CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Microbial seed

Microbial seed was obtained from an upflow anaerobic sludge blanket system (UASB), wastewater treatment processes of Cho Heng Rice Vermicelli Factory Co. Ltd., Nakhonpathom Province, Thailand. The wastewater treatment process was operated under steady state conditions (Table 3.1); therefore it was ensured the variation of microbial seed composition obtained from the plant from time to time is negligible.

Table 3.1 Characteristics of influent water after using the UASB of Cho Heng Rice Vermicelli factory (Cho Heng, 2010)

Parameter	Effluent		Thai Industrial
	Range	Average	Effluence Standard
BOD (mg L^{-1})	3 – 16	11	< 60
T-COD (mg L^{-1})	18 – 50	40	< 120
SS (mg L^{-1})	6 – 23	14	< 50
Temperature	33 - 35°C	-	< 40°C
pH	7.6 - 7.9	-	5.5 - 9.0
Odor	Less Small		
Space	2 Acres		
Methane Gas (m^3 d^{-1})	Reuse (Generator or Boiler) (1,800 - 2,000 m^3 d^{-1})		
Sludge	Little		
Investment	Very High		

BOD (Biochemical oxygen demand); T-COD (Total chemical oxygen demand); SS (Total Suspended solids)

The granules were washed with 0.5 % w v^{-1} NaCl solution twice to remove methanogenic bacterial group inside communities (Fox *et al.*, 1994), and subsequently

resuspended in deionized water around 2 h to eliminate salt residues from the cells. The final biomass was adjusted to 0.2 % w v^{-1} starter seeds (2 g L^{-1}) after the determination of the sludge by wet weight (Liu *et al.*, 2000) subsequently were transferred to 1-L bottle (Duran, Germany) containing 500 mL of medium for preparing the starter culture. The inoculated flasks were incubated on rotary shaker (200 rpm) for 18-24 hours in the range bacterial ready to grow fully. These cultures in flasks were grown and kept as stock cultures at 30°C and maintained and 4°C.

3.1.2 Cultivation medium

The media for all experiments (Figure 3.1A) were modified from Almeida *et al.* (2007). One liter of the medium contained 6 g Na_2HPO_4, 3 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 g yeast extract, and 5 g tryptone. The medium was supplemented with purified commercial – glycerol grade (Qrec, purity 99.5%, New Zealand). Chemicals and reagents were of analytical grade.

In the experiment with crude glycerol (Figure 3.1B), the cultivation medium contains (per L) 2.3 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.3 g $NaHCO_3$, 0.1 g $CaCl_2.2H_2O$, and 1 mL trace element solution (0.58 g $ZnSO_4.7H_2O$, 3.96 g $MnCl_2.4H_2O$, 0.6 g H_3BO_3, 5.56 g $FeSO_4.7H_2O$, 5.62 g $CoSO_4.7H_2O$, 0.34 g $CuCl_2.2H_2O$, 0.04 g $NiCl_2.6H_2O$, 0.06 g $NaMoO_4.2H_2O$ dissolved in 0.5 N HCl per liter) (Serafim *et al.*, 2004). Crude glycerol obtained from the Elstar Oils Company (Malbork, North Poland)was added to the medium to obtain a final concentration of 5% v v^{-1}. $(NH_4)_2SO_4$ was used to adjust and keep the carbon-to-nitrogen ratio at a constant level (C/N = 10).

