon source and ammonia was never exist together during SBR N_D- operation different from SBR N+ and SBR-, probably microorganisms incompetent to accumulate polymer in the absence of ammonia were not selected.

Table 6.20: Relationship between polymer accumulation and bacterial species in SBR N_D-.

	q_P	$Y_{P/S}$	Δ PHA
PCA			
Caulobacteraceae			0.604
clone Y	-0.637		-0.608
Alphaproteobacteria			
Thauera			
clone L	0.648	0.783	0.747
clone Z	0.702	0.794	0.765
Zoogloae			
Rhodocyclaceae			
clone M	-0.888	-0.913	-0.896
clone O	0.75	0.67	0.771
Betaproteobacteria			
Planctomycetes			
Flavobacterium			
Sphingobacterium			
Bacteroidetes			
clone D	0.919	0.869	0.895
clone E			0.636
clone P	-0.849	-0.802	-0.839
Verrucomicrobiae	0.689	0.645	0.749

6.8 Significance of Delayed Nitrogen Feeding

The greatest drawback for biodegradable biopolymers use as petrol-based polymers substitutes is the production cost. PHA production processes based on mixed microbial cultures are being investigated as a possible technology to decrease production costs, as no sterilization is required and bacteria can adapt quite well to the complex substrate present in low-cost substrates (Dias et al., 2006). Among many process, Aerobic Dynamic Feeding is the most promising one cause the highest polymer content and polymer yield were obtained for this process (Serafim et al. 2004). The authors stated that the highest polymer yield was obtained when no ammonia was supplied, which was confirmed also in this study. Furthermore results

obtained in this study also showed that sludge history has a great impact on polymer production. Similarity between C/N ratio during biomass enrichment and batch experiments improved polymer production. Accordingly a biomass enrichment strategy in which substrate and ammonia did not exist together may be a promising alternative for PHA production by mixed cultures. In this study the highest polymer yield and the highest polymer content were obtained for biomass enriched under aerobic dynamic conditions with delayed nitrogen feeding. The superiority of this process is its ability to increase accumulation by limiting growth occupying both internal, such as insufficient amount of enzymes and RNA, and external, such as lack of nutrients, factors together during biomass enrichment. Optimization of this strategy may produce higher polymer productivity and polymer content. Carbon to nitrogen ratio, organic loading rate, sludge retention time, pH, and nitrogen adding time can be some parameters to be optimized.

Feeding control based on Dissolved Oxygen is another feeding strategy for PHA production. In this control strategy, the DO is kept constant at a desired set point and carbon source is fed pulsewise. A new pulse is fed whenever a sharp increase in DO concentration is detected. This strategy was reported to extend feast phase while avoiding acetate limitation. It also allows higher organic loading without substrate inhibition. Serafim et al. (2004) obtained a PHB content of 65% using this feeding strategy. Combination of pulsewise feeding control and delayed nitrogen feeding strategy may produce better polymer production.

Many industrial wastewaters requiring nutrient addition for biological treatment, such as pulp and paper, palm oil, and wine, may be potential carbon sources for this process. Generally fermented wastewaters were used for PHA production because of their higher VFA contents. As reported before, (Albuquerque et al., 2007; Bengtsson et al., 2008) from these wastewaters, it is also possible to produce a carbon source with higher VFA content and a convenient C/N ratio (100/0.03).

Delayed nitrogen feeding strategy, which was proposed first time in this study, may be a strong alternative for other dynamic feeding strategies if it is optimized and combined with pulsewise feeding control, and can be applied for PHA production from fermented wastewaters by mixed microbial cultures.

7. CONCLUSIONS

Polyhydroxyalkanoates (PHA) are polyesters completely biodegradable, biocompatible and with wide variety of material properties for a significant number of industrial applications. PHAs are accumulated intracellularly by a wide variety of microorganisms, acting as carbon and energy sources. Because no sterilization is required and bacteria can adapt quite well to the low-cost and complex substrates, PHA production processes based on mixed microbial cultures are being investigated as a possible technology to decrease production costs. The process known as ‘‘feast and famine’’ or as ‘‘aerobic dynamic feeding (ADF)’’ could be competitive to pure culture PHA production when fully developed.

