EFFECT OF NITRITE AND pH ON THE ACCUMULATION OF POLYHYDROXYALKANOATES (PHAs)

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by

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LIST OF SYMBOLS

	Symbol	Unit
$Y_{PHA/CDW}$	PHA content	g PHA/g CDW
T	Temperature	^{o}C
t	Time	hours

NOMENCLATURE

ADD	Aerobic Dynamic Discharge
ADF	Aerobic Dynamic Feeding
CDW	Cell Dry Weight
COD	Chemical Oxygen Demand
FA	Free Ammonia
FNA	Free Nitrous Acid
IFAS	Integrated Fixed-Film Activated Sludge
MCL	Medium-chain length
MLSS	Mixed Liquor Suspended Solid
MMC	Mixed Microbial Culture
NOB	Nitrite Oxidizing Bacteria
OLR	Organic Loading Rate
PHA	Polyhydroxyalkanoate
SBR	Sequencing Batch Reactor
SCL	Short-chain length
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solid

KESAN NITRIT DAN pH TERHADAP PENGUMPULAN POLIHIDROKSIALKANOAT (PHA)

ABSTRAK

Plastik sintetik yang terbuat dari bahan api fosil biasanya digunakan dalam kehidupan manusia kerana kelebihannya dari segi kekuatan, ringan dan mempunyai daya tahan yang tinggi. Penghasilan plastik dari bahan berasaskan bio seperti polihidroksialkanoat (PHA) adalah salah satu kaedah yang dapat menggantikan polimer yang diperoleh daripada petroleum konvensional. Walau bagaimanapun, terdapat beberapa faktor yang boleh mempengaruhi pengumpulan PHA. Tujuan kajian ini adalah untuk mengkaji kesan pH dan kepekatan nitrit terhadap pengumpulan PHA. Eksperimen dijalankan selama 6 jam dalam satu kitaran dengan menggunakan bioreaktor yang mempunyai isipadu 2 L. Sampel 1 L yang terdiri daripada mikroorganisma PHA yang terkumpul dalam bentuk granular diambil dari reaktor induk yang mampu menampung 8 L sampel dan dicairkan dengan menambah 1 L air untuk menghasilkan larutan yang terdiri daripada air dan granular. 2 L larutan tersebut dimasukkan ke dalam 2 L bioreactor beserta dengan larutan nitrit yang mempunyai kepekatan bermula dari 30 mg / L, 60 mg / L, 90 mg / L, 120 mg / L dan 150 mg / L untuk setiap kitaran. Nilai pH dimalarkan pada 7.5. Sampel diambil pada 0, 0.5, 1.0, 1.5, 2.0, 4.0 dan 6.0 h untuk analisis COD, nitrit (NO_2^-), FNA, dan PHA. Langkah yang sama diulang dengan mengubah pH larutan dari 6.0, 6.5, 7.0, 7.5 dan 8 untuk setiap kitaran 6 jam tanpa tambahan nitrit untuk mengkaji kesan pH pada pengumpulan PHA. Larutan stok natrium hidroksida, NaOH dan asid asetik, CH_3COOH digunakan untuk mengawal nilai pH. Pengumpulan PHA diperhatikan lebih tinggi pada pH 7.0 dan kepekatan nitrit sebanyak 30 mg / L. Kesan nitrit

dan pH ditentukan oleh kehadiran asid nitrous bebas (FNA) dalam sampel. Oleh itu, kesan nitrite dan pH terhadap pengumpulan PHA di dalam mikroorganisma akan ditentukan oleh nilai FNA di dalam sampel. Semakin rendah nilai FNA di dalam sampel, semakin tinggi pengumpulan PHA di dalam mikroorganisma

EFFECT OF NITRITE AND pH ON THE ACCUMULATION OF POLYHYDROXYALKANOATES (PHAs)

ABSTRACT

Synthetic plastic which is made from fossil fuel is commonly used in human life due to its advantages in term of strength, lightness and durability. Producing plastics from bio-based materials such as polyhydroxyalkanoate (PHA) is one of the method that are able to replace conventional petroleum-derived polymers. However, there are some factors that can affect the accumulation of PHA. The purpose of this study is to investigate the effect of pH and nitrite concentration on the accumulation of PHA. Experiment are conducted for 6 hours operation cycle by using 2 L bioreactors. 1 L sample which consists of PHA-accumulating microorganisms in granules form are taken from 8 L parent reactor and dilute with 1 L water to form mixed liquor. 2 L of mixed liquor are added into the 2 L bioreactor together with the specific concentration of nitrite starting from 30 mg/L, 60 mg/L, 90 mg/L, 120 mg/L and 150 mg/L for each cycles by controlling the value of pH at 7.5. Sample are taken at 0, 0.5, 1.0, 1,5, 2,0, 4.0 and 6.0 h for COD, nitrite (NO_2^-), FNA, and PHA analysis. Similar step is repeated by changing the pH of the mixed liquor from 6.0, 6.5, 7.0, 7.5 and 8 for each 6 hours cycle without any addition of nitrite. Stock solution of sodium hydroxide, NaOH and acetic acid, CH_3COOH were used to control pH value. Accumulation of PHA is observed to be higher at pH of 7.0 and nitrite concentration of 30 mg/L. Effect of nitrite and pH were determined by the presence of free nitrous acid (FNA) in the sample. Hence, the effect of nitrite and pH value on the accumulation of PHA will be determined by the presence of FNA.