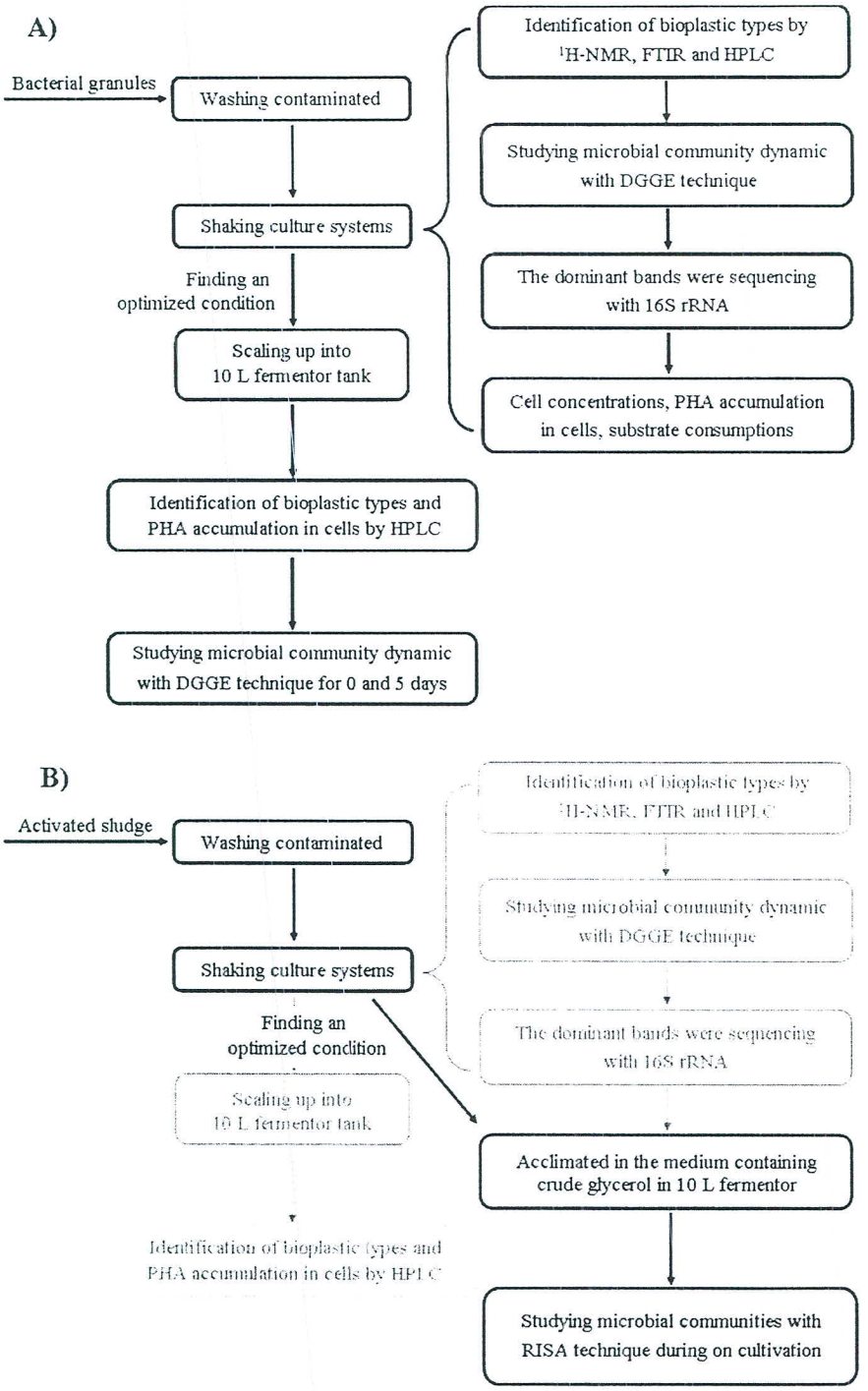

Figure 3.1 schematic diagram of proposed PHA production from the mixed culture was categorized into 2 main parts: (A) study of bacteria granules in various conditions and parameters throughout scaling up from pilot scale into 10-L under optimum conditions, while (B) study of bacterial communities composed of PHA-producing bacteria and bioplastic type from activated sludge under optimized conditions, cultivating on a 10-L bioreactor.

3.1.3 Reactors

The reactors could classify into 3 types, depending on the expected study at a time. Primary enrichment culture was used to find a suitable condition that increased the quantity of bacteria cells and PHB productions. The growth of the bacteria culture was confirmed in liquid medium supplemented with various glycerol concentrations. Inoculum of bacteria was prepared by using a loopful of bacteria into a test tube that contained 5-mL of medium containing 10, 30, 50, 60, 70 and 80 % (v v^{-1}) glycerol concentrations without glycerol as control and placed on the rotary shaker at 200 rpm, 30°C for 1 day. Then, 5-mL of suspension culture was then transferred to enrichment step prepared as 500-mL medium in 1-L bottle glass and shaking-incubated on the rotary shaker at 200 rpm, 30°C for a week (Figure 3.2).