In this study three lab scale sequencing batch reactors (SBRs) were operated under ADF conditions with synthetic wastewater mainly to investigate effect of ammonia availability during a cycle on selection of a culture with a high capacity for PHA accumulation. The response of enriched biomass in three enrichment reactors under ADF conditions was much faster than that of inoculum sludge, as deducible by the higher values of polymer storage rate, yield of polymer on substrate consumed, and amount of polymer stored. Corresponding values indicated that PHA storage ability increased continuously during enrichment of biomass under ADF conditions. Substrate was accumulated mainly in the form of HB when acetate was supplied as sole carbon source. Polymer storage ability of biomass was improved more under dynamic conditions with nitrogen deficiency when compared to that without nitrogen deficiency. In this study, in order to go one step beyond from enrichment of biomass with nitrogen deficiency, a new process was proposed. In this process, during a SBR cycle, microorganisms never encounter with carbon and nitrogen together. In the mentioned process, which was called as delayed nitrogen feeding, ammonia was fed to the reactor after substrate depletion so microorganisms were selected under conditions limiting growth on substrate by using internal and external factors together.

The experimental data showed that, throughout biomass enrichment, nitrogen restraint during substrate uptake stimulated polymer accumulation. Accordingly the highest amount of polymer accumulation and yield of polymer on substrate consumed were obtained for delayed nitrogen feeding process and the lowest values were obtained for enrichment under dynamic conditions without nitrogen deficiency. However specific polymer storage rates obtained for delayed nitrogen feeding process was lower than corresponding values obtained for two other process. The highest specific polymer rate was obtained for biomass enriched under nitrogen deficient conditions.

This study also represents the changes in bacterial diversity throughout operation periods of SBRs. The bacterial community of the inoculum sludge investigated by construction of 16S rRNA clone library and subsequent sequencing analysis was consisted of a heterogeneous microbial community, rich in different bacterial species. According to rarefaction analysis, 94% of the species were determined and considering all the sequences, members of *Proteobacteria* dominated in the inoculum sludge bacterial clone library with 14.5% belonging to *α-proteobacteria*, and 53% belonging to *β-proteobacteria*. *Proteobacteria* followed by *Verrucomicrobiae* (14.5%), *Bacteroidetes* (13.3%) and *Planctomycetes* (4.8%). Among the members of *β-proteobacteria* class, *Rhodocyclaceae* (42.2%) was the most predominant family represented by clones. Other members of this class such as *Rubrivivax* (10.8%), and *Alcaligenaceae* (1.2%), belonged to *Burkholderiales* order. Among the members of *Rhodocyclaceae*, *Zoogloea* was the most predominant genus with 19.3%, and followed by *Thauera* (15.7%), and *Azoarcus* (6%). Changes in relative abundances of these species during operation of SBRs were monitored by a semi-quantitative method, denaturing-gradient gel electrophoresis (DGGE). Although changes in bacterial diversity during operation of three SBRs were different in details, species belonging to *Rhodocyclacea* family in *Betaproteobacteria* phylum and especially *Zoogloea* genus was always predominant in three reactors. It is concluded that contribution of the species belonging to phyla *Planctomycetes* and *Bacteroidetes* to PHA accumulation were paltry. Correlation between relative intensity of DGGE bands representing certain species and parameters representing reactor performance such as polymer storage rate, polymer storage yield, and amount of polymer accumulated were also calculated to describe degree of relation between microbial

species and polymer storage. Relatively lower correlations were obtained between changes in community structure and polymer accumulation for delayed nitrogen feeding process. Relative abundance of *Rhodocyclaceae* genus was observed to correlate with polymer accumulation in both SBRs operated with and without nitrogen deficiency. Species belonging to *Caulobacter* and *Azospirillum* in *Alphaproteobacteria*, which were reported as PHA producers before, correlated positively with polymer production in SBRs operated without and with nitrogen deficiency respectively. Close relative of another PHA producer, *Alcaligenes latus*, was observed to correlate with PHA production in both reactors. Statistical analysis of results obtained from DGGE indicates that changes in relative abundance of bacterial species in the activated sludge were more significant than changes in number of species during SBR operations. Results obtained in this study by combination of DGGE, cloning, and sequencing were evaluated statistically to contribute the limited information in the literature about community structure of biomass selected under ADF conditions. Confirmation of the obtained results by RNA based methods may produce more definite results.

In this study, effect of substrate loading, C/N ratio, sludge origin, and substrate shift on polymer accumulation by three different sludges were also investigated by variety of batch experiments carried out under different conditions.

In general, concentrations of polymer accumulated by three different sludges increased directly with substrate loading however the highest polymer accumulation obtained for the sludge enriched under delayed nitrogen feeding conditions. Accordingly highest sludge polymer content, 47.1% on COD basis, was obtained for biomass enriched under delayed nitrogen feeding conditions. The specific acetate uptake rate observed to increase directly with substrate loading for batch experiments carried out with three different sludges however higher uptake rates were observed for sludge enriched without nitrogen deficiency. Relation between polymer storage rate and substrate loading was determined to depend strongly on nitrogen availability during batch test. Substrate loading caused an increase in the specific polymer storage rate during the batch tests where nitrogen does not exist, however it caused a decrease in specific polymer storage rate if nitrogen available. Yield of polymer on substrate consumed decreased directly with substrate loading for three different sludges. Difference between higher and lower polymer yields obtained was small for

batch experiments performed without nitrogen limitation. On the other hand polymer yield decreased considerably with substrate loading where nitrogen was deficient or was not exist. The highest polymer yield (0.71) was obtained during batch tests performed with sludge enriched with delayed nitrogen feeding and the lowest substrate loading applied. Polymer yield increased with substrate concentration if nitrogen concentration kept constant and decreased with substrate concentration if C/N ratio kept constant.

Experimental results indicated that the sludge enriched under nitrogen deficient conditions accumulated more polymer than the one enriched under delayed nitrogen feeding conditions during batch test where applied nitrogen regime was similar to former one and vice versa. Evaluation of the results obtained from batch experiments showed that neither enrichment strategy nor conditions during batch experiment enough by itself to obtain high polymer accumulation. Harmony between conditions applied during SBR operation and batch experiments may be considered as a third factor affecting polymer storage.

Substrate was accumulated mainly in the form of HB and HV when a mixture of acetate and propionate was supplied. During the batch experiments carried out with all sludges lower amount of polymer content, lower polymer accumulation, and lower specific productivity were obtained for acetate/propionate mixture was used as carbon source instead of acetate. Yield of PHA on acetate/propionate mixture consumed by sludge enriched under delayed nitrogen feeding was similar to that on acetate as carbon source. Amount of PHV accumulated by the sludge enriched under delayed nitrogen feeding conditions was also higher than that accumulated by sludge enriched under nitrogen deficient conditions and noticeably higher than that accumulated by sludge enriched without nitrogen deficiency. Results indicated that sludge enriched under dynamic conditions with delayed nitrogen feeding capable of accumulate more PHV.

Delayed nitrogen feeding strategy was proposed first time in this study. Generally higher polymer content, higher polymer yield and higher polymer uptake rate were obtained for biomass enriched under delayed nitrogen feeding. If it is optimized and combined with pulsewise feeding control it can be a stronger alternative to industrial production of PHAs achieved by pure cultures. After a fermentation process, nutrient

deficient wastewaters can be used as carbon source in this process for PHA production.

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