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POLYHYDROXYALKANOATES (PHAS) PRODUCTION FROM AEROBIC-MIXED CULTURES

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Abstract: This study assessed the optimal conditions for PHA production using saponified fatty acid, derived from sunflower oil (SO). A fed-batch reactor was used to produce PHA. A mixture of sewage and semi-treated oil palm effluent from a facultative pond was cultured for six months to obtain a steady-state condition. The culture was then transferred to the fed-batch system. SO mainly contains long-chain-fatty-acid (LCFA) with unsaturated fatty acid fractions of $C_{14:1} - C_{18:3}$, therefore the PHA production was assessed under feast-famine condition. The main purpose of supplementing saponified SO was to improve the production of PHA constituents (copolymer of hydroxyl-unit) such as hydroxybutyrates (HBs), hydroxyvalerates (HVs) and hydroxyhexanoates (HHs) in mixed cultures. Fed-batch operation under aerobic-mixed cultures increased the PHA production up to 33% of the dried cell. Although sludge submitted to aerobic condition in mixed cultures could improve the PHA production, the production rates are still low. This study found that the HBs constituent in the sludge is always higher compared to HVs and HHs. Saponified SO has high specific PHA storage rates (q_p^{feast} = 0.5 C-mol/C-mol. h) which are comparable to other vegetable oils (e.g. corn oil, soy bean oil, etc.)

Keywords: *Aerobic-Mixed Cultures; PHAs Constituents; Saponified Fatty Acid; Sunflower Oil.*

Abstrak: Kajian telah dijalankan bagi menilai keadaan optima penghasilan PHA menggunakan asid lemak tersaponifikasi yang diubahsuai daripada minyak bunga matahari (SO). Sebuah reaktor suapan-kelompok telah digunakan untuk menghasilkan PHA. Campuran daripada airsisa dan efluen separa olahan daripada kolam pengoksidaan dikulturkan selama enam bulan untuk menghasilkan keadaan '*steady-state'*. Kultur tersebut kemudiannya dipindahkan ke sistem suapan-kelompok. SO mengandungi asid lemak rantaian panjang dalam kumpulan asid lemak tak tepu ($C_{14:1} - C_{18:3}$), oleh itu penghasilan PHA telah diteliti semasa keadaan '*feast-famine'*. Tujuan utama membekalkan SO yang tersaponifikasi adalah untuk menambah kualiti kandungan PHA (kopolimer unit hidroksil) seperti hidroksibutirat (HB), hidroksivalerat (HV) dan hidroksiheksanoat (HH) di dalam kultur campuran. Operasi suapan berkelompok menggunakan kultur campuran secara aerobik dapat meningkatkan penghasilan PHA sehingga 33% daripada

berat sel kering. Walaupun enapcemar yang dikulturkan secara campuran dapat meningkatkan kualiti penghasilan PHA, kadarnya masih rendah. Kajian ini mendapati kandungan HB dalam enapcemar sentiasa lebih tinggi berbanding HV dan HH. SO yang tersaponifikasi mempunyai kadar simpanan spesifik PHA yang tinggi (q_p^{feast} = 0.5 C-mol/C-mol. h) dan setanding dengan minyak sayuran lain (seperti minyak jagung, minyak soya, dan sebagainya)

Kata Kunci: *Kultur Campuran Aerobik; Kondungan PHA; Asid Lemak Tersaponifikasi; Minyak Bunga Matahari.*

1.0 Introduction

Attempts to produce polyhydroxyalkanoates (PHAs) using aerobic-mixed culture have been motivated by the presence of PHAs in wastewater treatment plant which act as a metabolic intermediate. Accumulation of PHAs takes place mainly during the removal process of pollutants (e.g. COD or BOD removal). The mechanisms of PHAs storage can be described by the ability of microorganisms in storing and consuming substrate during unbalance growth; feast (availability of substrates) and famine (exhausted of substrates) conditions (van Loosdrecht et al., 1997).

There are three kinds of polymer produces as intracellular storage inside microorganisms, namely glycogen, PHAs and polyphosphate (Poly-P). However, only glycogen and PHAs are the main reported bacterial storage polymers, while Poly-P is recognized as reducing sink power to balance internal energy (van Loosdrecht et al., 1997). The mechanisms of storing activity in activated sludge bacteria have been frequently reported (e.g. Beccari et al., 1998; Wu et al., 2000; van Loosdrecht and Heijnen, 2002; Zinn et al., 2003; Salehizadeh and van Loosdrecht, 2004; Serafim et al., 2004).

PHAs are recognized as biodegradable plastic raw material because they posses material properties similar to various synthetic thermoplastics and elastomers that are widely available in the market (e.g. polypropylene (PP), polyethylene (PE) or synthetic rubber). The advantage of using PHAs is that upon disposal, they are completely decomposed to water and carbon dioxide (and methane under anaerobic conditions) by microorganisms (Mergaert et al., 1992; Brandl et al., 1995; Krishna and van Loosdrecht, 1999; Blaylock, 2002). However, a large scale use of PHA as a substitute for conventional plastic has been hampered by its high production cost compared with petrochemical-based polymers (Lee et al., 2000).

Since the first finding of PHA by Lemoigne in 1926 (Lee, 1996a, 1996b; Lentz and Marchessault, 2005), more than 100 different monomer units have been identified as constituents of PHA involving over 300 different microorganisms. These include 3-hydroxyalkanotes of 3-12 carbon atoms with large variety of R-pendant groups, 4-hydroxyalkanoates of 4-8 carbon atoms, 5-hydroxypentanoates, 5-hydroxyhexanoate and 6-hydroxydodecanoate. However, only a few of these PHAs have been produced commercially. Poly(3-hydroxybutyrate) [P(3HB) or PHB] is a homopolymer of 3-hydroxybutyrate acid and is the first to be commercially manufactured (Doi, 1990). Since PHB is a highly crystalline and brittle homopolymer and has limited application,

the consortiums of HVs and HHs have been developed (Holmes, 1988; Choi and Lee, 1999). The poly(3-hydroxybutyrate-co-hydroxyvalerate) [P(3HB-co-3HV) or PHB-co-HV] has better properties than PHB because it is more flexible and stronger (Holmes, 1988; Doi, 1990; Lee, 1996a)

This study aimed at investigating the potential of saponified SO in inducing the PHA production. Typically, the PHA production can be induced by providing the feast and famine conditions which always occur in biological treatment plants. The objective was to define optimum operating conditions for improving the percentage of PHA in the biomass, and to characterize the composition of the PHAs, i.e. PHV, PHB and PHH concentrations as the polymers stored in the cells. However, previous works on biodegradable materials were mostly confined to PHB constituents derived from biomass cells (Poirier et al., 1995; Pereira et al., 2003). Therefore, a sequencing batch reactor (SBR) has been developed similar to the typical biological treatment plant to investigate the potential of other PHA constituents (HV and HH monomers). Four operating conditions have been applied, i.e. the control of the nutrient feeding (C/N ratio), air flowrate, temperature effect and cycle length. The kinetics degradation of accumulated PHA production was also examined in order to optimize the operating conditions. PHA production obtained from this saponified fatty acid was expected to be lower than those obtained in a single culture of fatty acid (acetate or propionate) (Hassan et al., 1997a, 1997b, 2002).