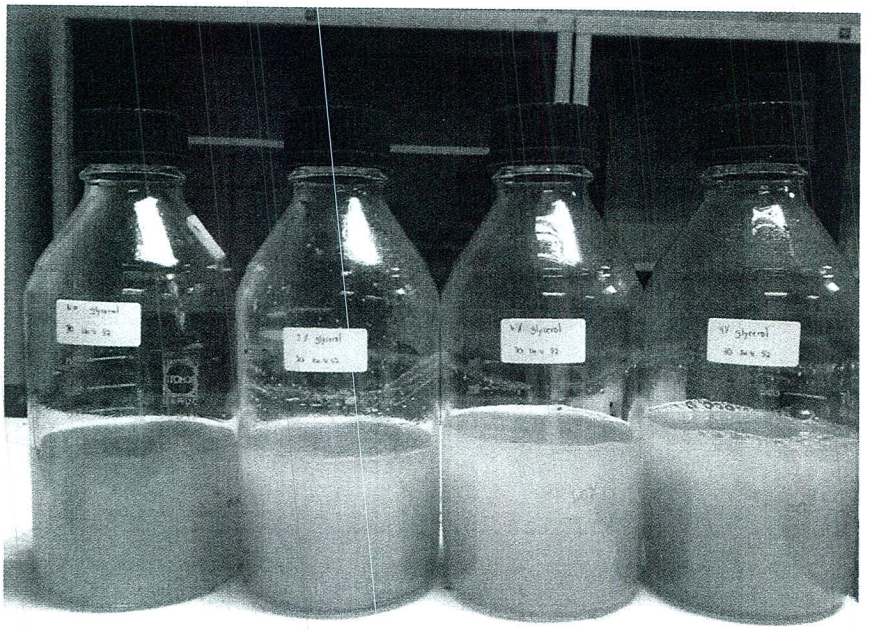

Figure 3.2 Bacterial cultures in 1-L bottles with different glycerol concentrations of 3, 6 and 9% v v^{-1} after 5 days of incubation.

Optimal conditions of PHA production obtained in 1-L bottle experiments was selected to upscale in 10-L reactor, which was designed and constructed by B.E. Marubishi (Thailand) Co., Ltd (Figure 3.3).

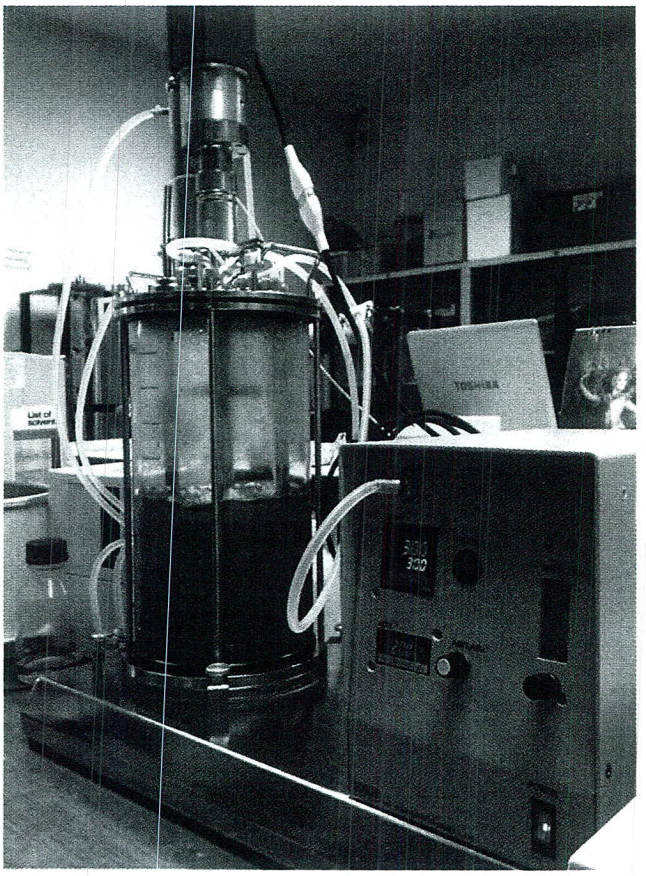

Figure 3.3 The medium was contained in 5-L working volume in 10-L fermentor tank

In the final work was done in Poland, the experiments were set up separately and studied in Thailand. Fed-batch cultures were carried out in a 10-L fermentor tank, as shown in Figure 3.4 (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ, USA) containing 2 L inoculum in the concentration 1.09 g L^{-1} that not only bacterial source, operation system also cultivation condition was different from those used in experiments in Thailand. The fermentation proceeded for 5 days. During this period, the feeding medium was consecutively added using a PP-1B-05A peristaltic pump (Zalimp, Warsaw, Poland) at a flow rate of 1-L per 24 h. Two phases of different lengths were carried out each day. During the first 6 h phase, the feeding solution consisted of methanol, ammonium and micronutrients, whereas during the second stage (18 h), the medium contained only methanol and micronutrients. After obtaining 7-L of culture, the process was discontinued and 6-L of biomass of concentration 1.86 g L^{-1} was harvested for PHA extraction (Ciesielski *et al.*, 2006). The remaining biomass of volume 1-L was used as inoculum for the next fermentation.

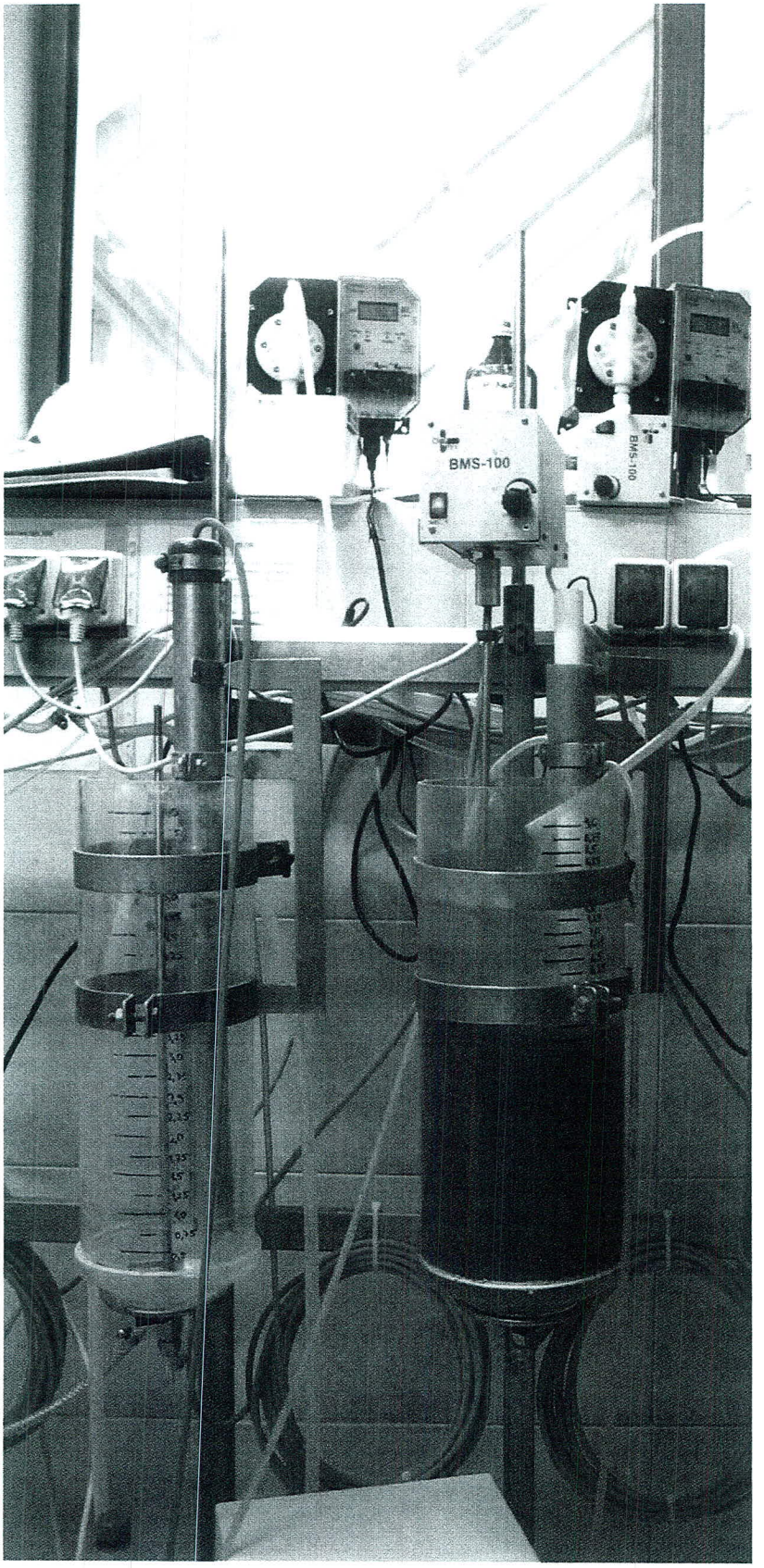

Figure 3.4 Activated sludge in 10-L bioreactor

3.2 Experimental setup

The overall research is shown in Figure 3.1. There was a definite need for the optimization of the entire PHA production process, and that there was definite quantifiable PHA accumulation in cells and identified PHA types, especially after scaling up into 10-L bioreactor processing system. The mixed bacterial growth was monitored *via* the spectrophotometer as culture turbidity (OD_{600} nm) and total cell dried weight. Cell growth in the presence of glycerol used was also monitored to distinguish bacteria of which glycerol could serve as a growth substrate (control). The cultivations were grown and incubated at 200 rpm, 30°C for a week. Liquid samples were collected periodically for analysis of bacterial growth, cell dried weight, glycerol concentrations, microbial community dynamic and PHA accumulation in the cells. Subsequently, a high PHA production in shaking flask system was applied into a large scale of 10-L reactor, using the similar optimized condition setup. For reactor, the production and type of PHA as well as substrate consumptions were determined through using the HPLC. Then changing bacteria communities was determined by DGGE technique in order to differentiate from dominant bands of before and after scaling up into 10-L bioreactor (Figure 3.1A).

Figure 3.1B illustrates the PHA-producing mixed culture which passed the preliminary test the capable of producing PHA, using crude glycerol from by-product of biodiesel production. Then, the research was studied fermentation process in 10-L fermentor and investigated bacterial communities within cultivation time (Figure 3.1B).

3.3 Analytical methods

3.3.1 Biomass yield

Samples taken from the shaking flask were immediately chilled at 0°C by placement in an ice bath. One liter of cell suspension was centrifuged at 9,000 rpm 15 min at 4°C, washed twice with deionized water, recovered by centrifugation, and dried at 85°C for 36 h (until to weight constant). Cell concentration, defined as the amount (dry weight) of cells per liter of culture broth, was determined by weighing dry cells as described previously (Lee *et al.*, 1994).

3.3.2 Quantification of glycerol and glucose

In order to measure the glycerol and glucose utilization during PHA production, liquid samples from the shake flask would be collected periodically and determined with high performance liquid chromatography (HPLC) technique. Each sample was filtered through a non-sterile 0.45 μm nylon membrane filter to remove cells and solids. The glycerol concentrations were analyzed using high pressure liquid chromatography, HPLC (Agilent LC1200 Series, USA) equipped with refractive index detector (RI). Sample was injected to HPLC column (Aminex HPX-87H column, 300 x 7.8 mm) and eluted by 5 mM H_2SO_4 with a flow rate 0.60 mL min^{-1} and the column temperature was 55°C (Koller *et al.*, 2005). The calculations were shown in Appendix B-3.

3.3.3 PHA structure

One liter of cell suspension was harvested by centrifugation, washed with 30% methanol solution in water, dried until to weight steady and weighed. One gram of dried cells was suspended in 100-mL chloroform (about 1:100) at room temperature for 24 h (Green *et al.*, 2002). The mixture was filtered and the filtrate was concentrated by evaporation. The polymer was reprecipitated in methanol and dried in order to determine PHA yield (Takagi *et al.*, 2004). The partially purified PHA subjected to Fourier-transformed infrared spectrometry (FTIR) and Proton nuclear magnetic resonance (^{1}H-NMR) analyses.

Cells from shaking flask cultures were separated by centrifugation, washed with distilled water and spread on KRS-5 windows. Cells grown on petri dishes were directly applied to the window. After air-drying or irradiation with infrared light, the dried cells were subjected to FTIR. