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省エネルギー型活性汚泥プロセスから発生した PHA 蓄積余剰汚泥の嫌気性消化

Anaerobic Digestion of Excess Sludge Accumulating Polyhydroxyalkanoate Generated from Energy Saving Activated Sludge Process

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CHAPTER ONE

INTRODUCTION

1.1 General

The activated sludge process is the most commonly used biological process for municipal and industrial wastewater treatment. It is an aerobic process where influent wastewater and activated sludge are mixed together in an aeration tank which is coupled to a settling tank. When the aerobic reaction is complete, the activated sludge and treated water are separated, and the treated wastewater is discharged to the natural environment. A small portion of the settled sludge is removed from the system, while the major portion is recycled to the aerobic reactor as return sludge (Figure 1.1a). The activated sludge process, though widely successful, yet there is scope of improvement by addressing some known issues. The ecology of the system can change from day to day, leading to significant problems, such as sludge bulking. It makes the process difficult to capture and recycle the microorganisms fast enough for maintaining the desired large biomass concentration in the reactor. Considering all the issues related to the performance of activated sludge process, researchers have proposed different alternatives as outlined by Metcalf and Eddy (2004).

One of the principal issues associated with this process is the high aeration energy requirement. Energy is only one of the costs pertaining to the construction and operating a treatment plant. The major treatment related energy used would be primary tank sludge management, i.e. pumps and scrapers; aeration and/or mixing in secondary treatment tanks and related sludge recirculation pumping and transfer pumping and sludge management (after dewatering: composted or incinerated, or anaerobic/aerobic digestion). The choices of sludge management alternatives do have a significant impact on the plant's energy use. The increase in water consumption causes an increase in sewage and resource/energy consumption in sewage treatment. Total electricity consumption in sewage treatment facilities in Japan has increased annually. The electricity consumption per unit volume of sewage has increased in the past ten years from 2.6×10^9 kWh in 1982 to 4.8×10^9 kWh in 1994. In Japan, the energy consumption per unit volume

of wastewater is 0.45 $kWhm^{-3}$ and about two-third of which is utilized for oxidizing the organic matter (Goto et al., 2001). There is a strong correlation between electricity consumption per unit volume of sewage and facility scale. The smaller the facility scale, the higher the electricity consumption per unit volume of sewage (Goto et al., 2001).

Energy efficiency is one of the important factors that should be taken into account when selecting a treatment method of wastewater. As a new approach to reducing resource/energy consumption in sewage treatment facilities, development of new treatment technologies and improvement of treatment processes have been investigated. Energy consumption could be improved significantly by replacing existing treatment processes with newly developed technologies. However, improvement cannot be expected because most facilities have been using an aerobic biological treatment process that is difficult to change. The most realistic and practical approach in reducing the resource/energy consumption is the selection of an optimum treatment method and optimum operating conditions. But sustainable solutions for sewage treatment require modification of existing treatment process to decline energy requirements. In this aspect, two stage energy saving activated sludge process was initiated (described in section 1.2) which allows achieving aeration-saving and excess biomass recovery treatment (Oshiki et al., 2009). The recovered excess biomass composed of excess sludge accumulating PHA. It is expected that PHA rich excess sludge in anaerobic digester could be degraded rapidly and be produced excess energy through excess methane production than only excess sludge otherwise the prescribed process will produce more waste sludge.

Polyhydroxyalkanoate (PHA) is known as a temporal carbon storage material of microorganisms in activated sludge (Satoh et al., 1998). Activated sludge contains a mixed cultures of microorganisms used in the treatment of wastewater. Within this sludge, the competition between microorganisms for food is severe, and PHA often appears to be used by microorganisms as temporal carbon storage in order to survive effectively in such an environment. A wide variety of microorganisms accumulate PHA within cells as an intracellular storage material of carbon and energy (Akar et al., 2006; Dionisi et al., 2006). It is also regarded as new environmental-friendly biodegradable plastic. PHA is composed mainly of poly-beta-hydroxybutyric acid (PHB) and poly- beta-hydroxyvaleric acid (PHV) where PHB is the most

common and best characterized lipid like polymer stored by bacteria (Serafim et al., 2004). Their presence and relative proportions depend on the type of carbon substrate used by the microorganism. PHA accumulates as carbon/energy or reducing-power storage material in microbial cells. PHA is synthesized and accumulated as intracellular granules usually when there is an essential growth-limiting component such as nitrogen, phosphate, sulfur, oxygen or magnesium in the presence of excess carbon source (Lee, 1996a; Poirier et al., 1995). In recent years many studies have been conducted on the production of PHAs by activated sludge exposed to excess carbon supply. Under these dynamic conditions, sludge submitted to successive periods of external substrate accessibility (feast) and unavailability (famine) generates a so-called unbalanced growth (Serafim et al., 2004). During the excess of external carbon substrate, the uptake is mainly driven to PHA storage and, to a lesser extent, to biomass growth. After substrate exhaustion, the stored polymer can be used as an energy and carbon source. Under these conditions, activated sludge is able to accumulate PHAs up to 50% of cell dry weight (Beccari et al., 1998). Satoh et al. (1998) reported that 62% of PHA content was observed in the activated sludge acclimatized in microaerophilic-aerobic process, where a limited amount of oxygen is supplied to the anaerobic phase of the anaerobic and aerobic process. They also reported that PHA is produced more under aerobic conditions than under anaerobic condition because much more energy and reducing power are generated under aerobic conditions than under anaerobic condition. It is well known that production and storage of PHAs are integral and essential parts of the enhanced biological phosphorus removal (EBPR) mechanisms. These bacterial PHAs are used for the manufacture of biodegradable plastics. Plastics production is typically accomplished using pure cultures with the goal of maximizing the amount of stored PHAs at the end of a nutrient limited or oxygen limited phase. Thus, for EBPR the microbial cells are harvested when phosphorus storage is at a maximum, and for plastics production the cells are harvested when PHA storage is at a maximum. PHA has attracted industrial interests having similar mechanical properties to those of polypropylene, with the additional advantage of being completely biodegradable, biocompatible, and produced from renewable resources (sugars and fatty acids) (Serafim et al., 2004) . Plastics produced from PHA have been reported to be truly, fully biodegradable. In addition, the degradation product of PHA is a common intermediate compound in all higher organisms. Therefore, it is plausible that it is biocompatible to animal tissues and PHA may be use in surgical applications without any toxicity.

Most of the studies on utilization of PHA were reported to produce biodegradable plastic which was very expensive. The price of PHA depends on the substrate cost, PHA yield, and on the downstream process used for polymer extraction (Lee, 1996b). Until now the main barrier to the replacement of synthetic plastics by PHAs has been the cost difference (€9/kg for PHAs vs. €1/kg for synthetic plastics. Consequently, the degradation of PHA rich excess sludge produced from energy saving activated sludge process in anaerobic digester needs to be assessed.

Anaerobic digestion is one of the oldest and most traditional processes for reducing the volume of sewage sludge. The technique of anaerobic digestion offers many advantages in the effective treatment of a board range of organic waste. It attracts interest due to its capability of converting volatile solids contained in the sludge to methane (biogas), an environmentally sound energy source. Generally, four stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) are involved in anaerobic digestion, and the hydrolysis stage is considered as the rate limiting step for excess sludge digestion. It may lead to environmental benefits with regard to waste treatment, pollution reduction, energy production and improvements in agricultural practices. To monitor the anaerobic process, the activity of different groups of organisms can be measured indirectly, e.g by the gas production rate, or by the accumulation of intermediates of anaerobic degradation, reflecting the current metabiloc status of the active organisms in the system (Gujer and Zehnder, 1983). There are several suggestions in the literature as to what to measure. Some of the more commonly used indicators include volatile fatty acids (VFA), alkalinity, pH, gas production rate and the amounts of hydrogen, methane and carbon dioxide in the gas (Ahring et al., 1995). Anaerobic digestion of waste and wastewater can be performed in batch, or in continuous processes. In normal batch digestion, reactors are filled once with fresh waste, with or without the addition of inoculums, and also allowed to go through the degradation process leading to the formation of biogas (Parawira et al., 2004). Anaerobic batch digestion experiments are useful because they can be performed quickly with simple and inexpensive equipment, and are useful in assessing the rate at which a material can be digested.

Although a considerable amount of research has been performed on the degradation of PHA in aerobic conditions but no work has already been done on degradation of excess sludge accumulating PHA under anaerobic digestion through methane production. Therefore, in this

study, excess sludge accumulating PHA was subjected to batch experiments under anaerobic digestion to evaluate the degradation of excess sludge accumulating PHA and excess methane production compared with excess sludge.

1.2 Two Stage Energy Saving Activated Sludge Process

The two stage energy saving activated sludge process, named as Final AeRation of Excess Sludge with Excess Loading (FAREWEL) process was initiated by Oshiki et al. (2009). The first stage is the conventional activated sludge process and the second is FAREWEL process (Figure 1). In the second stage, the influent is treated by excess sludge discharged from conventional process, and the excess sludge is again fed with influent (final feed) and aerated shortly. The treatment allows microorganisms absorb organic matter in influent as temporal carbon storage materials such as polyhydroxyalkanoate (PHA). As the FAREWEL process is concluded without the consumption of temporal carbon storage materials, oxygen consumption should be less than conventional activated sludge process. Oshiki et al. (2009) estimated the effect of reduction of aeration volume and the increase of biomass recovery by performing batch experiments. The results indicated that one-tenth of aeration volume can be reduced and three-tenth of extra biomass can be recovered in FAREWEL process.

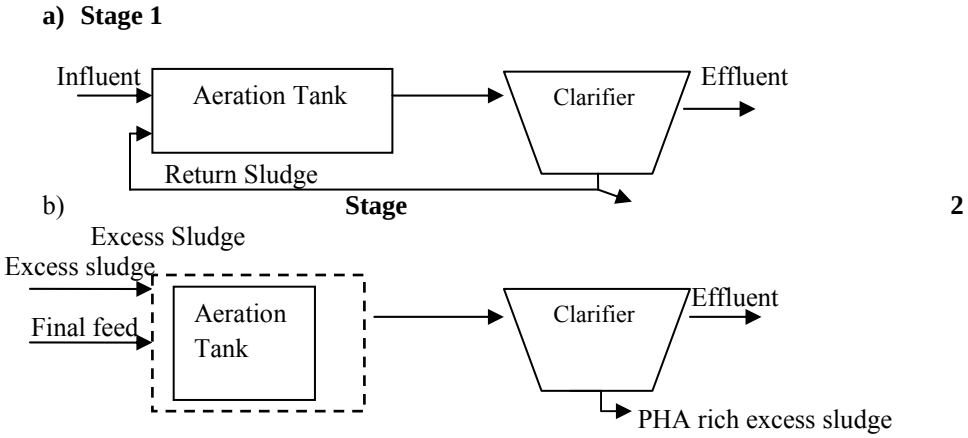


Figure 1.1: Two stage activated sludge process, *a) stage 1*: conventional activated sludge process; *b) stage 2*: FAREWEL process.

1.3 Objectives

In energy saving FAREWEL process, excess sludge will be allowed to accumulate temporal carbon storage materials such as PHA before it is sent to sludge treatment process. The higher accumulation of PHA in FAREWEL process leads to excess sludge with higher PHA accumulation; reduction of which under anaerobic digestion will definitely improve the sludge treatment process. The degradation of the temporal carbon storage materials will be better than that of excess sludge under anaerobic digestion; otherwise, the sludge treatment process will be overloaded.

General objective

- To study the feasibility of energy-saving FAREWEL process

As FAREWEL process can reduce the consumption of energy and produce excess amount of PHA rich excess sludge, so it is very important to assess the degradation rate of PHA through excess methane production under anaerobic digestion. It was thought that anaerobic digestion of excess sludge accumulating PHA to produce excess methane will ultimately facilitate the higher recovery of usable bio-energy.

Specific objective

- To evaluate the degradation of excess sludge accumulating PHA under anaerobic digestion through excess methane production compared to excess sludge without PHA.

To achieve the objectives, excess sludge was collected from a laboratory scale reactor and PHA in excess sludge was generated through introducing external carbon substrate in aerobic condition for 5h. After which excess sludge with and without PHA along with anaerobic digested sludge were subjected to anaerobic digestion in batch experiments and evaluated the degradation of PHA and methane production. Two experiments were carried out *viz.* first one is for evaluation of degradation of excess sludge accumulating PHA under anaerobic digestion through methane production, and second for re-evaluation of degradation of PHA under anaerobic digestion.

1.4 Outline of Dissertation

This dissertation contains six chapters and the contents of each chapter are as follows:

Chapter two presents the review of literature of the whole study. The relevant background information and concise findings by other researchers are described here.

Chapter three describes the detailed methodology of the experiment. There are two experiments; the methodology of each experiment is described separately.

Chapter four discusses the results and discussion of the experiments. Results and discussion for each experiment has been described separately. Finally, a comparison between the results of two experiments has been illustrated and an overall discussion is incorporated.

Chapter five presents the overall conclusions of the outcome of the research and concluding remarks for further study.

Chapter six includes all relevant references.

CHAPTER TWO

REVIEW OF LITERATURE

This chapter includes a brief review on polyhydroxyalkanote (PHA) and anaerobic sludge digestion processes. The sub-sections on PHA illustrate its role as storage of carbon in microorganisms, its metabolism, and its accumulation by various activated sludge processes and application of PHA as biodegradable plastic. The sub-sections of anaerobic digestion contain different steps of the process: hydrolysis of polymeric molecules and fermentation, acetogenesis and methanogenesis as well as effects of different environmental conditions such as pH, alkalinity, temperature, nutrients, and toxicity of free ammonia and sulfide.

2.1 Polyhydroxyalkanote (PHA)

PHA is a polymer of hydroxyalkanoate that are accumulated as carbon/energy storage in microbial cells. It is typically formed as intracellular granules under limitation of essential growth factors such as nitrogen, phosphate, sulfur, oxygen or magnesium in the presence of excess carbon source (Lee, 1996b). Depending on the feeding materials (carbon), many microorganisms can include a broad variety of 3-hydroxy fatty acids in the PHA. One of the most well known PHA is composed of 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV). Other monomeric units (different 3-hydroxyalkanoates) are also incorporated into PHA. PHB was first recognized in 1926, and now, more than 90 different monomer units have been recorded as constituents of PHA in more than 300 different microorganisms that are known as synthesize PHAs only a few bacteria have been employed for the production of PHAs. (Lee, 1996b). Activated sludge accumulates PHA to around 20% of dry weight under anaerobic conditions. Activated sludge acclimatized in the microaerophilic-aerobic process accumulated PHA of as much as 62% of sludge dry weight (Satoh et al., 1998). According to Lafferty *et al*. (1988), the copolymer of 3HB and 3HV has physical properties, including tensile strength and flexibility, similar to polyethylene and polystyrene.

PHA is of interest because it possesses thermoplastic characteristics. Plastic materials made of PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments. More than 80 hydroxyalkanotes have been detected as constituents of PHAs (Lee, 1996a). Only two forms of PHAs, i.e., PHB homopolymer and 3HB-3HV copolymer are commercially produced by Zeneca Bio Product, UK. Their presence and relative proportions depend on the type of carbon substrate used by the microorganism. A copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate is industrially produced by Metabolix (Cambridge, MA) using a pure culture of *Ralstonia eutropha* with propionic acid and glucose as substrates. The copolymer has improved mechanical properties relative to the homopolymer. The amount of PHA accumulated by this strain can be as high as 80% of the cell dry weight. Most of the studies on the accumulated PHA aimed at producing biodegradable plastic which is rather an expensive alternative (Lafferty *et al*. ,1988; Anderson and Dawes, 1990; Doi 1990; Lee, 1996a,b; Steinbuchel,1996; Braunegg *et al*., 1998 and Salehizadeh and Van Loosdrecht, 2004). No work has been reported on the degradation of excess sludge accumulating PHA under anaerobic condition aiming at the production of bio-gas, methane.

2.1.1 Structure of PHA

Figure 2.1 shows the structure of copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate. These monomeric units are thought to be randomly incorporated into PHA. Side chains, methyl or ethyl in Fig.2.1 are replaced by other chemical groups. PHAs which contain 4-hydroxybutyrate and 5-hydroxyvalerate are also reported.