2.0 Methodology

The experiments were performed in a double-jacketed laboratory bioreactor with working volume of 2 L. The operating condition comprises different cycle phases (Table 1). The operating principles of a batch activated sludge system are characterized by three discrete periods: fill, react and drawing. In order to control the fast uptake and storing capacity, the system was operated in continuous reaction period, which means no settling or allowing an idle phase to occur (HRT = SRT). The cultivation was maintained in a single fed-batch reactor and operated in two-steps condition; growth (with nutrient supply) and accumulation phase (without nutrient supply).

Table 1**:** Operating phase of fed-batch reactor with SO as substrate

Experiment	Operating phase (hr)			
	Aerobic mineral feed	Aerobic feed	Aerobic react	Draw/discharge
Growth phase	5 min	1	1-47.9	47.9-48
C/N ratio, CN_{so}	no fill	1	1-47.8	47.8-48
Air flowrate, Air_{so}	no fill	1	1-47.8	47.8-48
Temperature, T_{so}	no fill	1	1-47.8	47.8-48
Cycle length, HRT_{so}	no fill	1	up to 95.8	up to 96

A mixed culture of sewage from wastewater and facultative ponds was prepared as inoculums at 1:2 ratio. The steady-state period was determined based on the profiles of total organic carbon (TOC), cell dried weight (CDW) or dissolved oxygen (DO). The pH was maintained at 7.00 ± 0.1 using 2N HCl or 2N NaOH in both growth and accumulation phases. The temperature was controlled at 30°C using a thermostat bath. The well-aerated fed-batch reactor during the growth phase was operated with airflow of 2.39 L/min, controlled by a mass-flow controller and stirred with two flat geometry six-bladed turbines. However, during the accumulation phase, the airflow was reduced to as low as 0.42 L/min. The airflow of 0.42 L/min was chosen because at this flow rate the oxygen saturation can be maintained at approximately 8%. At low oxygen saturation (< 10%), the biomass will utilize low energy which favours PHA production. The schematic diagram of fed-batch reactor is shown in Figure 1.

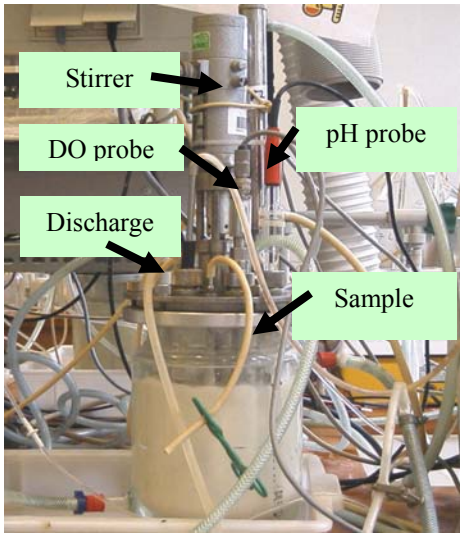


Figure 1: SBR used in this study

2.2 Inoculation and fed-batch technique

The wastewater (pre culture of SO, sewage and distilled water) was inoculated by growing biomass in aerobic reactor and subsequently cultivated for approximately 36 hr at 30°C. A portion of the culture medium was transferred to the fed-batch reactor at 10% of the working volume after the cells had reached the late exponential stage. In general, the proliferation of growth phase (under non limiting condition) was examined first to ensure the dynamic population of bacteria growth. Then, the accumulation process was performed to 'select' the potential of PHA producers. The sludge from residual biomass was collected to quantify the PHA content. The experimental procedures and processes are shown in Figure 2.