The lower the value of FNA in the sample, the highest the PHA will be accumulated in the microorganism

CHAPTER ONE

INTRODUCTION

1.1 Polyhydroxyalkanoates (PHAs) as Raw Material for Producing Synthetic Plastics

Synthetic plastic has been widely used around the world to improve the quality of human life replacing commodity like glasses or paper in packaging (Możejko-Ciesielska and Kiewisz, 2016b) or other sectors especially in manufacturing. Synthetic plastics have comprehensive features of strength, lightness and durability even being non-biodegradable where 98% of the total production corresponded to fossil fuel-based plastics obtained from petroleum (Możejko-Ciesielska and Kiewisz, 2016a). According to data from PlasticsEurope organization, world and European, plastics production from 2002-2013 which includes plastic materials such as thermoplastics, polyurethanes, thermosets, adhesives, coatings, sealants, and PP-fibres continuous to growth for more than 50 years. Data from this organization also state that, global plastics production rose to 299 million tonnes in 2013 as compared to 288 million tonnes in 2012, with 3.9% increasing after 1 years (PlasticsEurope, 2015). The increases in production of plastics especially in Europe had showed a positive impacts in its demand for five well-known country such as Germany which at first ranking with 25.4% demand, followed by Italy, France, United Kingdom (UK) and Spain (PlasticsEurope, 2015). In fact, growing trend in the production of plastics is expected to increase at least until 2020 (Anjum et al., 2016) due to the case study that happened during 2016 with increasing in the production of plastic materials by 4 % (Muhammadi et al., 2015).

Even though plastics are used for many applications, the usage of petroleum plastics has contribute to major environmental issues such as global warming, human health risks or ecosystem toxicity (Harding et al., 2007). Disposal of plastic waste into environment ended up in the waste upstream resulting in a significant burden on plastic waste management (Możejko-Ciesielska and Kiewisz, 2016a) because plastic materials are resistant to microbial degradation (Możejko-Ciesielska and Kiewisz, 2016a). Depletion of petroleum resources, escalation of crude oil and negative impacts of petroleum to the environment had encouraged researchers to propose sustainable alternative sources to produce biodegradable plastics from biological derived polymers which are more eco-friendly (Możejko-Ciesielska and Kiewisz, 2016a). Due to its advantages, it is predicted that the production of bio-based polymers will triple from 5.1 million tonnes in 2013 to 17 million tonnes in 2020 (F. Aeschelman and Carus, 2015).

Bio-based materials such as polylactide, polysaccharides, aliphatic polyesters and polyhydroxyalkanoates (PHAs) which possess similar physicochemical properties as conventional plastics (Castilho et al., 2009) are seen to be able to replace conventional petroleum-derived polymers (Anjum et al., 2016). They can be produced naturally by variety of microorganisms and plants. Biopolymers are biocompatible because they have no adverse effects on biological systems. It is believed that biopolymers of bacterial origin are produced as a result of their defence mechanism or as storage material which are then decompose (Anjum et al., 2016). Among all of these biopolymers, polyhydroxyalkanoates (PHAs) are chosen as the best biopolymers due to its great potential such as high biodegradability, their recyclable nature (Anjum et al., 2016), sustainability and environment-friendly properties (Salehizadeh and Van Loosdrecht, 2004). PHA are family of biopolyesters with molecular

weight in range of 5 x 10^4 to 2 x 10^7 which can be synthesized by various bacteria as carbon and energy stores (Tan et al., 2017). According to Możejko-Ciesielska and Kiewisz (2016b), PHA have been observed in 1888 by Beijerincka. However, the role and composition of these biopolymer cannot be defined properly by him. French researcher Lemoigne obtained the poly-3-hydroxybutyric acid (P(3HB)) from Bacillus ,egaterium in year 1926. After that, in 1958, due to the problem faced by Beijerincka, Macrae and Wilkinson have proved that PHAs in bacterial cells play the role as the reserved materials of carbon and energy and they are collected only in an increased carbon to nitrogen ratio (Możejko-Ciesielska and Kiewisz, 2016b). In beginning of year 1959, many companies start to grab this chances to commercialize PHAs as environmentally bioplastics which is fully independent from petroleum sources. PHA have much wider diversity in monomers with over 150 structural variations as compared to other bio-based polymers. Common PHA monomer are 3-hydroxypropionate (3HP or C3), 3-hydroxybutyrate (3HB or C4), 3-hydroxyvalerate (3HV or C5), 3-hydroxyhexanoate (3HHx or C6), 3-hydroxyoctanoate (3HO or C8), 3-hydroxydecanoate (3HD or C10), 3-hydroxydodecanoate (3HDD or C12), 3-hydroxytetradecanoate (3HTD or C14), and 4-hydroxybutyrate (4HB) (Wang et al., 2014, Steinbüchel and Valentin, 1995). PHA can be divided into short-chain length (SCL) and medium-chain length (MCL), consisting of monomers in range of three to five (C3-C5) and C6 to C14 of carbon atoms respectively (Witholt and Kessler, 1999).

1.2 Problem Statement

Commercial PHA production use expensive raw materials and chemical as sources of organic matter (Rodriguez-Perez et al., 2018) which cause most of plastic industry need to be careful in choosing PHA as their raw materials since it contribute to high production costs.