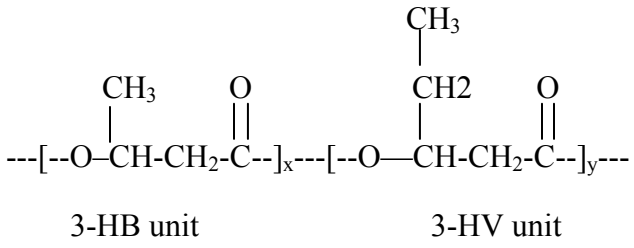


Figure 2.1: Structure of Copolymer 3HB-3HV

2.1.2 Storage of PHA in microorganisms

PHA can serve as a carbon or energy source for microorganisms during starvation periods. In EBPR process, PHA is formed and stored during the anaerobic period by polyphosphate accumulating organisms (PAOs) when no electron acceptors are available for growth mechanisms. The accumulation of PHA can be inspired under unbalanced growth conditions, i.e., when nutrients such as nitrogen, phosphorus or sulfur become limiting, or when oxygen supply is low (Lafferty *et al*., 1988). According to Doi (1990), when growth conditions are unbalanced, acetyl-CoA do not enter the tricarboxylic acid (TCA) cycle due to inhibition with high concentrations of NADH. The high concentration of NADH is resulted from the termination of protein synthesis. The high concentrations of NADH inhibit enzyme citrate synthase, one of the key enzymes of the TCA cycle, leading to an increase in the level of acetyl-CoA. Acetyl-CoA is then used as substrate for PHA biosynthesis by a sequence of three enzymatic reactions (Figure 2.2).

High intracellular concentrations of CoA-SH inhibit enzyme 3-ketothiolase, one of the three enzymes of PHA biosynthesis. When the entry of acetyl-CoA to the TCA cycle is not restricted, citrate synthase consumes acetyl-CoA, and as a result, CoA-SH concentration increase, and PHA synthesis is inhibited (Doi, 1990).

2.1.3 PHA biosynthesis pathways

Figure 2.2 shows a schematic of the PHA biosynthesis pathway (Doi, 1990 and Anderson and Dawes, 1990). Most of the organisms synthesize PHA using this pathway. The biosynthesis pathways of *R. eutropha*, *Zoogloea ramigera*, and *Azotobacter beijerinckii* are well established (Doi, 1990). Firstly, a substrate is condensed to acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA is subjected to a sequence of three enzymatic reactions for PHB synthesis.

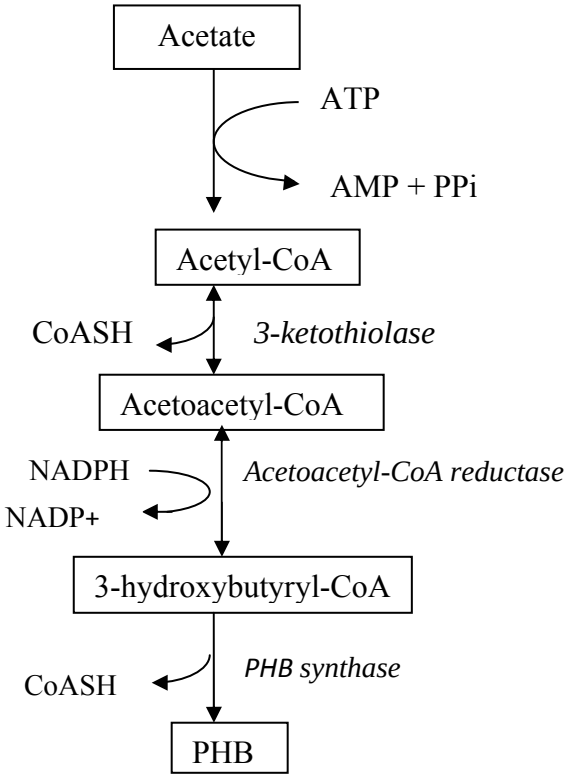


Figure 2.2: PHA biosynthesis pathway

2.1.4 PHA Metabolism

Figure 2.3 illustrates cyclic metabolism of PHA. 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" (Doi, 1990). He 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., 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.

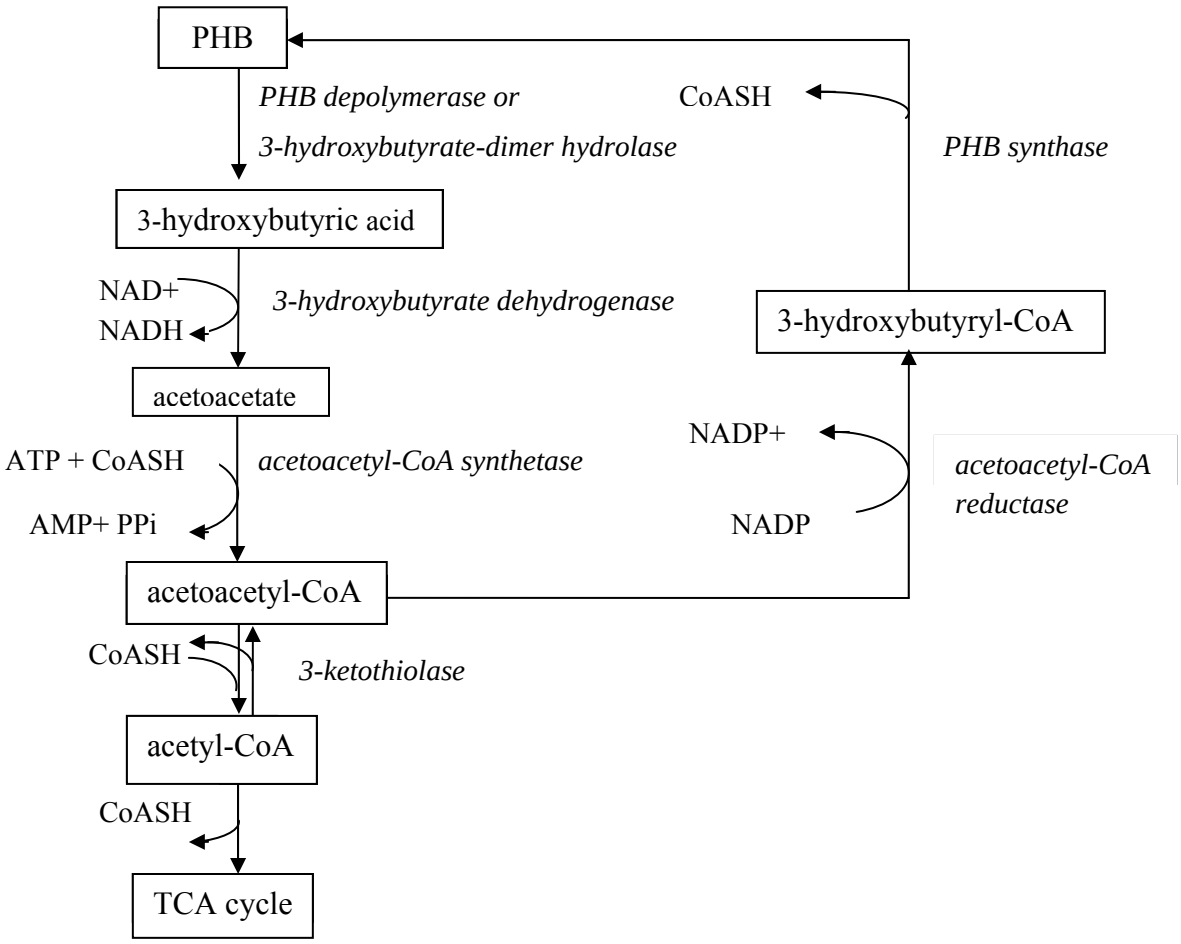


Figure 2.3: Cyclic nature of PHA metabolism

2.1.5 PHA Accumulation by Activated Sludge

Activated sludge is able to store PHA as carbon and energy storage material under unbalanced conditions arising from an intermittent feeding. There is a strong competitive benefit observed where microorganisms are able to quickly store carbon substrate over organisms without the capacity of substrate storage. Activated sludge submitted to aerobic dynamic feeding conditions showed a good and stable capacity to store PHB. In the last decade, considerable efforts have been devoted to the establishment of a novel PHA production system by using mixed bacterium culture available in waste. Many researchers (Ueno et al., 1993, Saito et al., 1995, Hu et al., 1997,

Satoh et al., 1998, Chua et al., 1999 and Takabatake et al.,2000, 2002) have been concentrating on activated sludge as biomass for PHA production.

2.1.6.1 Anaerobic-Aerobic Activated Sludge Process /EBPR

The significant role of PHA was illustrated after the invention of Anaerobic-Aerobic Activated Sludge Process in the middle of 1970s. This process is able to remove not only organic pollutants but also phosphorus. In EBPR process activated sludge and influent wastewater are mixed under anaerobic conditions, where no oxygen, nitrate and nitrite exist. Then the mixture of wastewater is treated aerobically. In the subsequent sedimentation tank, treated water and activated sludge are separated gravimetrically. Most of the collected activated sludge is returned to reactor as activated sludge. The rest of the activated sludge is taken out as excess sludge.

The EBPR process is attributed to the domination of microorganisms often referred to as PAOs (Mino et al., 1998). When PAOs are contacted with sewage under anaerobic condition, they take up organic substrates such as short chain fatty acids while consuming the polyphosphate as energy source. PAOs temporarily store the organic substrates until the condition becomes aerobic. When they come to the aerobic tank, they grow and regenerate polyphosphate while aerobically utilizing the temporal carbon storage. PHA is supposed to be one of the most important carbon storage materials formed as a result of anaerobic substrate uptake.

Salehizadeh and Van Loosdrecht (2004) have reviewed the production of PHA by mixed cultures. According to reviewed article, PAOs and the glycogen-accumulating organisms (GAOs) are capable of anaerobic storage of carbon source in mixed culture. PAOs are probably the most widely recognized for producing PHA, glycogen and polyphosphates. PAOs are thought to accumulate polyphosphate under aerobic conditions and then to use it for the uptake of organic materials under anaerobic conditions. GAOs were recognized as competitors of PAOs and they rely on fermented substrates (e.g., glucose), and store the fermentation products inside the cell rather than excreting. These organisms can also use internal stored glycogen for fermentation to PHB. The energy released in the glycolysis process is subsequently used to accumulate

fermentation products (e.g., acetate) in the form of PHB. PAOs and GAOs multiply in systems where the substrate is present regularly while an electron acceptor is absent.

Both PAOs and GAOs can take up acetate 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. Two metabolic models, Comeau et al. (1986) and Mino et al. (1987, 1996) have been proposed for EBPR. The main difference in their models was the source of electrons for formation of the PHA. Comeau et al. (1986) proposed the oxidation of substrate in the TCA cycle. Mino et al. (1998) measured the anaerobic decrease of intracellular carbohydrate and indicated that the conversion of glycogen to acetyl-CoA delivered the essential reduction of power for forming the PHA.

2.1.6.2 Conventional Activated Sludge Process

Conventional activated sludge process was developed in 1914 with the main function to remove the carbonaceous organic matter in wastewater. It is the most widely used biological process for municipal and industrial wastewater treatment. It is an aerobic process carried out in an aeration tank where waste water and activated sludge are mixed thoroughly and oxidized. Then the sludge is transferred to the settling tank for gravimetric settling. After that, water is discharged to the natural environment and a small portion of settled sludge is withdrawn as excess sludge and the rest amount is sent to the aeration tank as return sludge, known as activated sludge.

It is reported that PHA might possibly play an important role in conventional activated sludge process without anaerobic zones. It is explained that PHA is a storage material, which is very easily generated from volatile fatty acids. Hence taking up acetate followed by PHA accumulation must be much faster than taking up acetate while generating cellular constituents such as proteins or DNAs (Van Loosdrecht et al., 1997, Satoh et al., 1998).

2.1.6.3 Microaerophilic-aerobic Activated Sludge Process

In order to increase the PHA content of activated sludge, Satoh et al. (1998) introduced a new activated sludge process named “ microaerophilic-aerobic” activated sludge process. The

anaerobic-aerobic activated sludge process enriches PHA accumulators that are capable of accumulate glycogen and/or polyphosphate for anaerobic substrate uptake. By introducing small amounts of oxygen into the anaerobic zone of the anaerobic-aerobic process, it was expected that the accumulation of PHA accumulators that are not capable of accumulate glycogenand/or polyphosphate may be promoted. Satoh et al. (1998) reported that 62% of PHA content was observed in the activated sludge acclimatized in microaerophilic-aerobic process, where a limited amount of oxygen is supplied to the anaerobic phase of the anaerobic and aerobic process. They also reported that PHA is produced more under aerobic conditions than under anaerobic condition because much more energy and reducing power are generated under aerobic conditions than under anaerobic condition.

2.1.6.4 Final Aeration of Excess Sludge with Excess Loading (FAREWEL) Process

FAREWEL process allows achieving aeration-saving and biomass recovery treatment. There are two stages in this treatment system. The first is the conventional activated sludge process and the second is FAREWEL process. In the second stage, the influent is treated by excess sludge discharged from conventional process, and the treatment is optimized when the maximum amounts of temporal carbon storage materials i,e, PHA are accumulated. As the FAREWEL process is concluded without the consumption of temporal carbon storage materials, oxygen consumption should be less than conventional activated sludge process. Oshiki et al. (2009) estimated the effect of reduction of aeration volume and the increase of biomass recovery by performing batch experiments. The results indicated that one-tenth of aeration volume can be reduced and three-tenth of extra biomass can be recovered in FAREWEL process. Higher productions of excess sludge generates the higher accumulation of PHA in FAREWEL process

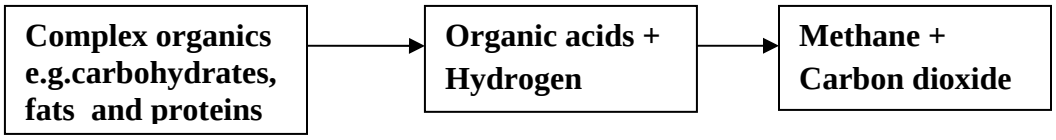
2.1.7 Applications of Biodegradable Plastics Produced from PHA

The possible applications of bacterial PHA is directly connected with their properties such as biological degradability, thermoplastics characteristics, piezoelectric properties, and depolymerization of PHB to monomeric D(-)-3-hydroxybutyric acid. The applications of bacterial PHAs have concentrated on three principal areas: medical and pharmaceutical,

agricultural, and commodity packaging (Lafferty *et al*., 1988). Some more applications of PHAs have been listed in (Lee, 1996a).

2.2 Anaerobic digestion

Anaerobic digestion is an effective method of treating agricultural, industrial and domestic wastes. It is typical anaerobic ecosystem where complex organic polymeric substances are enzymatic broken down into the final end products of methane (CH_4) and carbon dioxide (CO_2) by the action of different microbial populations in the absence of free oxygen. The simplied steps are as follows:



2.2.1. *Hydrolysis and Fermentation*

Hydrolysis is the first step in the anaerobic digestion in which particulate material is converted to soluble compounds that can be hydrolyzed further to simple monomers. It breaks down the complex organic compounds (such as carbohydrates, fats and protein) into their monomers (simple sugars like glucose). The breakdown of organic polymers is performed by extracellular enzymes, which are produced by both facultative and strictly anaerobic bacteria. The monomers resulting from hydrolytic bacteria and then fermented to volatile fatty acids (VFAs) such as acetic, propionic and butyric acids and alcohols, CO_2, H_2 and some lactic acid. Hydrolysis is regarded as the rate limiting step for insoluable polymers. For carbohydrates, cellulose is considered to be the rate limiting step but not starch which can be easily hydrolysed . Hydrolysis of carbohydrates under anaerobic conditions is generally faster than the hydrolysis of protein. Temperature and pH are two environmental factors affecting hydrolysis. Hydrolysis of cellulosic materials by enriched cultures at pH 6.7 was faster than at pH 5.1 and 5.2 (Eastman and Feruson, 1981). When experiments conducted at a neutral pH and temperatures between 20 and 45^0C, temperature optimum was found to be 40^0C. Carbohyrates such as starch and sugars are most commonly fermented by *Bacteriodes, Clostridia, Butyrivibrio, Selenomonas, Micrococcus* and *Lactobacillus*. Sugars are common energy sources for fermentative microorganisms.

Biochemical pathway occurring within the cell during this breakdown is generally via pyruvate. Pyruvate is metabolized primarily to acetate, formate, hydrogen and carbon dioxide. In this metabolism primarily other products may also be found such as propionate, butyrate, succinate, ethanol, and lactate.

Pyruvic acid is a result of breakdown of sugars through the Embden-Meyerhof-Parnas (EMP) or glycolysis pathway. The glycolytic reactions in glucose fermenting bacteria also produce electron equivalents in the form of NADH which are required to be re-oxidized in order to continue substrate degradation. One pathway for the bacteria to regenerate these reducing equivalents is via anaerobic respiration using inorganic electron acceptors. In the absence of external inorganic acceptors, NADH is commonly recycled through H^+ to produce H_2 or through pyruvate to produce lactate, propionate or butyrate. The type of end product produced depends on the bacterial types and thermodynamic conditions (Mosey, 1983; Mclnerney and Beaty, 1988).