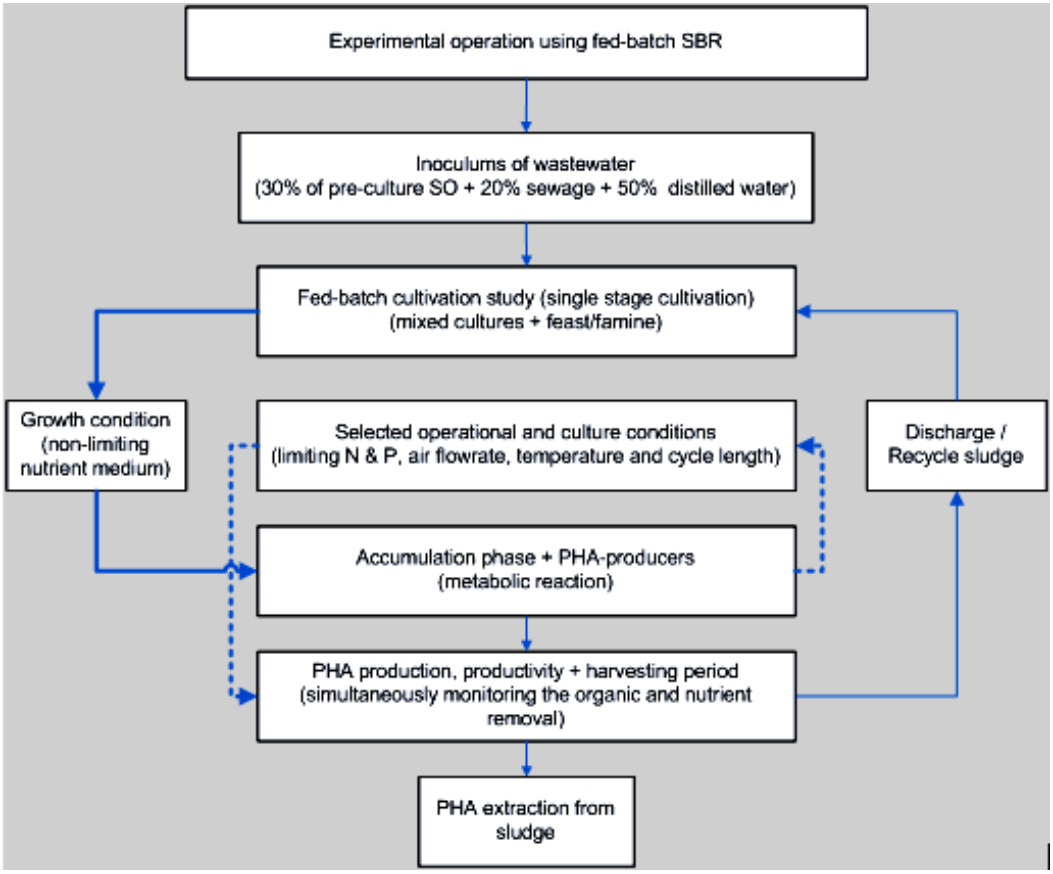


Figure 2: Experimental set-up and processes for PHA extraction from sludge

Note: ——— non-limiting nutrient ▪▪▪▪▪▪▪ accumulation process

The cultures for fermentation were prepared for at least 24 hours in 2 L flasks. Each culture contains 1.1 L seed of inoculum, 0.8 L of mineral solution and 0.1 L of SO as carbon sources. The steady-state condition as indicated by consistent concentrations of DO, TOC or CDW was obtained after six months of fermentation. The samples were then collected for analysis.

Since the bacteria in the activated sludge degrade the organic compounds, the substrate concentration in the bulk liquid decreases over time. The feeding of the bacteria and the removal of effluent and sludge were accomplished by using peristaltic pumps. To prevent the possible influence of nitrification on the measurements, at least 100 mg of allylthiourea (ATU) was added to the reactor before each sampling cycle. Reactors and tubes were periodically cleaned to avoid proliferation of bacteria on the lines and walls. The length of the feast period in the pulse-fed systems was estimated from a DO continuous measurement system (ISTEK® and DAPS, Korea). With the presence of external substrate (feast phase), bacteria consumes oxygen to degrade the organic matter, thus the DO in the

reactor decreases. Once the external substrate is depleted, the DO starts to increase and the famine phase begins.

Concentration of SO in the culture broth was determined by taking 2 ml of the culture broth into a screwed tube and then mixed with 5 ml hexane. After vigorously shaked for 1 min, 1 mL of hexane layer was transferred to pre-weighted tube, and dried at 37^{o}C until the hexane phase evaporated. The SO concentration was estimated from the amount of hexane extract using a predetermined calibration curve that was computed by Curve Expert software. In order to examine the constituent of SO compounds, an analysis of fatty acid was performed. Fatty acid profile in SO was measured by gas liquid chromatography as described by Guinda et al. (2003). A gas-liquid chromatograph (Model HP 5890, Series II, Hewlet Packard, Palo Alto, CA, USA) equipped with a flame ionization detector was used to analysed fatty acids as methyl esters. Chromatography analysis was performed using a 60-m capillary column with 32 mm inner diameter and 20 mm thickness impregnated with Sp 2330 FS (Sepelco Inc. Bellefonte, Palo Alto, CA, USA). The injector and detector were maintained at 250^{o}C and 275^{o}C, respectively. Nitrogen was used as the carrier gas, and the split ratio was 29:1. Within 40 min, the temperature was automatically adjusted as follows: initial temperature was 160^{o}C for 5 min, increased by 6^{o}C/min to 195^{o}C, increased by 4^{o}C/min to 220^{o}C, further increased by 2^{o}C/min to 230^{o}C, hold constant for 12 min, and finally reduced by 14^{o}C/min to 160^{o}C. The fatty acid compositions in SO are shown in Table 2.

Table 2: Fatty acids composition in SO found in this study

Compound (mol-%)	Omega Name	Trivial Name	Nomenclature	Group
±6	$C_{16:0}$	Palmitic Acid	MCFA	Saturated
±5	$C_{18:0}$	Stearic Acid	LCFA	Saturated
±29	$C_{18:1}$	Oleic Acid	LCFA	Unsaturated
±58	$C_{18:2}$	Linoleic Acid	LCFA	Unsaturated
±0.3	$C_{20:0}$	Arachidic Acid	LCFA	Saturated
±0.7	$C_{22:0}$	-	LCFA	Saturated

Note : LCFA : long-chain-fatty-acid
MCFA : medium-chain-fatty-acid

2.3 Analytical Procedures

Samples for analysis of ammoniacal-nitrogen (NH_4-N), phosphate-phosphorus (PO_4-P), TOC, chemical oxygen demand (COD) and volatile fatty acid (VFA) were immediately centrifuged in a high rotation. The slurry was then filtered through 0.45 μm membrane filter to separate the bacterial cells from the liquid. Then, the supernatant was stored in a refrigerator for TOC, COD and PHA analyses and in a freezer for the determination of VFA, VSS, CDW, NH_4-N, NO_3^-, PO_4-P and COD. NH_4, VSS, PO_4, NO_3 and COD were analysed according to the Standard Methods (APHA et al., 2002). The supernatant of

VFAs was measured according to the type of carbon chains. Acetic acid (HAc), propionic acid (HPr), and butyric acid (HBt) were measured with GC and a flame ionization detector (FID) by direct injection of acidified aqueous samples at pH 2 to 3 in a Supelco fused-silica capillary column (ø 0.25 mm x 25 mm). The CDW, volatile suspended solids (VSS) and ash content of the biomass were determined according to the Dutch Standard Method (NNI, NEN6621, 1982) The PHA content of the washed and dried biomass was determined by extraction, hydrolyzation, and esterification in a mixture of hydrochloric acid, 1-propanol, and dichloroethane at 100^{o}C. The resulting organic phase was extracted with water to remove any free acids. The proplyesters (mixtures of PHA and other organic polyesters) were analyzed by GC. Benzoic acid was used as an internal standard. The PHA content was given as a percentage of the total biomass dry weight (% PHA).