Many alternatives have been proposed to enhance the profitability of the system and to facilitate the demand of PHA in the market as one of the raw materials in producing biodegradable plastics. Production of PHA requires high cost due to the complexity of the process and types of substrate used. One of the most popular method to reduce the production cost is the uses of industrial by-products and/or waste streams such as waste plant oils or wastewater, agriculture feedstock (Rodriguez-Perez et al., 2018) as a substrate or source of carbon (Tan et al., 2017). Wastewater is chosen as an alternatives to study the accumulation of PHA at lab scale due to its advantages in term of its availability and able to provide sufficient requirement in term of carbon sources and nutrients. Bacteria or microorganisms that is being used in biological wastewater treatment to treat any contaminants or undesired nutrients contain PHA as energy store. After being used in treating wastewater, bacteria or biomass will settle down in the container. Without knowing the presence of PHA in the microorganisms, most of bacteria will only be used for treating purposes and recycled for a few times. After several study was carried out, most of them had found the present of PHA in bacteria that can be extracted out (Werker et al., 2014) to be used for other purposes.

Even though municipal wastewater is chosen as substrate to accumulate PHA in the microorganisms, sometimes it might lead to some major drawbacks such as tendency for PHA inhibition by any substances or materials in the culture itself. According to study conducted by Bengtsson et al. (2017), the production of PHA from wastewater using mixed microbial culture is commonly carried out through a sequence of process elements such as acidogenic formation for production of volatile fatty acids (VFAs), enrichment and production of biomass with accumulation potential with concurrent wastewater treatment, accumulation of PHA in the biomass in order to enhance the biomass PHA content, and

downstream polymer recovery. It has been found that, the readily biodegradable organic fraction of municipal wastewater may also promote enrichment of PHA-storing organisms. When this occur, biomass is exposed to 'feast and famine' conditions with respect to the carbon sources (Bengtsson et al., 2017). Municipal wastewater also contain higher amount of nitrogen to those required for heterotrophic microbial growth and this nitrogen excess need to be removed in order to meet the criteria of treated water before it being discharged. Ammonia in the municipal can also affect the accumulation of PHA by the oxidation of its component into nitrite by the action of AOB before it further oxidize into nitrate and being remove as nitrogen as at the end of the cycle. Recently, study on the effect of nitrite in the accumulation of PHA in municipal wastewater is still limited and further research need to be conducted whether this component may affect the yield of PHAs. Operating conditions are fundamental when involving wastewater treatment and accumulation of PHA. For example, there are many research conducted to study the effect of this factor on the accumulation of PHA such as pH control (Chua et al., 2003, Guerra-Blanco et al., 2018, Montiel-Jarillo et al., 2017) , aeration, substrate composition (Albuquerque et al., 2011, Martins et al., 2011, Venkateswar Reddy and Venkata Mohan, 2012), temperature (Johnson et al., 2010) and others. Even though study on PHA has become one of the favorite topic by most of the researchers, further study at various pH value (acidic to alkaline) need to be carried out.

1.3 Objectives

Accumulation of PHA has been affected by certain conditions. Hence, objective of this study are listed below in order to investigate the effect of these conditions to PHA :

1.3.1 To study the effect of pH value on the accumulation of PHA

1.3.2 To study the effect of nitrite (NO_2^-) concentration on the accumulation of PHA

CHAPTER TWO

LITERATURE REVIEW

2.1 Process for Production of Polyhydroxylalkanoates (PHA)

Polyhydroxylalkanoates (PHA) has become one of the famous topic due to its advantages especially as biodegradable polyesters. PHA is added-value product produced from the biological wastewater treatment. Researchers have find this as one of the chances that can contribute to minimize the use of petroleum resources and problem related to growing number of synthetic plastics which can affect the climate change (Możejko-Ciesielska and Kiewisz, 2016b). Based on study reported by (Możejko-Ciesielska and Kiewisz, 2016b), PHA can be produced in the presence of excess carbon sources in PHA-accumulating microorganisms. Most commonly, acetate will be used as one of the famous carbon sources (Guerra-Blanco et al., 2018) that plays an important role in controlling the accumulation of PHA. This valuable product can be synthesized in various process or method depends on the type of bacteria being used and operating conditions such as pH, organic loading rate, substrate concentration and temperature

Process being used in treating wastewater or producing PHA will affect the amount of this valuable product since the activity of the biomass will give an important measurement on the PHA productivity. Combination of municipal wastewater treatment and enrichment for PHA accumulation by using integrated fixed-film activated sludge (IFAS) with biofilm carrier media was investigated by Bengtsson et al. (2017) even though it was possible to enrich for PHA-storing microorganisms while treating industrial wastewater especially when

it having relatively high volatile fatty acid (Bengtsson et al., 2008). Research conducted by Anterrieu et al. (2014) use sugar industry process water as a sample to apply the combination of enrichment for PHA accumulation with biological nitrogen removal by nitrification/denitrification. The main difference between both of these researchers is they are using different sample with different nitrogen content in order to investigate the effect to the nitrogen removal and its significance to the production of PHA. According to (Bengtsson et al., 2017), pilot scale investigation (500-800 L) by using IFAS system can lead to higher total nitrogen removal and total chemical oxygen demand up to about 83% and 80% respectively. Biomass produced from this research are able to accumulate up to 49% PHA of volatile suspended solids (VSS).

Another common process involve in accumulation of PHA is aerobic dynamic feeding (ADF) (Chen et al., 2016, Gobi and Vadivelu, 2014, van Aalst-van Leeuwen et al., 1997, Majone et al., 1996, Serafim et al., 2004b). Feast and famine regime is commonly describe the behavior of ADF process. According to van Aalst-van Leeuwen et al. (1997), Majone et al. (1996) and Chen et al. (2016) microorganisms are cultivated in dynamic condition of alternating presence (feast phase) and absence (famine phase) of substrate culture (carbon sources), designated as the feast-famine regime or aerobic dynamic feeding mode. During feast phase, organisms will use external substrate and mainly driven toward internal polymer storage which further be used as a carbon and energy sources after substrate exhaustion (famine phase) (Chen et al., 2016). This regime continue to occur throughout the process until certain period of time. ADF also been applied by Gobi and Vadivelu (2014) in sequencing batch reactor (SBR) as a strategy for producing granules in accumulation of PHA in aerobic granules. The main reasons ADF strategy was applied by most researchers is due

to the challenges given by mixed culture that can affect the amount of PHA being produced as much as pure culture strain for given volume (Campos et al., 2014). According to Johnson et al. (2009) and Serafim et al. (2004b), ADF strategy is needed in order to optimize the PHA accumulation because not all the myriad types of microorganism in a mixed culture can accumulate PHA. The implementation of ADF process was proven by Johnson et al. (2009) as one of the successful implementation in which 89% of the cell dry weight (CDW) of the mixed culture consists of PHA. For Tay et al. (2001), implementation of ADF strategy will lead to the formation of granules under aerobic condition which can help to induce hydrophobicity between cells and thus enhancing the production of PHA (Gobi and Vadivelu, 2014, Tay et al., 2001)

Aerobic dynamic discharge (ADD) process was another process developed to improve the efficiency of PHA accumulation cultures as compared to conventional aerobic dynamic feeding (ADF). ADD process involve physical selective pressure to favor the accumulation of PHA-accumulating bacteria in mixed microbial cultures (MMCs) (Chen et al., 2015a). According to study conducted by Albuquerque et al. (2011), the ADF selection mode imposes a well-documented selective pressure. But, stability problems had occurred in this process such as sludge bulking which is mostly happened during PHA accumulating culture enrichment process (Jiang et al., 2011, Shahhosseini, 2004, Martins et al., 2011, Tamis et al., 2014, Wen et al., 2012). Due to the problem occurred in the ADF process, ADD mode was proposed as an improvement of the ADF especially for accumulating mixed microbial culture (MMC) selection and enrichment by adding physical selective pressure in order to speed up the enrichment (Chen et al., 2015b, Chen et al., 2016). Research conducted by Chen et al. (2015b) also focusing on the comparison between ADF and ADD process

based on his study, cultures selected under the ADD mode showed a better PHA producing potential than ADF modes in terms of both PHA content and selection time which can be explained by altered physical selective pressure. Even though, ADD mode proposed a better PHA accumulation in MMC, it uses still unfavorable because ADD is a new process and need time for other people to implement it.

2.2 Culture Conditions for Accumulation of PHA

2.2.1 Effect of Carbon Sources on the Accumulation of PHA

Carbon sources is one of the important factor in controlling the amount of PHA being produced since it is needed by biomass. Carbon sources can be obtained naturally or artificially by using chemical substances. Acetate and butyrate is one of the artificial carbon sources use in research conducted by Guerra-Blanco et al. (2018). Xylose study conducted by Huang et al. (2016) is a new method to reduce the cost of substrate but still producing PHA at higher efficiency. Xylose contain most highest of lignocellulose hydrolysates (Zhou et al., 2011a, Pan et al., 2012) and is the second most abundant sugar in nature (Lopes et al., 2009). Due to this reasons, it is have a potential to be used as carbon sources or substrate in order to enhance the production of PHA. Another study conducted by Wang and Nomura (2010), they are more focusing on the behavior of microorganisms (Pseudomonas putida KT2440) which grown at different carbon sources such as glucose, glycerol, citrate or fatty acid (lauric acid). All of these carbon sources have different carbon number which give impact on the chain length of these biodegradable polymers.

Most of researchers focus on the types of carbon sources use to increase the production of PHA instead of microorganisms itself. However, study conducted by Taguchi et al. (2003) showed a bit interesting in which adaptation of microorganisms toward carbon

sources is chosen as one of the topic in producing PHA. According to Taguchi et al. (2003), Ralstonia eutropha is one of the best known bacteria among PHA-producing microorganisms and has several advantages for efficient PHA production as a recombinant host strain. One of the reason why this researcher use R. eutropha is because it can intrinsically synthesize PHA from various renewable carbon sources, sugars and plant oils. Carbon sources can be obtained from any material that have carbon chain. Mannitol rich ensiled grass press juice (EGPJ) is another carbon sources that can be used in producing PHA through high cell density cultivation. According to Cerrone et al. (2015), EGPJ able to produce 44.5 g/L CDW containing 33% polyhydroxybutyrate (PHB) from Burkholderia sacchari IPT101 in 36 h, while Pseudomonas chlororaphis IMD555 produced a CDW of 37 g/L containing 10% of medium chain length polyhydroxyalkanoates (mcl-PHA) in 34 h.

2.2.2 Influence of Temperature on the Production of PHA

Temperature is one of the factor that can manipulating the accumulation of PHA because it will control the behavior of the culture medium as well as biomass. In wastewater treatment especially for removal of nitrogen, temperature will determine the amount of free ammonia (FA) and free nitrous acid (FNA) in which can inhibit the accumulation of PHA at the end of the treatment process. The role of FNA was been deeply discussed by Zhou et al. (2011b) in their research. For example, research carried out by De Grazia et al. (2017) described about the effect of temperature on mixed microbial culture (MMC) of PHA production while treating starch industry wastewater. Even though research study by Morgan-Sagastume and Allen (2003) do not focus on the production of PHA, it can also be refer due to the basic information that is very useful in handling biological wastewater treatment process. in this study, Morgan-Sagastume and Allen (2003) more focus on the

effects of temperature transient conditions on aerobic biological treatment of wastewater. Understanding on the biological wastewater treatment is compulsory especially when dealing with biomass and PHA because this treatment will determine the production of PHA at the end of the process.