Lactic acid is the most common product in the fermentation of sugars. In natural mixed population fermentations, homofermentive bacteria such as *Lactobacillius curratus* and *L. platarum* initiate acidification by following way:

$$C_6H_{12}O_6 \longrightarrow 2CH_3CHOHCOO^- + 2H^+$$

The heterofermentive bacteria such as *L. buchneri* and *L. brevis* convert glucose according to the following reaction:

$$C_6H_{12}O_6 \longrightarrow CH_3CHOHCOO^- + CH_3CH_2OH + CO_2 + H^+$$

The volatile fatty acids (VFA) produced by anaerobic fermentative bacteria grown on glucose are mainly acetic, propionic and butyric acids. Acetic acid is the most abundant followed by propionic and butyric acids (Toerien and Hattingh, 1969). The conversion reactions followed as follows:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

$$3C_6H_{12}O_6 \longrightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2O$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

Under stable conditions, the first reaction to produce acetic acid from glucose is the preferred reaction (Mosey, 1983).The second intermediate metabolic product, propionic acid, may also be formed by the following reactions:

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O$$

$$3CH_3CHOHCOOH + H_2 \longrightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + 2H_2O$$

The second reaction is a result of the work done by *Propionibacterium* consuming lactate, produced by lactic acid bacteria.

Propionic acid may also result from metabolism of long chain fatty acids that contain odd numbers of carbon atoms as an end product. Firstly, odd numbered fatty acids are metabolized through β-oxidation. Then, the three carbons remaining as propionyl-CoA are converted to succinyl-CoA and oxidized to CO_2 through the Tricarboxylic Acid (TCA) cycle. Because anaerobic bacteria cannot use the TCA cycle as a complete pathway, propionic acid would be produced as an end product.

Another end product, butyric acid is a result of anaerobic metabolism of *Clostridum* species bacteria, known as the butyric clostridia, such as *Clostridum butyricum*, *C. tyrbutyricum*.

2.2.2 Acetogenesis

The fermentation products such as propionic acid and butyric acids as well as ethanol need to be converted to a simpler product, i. e. acetic acid before being utilized by methanogenic bacteria. The bacteria responsible for the conversion are known as acetogenic bacteria (or called H2 producing bacteria). Two common types in anaerobic digestion are the alcohols and the fatty acids degrading acetons such as *Acetobacterium, Acetobacter, Syntrophobacter, Syntrophomonas* and *some Desulfovibrio* species. These bacteria grow very slowly due to the low free energy available from their metabolic substrate degradation with doubling time ranging from 1.5 to 4 days (Lawrence and McCarty, 1969).

Acetogenesis could perform efficiently when the acetogens grow synthropically with methanogens since methanogenic bacteria maintain the acetogenic end products at a low level leading the reaction in a thermodynamically favourable direction. For example *Syntrophomonas,*

Syntrophobacter and *Desulfovibrio* have been co-cultured with species of *Metanobacterium* or *Metanospirillum*. Another group of acetogens known as H_2-acetigenic and homoacetogenic bacteria convert H_2 and CO_2 to acetate, according to the reaction:

$$2CO_2 + 4H_2 \longrightarrow CH_3COOH + 2H_2O$$

2.2.3 Methanogenesis

Methanogenesis is the final step in anaerobic digestion to produce methane and carbon dioxide from acetate and H_2 produced in acetogenesis step. In all anaerobic ecosystems, methanogenesis is carried out by methanogenic bacteria. These bacteria are the most sensitive bacterial group in the anaerobic digester ecosystems to oxygen and pH (Zehnder and Wuhrmann, 1977; Barredo and Evison, 1991). Methanogens are able to metabolise a very narrow range of substrates. Almost all methanogens (except 4 species which include *Methanotrix soehngenii*) can grow on H_2 and CO_2 while some genera can use formate. Formate utilizing genera include *Methanobacterium, Methanobrevibacter, Methanococcus, Methanomicrobium, Methanogenium* and *Methanospirillum*. *Methanosarcina* and *Methanothrix* are two bacterial groups, which can utilize acetic acid and present in high numbers in anaerobic digesters (Zehnder, 1978; Smith and Mah, 1980) but they cannot use formate. The former is present abundantly in unstable digesters, containing high acetate concentrations whereas the later is dominant in stable systems, containing less acetate. There are two types of methanogenic bacteria, i.e. aceticlastic methangenic and H_2 utilizing bacteria. These methanogens play an important role to control the pH during fermentation process by the removal of acetate to form CO_2 and CH_4 (Mosey, 1983).They are responsible for 60-70% of the methane produced in anaerobic digesters (Smith and Mah, 1980) with the reaction that proceeds as follows:

$$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3^-$$

The H_2-utilizing methanogenic bacteria, is responsible for 30% of the total methane produced in anaerobic digesters. The H_2 concentration strongly affects the metabolic pathways used by the fermentative bacteria and is responsible for the types of end –products formed. The inhibition of some H_2-producing reactions by H_2 concentration has been known as one of the major causes of VFA accumulation leading to failure in digester operations (Harper and Pohland, 1986). Only at very low levels of H_2 concentration through interspecies H_2 transfer degradation of VFAs such as

propionate can occur. Minimum doubling times for the H_2-utilising methanogens are in the range of 6 to 12 hours (Smith and Mah, 1978; Gujer and Zehnder, 1983).

2.2.4 Requirements for anaerobic treatment process

2.2.4.1 pH and Alkalinity

The desired pH for anaerobic treatment is between 6.6 and 7.6. Values outside this range can be quite detrimental to the process, particularly to methanogenesis. The main problem generally is to maintain the pH above 6.6, because organic acids produced as intermediates in the process during start up, over load, or other unbalance can cause a rapid pH drop and cessation of the methane production (Rittmann and McCarty, 2001).

With the high CO_2 content (typically 30-50%) in the gas produced in anaerobic treatment, alkalinity concentrations in the range from 2000 to 4000 mg/L as $CaCO_3$ are typically required to maintain the pH at or near neutral. The alkalinity may be generated in some cases by the degradation of protein and amino acids (Metcalf and Eddy, 2004). The requirement to purchase chemicals for pH control can have a significant impact on the economics of anaerobic treatment. Alkaline materials often are added to provide adequate buffer when it is not present in a wastewater or to prevent an excessive drop in pH during unbalanced conditions. Common materials used for the purpose are lime ($Ca(OH)_2$), sodium bicarbonate ($NaHCO_3$), soda ash (Na_2CO_3), sodium hydroxide (NaOH), ammonia (NH_3), or ammonium bicarbonate (NH_4HCO_3). Generally, lime, sodium hydroxide, and ammonia are the cheapest of these chemicals and thus the ones selected.

2.2.4.2 Temperature

Temperature affects reaction rates considerably. In anaerobic treatment, the slow growth rate of microorganisms most critical to the process makes temperature all the more important for reactor design. Growth rates in general roughly double for each 10^oC rise in temperature within the uusual mesophilic operational range from 10 to 35^oC. Growth rates generally do not change between 35 and 40^oC, but denaturation of proteins at higher temperatures slows growth rates for

mesophilies. However, different mixed cultures adapted to thermophilic temperatures have optimum temperatures in the 55 to 65^{o}C range. Thermophilies do not fuction as well at the intermediate temperature of 40-45^{o}C as do mesophilic organism. Thus, one must make the decision to operate at the lower mesophilic range with a temperature optimum of 55 to 60^{o}C (Rittmann and McCarty, 2001).

With dilute wastewaters at ambient temperature, the methane produced may be insufficient to raise the wastewater temperature, and operation at ambient wastewater temperature may be the economical option. With more concentrated wastewaters that produce larger volumes of methane per unit volume of reactor or with high temperature wastewaters, operation at the mesophilic optimum of 35oC or at thermophilic temperatures is the best option. With the latter, the rates are typically 50 to 100 percent higher than at the optimum mesophilic temperature. Thus, the advantage of the higher temperature is faster reactions and smaller required tank volumes. The disadvantages are a greater energy cost to maintain the higher temperature and the risk of a rapid loss in treatment capacity due to failure of the reactor heating system.

2.2.4.3 Nutrients

Though anaerobic processes produce less sludge and thus require less nitrogen and phosphorus for biomass growth, many industrial wastewaters may lack sufficient nutrients. Thus, the addition of nitrogen and/or phosphorus may be needed. Depending on the characteristices of the substrate and the SRT value, typical nutrient requirements for nitrogen, phosphorus, and sulfer are in the range from 10 to 13, 2 to 2.6, and 1 to 2 mg per 100 mg of biomass, respectively. The values for nitrogen and phosphorus are consistent with the values for these constituents estimated on the basis of the composition of the cell biomass. Further, to maintain maximum methanogenic activity, liquid phase concentrations of nitrogen, phosphorus, and sulfur on the order of 50, 10, and 5mg/L, respectively are desirable (Metcalf and Eddy, 2004) .

2.2.4.4 Ammonia Toxicity

Ammonia is produced anaerobically from the degradation of proteinaceous wastes. Ammonia, which is a base, combines with carbon dioxide and water to form ammonium bicarbonate, the bicarbonate being the natural pH buffer. It is ammonia, not the ionized ammonium ion that more

often causes inhibition. The NH_4^+ nitrogen concentration found to cause inhibition was much higher, about 3,000 mg/L. At high ammonia nitrogen concentrations, whether NH_3 or NH_4^+ is more inhibitory depends upon system pH. The release of ammonia nitrogen from protein increases the bicarbonate alkalinity concentration. As a result, the pH can increase too. It is not uncommon with high concentration proteinaceous wastes that treatment pH can be quite high, approaching 8.0. If the total ammonia nitrogen concentration (NH_3+$NH_4^{+)}$ were 2,000 mgN/l and the pH were 8.0, the NH_3 nitrogen concentration would be 110 mg/L, which is in the inhibitory range. The response of the anaerobic treatment system to such inhibition is to reduce the rate of consumption of the volatile acid intermediates, which in turn drives the pH lower so that inhibition is reduced. The best control of ammonia toxicity is to reduce the waste's N concentration through dilution, Hydrochloric acid addition to reduce pH somewhat could also be used if NH_3 toxicity was the problem, but it does not work if NH_4^+ is the cause of toxicity (Rittmann and McCarty, 2001).

2.2.4.5 Sulfide production

The concentration of oxidized sulfer compounds in the influent wastewater to an anaerobic process is important. High concentrations can have a negative effect on anaerobic treatment. Sulfate reducinf bacteria compete with the methanogenic bacteria for COD and thus can decrease the amount of methane gas production. While low concentrations of sulfide (<20mg/L) are needed for optimal methanogenic activity, higher concentrations can be toxic. Methanogenic activity has been decreased by 50% or more at H_2S concentrations ranging from 50 to 250mg/L (Metcalf and Eddy, 2004).

CHAPTER THREE

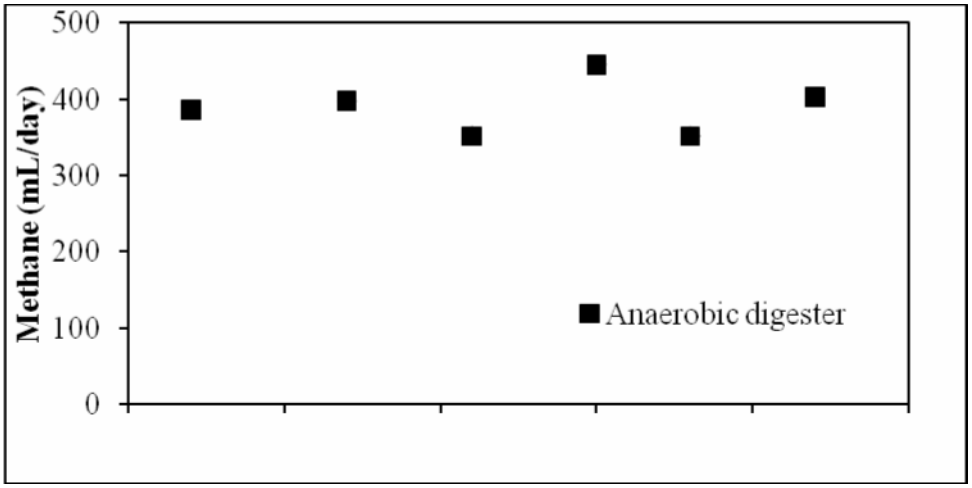
MATERIALS AND METHODS

In this chapter, the detailed methodology of the whole experiment is described. The study was accomplished by conducting two experiments to fulfill the objectives described in section 1.3. Experiment I was performed to evaluate the degradation of excess sludge accumulating PHA under anaerobic digestion compared with excess sludge and also digested sludge (control). Experiment II was to performed to examine the reproducibility of Experiment I. Thus, experiment II was as similar to experiment I except that control was omitted. Section 3.1 describes the operation and performance of anaerobic digester, from which anaerobic sludge was obtained for batch experiments. Sections 3.2 and 3.3 are on the methodology of batch experiment, including the preparation of inoculum, substrates, experimental set up and preparation of anaerobic sludge and excess sludge (with or without PHA) mixtures. Section 3.4 is on the detailed analytical methods.

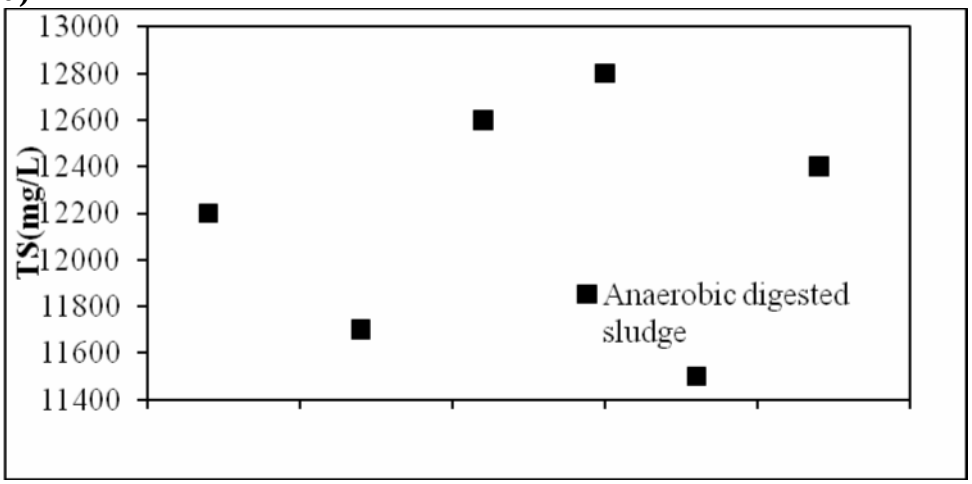
3.1 Anaerobic Digester

3.1.1 Operation of anaerobic Digester

A laboratory scale anaerobic digester with a volume of 1.5L was operated at 37^{o}C. The loading rate of the digester was 1.0/kgVSS/m^{3}/day and sludge retention time (SRT) was 23 days. For monitoring the digester gas production and composition, total solids, volatile solids, total COD_{cr}, pH, organic acids, NH_3-N were analyzed periodically. Concentrated excess sludge from laboratory scale sequential batch activated sludge reactor was used as feed for anaerobic digester. Digested sludge collected from an anaerobic sludge digester at a full scale domestic wastewater treatment plant was used as the inoculum. It was started on 30 May 2009. Anaerobic sludge for the batch experiments were taken from this reactor on 28 April 2010 for Experiment I and 3 June 2010 for Experiment II, respectively.



a)



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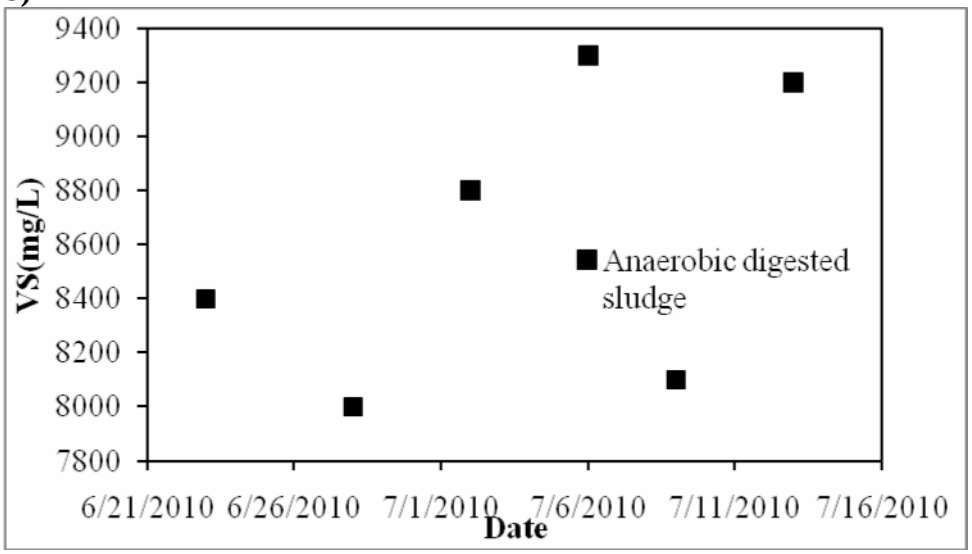

c)

Figure 3.1: a) Methane production in anaerobic digester, b)TS, and c)VS concentration of excess sludge and anaerobic digested sludge.

