ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

FACTORS AFFECTING THE SELECTION OF POLYHYDROXYALKANOATE (PHA) STORING MICROORGANISMS

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Date of submission : 8 May 2006
Date of defence examination: 16 June 2006

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JUNE 2006

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

POLİHİDROKSİALKANAT (PHA) DEPOLAYAN MİKROORGANİZMALARIN SEÇİMİNİ ETKİLEYEN FAKTÖRLER

YÜKSEK LİSANS TEZİ

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Tezin Enstitüye Verildiği Tarih : 8 Mayıs 2006
Tezin Savunulduğu Tarih : 15 Haziran 2006

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HAZİRAN 2006

ACKNOWLEDGEMENT

Firstly I would like to thank my supervisor, dear Prof. Rüya Taşlı Toraman for her great support and help during my study. She showed an incredible understanding and a real concern to me all the time.

Also I am grateful to our programme coordinator Prof. Nazik Artan for her guidance during my master studies. I would especially like to thank to Ass. Prof. Dr. Nevin Özgür Yağcı for her support, kindness and help for all my experimental and every stage of my studies.

I would like to thank to all my friends and assistant professors especially Ass. Prof. Dr. Özlem Karahan, for their help in Environmental Engineering, Biotechnology, Sedat Üründül Laboratory during all my experimental studies. Also I would like to thank to Bertan Başak for his assistance in my experiments and Ass. Prof. Dr. Gülsüm Emel Zengin for her opinions during my study.

I am very grateful to my dear friend Barış Koban for his endless support and understanding during my thesis and all my studies.

I would like to give my special thanks to my uncle Cesim Kaya and his family (Nevin, Gizem and Sıtkıcan) for their amazing support and love.

Finally I would like to give special thanks to my beloved dear mother İnci Kayaalp and my father Rafet Kayaalp for their endless love and support during my whole education and my life.

May, 2006 Oya Kayaalp

CONTENTS

NOMENCLATURE

COD	: Chemical Oxygen Demand, mg/l
EBPR	: Enhanced Biological Phosphate Removal
GAO	: Glycogen Accumulating Organisms
m	: Number of Cycles in a Day
MCFA	: Medium Chain Fatty Acids
n	: Number of Reactors
PAO	: Phosphate Accumulating Organisms
PHA	: Polyhydroxyalkanoate
PHB	: Polyhydroxybutyrate
PHV	: Polyhydroxyvalerate
PO_4-P	: Phosphate, mg/l
SBR	: Sequencing Batch Reactors
SCFA	: Short Chain Fatty Acids
SS	: Suspended Solids, mg/l
SVI	: Sludge Volume Index
T_A	: Duration of Aeration Period, T
T_{AN}	: Duration of Anaerobic Period, T
T_C	: Total Cycle Time, T
TCA	: Tricarboxylicacid
T_I	: Duration of Idle Phase, T
T_R	: Reaction Time, T
T_S	: Duration of Settling Phase, T
T_W	: Durarion of Withdrawal Phase**,** T
VFA	: Volitile Fatty Acid
V_F	: Fill Volume, l
Vo	: Initial Volume, l
V_T	: Total Volume, l
VSS	: Volatile Suspended Solids, mg/l
θ_X	: Total Sludge Age, days

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FIGURE LIST

FACTORS AFFECTING THE SELECTION OF POLYHYDROXYALKANOATE (PHA) STORING MICROORGANISMS

SUMMARY

Plastics are polymers and their usages are increasing day by day. Their degradation in the environment takes very long time so the pollution caused by this has become a bigger problem to the world. Polyhydoxyalkanoates (PHAs) are polymers which are biodegradable in the environment. Consequently it will be an important development for the environment of their usage for plastic production. There are many researches in the world about this subject. In this study PHAs polymers' production from activated sludge systems will be explained by the experiments.

PHA storing microorganism can be produced by different metabolic ways. In this study Enhanced Biological Phosphorus Removal (EBPR) mechanism was used to produce PHA storing microorganism from activated sludge systems which is capable of phosphorus removal.

From this point of view Sequencing Bath Reactor Systems (SBR) were used as the most appropriate system for EBPR mechanism. By using SBR system, reactors were operated under different operational conditions such as temperature, substrate types, the amount of phosphorus added to the systems. These parameters and their effects for PHA storage were investigated.

Systems were all operated under anaerobic/aerobic conditions and fed with synthetic wastewater consist of acetic acid, ethanol, propionic acid, glutamic acid, glucose and sodium acetate in different variations.

According to the experimental results expected PO_4-P release and uptake did not occur in the system. Some of the SETs resulted with the death of the microorganisms because of the low temperatures and overflows of the reactors. Under aerobic conditions sometimes dissolved oxygen level decreased because of the diffusers or under anaerobic conditions dissolved oxygen was measured because of the reactor structure. Under these circumstances the anaerobic/aerobic conditions were not provided and as a result PO_4-P release and uptake could not be observed in the reactors. So microorganisms were not capable of PHA production.

Also excess amount of carbon entrance to the aerobic zone was observed because organic matter was not depleted under anaerobic conditions. This condition together with low dissolved oxygen concentration resulted with the production of filamentous bacteria. So the settling properties of the system was deteriorated and caused the wash out of the sludge most of the times.

However, even in the SETs where the most of the conditions are favorable for the growth of the PAO bacteria (favorable temperature, appropriate aeration etc.), P

release in the anaerobic conditions and the following aerobic phosphorus uptake were not observed in the system indicating that the seed sludge did not contain PAOs. Further investigations with different seed sludge should be performed.

This study can be guidance for further researches to compare the results.

POLİHİDROKSİALKANAT (PHA) DEPOLAYAN MİKROORGANİZMALARIN SEÇİMİNİ ETKİLEYEN FAKTÖRLER

ÖZET

Plastikler tüm dünyada kullanımı gittikçe artan polimerlerdir. Doğada ayrışmalarının çok uzun yıllar alması sebebiyle de oluşturdukları kirlilik giderek büyüyen bir çevre sorunu haline gelmektedir. Polihidroksialkanat (PHA) olarak isimlendirilen polimerler ise doğada biyolojik olarak ayrışabilen maddelerdir. Dolayısıyla plastik olarak kullanılmak üzere üretimlerinin gerçekleştirilebilmesi çevre açısından çok önemli bir gelişme olacaktır. Halen dünyada ve literatürde bu konu ile ilgili çalışmalar yapılmaktadır. Bu çalışmada PHA adı verilen polimerlerin aktif çamur sistemlerinden elde edilebilmesi için yapılan deneyler anlatılmıştır.

PHA depolayan mikroorganizmalar çeşitli metabolik yollardan elde edilebilirler. Bu çalışmada Biyolojik Aşırı Fosfor Giderme (BAFG) mekanizmasından yararlanılarak fosfor giderimi yapan bir aktif çamurdan PHA depolayan mikoorganizma üretimi gerçekleştirilmeye çalışılmıştır.

Bu noktadan yola çıkılarak BAFG için en uygun sistemlerden biri olan Ardışık Kesikli Reaktör (AKR) Sistemi kullanılmıştır. AKR sistemi kullanılarak reaktör farklı işletme koşulları altında çalıştırılmış, sıcaklık, kullanılan organik madde çeşidi, sisteme ilave edilen fosfor miktarı… vb parametreler incelenerek bunların PHA depolanmasına olan etkileri araştırılmıştır.

Sistem anaerobik/aerobik şartlar altında işletilmiş olup organik madde kaynağı olarak değişik varyasyonlarda asetik asit, glikoz, glutamik asit, etanol, propiyonik asit ve sodyum asetat içeren sentetik atıksu ile beslenmiştir.

Elde edilen sonuçlar incelendiğinde, öncelikle fosfor salımı ve giderimi yapması beklenen sistemin bu koşulu gerçekleştiremediği gözlenmiştir. Bazı SET'lerde, laboratuar koşullarında ani sıcaklık değişimleri ve taşmalar sistemdeki mikroorganizmaların ölümü ile sonuçlanmıştır. Aerobik fazda çalışması gereken sistemde difüzörler sebebiyle zaman zaman oksijende azalma görülmüş veya anaerobik fazda reaktör yapısı sebebiyle çözünmüş oksijen tesbit edilmiştir. Bu koşullar istenilen anaerobik/aerobik şartları tam olarak sağlayamadığından fosfor salımı ve giderimi gerçekleştirilememiş ve PHA depolayan mikroorganizma üretimi sağlanmamıştır.

Ayrıca organik maddenin anearobik fazda tüketiminin olmaması sebebiyle aerobik faza organik madde kaçışı gerçekleşmiştir. Aerobik bölgeye karbon kaynağı geçişi, düşük oksijen konsantrasyonu gibi filament bakterilerin üremesini sağlayan koşulların varlığı ile sistemde dominant hale gelmeleri söz konusu olmuştur. Bu nedenle sistemin çökelme özellikleri bozularak çoğu kez tüm çamurun yıkanarak sistemden kaçması ile sonuçlanmıştır.

Bununla birlikte, PAO bakterilerinin büyümesi için uygun şartlarda (uygun sıcaklık ve havalandırma gibi.) çalıştırılan Set'lerde bile anaerobik şartlarda fosfor salımı ve aerobik şartlarda fosfor alımının olmaması aşı çamurunda fosfor gideren bakteri bulunmadığını düşündürmüştür. Bu yönde farklı aşı çamurları ile değişik çalışmaların yapılması gerekmektedir

Bu çalışma, daha sonra yapılabilecek denemelerde sonuçların değerlendirilebilirliği açısından ileriye yönelik bir kaynak teşkil etmektedir

1. INTRODUCTION

Synthetic polymers consisting petroleum origin have been used for years all over the world. This usage causes very important problems such as pollution. In order to reduce these polymers scientists are trying to develop new technologies in recent years. Biodegradable polymers are one of the most important materials that could be a solution to this problem. So there has been a significant increase of the interest of the biodegradable polymers recently.

Plastics as synthetic polymers are utilized in almost every manufacturing industry in the world. Most of today's plastics and synthetic polymers are produced from petrochemicals. 99.5% of current plastics are made from fossil fuel, which are non-biodegradable. About 34 million ton of plastics are produced by the plastic industry a year. Improperly disposed plastic materials are a significant source of environmental pollution. Plastics are persistent in the environment since they are resistant to microbial degradation. On the other hand, biodegradable plastics are eco materials which are degraded by microorganisms in the environment. Nowadays, the development of biodegradable plastics is also attracted interests of researchers in terms of their renewability, sustainability, economic continuity and product performance. And also there is an increasing environmental concern in the present society, because of biodegradability and recyclability of biodegradable plastics.

Microbial polyester has been examined in both biological and polymer sciences since 1925. That year Lemogine isolated poly (3-hydroxybutyrate) from *Bacillus megaterium.* It was explored that a wide variety of prokaryotic organisms accumulate poly (3-hydroxybutyrate) as a storage material. Between the years 1960 to 1970 the role and the metabolism of this polymer was investigated and in the year 1973 it was discovered by E.A.Dawes and P.J.Senior (1973). A second important

discovery instead of poly (3-hydroxybutyrate), poly (3-hydroxyalkanoate) was found in 1974. The most important developments about poly (3-hydroxybutyrate) and poly (3-hydroxyalkanoate) was controlled fermentation for the production of microbial co-polyesters with a wide range of compositions. The poly (3-hydroxyalkanoate) family is important because they are thermoplastics which have biodegradable and biocompatible properties. They are produced by different kinds of carbon substrates with the help of microorganisms. PHAs are found in cells as granules like glycogen and polyphosphate.

In the recent years industrial interest has been started to these microbial polyesters as large scale biotechnological products. The biosynthesis, structure and properties of poly 3-hydroxyalkanoates (PHAs) have been investigated in both academic and industrial research centers. The researches and developments are mostly done in biological and polymer sciences.

PHA is one of the biodegradable plastics produced mainly by bacteria. PHAs are bacterial reserve materials which are accumulated when a carbon source is available in abundance, while growth is limited by the lack of an essential nutrient. PHAs offer a better alternative to these petroleum based polymers because PHAs are synthesized from renewable resources (mainly agriculture and industrial wastes). In the last three decades, PHA has attracted industrial interest.

PHAs are an intracellular storage material synthesized by a variety of bacteria which have some properties similar to synthetic plastics. Plastics made of PHAa are completely biodegradable (Page, 1995). Because of their similar material properties to conventional plastics and complete biodegradability under natural environment upon disposal PHAs are supposed to be substitutes for non-degradable petrochemical derived plastics, (Lee and Choi, 1998).

But despite all these advantages of PHAs, the production of biodegradable plastics on a large scale is limited. Because the substrate is expensive and polymer production is low .Also the cost of maintaining a pure culture is a problem. According to Yamane (1993), higher production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional petroleum based plastics in the commercial marketplace. For economical PHA

production a mixed culture that can store high PHA content with an inexpensive substrate should be used.

According to Satoh, *et al*. (1998) the basic feasibility by showing that conditions could be used to increase the percent PHA in activated sludge by using sodium acetate as the main organic substrate.

PHA accumulating microorganisms can be produced in activated sludge systems by using biological nutrient removal mechanism. This has the following advantages compared with that using pure culture: first, this system leads to production of valuable materials (PHA) from wastes such as sewage, wastewater and activated sludge, and second, cost of PHA production could be lowered because the production system is simpler and the raw materials are cheaper.

For this reason, there are some researches to obtain PHAs by using the biomass from the wastewaters. If that can be proven, wastewater can be suitable raw material for biodegradable plastics. Also it can be an economical solution to waste management and wastewater treatment fields. But the knowledge of PHA production by using activated sludge bacterial cultures is not well known.

2. LITERATURE REVIEW

2.1 Storage Polymers in Microorganisms

PHA Polymers are produced by different kinds of microorganisms which can store carbon and energy under limited conditions such as glycogen and polyphosphate. There are some essential elements for growth. They are oxygen, nitrogen, phosphorus, sulfur and carbon. When these elements are limited in the cell, PHA can be stored. Phosphorus storage occurs in the form of Poly-P and carbon and energy storage occurs in the form of PHA polymers and glycogen. These products are stored as granules. They exist in low solubility in water so they don't effect the osmotic balance in the cell (Lee and Choi, 1999).

There are four different types of storage polymers in microorganisms which are carbohydrates, polyphosphate, nitrogen reserves and lipids (PHAs).

2.1.1 Carbohydrates

In carbohydrate reserves there are glycogen and glycogen-like materials. These materials can be accumulated by a wide variety of microorganisms.

Glycogen is an intracellular carbohydrate present in cytosol in form of granules. It is a readily mobilized form of glucose. It serves as a carbon, energy and reducing power source for biological phosphate removing process (Yağcı, 2004).

In 1973 Dawes and Senior and in 1984 Preiss did some researches about bacterial species capable of accumulating glycogen and glycogen-like reserves. Their results are summarized in Table 2.1 (Punrattanasin, 2001).

Table 2.1 Microorganisms Capable of Glycogen and Glycogen-Like Reserve Accumulations, (Punrattanasin, 2001).

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

2.1.1.1 Factors affecting carbohydrate accumulation

According to Dawes (1985) glycogen and glycogen-like materials are accumulated in cells when nitrogen is limited when there is an excess of carbon available. In 1984 Preiss discovered that glycogen was accumulated not only when nitrogen was limited also it can be accumulated under sulfur limiting-or phosphorus limiting conditions or when pH for growth is unfavorable. But nitrogen limiting condition was reported to be the most stimulating condition for glycogen accumulation in many organisms.

2.1.1.2 Carbohydrate reserves and glycogen

According to Dawes and Senior (1973) and Preiss (1984), organisms capable of carbohydrate reserves accumulation tend to prolong their viability during starvation periods by utilizing the storage reserve as sources of carbon and energy. Like PHA and poly-P, glycogen is an essential part of the metabolism of PAOs in BNR systems (Satoh *et. al*, 1992).

The most resent biochemical model for BNR systems is Mino's Model. According to Mino (1987), reducing equivalents (NADH) produced during the degradation of glycogen are used for PHA biosynthesis during the anaerobic period.

Hood and Randall found out that the replenishment of glycogen occurs during the aerobic period as PHAs provide a carbon skeleton for its synthesis. In 1994 Smolders *et al.* discovered that energy obtained during glycogen degradation is also used for the uptake of organic substrate during the anaerobic period resulting in a decrease in the required energy for substrate uptake by the hydrolysis of poly-P.

In both models Smolders and Mino, the reducing power for PHA formation is only generated by the breakdown of glycogen through glycolysis. In 1997 Liu *et al.*reported that PAOs can store energy in the form of polyphosphate and that glycogen storage is of minor importance for EBPR. In the aerobic phase, anaerobically consumed glycogen is replenished while PHA is consumed. In 1994 Smolders developed a structured metabolic model of the aerobic phase including glycogen synthesis. In this model glycogen is produced from oxaloacetate in glyconeogenesis where oxaloacetate is produced from PHA through the glyoxylate cycle.

2.1.2 Poly–Phosphates

Polyphosphates are linear polymers formed by the linkage of orthophosphates by energy-rich phosphoanhydride bonds (Kulaev and Vagabov, 1983). According to Dawes (1985), the poly-P bacteria have a chain length that varies from 2 units (pyrophosphate) to 10 000 units. Polyphosphates serve as an energy source for organisms also they provide a source of phosphorus for some important metabolic processes, i.e., nucleic acid and phospholipid synthesis (Dawes, 1985). But the role of poly-P differs according to the type of the microorganism. For example *Hydrogemonas* and *Enterobacter aerogenes* do not appear to use poly-P as the energy source when *Rhodospirillium rubrum*, *Entamoeba hystolytica*, *Propionibacteruim shermanii*, *Acetobacter xylinum*, and *Bacteriodes symbiosus* appear to possess enzymes for energy production using poly-P. According to Kulaev and Vagabov (1983) the extensive study of poly-P metabolism have been performed

only in mycobacterium, corynebacteria, propionic-acid bacteria, streptomycetes, *Aerobacter aerogenes*, and *E. coli*.

Kulaev and Vagabov (1983) reported some enzymes involved in biosynthesis and degradation of poly-P. These enzymes are summarized below.

1. Polyphosphate: ADP phosphotransferase or polyphosphate kinase

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

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

2. Polyphosphate: adenosine monophosphate phosphotransferase

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

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

3. Polyphosphate (metaphosphate)-dependent NAD_+ kinase

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

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

4. Polyphosphate: D-glucose 6-phosphate phosphotransferase

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

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

5. 1,3-diphosphoglycerate: polyphosphate phosphotransferase

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

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

6. Polyphosphate polyphosphohydrolases or polyphosphate depolymerase

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

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

7. Polyphosphate phosphohydrolases or polyphosphatases

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

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

For BNR systems, poly-P plays an essential part for the metabolism of PAOs. By the degredation of poly-P ATP is produced. That ATP is used for the uptake and storage of organic substrates under anaerobic conditions. As a result of poly-P degradation, orthophosphate is released into the medium as observed during the typical anaerobic P release in BNR systems.

The ability of the PAOs to utilize poly-P during anaerobic period gives them competitive advantage over other bacteria in activated sludge systems. Van Groenestijn *et al*. (1987) studied the production of ATP from poly-P in *Acinetobacter*

strain 210A. As a result of their study they reported that ATP produced by this organism is due to a combined reaction of polyphosphate: AMP phosphotransferase and adenylate kinase. The activities of the enzymes polyphosphate glucokinase and polyphosphate dependent NAD kinase were not detected in this organism.

Mino *et al.* (1985a, 1985b) studied the fraction of intracellular polyphosphates from BPR sludge using a modification of the Schmidt-Thannhauser-Schneider (STS) method. According to this method high and low molecular weight polyphosphates can be separately determined. Low molecular weight poly-P was degraded during the anaerobic period, while it was synthesized during the aerobic period. It was suggested that low molecular weight poly-P was used as an energy source during the anaerobic period when high molecular weight poly-P serves as a source of phosphorus for cell growth. They also suggested that high molecular weight poly-P can provide phosphorus for other reactions. For example the formation of low molecular weight poly-P during aerobic periods.

2.1.3 Nitrogen reserves

Cyanophycin and phycocyanin are two types of nitrogen reserves reported in cyanobacteria and they are utilized under nitrogen limiting conditions. Generally, microorganisms accumulate insignificant amount of nitrogen reserves.

2.1.4 Lipids

Microorganism can produce one or more intracellular compounds when they have enough sources of carbon and energy. Some yeasts and fungi accumulate large amounts of oil or lipid while bacteria accumulate polyhydroxyalkanoates (PHAs). Some microorganisms excrete large amounts of polysaccharides into their growth medium and accumulate them as intracellular reserve compounds. PHAs are produced by different kinds of carbon substrates with the help of microorganisms while growth is limited by the lack of an essential nutrient

PHAs that are accumulated by the bacteria as storage products will be explained and discussed in detail in the following section.

2.2 Polyhydroxyalkanoates (PHAs)

PHAs are intracellular carbon and energy reserve compounds produced by many bacteria. In this section the production, the history, the metabolism, the pathways, the structure, the factors affecting the storage, the properties of PHAs and the usage of PHAs as biopolymers and their applications will be examined.

2.2.1 The History of PHAs

The microbial PHA family of polyesters is thermoplastics which have biodegredable and biocampatible properties. This property makes them very important nowadays because of the plastics which are highly pollutant for the environment. Scientists from different research centers such as academic and industrial are developing new technologies for producing PHAs. Although this seems to be a new technology; as biodegredable materials, microbial polyesters has been examined in both biological and polymer sciences since 1925.