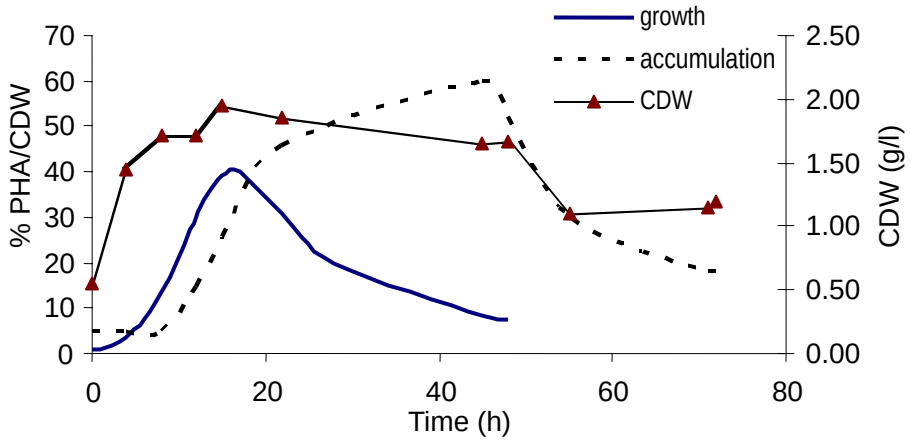
In order to evaluate the kinetic relation needed to describe the degradation rate of PHA in the famine period as a function of the PHB content of the cells, a general differential equation of chemical reaction kinetics was used, which often applied for microbial processes (Beun et al., 2000a, 2000b):

$$\frac{df_{PHB}}{dt} = -k \bullet f_{PHB}^{n} \quad (1)$$

3.0 Results and Discussion

3.1 Preliminary Experiment

Figure 2 shows the PHA accumulation rate during the growth phase, which is most rapid between 10 to 20 hours. The increasing rate of PHA production during the growing phase might be attributed to the uptake of fatty acid. The accumulations of short fatty acids, such as acetic and butyric acids were expected to decrease first, followed by medium and long-chain fatty acids. Therefore, long-chain fatty acids (e.g. oleic, palmitic, etc.) were initially converted into other by-products by bacteria due to the tricarboxylic acid (TCA) cycles, or metabolites enzyme (e.g. ATP/NADH). Bacteria will then utilize the by-products (e.g. HAc, HPr, etc.) as energy or store as polymers (e.g. PHA, glycogen). However, since most of the systems operate in a low nutrient concentration, the metabolic pathways preferred storage activity over growth. Eventually, when the PHA productions reach a maximum, the production rate of PHA decreases. This is due to the internal utilization of carbon source in famine period. Active biomass and PHA contents were high during the growth stage, as observed at the beginning of 20 hours (Figure 2). The maximum PHA content during accumulation phase was detected at 48 hour amounting to 60% of the CDW (CDW concentration of 1.35 g/L).



Note : % PHA/CDW = percent of PHA in cell dried weight

CDW = cell dried weight

Figure 2: Overall percentage of PHA production in cell acclimatized during growth and accumulation condition.

The biomass utilized acetic acid (HAc) up to 88% more than propionic (HPr) and butyric acids (HBt) (Table 3). As reported earlier (Chua et al., 1997; Lee et al., 2000; Wu et al., 2000; Chua et al., 2002), acetic, propionic and butyric acids were released into the medium from β-oxidation at certain concentration and taken up again at low concentration. Although SO contains high LCFA, SO could be easily degraded via β-oxidation by sequential removal of carbon-chain to VFAs (Lee and Yu, 1999). Most of the SO components have long-chain-fatty acid (LCFA) with 14 to 18 carbon atoms. Therefore, when the LCFA is converted to VFAs, the by-products of fatty acids will be taken up by biomass. Usually, excessive substrate uptake will not directly produce high PHA. However this study showed contradictory results. For example, the PHB was able to yield over 95% acetate consumption rate ($Y^{max}_{PHB/Ac}$) during the accumulation phase compared to only 62% during the growing phase. Meanwhile, the biomass cell could not utilize the VFAs (i.e. HPr, HBt and HAc) during the growth and accumulation phase. Only 4 to 11% of polymer storage per total VFAs was produced during the feast period. Therefore, VFAs was found to be a limiting factor for producing polymer storage in biomass.

Table 3: VFAs utilization during the growth and accumulation phases

Conditions	VFAs						
	HAc			HBt		HPr	
	HAc (mM/h)	$Y^{max}_{PHB/HAc}$ (%) *	% HAc/ total VFA	HBt (mM/h)	%HBt/ total VFA	HPr (mM/h)	%HPr/ total VFA
growth	20	62	84	0.23	11	0.08	4
accumulation	64	96	88	0.14	6	0.4	6

* data during initial HAc consumed

3.2 Specific Rates of PHA Production and Substrate Uptake

In general, the efficiency of PHA production in this study is significantly influenced by the level of DO (%PHA can reach $14.43 \pm 2.72\%$) and cycle length (%PHA = $33.77 \pm 3.10\%$) as depicted in Table 4. It is also shown that PHA could be operated using three limiting conditions: N, P and control of air flowrate. The experimental control of C and N has been proven suitable for inducing PHA production. Similar result was obtained by Chua et al. (2002) who induced PHA accumulation in activated sludge under four different C/N ratios, i.e. 24, 48, 96, and 144. The maximum specific polymer yield of 0.374 g polymer/g cell (or 37.4% PHA) was obtained at the highest C/N ratio of 144. However, the results of specific polymer yield in their study could exceed 0.6 g polymer/g cell (at C/N = 45 g TOC/g N). But the PHA production was low (only 9% of CDW) because the PHA content of the biomass was low compared to the preliminary results (50% of CDW) (Chua et al., 1997). However, this study found that temperature has a moderate effect on the PHA production, which recorded between 10 to 20% per CDW at temperature between 15 and 30°C. As a conclusion, the study indicated that the temperature effect was not the major effect of PHA production if the system was conducted between 25 – 30°C. However, the highest PHA production was obtained at 30°C, which suitable for tropical operational conditions.

The influence of temperature on the PHA production was assessed by computing the stoichiometric and kinetic rate of the substrate. The temperature has a moderate effect on aerobic stoichiometric coefficients, such as PHA, PO_4 (P) and NH_4 uptake per amount of O_2 consumed (Table 5). The PHA consumption over oxygen consumption ratio (PHA/oxygen) was highest at 20°C. However, at 15°C, the PHA/oxygen ratio is 46% lower than at 25°C. The ratio at 30°C was lower than at 20°C. Although the solubility of oxygen is typically higher at low temperature, the effect was most obvious at temperature below 20°C. A low ratio of PHA over oxygen consumptions (mg/mg O_2) suggests a low decomposition of PHA during O_2-electron consumption. The NH_4/oxygen ratio shows a consistent trend due to small differences in the measured values. Other researchers also found that the coefficient rates for P, NH_4 and PHA consumption per active biomass were strongly influenced by temperature

variation (Krishna and van Loosdrecht, 1999; Dircks et al., 2001; Chinwetkitvanich et al., 2004). Between 20 and 25°C, the overall trend of the oxygen consumption rate sharply changed during aerobic batch experiments. It can be concluded that the remaining part of the aerobic phase oxygen is not used for poly-P formation, but only for biomass growth and maintenance. As a result, the P-uptake over active biomass rate (mg P/mg. h) was significant only for temperature above 15°C.