Another study was conducted by Ocampo-López et al. (2015) in modelling of microbial growth and ammonia consumption at different temperatures for accumulation of PHA biopolymer. In this study, Ocampo-López et al. (2015) try to come out with new mixed mathematical model comprising a logistic model, and a magnetic saturation model which can be used to estimate the ammonia consumption under limiting conditions. This model representing the influence of temperature on cell growth and nitrogen consumption during the PHA production. Even though there are research conducted by Divyashree et al. (2009) in order to proposed a kinetic model for PHA biosynthesis in B. flexus, author only conducted this study at constant temperature of 30 ^{o}C and do not relate the behavior of the nitrogen consumption in the PHA production a different temperatures. Choma et al. (2000) also conducted research on the effect of temperature on the maximal specific growth rate of B. cereus at temperature between 5 ^{o}C and 40 ^{o}C in order to form mathematical model.

2.2.3 Effect of Aeration on the PHA Accumulation

Study on aeration in the accumulation of PHA become one of the popular topic since aeration will also affect the yield of PHA. For example, research conducted by Vjayan and Vadivelu (2017) discussed about the effect of famine-phase reduced aeration on the PHA accumulation in aerobic granules. This work is a continuity to the previous work that are more focusing on the formation of aerobic granules in ADF strategy (Gobi and Vadivelu, 2014). From this study, Vjayan and Vadivelu (2017) were found that, variation in aeration

rates especially during famine period, do not shows obvious effect in terms of chemical oxygen demand removal and PHA content. Most aeration rates show similar COD removal and PHA content. But, according to them, the decrease in famine-period aeration rates accelerated the maximum PHA accumulation together with increase in granular size and settling ability. According to this work, when 2 L/min aeration rate was applied, the PHA content of the aerobic granules in the reactor increased with the decrease in COD concentrations. Vjayan and Vadivelu (2017) also specified that, during COD uptake, the COD concentration was well below saturation level despite that a constant aeration of 2 L/min was provided. When aeration rate is change to 1.0 L/min, 0.5 L/min and 0 L/min, it is showed that these three aeration rates demonstrated a trend similar to that of 2 L/min. This statement is supported with only one previous study conducted by Gobi and Vadivelu (2015) in which they investigate the effect of aeration intensity on PHA accumulation in aerobic granules. According to Gobi and Vadivelu (2015), they concluded that increasing the aeration intensity did not affect the maximum amount of PHA accumulated in the granules, but it did accelerate the PHA accumulation process.

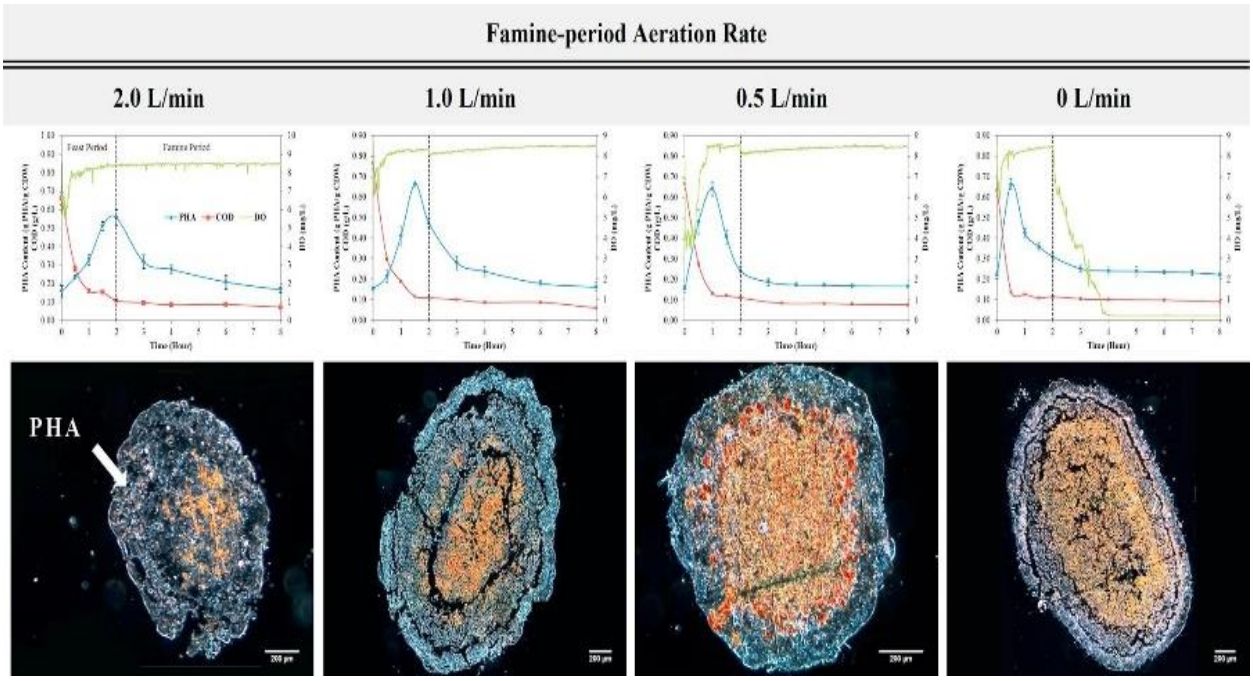

Figure 2.1 Effect of Aeration Rate on the Accumulation of PHA (Vjayan and Vadivelu, 2017)