First in 1925 Lemogine explored that a wide variety of prokaryotic organisms accumulate poly (3hydroxybutyrate) as a storage material. He was a microbiologist working at the Pasteur Instıtute in Paris. He isolated the polymer from *Bacillus megaterium* by chloroform extraction and discovered that it was a polyester of 3-hydroxybutyric acid, P(3HB). He also discovered that unlike other biological polymers such as proteins and polysaccharides, P(3HB) is a thermoplastic which has a melting point of 180 ºC.

Between the years 1960 to 1970 the role and the metabolism of this polymer was investigated and in the year 1973 it was discovered by E.A.Dawes and P.J.Senior (1973).

A second important discovery instead of poly (3-hydroxybutyrate), poly (3-hydroxyalkanoate) was found in 1974 by Wallen and Rohwedder. They isolated PHAs by chloroform extraction of activated sludges. They also discovered that the polymer had four different monometric units: 3-hydroxybutyrate, 3-hydroxyvalerate (as the main component), 3-hydoxycaproate and 3-hydroxyheptanoate.

The most important developments about poly (3-hydroxybutyrate) and poly (3-hydroxyalkanoate) was controlled fermentation for the production of microbial copolyesters with a wide range of compositions.

In 1981 a new controlled fermentation process was developed by Imperial Chemical Industries (Holmes *et al.* ,1981).In this process P(3HA) could be produced by feeding bacterial monocultures made of carbon sources.From propionic acid and glucose a copolymer of 3-hydroxybutyrate, 3-hydroxyvalerate P(3HB-co3HV) was produced by *A.eutrophus* . It was a commercially produced product under the trade name of Biopol,(Holmes,1985).

In 1988 it was discoverd that a new copolymer of 3- and 4- hydroxybutyrate P(3HB-4HB) could be produced by *A.eutrophus* from 4-hydroxybutyric acid (Doi and Kunioka,1988).

In 1995 Page showed that plastics made of PHAs are biodegredable in both aerobic and anaerobic environments.

PHA can be accumulated up to 80% dry weight by a bacterial strain called *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) ,(Lee, 1996).By using this *Ralstonia eutropha,* Imperial Chemical Industries (ICI) , Zeneca Bio Products from Billingham, England is producing biodegredable plastic which they sell them under the name of "Biopol" since 1996. It will be rewieved on the next sections.

In 1996 Lee found out that PHAs have 80 different forms in bacterias. But they are mostly formed by poly-beta-hydroxybutyric acid (PHB) and poly-betahydroxyvaleric acid (PHV).

There are some rewievs about PHAs, the factors which effect their production and composition ,also applications of biodegredable plastics produced from PHAs by Lafferty *et al*. (1988), Anderson and Dawes (1990), Doi (1990), Sasikala and Ramana (1996), Lee (1996), Steinbuchel (1996) and Braunegg *et al*. (1998).

Since the importance of the biodegredable polymers have been discovered, PHAs had become a valuable material for both the industries and academic studies.

2.2.2 Biodegredable plastics

The importance of the biodegredable plastics were explained in introduction part in Chapter 1. Bioplastics are special types of biomaterials. They are polyesters, produced by a range of microorganisms, cultured under different nutrient and environmental conditions (Madison and Huisman, 1999).

The number and size of the granules, the monomer composition, macromolecular structure and physico-chemical properties vary, depending on the producer organism (Anderson and Dawes, 1990). They can be observed intracellular as light-refracting granules or as electron lucent bodies that, in overproducing mutants, cause a striking alteration of the bacterial shape.

This can be seen in Figure 2.1 (Jose M Luengo, Belein Garcia, Angel Sandoval, German Naharro and Elias R Olivera, 2003).

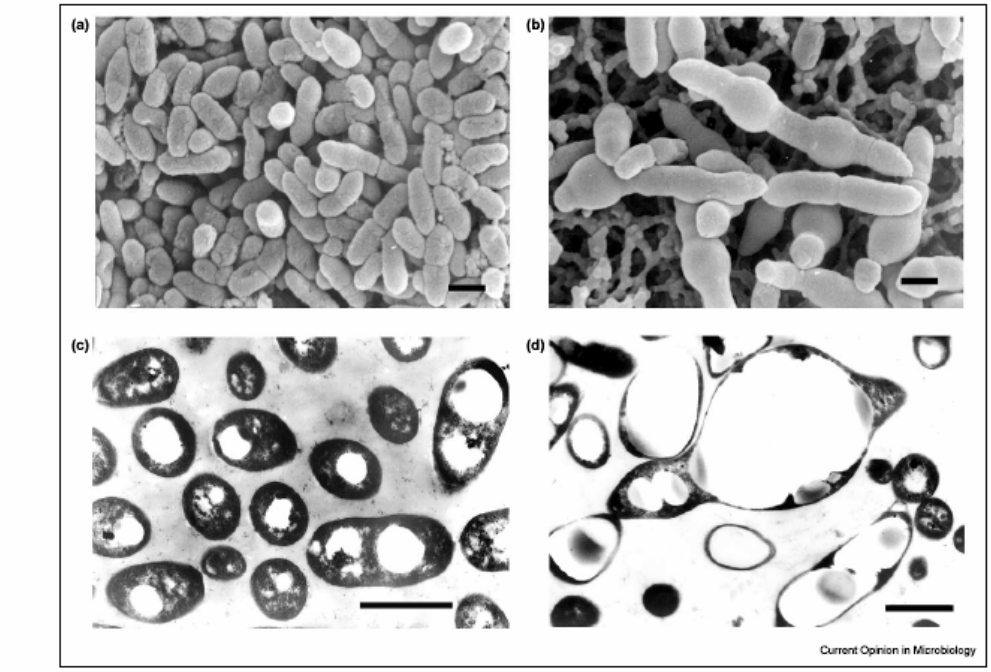


Figure 2.1 Bioplastics Scanning (a,b) and transmission (c,d) electron microphotographs of P. putida U (a,c) and its DfadBA β-oxidation mutant (b,d) cultured in a chemically defined solid medium containing 7-phenylheptanoic acid (5 mM) as a source of aromatic PHAs and 4-hydroxyphenylacetic acid (5 mM) as an energy source. Bar ¼ 1 mm.

2.2.3 Applications of bioplastics from PHAs

PHAs can be used in different application areas as bioplastics (Holmes, 1985). According to Lafferty *et al.* (1988), the possible applications of bacterial PHA depends on their properties such as biological degradability, thermoplastics characteristics and depolymerization of PHB to monomeric D(-)-3-hydroxybutyric acid.

The main application areas of bacterial PHAs have focused on 3 areas: medical and pharmaceutical, agricultural and commodity packaging (Holmes, 1985; Huang *et. al*, 1990; Lafferty *et al*., 1988; Lee, 1996). Laferrty *et al*. (1988) told that the most advanced development of bacterial PHAs is in the medical field, especially pharmaceutical applications.

1) Medical and pharmaceutical applications

D(-)-3-hydroxybutyric acid is the degradation product of P(3HB). It is a common intermediate metabolic compound in all higher organisms (Lafferty *et al*., 1988 and Lee, 1996). It is biocompatible to animal tissues and P(3HB) can be implanted in animal tissues without any toxic. Some possible applications of bacterial PHAs in the medical and pharmaceutical applications include: biodegradable carrier for long term dosage of drugs inside the body, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, and stimulation of bone growth and healing by piezoelectric properties.

Using biodegradable plastics during implantation have a serious advantage which is the biodegredation of the material. For example the need for surgical removal is not necessary.

2) Agricultural applications

PHAs are biodegraded in soil. Therefore, the use of PHAs in agriculture is very promising. They can be used as biodegradable carrier for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation. At the end of the harvesting season there is no need to remove the biodegradable items.

3) Biodegradable commodity packaging

According to Lafferty *et al*. (1988), PHB homopolymer and PHB-PHV copolymer have some properties, i.e., tensile strength and flexibility, similar to polyethylene and polystyrene. Holmes *et al*. (1981) reported that PHAs can be used in extrusion and moulding processes and blended with synthetic polymer, such as chlorinated

polyethylene, to make heteropolymers. Also the property of some conventional polymers can be improved by making some additions to PHAs.For example addition of a small amount of PHA reduces the melt viscosity of acrylonitrile.

In 1994 Tsuchikura told that "BIOPOL" with high PHV content is more suitable for extrusion blow moulding and extrusion processes such as films, sheets, and fibers, while "BIOPOL" with low PHV content is more suitable for general injection moulding processes.

According to Lafferty *et al*. (1988), one particular property of PHB films that make it possible to be used for food packaging is the relatively low oxygen diffusivity.

The Economics of PHAs

There are three main problems in PHA production. These are: the production of biodegradable plastics on a large scale is limited, the substrate is expensive and polymer production is low and the cost of maintaining a pure, culture is difficult.

According to Yamane (1993), higher production costs, especially raw material costs, make it difficult for PHA biodegradable plastics to compete with conventional petroleum based plastics in the commercial marketplace

There are three factors responsible for the high costs: Carbon substrate; expensive carbon sources like sugars,(mixed cultures and raw substrates could be a solution), extraction step of PHAs and organisms: pure cultures and genetically modified microorganisms with the additional step of sterilization. For economical PHA production a mixed culture that can store high PHA content with an inexpensive substrate should be used.

There are some ways for the usage of inexpensive carbon sources for PHA production. Low prised sources could be used such as glucose and sucrose. Also plant oils or fatty acids can be used for carbon sources. Agricultural or food industrial waste materials can be used again as an inexpensive carbon source for PHA producing bacteria. Another different source could be carbon dioxide which is in the atmosphere (Tsuge, 2002).

To obtain PHAs by using the biomass from the wastewaters could also be an economical solution to waste management and wastewater treatment fields. But the

knowledge of PHA production by using activated sludge bacterial cultures are not well known. In Figure 2.2 a schematic of activated sludge process is shown.

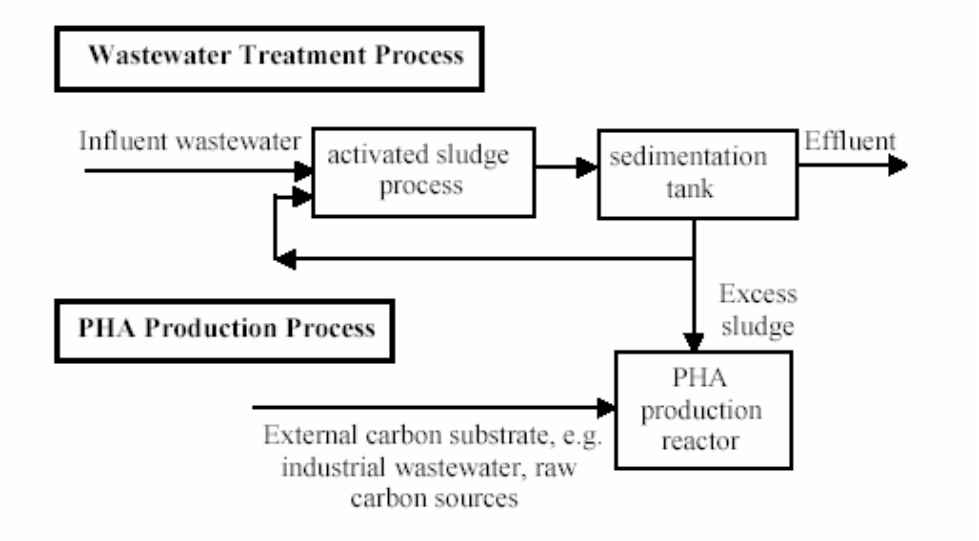


Figure 2.2 PHA Production System by Using Activated Sludge Treating Wastewater

2.2.4 The Microbial structure of PHAs

Poly (3-hydroxyalkanoates) are the first examples of microbial copolyesters of 3-hydroxyalkanoic acids. As it is mentioned previously Wallen and Rohwedder isolated Poly (3-hydroxyalkanoates) by chloroform extraction of activated sludge (1974). In the activated sludge the copolyester P(3HA) was 1.3 % of its dry weight. Wallen and Rohwedder discovered that the polymer had four different monometric units: 3-hydroxybutyrate, 3-hydroxyvalerate (as the main component), 3-hydoxycaproate and 3-hydroxyheptanoate. The general structures of PHAs are shown in Figure 2.3 and four different monometric units are shown in Figure 2.4 below.

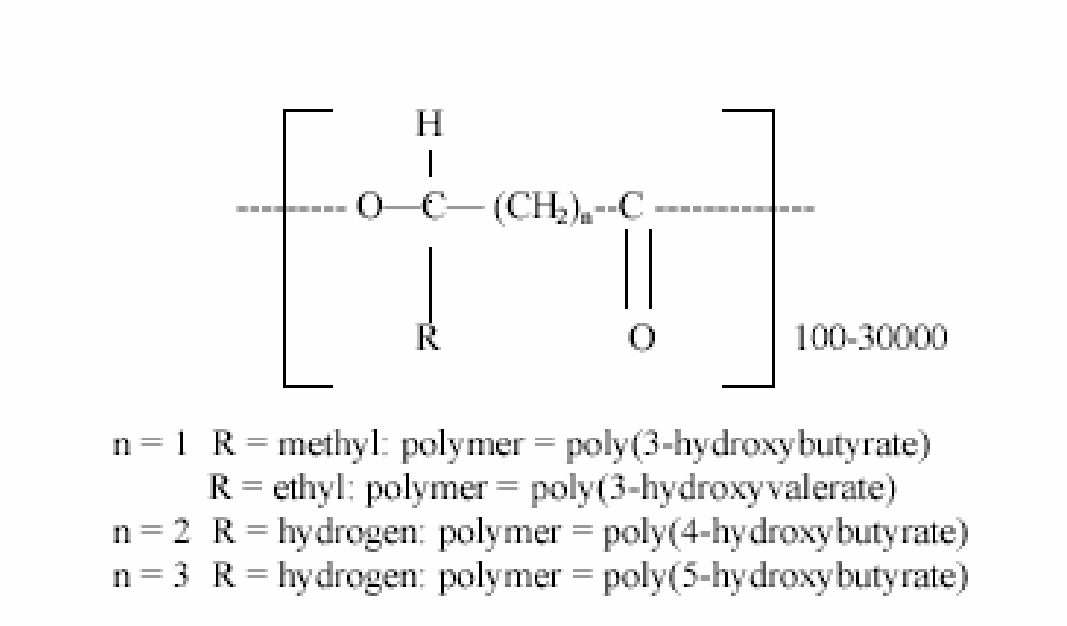


Figure 2.3 The General Structure of PHAs

In 1981 a new controlled fermentation process was developed by Imperial Chemical Industries (Holmes *et al.*, 1981).In this process P(3HA) could be produced by feeding bacterial monocultures made of carbon sources. From propionic acid and glucose a copolymer of 3-hydroxybutyrate, 3-hydroxyvalerate P(3HB-co3HV) was produced by *A.eutrophus* . It was a commercially produced product under the trade name of Biopol, (Holmes, 1985).

In 1988 it was discovered that a new copolymer of 3- and 4- hydroxybutyrate P(3HB-4HB) could be produced by *A.eutrophus* from 4-hydroxybutyric acid (Doi and Kunioka,1988).

By the time that PHBs were discovered by Lemoigne in 1925, many scientists such as microbiologists and biochemists showed an interest about PHB's metabolism. But the general knowledge was not known until 1973. Dawes and Senior summarized a rewiev about P (3HB).

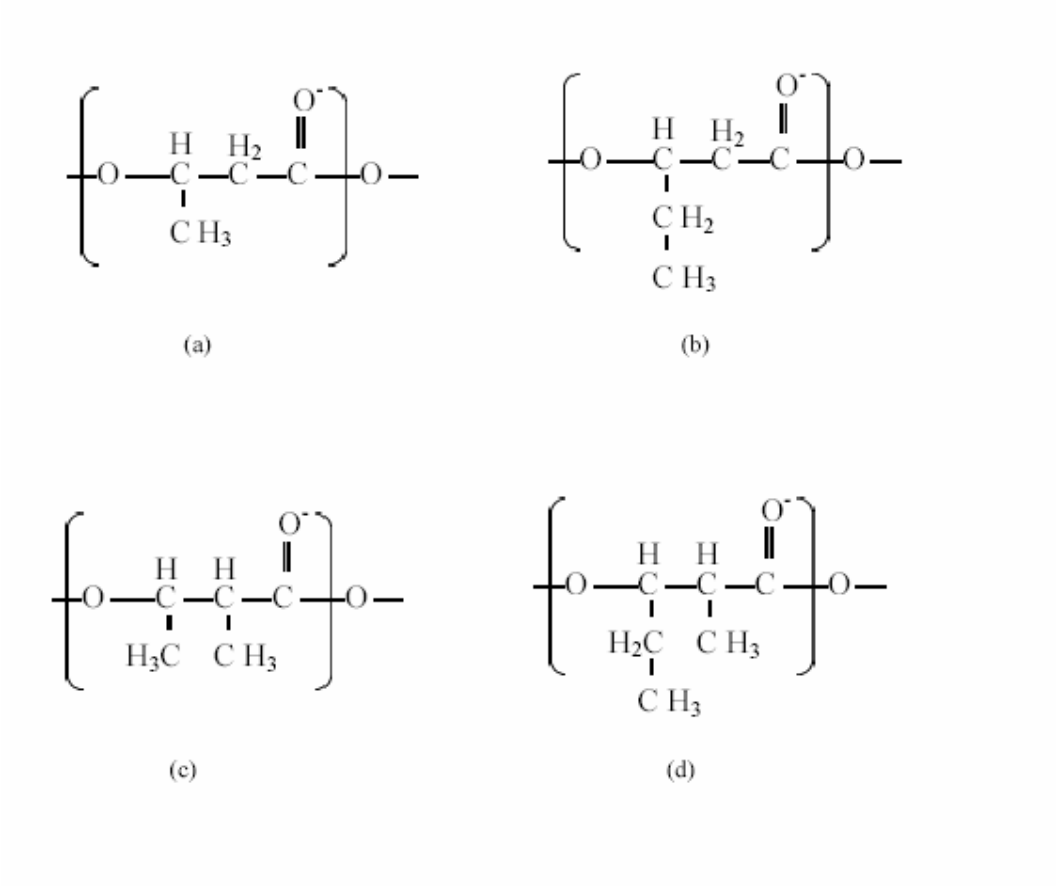


Figure 2.4 Molecular formula of PHA units: (a) hydroxybutyrate; (b) hydroxyvalerate; (c) hydroxymethylbutyrate; (d) hydroxymethylvalerate (Lee and Choi, 1999).

Many different kinds of prokaryotic microorganisms such as bacteria accumulate P (3HB) 30-80 % of their cellular dry weight if growth is limited by the deplatition of a nutrient such as nitrogen, phoshorus, oxygen, sulfur or magnesium. P (3HB) is found in granules in the cytoplasmic fluid of the cell. Their diameters are 0.3 – 1.0 µm. According to Byron having a diameter range of 0.2 – 0.5 µm were observed in *Alkaligenes eutrophus* (Byrom, 1994). The molecular weights of the polymers are in range at 2×10^5 to 3×10^6 daltons. Their molecular weight depends on the type of the microorganism and growth conditions (Byrom, 1994).

The structure of Copolymer 3HB-3HV is given in Figure 2.5 and the Structure of Copolymer 3HB-4HB is given in Figure 2.6.

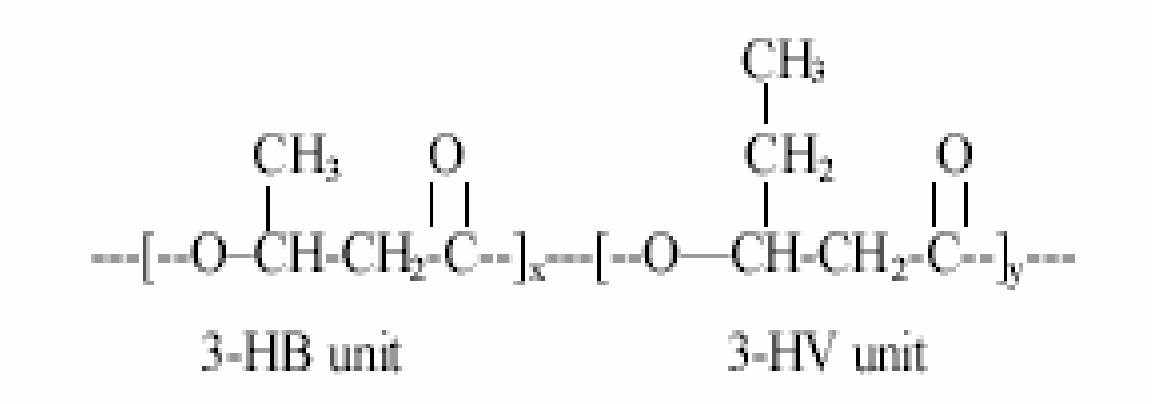


Figure 2.5.The structure of Copolymer 3HB-3HV

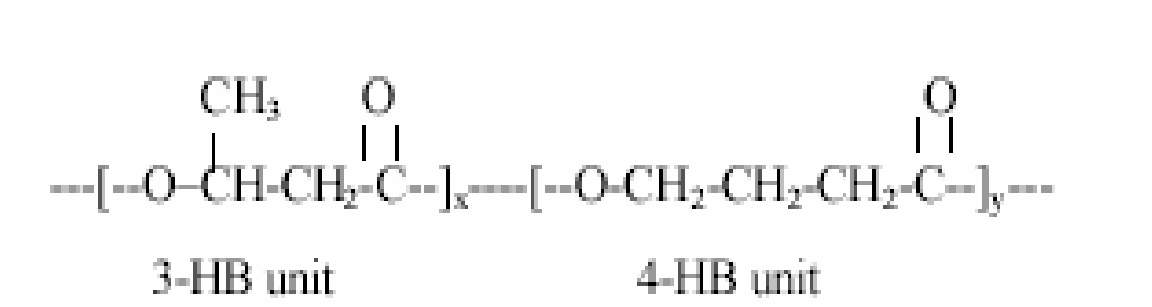


Figure 2.6 The Structure of Copolymer 3HB-4HB

2.2.4.1 The Pathways of PHAs

The biosynthetic routes to PHA monomers rely on important pathways such as the tricarboxylic acid (TCA) cycle, fatty acid degradation (b-oxidation) and fatty acid biosynthesis for precursors, and involve central metabolites such as acetyl-CoA and cofactors such as NADPH .