Table 4: Specific PHA production and substrate uptake rates at different experimental conditions

Experiment	Cycle length (h)	Temp (°C)	(-) q_s^{feast}, Cmol/Cmol. h	q_p^{feast}, Cmol/Cmol.h	$q_p/-q_s^{feast}$ Cmol/Cmol	%PHA/CDW (mean value)
$^{A1}CN_{so}$-15	24	30	0.135	0.064	0.48	3.33 ± 0.09
$^{A2}CN_{so}$-22	24	30	0.409	0.222	0.64	5.39 ± 0.08
$^{A3}CN_{so}$-45	24	30	0.661	0.461	0.70	9.06 ± 0.39
$^{B1}Air_{so}$-0.42	48	30	0.164	0.094	0.580	14.43 ± 2.72
$^{B2}Air_{so}$-0.94	48	30	0.140	0.015	0.110	13.44 ± 1.22
$^{B3}Air_{so}$-1.20	48	30	0.089	0.014	0.157	9.54 ± 1.28
$^{B4}Air_{so}$-2.50	48	30	0.270	0.030	0.110	9.31 ± 1.40
$^{C1}T_{so}$-15	48	15	0.263	0.035	0.130	10.47 ± 0.21
$^{C2}T_{so}$-20	48	20	0.545	0.434	0.796	16.08 ± 1.16
$^{C3}T_{so}$-25	48	25	0.653	0.121	0.186	18.96 ± 0.64
$^{C4}T_{so}$-30	48	30	0.507	0.120	0.236	20.49 ± 1.49
$^{D1}HRT_{so}$-53.3	24	30	0.120	0.024	0.203	11.41 ± 0.68
$^{D2}HRT_{so}$-80.0	36	30	0.126	0.083	0.660	22.31 ± 1.85
$^{D3}HRT_{so}$-106.7	48	30	0.288	0.206	0.720	33.77 ± 3.10
$^{D4}HRT_{so}$-213.3	96	30	0.249	0.216	0.870	33.73 ± 2.36

Note: [^{A1} = 15 g TOC/g N, ^{A2} = 22 g TOC/g N, ^{A3} = 45 g TOC/g N]; [^{B1} = air, 0.42 l/min, ^{B2} = air, 0.94 L/min, ^{B3} = air, 1.20 L/min, ^{B4} = air, 2.50 L/min]; [^{C1} = temp, 15°C, ^{C2} = temp, 20°C, ^{C3} = temp, 25°C, ^{C4} = temp, 30°C]; [^{D1} = HRT, 53.3 h, ^{D2} = HRT, 80 h, ^{D3} = HRT, 106.7 h, ^{D4} = HRT, 213.3 h]

As concluded from previous studies, an activated sludge can accumulate PHA around 20% under anaerobic conditions (temperature of 20°C) and up to 33% under aerobic conditions in anaerobic-aerobic system (temperature of 15°C) for PAOs cultures (Brdjanovic et al., 1998). The PHA content of activated sludge can also be increased up to 62% by applying a microaerophilic-aerobic sludge process using moderate temperature (~20°C) (Satoh et al., 1998, Takabatake et al., 2002). When compared with pure culture, more than 80% PHA of cell dry weight can be achieved (Lee and Yu, 1999, Lee et al., 2000). However, in this study on temperature variations, the PHA content can only reach to 21% of CDW indicating that temperature is not the solely influence.

Table 5: Derivation of stoichiometric and kinetic parameters for aerobic batch experiments at 15, 20, 25 and 30°C and HRT of 96.6 hours

Parameter	Unit/°C	15	20	25	30
		Stoichiometric parameters			
PHAconsumption/O_2 consumption	mg/mg O_2	2.34±1.24	5.06±1.75	4.07±2.09	3.21±0.29
PHA/O_2 relative to 20°C		0.46	1.00	0.80	0.63
O_2/PO_4 ratio	mg O_2/mg P	0.03±0.01	0.98±0.21	0.87±0.15	0.12±0.05
P-uptake/O_2 consumption	mg P/mg O_2	0.63±0.22	1.45±0.44	1.99±0.11	0.84±0.18
NH_4 consumption/ O_2 consumption	mg/mg O_2	0.06±0.17	0.076±0.03	0.12±0.02	0.12±0.03
		Kinetic parameters			
P-uptake/active biomass rate	mg P/mg. h	0.006±0.003	0.023±0.02	0.044±0.04	0.012±0.02
O_2 consumption/ active biomass rate	mg O_2/mg. h	0.008±0.002	0.013±0.03	0.012±0.01	0.011±0.01
NH_4 consumption/ active biomass rate	mg/mg. h	0.0001±0.00	0.001±0.03	0.002±0.01	0.002±0.05
PHA consumption/ active biomass rate	mg/mg. h	0.027±0.08	0.057±0.09	0.064±0.08	0.076±0.08

Based on the results in Table 6, PHA accumulation inside the biomass components was strongly influent by specific growth rate (μ). The accumulation of PHA was most significant for specific growth rate between 0.015 and 0.065 h^{-1}. The table showed that acclimatization of μ in CN_{so}-45 occurred in both feast and famine conditions. Since the mixed cultures were always inconsistent, the growth rate during famine condition could also affect the PHA accumulation. The prolonged depletion of external substrate would lead to a faster growth of PHA-producers (Lemos et al., 1998; van Loosdrecht and Heijnen, 2002). The bacterium will then utilize residual protein from dead organisms to produce small quantity of PHAs. Therefore, the potential of PHA production rate at famine period (30 percent of the overall famine period) can be examined. For example, at the highest specific growth rate (μ^{famine}= 0.089 h^{-1}), about 0.51 C-mol/C-mol of specific PHA production rate ($q_p^{famine}/-q_s^{famine}$) was recorded (data not shown). The results also showed that the growth rate, substrate uptake rate ($-q_s$), product formation (q_p) and the biomass rates ($q_p/-q_s$) increased when the system is under high C/N ratio. Both temperature (T_{so}) and cycle length (HRT_{so}) studies indicated that the maximum PHA production can be induced up to 620.41 (at 15°C) and 387.95 C-mM (at HRT = 213.3 h), respectively. The accumulation of PHA was significant for a specific growth rate between 0.015 and 0.065 h^{-1}. Furthermore, these specific growth rates for feast phase should be higher than obtained in famine phase as reported by Wong (2001), Yu (2001) and Zinn et al. (2003). It is also observed that the production of PHA up to

287.95 C-mM can be achieved when μ^{famine} is maintained at 0.003 h^{-1}. Therefore, storage of substrate and balanced growth is a better strategy for biomass cultivation than alternating the period of fast growth (balanced growth) with periods of starvation (famine).