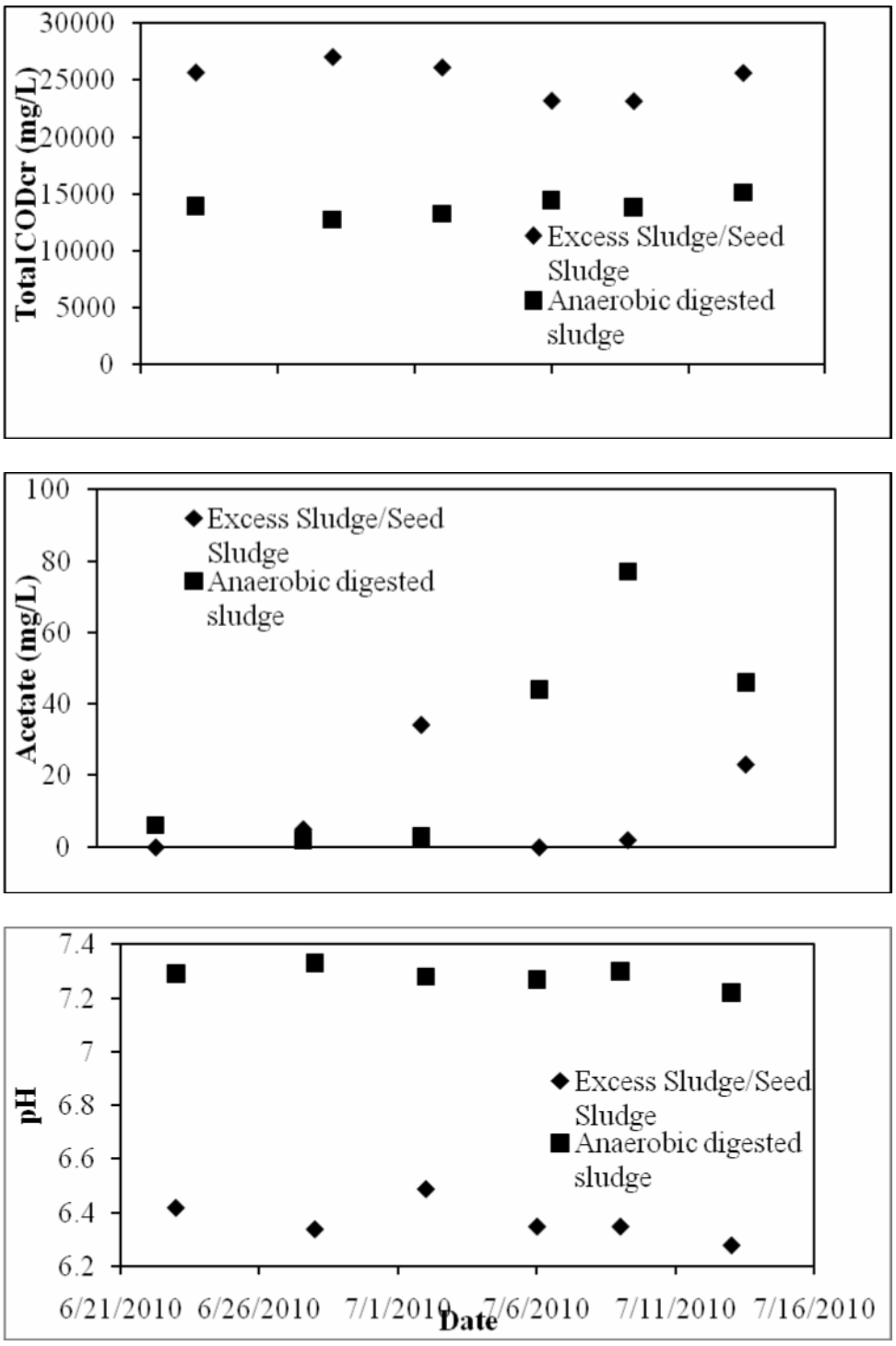

Figure 3.2: a) Total COD_{cr}, b) Acetate, and c) pH of excess sludge and anaerobic digested sludge.

3.1.2 Performance of digester

The anaerobic digester was monitored two times per week and methane production, TS, VS, total COD_{cr}, acetate formation, and pH etc. were analyzed. Methane gas production /day, TS and VS concentration are presented in Figure 3.1. The methane production/day was found almost stable in the digester. The TS, and VS were in digested were ranged from 11000-13000 mg/L and 8000-9000 mg/L, respectively. Figure 3.2 showed the total COD_{cr}, acetate, and pH of excess sludge and anaerobic digested sludge. The total COD_{cr}, and pH were found in stable condition and pH in anaerobic digester was in normal range. Aceate formation were increasing in both sludge. There was no inhibitory effect in anaerobic digester. The concentration of NH_3-N was ranged from 500 to 700 mg/L but the inhibitory range of NH3-N is 1500-3000 mg/L (Metcalf and Eddy, 2004). The performance of digester during experimental period was not shown.

3.2 Experiment I: Evaluation of degradation of excess sludge accumulating PHA under anaerobic digestion through methane production

Experiment I was carried out to evaluate the degradation of excess sludge accumulating PHA under anaerobic digestion through methane production and was conducted in April-May, 2010.

3.2.1 Inoculum

Anaerobically digested sludge (DS) maintained in the laboratory as described in 3.1 was used as inoculum. Prior to start up of the experiment, the total solids (TS) and volatile solids (VS) of the digested sludge were measured by following the Standard Methods (APHA, 2005). The TS and VS were found 16100 mg/L, and 9750 mg/L, respectively.

3.2.2 Collection of Excess sludge

Excess sludge (2.8L) at the end of aerobic phase was collected from a laboratory-scale reactor showing a good capacity of enhanced biological phosphorus removal (EBPR). MLSS Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) of collected sludge were measured following the standard methods. PHA was also quantified by the gas chromatographic method as per procedures stated by Oshiki et al. (2008). The MLSS, MLVSS and PHA were recorded 2120 mg/L, 1960 mg/L, and PHA 41 mg/L, respectively.

3.2.3 Preparation of Substrates

3.2.3.1 Excess Sludge (ES)

The collected sludge suspension (1.4L) was centrifuged at 3500rpm for 10 minutes and the supernatant was decanted. The supernatant were decanted (Figure 3.3). The sludge pellet was suspended with effluent of approximately 250 ml. TSS and VSS concentrations were measured by following the Standard Methods (APHA, 2005). The TSS and VSS were 22400 mg/L, and 13600 mg/L, respectively. The prepared sludge was then kept at 4^{o}C.

3.2.3.2 Preparation of Excess Sludge Accumulating PHA (ES+PHA)

The collected excess sludge suspension (1.4L) was taken into a glass beaker. Then acetate was fed at final concentration 1000 mgC/L and incubated for 5h under aerobic condition by air bubbling. After 5h, the MLSS, MLVSS and PHA were also measured and the values were 2480 mg/L, 2300 mg/L, and 605 mg/L, respectively.

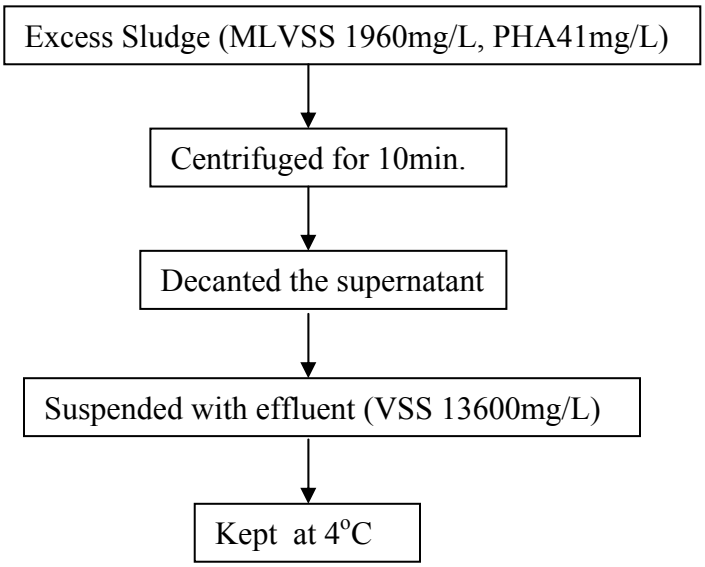

Figure 3.3: Outline of preparation of excess sludge for experiment I

After that, the sludge was centrifuged at 3500rpm for 10 minutes and the supernatant was decanted (Figure 3.4). Then sludge pellet was suspended with effluent of approximately 250ml. TSS and VSS concentrations were measured and the values were 32200mg/L, and 19200mg/L, respectively. The prepared sludge was then kept at 4^{o}C.

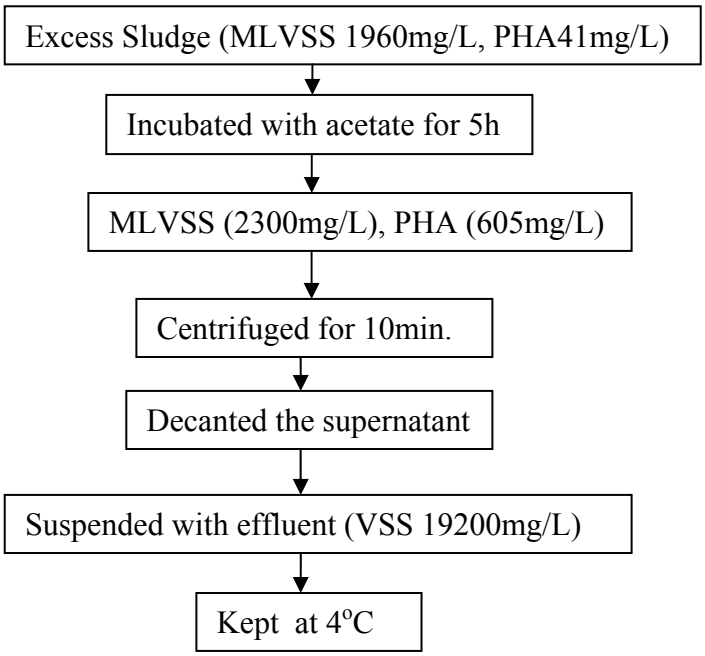

Figure 3.4: Outline of excess sludge accumulating PHA production for experiment I

3.2.4 Experimental Set Up

The batch experiment was conducted in three different groups viz. T0 (DS) (Control), T1 (DS+ES) and T2 (DS+ES+PHA). The tests were performed in 10-ml vials with a liquid volume of 7 ml. A total of 69 vials were used for the experiment and each group had 23 vials. Inoculum and substrates were mixed at VS ratio of 1:1 (Figure 3.5). Each vial of T0 group contained 34 mg VS of DS. The vials of T1 group had 34 mg VS of DS and 34 mg VS of ES. The vials of T2 also contained 34mgVS of DS and 34mg VS of ES+PHA.

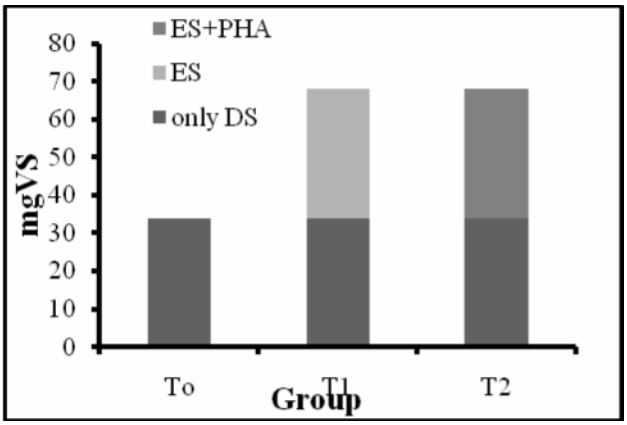

Figure 3.5: Mixing ratio of VS in different group

3.2.5 Preparation of Mixtures in vials

Sodium bicarbonate at a concentration of 14 g/L was prepared to provide buffering capacity. DS (300 ml) was taken from the laboratory scale anaerobic digester and mixed thoroughly by a magnetic stirrer. 1ml of bicarbonate solution and 3.5 ml of DS (VS 9750 mg/L) were poured into all the vials individually. Then for T0 group, 2.5 ml of effluent was added to each of 23 vials. For preparing T1, 2.5 ml of ES (13600 mg/L) was mixed with each of another 23 vials. For T2 group, 1.77 ml of ES+PHA (19200 mg/L) and 0.73ml of effluent were mixed with each of the rest 23 vials. Then all the vials were closed tightly with butyl rubber and aluminum caps. After that, nitrogen gas was purged into all the vials with help of 20 gauge needles for 45 minutes. After purging, all the vials were mixed thoroughly and incubated at 37^{o}C under stirring conditions (200 rpm). Gas composition and chemical analysis were performed on days 0, 2, 5, 8, 12, 16, 19, 22, 26, 29 and 34. For daily gas measurement three vials were used, and two vials were used for measuring gas composition. Chemical analyses were performed in duplicate.

3.3 Experiment II: Re-evaluation of degradation of PHA under anaerobic digestion

Experiment II was performed to examine the reproducibility of the data obtained from experiment I. The experiment was conducted in June, 2010. In this experiment, the procedure of preparation of substrates and experimental set up were almost same as experiment I. But the t values of MLSS, MLVSS, TSS, VSS, TS, VS, PHA etc. were dissimilar and the observational days were also different.

3.3.1 Inoculum

Anaerobically digested sludge (DS) was collected from the same digester. The TS and VS were found 10150 mg/L and 7350 mg/L respectively.

3.3.2 Collection of Excess sludge

Excess sludge (1L) at the end of aerobic phase was collected from a laboratory-scale reactor showing a good capacity of enhanced biological phosphorus removal (EBPR). MLSS and

MLVSS and PHA of collected sludge were measured. The MLSS, MLVSS and PHA were recorded 1960mg/L, 1560mg/L and PHA 26mg/L respectively.

3.3.3 Preparation of Substrates

3.3.3.1 Excess Sludge (ES)

The collected sludge suspension (0.5L) was centrifuged at 3500rpm for 10 minutes and the supernatant was decanted. Then the sludge suspension was centrifuged at 3500rpm for 10 minutes. The supernatant was decanted (Figure 3.6). The sludge pellet was suspended with osmolysis water of approximately 50 ml. TSS and VSS concentration were measured by following the standard methods. The TSS and VSS were 13500 mg/L, and 12000 mg/L, respectively.

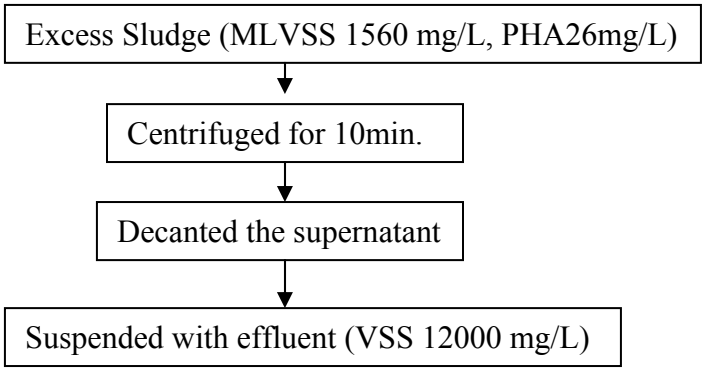

Figure 3.6: Outline of preparation of excess sludge for experiment II

3.3.3.2 Preparation of Excess Sludge Accumulating PHA (ES+PHA)

The collected excess sludge suspension (0.5L) was taken into a glass beaker. Then acetate was fed at final concentration 1000 mgC/L and incubated for 5h under aerobic condition by air bubbling. After 5h, the MLSS, MLVSS and PHA were also measured and the values were 2200 mg/L, 2040 mg/L and 307 mg/L respectively. After that, the sludge was centrifuged at 3500rpm for 10 minutes and the supernatant was decanted (Figure 3.7). Then sludge pellet was suspended with osmolysis water approximately of 50 ml. TSS and VSS concentration were measured the values were 19500 mg/L and 17750 mg/L respectively.

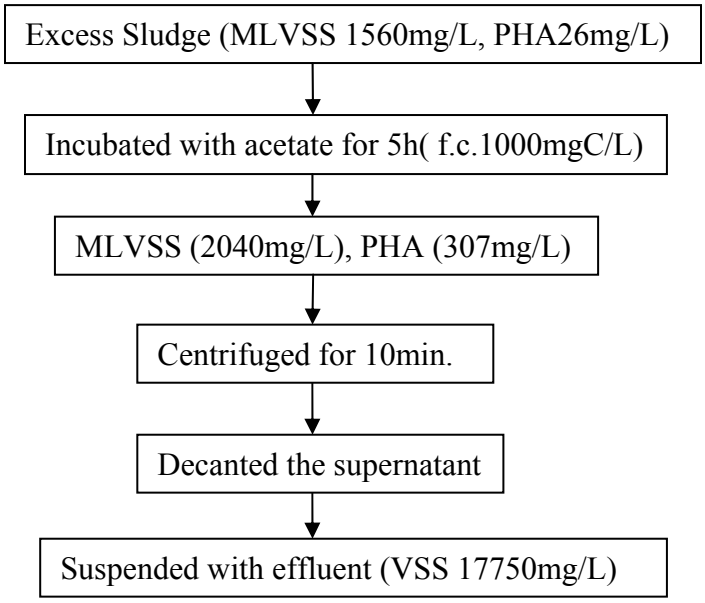

Figure 3.7: Outline of excess sludge accumulating PHA production for experiment II

3.3.4 Experimental Set Up

The batch experiment was conducted into two different groups viz. T1 (DS+ES) and T2 (DS+ES+PHA).The tests were performed in 10-ml vials with a liquid volume of 7.5 ml. A total of 26 vials were used for the experiment and each group had 13 vials. Inoculum and substrate were mixed at VS ratio of 1:1. Sodium bicarbonate at a concentration of 14 g/L was prepared to provide buffering capacity. DS (150 ml) was taken from the laboratory scale anaerobic digester and mixed thoroughly by magnetic stirrer. Each vial of T1 group contained 29.4 mg VS of DS and 29.4mg VS of ES and T2 also had 29.4 mg VS of DS and 29.4 mg VS of ES+PHA. For preparing the mixtures, at first 1ml of bicarbonate solution and 4.0 ml of DS (VS 7350 mg/L) were poured into all the vials individually. For preparing T1 group, 2.45 ml of ES (VSS12000 mg/L) and 0.05ml water were mixed with each of 13 vials. For T2 group, 1.656 ml of ES+PHA (VSS 17750 mg/L) and 0.844ml of water were mixed with each of the rest 13 vials. Then all the vials were closely tight with rubber and aluminum caps. After that, nitrogen gas was purged into all the vials with help of 20 gauge needle for 45 minutes. After purging N_2 gas, all the vials were mixed thoroughly and incubated at 37^{o}C under stirring conditions (200 rpm). Gas composition and chemical analysis were performed on days 0, 1, 4, 7, 11, and 14. For daily gas measurement three vials were used and two vials were for measuring gas composition. Chemical analyses were performed in duplicate.

3.4 Analytical Methods

3.4.1 Gas Production and Composition

Gas production was measured using a syringe equipped with a 22 gauge needle. Prior to biogas release the vials were shaken for 15s, in order to reach the equilibrium between gases present in both the gas and liquid phase. Methane was analyzed by gas chromatography (GC-3200D) at a flow rate of 20 ml min^{-1} and argon as the carrier gas. Two replicates (0.5 ml) from each treatment were manually injected with a SUS column (molecular sieve 5A 30/60, 3mm i.d.x 2m). The temperatures of injector, oven and detector were 50^{o}C.The values of methane production were corrected for the standard temperature and pressure conditions (STP).

3.4.2 pH

The pH meter was calibrated with two standard solution where pH 6.86 & 4.01. Prior to measurement, the liquid in vial was mixed and pH meter (HM 30G) was placed inside the vial and recorded the value.

3.4.3 Total Chemical Oxygen Demand ($TCOD_{Cr}$)

Total COD_{Cr} was measured by using HACH company kit followed the colorimetric determination method. The samples were diluted 40 times and 2 ml diluted samples were poured into the HACH kit and for zero sample 2 ml Milli Q water in another kit and then mixed thoroughly . Then the kits were kept into the oven at 150^{o}c for 2hours. After 2 hours the kits were placed in the rack for getting room temperature. The values were measured by data logging spectrophotometer (HACH, DR/2010). The wave length was set at 620 nm.

3.4.4 Total Solids (TS) and Volatile Solids (VS)

TS and VS were also measured according to Standard Methods (APHA, 2005). At first empty weight of porcelain crucibles were measured (A). Then the samples were shaked thoroughly into the vials and 1ml sample was placed into the dish and oven dried at 105^{o}C for drying approx.10 hours. After drying, the dishes were kept in a dessicator for cooling. The dishes were weighed again (B) and calculated the TS by using the following equation:

$$\text{mg TS/L} = \frac{(B-A)*1000}{sample\ volume,ml}$$

For measuring the VS, the measured dishes (B) were kept into the muffle furnace at 600oc for 30minutes, then cooled in 105oC in the oven and then the dishes were placed in a dessicator for cooling. The dishes were then weighed (C) and Vs was calculated as follows:

$$\text{mg VS/L} = \frac{(B-C)*1000}{sample\ volume,ml}$$

TS and VS measurement were performed in duplicate for each treatment.

3.4.5 Preparation of Samples for VFAs and NH_3-N Measurements

For liquid-phase analysis, 2ml samples from each vial were centrifuged for 2 minutes in high speed micro-centrifuge (13000rpm) MX 150 (TOMY). Samples from the supernatant were taken for analysis of VFA, and NH_3-N measurement. The supernatant were collected and filtered using a 0.2 µm membrane. The solids were stored at 80^{o}C for microbial analysis.

3.4.6 Volatile Fatty Acids (VFAs) Analysis

VFA concentrations were analyzed by HPLC Shimadzu CT010A, SCR 101H column, with 0.025% sulfuric acid as eluent, with an elution rate of 1 ml/min, and an operating temperature of 60^{o}C. A UV detector (L-2400, Hitachi, Japan) set at 210 nm was used. The filtrated samples (1.5ml) were poured into small sized glass vial (2ml) and kept into the rack of Auto sampler L2200, Hitachi, Japan. 50 µl sample was injected by auto sampler into the column from each vial. The peaks were processed by using the standard samples peaks.

3.4.7 Ammonia-Nitrogen (NH_3-N)

NH_3-N was measured by using HACH company kit followed by the Salicylate Method. At first the filtrated supernatants were diluted 80 times and 0.1 ml diluted samples were poured into the HACH kit and for zero sample, 0.1 ml of ammonia free water into another. Then Ammonia Salicylate Reagent powder Pillow for 5 ml sample was added to each vial. After that, Ammonia Cyanurate Reagent Powder Pillow for 5 ml sample was also added to each vial. All the vials were capped tightly and shaked thoroughly to dissolve the powder. The values of NH_3-N were

measured by data logging spectrophotometer (HACH, DR/2010). The wave length was set at 655 nm.

3.4.8 PHA Measurement

PHA was quantified by the gas chromatographic method as per procedures stated by Oshiki *et al.* (2008). Ten milliliter of activated-sludge-mixed liquor collected from the activated sludge reactor was centrifuged at 3500 rpm for 5 min, and the supernatant was decanted. For batch experiment sample 1 ml of mixed sample from each treatment was poured into bottle. For the preparation of standard sample, pure PHB and chloroform was mixed at 1:1 and mixed thoroughly and then 1 ml of sample was poured into bottle and opened the cap of the bottle for a certain period to evaporate the chloroform. The sample was frozen in a freezer and dried in a lyophilizer (consists of a high vacuum pump and a cold trap). After the addition of 2ml of chloroform and acidified methanol (10% sulfuric acid with around 100mg/L of benzoic acid), the content in the tube was mixed well, and was digested at 100^{o}C for 1 day in a sealed test tube with a teflon-linered screw cap. Then, it was cooled to room temperature, 1ml aqueous ammonia solution (28%) was added to it, the tube was shaken, and, the methyl ester of PHA monomers in the chloroform layer was collected by using pastured pipette. The chloroform phase was washed again with 0.5ml water, and then injected to a gas chromatograph (GCMS-QP2010Plus) equipped with a column Neutrabond-1 (GL Science Company, 30m length, 0.25 mm internal diameter and 0.4m film thickness). An autoinjector (AOC-29i) was used for the injection of samples. The temperature of the injector was set at 180^{o}C with split mode. Column temperature was kept at 60^{o}C. The injection volume was 0.1ml with a split ratio of around 1:20. Helium gas was used as the carrier gas at a flow rate of 2ml/minute, and as the make-up gas at a flow rate of 45ml/minute. Methyl benzoate was used as the internal standard. Poly 3-hydroxybutyrate (Sigma, USA) was used as the standard for the quantification of 3HB. As acetate was used as the carbon source in this study, the monomeric unit of the PHA produced was almost all 3HB.

The following equation was used to calculate the PHB:

$$\frac{Area\ 3HB,Standard / Amount\ 3HB,Standard}{Area\ IS,Standard / Amount\ IS,Standard} = \frac{Area\ 3HB,Sample / Amount\ 3HB,Sample}{Area\ IS\ sample / Amount\ IS,Standard}$$

Where,

Area 3HB, std.: 3HB peak area of the standard chromatogram

Amount 3HB, std.: Amount of 3HB in the standard tube, or 1mg

Area IS std.; Internal Standard peak area of the standard chromatogram

Amount IS std.: amount of internal standard in the standard tube (200mg/L*2ml)

Area 3HB sample: 3HB peak area of sample chromatogram

Amount 3HB, sample: Amount of 3HB in the sample tube (unit in mg)

Area IS, sample: Internal standard area of the sample chromatogram

Amount IS, sample: Amount of internal standard in the sample tube (200mg/L*2)

Thus, the concentration of 3HB in sample = $Amount\ 3HB,sample * 1000/10$ [mg/L]

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Experiment I: Evaluation of degradation of excess sludge accumulating PHA under anaerobic digestion through methane production

The cumulative methane gas production (ml/g VS of DS) at different group during incubation is presented in Figure 4.1. The highest yield of methane was found in T2 (DS+ES+PHA) (213ml/g VS of DS) followed by T1 (DS+ES) (170 ml/g VS of DS) and T0 (only DS) (31ml/g VS of DS). The methane gas production was found to be increased (25%) in T2 compared to T1. The total amount of methane in T1 and T2 was 182 ml and 139 ml respectively compared to T0 (control). Thus, it is revealed that excess sludge accumulating PHA can produce excess methane under anaerobic digestion compared to excess sludge.

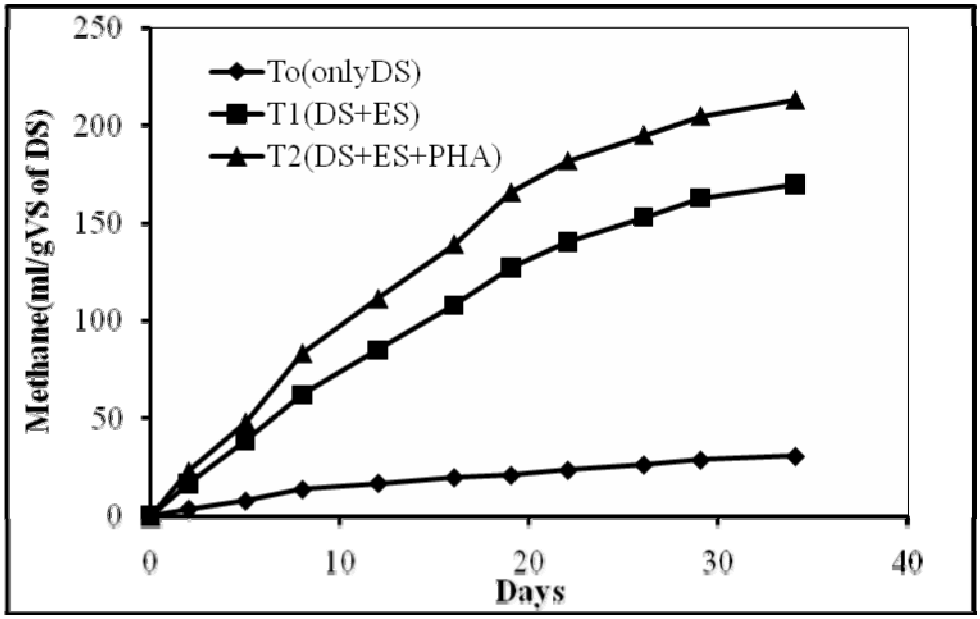

Figure 4.1: Cumulative methane gas production (ml/g VS of DS) at different group during anaerobic incubation

Figure 4.2 shows the total COD_{cr} of different groups during incubation. Total COD_{cr} was found to be decreased from the day of incubation to last day in all the groups. Total COD_{cr} in T0, T1 and T2 at the time of incubation were recorded 7440 mg/L, 14300 mg/L and 14600 mg/L and in the last day 6020 mg/L, 10120 mg/L and 10060 mg/L respectively. The total COD reduction rate in T0, T1 and T2 were 19%, 29%, and 31%. When first order kinetics was applied to model the degradation of total COD, the reduction rate parameter were 0.006 (r^2=0.87) in T0, 0.011 (r^2=0.99) in T1 and 0.012 (r^2=0.93) in T2. As a result, it can be stated that the total COD_{cr} was reduced in excess sludge accumulating PHA compared to excess sludge under anaerobic digestion.

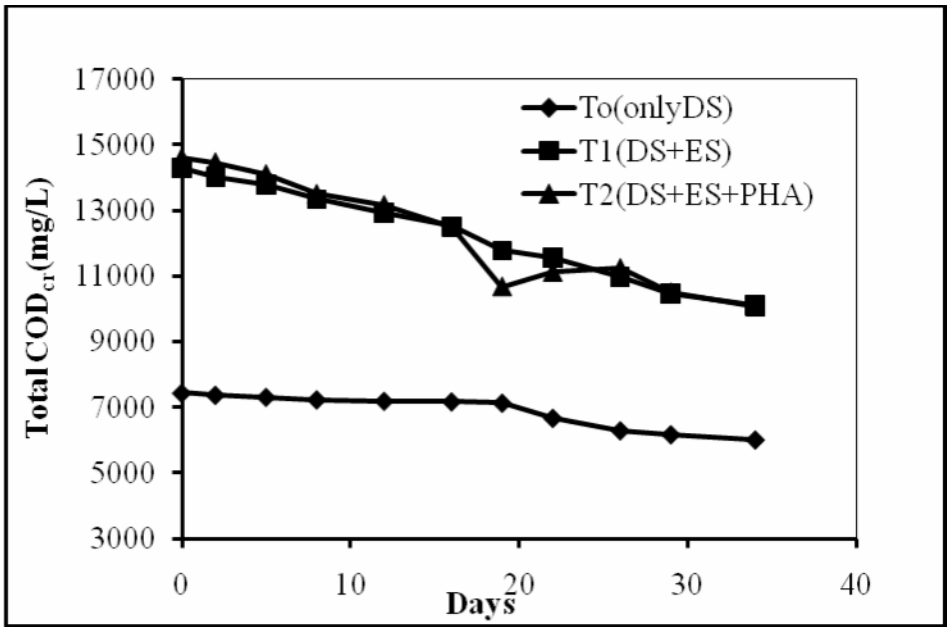

Figure 4.2: Total COD_{cr} during incubation anaerobic at different groups.

TS and VS concentration during incubation at different group is shown in Figure 4.3. TS and VS concentration were found to be reduced in all the groups from the day of incubation to last day. During start up of the experiment the TS concentration of T0, T1, & T2 were 16550 mg/L, 20700 mg/L & 21000 mg/L and VS were 7400 mg/L, 11250 mg/L, & 17900 mg/L respectively. After the end of the experiment, TS of T0, T1, & T2 were 14,400 mg/L, 17350 mg/L & 17900mg/L and the VS were 6300 mg/L, 8950 mg/L, & 8950 mg/L respectively. The percentages of reduction of TS concentration in T0, T1 and T2 were 13, 16, and 15 respectively. In case of VS concentration, the percentages of reduction in T0, T1, and T2 were 15, 25 and 22. The declining

of TS and VS concentration were found rapid in T1 (DS+ES) compared toT2 (DS+ES+PHA) under anaerobic digestion.

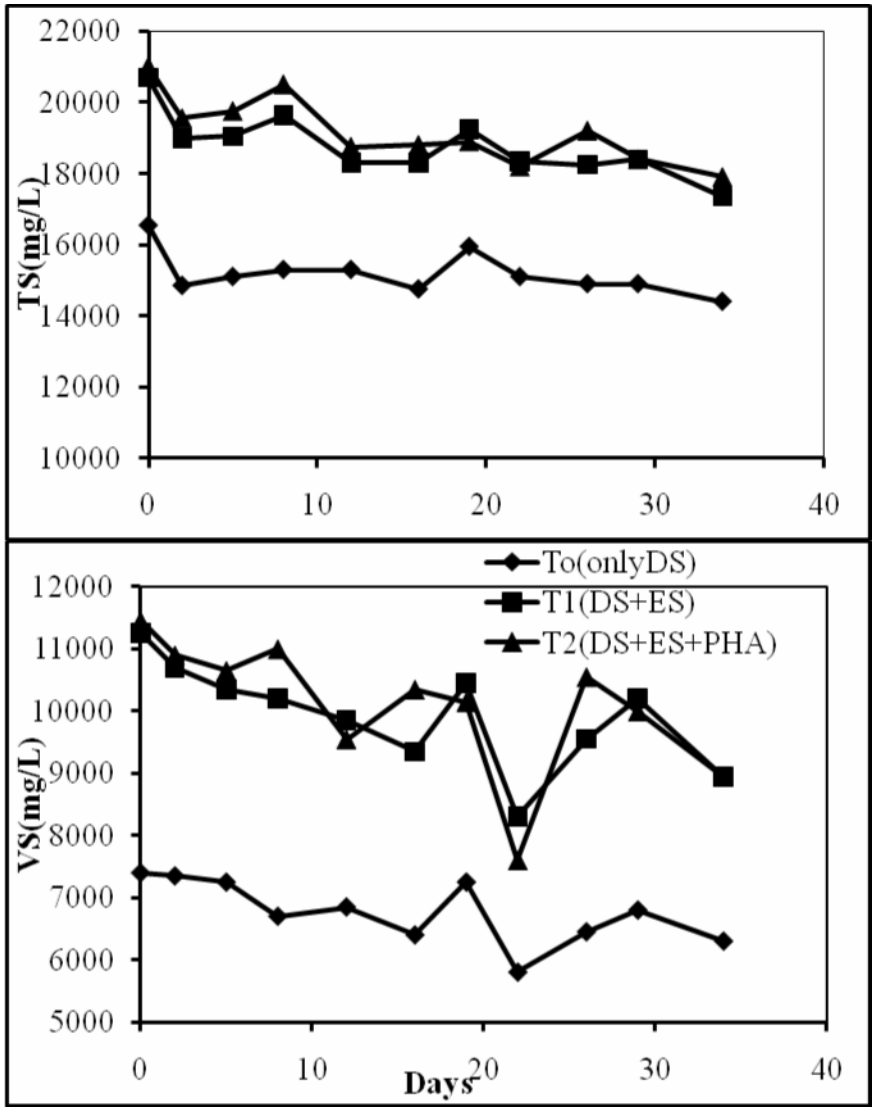

Figure 4.3: TS and VS concentration during anaerobic incubation at different groups.

Figure 4.4 shows the acetate concentration during incubation at different groups. After the start up of the experiment, the acetate concentration were increased rapidly in T0, T1 and T2 group upto 12 days and then decreased. In T0, the acetate concentration was found highest (70 mg/L) in day 2 whereas the concentration in T1 (723.5 mg/L) and T2 (987 mg/L) were found in day 12. After that, the concentration were declined and at the last day of observation, the concentration

in T0, T1, and T2 were recorded 0 mg/L, 177 mg/L, and 275 mg/L respectively. It is clear from the Figure 4.4 that the acetate formation was found to be the highest in T2 group (DS+ES+PHA) in comparison to T1(DS+ES). It was also observed that a considerable amount of other volatile fatty acids (propionate, butyric acid and valeric acid) were also found in T1 and T2 group but greater amount was found in T2 than T1.

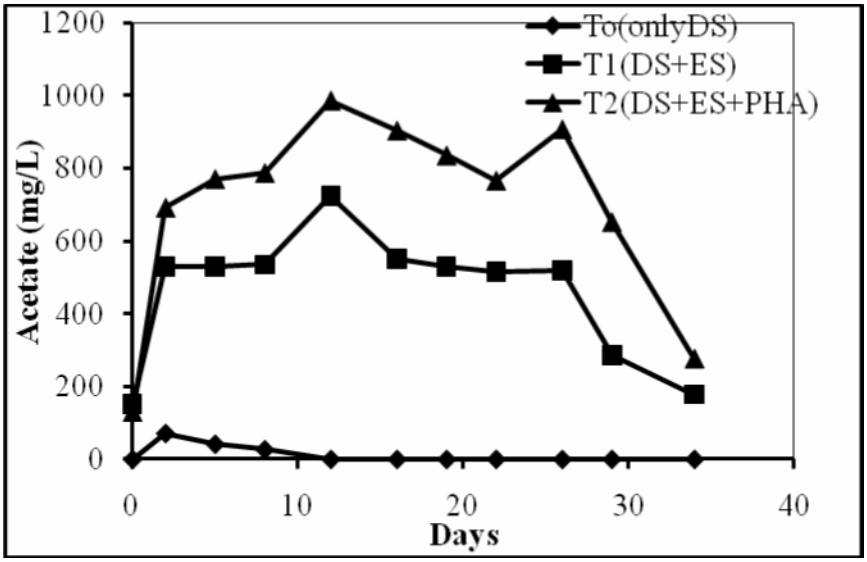

Figure 4.4: Acetate formation during anaerobic incubation at different groups

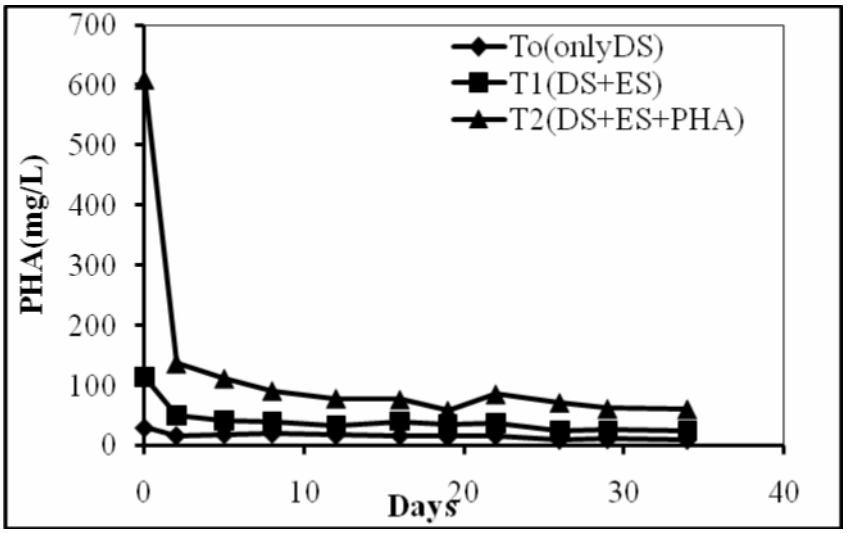

Figure 4.5: PHA degradation during anaerobic incubation at different groups

PHA degradation during incubation at different group is illustrated in Figure 4.5. After the start up of the experiment, PHA was degraded sharply within first 2 days in both T1 and T2 and then slowly degraded. But a little amount of PHA (30 mg/L) was initially found in T0 (control) which was also degraded and then stabilized. At the time of incubation, the concentration of PHA in T1 & T2 were found 114 mg/L & 609 mg/L respectively and in second day the concentration in T1 & T2 were recorded 50 mg/L & 137 mg/L respectively. Within two days the degradation of PHA in T1 and T2 were 56% and 77.5%. Apparently, the degradation was very rapid in first two days of incubation. At the final day the PHA in T1 and T2 were found 25 mg/L and 61 mg/L respectively. It is clear that after second to final day, the degradation was very slow and like steady condition. The degradation of PHA at the time of incubation to final day in T1 and T2 were 78% and 90% respectively. Consequently, it was revealed that initially, the PHA content were degraded rapidly in T2 (DS+ES+PHA) and then the rate of degradation diminished to a steady state. Moreover, initial rapid drop of PHA was also observed in T0 and T1.

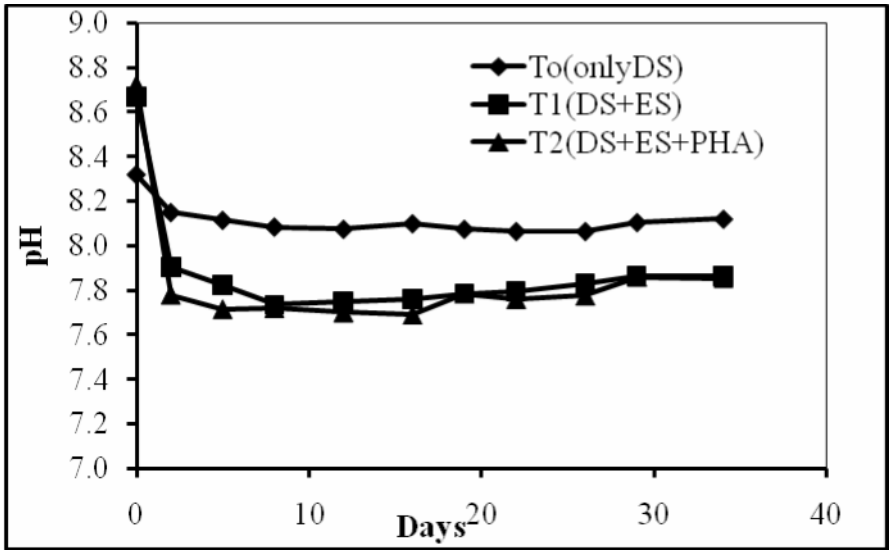

Figure 4.6: pH during anaerobic incubation at different groups

In anaerobic digestion process, pH and NH_3-N are one of the key indicators to monitor the volatile fatty acid formation and the effect of inhibition respectively. pH during incubation at different group was shown in Figure 4.6 . During the start up of the experiment pH in T0, T1 and T2 were 8.3, 8.1, and 8.7 respectively. After incubation the pH was dropped and finally stable as well as ranged from 7.7-7.9 in T1 & T2 and 8.1 in T0. Thus it can be noticed that during the whole experiment pH was in better condition. The desired pH for anaerobic group is

between 6.6 and 7.6. Values outside this range can be quite detrimental to the process, particularly to methanogenesis (Rittmann and McCarty, 2001).

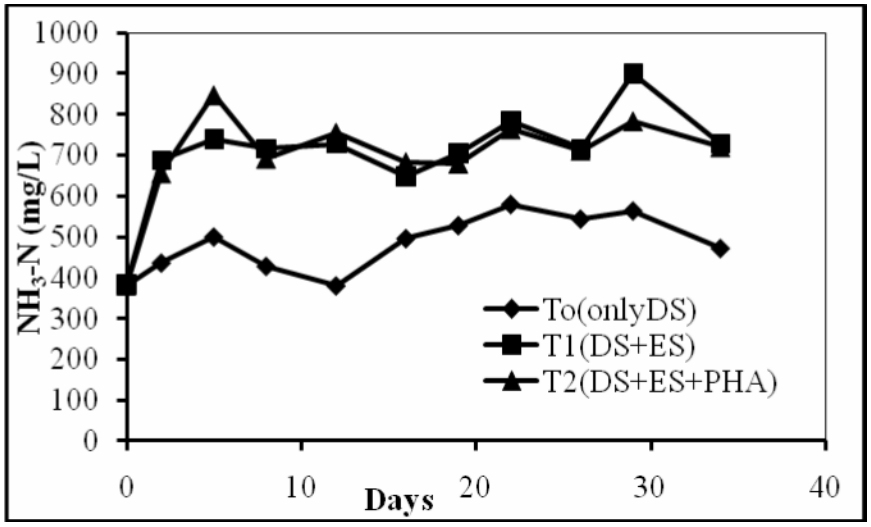

Figure 4.7: NH_3-N concentration during incubation at different groups

Figure 4.7 shows the ammonia- nitrogen concentration during incubation at different group. At the time of incubation, the NH_3-N in T0, T1 and T2 were found 380 mg/L, 384 mg/L and 380 mg/L respectively and then the concentration were increased in all the groups. At the final observational day NH3-N in T0, T1, and T2 were recorded 428 mg/L, 728 mg/L, and 720 mg/L respectively. The NH_4^+ nitrogen concentration found to cause inhibition was much higher, about 3,000 mg/L. If the total ammonia nitrogen concentration (NH_3+$NH_4^{+)}$ were 2,000 mgN/l and the pH were 8.0, the NH_3 nitrogen concentration would be 110 mg/L, which is in the inhibitory range (Rittmann and McCarty, 2001). During the experiment, the NH_3-N concentrations in all groups were found within the normal range and there was no inhibitory effect.

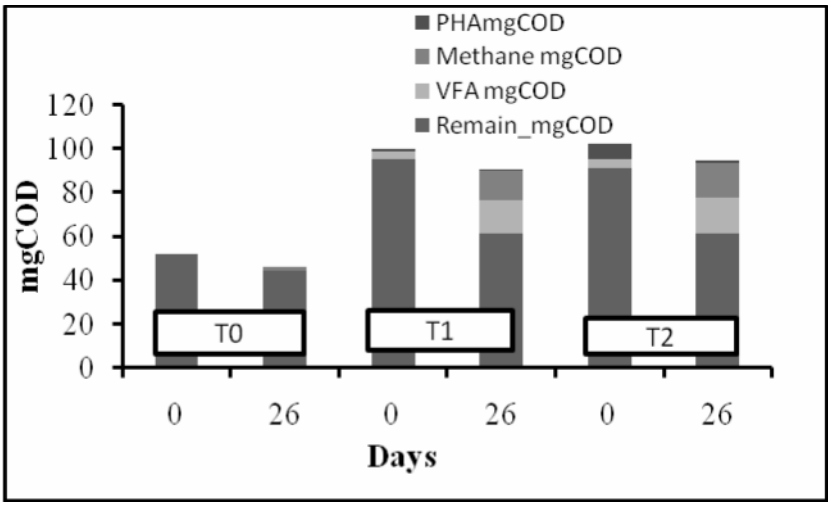

Figure 4.8: Mass balance of T0, T1 and T2 at day 0 and 26.

Figure 4.8 shows the mass balance of different groups at day 0 and 26. There were differences in balance between day 0 and 26 in all groups. But the differences were not significant. The differences mass balance between day 0 and 26 was 11.5% in T0 group, 10% in T1 and 7% in T2. Thus, the difference was in error range. From the mass balance analysis, it can be stated that the quality of the experiment was good, though the error was within around 10%.

From the present study, it was revealed that PHA accumulated in excess sludge was initially quickly degraded, and within only 2 days, more than 77% of PHA was degraded. On the other hand, the degradation of PHA was slowed down after day 2, and even after 34 days, 10% of PHA remained undegraded. This means that not all of the temporal carbon storage materials are degraded, and will cause increase of anaerobic digested sludge. As the total COD_{Cr} degradation was comparatively better T2 than T1 so, the increase of anaerobic digested sludge will be small. It was observed from the anaerobic digestion of PHA-enriched excess sludge that the degradation of PHA was enhanced within the first 5 days whereas acetate formation was increased within first 8 days but methane gas generation was rose after day 18. But the results are compatible in anaerobic digestion process because the methanogens are very slow growing.

4.2 Experiment II: Re-evaluation of degradation of PHA under anaerobic digestion

The cumulative methane gas production (ml/g VS of DS) at different group during incubation is presented in Figure 4.9. The highest yield of methane at the final day was found in T2 (DS+ES+PHA) (99 ml/g VS of DS) followed by T1 (DS+ES) (76.5 ml/g VS of DS). The methane gas production was found to be increased (29%) in T2 compared to T1. Thus, it is revealed that excess sludge accumulating PHA can produce excess methane under anaerobic digestion compared to excess sludge.

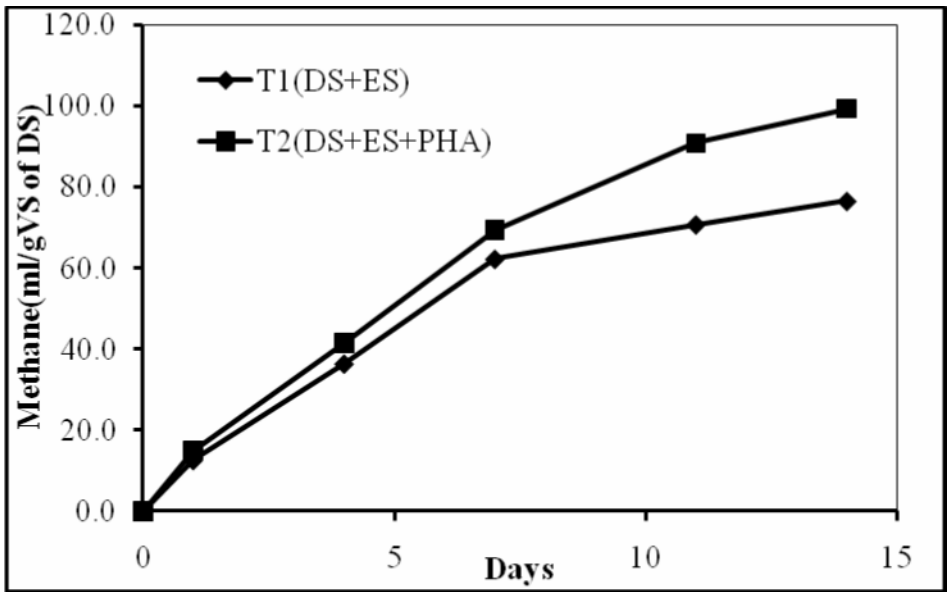

Figure 4.9: Cumulative methane gas production at two different groups during anaerobic incubation

Figure 4.10 shows the total COD of different group during incubation. Total COD was found to be decreased from the day of incubation to last day in all the groups. Total COD in T1 and T2 at the time of incubation were 11360 mg/L and 11740 mg/L and in the last day 8720 mg/L, and 8760 mg/L respectively. The total COD reduction rate in T1 and T2 were 23%, and 25%. When first order kinetics was applied to model the degradation of total COD, the reduction rate parameter were 0.0178 (r^2=0.94) in T1 and 0.0218 (r^2=0.97) in T2. Thus, it can be stated that the total COD was reduced in excess sludge accumulating PHA (T2) compared to excess sludge (T1) under anaerobic digestion.

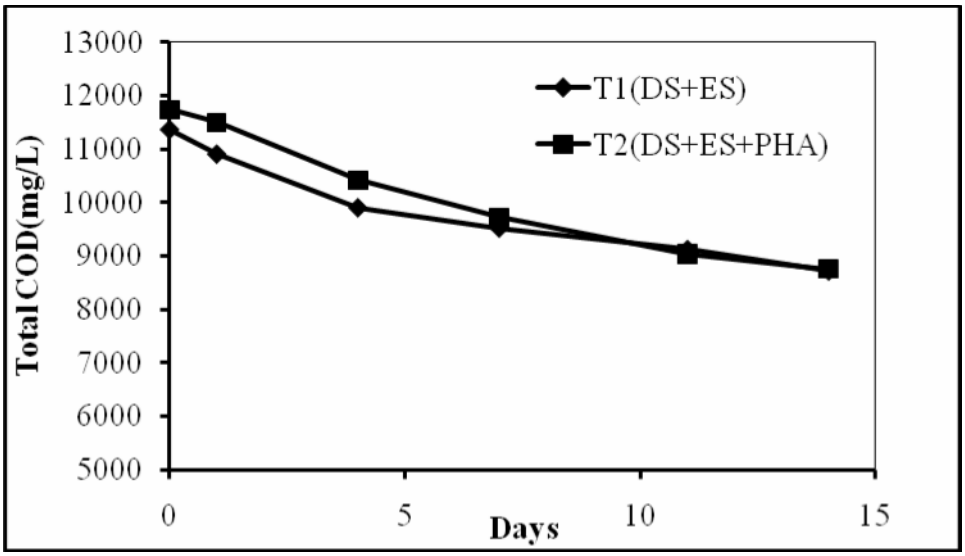

Figure 4.10: Total COD (mg/L) during incubation at different groups.

TS and VS concentration during incubation at different group is shown in Figure 4.11. TS and VS concentration were found to be reduced in all the groups from the day of incubation to last day. During start up of the experiment the TS concentration of T1 & T2 were 16125mg/L & 16800 mg/L and VS were 8925 mg/L, & 9950 mg/L respectively. After the end of the experiment, TS of T1, & T2 were 14825 mg/L, &14125 mg/L and the VS were 7575 mg/L, & 6950 mg/L respectively. The percentages of reduction of TS concentration in T1 and T2 were 8, and 16 respectively. In case of VS concentration, the percentages of reduction in T1, and T2 were 15, and 30. So, it can be noticed that the declining of TS and VS concentration were rapid in T2 (DS+ES+PHA) compared to T1 (DS+ES) under anaerobic digestion and the reduction was almost double in T2 than T1, but the measurement was more susceptible to error..

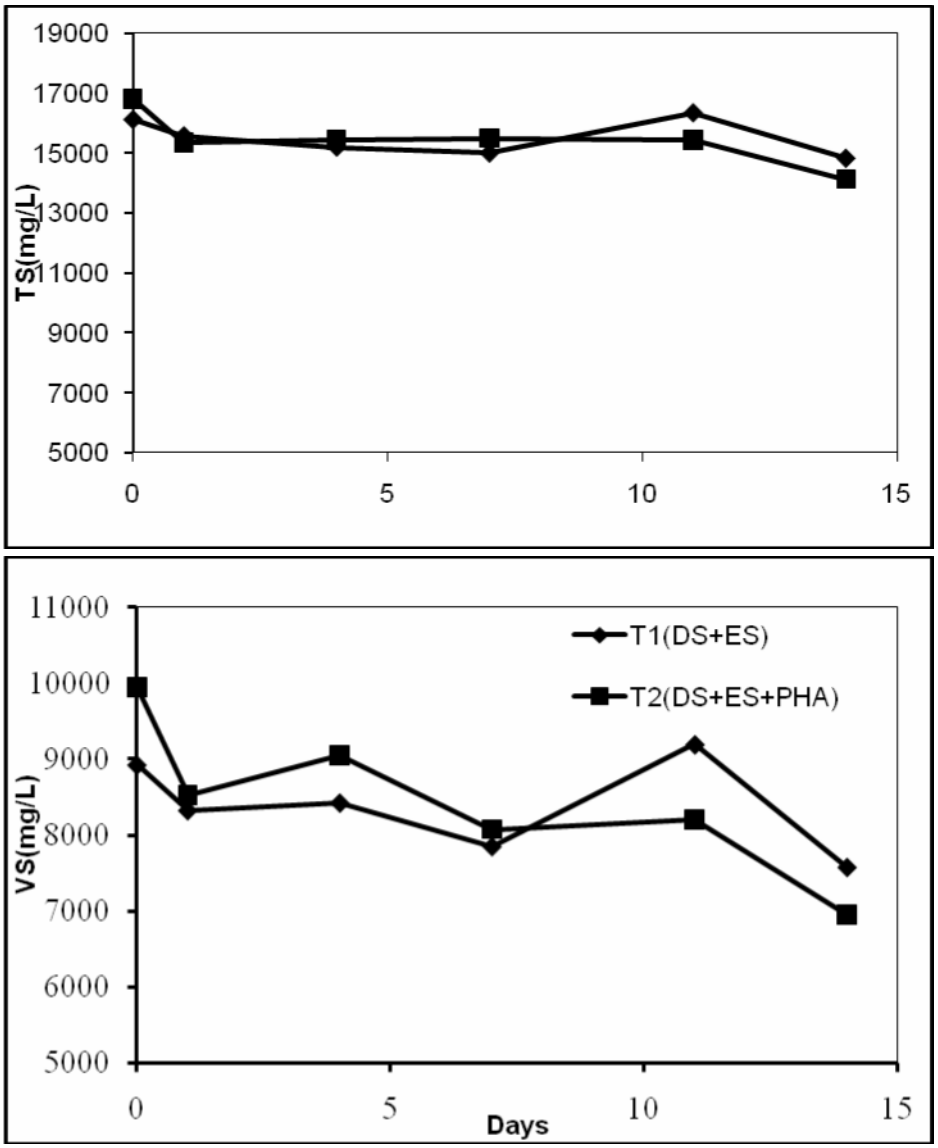

Figure 4.11: TS and VS concentration during anaerobic incubation at different groups.

Figure 4.12 shows the acetate concentration during anaerobic incubation at different groups. During the time of incubation the acetate concentration were found almost the same in both T1 and T2. After the start up of the experiment, the acetate concentration were increased rapidly in T1 and T2 group up to day 4, and then decreased. The highest acetate concentration in T1 and T2 were found 180 mg/L and 452 mg/L respectively in day 4. After that, the concentration was declined and at the last day of observation, there were no acetate recorded in both groups. It is clear from the Figure 4.4 that the acetate formation was found to be the highest in T2 group

(DS+ES+PHA) in comparison to T1(DS+ES). It was also observed that a considerable amount of propionate were also found at first two observational days in both group and then reduced.

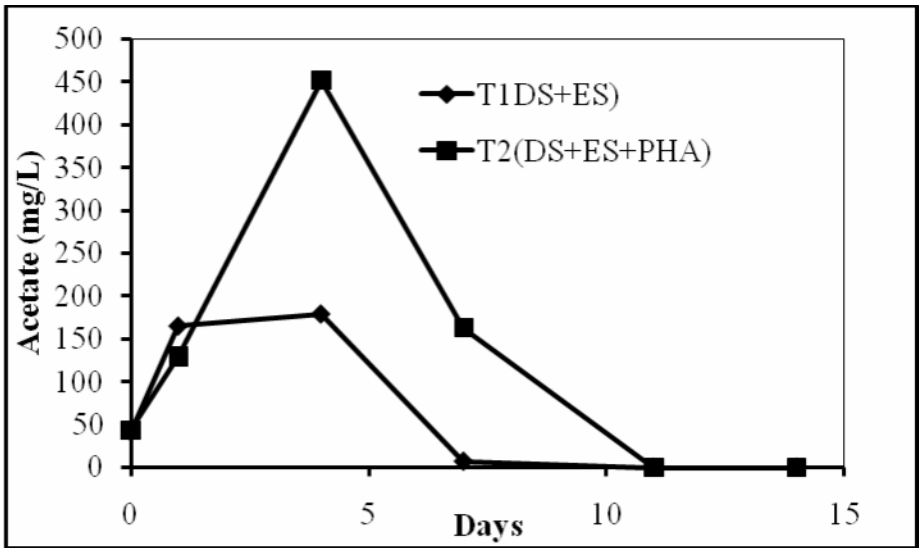

Figure 4.12: Acetate formation during anaerobic incubation at different groups

PHA degradation during anaerobic incubation at different group is illustrated in Figure 4.13. After the start up of the experiment, PHA was degraded sharply within first day in both T1 and T2 and then slowly degraded. At the time of incubation, the concentration of PHA in T1 & T2 were found 92 mg/L & 1004 mg/L respectively and after one day the concentration were recorded 45 mg/L & 1515 mg/L respectively. Within a day the degradation of PHA was 50%. Apparently, the degradation was very rapid in first day. At the final day the PHA in T1 and T2 were found 29 mg/L and 154 mg/L respectively. It is clear that after second to final day, the degradation was slow and like steady condition. The degradation of PHA at the time of incubation to final day in T1 and T2 were found 68% and 85% respectively. Consequently, it was revealed that the PHA content were degraded rapidly in T2 (DS+ES+PHA) compared with T1 (DS+ES) and initially the degradation was very rapid and then slow and steady.

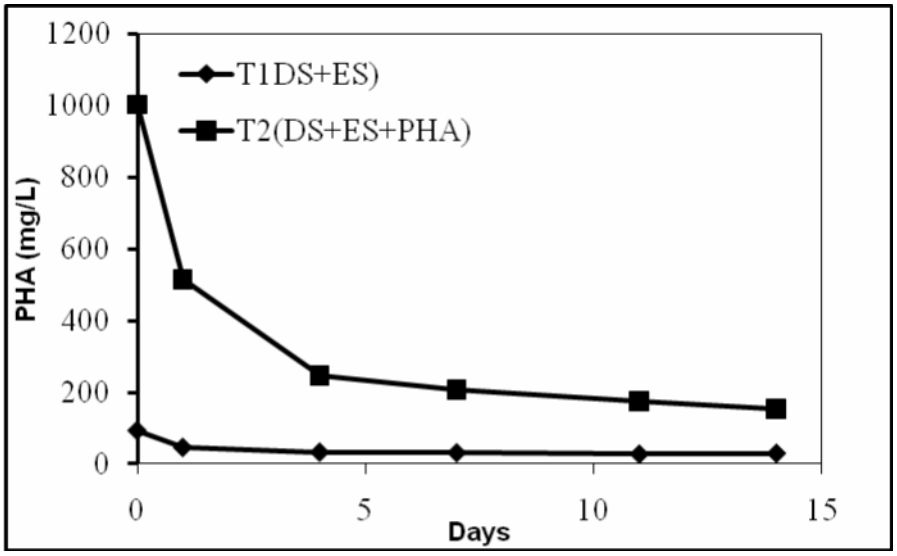

Figure 4.13: PHA degradation during anaerobic incubation at different groups

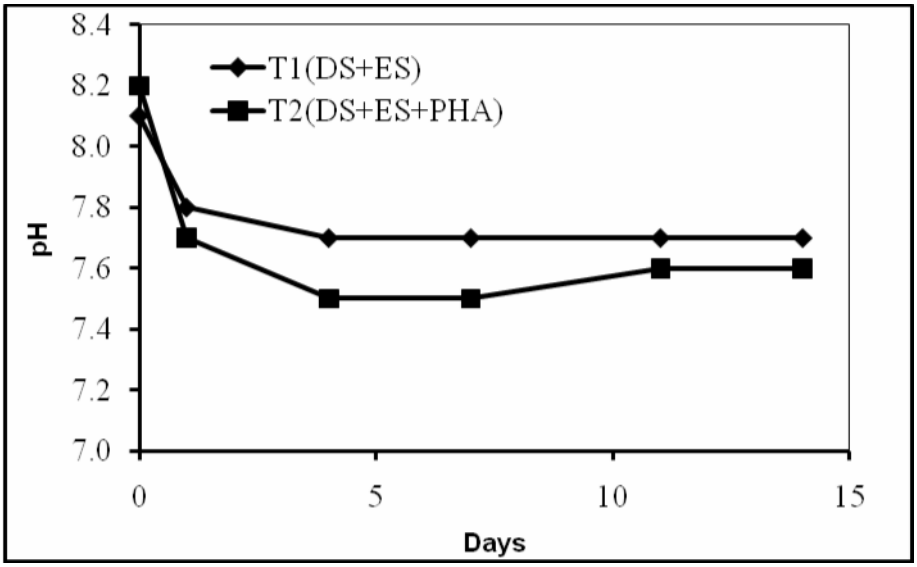

Figure 4.14: pH during anaerobic incubation at different groups

pH during incubation at different group is shown in Figure 4.14 . During the start up of the experiment pH in T1 and T2 were 8.1, and 8.2 respectively. After incubation the pH was dropped and finally stable as well as ranged from 7.6-7.7 in T1 & T2. Thus it can be noticed that during the whole experiment pH was found in normal range.

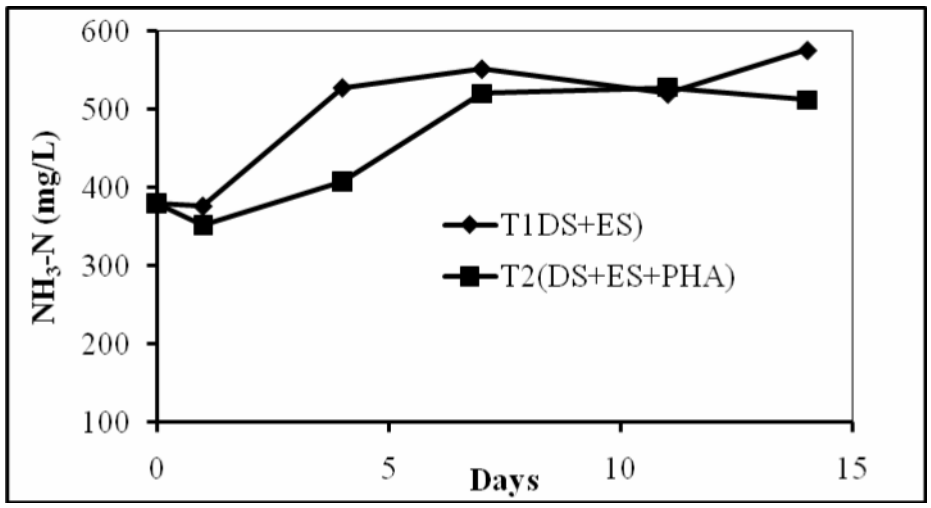

Figure 4.15: PHA degradation during incubation at different groups

Figure 4.15 shows the ammonia- nitrogen concentration during incubation at different group. At the time of incubation, the NH_3-N was found 380mg/L in both groups and then the concentration were increased in all the groups. At the final observational day NH_3-N in T1, and T2 were recorded 512mg/L, and 576mg/L respectively. During the experiment, the NH_3-N concentrations in all groups were found within the normal range and there were no inhibitory effect.

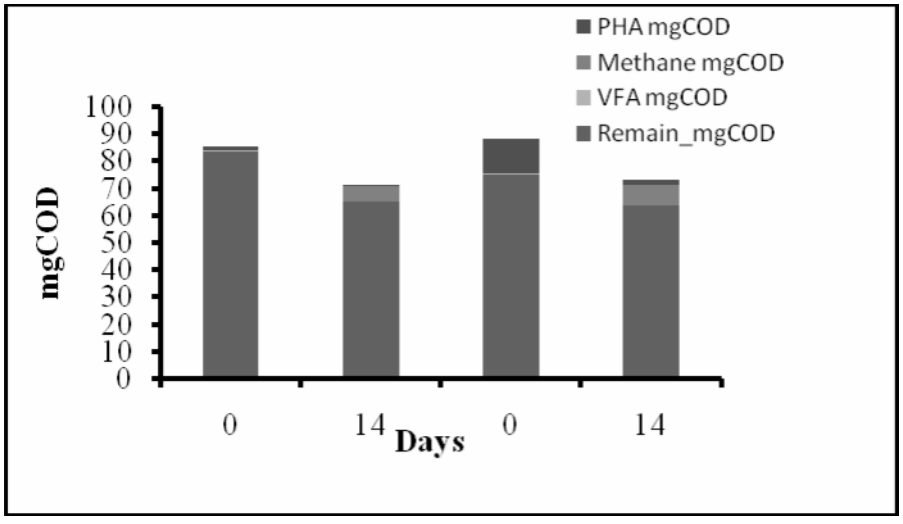

Figure 4.16: Mass balance of T1 and T2 at day 0 and 14

Figure 4.16 shows the mass balance of different groups at day 0 and 14. There were differences in balance between day 0 and 14 in all groups. The differences mass balance between day 0 and

26 was 17% in both group. From the mass balance analysis, it can be stated that the quality of the experiment was more susceptible to error.

It was observed from the present study that, initially, PHA enriched in excess sludge was rapidly degraded, and within only 4 days, more than 75% of PHA was degraded. The degradation of PHA was sturdy after day 4, and even after 14 days, 15% of PHA remained undegraded. As all the temporal carbon storage materials are not degraded, which will cause increase of anaerobic digested sludge. It is also observed from the anaerobic digestion of PHA-enriched excess sludge that the degradation of PHA was enhanced within the first 4 days whereas acetate formation was increased following the same approach but methane gas generation was rose after day 7. This affinity of such results is common in anaerobic digestion process.

4.3 Comparison between Results of Two Experiments

From the both experiments it was confirmed that initially, PHA was degraded very rapidly and then the degradation was slow and stable. The experiment I showed the reduction of PHA up to 16 days in T2 (DS+ES+PHA) was 87% whereas in experiment II, it was 85% within 14 days. On the other hand, in both experiments, excess methane was also found in T2 group. The methane production in T2 group was found to be increased 29% compared to T1 (DS+ES) in both experiments, where experiment I illustrated up to 16 days outcome and experiment II 14 days. Total COD_{cr} and VS were also declined rapidly in both experiments.

In the present both studies, it can be stated that PHA accumulated in excess sludge was initially quickly degraded, and more than 75% of PHA was degraded under anaerobic digestion. Thus, PHA enriched excess sludge generated from energy saving FAREWEL process can be degraded better than that of excess sludge: otherwise, the sludge treatment process will be overloaded. This outcome supports the feasibility of FAREWEL process where temporal carbon storage materials formed which may be easily degraded and converted to methane gas. On the other hand, the degradation of PHA was slowed down after day 6 in experiment I and day 4 in experiment II, and after 34 days 10% of PHA in experiment I and after 14 days 15% of PHA in experiment II remained undegraded. This means that not all of the temporal carbon storage materials are

degraded, and will cause increase of anaerobic digested sludge that is to be finally disposed. But the increase of anaerobic digested sludge will be small in FAREWEL process.

It was also observed from the anaerobic digestion of PHA-enriched excess sludge that the degradation of PHA was enhanced within the first 2 days in experiment 1 and first 4 days in experiment II whereas acetate formation was increased within first 12 days in experiment I and first 4 days in experiment II but methane gas generation was rose after day 18 in experiment I and after day 7 in experiment II. This behavior is compatible in anaerobic digestion process because microorganisms involves in hydrolysis and fermentation steps grow relatively rapid and forms acetate but the archeal methanogens which convert acetate to methane are more slowly growing and tend to be rate limiting (Rittmann and McCarty, 2001). Though much research has been performed on degradation of PHA in aerobic condition but information on anaerobic digestion is very limited. Doi (1990) described a cyclic nature of PHA metabolism in aerobic condition where PHA was subsequently converted to acetoacetate, acetoacetyl-CoA, acetyl Co-A, and finally, to tri carboxylic cycle (TCA). From the study, it can be stated that under anaerobic digestion PHA is firstly hydrolyzed and converted to acetyl Co-A where acetyl Co-A is utilized by fermenting bacteria and then produce acetate and finally, archeal methanogens use acetate and convert it to methane gas, which need further study to confirm .

CHAPTER FIVE

CONCLUSIONS AND CONCLUDING REMARKS

5.1 Conclusions

From both experiments, it can be concluded that that temporal carbon storage material in excess sludge can produce excess methane (25% in experiment I and 29% in experiment II) compared to excess sludge alone under anaerobic digestion. It is clearly revealed that initially PHA degradation was rapid in PHA-enriched excess sludge which was 77.5% in experiment I within 2 days and 75% in experiment II within 4 days and then the rate of degradation diminished to a steady state in both experiments, so the metabolic pathway of PHA degradation under anaerobic digestion needs to be assessed. Finally, it can be stated that the temporal carbon storage materials accumulated in excess sludge generated from FAREWEL process can produce excess methane and initially, the degradation can be rapid till it reached a low rate steady state which will slightly increase the volume of anaerobic digested sludge and needs to be disposed.

5.2 Concluding Remarks

As has been discussed in Chap.4, the followings basic studies are needed.

1) As PHA was degraded rapidly within first couple of days and then the degradation was found slow and sturdy under anaerobic digestion, so it is very important to evaluate the mechanism of metabolism of excess sludge accumulating PHA.
2) Since methane gas production was high in excess sludge accumulating PHA and the degradation of PHA was also speedy under anaerobic digestion, microbial community analysis is also vital to observe the change of microbes during different observational days.

Further, in the present study, anaerobic digestion of excess sludge which has accumulated temporal storage material PHA was found to be effectively performed. Thus, here is the final remark.

3) The primary objective of the experiment was to study the feasibility of FAREWEL process from which a significant amount of excess sludge accumulating PHA produced. As excess methane was produced and the PHA was also rapidly degradable, so a pilot scale experiment was indispensable for the reduction of excess sludge accumulating PHA under anaerobic digestion.

CHAPTER SIX

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APPENDIX

The following experiment was performed on November 2009. During this experiment, some problems identified and for next experiments, some improvements were done. This appendix includes the specific objectives of the study, a brief description of materials and methods, results and discussion, problems and improvements.

Specific Objectives of the study

- To study the degradability of excess sludge accumulating PHA under anaerobic digestion through excess methane production.
- To compare the degradability of intracellular and intercellular PHA under anaerobic digestion.

Materials and Methods

Inoculum

Sludge from anaerobic digester treating excess sludge in the laboratory was used as inoculum at a VS ratio of 1 (substrate: inoculum). TS and VS of inoculum were 19200 mg/L and 16000mg/L respectively.

Substrates

Excess sludge was collected from Kasumigaura sewage treatment plant in November, 2009. Two liters of excess sludge suspension was taken and centrifuged. Then sludge pellet was suspended with effluent. TSS and VSS concentration were measured (Table 1). For PHA production, another two liters of collected excess sludge suspension was put into glass beaker. Acetate was fed at final concentration 425mg/L and incubated for 2.5h under aerobic condition by air bubbling. Chemical staining with Nile Blue A and microscopic observation was performed to confirm the PHA accumulation in excess sludge. After incubation, the activated sludge was centrifuged and washed three times with effluent and then sludge pellet was suspended in effluent. TSS and VSS concentration were measured (Table 1). PHA granules extracted from PHA-accumulating micro-organisms were collected from private company.

Table 1: Concentraton of TSS and VSS in inoculum and substrates

Inoculum/Substrates	**TSS (mg/L)**	**VSS (mg/L)**
Excess Sludge (ES)	18800	14000
ES accumulating PHA	19600	14400

Experimental Set Up

The tests were performed in 10-ml vials with a liquid volume of 6.5ml and nitrogen atmosphere. Inoculum and substrates were mixed at VS ratio of 1:1 and the following treatments were used in the experiment (Table 2) .

Table 2: Different treatments of experiment

Group	Treatments
T0	DS only
T1	DS + ES (1:1 VSS)
T2	DS + ES accumulating PHA (1:1 VSS)
T3	DS + (ES + PHA granule 1:1 VSS) (1:1 VSS)

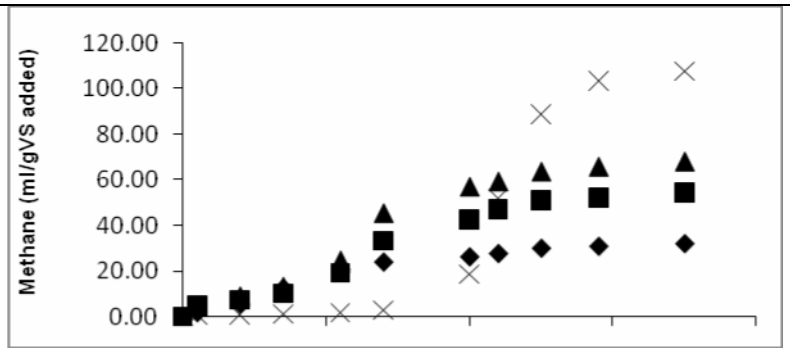
Bicarbonate at a concentration of 14g/L was added to provide buffering capacity. The vials were incubated at 37^{o}C under stirring conditions (200 rpm). Gas composition and chemical analysis were performed on days 0, 1, 4, 7, 11, 14, 20, 22, 25, 29 and 35. Chemical analyses were performed with duplicate. For daily gas measurement five vials were used and two vials were used for measuring gas composition.

Analysis

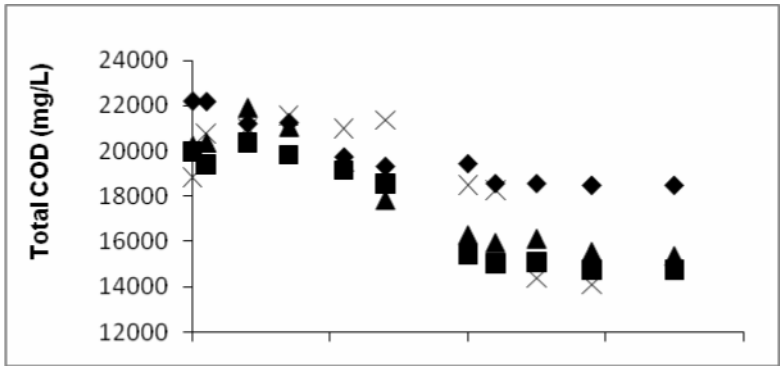
Biogas production was measured using a glass syringe equipped with a 25 gauge needle. Prior to biogas release the vials were shaken for 30s, in order to reach the equilibrium between gases present in both the gas and liquid phase. Gas composition was analyzed periodically using GC-3200. For liquid-phase analysis, 2ml samples from each vial were centrifuged for 2 minutes in micro-centrifuge MX 150 (TOMY). Samples from the supernatant were taken for analysis of VFA, and NH_3-N measurement. The solids were washed twice with Phosphate-Buffered Saline and stored at 80^{o}C for microbial analysis. VFA concentrations were measured using HPLC (L-2400, Hitachi, Japan). Total COD and NH_3-N were measured by using HACH company kit. TS and VS were also measured according to standard methods. pH was measured with a HM 30G pH-meter . The determination of PHA was performed by gas chromatography after methanolytic decomposition as described in Satoh et al. 1992. A gas chromatograph GCMS-QP2010 (Shimadzu, Japan) with a column Neutrabond-1 (GL Science, Japan, 30m length, 250 mm internal diameter, 0.4 mm film thickness) was used. The detector and injector temperatures were 250^{o}C and 180^{o}C, respectively.

Results

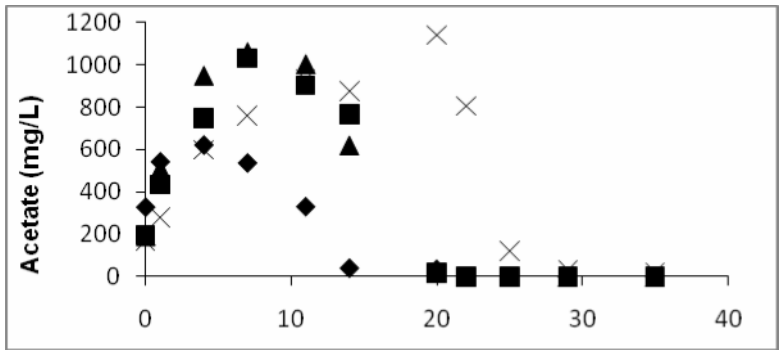
The methane production was found the highest in T3 (108 ml/gVS of DS added) treatment followed by T2 (68ml/gVS of DS added) and T1 (54ml/gVS of DS added) (Fig. 1a). In case of T2 treatment methane production were found highest after 10 days of start up of experiment whereas T3 treatment showed after 20 days. Apparently, excess methane productions were found in both intra and inter cellular PHA but the amounts of PHA in both treatments were not equal. T3 treatment was performed to examine the present experiment by distinguishing the degradability of inter cellular PHA. Total COD (Fig. 1b) of all treatments were decreased. After the start up of the experiment, in case of T0, T1 and T2 treatment the acetate concentration were increased very rapidly upto 10 days but in case of T3 it was increased after 20 days (Fig.1c).



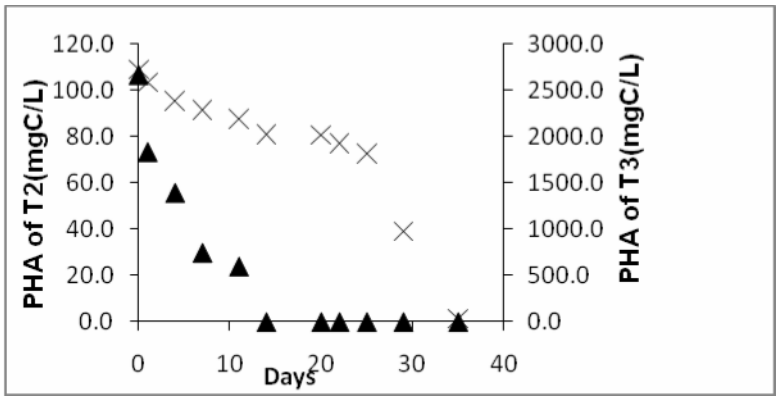
a)



b)



c)



d)

Fig.1: a) Methane production, b) degradation of Total COD, c) Acetate, and d) PHA during incubation of -♦- T0(DS), -■--T1(DS+ES),-▲-T2(DS+ES+PHA), -×-T3(DS+ES+PHA powder).

It was also observed that a considerable amount of other volatile fatty acids (propionate, butyric acid and valeric acid) were also found in T3 treatment. In case of PHA measurements, T0 and T1 did not show any PHA concentration. But in T2 PHA was degraded rapidly (Fig.1d) where as PHA in T3 was degraded slowly but after 20 days it was degraded rapidly and methane gas was formed. Thus, it is assured that that the degradation of intracellular PHA (T2) was rapid compared to intercellular PHA (T3).

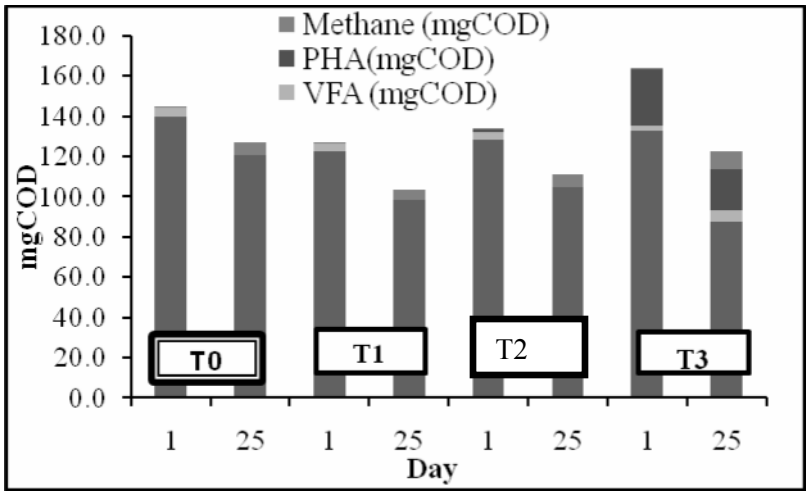


Fig. 2 Mass balance of T0, T1, T2 and T3 at day 0 and 25

Figure 2 shows the mass balance of T0, T1, T2 and T3 at day 0 and 25. The mass balance was not good which is susceptible to more error. From the mass balance, it was found that gas production was low and it could be occurred either leakage of caps of vials or inaccurate gas measurement.

Problem identified during the experiment:

- The caps of the vials were not tight properly, leakage of gas occurred. It is because of the improper use of instrument which is used for making tight of the caps.
- For measuring COD, and VS pipetting is important. Due to improper pipetting the results of the COD& VS fluctuated too much.
- In PHA measurement, some difficulties found in preparing standard sample and calibration curve. During preparing of the mixtures of PHB and chloroform, uniform mixtures did not make. That's affect the calibration curve.

Improvements:

- Before making tight, instrument was calibrated and practiced.
- Auto micro-pipette was used during the experiment. Same pipette was used in each observational day.
- PHB can mix with chloroform. But only shaking it cannot mix. After taking the chloroform and PHB powder, the tube was warmed during mixing and it mixed uniformly. Finally, the calibration curve was accurate.