2.2.4 Effect of pH on the Accumulation of PHA

Effect of pH on the accumulation of PHA plays an important role in determining the amount of PHA that is being produced (Wei et al., 2011). According to research conducted by Guerra-Blanco et al. (2018), the monomeric composition and accumulation of PHAs produced by microorganisms can be regulated by controlling the culture condition especially related to pH and substrate composition. In this study, three photoheterotropic mixed culture with different substrate (Acetate & Butyrate) namely as C2, C4, and C5 are used to study the effect of pH on these consortia and the amount of PHA yield. Among these consortia, only C2 and C4 are able to produce PHA in which C4 showed the highest production of 44% of the cell dry mass (CDM) (Guerra-Blanco et al., 2018). Another study reported by Montiel-Jarillo et al. (2017) also investigate the effect of pH and nutrients (phosphorus and nitrogen) on the production of PHA in mixed culture. In this study, different pH was applied in a

sequencing batch reactor (SBR) by MMC which is operated under feast/famine regime. Operating like other process, this researcher start enrichment step with pH of 7.5 and also without control with averaged value of 9.0 (Montiel-Jarillo et al., 2017). In this study, it is found that between pH 8.8 to 9.2, PHA accumulation was 44% g PHA g^{-1} VSS, 39% g PHA g^{-1} (pH 7.5) and 31% g PHA g^{-1} VSS (pH 8.5). Study on this effect to the production of PHA are contradictory. For example, according to Wei et al. (2011), pH range of 6.0 to 7.5 is suitable to the production of PHA and cell growth. Another study reported by Venkata Mohan and Venkateswar Reddy (2013), neutral pH would be better as enzymes involved in PHA production are active at pH 7. Other studies conducted by Chua et al. (2003), Serafim et al. (2004b) and Villano et al. (2010) report that a PHA content would be higher at pH range of 8.0-9.0. Study on the effect of pH in the production of PHA is still limited especially when there are two process involve; biological municipal wastewater treatment and PHA accumulation. Even though, nitrogen removal be one of the famous topic discussed in most researchers and its advantages towards the industry, they are only focusing on one topic and do not considering the production of this added-value biopolymer.

2.2.5 Effect of Nitrite on the Accumulation of PHA

Nitrite, NO_2^- is one of the component that will manipulate the accumulation of PHA in the wastewater treatment. Mostly, NO_3^- was found during the nitrification/denitrification process by the action of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) respectively. Ammonia need to be removed from the wastewater since it will cause eutrophication problem to occur when it released into water stream. Many researchers has found the benefits of ammonia removal from the wastewater. Research conducted by Bettazzi et al. (2010) is one of the example that relate the presence of nitrite in the wastewater and its

affect to the Anammox bacteria. Even though this study more focus on the nitrite inhibition and Anammox process, it have relation to the accumulation of PHA because it use microorganisms to treat the wastewater.

Study on NO_2^- is more on the Anammox process and less focusing on the effects of this compound to the accumulation of PHA.

Free ammonia (FA) and free nitrous acid (FNA) is commonly indicator that can be used to relate the effect of NO_2^- to the accumulation of PHA. For example, research conducted by Qian et al. (2017) discussed about the effects of free ammonia on AOB and NOB under anaerobic condition. Both of these bacteria will be affected in term of their sensitivity to the FA. According to this study, NOB was more sensitive to the FA anaerobic treatment as compared to AOB. This results indicate that, FA will affect the behavior of the bacteria and the accumulation of PHA. Research conducted by Zhou et al. (2011b) briefly explained about the role of FNA and FA in wastewater treatment plants and this work support the previous work conducted by Qian et al. (2017) in which FNA and FA may possibly affect the behavior of AOB and NOB. In this study, author said that further research is necessary before the FNA inhibition mechanism can be more effectively used to optimize wastewater treatment plant bioprocess. Currently, most of the nitrite study is only focus on the Anammox process and its affect to the nitrogen removal and less study relate the effects of nitrite, FA and FNA to the accumulation of PHA.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.0 Materials and Apparatus

Materials used in this research are listed on the Table 3.1 below. All materials listed on the table are according to the whole of experiment conducted which is 12 different experiments. There are some materials that need to be conducted properly due to it hazardous. In study the accumulation of PHA, preparation of sample for test need to conduct properly and faster because it will undergo complex procedure. Other than that, apparatus also need to be prepared in large quantity in order to reduce time consuming throughout the experiment. Apparatus that will be used are listed on Table 3.2 below.

Table 3.1 List of materials

Materials	**Function**
Ice-cold methanol	To be used in PHA extraction
Sodium nitrite	As a sources of nitrite ion
Nitrite reagent	To determine concentration of nitrite
Partially-treated POME	As a feed/carbon sources
Chloroform	To be used in PHA extraction
Sodium hypochlorite	To be used in PHA extraction
High range COD test kit	To measure COD of the sample

Table 3.2 List of apparatus

Apparatus	Function
2 L reactor	Container to conduct experiment
1 L beaker	For transferring POME or microorganisms
5 L jug	To fill up tap water for dilution purposes
Filter paper	To remove cell debris
25 mL beaker	PHA extraction process
Pipette	For precipitation process
Test tube	For measuring concentration of nitrite
Vacuum pump	For MLSS & MLVSS measurement
Eppendorf 5702 Series Centrifuge	To separate microorganism from culture

3.1 Methodology

3.1.1 Aerobic granules and wastewater

The aerobic granules used in this study were obtained from a parent sequencing batch reactor (SBR) that already operated in continuous 6-h operational cycle under an organic loading rate (OLR) of 2.25 kg chemical oxygen demand (COD)/m^3 day. SBR was supply with constant aeration rate at about 3 L/min throughout the duration, with a COD removal more than 85%. By referring to (Gobi and Vadivelu, 2014), detailed explanation of the development and performance of aerobic granules in the main SBR can be found in term of its formation. Palm oil mill effluent (POME) was used as the sole feed in this study and it will be used to study the effect of nitrite, NO_2^- towards the accumulation of PHA. POME contains volatile fatty acids (VFAs) that are used for PHA production. The POME was

allowed to settle and the supernatant was collected, filtered using cloth filter to remove debris and stored at 4 °C until further use.