Table 6: Accumulation of PHA content in various operational conditions under acclimatization of biomass concentration and specific growth rate

Experiment	Variable	Biomass, C_x (C-mM)		Specific growth rate, μ (h^{-1})		PHA^{max} (C-mM)
		feast	famine	feast	famine	
CN_{so}-15	C/N	352.57	418.00	0.067	0.042	18.50
CN_{so}-22		943.00	1000.57	0.077	0.042	25.10
CN_{so}-45		164.29	481.43	0.136	0.089	31.34
Air_{so}-0.42	Air flowrate	1142.86	1251.43	0.018	0.004	40.36
Air_{so}-0.94		1891.43	3251.43	0.029	0.014	51.81
Air_{so}-1.20		2088.57	2411.43	0.032	0.006	47.21
Air_{so}-2.50		994.29	2171.43	0.066	0.003	29.44
T_{so}-15	Temp	2615.71	311.43	0.026	0.015	620.41
T_{so}-20		2894.29	338.57	0.015	0.021	398.27
T_{so}-25		2517.14	1290.00	0.011	0.016	361.44
T_{so}-30		2595.71	1151.43	0.043	0.019	383.86
HRT_{so}-53.3	Cycle length	1998.57	310.00	0.030	0.042	265.19
HRT_{so}-80.0		1957.14	247.14	0.020	0.005	255.51
HRT_{so}-106.7		1927.14	364.29	0.021	0.015	387.95
HRT_{so}-213.3		1925.71	181.43	0.065	0.006	387.95

3.3 Optimization of PHA Production on Selected Operational

The optimization of PHA production was conducted individually by stimulating the "standard" productivity of PHA (Δf_{PHA}) using SO as substrates. An analysis of residual biomass and PHA production was carried out in order to determine the standard Δf_{PHA}. The rate of PHA production is strongly correlated with C/N ratio, air flowrates, HRT=SRT and temperature with r^2 ranging from 0.79 to 0.95 (Figure 3). The final formula obtained from different experimental conditions showed that the PHA production increases at high C/N ratio (150 - 200 g TOC/g N) and HRT=SRT (90 h). Unfortunately, the efficiency decreased when exposed to the high air flowrate (> 1 L/min) and temperature (> 25^{o}C). At air flowrate greater than 2 l/min, excessive oxygen saturation occurs, which is not favourable for PHA inclusion. Whilst a higher temperature will enhance biomass production, the PHA inclusion tends to decrease. This finding concurred with Chua et al. (1997), Chung et al. (1997) and Dionisi et al. (2001).

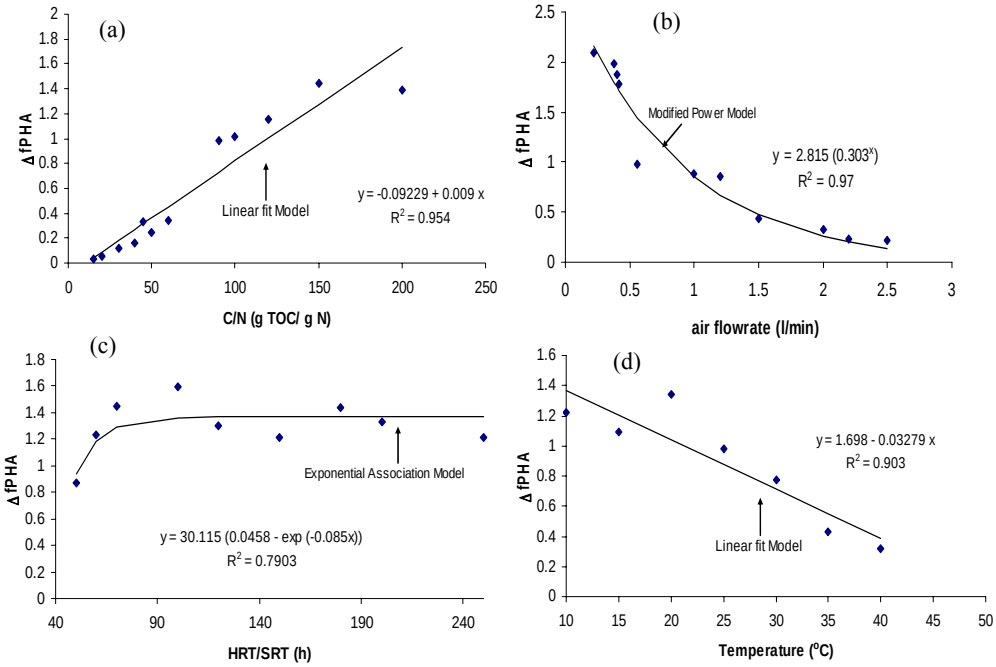


Figure 3: Dependence of PHA amount produced on (a) C/N ratio, (b) air flowrates, (c) HRT=SRT and (d) temperature. Notes: (♦) experiments used for fitting the points, (-) model equation developed from fittings

3.4 Fatty Acid Uptakes for PHA Constituents

The patterns of selected fatty acid distributions are shown in Figure 4. In general, the maximum fatty acid was observed at the 10th hours, before depleting. The pH dropped slightly from 6.9 to 5.2 due to the accumulation of organic acids. However, the medium was adjusted to pH 7.0±0.22 between the 20th and 44th hours. HPr was the highest fraction of VFAs followed by HBt and then HAc. The initial consumption of fatty acid via biomass uptake rate is always driven by TCA cycles. A different type of intracellular polymer (i.e. PHAs and glycogen) was formed at various alkanoic acid compositions. Since the degradation of fatty acid from SO is slower than acetate or butyrate, a higher production of co-monomer, such as hydroaxyhexanoate (HH) and hydroxyvalerate (HV) than HB can be expected. However, as reported by Lee (1996b) co-monomers of HH and HV are useful in restructuring the brittleness of PHB. The present results confirmed that a fatty acid constituent is a precursor to produce different types of PHA (e.g. PHV and PHH) (Khanna and Srivastava, 2004).