Metabolic routes towards substrates for PHA synthesis is given in the Figure 2.7 (Rehm, Steinbuchel, 1999)

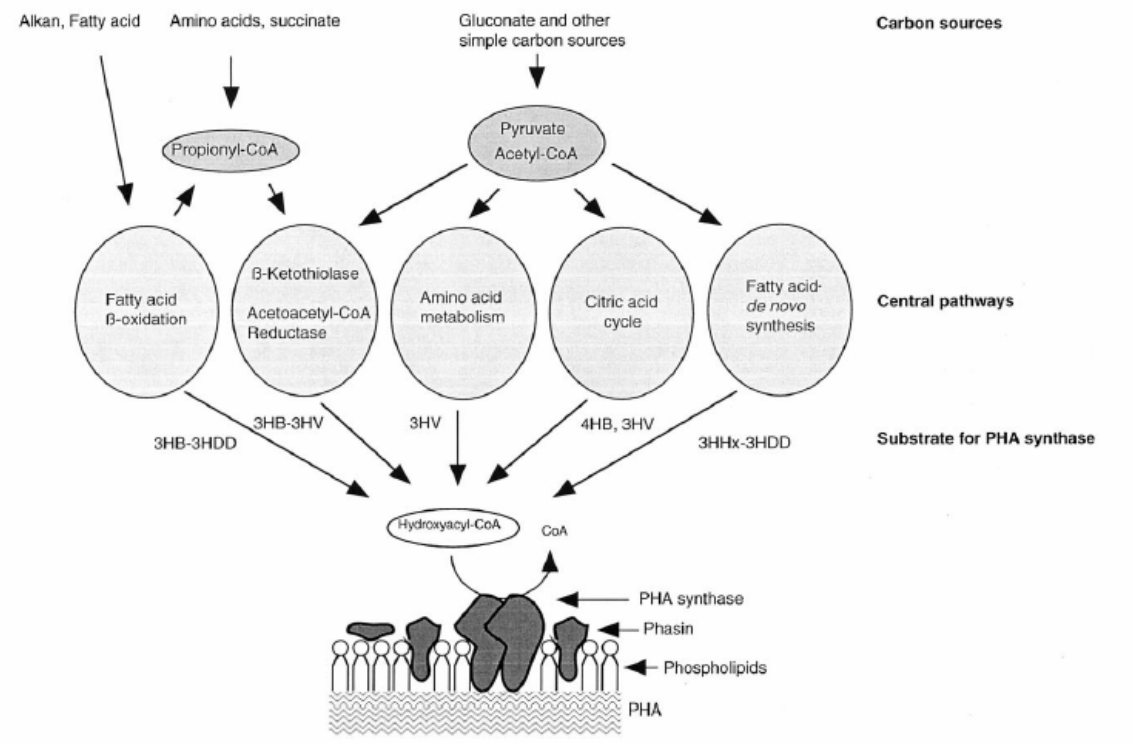


Figure 2.7 Metabolic Routes for PHA Synthesis

There are four different biosynthetic pathways for PHAs. These are summarized below.

1) *R. eutropha* PHA biosynthetic pathway

This is the most commonly used pathway from the microorganisms. The biosynthesis pathways of *R. eutropha*, *Zoogloea ramigera*, and *Azotobacter beijerinckii* are established by Doi, 1990. At first a substrate is condensed to acetyl-coenzyme A (acetyl-CoA). Then to synthesize one mole of PHB two moles of acetyl-CoA are used. Acetyl-CoA is subjected to a sequence of three enzymatic reactions for PHB synthesis. In Figure 2.8 a schematic of PHB biosynthesis by these organisms is shown (Doi, 1990 and Anderson and Dawes, 1990).

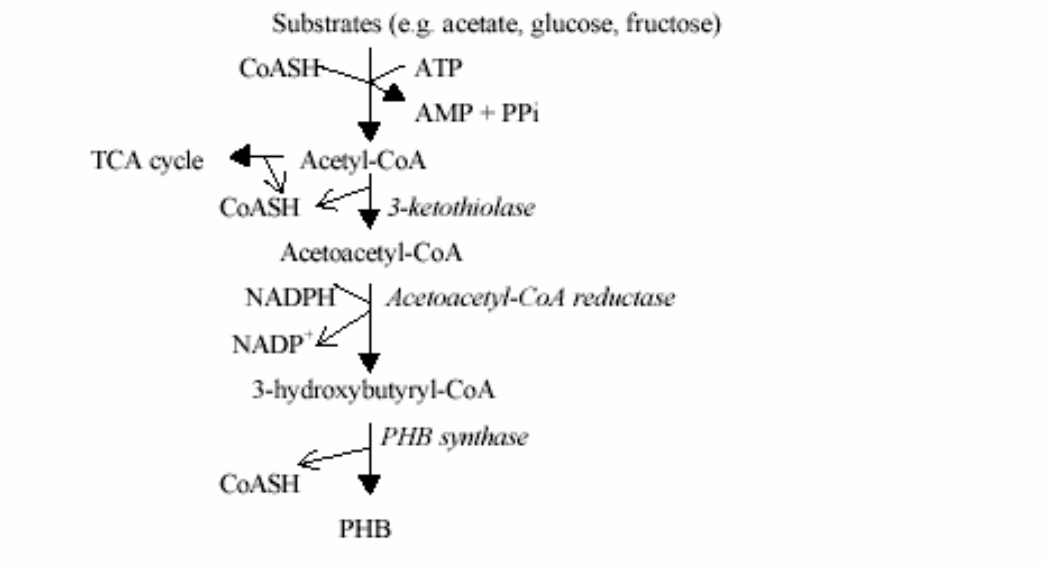


Figure 2.8 PHA Biosynthesis Pathway of *R. eutropha*, *Zoogloea ramigera*, and *A. beijerinckii*

PHB-PHV copolymer is formed when propionic acid is used as a sole substrate. Acetyl-CoA is formed by the elimination of carbonyl carbon from propionyl-CoA. Two moles of acetyl-CoA are used to form a HB unit of the copolymer, while a HV unit is formed by the reaction of acetyl- CoA and propionyl-CoA. In Figure 2.9 the biosynthesis pathway of PHB-PHV copolymer by *R. eutropha* is shown (Doi, 1990).

According to Doi (1990), the degradation of PHA by *R. eutropha* can occur simultaneously with its biosynthesis under nitrogen limitation. This observation is called "a cyclic nature of PHA metabolism". The author reported that the composition of polymer was changed from PHB homopolymer to PHB-49%PHV copolymer when the substrate was changed from butyric acid to pentanoic acid after 96 hours of nitrogen limitation accumulation period, i.e., there was a replacement of PHB by PHB-PHV. Likewise, when *R. eutropha* with a PHV fraction of 56% of its PHA content was fed with butyric acid as a sole substrate under nitrogen limitation, the PHA composition changed markedly, i.e., the fraction of PHV decreased from 56% to 19% after 48 hours. These findings show the simultaneous synthesis and degradation of PHA, i.e., the cyclic nature of PHA metabolism. Figure 2.10 illustrates cyclic metabolism (Doi, 1990).

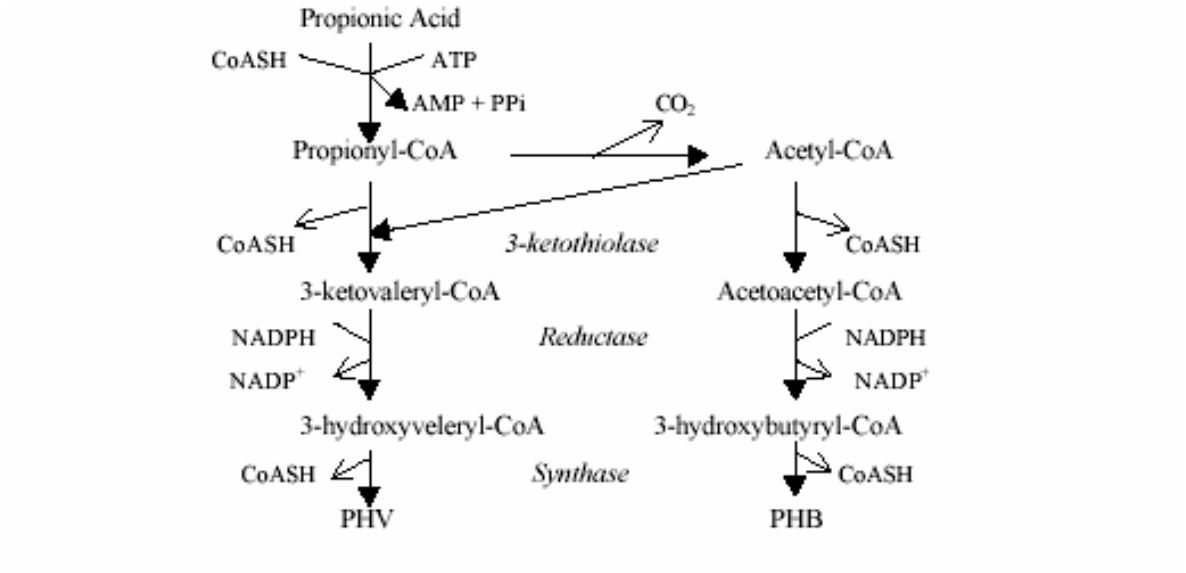


Figure 2.9 Biosynthesis Pathway of PHB-PHV Copolymer by *R. eutropha*

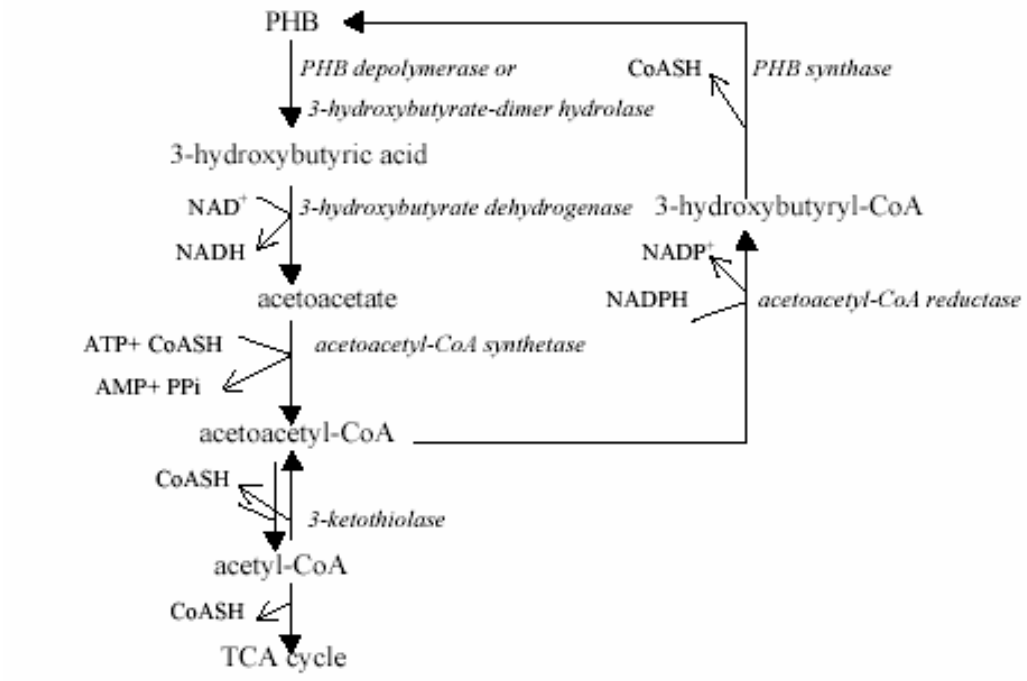


Figure 2.10 Cyclic Nature of PHA Metabolism

2) *Rhodospirillum rubrum* PHA biosynthetic pathway

This pathway is almost similar to the *R. Eutropha* pathway.The difference of this pathway is two enoyl-CoA hydratases are also involved in the second step of catalyzing the conversion of L-3-hydroxybutyryl-CoA to D-3-hydroxybutyryl-CoA via crotonyl-CoA (Anderson and Dawes, 1990; Doi, 1990, Lee, 1996).
A simple schematic of this pathway is shown as:

acetate →acetyl CoA →acetoacetyl CoA →L-3-hydroxybutyryl-CoA →crotonyl CoA →

D-3-hydroxybutyryl-CoA → PHB.

3) *Pseudomonas oleovorans* PHA biosynthetic pathway

This pathway of biosynthesis is found in *P. Oleovorans* and most pseudomonads from the rRNA homology group I (Lee, 1996). These organisms produce medium-chain-length (MCL) PHAs (from C6-C9) from MCL-alkanes, alcohols, or alkanoates.

According to Doi (1990), productions of short-chain-length (SCL) (from C4 and C5) PHAs, i.e., PHB homopolymer and PHB-PHV copolymer, could also be produced by these organisms. Although they could be produced, the productions were less than 1.5%.

This PHA biosynthesis involves the cyclic-β-oxidation and thiolytic cleavage of fatty acids, i.e., 3- hydroxyacyl-CoA, and intermediates of the β-oxidation pathways, are used for PHA biosynthesis.

The fatty acid and fatty acid -β-oxidation cycles can be seen in Figure 2.11 (Aldor and Keasling , 2003) .

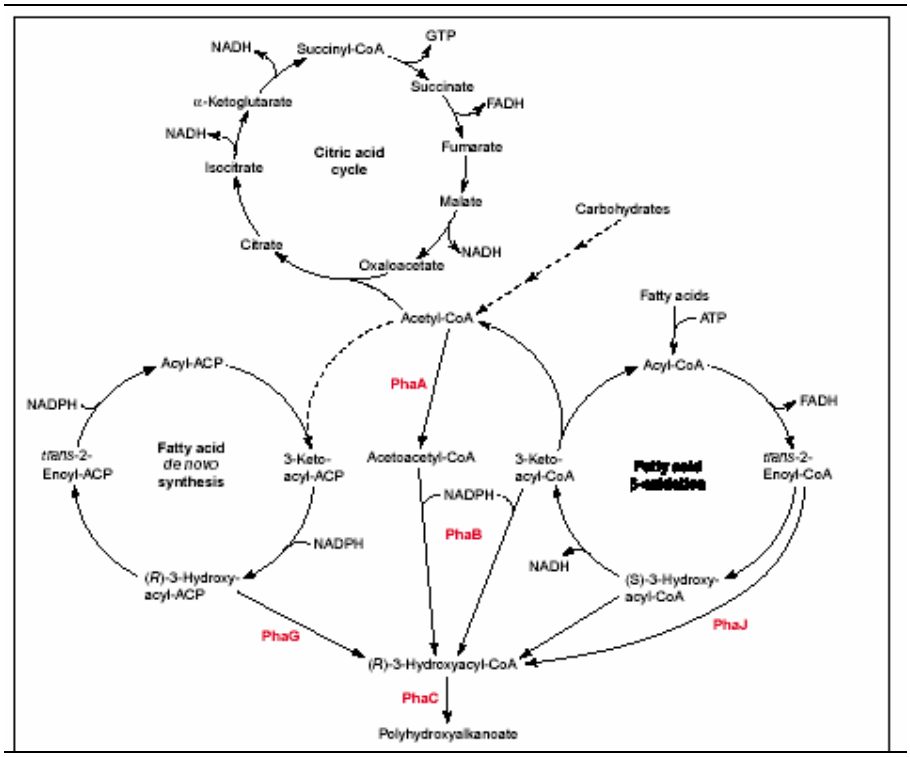


Figure 2.11 The biosysnthesis in the context of microbial mechanism.

In the Figure 2.11 the major enzymes are PhaA (3-ketothiolase),PhaB ((R)-3-ketoacyl-CoA reductase for PHB biosynthesis, this enzyme is acetoacetyl-CoA reductase), PhaG ((R)-3-hydroxyacyl ACP:CoA transacylase),PhaC (PHA synthase or Polymerase) and PhaJ ((R)-specific enoyl-CoA hydratase). In th Dot lines intermediate metabolic reactions are not shown.

4) *P. aeruginosa* PHA biosynthetic pathway

Most pseudomonas from the rRNA homology group except *P. oleororans* also produces MCL-PHAs using this pathway. The pathway used in these organisms is called the *P. aeruginosa* PHA biosynthetic pathway. According to Steinbuchel (1996) MCL-PHAs produced by this pathway are from substrates like gluconate or actetate. PHA is synthesized from acetyl-CoA via fatty acid synthetic pathways shown in Figure 2.11.

These are commonly known biosynthesis pathways of PHA family.

2.2.4.2 The Number of carbon atoms in PHAs

It is told that PHAs are produced of different number of carbon atoms. So in this section this will be explained briefly.

The majority of PHAs are composed of R(-)-3-hydroxyalkanoic acid monomers ranging from C3 to C14 carbon atoms with variety of saturated and unsaturated and straight or branched chain containing aliphatic or aromatic side groups (Doi *et al*,1992 and DeSmet *et al*,1983).

PHAs can be determined into two groups based on the number of carbon atoms in the monomer units. This grouping is due to the substrate specificity of the PHA synthesis that only accept (3-HAs) of a certain range of carbon length (Anderson and Dawes, 1990).

1. The Short Chain Length PHAs (SCL) :

They contain C_3-C_4 atoms. In the PHA synthesis of *A.eutrophus* only polymerize 3Has (SCL) can be synthesized.

2. The Medium Chain Length PHAs (MCL) :

They contain C_6 –C_{14} atoms. P.oleovorans can polymerize 3HAs (MCL). It is reported that a lot of PHAs (MCL) contain functinal groups like olefins, branched alkyls, halogens, aromatic and cyano (Fritzsche,1990 ; Huijberts,1992 ; Kim,1992 ; Hazer,1994).

2.2.4.3 The Metabolic engineering of PHA production

Metabolically engineered microorganisms can be used to produce PHAs. There are some approaches given for this production and they will be explained shortly. Figure 2.12 shows a schematic of various strategies for the metabolic engineering of PHAs.

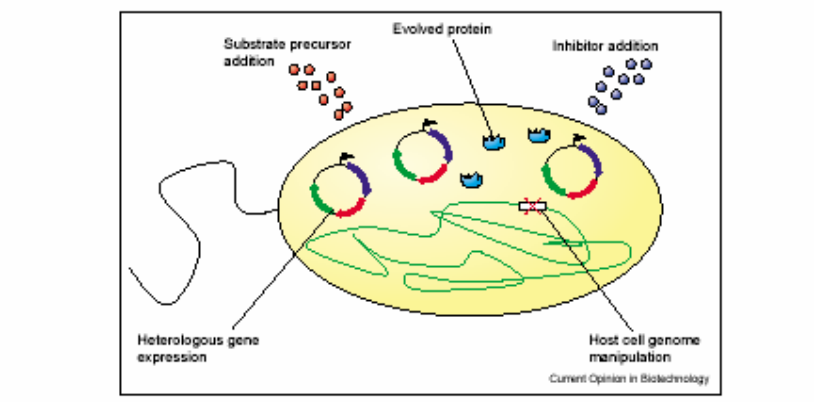


Figure 2.12 Schematic of various strategies for the metabolic engineering of PHAs. (Aldor and Keasling, 2003).

The External Substate Usage

The simplest metabolic engineering strategy is to manipulate the carbon source(s) supplied to the host to control and direct carbon flux through the precursor and polymer biosynthesis enzymes. This strategy has traditionally been exploited to modulate polymer composition by varying the feed ratio of different substrate precursors

Inhibitor Edition

As an adddition to external substrates inhibitors are materials that canalize precursors to PHA synthesis. These compounds are useful for the incorporation of MCL monomers derived from β- oxidation into PHAs.

Recombinant Gene Expression

The most important PHA biosynthetic enzymes are shown in Figure 2.11. *R. eutropha, Pseudomonas putida, Pseudomonas oleovorans and Escherichia coli* are some microorganisms which are recombinant for PHA synthesis. Many genome sequencing projects have been done about the newly identified genes.

It is a possible way to express the genes by giving them the required enzymes for PHA biosynthesis in *E.coli* and other bacterias which don't produce PHA under normal conditions. Theoritically it is possible to produce PHA from any kinds of

microorganisms but there are some problems. Firstly the key genes required for biosynthesis of a polymer should be identified and cloned.

The host organism determines the range of substrates that can be used. So for large scale production of PHA the host organism is very important. Also productivity, maximum polymer content and downstream processing are other important subjects to be considered.

E.coli is a proper host for PHA production in recombinant microrganism. Its genetics, metabolism and the range of substrates that it can utilize are well characterized.

R.eutropa is known as the best PHA producing organsim but *E.coli* grows more rapidly than *R.eutropha* and produces more polymers. But it is not stable. Some of the *E.coli* strains require a more complex and so more costly medium than that used for *R.eutropa*. Most of the studies with *E.coli* PHB was produced rather than the other co-polymers (Anderson and Wynn, Basics of Biotechnology).

Host Cell Genome

Host cell genome manipulation can eliminate competing pathways or modify native regulation for improved function of desirable pathways (Aldor and Keasling, 2003).

Protein Engineering of PHA Enzymes

Nowadays metabolic engineers are able to optimize enzyme performance to obtain desirable polymer properties and yield by the help of protein engineering, mutagenesis and molecular evolution.

2.2.5 The Metabolism of PHA production

2.2.5.1 PAO/GAO System

In mixed cultures where anaerobic/aerobic process is available, PHA plays an important role. Electron donor and acceptors are seperated in these processes (Satoh et al., 1998b).

There are two types of microorganisms capable of anaerobic storage of carbon source in mixed culture (Chech and Hartman, 1990, 1993): (i) the

polyphosphateaccumulating organisms (PAO) and (ii) the glycogen-accumulating organisms (GAO).

In this study, the mechanism of the enhanced biological phosphorus removal (EBPR) and PHA biosynthesis from PAO metabolisms will be examined. So PAO mechanisms and enhanced biological phosphorus removal (EBPR) metabolisms are going to be explained in detail. Also glycogen accumulating organism (GAO) mechanisms will be summarized together with PAO to show the relationship between these two.

Phosphorus removal from wastewater has become important in order to protect water bodies from eutrophication such as lakes and largely enclosed bays where water is relatively stagnant (Lee, Rast, Jones, 1978).

Biological phosphorus removal process has been attracting much attention because of its low capital and operational costs compared with chemical precipitation processes (Chen, 1975 and Osborn, 1978). Release of phosphorus, uptake of organic substrates and accumulation of PHA by bacteria occur simultaneously under anaerobic condition (Marais, Loewenthal, Siebritz, 1983). In the subsequent aerobic condition, more phosphorus can be taken up than the previously released phosphorus. In this anaerobic/aerobic cycle microorganism population which is capable of storing phosphorus in their cells is selected. By this processes phosphorus can be removed from the wastewater. The stored phosphorus is in the form of polyphosphate (poly P).

Under anaerobic conditions when the influent wastewater is added to the sludge phosphorus accumulating organisms -poly P- gets an advantage because they can utilize more energy than other microorganisms to uptake substrates by breaking down the accumulated poly P. This substrate is then accumulated as PHAs. When the anaerobic conditions finish aerobic conditions start to supply energy and metabolic intermediates for cell growth and for regeneration of poly P.

Marais, Loewenthal, Siebritz (1983) found out that the energy gained by the degradation of poly P under anaerobic condition is utilized for the uptake and storage of suitable substrates such as fatty acids, particularly acetate. From complex substrates acidogenic bacteria produces fatty acids. Then fatty acids are converted

into osmotically inert PHA as a reserve material by the poly P accumulating bacteria (Comeau, Oldham, Hall, 1987) and (Fukase, Shibata, Miyaji, 1982).

The conversion of substrates into PHA requires reducing equivalents in the form of NADH or NADPH, which were suggested to be produced either by oxidation of acetate in the TCA cycle (Comeau, Hall, Hancock, Oldham,1986) and (Matsuo,1985) or by oxidation of glycogen (Mino, Matsuo,1984) and (Mino, Arun, Tsuzuki, Matsuo,1987). Because of the fact that succinate dehydrogenase can not operate due to the lack of the regeneration of FAD, the TCA cycle doesn't function anaerobically. But, in 1986 Comeau *et.al*. and in 1985 Matsuo discovered that there was a possibility that the TCA cycle could operate under the anaerobic conditions. In 1982 Florentz and Hartemann found out that some of the TCA cycle enzymes retain their activities under anaerobic conditions but it is not certain if the TCA cycle is active under the anaerobic conditions or not.

In 1984 Mino and Matsuo said that reducing power generated during glycoysis is coupled to the acetate uptake under anaerobic condition.

In 1987 and 1988 Mino, Arun, Tsuzuki and Matsuo observed that the amount of intracellular glycogen decreases under anaerobic conditions.One year later in 1989 Bordacs and Chiesa reported that 14 C-labelled acetate was not converted to CO_2 under anaerobic condition, which indicates that acetate is not degraded through the TCA cycle under this condition. In this process 1 mole of glucose yields 4 moles of NADH and 2 or 3 moles of ATP by glycogen degradation. As well as energy, glycogen serves as a source of reducing power that can be used for the uptake of acetate followed by conversion into PHA (Mino, Arun, Tsuzuki and Matsuo, 1987).

Kulaev from UK suggested that under aerobic condition, phosphorus is ingested rapidly and ATP is formed by utilizing the energy produced by the aerobic decomposition of intracellular PHA. Then, a portion of ATP is converted back to poly P by polyphosphate kinase for the generation of ATP/ADP ratio in the cell (Kulaev, 1979).

Figure 2.13 shows the proposed mechanism during the phosphorus removal process and the relationships between phosphorus, acetate and P(3HB) under (a) anaerobic condition and (b) under aerobic conditions.(Wentzel, Lotter, Loewenthal, Marais,1986).

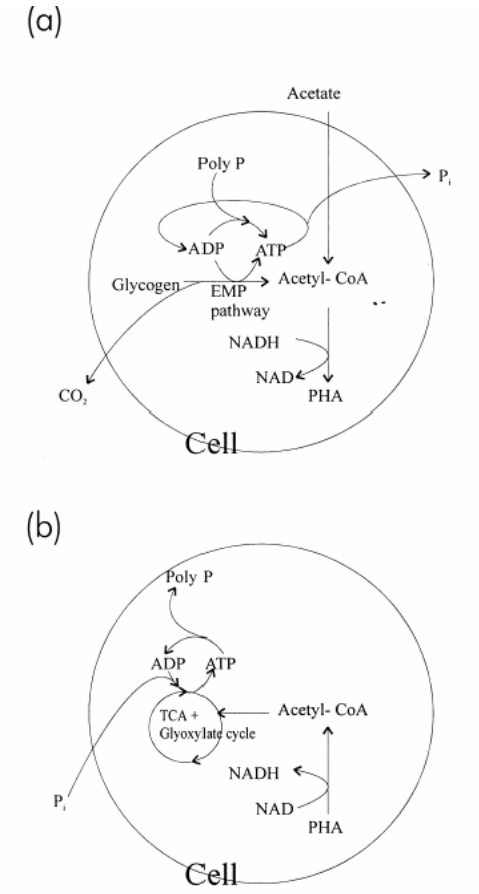


Figure 2.13 The proposed mechanism during the phosphorus removal process and the relationships between phosphorus, acetate and P(3HB) under (a) anaerobic condition and (b) under aerobic conditions.

So these are the characteristics of conventional activated sludge processes for phosphorus removal. Also there are some researches about the uptake mechanisms of acetate, propionate and acetate in anaerobic sludge (Satoh, Mino, Matsuo, 1992). The cells take up these acids and convert them into PHAs composed of 3-hydroxybutyrate, 3-hydro- xyvalerate, 3-hydroxy-2-methylbutyrate and 3-hydroxy-2-methylvalerate via acetyl-CoA and propionyl-CoA. For the production of PHA a reducing power is necessary. This can be supplied from the breakdown of glycogen. According to Fukase, Shibata and Miyaji (1985) and also Cech and Hartman (1990), if the glycolysis of intracellular carbohydrates such as glycogen is adequately coupled with propionate fermentation pathway and PHA accumulation, bacteria can obtain energy under anaerobic condition without utilizing poly P. But in this

metabolism some bacteria other than poly P accumulating ones, can survive in the anaerobic/aerobic conditions.

If this situation occurs in the anaerobic/aerobic phase it causes deterioration of the phosphorus removal. When PHA is utilized for the regeneration of poly P, biological phosphorus removal can be enhanced by metabolically engineering poly P accumulating bacteria with respect to their PHA biosynthetic pathways.

As it can be understood from these explanations that PAOs are probably the most widely recognized for producing storage polymers (PHA, glycogen and polyphosphates). PAOs and GAOs proliferate in systems where the substrate is present regularly while an electron acceptor is absent (Cech and Hartman, 1993). 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. 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 hydroxybutyl-CoA and finally polymerization to PHB (Figure 2.14).

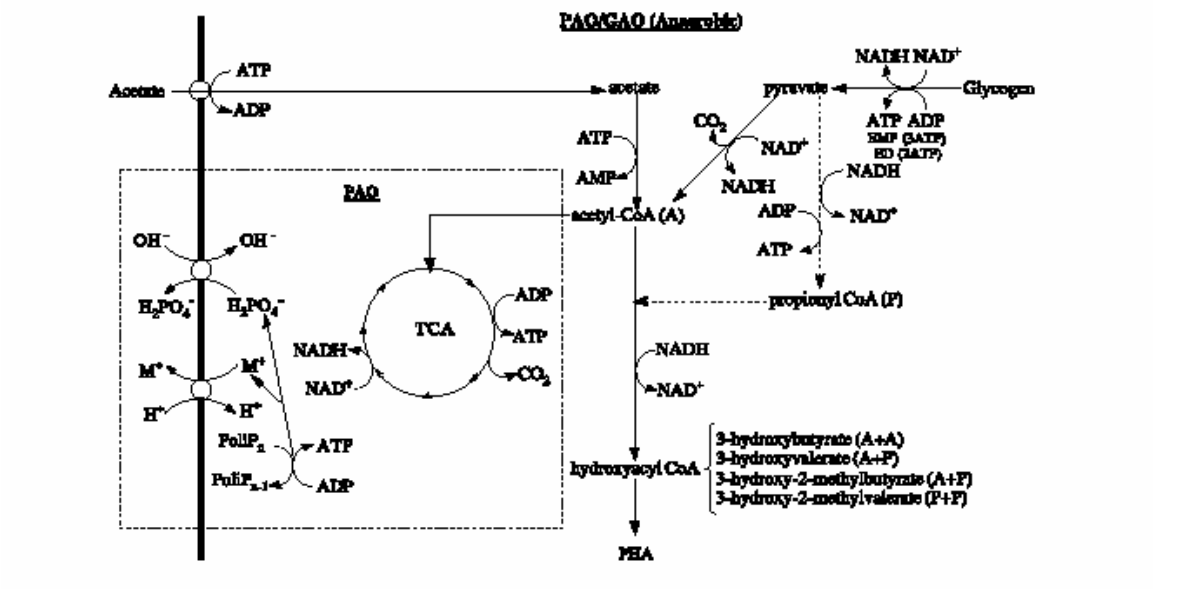


Figure 2.14 PAO/GAO System Pathways

PAOs and GAOs use the conversion of glycogen to PHA in order to produce ATP and NADH. PAOs use this conversion mainly to produce reducing power, whereas in

GAOs, this conversion is predominantly used for ATP production. GAOs use Embden–Meyerhoff–Parnas pathway (EMP) (Filipe et al., 2001a) for glycogen hydrolysis, whereas PAOs use the Entner– Doudoroff (ED) pathway (Maurer et al., 1997; Hesselmann et al., 2000). The presence and relative proportion of different PHAs is dependent on the type of carbon substrate available. The different polymers are formed to allow the cells to balance the redox equivalents produced and needed in conversion of substrate to PHA. Satoh et al. (1992) demonstrated the formation of other PHAs containing monomeric units HV, 3H2MB (3-hydroxy-2-methyl butyrate), and 3H2MV (3-hydroxy-2-methyl valerate).

Satoh et al. (1994) and Liu et al. (1994) explained the existence of HV formation in polyphosphate-independent anaerobic and aerobic activated sludge. According to HV fermentation, glycogen is converted to PHA through propionyl-CoA in order to satisfy the redox balance. Due to the fact that the organisms are subjected to rapid changes between aerobic and anaerobic conditions, the enzymes of intermediary cell metabolism are stil active under anaerobic condition. This also leads to formation of PHV when acetate is the sole substrate for PAOs (Pereira et al., 1996).

2.2.5.2 Microaerophilic–aerobic system

It is well known that under anaerobic–aerobic conditions PHA accumulation of the microorganisms are available. However there is no guarantee that anaerobic–aerobic system of the activated sludge process is the best way for PHA accumulating microorganisms. According to Ueno et al. (1993) and Saito et al. (1995) more PHB is accumulated under aerobic conditions than in anaerobic conditions. In 1998 Satoh found a new process for PHA production. This is microaerophilic– aerobic process where a limited amount of oxygen is given to the system under the anaerobic zone. In this process microorganisms are able to take organic subsrates by obtaining energy through oxidative degradation of some part of the organic substrates. When the oxygen is enough, the microorganism can take energy for assimilative activities which are the production of protein, glycogen and other cellular components. But if the amount of the oxygen provided to the system is limited or controlled the microorganism can accumulate PHA because of the suppressed assimilative activity. Under this conditons PHA accumulators are selected regardless of the ability of

microorganisms to accumulate poly-P or glycogen. The selected PHA accumulators will have a lower tendency to accumulate glycogen.

Van Aalst-van Leeuwen et al. (1997) and Beun et al. (2000a,b) formulated a metabolic model of PHB metabolism. According to their model the following reactions take place: synthesis of acetyl-CoA from acetate, anabolism reactions, catabolism reactions, electron transport phosphorylation, synthesis of PHB from acetyl-CoA, and the synthesis of acetyl-CoA from PHB. The aerobic metabolism for this system and for feast and famine system which will be explained is given in Figure 2.15.

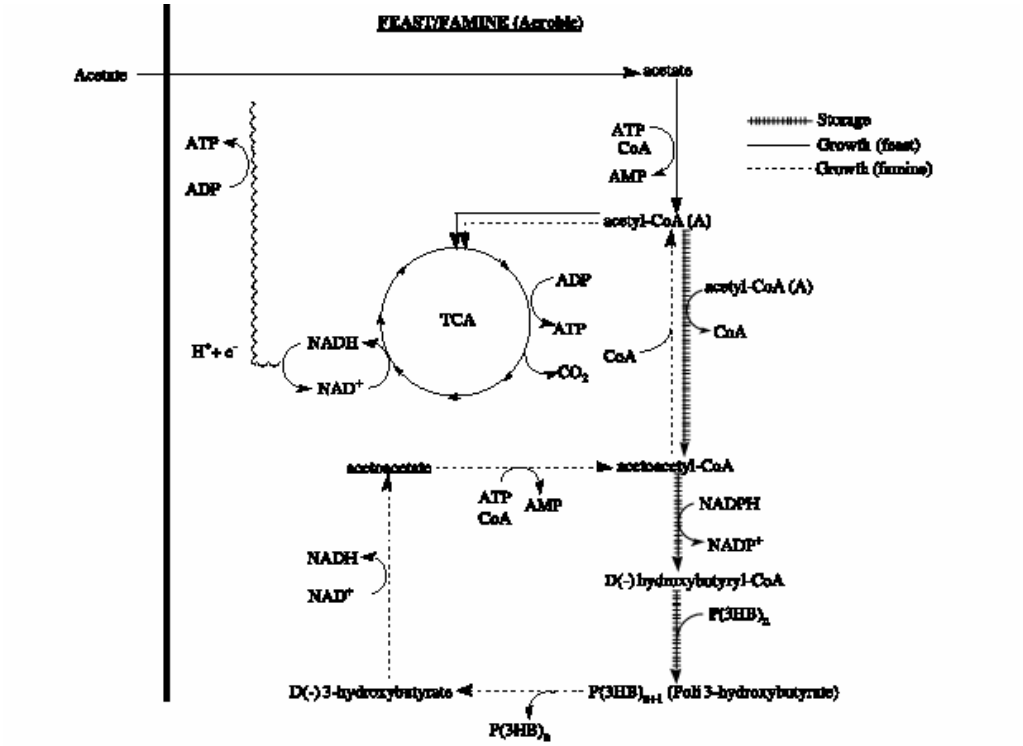


Figure 2.15 The Aerobic Metabolism of PHB Production in Both Microaerophilic and Feast and Famine Conditions (Salehizadeh and Van Loosdrecht, 2004).

2.2.5.3 Feast and famine metabolism (Dinamic conditions)

Activated sludge processes are highly dynamic conditions with respect to the feed regime. In this process for the biomass there is an available external substrate period called feast and no available external substrate called famine period. They are generally defined as unbalanced growth conditions.

When external substrate is added to the system growth of biomass and storage of polymer occur under dynamic conditions. When all the external substrate is consumed, stored polymer can be used as carbon and energy source where there is no limiting. This can be seen in Figure 2.15.

When substrate is present for a long time physiological adaptation occurs, and growth becomes more important than storage.

The ability to store internal reserves gives to these microorganisms a competitive advantage over those without this ability, when facing transient substrate supply (Salehizadeh and Van Loosdrecht, 2004).

In this section the systems for PHA production mechanism are explained. The most promising system for PHA production for industries is the feast and famine approach because the carbon substrate turns into PHA and not to glycogen or other intracellular materials.

3. EXPERIMENTAL APPROACH

3.1 Planning of the Experimental Study

The aim of this study is to produce biomass that can store PHA (Polyhydroxyalkanoates). This biomass will be used in the production of biodegradable plastics. For this purpose, conditions favoring the selection of PHA storing microorganisms in mixed culture will be investigated. According to this aim Sequencing Batch Reactor (SBR) systems are going to be used in these experiments. SBR Systems are systems that allow investigating the complex mechanisms by using different operational conditions. In the next section, the reasons for choosing SBRs will be explained.

The first SET (SET 1) was seeded with sludge which was taken from a treatment plant treating domestic sewage from Fatih University.

The following SETSs (SET 2, SET 3, SET 4, SET 5, SET 6 and SET 7) were seeded with sludge which was taken from Paşaköy Advanced Biological Wastewater Treatment Plant in Istanbul.

Different operational conditions will be investigated to reach the aim of this study. For example temperature, the synthetic wastewater characteristics (carbon sources), anaerobic/aerobic time periods, the ratios of micro nutrients which were added to the systems.

Batch experiments will be performed with the sludge which was taken from the SBRs under different operational conditions. Bioreactor will be used for the batch experiments to produce PHAs.

In this study PAO/GAO System is selected to produce PHA so PO_4-P will be an important parameter which must be measured carefully. Also to examine the consumed substrate, COD (Chemical Oxygen Demand) should be measured. In a biological system suspended solids (SS) and volatile suspended solids (VSS) should be examined for the maintaining.

According to the results of the different operational conditions the most appropriate way for the production of PHAs will be investigated.

3.1.1 Sequencing batch reactors (SBR)

3.1.1.1 Process description

A general description of the SBR process, in a way that may be readily utilized in relevant stoichiometric relationships and mass balance equations, can be made by identification and appropriate mechanistic definition of the set of operation parameters reflecting basic differences of the process with the continuous-flow activated sludge system (Artan and Orhon, 2005). But basically the Sequencing Batch Reactor (SBR) Systems are activated sludge systems which are operated as fill and draw mechanisms.

Generally SBR systems consist of one tank or some parallel tanks. The main difference between the conventional activated sludge systems and SBRs is in SBR instead of area, time is the important factor. In SBRs all the reactions such as feeding, mixing, aeration, settling occur in the same tank. But in conventional activated sludge systems all the reactions take place in different tanks. In SBR systems time periods have an important role for treatment.

At very early 1914s and 1920s activated sludge systems that were operated were mostly designed as fill and draw systems. But after the development of continuous systems SBR systems were not used much until 1960s.

The Definition of Time Periods in Process Descriptions

In a typical SBR tank there are 5 basic operation conditions: Fill (feeding), Reaction, Settling, Withdrawal and Idle.

First of all the tank is fed with the wastewater and this wastewater is mixed with the biomass which is in the tank from settling part of the last cycle. This is the filling (feeding) part. Then the reaction phase starts, aerobic, anaerobic/aerobic, anaerobic/anoxic…etc. In this phase the biological reactions take place. After this the microorganisms are settled and separated from the treated water. This is called settling. The treated water is discharged and the system is left for an idle phase. The total time of these phases shows the total time of one cycle.

This can be shown as

$$Tc = T_F + T_R + T_S + T_{W+I} \quad (3.1)$$

In this formula ;

Tc : The Total cycle time

T_F : The filling time

T_R : The reaction time

T_S : The settling time

T_{W+I} : The Withdrawal and Idle time

Sometimes the reaction time can be defined in two different parts such as anaerobic and aerobic parts. If this is formulated, it can be written as

$$T_R = T_{AN} + T_A \quad (3.2)$$

T_{AN} : The anaerobic reaction time

T_A : The aerobic reaction time

The Definition of Volumes in Process Descriptions

The total volume of the reactor is represented with V_T and the volume of the reactor at the beginning is represented with V_0. The time for filling the reactor from V_0 to V_T is called T_F (the filling time) and the filling volume is called V_F which can be found from equation (3.3) given below.

$$Q = m. V_F \quad (3.3)$$

In equation (3.3) m represents the number of the cycles in a day (cycle frequency) where Q is the daily volumetric flow rate of the wastewater treated from the SBR. The total cycle time of the SBR can be calculated from equation (3.4).

$$T_C = 1/m \quad (3.4)$$

Number of tanks is shown as n which can be seen in (3.5).

$$T_F = \frac{T_C}{n} = \frac{1}{mn} \tag{3.5}$$

3.1.1.2 The Phase definitions of sequencing batch reactor (SBR) systems

These operational phases are controlled by timers. Each phase is explained below (Irvine, 1989)

The Filling Phase

In a filling phase wastewater is added to the biomass left in the tank either in three ways;

1. Static filling without aeration or mixing,
2. Filling by mixing,
3. Filling by aeration.

The initial volume, V_0 should be at least 25% or at most 70% of the total volume, V_T.

The Reaction Phase

The Reaction phase starts with the filling period. If oxygen is needed then aeration should take place. But if it is going to be a system without oxygen then there will be only mixing phase with no aeration. To control the sludge age sludge should be disposed at the end of the reaction phase.

The Settling Phase

The settling takes place in the tank. Until the settling is completed there are no input or output activities in the tank. Settling time is usually between 0.5 to 1.5 hours. During the disposal of the treated water, the microorganisms settle down at the bottom of the tank.

The Withdrawal and Idle Phase

The disposal mechanism can be in many different ways. Mostly it is a pipe which has been fixed to the tank at a constant level. The flowrate is regulated by a pump in the system. The total time for the withdrawal mechanism is approximately between 5% - 30% of the total cycle time.

In the idle phase the system is ready to have wastewater again.

3.1.1.3 The Main advantages of sequencing batch reactors

According to the operational conditions of the SBRs, these systems are more useful than the conventional activated sludge systems. The main advantages of SBRs are given listed below. (Akora and others, 1985)

- According to the daily or seasonal wastewater charges (loads), number of the reactors can be added or displaced.
- The biomass can be kept in the system without any hydraulic fluctuation.
- The effluent is periodically disposed so that until the discharge limits water can be kept in the reactor without changing the total cycle time.
- Reaction and Settling phases occur in the same tank which causes easier applicability in practice.
- By the help of changing the operational conditions in the filling phase the growth of microorganisms with filamentous could be controlled.
- The activated sludge mixture is always in the reactor so there is no need for activated sludge recycle pump.
- In SBRs total cycle time and the operational conditions can be changed easily. Generally total cycle time is between 3 to 24 hours.
- The SBRs have time flexibility. So they can be operated as low energy, more sludge and human sources or high energy, less sludge and less human source.(Irvine, 1989)

3.1.1.4 The Development of SBR systems

Irvine and Davis defined the operational conditions of SBR Systems in the early 1970s. After that these systems had an opportunity to be applied in many countries.

In their researches Dennis and Irvine had discovered that the characteristics of the sludge settling depends on filling /reaction ratio and long mixing time periods during filling, causes a degrease in the sludge volume index (SVI), (Dennis and Irvine, 1979).

Wanner had compared a continuous activated sludge system and SBR system and found out that in SBRs filamentous microorganisms had not seen much and settling properties were better although the carbon and nutrient removals were same (1992). It is said that in SBRs SVI is around 100 mg/g when the SVI is between 100-200 mg/g in continuous systems.

Ketchum had discovered that in SBR systems investment costs are lower than the costs in conventional systems. But operational and maintenance costs are nearly the same in both systems (Ketchum, 1979). He also discovered that for chemical phosphorus removal, to do the third treatment in SBRs causes a better effluent quality with the help of cheap chemicals (Ketchum and Liao, 1979).

The SBR systems have developed according to the technology because different types of valves, timers, level sensors, pumps were found (Irvine and others, 1983, 1987).

But sometimes these properties might turn into disadvantages because the system requires complicated control strategies. Also the flexibility in the process could make system mechanics more complicated (Orhon and others, 1986).

In appropriate design and operational conditions nitrification and denitrification is possible in SBR systems and many researchers have determined it in their studies (Irvine and others, 1979; Alleman and Irvine,1980; Silverstein and Schroeder, 1983 ;Palis and Irvine ,1985). Also some of the researches showed that within the appropriate conditions SBRs gave successful results of treating wastewaters consisting of C, N, P (Bortone and others, 1992, 1994; Subramaniam and others, 1994)

SBRs are capable of enhanced biological phosphorus removal (EBPR) because of their operational conditions (Irvine and others, 1985). So there have been many researches about EBPR by the help of SBRs.

SBR can also be used for the treatment of industrial wastewaters such as winery wastewaters (Torrijos and Moletta, 1997), brewery wastewaters (Ling and Loo, 1999), food industry wastewaters (Raper and Green, 2001), dairy wastewaters (Mohseni and Bazari, 2000), slaughterhouse wastewaters (Belanger, 1986), piggery wastewaters (Lee, 1997), pulp and paper mill effluents (Franta and Wilderer, 1997) and tannery effluents (Carucci, 1999).

Enhanced Biological Phosphorus Removal in SBRs

SBRs play an important role in enhanced biological phosphorus removal (EBPR) systems. EBPR is done by adding an anaerobic phase to the operational phases. This phase generally occurs after filling. The sludge left in the reactor mixes with the wastewater in the filling period and no oxygen is given to the system. SBRs have easy conditions in both operating and controlling for this purpose they are ideal systems for EBPR, (Taşlı, 1996).

SBRs are also used for the removal of phosphorus and nitrogen together. By giving more time to aeration phase nitrification can be obtained and by giving more time to settling and idle phase denitrification can be obtained (Arora and others,1985).

In this study SBRs were used for PHA accumulating microorganism production in EBPR Systems which were explained in detail in Chapter 2.

3.1.2 Experimental design of SBRs

The SBR reactors which had been used in the system can be seen on Figure (3.1) with all the equipments that were used in the system. The two cylindrical bench-type Plexiglas reactors with 50 cm height and 17 cm diameter were used in the experiments. They were closed with a plexiglas cover. The systems were operated electronically with timers to control all the equipments such as air pumps, mixers, aerators, dosage, filling and discharge pumps. The SBRs run anaerobic/aerobic phases during the experiments.

The volume of the SBRs were 8 liters as working volume, 4 liters as discharge volume and 4 liters as filling volume with a ratio of Vo/V_F =0.5. Both reactors were operated in identical conditions. They were designed for four cycles a day with a total cycle time of 6 hours, (Tc=6h) .Five hours of this period was called a process phase (Tp=5 h) and the remaining hour is for settling, withdrawal and idle. The cycles started with an anaerobic phase of 2 hours for the first SET (T_{AN}=2 h) and 3 hours for the remaining six SETs (T_{AN}=3 h). The first 15 minutes were for filling process in all SETs,(T_F=15 min).

Figure 3.1 Experimental Design

The reactors were being mixed during the filling period. After the anaerobic periods aeration had started in the reactors. In the first SET, aeration took place for 3 hours,(T_{AN}=3 h). In the other SETs aeration period were 2 hours, (T_A=2 h). When 5 hours of process phase was finished the next three phase had started which were 30 minutes of settling (Ts=30 min), 15 minutes for withdrawal (Tw=15 min) and 15 minutes for the idle (Ti=15 min) for all SETs. As a result the total cycle time for the SETs can be defined as T_C which was shown in (3.1).

During the experiments the room temperature was 20±2ºC but in some of them temperatures degreased to 10 ºC.

All of the systems were operated with a sludge age of 10 days, (θx = 10 d). The sludge was discharged at the end of the aeration phase once a day for 5 minutes. pH was not controlled during the experiments but was measured rarely. But in SET 6 and SET 7 pH was measured during the experiments. The measurements were made at steady- state conditions. Operating parameters of the reactors will be explained in next section.

3.1.3 Operational conditions

In EBPR systems the main subject is the storage of phosphorus in microorganisms. So that removal efficiency of the system is directly proportional to the wasted sludge from the system. For this reason to increase the amount of the wasted sludge, the system has to work in lower sludge ages (θx). In all SETs the sludge age was selected as 10 days and the wasted sludge was 800 ml/day. In SBR systems in order to keep the sludge age low, cycle numbers can be increased. By increasing the number of cycles, also the number of anaerobic/aerobic phases will increase. So loading /day increases which could accelerate the Poly-P microorganisms dominant in the system. According to these informations it is appropriate to work at maximum cycle numbers a day.

To maintain the steady state conditions in the system the wasted sludge was discharged at the end of one cycle a day.

In all the SBR systems that were used in the experiments, anaerobic/aerobic phases were used.

By changing the T_{AN} and T_A and the characteristics of the wastewater, the experiments were done in both SBRs and in bioreactors for batch experiments. The operational conditions and parameters of the SETs are given in Table (3.1).

There were seven different conditions that were defined as SETs. They are called SET 1, SET 2, SET 3, SET 4, SET 5, SET 6 and SET 7. The day that every reactor had started was called Day 1 in all systems. Each reactor's operational conditions, experimental data and results will be explained.

3.1.3.1 Characteristics of Synthetic Wastewater

The SETs (1-6) were fed with synthetic wastewater compound as an carbon source consisting of acetic acid, propionic acid, ethanol, glutamic acid and glucose. But SET 7 was fed with only sodium acetate. Also SET 5 was fed with a compound consist of acetic acid, propionic acid, ethanol and glucose without glutamic acid. Their influent COD's remain the same as 400mg/L.

Table 3.1 Operational Conditions for SETs

PARAMETER	SYMBOL	VALUE	UNIT
Cycle Time	Tc	6	h
Duration of filling period	T_F	15	min
Duration of anaerobic (mixing) period[1]	T_{AN}	2-3	h
Duration of aerobic (aeration) period[2]	T_A	3-2	h
Duration of settling period	T_S	30	min
Duration of withdrawn period	T_w	15	min
Duration of idle period	Ti	15	min
Initial volume	V_0	4	l
Total volume	V_T	8	l
V_F/V_0	-	-	0.5
Hydraulic Retention Time	HRT	12	h
Sludge Retention Time	SRT	10	days
Temperature[3]	t	20 ±2	^OC

1. Duration of anaerobic phase was 2 or 3 hours in SETs.
2. Duration of aerobic phase was 2 or 3 hours in SETs.
3. In some of the SETs temperature was 10 ±2 ^OC.

SETs 1, 2, 3, 4 and 6 were fed with a compound called Carbon source 1.

SET 5 was fed with a compound called Carbon source 2.

SET 7 was fed with a compound called Carbon source 3.

All of them are given in Table 3.2, Table 3.3, Table 3.4.

Addition to these compounds two different kinds of solutions were also used in the reactors. For receiving a constant level of influent phosphorus Solution A_1 and A_2 was used. Their compounds and ratios are given in Table 3.5 and Table 3.6.

To maintain the phosphorus removal mechanism there are some necessary micro nutrients. For this purpose Solution B was also used. The chemical compounds which had been used in Solution B are given in Table 3.7.

The carbon sources and the solutions were added to the system from different pumps and sources to prevent any biological activity in feeding which could cause a reduction in the influent COD.

Carbon sources were put into a bottle with a closed cap and kept at a room which has a temperature of 4ºC. Solutions A and B were put into a bucket and mixed with tap water and kept at room temperature.

Table 3.2 Carbon Source 1

Organic Compound	Amount (/L)	COD (mg)
Acetic acid	10 ml	10000
Propionic acid	3.1 ml	4000
Ethanol	1.68 ml	2000
Glutamic acid	3.62 g	4000
Glucose	4.54 g	4000

Table 3.3 Carbon Source 2

Organic Compound	Amount (/L)	COD (mg)
Acetic acid	14 ml	14000
Propionic acid	3.1 ml	4000
Ethanol	1.68 ml	2000
Glucose	4.54 g	4000

Table 3.4 Carbon Source 3

Organic Compound	Amount (/L)	COD (mg)
Sodium Acetate	30.75 ml	24000

Table 3.5 Solution A_1

Compound	Amount (g/L)
K_2HPO_4	30
KH_2PO_4	15
NH_4Cl	200

Table 3.6 Solution A_2

Compound	Amount (g/L)
K_2HPO_4	60
KH_2PO_4	30
NH_4Cl	200

Table 3.7 Solution B

Compound	Amount (g/L)
$MgSO_4.7H_2O$	15 g/l
$FeSO_4.7H_2O$	0.5 g/l
$ZnSO_4.7H_2O$	0.5 g/l
$MnSO_4.H_2O$	0.412 g/l
$CaCl_2$	2 g/l

3.2 Experimental Plans

3.2.1 SBR Experiments

3.2.1.1 Daily and cycle measurements

At the beginning of the study a carbon source mixture which is shown in Table 3.2 was selected (Orhon). Then two different carbon sources were used in the SBR systems. They are given in Table 3.3 and Table 3.4. It will be explained which one was used, in the next chapter.

Solution B was used for all of the SETs.

Solution A_1 was used at the first two SETs and then A_2 was used for the remaining sets.

Al the experiments were done at the same cycle of everyday in which the sludge was wasted. That cycle was called the 4^{th} and the last cycle.

For the experiments samples were taken from mixed liquor at the end of aeration phase of cycle 3, feeding end, mixing end and aeration end of cycle 4. Also samples were taken from the influent wastewater. This is called as one set. One set can be summarized as;

1. The end of aeration phase of cycle 3, (the end of the previous cycle),

2. The end of feeding phase (after filling),

3. Stock wastewater (influent),

4. The end of anaerobic phase (mixing end),

5. The end of aerobic phase (aeration end),

In a set phosphorus amounts, COD and in some of them pH was measured. It is shown in Table 3.8 below.

Table 3.8 Daily Monitoring of SBR Experiments

Measurement Point	SS	VSS	PO_4-P	COD	pH
1 Aeration End 1			x	x	x
2 Feeding End			x	x	x
3 Influent			x		
4 Mixing End	x	x	x	x	x
5 Aeration End 2			x	x	x

The COD and phosphorus experiments were done by filtered samples. For filtering "Millipore Millex –HV, Hydrophilic PVDF 0.45 µm" filters were used and one filter was only used for one time.

Adding to these experiments, anions and sometimes cations were measured for the micro nutrients. They were also measured by filtered samples. The anions and cations were analyzed in Ion Chromatograph.

Sludge Volume Index (SVI) was not measured during the experiments. It was only monitored.

3.2.2 Batch experiments

To produce microorganisms which can store Polyhydroxyalkonates (PHA) some batch experiments were done with the wasted sludge discharged from the SBRs at the end of the aerobic phase. For the experiments a bioreactor called " Bioflow 110/Fermentaor " was used. It is shown in Figure 3.2.

For Batch experiments only sodium acetate or acetic acid was used as a carbon source. Different amounts of COD were given to the system in every set. The SS and VSS results, PO_4-P amounts, the other micro nutrients and PHA experiments were done from batch tests. They are shown in Table 3.9 below. For measurements on the 0, 15, 30, 60, 90, 120 and 180th minutes samples were taken from the bioreactor. The results will be given in Chapter 4.

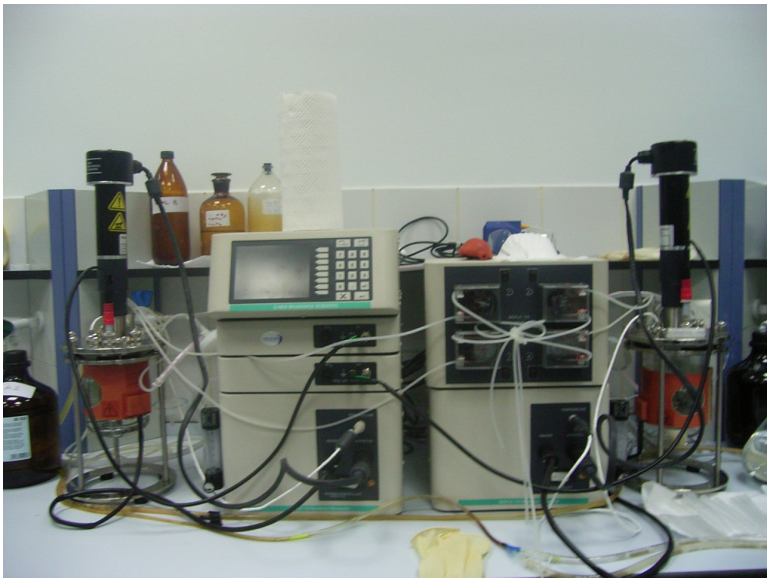


Figure 3.2 The Bioreactor

Table 3.9 Daily Monitoring of Batch Experiments

Measurement minutes	SS	VSS	PO	COD	pH	PHA
0	x	x	x	x	x	x
15			x	x	x	x
30			x	x	x	x
60			x	x	x	x
90			x	x	x	x
120			x	x	x	x
180			x	x	x	x

No inorganic solutions were added to the system because the sludge was taken from the SBRs. So it was accepted that there was enough micro nutrients in the sludge.
For the anaerobic experiments to eliminate the oxygen in the sludge N_2 was passed through it and put into the bioreactor for batch tests.

3.3 Analysis Methods

3.3.1 SS and VSS Analysis

The SS and VSS experiments were done with filtration set by using AP40 filters according to the Standard Methods, 1998. At first the filters were left into the sterilizer for an hour and then 10 ml of the sample taken from the mixing phase was filtered by the set. The filter paper with sample was left again to the sterilizer for one

hour. The filter was left into the desicator for 15-30 minutes. After this period SS was measured. To find VSS the filter was left into the oven for 30 minutes. Then the VSS was measured.

3.3.2 pH Analysis

At the beginning of the daily experiments in SBRs pH was not controlled but measured rarely .In the SET 6 and SET 7 it was measured.
To measure the pH, pH meter was used.

3.3.3 COD Analysis

All the COD s were performed with closed reflux method according to the Standard Methods, 1998. 1- 2.5 ml filtered samples were used for the experiments. 1.5 ml of potassium dichromate (KCr_2O_7) and 1.5 ml sulphuric acid with silver (Ag_2SO_4) were added to the COD tube over the sample. This waited in the thermal block for two hours at 150 ° C. After cooling titration part took place. Ferroin was used for indicator and DAS ($(NH_4)Fe(SO_4).6H_2O$) was used for titration. For daily experiments in SBRs COD was mostly measured.

COD samples were heated with "VLM $_{HP2}$ "thermal block and titrations were done in Environmental Biotechnology Laboratory. They are shown in Figure 3.3 and Figure 3.4 below.

Figure 3.3 COD Thermal Block

Figure 3.4 COD Titration Device

3.3.4 PO_4-P Analysis

PO_4-P analyses were done in two ways. One of them was Tin chloride ($SnCl_2$) method which was used for daily measurements and the other way was with Ion Chromatograph "DİONEX".

For $SnCl_2$ Method ammonium molibdate was also used for coloring. 5 ml of a sample was taken and put into a pot. Then 0.5 ml of $SnCl_2$ and 4 ml of ammonium molibdate was added to the pot. After 10-12 minutes samples were put into a spectra photometer "Pharmacia LKB" with a wave light of 690 nm which can be seen in Figure 3.5.

3.3.5 Anions and cations analysis

The Cation and Anion Analysis were done in Ion Chromatograph "DİONEX.

Figure 3.5 The Spectra photometer for PO_4-P Analysis

3.3.6 PHA Analysis

PHA Analyses were done in the bioreactor with the sludge taken from the SBR systems. For measuring PHA 10 ml of samples were taken and a few drops of formaldehyde was added to the PHA tubes to prevent biological activity. Then samples were taken and put into the centrifuge device to discard liquid phase. After that 4.5- 5 ml of phosphate buffer was added to the PHA tubes. The phosphate buffer includes:

PO_4 Buffer : (K_2HPO_4 = 0.58 g/l) + (KH_2PO_4 = 0.23 g/l)

Then the samples were put into vortex and centrifuged again. After centrifugation the water was wasted and the remained sludge was freezed at -20 ºC to increase the extraction efficiency.

The samples were freeze dried for 48 hours at -50ºC in the freeze drier "Thermo Savant Modulyo D-230 " which is shown in Figure 3.6 .

Figure 3.6 Freeze Dryer

Then the freeze dried samples were heated at 100°C for 2 hours by adding 1.5 ml hydrochloric acid and 1.5 ml 1,2 dichloro ethane for extraction. They were cooled down and added 3 ml of water. After that they were centrifuged for 5 min at 2500-3000 rpm.

Then they were measured in GC.

4. EXPERIMENTAL RESULTS

In this study 7 different SETS were carried out. The daily results and cycle measurements were measured from the SBRs and the PHA measurements were performed by batch experiments with the sludge taken from the SBRs. So the results will be examined in batch, cycle and daily measurements. Each SET refers to a different operational condition of the SBRs.

In Tables there are some abbreviations which are shown below.

A.E.1 : Aeration End of the previous cycle,

Inf. : Influent of the wastewater,

F.E. : Feeding End,

M.E. : Mixing End, The end of the anaerobic period,

A.E.2 : Aeration End of that cycle.

4.1 SBR Experiments

4.1.1 Daily measurements

There were seven different SBRs operated during the SETs. Each of them will be explained in detail.

4.1.1.1 SET 1

The sludge was taken from a treatment plant treating domestic sewage from Fatih University.

The first SET was operated 114 days. The sludge age of the reactor was selected as ten days ($\theta x = 10$ days). On the 10 th day 1000 ml of sludge was disposed and till the 13 th day 800 ml of sludge was disposed daily.

On the 68 th day of the measurements the room temperature had fallen to 10 ^{o}C and this condition continued until the 78 th day.

Then the room temperature remained at 17 ^{o}C. When the temperature decreased the MLSS had also decreased and parallel to this turbidity had increased.

For receiving a constant level of influent phosphorus Solution A_1 was used in SET 1. Also to maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.5 and 3.7. 2 mg/ l of Sol B and 1 mg/l Sol A_1 were added to the tab water per liter.

Carbon source 1 which is given in Table 3.2 was used as a carbon source to achieve an influent of 400 mg/l COD.

SET 1‘s operational conditions are given in Table 4.1.

To control of biological systems SS and VSS was observed. To achieve a PAO system, phosphorus was measured in daily experiments. COD was measured and pH was not controlled during the experiments.

The daily measured experimental results are given in Table 4.2.

Table 4.1 Operational Conditions of SET 1

PARAMETER	SYMBOL	VALUE	UNIT
Cycle Time	Tc	6	h
Duration of filling period	T_F	15	min
Duration of anaerobic (mixing) period	T_{AN}	2	h
Duration of aerobic (aeration) period	T_A	3	h
Duration of settling period	T_S	30	min
Duration of withdrawn period	T_w	15	min
Duration of idle period	Ti	15	min
Initial volume	V_0	4	l
Total volume	V_T	8	l
V_F/V_0	-	-	0.5
Hydraulic Retention Time	HRT	12	h
Sludge Retention Time	SRT	10	days
Temperature *	t	20 ±2	^{O}C

*For 10 days the temperature was around 10 ºC

Table 4.2. Daily Experimental Results for SET 1 (1)

PARAMETER		**PO_4 – P (mg/l)**					**COD (mg/l)**					**SS (mg/l)**	**VSS(mg/l)**	**VSS/SS**
SAMPLE PLACE		A.E 1	Inf	F.E.	M.E	A.E.2	A.E 1	Inf	F.E.	M.E	A.E.2	M.E	M.E	M.E
DAYS	1											1095	1000	0,913
	6											1882	1676	0,89
	10											1930	1800	0,933
	13											1990	1820	0,915
	14											1650	1570	0,952
	15							400		444	209	1670	1590	0,952
	16											1570	1510	0,962
	17											1610	1460	0,907
	20											1440	1380	0,958
	21											1500	1280	0,853
	22											1667	1533	0,919
	23		23,01		31,11	23,11						1360	1260	0,927
	24											1570	1540	0,98
	27											1450	1310	0,904
	28											1470	1430	0,973
	29											1290	1180	0,915
	31											1440	1340	0,931
	34											1580	1550	0,981
	35											1670	1570	0,94
	38		11,96		10,91	8,64						1260	1200	0,952
	41		6,61		24,09	7,12						1550	1440	0,929

Table 4.2. Daily Experimental Results for SET 1 (2)

PARAMETER		PO_4 – P (mg/l)					COD (mg/l)					SS (mg/l)	VSS(mg/l)	VSS/SS
SAMPLE PLACE		A.E 1	Inf	F.E.	M.E	A.E.2	A.E 1	Inf	F.E.	M.E	A.E.2	M.E	M.E	M.E
DAYS	42				2,32	9,55						1550	1440	0,929
	43				6,18	4,42						1580	1560	0,987
	48											1550	1440	0,929
	57											1690	1540	0,911
	58		7,32		5,18	4,28						1550	1500	0,967
	59		7,36		5,18	4,28						1390	1360	0,978
	62		7,06		5,8	4,35						1720	1680	0,977
	63		7,55		7,31	2,08						1710	1530	0,895
	64		5,64		4,3	2,74						1450	1400	0,965
	65		8,46		3,13	3,92						1620	1500	0,926
	66		7,18		4,8	4,2						1660	1590	0,958
	69											1360	1320	0,971
	71		6,66		5,07	2,05						1500	1480	0,987
	73											1410	1330	0,943
	77		7,99	6,01	5,82	4,62		400	267	214	60	730	710	0,973
	77		7,87	6,2	6,08	4,45		400	206	204	35			
	78		7,9	6,17	5,99	4,7		400	215	200	34	720	690	0,958
	78		7,95	5,65	6,35	5,13		400	223	216	77			
	84											460	420	0,913
	94											600	590	0,983
	97											960	950	0,989
	104		6,52	4,27	4,37	0,71		400	208	153	50	1870	1860	0,995
	111											970	950	0,979

4.1.1.2 SET 2

The sludge was taken from Paşaköy Advanced Biological Treatment Plant which was treating domestic sewage .

SET 2 was operated for 54 days. The sludge age of the reactor was selected as ten days (θx = 10 days) and discharged from the first day of the SBR. But from the 13 th day to 35th day no sludge was disposed.

The reactor was set up at 8 ^{o}C because of the conditions in the laboratory until the 26 th day. Then it was around 24 ^{o}C.

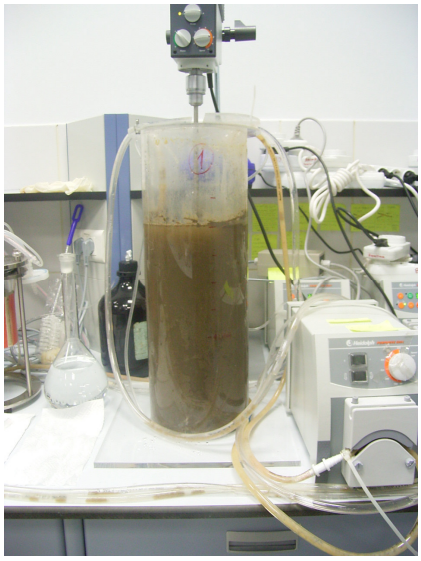
For receiving a constant level of influent phosphorus Solution A_1 was also used in SET 2. The influent phosphorus was 8 mg/l. To maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.5 and 3.7. 2 mg/ l of Sol B and 1 mg/l Sol A_1 were added to the tab water per liter.

The influent COD was 400 mg/l and Carbon source 1 that was shown in Table 3.2, was used for this SET.

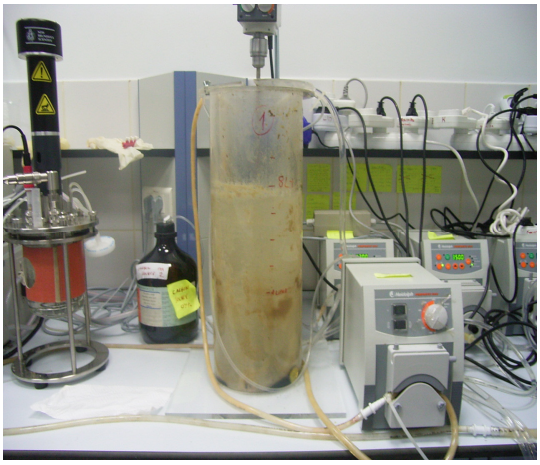
The operational conditions of SET 2 are the same as SET 1 which is given in Table 4.1 except for the first 26 days the temperature was around 8 ^{O}C.

For SET 2 phosphorus, COD, SS and VSS parameters were measured. The daily experimental results are shown in Table 4.3.

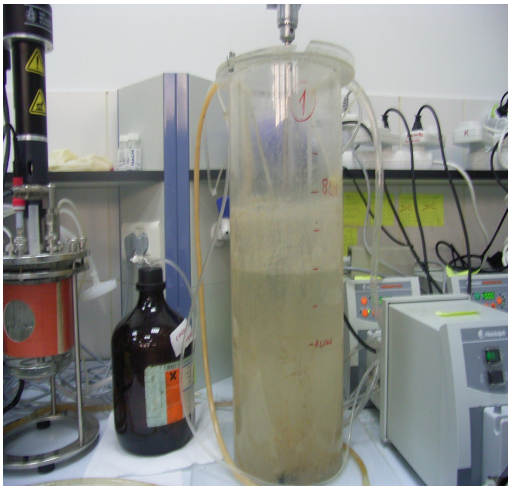
In Figure 4.1 the pictures of SBR is shown on different days such as Day 1, Day 24, Day 42 and Day 54.



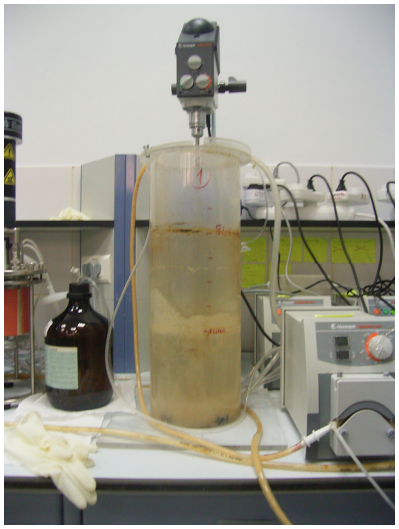
(a) Day 1, Mixing



(b) Day 24, Mixing



(c) Day 42,Mixing



d)Day54,Settling

Figure 4.1 The Pictures of SBRs in SET 2 (a) Day 1 , (b) Day 24 ,(c) Day 42 , (d) Day 54

Table 4.3 Daily Experimental Results of SET 2

PARAMETER		**PO4-P (mg/l)**				**COD (mg/l)**				**SS (mg/l)**	**VSS(mg/l)**	**VSS/SS**
SAMPLE PLACE		Inf	F.E.	M.E	A.E.2	Inf	F.E.	M.E	A.E.2	M.E	M.E	M.E
DAYS	1	10,56		10,15	8,2					1762	1040	0,59
	11	7,26	4,92	10,99	3,89	400	214	199	67	560	360	0,643
	12	7,2	5,95	4,08	3,42	400	281	225	144	470	220	0,468
	13	7,6	6,15	7,18	4,54	400	227	105	69	330	270	0,818
	15	5,2	5,54	4,97	3,74							
	25									600	50	0,083
	40											
	41	11,3	9,74	10,2	9,53	400	419	401	302	310	290	0,935
	43									620	600	0,968
	48					400	220	275	45			
	49	8,25	7,62	7,1	6,89	400	222	215	102	750	710	0,947

4.1.1.3 SET 3

The sludge was taken from Paşaköy Advanced Biological Treatment Plant treating domestic sewage.

SET 2 and SET 3 was operated parallel so as a result of this situation most of their conditions were same.

SET 3 was operated for 54 days. The sludge age of the reactor was selected as ten days (θx = 10 days) and discharged from the first day SBR. But from the 13 th day to 35th day no sludge was disposed.

The reactor was set up at 8^{o}C because of the conditions in the laboratory until the 26 th day. Then it was around 24^{o}C.

For receiving a constant level of influent phosphorus Solution A_2 was used in SET 3. The influent phosphorus was 16 mg/l. To maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.6 and 3.7. 2 mg/ l of Sol B and 1 mg/l Sol A_1 were added to the tab water per liter.

The influent COD was 400 mg/l and Carbon source 1 that was shown in Table 3.2, was used for this SET.

The operational conditions of SET 3 are same as SET 1 and SET 2 which is given in Table 4.1.

For SET 3 phosphorus, COD, SS and VSS parameters were measured. The results are shown in Table 4.4.

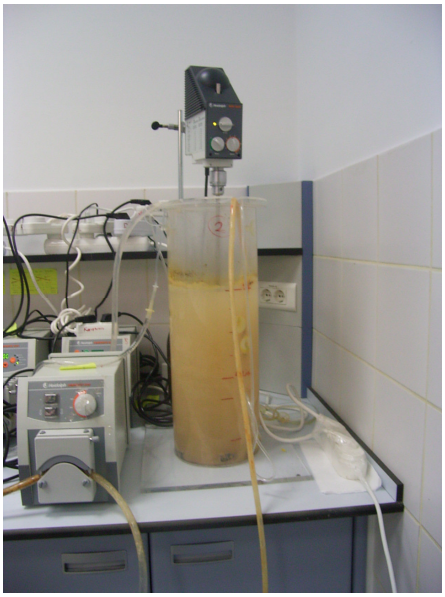
The pictures of SBR in SET 3 are given as Day1 in (a) ,Day 24 in (b) and the last day, Day 54 .

Table 4.4 Daily Experimental Results of SET 3

PARAMETER		**PO4-P (mg/l)**				**COD (mg/l)**				**SS (mg/l)**	**VSS(mg/l)**	**VSS/SS**
SAMPLE PLACE		Inf	F.E.	M.E	A.E.2	Inf	F.E.	M.E	A.E.2	M.E	M.E	M.E
DAYS	1	14,5	12,78	11,3	10,9					1850	1078	0,583
	11	14,6	12,6	6,66	9,95	400	224	277	32	1110	940	0,847
	12	16,76	13,33	12,33	12,87	400	199	198,6	32	940	860	0,915
	13	5,36	21,6	13,9	12,1	400	141	36	21	720	700	0,972
	15	15,18	7,59	6,65	7,84							
	25									1190	810	0,681
	40									1750	1730	0,989
	41	17,6	16,12	18,28	16,48	400	252	245	13	1380	1360	0,986
	43									1340	1300	0,97
	48					400	220	220	52			
	49	12,28	14,08	10,82	10,08	400	210	282	72	880	800	0,909

(a) Day 1, Mixing

(b) Day 24, Mixing



(c) Day 54, Mixing

Figure 4.2 The Pictures of SBRs in SET 3,(a) Day 1 , (b) Day 24 ,(c) Day 54

4.1.1.4 SET 4

The sludge was taken from Paşaköy Advanced Biological Treatment Plant treating domestic sewage.

SET 4 and SET 5 was operated parallel so as a result of this situation most of their conditions were same.

SET 4 was operated for 15 days. The sludge age of the reactor was selected as ten days ($\theta x = 10$ days) and discharged from the first day SBR.

The reactor was set up at 20 ^{o}C under the laboratory conditions .

For receiving a constant level of influent phosphorus Solution A_2 was used in SET 4. The influent phosphorus was 16 mg/l. To maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.6 and 3.7. They were the same amounts with SET 1, 2, 3.

The influent COD was 400 mg/l and Carbon source 1 that was shown in Table 3.2, was used for this SET.

The operational conditions of SET 4 are given in Table 4.5. .

For SET 4 phosphorus, COD, SS and VSS parameters were measured. The daily measurements experimental results are shown in Table 4.5.

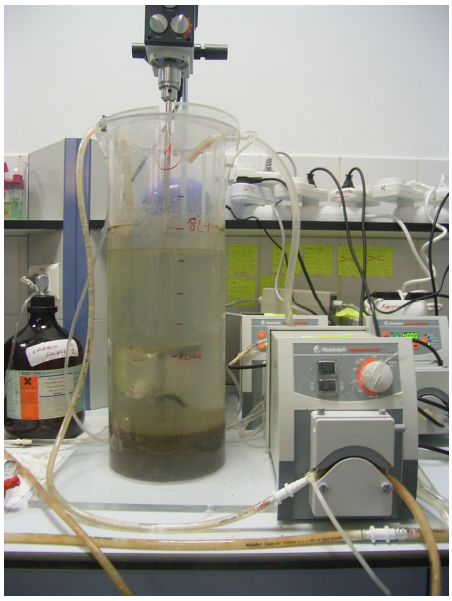
The pictures of SBR are shown in Figure 4.3.

Table 4.5 Operational Conditions of SET 4

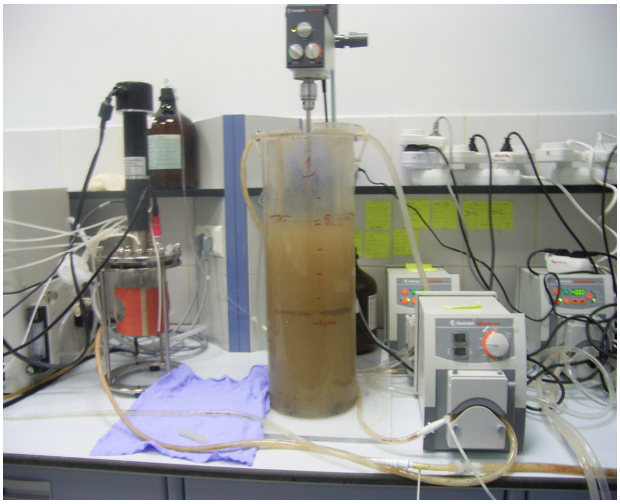
PARAMETER	SYMBOL	VALUE	UNIT
Cycle Time	Tc	6	h
Duration of filling period	T_F	15	min
Duration of anaerobic (mixing) period	T_{AN}	3	h
Duration of aerobic (aeration) period	T_A	2	h
Duration of settling period	T_S	30	min
Duration of withdrawn period	T_w	15	min
Duration of idle period	Ti	15	min
Initial volume	V_0	4	l
Total volume	V_T	8	l
V_F/V_0	-	-	0.5
Hydraulic Retention Time	HRT	12	h
Sludge Retention Time	SRT	10	days
Temperature	t	20 ±2	^{O}C

Table 4.6 Daily Experimental Results of SET 4

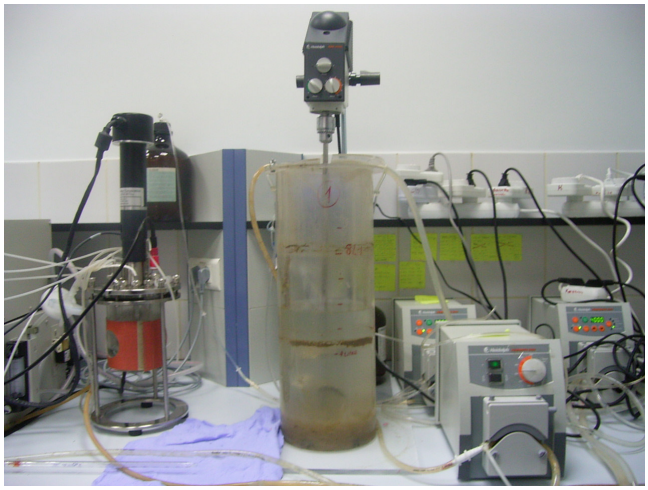
PARAMETER		**PO4-P (mg/l)**				**COD (mg/l)**				**SS (mg/l)**	**VSS(mg/l)**	**VSS/SS**
SAMPLE PLACE		Inf	F.E.	M.E	A.E.2	Inf	F.E.	M.E	A.E.2	M.E	M.E	M.E
DAYS	1									1500	890	0,593
	3	16,5	14,51	16,97	12,5	400	468	365	237	1571	850	0,541
	9	16,05	14,74	14,13	12,56	400	305	338	261	770	710	0,922
	10	16,5	14,63	15	14,6	400	320	358	36	600	570	0,95
	11	16,43	15,2	13,8	12,7	400	395	430	215	470	410	0,872



(a) Day 4 ,Settling



(b) Day 9 ,Mixing



(c) Day 9,Settling

Figure 4.3 The Pictures of SET 4, (a) Day 4, Settling , (b) Day 9, Mixing, (c) Day 9, Settling

4.1.1.5 SET 5

The sludge was taken from Paşaköy Advanced Biological Treatment Plant treating domestic sewage.

SET 4 and SET 5 was operated parallel so as a result of this situation most of their conditions were same.

SET 5 was also operated for 15 days. The sludge age of the reactor was selected as ten days ($\theta x = 10$ days) and discharged from the first day SBR.

The reactor was set up at $20^{o}C$ under the laboratory conditions.

On the 9^{th} day the reactors had overflowed for 2 times.

For receiving a constant level of influent phosphorus Solution A_2 was used in SET 4. The influent phosphorus was 16 mg/l. To maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.6 and 3.7.

The influent COD was 400 mg/l and Carbon source 2 that was shown in Table 3.3, was used for this SET. In carbon source 2 there was o glutamic acid.

The operational conditions of SET 5 was the same as SET 4 .They are given in Table 4.5..

For SET 5 phosphorus, COD, MLSS and MLVSS parameters were measured. The results are shown in Table 4.7.

The pictures of SBR are shown in Figure 4.4.

Table 4.7 Daily Experimental Results of SET 5

PARAMETER		**PO4-P (mg/l)**				**COD (mg/l)**				**SS (mg/l)**	**VSS(mg/l)**	**VSS/SS**
SAMPLE PLACE		Inf	F.E.	M.E	A.E.	Inf	F.E.	M.E	A.E.2	M.E	M.E	M.E
DAYS	1									1500	890	0,593
	3	24,5	15,44	15,08	14,18	400	349	315	265	1500	680	0,453
	9	16,2	13,56	12	12,3	400	240	368	12	460	430	0,935
	10	16,1	17,7	12,9	12	400	190	220	46	270	210	0,777
	11	14,4	12,6	12,1	10,9	400	224	279	80	40	20	0,5

(a) Day 4, Settling

(b) Day 4,Mixing

(c) Day 9, Mixing

Figure 4.4 The Pictures of SET 5

(a) Day 4,Settling , (b) Day 4,Mixing ,(c) Day 9 ,Mixing

4.1.1.6 SET 6

The sludge was taken from Paşaköy Advanced Biological Treatment Plant treating domestic sewage.

SET 6 and SET 7 was operated parallel so as a result of this situation most of their conditions were same.

SET 6 was also operated for 30 days. The sludge age of the reactor was selected as ten days ($\theta x = 10$ days) and discharged from the first day SBR.

The reactor was set up at $20^{o}C$ under the laboratory conditions.

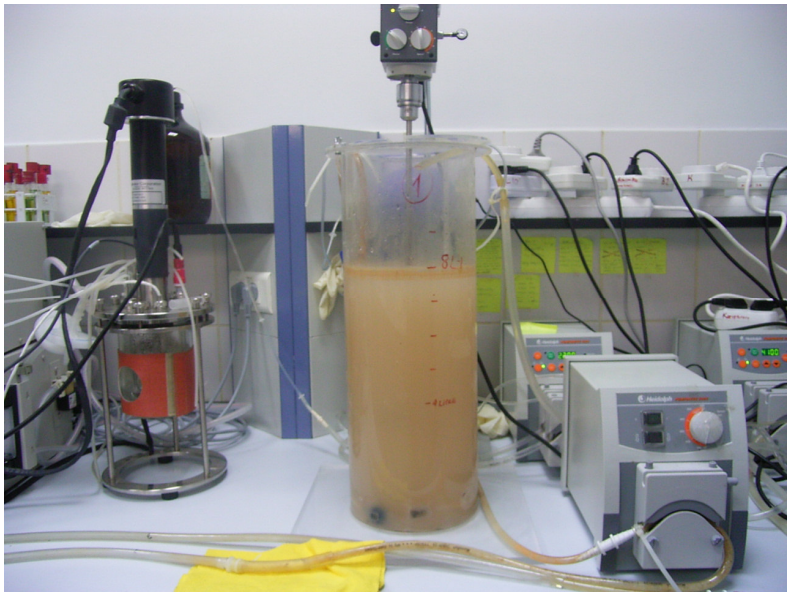
For receiving a constant level of influent phosphorus Solution A_2 was used in SET 6. The influent phosphorus was 16 mg/l. To maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.6 and 3.7.

The influent COD was 400 mg/l and Carbon source 1 that was shown in Table 3.2, was used for this SET.

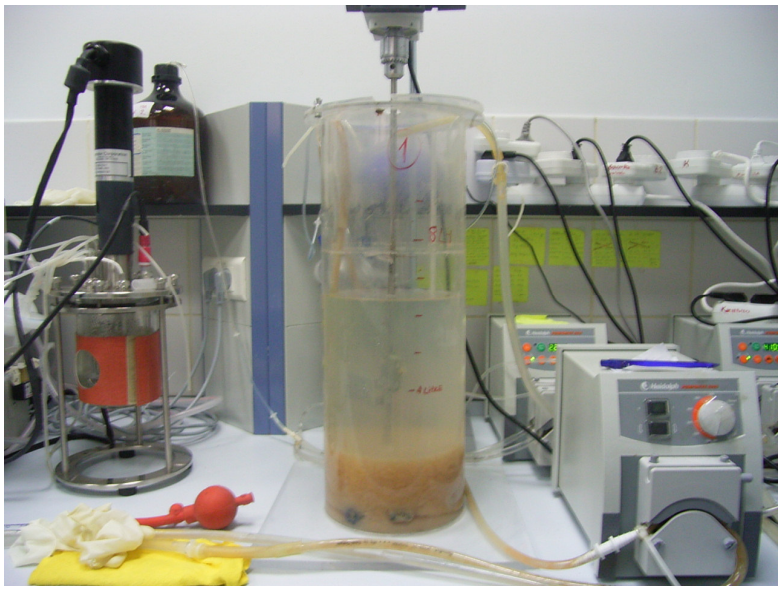
The operational conditions of SET 6 were the same as SETS 4 and 5 given in Table 4.5.

For SET 6 phosphorus, pH, COD, SS and VSS parameters were measured. The results are shown in Table 4.8.

The pictures of SBR are shown in Figure 4.5.

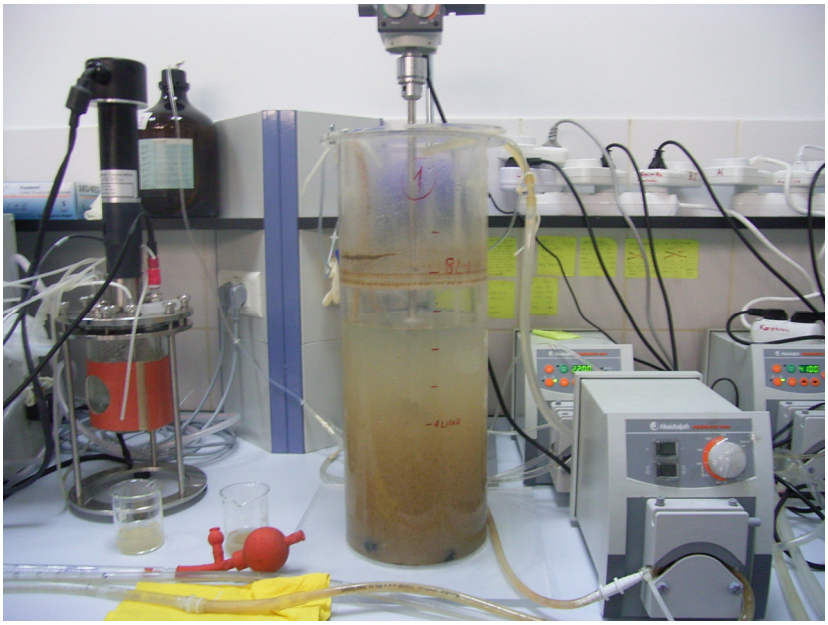


(a) Day 10, Mixing



(b) Day 10, Settling

Figure 4.5 The Pictures of SET 6, (a) Day,10 Mixing and (b) Day 10, Settling



(a) Day 22, Settling

(b) Day 30, Mixing

Figure 4.6 The Pictures of SET 6, (a) Day 22, Settling and (b) Day 30, Mixing

Table 4.8 Daily Experimental Results of SET 6

PARAMETER		Ph					PO_4 - P (mg/l)					COD (mg/l)					SS (mg/l)	VSS (mg/l)	VSS/SS
SAMPLE PLACE		A.E 1	Inf	F.E.	M.E	A.E.2	A.E 1	Inf	F.E.	M.E	A.E.2	A.E 1	Inf	F.E.	M.E	A.E.2	M.E	M.E	-
DAYS	2	7,34	7,03	7,35	7,07	7,39	14,69	13,79	14,43	13,77	13,56	155,6	400	259,4	242,1	201,7	1620	900	0,555
	7	7,02	-	7,05	6,75	7,01							400				1330	1190	0,897
	8						12,56	16,66	16,56	13,77	12,64	-	400	167,9	116,8	43,8	1230	1100	0,894
	9				6,97					15,74			400						
	10												400				1220	1140	0,934
	11	7,35	-	5,28	6,04	6,63	12,03	14,01	14,01	13,23	10,72	236,5	400	248,2	154,8	35	1230	1150	0,935
	18	6,7	3,03	1,54	6,4	6,73	5,44	9,21	9,23	10,64	5,44	36,5	400	416	226,3	72,9	830	760	0,916
	21	7,2	-	7,58	7,36	7,6	6,31	9,69	11,16	11,69	10,14	423?	400	248,2	240,9	29,2	1040	990	0,952
	23	7,32	-	6,91	7,41	6,79	6	8,74	8,28	6,1	6,38	15,4	400	161,3	145,9	7,8	790	720	0,911
	25	6,79	-	6,82	6,69	6,75	6,9	9,59	7,92	8,66	6,64	76,8	400	376	222,6	92,1	490	470	0,959
	28	7,2	-	7,31	7,03	7,18	7,59	11,3	10,59	10,87	8,38	23	400	214,9			560	510	0,911

The sludge was taken from Paşaköy Advanced Biological Treatment Plant treating domestic sewage.

SET 7 was operated for 30 days. The sludge age of the reactor was selected as ten days ($\theta x = 8$ days) and discharged from the first day SBR.

The reactor was set up at $20^{o}C$ under the laboratory conditions.

For receiving a constant level of influent phosphorus Solution A_2 was used in SET 7. The influent phosphorus was 16 mg/l. To maintain the phosphorus removal mechanism the necessary micro nutrients were added to the system with Solution B. Their compounds are given in Tables 3.6 and 3.7.

The influent COD was 400 mg/l and Carbon source 3 that was shown in Table 3.4, was used for this SET.

The operational conditions of SET 7 were the same as SETS 4, SET 5 and SET 6 given in Table 4.5.

For SET 7 phosphorus, pH, COD, SS and VSS parameters were measured. The results are shown in Table 4.9.

The pictures of SBR are shown in Figure 4.7 and Figure 4.8 on different days.

Figure 4.7 The Pictures of SET 7, Settling and Day 22 Settling

(a) Day 22, Mixing

(b) Day 30, Mixing

Figure 4.8 The Pictures of SET 7, (a) Day 22, Mixing and (b) Day 30, Mixing

Table 4.9 Daily Experimental Results of SET 7

PARAMETER		Ph					PO_4 - P (mg/l)					COD (mg/l)					SS (mg/l)	VSS (mg/l)	VSS/SS
SAMPLE PLACE		A.E 1	Inf	F.E.	M.E	A.E.2	A.E 1	Inf	F.E.	M.E	A.E.2	A.E 1	Inf	F.E.	M.E	A.E.2	M.E	M.E	-
DAYS	2	7,67	7,28	7,34	7,41	7,72	18,21	19,51	19,91	19,87	18,85	149,8	400	236,3	247,8?	219	1510	600	0,397
	7												400				1930	1490	0,772
	8	7,7	-	7,53	7,49	7,75	13,1	16,72	15,46	15,99	13,2	-	400	153,3	146	14,6	1460	1240	0,849
	9				7,51					16,97			400						
	10												400				1540	1320	0,857
	11	7,69	-	7,35	7,36	7,49	13,33	14,36	13,54	14,87	11,99	90,5	400	210,2	210,2	26,3	1660	1540	0,928
	18	7,4	7,7	6,79	7,49	7,64	6,95	9,54	8,33	9,99	6,9	94,9	400	204,4	233,6	43,8	1130	1040	0,92
	21	7,45	-	7,6	7,41	7,73	8,2	9,36	8,41	8,85	8,05	131,4	400	525,5	226,3	14,6	750	520	0,693
	23	7,17	7,35	7,48	7,58	7,38	6,9	10,05	8,41	8,64	7	23	400	238	92	30,7	1250	1230	0,984
	25	7,37	7,35	7,47	7,25	7,28	7,54	8,79	8,41	7,95	6,77	38,4	400	207,3	191,9	61,4	1010	960	0,95
	28	7,73	7,83	7,71	7,36	7,67	7,59	11,3	10,59	10,87	8,38	30,7	400	238	235,7	76,2	900	770	0,855

4.1.2 Cycle measurements

Besides daily measurements also cycle measurements were done. In this section an example for these measurements will be shown with graphics for each SET.

4.1.2.1 SET 1

The experiments were done on the 78th day. The results are in Table 4.10 and Figure 4.9 given below.

Table 4.10 Cycle Measurements of SET 1

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	7,9	400		
Time (min)	0	4,6	38		
	15	6,17	215		
	30	6,10	212		
	60	6,18	208		
	90	6,03	202		
	120	5,99	200	720	690
	180	5,3	132		
	240	4,9	86		
	300	4,7	34		

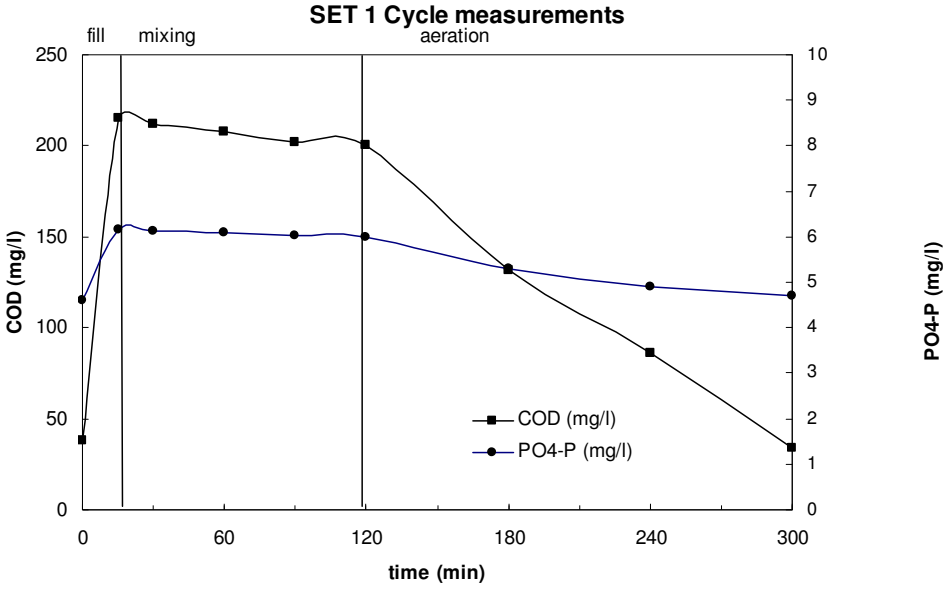


Figure 4.9 Cycle measurements of SET 1

In SET 1 Solution A_1 which had an influent of 8 mg/l. phosphorus, was used. The measured influent phosphorus was 7,9 mg/l and influent COD was 400 mg/l. The cycle measurements were performed for 5 hours. The anaerobic phase took place for 2 hours. The initial phosphorus amount was 4,6 mg/l and the initial COD was 38 mg/l in the reactor at the beginning of the cycle. After feeding phosphorus amount increased to 6,17 mg/l. COD was 215 mg/l in the reactor. As it can be seen from the Figure 4.9 that phosphorus was not released in the anaerobic zone. At the end of the anaerobic phase it was 5,99 mg/l. Parallel to this situation COD was not depleted either in the anaerobic zone. 215 mg/l COD decreased to 200 mg/l at the end of 2 hours. But as it can be observed from the measured values of the experiments COD was depleted from 200 mg/l to 34 mg/l in the aerobic phase. Also phosphorus values decreased from 5,99 mg/l to 4,7 mg/l at the end of the aerobic phase. During cycle measurements of SET 1 on the 78th day of the reactors Suspended solids value was 720 mg/l and Volatile Suspended Solids was 690 mg/l. As a result of this cycle measurements it can be seen that COD was depleted in the aerobic phase instead of anaerobic phase and phosphorus release did not occur in the anaerobic phase.

4.1.2.2 SET 2

The experiments were measured on the 14th day of the reactors. The results are given in Table 4.11 and Figure 4.10 below. Suspended solids value was 520 mg/l and Volatile Suspended Solids was 480 mg/l in cycle measurements.

Table 4.11 Cycle Measurements of SET 2

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	7,12	400		
Time (min)	0	3,35	78		
	15	6,15	227		
	30	6,12	220		
	60	6,56	157		
	90	7,22	122		
	120	7,18	105	520	480
	180	6,78	77		
	240	5,4	72		
	300	4,54	69		

Solution A_1 was also used in this SET and as a result in SET 2 the influent PO_4-P was 7,12 mg/l and the effluent PO_4-P was 3,35 mg/l coming from the previous cycle. After 15 minutes of filling period the PO_4-P was 6,15 mg/l. Although PO_4-P was decreased 0,03 mg/l after 30 minutes, at the end of 60 minutes, it increased from 6,12 mg/l to 6,56 mg/l. The increase of PO_4-P continued during the next hour and half an hour later it increased to 7,22 mg/l. As it can be seen from the Figure that at the end of the anaerobic phase PO_4-P was 7,18 mg/l. As parallel to this COD depletion occurred about 122 mg/l. At the end of the filling period it was 227 mg/l and one hour later it decreased to 157 mg/l. At the end of the anaerobic phase it became 105 mg/l.

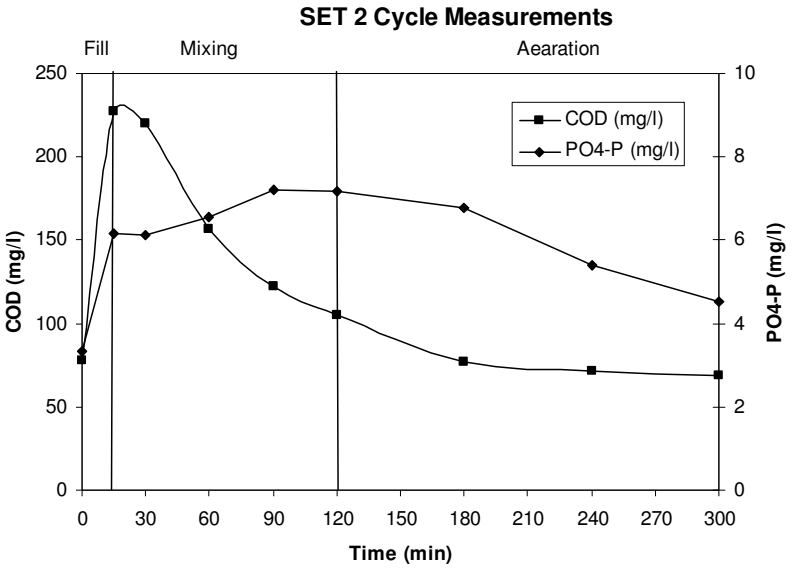


Figure 4.10 Cycle Measurements of SET 2

As it can be seen from the Figure COD was not much left for the aerobic phase. Only 36 mg/l of COD was depleted in the aerobic phase. To give more detail, it was 105 mg/l at the beginning of the aerobic phase. For the next hours COD was 77 mg/l, 72 mg/l and finally 69 mg/l at the end of aerobic phase. PO_4-P also increased during aerobic phase. At the end of the aerobic phase it became 4,54 mg/l as an effluent value.

4.1.2.3 SET 3

The experiments were done on the 40th day. The results are in Table 4.12 and Figure 4.11 given below.

Table 4.12 Cycle Measurements of SET 3

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	17,6	400		
Time (min)	0	15,2	35		
	15	16,12	252		
	30	16,58	262		
	60	17,42	242		
	90	17,55	250		
	120	18,28	245	1380	1360
	180	18,22	76		
	240	16,35	38		
	300	16,48	13		

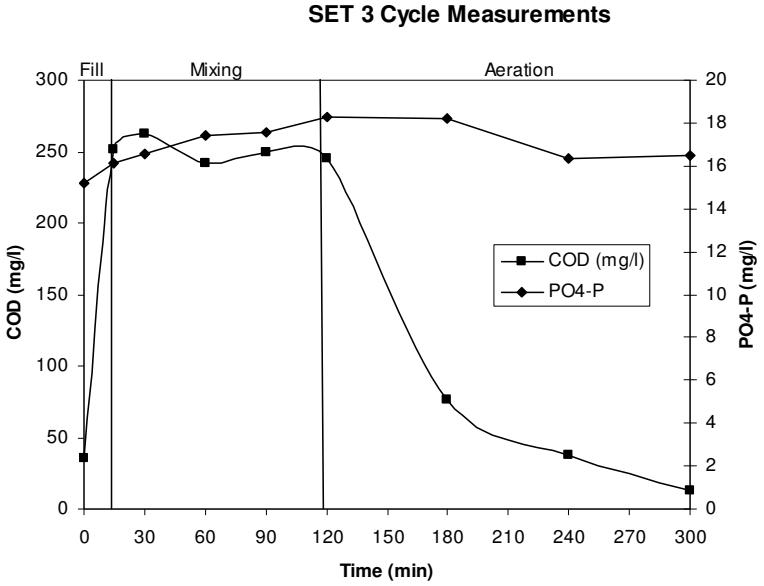


Figure 4.11 Cycle Measurements SET 3

In SET 3 as it was explained previously Solution A_2 was used as PO_4-P source. So the initial PO_4-P was doubled. The influent PO_4-P was 17,6 mg/l. The effluent value of PO_4-P was 15,2 mg/l which was coming from the previous cycle. At the end of the

filling period the PO_4-P value became 16,12 mg/l. As it can be seen from the figure, 2,16 mg/l PO_4-P was released during anaerobic phase. At the end of anaerobic phase the PO_4-P was 18,28 mg/l. However COD depletion was not much when it is compared to PO_4-P. The COD value after filling was 252 mg/l and only 17 mg/l COD was depleted during the anaerobic phase. But in aerobic phase COD depletion was 245 mg/l to 13 mg/l. The PO_4-P value decreased from 18,22 mg/l to 16,48 mg/l.

4.1.2.4 SET 4

The experiments were done on the 12th day. The results are in Table 4.13 and Figure 4.12 given below.

Table 4.13 Cycle Measurements of SET 4

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	14,8	400		
Time (min)	0	12.5	212		
	15	15,8	320		
	30	15,5	312		
	60	15,7	312		
	90	14,3	316		
	120	13,87	340		
	180	13,12	298	420	390
	240	13,18	255		
	300	12,9	206		

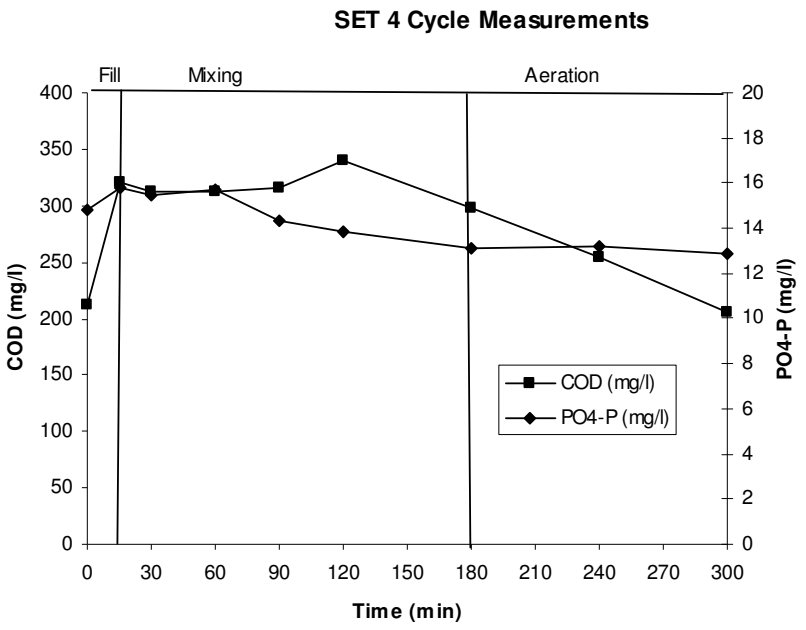


Figure 4.12 Cycle Measurements of SET 4

As SET 3, A_2 was used in SET 4.and the influent PO_4-P was 14,8 mg/l which can be seen in Table 4.13. The PO_4-P value of the previous cycle was 12,5 mg/l. After filling period it reached 15,8 mg/l. However no decrease in the PO_4-P amount was observed during anaerobic period. At the end of anaerobic period PO_4-P was 13,12 mg/l and at the end of the aerobic period it was 12,9 mg/l. COD values had interesting results. At the end of the filling phase it was 320 mg/l and increased to 340 mg/lat the end o the second hour of the anaerobic phase. But at the end of the anaerobic phase it became 298 mg/l. As a result only 92 mg/l of COD could be depleted during aerobic phase. During the day that the cycle measurements were performed suspended solids was 420 mg/l and volatile suspended solids was 390 mg/l.

4.1.2.5 SET 5

The experiments were done on the 11th day. The results are in Table 4.14 and Figure 4.13 given below. On the day which cycle measurements were performed, Suspended solids value was 40 mg/l and Volatile Suspended Solids was 20 mg/l. The influent PO_4-P was 14,4 (mg/l). As it can be seen from the figure very little phosphorus release occurred. After an hour, in the anaerobic period the PO_4-P became 13,2 (mg/l). At the end of the anaerobic phase it was 12,1 mg/l. Only 1,2

mg/l PO_4-P was removed at the end of aerobic phase and the value was 10,9 mg/l. By investigating the figure it can be seen that 224 mg/l COD increased to 279 mg/l COD at the end of the anaerobic phase. However COD was depleted under aerobic conditions .It decreased to 80 mg/l at the end of the aerobic phase of the cycle.

Table 4.14 Cycle Measurements of SET 5

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	14,4	400		
Time (min)	0	12,9	46		
	15	12,6	224		
	30	12,7	221		
	60	13,2	210		
	90	12,7	222		
	120	13,1	217		
	180	12,1	279	40	20
	240	11,5	106		
	300	10,9	80		

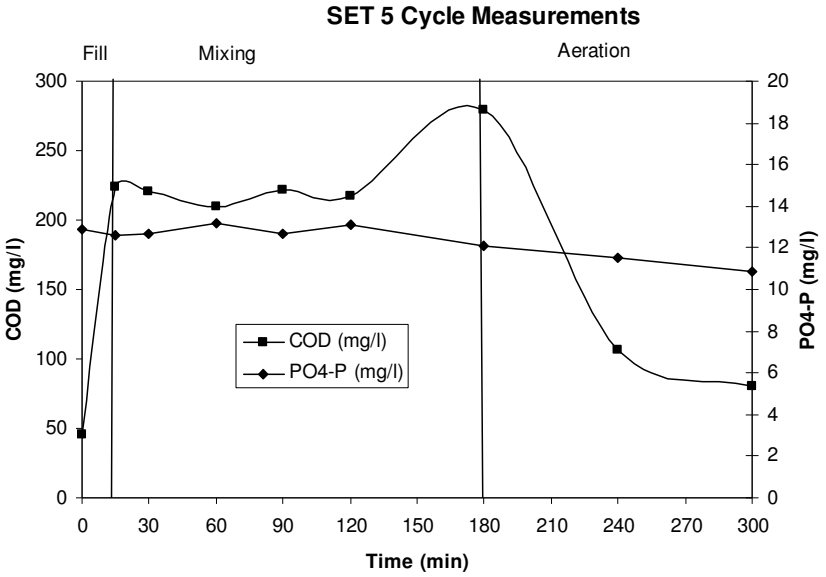


Figure 4.13 Cycle Measurements of SET 5.

4.14 given below. In SET 6 Solution A_2 which had an influent of 16 mg/l. phosphorus, was used, as a result the influent was PO_4-P 14,01 mg/l. The previous cycle's effluent PO_4-P was 12,03 (mg/l). After filling period only 0,35 mg/l PO_4-P was released in the anaerobic phase. After 30th minute it started to decrease. At the end of the anaerobic phase PO_4-P was 13,23 (mg/l). When the aerobic phase was completed PO_4-P was 10,72 (mg/l). COD was 248 mg/l at the end of filling phase. It decreased during the anaerobic phase. When the anaerobic phase ended it was 154 mg/l. The COD depletion was more in aerobic phase. In 2 hours 119 mg/l COD was depleted as it can be seen in figure. The Suspended solids value was 1230 mg/l and Volatile Suspended Solids was 1150 mg/l.

Table 4.15 Cycle Measurements of SET 6

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	14,01	400		
Time (min)	0	12,03	236		
	15	14,01	248		
	30	14,36	222		
	60	13,52	218		
	90	13,56	193		
	120	13,45	185		
	180	13,23	154	1230	1150
	240	10,65	92		
	300	10,72	35		

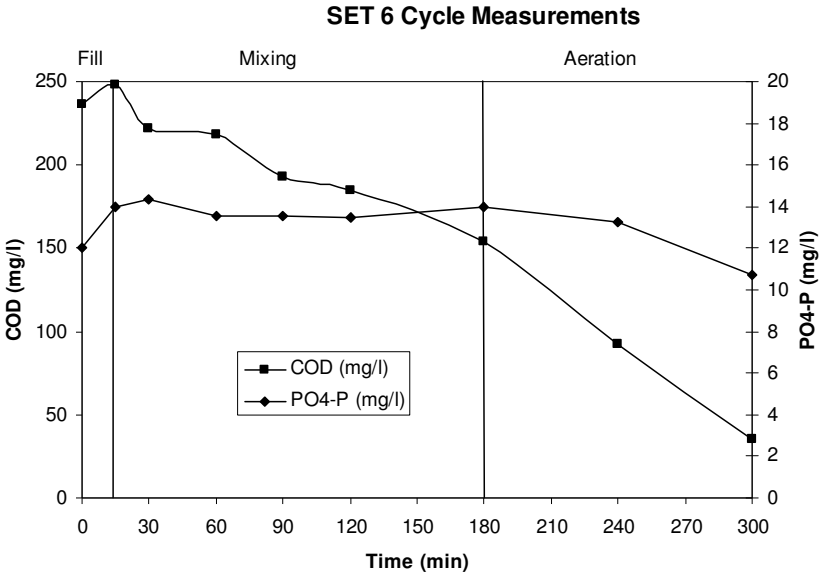


Figure 4.14 Cycle Measurements of SET 6

4.1.2.7 SET 7

The experiments were done on the 12th day. The results are in Table 4.16 and Figure 4.15 given below. In SET 7, very little PO_4-P release occurred. The influent PO_4-P was 16,76 (mg/l) and 13,1 mg/l PO_4-P was left in the reactor from the previous cycle. At the end of filling period PO_4-P was 16,46 (mg/l) and it increase from 16,13 to 16,89 mg/l. Two hours later, it decreased to 13,5 mg/l. as an effluent of the cycle. COD depletion did not occur in the anaerobic zone as it can be seen from the figure. The filling end COD was 221 mg/l and it could only decreased to 212 mg/l at the end of the anaerobic phase. However COD depletion occurred in the aerobic phase. 194 mg/l COD was depleted in the aerobic zone. Suspended solids value was 1440 mg/l and Volatile Suspended Solids was 1230 mg/l.

Table 4.16 Cycle Measurements of SET 7

PARAMETER		PO_4-P (mg/l)	COD (mg/l)	SS(mg/l)	VSS (mg/l)
Sample Place	Influent	16,76	400		
Time (min)	0	13,1	34		
	15	16,46	221		
	30	16,13	221		
	60	16,62	206		
	90	16,34	218		
	120	16,77	210		
	180	16,89	212	1440	1230
	240	15,3	75		
	300	13,5	18		

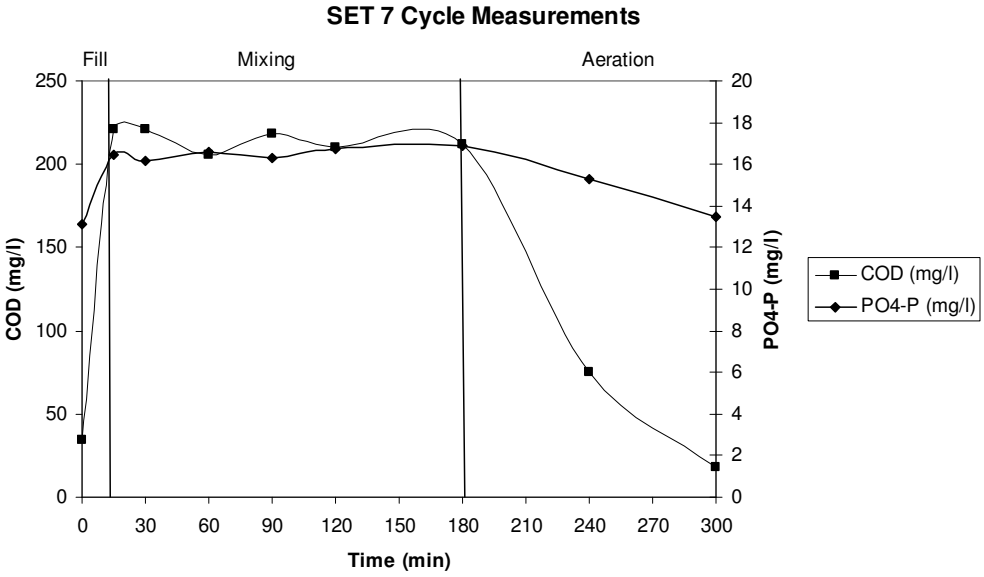


Figure 4.15 Cycle Measurements of SET 7

4.2 Batch Experiments

Besides SBR experiments, three batch experiments were also performed in order to observe the PHA storage capacity of the sludge. Two of them were performed with the sludge taken from the end of aerobic phase of SBR operation during SET 1 and SET 3. One of them was performed with the original sludge from the Paşaköy Plant .

4.2.1 SET 1 Batch Experiments

The batch experiments of SET 1 were performed on the 66th day. Acetic acid was used as a carbon source to achieve an influent COD of 600 mg/l. The VSS concentration used in the bioreactor was 800 mg/l. Anaerobic conditions were maintained in the bioreactor by purging N_2 gas throughout the batch experiment. Samples were taken every half an hour for PHA, VSS and PO4-P analysis. COD measurements were not conducted in SET 1.

The results of batch experiments are shown in Table 4.17.

Table 4.17 SET 1 Batch Results

Time (min)	SS (mg/l)	VSS (mg/l)	PO_4-P (mg/l)	PHA	
				as PHB(mg/l)	as COD(mg/l)
0	2500	2470	4,74	26,66	44,64
30	1180	1140	5,32	16,6	27,8
60	1020	900	6,03	11,27	18,88
90	830	820	6,05	17,07	28,59
120	800	790	6,08	4,72	7,89
180	1080	1020	6,03	9,73	16,28

Although PHB storage was expected from the sludge during anaerobic conditions, as can be seen from the table, there was PHB utilization instead of storage. So the results of PHB measurements are not representative to interpret them into meaningful comment. The only conclusion to these results could be that PHB measurements are not reliable enough.

4.2.2 Batch Experiment with Wastewater Treatment Plant Sludge

This batch experiment was performed with the sludge taken from Paşaköy Biological Treatment Plant. Influent COD was selected as 500 mg/l and sodium acetate was used for the COD supplement during the experiment. Anaerobic conditions were maintained during the batch experiment.

The results of the PO_4-P and COD measurements are shown in Table 4.18 and in Figure 4.16 and 4.17. As it can be seen from results, there was very little amount of PO_4-P in the sludge. However, as it can be seen from the figure 4.17, the PHB amounts increased while the COD amounts were decreasing. PHB results compared with the COD results are shown in the Figure 4.17. At the beginning of the batch experiment the PHB amount was only 2,40 mg/l but at the end of 180^{th} minute PHB amount had become 10,43 mg/l. Parallel to this situation COD was depleted about 110 mg/l in 3 hours and decreased to 468 mg/l at the end of the experiment. The influent COD was measured as 564 mg/l.

Table 4.18 PO_4-P and COD Measurements of Batch Experiments

Time (min)	COD (mg/l)	PO_4-P (mg/l)	PHA	
			asPHB(mg/l)	as COD(mg/l)
0	564	0,44	2,40	4,12
15	524	0,56	2,42	4,06
30	502	0,87	3,30	5,33
60	498	0,41	4,86	8,14
90	489	1,2	5,88	9,85
120	468	0,46	7,89	13,22
180	454	1,00	10,43	17,42

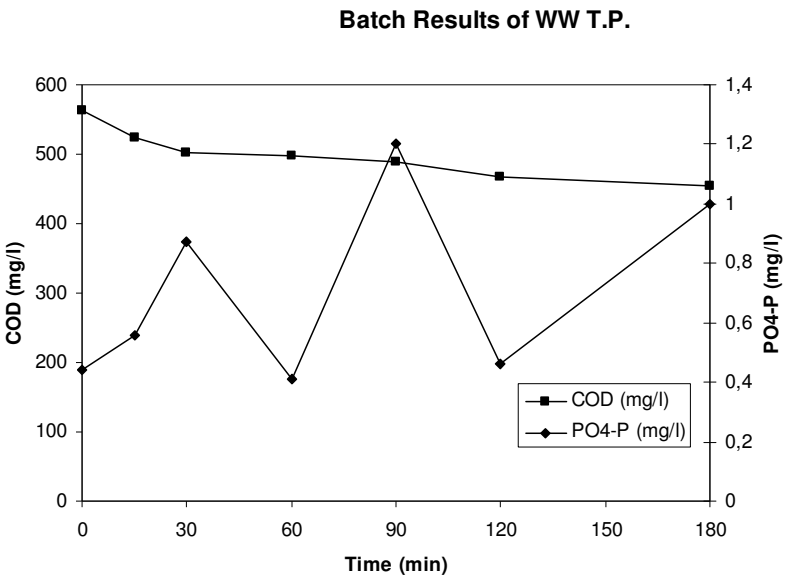


Figure 4.16 The results of batch experiment of ww sludge

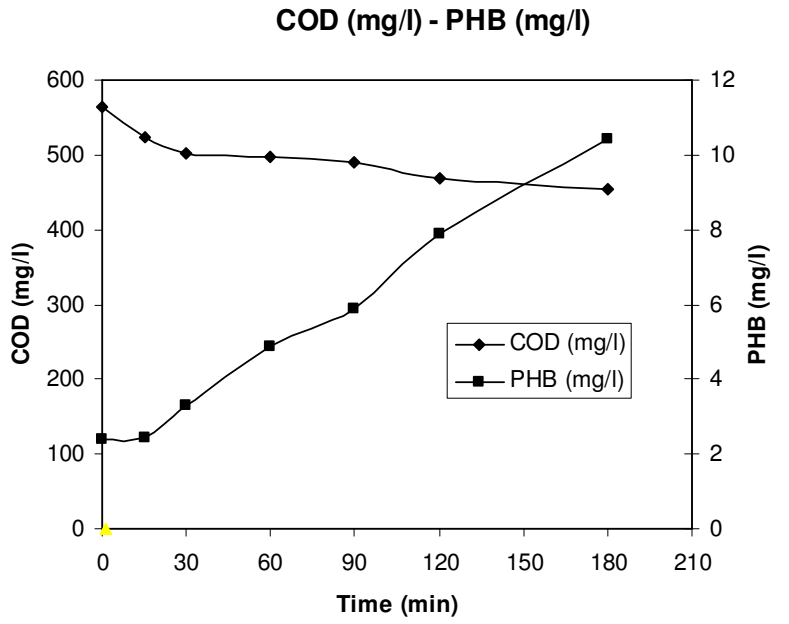


Figure 4.17 PHB and COD results of ww sludge

4.2.3 SET 3 Batch Experiments

This was performed with the sludge taken from the end of aerobic phase of SBR operation during SET 3. Influent COD was selected as 500 mg/l and sodium acetate was used for the batch experiment. The results of the PO_4-P and COD measurements are shown in Table 4.19, Figure 4.18 and 4.19. The experiments were conducted with the wasted sludge on the 54^{th} day of the reactor under aerobic conditions.

Table 4.19 PO_4-P and COD Measurements of Batch Experiments from SET 3

Time (min)	COD (mg/l)	PO_4-P (mg/l)	PHA	
			asPHB(mg/l)	as COD(mg/l)
0	500	11,2	13,99	23,43
15	570	10,8	20,42	34,19
30	505	10,6	15,49	25,93
60	363	10.0	23,32	39,05
90	205	9,9	85,36	142,93
120	119	0,5	60,10	100,64
180	82	5,75	-	-

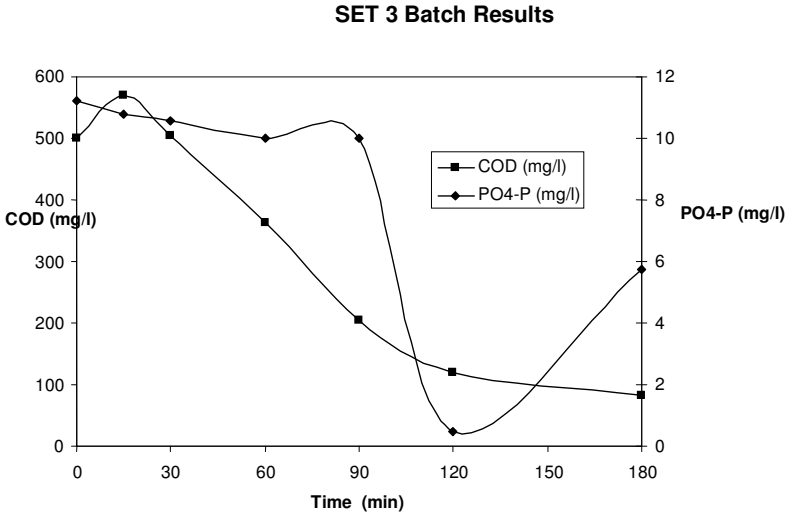


Figure 4.18 The Batch Results of SET 3

The PO_4-P was 11,2 mg/l at the beginning of the batch experiments. After 15 minutes from the feeding it decreased to 10,8 mg/l and for the next 15 minutes it continued decreasing to 10,6 mg/l. At the end of the first hour of the batch experiment it PO_4-P decreased to 10.0 mg/l and it was stabled that point until the 90th minute. Then it decreased to 5,75 at the end of the batch test.

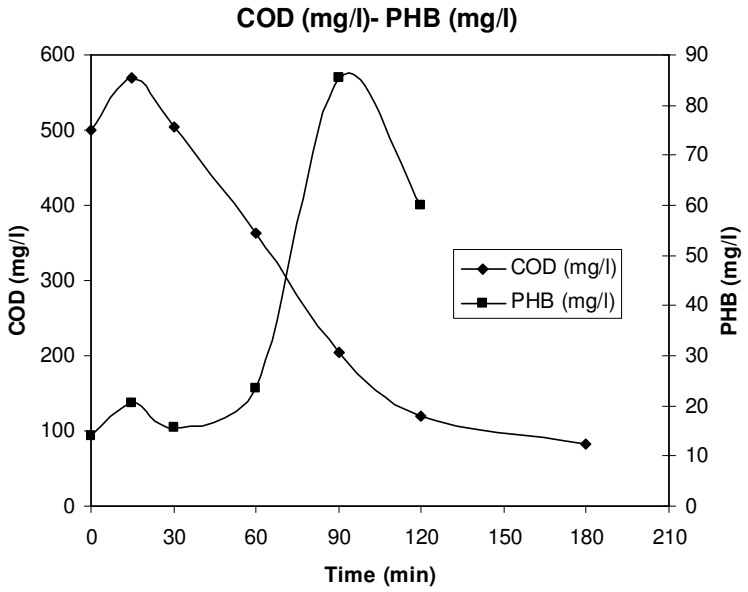


Figure 4.19 PHB Results compared to COD in SET 3

As it can be seen from the figure 4.19, COD was not depleted in the first 15 minutes of the experiments moreover it was measured as 570 mg/l. Then the COD utilization started to the parallel with PO_4-P depletion. At the end of the 3rd hour of the experiment 418 mg/l COD was depleted by the microorganisms.

According to the batch experiment results from SET 3, PHB amounts increased until the 90th minute. Then it started to decrease. In Figure 4.19 PHB production and COD depletion can be observed.

5. CONCLUSION

Recently, biodegradable plastics are developing subjects all over the world. The production of PHAs biologically from activated sludge is one of the alternatives for producing biodegradable plastics. Enhanced biological phosphorus Removal (EBPR) activated sludge systems were selected for this study to produce PHAs.

Experimental study using Sequencing Batch Reactors (SBR) have been conducted to investigate the factors affecting the selections of PHA storing organisms under different operational conditions. There were 7 different conditions used in SBR systems. According to the experimental results obtained from SBR operations, expected PO_4-P release and uptake did not occur in the system. No remarkable COD depletion was observed during anaerobic condition as well, it was depleted only under aerobic conditions. These results could be attributed to several reasons:

First of all anaerobic/aerobic phases did not work properly. Because of the reactor shape and mixing conditions dissolved oxygen concentration in the mixing period might cause the condition not to be fully anaerobic.

In the aerobic phase, aeration conditions sometimes were not executed properly because of the clogging of diffusers. Improper aerating and the COD entrance to the aerobic phase since not all the COD was utilized in the anaerobic phase are the conditions favoring filamentous bacteria growth. In this case, instead of very slow growing PHA storing PAO bacteria, filamentous bacteria were dominant in the system. This result also explains the reason that sludge settling was deteriorated after 10-20 days in all of the SETs and the sludge was washed out from the reactor.

In some of the SETs the sludge escaped from the reactor because of the mechanical problems at the pumps. This also caused a decrease in microorganism concentration in the reactor. So their growth has been adversely affected.

Environmental Biotechnology Laboratory where all the experiments were performed, the temperature was not maintained in constant values because of air conditioner

problems. As a result, most of the time during experimental study, working temperature was between 7-10°C. This was very low causing bacteria growth took place under undesirable conditions. So the dramatic temperature changes affected bacterial growth and they were completely dead because of very low temperatures in some of the SETs.

It is not likely that the growth of PHA accumulating microorganisms was affected by the carbon sources that were added to the system. A mixture of acetic acid, propionic acid, ethanol, glutamic acid and glucose was mostly used as a carbon source. In one SET only sodium acetate was used. In both case, anaerobic carbon uptake and PO_4-P release were not observed. This can also be attributed to the reason that excess amount of carbon entering to the aerobic zone favored the growth of filamentous bacteria growth causing the PAO bacteria not to have chance to grow in the system.

However, even in the sets where the most of the conditions were favorable for the growth of the PAO bacteria (favorable temperature, appropriate aeration etc.), P release in the anaerobic conditions and the following aerobic phosphorus uptake were not observed in the system indicating that the seed sludge taken from the Paşaköy Biological Treatment Plant did not contain PAOs. This result indicates the importance of the seed sludge and further investigations with different seed sludge should be performed.

When the batch experiment were evaluated, results showed that the aerobic PHA storage was higher compared to anaerobic batch experiments results, supporting the results observed in continuous SBR operation since there was no PAO in the system allowing anaerobic P release and PHA storage during the anaerobic conditions.

According to these results it can be said that this study could be used as guidance for further researches as it shows the results of conditions negatively affecting PHA accumulating organism growth. For further investigations, these conditions should be considered carefully and further studies should be focused on aerobic PHA storing mechanism.

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