3.2 Experimental setup

3.2.1 Varying the amount of nitrite ion (NO_2^-) with constant pH

To study the effect of nitrite in accumulation of PHA, another SBR with a height-to-diameter ratio of 10 and working volume of 2 L were used (small reactor). The reactor was inoculated with 1 L of mixed liquor containing aerobic granules taken from main SBR and 1 L tap water. The reactor were operated in 6-h operational cycle consisting of feeding, aeration, settling and decanting. During feeding, 0.5 L of feed (mixture of 400 mL POME and 30 mg/L sodium nitrite, $NaNO_2$) is added into the reactor to achieve a mixed liquor suspended solid (MLSS) content of approximately 2730 mg/L MLSS need to be calculated at the beginning of the experiment in order to know the concentration of the suspended solid in the mixed liquor. The pH of the mixture is control at 7 by using acetic acid and sodium hydroxide, NaOH. The volumetric exchange ratio was maintained at 25% of total volume (0.5 L) to establish an OLR of 2.25 kg COD/m^3 day. Fine air bubbles were supplied from the bottom of the reactor using air diffusers and controlled using a flow meter. The reactors were operated for 6 hours (In aeration phases). Concentration of the nitrite in the mixed liquor must be determined at the beginning and at the end of the experiment. Sample are collected at 0 h , 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h in feast-famine phases in order to study the manipulated variable (PHA, COD). After 6 hours operation, the mixture are let to settle for 1.5 min and then decanting for another 8.5 min. Data were collected after the reactors had established a steady-state operation. Repeat step by changing the concentration of $NaNO_2$ with 50, 70, 100 and 150 mg/L.

3.2.2 Varying the pH medium with constant concentration of sodium nitrate ($NaNO_2$)

To study the effect of pH on the accumulation of PHA, another SBR with a height-to-diameter ratio of 10 and working volume of 2 L were used (small reactor). The reactor was inoculated with 1 L of mixed liquor containing aerobic granules taken from main SBR and 1 L tap water. The reactor were operated in 6-h operational cycle consisting of feeding, aeration, settling and decanting. During feeding, 0.5 L of feed (mixture of 400 mL POME and 100 mg/L $NaNO_2$) is added into the reactor to achieve a mixed liquor suspended solid (MLSS) content of approximately 2730 mg/L MLSS need to be calculated at the beginning of the experiment in order to know the concentration of the suspended solid in the mixed liquor. Concentration of $NaNO_2$ is kept constant throughout the experiment. The pH of the mixture is set as manipulated variable starting from 3. It can be controlled by using acetic acid, CH_3COOH and sodium hydroxide, NaOH. The volumetric exchange ratio was maintained at 25% of total volume (0.5 L) to establish an OLR of 2.25 kg COD/m^3 day. Fine air bubbles were supplied from the bottom of the reactor using air diffusers and controlled using a flow meter. The reactors were operated for 6 hours (In aeration phases). Concentration of the nitrite in the mixed liquor must be determined at the beginning and at the end of the experiment. Sample are collected at 0 h , 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h in feast-famine phases in order to study the manipulated variable (PHA, COD). After 6 hours operation, the mixture are let to settle for 1.5 min and then decanting for another 8.5 min. Data were collected after the reactors had established a steady-state operation. Repeat step by changing the pH of mixed liquor with 5, 7, 9, and 11.

3.3 PHA extraction

3.3.1 Lyophilization process of aerobic granules

Lyophilization or freeze drying process need to be carried out first before PHA can be extracted from the microorganisms. In this process, water content is completely removed from the aerobic granules. Aerobic granules taken from SBR were immediately added with 2 drops of formaldehyde to stop all the biological activity that might affect the properties of the granules and thus inaccurate date will be collected. Then, the aerobic granules were inserted into a round bottom flask (or commonly known as freeze-drying flask) and placed in a freezer (-20oC) for about 30 min. After 30 min, the flask was taken out from the freezer and attached it to freeze drier (Telstar Cryodos, Spain). The frozen aerobic granules were freeze-dried at - 55^{o}C under vacuum condition- 1.6 mmHg with pressure of 1.2 mB for 24 hours. (*Precautions : it is important to lower the pressure to the range of a few millibars to ensure the ice form during freezing stages are sublimated. Pressure is controlled through the application of partial vacuum. The vacuum will speed up the sublimation process, making it useful as a deliberate drying process). The freeze-dried aerobic granules were later collected and subjected to PHA extraction process.*

3.3.2 Polyhydroxyalkanoate (PHA) extraction

The lyophilized granule obtained from freeze-drying process were subjected to PHA extraction using sodium hypochlorite (NaOCl) and choloroform ($CHCl_3$) dispersion method. There are two important stages in extraction of PAA which is digestion and dissolving step. The cells will first undergo digestion and followed by PHA dissolving step. At the end of dissolving step, dissolved PHA will be precipitated.

3.3.3 Cell Digestion step

Briefly, for each gram of lyophilized aerobic granules, 12.5 mL of NaOCl and 12.5 mL of CHCL3 was used. The mixture was vortexed or mixed and incubated in a water bath shaker at 37oC for 90 min. thereafter, the solution was centrifuged at 4400 rpm for 30 min. Upon centrifugation, three distinct layers were apparently formed. The top layer consists of NaOCl and the solution was pipetted out. The remaining cell debris (2^{nd} layer) and PHA-enriched chloroform (3^{rd} layer) fractions were filtered using a simple filtration method (using filter paper). The filtrate (chloroform + PHA) was subsequently subjected to PHA dissolving step.

3.3.1 PHA dissolving step

5 volumes of ice-cold methanol were added to the chloroform solution (or filtrate) to precipitate the PHA. The precipitated PHA was left in a fume hood to allow the excess solvent to evaporate from the solution. The precipitated PHA was then weighed and analyzed.

3.4 Analysis

3.4.1 PHA content

After PHA extraction process, PHA content can be determined by weighing the extracted PHA. The PHA content (YPHA/CDW) was calculated using equation shown below

$$Y_{\frac{PHA}{CDW}} = \frac{Total\ amaount\ of\ PHA\ (g)}{CDW\ of\ aerobic\ granule\ (g)} \quad (3\text{-}1)$$

Where

Cell dry weight (CDW) = Amount of freeze-dried aerobic granules used for PHA extraction process.

3.4.2 Free ammonia (FA) and free nitrous acid (FNA) analysis

FA and FNA need to be analyzed in order to study the amount of ammonium and nitrite that inhibit the accumulation of PHA. This can be performed by using formula as shown below (Park and Bae, 2009):

$$FA = \frac{17}{14} x \frac{(NH_4^+)\ x\ 10^{pH}}{e^{(6334/(273+T))} + 10^{pH}} \quad (3\text{-}2)$$

$$FNA = \frac{47}{14}\ x\ \frac{(NO_2^-)}{e^{(-2300/(273+T))}\ x\ 10^{pH} + 1} \quad (3\text{-}3)$$

3.4.3 COD analysis

COD can be analyzed by using COD test kit. Take 1 mL of sample and dilute it with 5 mL of distilled water. Shake it for a moment to ensure the solution mix properly. After that, take 2 mL of mixture and put it into high range test (or 0.2 mL for medium range test). Shake it properly and heat it up for 120 minutes at 150^{o}C. Next, let it cool at room temperature and test it by using COD test kit. Repeat step for different samples.

CHAPTER FOUR

RESULTS AND DISCUSSION

This chapter depicts the experimental results and discussion which consists of two main sections. The first section represents the effect of nitrite concentration on the accumulation of PHA and the second section will discuss about the effects of pH on the accumulation of PHA. Comparison of these variables with controlling experiment have been made in order to study any changes that occur during the experiment.

4.1 Effect of nitrite on the accumulation of Polyhydroxyalkanoate (PHA).

Nitrite, NO_2^- is one of the parameter that can affect the accumulation of PHA as discussed in Section 2.2.5. From the experiment that have been conducted, comparison with controlling experiment for each time at 0 h, 0.5 h, 1.0 h, 1.5 h, 2.0 h, 4.0 h and 6.0 h have been made in order to study if there are any changes especially in term of FNA that may affect the accumulation of PHA. FNA is very important parameter to be studied since it is related to the nitrite concentration. For controlled experiment, the procedure is same for both studies but there are no addition of nitrite, acetic acid and sodium hydroxide into the samples. When comparison is carried out in term of PHA accumulation between experimental and controlled data, there were some changes occurred in PHA content, COD, DO, FNA and nitrite concentration after certain period of time as shown in Table 4.1, Table 4.2, Table 4.3, Table 4.4 and Table 4.5 respectively. However, this section are more focusing on the effect of nitrite on the accumulation of PHA.

Accumulation of PHA depends on the several conditions such as nitrite and ammonium concentration, pH value, aeration rate, or any factors that might affect the behavior of the bacteria. This is because activity of PHA-accumulating microorganisms will manipulate the amount of PHA being produced. Most of the researchers have found that the presence of nitrite tend to inhibit the activity of the microorganisms and thus will affect the production of PHA. One of the study conducted by Zhou et al. (2011b) and Anthonisen et al. (1976) had proved this statement by considering the relationship between nitrite concentration and pH that may lead the reading of free nitrous acid (FNA).

Based on Figure 4.1 and Table 4.1, accumulation of PHA after 6 hours cycle for all nitrite concentrations show a positive increment as compared to controlled experiment even though there are some data that are not consistently increasing throughout the experiment. This changes happened due to the presence of nitrite that altered the activity of the microorganisms. The amount of aerobic granules that were taken out from bioreactor were not similar for each sample and such situation also affect the data. External factors such as enrichment culture condition in term of aeration rate, carbon sources, pH and temperature are kept constant or similar throughout the experiment. The highest amount of PHA produced is recorded when 30 mg/L of nitrite is added to the mixed liquor as compared to another concentrations. This might be happened due to the good physical properties of the microorganisms at the beginning of the experiment when it was taken out from the parent reactor. Physical properties of the microorganisms in term of its structure is also important to be discussed in this topic. Aerobic granules was being used in this experiment rather than conventional activated sludge due to its good characteristics which can enhance the accumulation of PHA. During conducting the first experiment with addition of 30 mg/L of nitrite into mixed liquor, microorganisms in large size as compared to another experiment.