It is possible that when the bacteria responsible for PHA accumulation are subjected to multiple nutrient limitations (N, P or O_2), they synthesize PHA using different biosynthesis pathways. Propionic acid is used less effectively when there is more than one limiting conditions. This could result in a lesser fraction of the available propionate being incorporated into the HV unit of the copolymer (Du et al., 2000, 2001). At the same time, the correlation between fatty acid constituents (VFAs) and the production of PHA is obvious, indicating that at temperature below 20°C, increments of VFAs and PHA storages are expected.

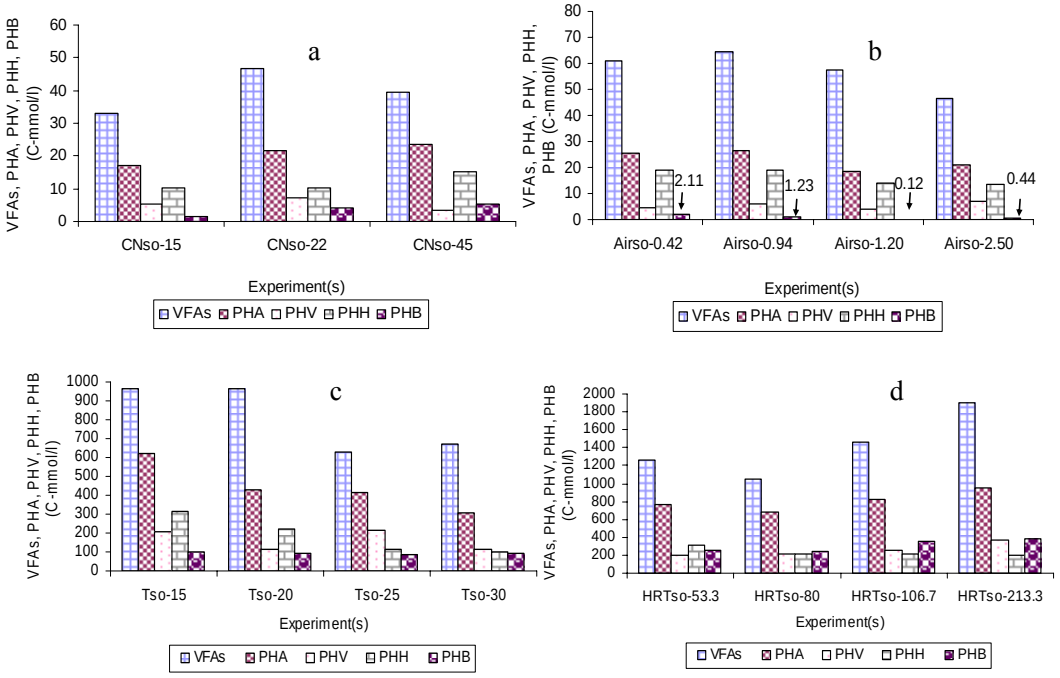


Figure 4: Summary on the VFAs uptake and PHA production at various conditions of (a) C/N ratio, (b) air flowrate, (c) temperature and (d) cycle length.

Different operating conditions (i.e. C/N ratios, air flowrate, temperature and cycle length) of the activated sludge mixed culture produced different rates of PHV, PHB and PHH, though the organic substrates used were the same. This is expected to occur since the substrates conversion can be changed when different VFAs are used. This difference is probably due to the fact that PHA carbon could be reproduced multiple times for a prolonged observation period (Liu et al., 2000). As shown in Figure 5, the PHA production could regenerate in any sequence phases, indicating that PHA-producers are available in alternate phases (growth and accumulation). In addition, the PHA production could regenerate many times as long as the CDW is maintained during

the limiting of nutrients. The increase in PHA production from 5% to 30% of CDW occurred when the CDW was maintained at 9 g/L. Therefore, the biomass concentration is usually significant in enhancing the accumulation of PHA.

Ren (2003) found that different bacteria were able to produce PHAs with different PHV/PHB compositions when growing on the same substrate. Anderson and Dawes (1990) studied the production of copolymer by the genera *Rhodococcus*, *Nocardia*, and *Corynebacterium*. They found that these genera had differences PHA accumulation and composition when grown on the same single carbon compounds. Therefore, the difference operating condition in this study seems to grow different group of PHA producing bacteria. The different PHA producing bacteria were responsible for both the differences in PHA production and constituents (%PHB and %PHV).

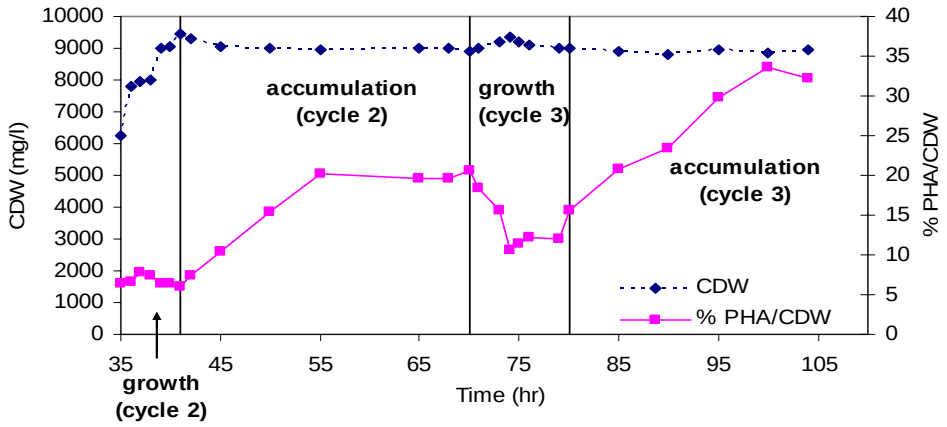


Figure 5: The PHA production and CDW concentration during reproducibility phase

3.5 Kinetic Rates of PHA Degradation

The assessment of PHA is always reported together with their degradation rates. Most studies found that the rate of PHA degradation occurred massively during the famine period (Beccari et al., 1998; Beun et al., 2000a, 2000b; Dionisi et al., 2001). In addition, the measurement of PHA degradation is useful to predict the appropriate configurations of batch study (e.g. cycle length and air flowrate). Figure 5 shows the best fit for all the data sets (CN_{so}-fit, Air_{so}-fit, T_{so}-fit and HRT_{so}-fit) which were calculated based on famine degradation of PHB fraction

Because of the different in f_{PHB} values for each data set at the start of the famine period, the independent data sets (time) were shifted so that they overlap and could be shown in Figure 6. The result suggests that PHB degradation is most affected by air flowrate with the fastest rate occurred when k = -0.48 h^{-1} and n = 0.503. On the other hand, the slowest PHB degradation was due to temperature effects with k = 0.00016 h^{-1} and n = 0.07. Due to this, the PHB degradation has been determined from the fastest to

slowest reaction as follows: Air_{so}-fit > HRT_{so}-fit > CN_{so}-fit > T_{so}-fit. It indicates that notable PHB degradation occurs under high air flowrate because the biomass cell has an external energy to consume the intracellular polymer (PHA) for maintaining their growths.

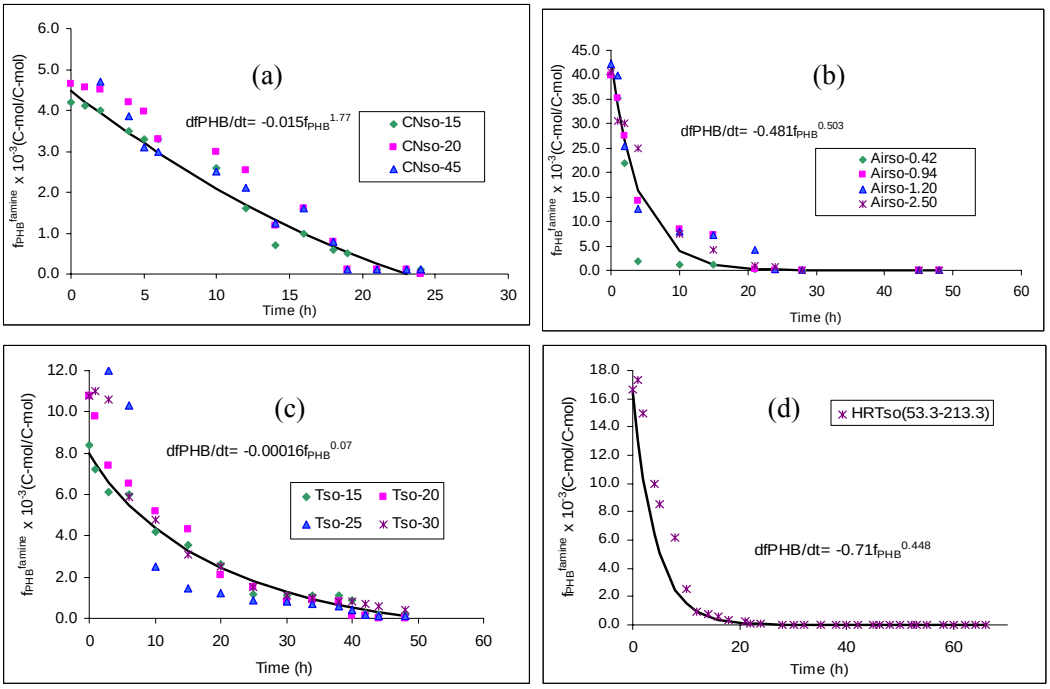


Figure 6: Curve estimate of PHB degradation using differential method

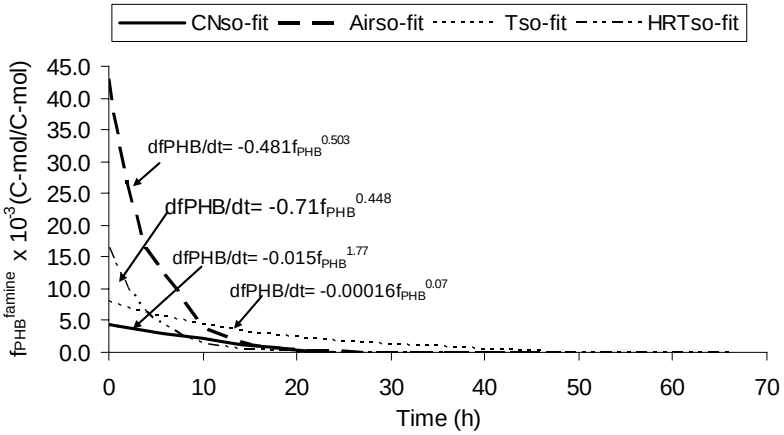


Figure 7: Degradation of PHB under different fitted conditions

5.0 Conclusions

The fed-batch cultivation of more than six months under aerobic dynamic conditions was able to produce PHA up to 34% of CDW. This study shows that biomass in an aerobic condition of mixed cultures could accumulate high amount of PHA by manipulating the cycle length. Major findings of this study are summarised as follows:

(a) A higher C/N ratio will increase the PHA production. However, if the ratio exceeds 400 g TOC/g N, the concentration will decrease immediately.

(b) Both temperature (T_{so}) and cycle length (HRT_{so}) are important for producing PHA. However, the stoichiometry of the aerobic processes was most significant between 15^{o}C and 30^{o}C. At the same time, the correlation between fatty acid conversions (VFAs) and the production of PHA is obvious, indicating that VFAs and PHA storages are expected to increase at temperature between 15^{o}C and 20^{o}C ,

(c) At specific growth rates in the feast period ($\mu^{feast} < 0.066\ h^{-1}$), the ratio of PHA over substrate consumption ($q_p/-q_s$) was quite high between 0.6 and 0.7 C-mol/C-mol. The results showed that the growth rate, substrate uptake rate ($-q_s$), product formation (q_p) and ratio of $q_p/-q_s$ increased when the system is under high C/N ratio.

(d) The optimum experimental conditions for PHA production was obtained at moderate C/N ratio (150 – 200 g TOC/g N), low air flowrate (< 0.5 l/min), sufficient cycle period (HRT = 90 h) and temperature between 25 ^{o}C and 30 ^{o}C.

(e) Degradation of internally stored PHB in the microorganism cells can be described by the first order kinetic rate. The degradation of PHB is mainly attributed to the air flowrate applied

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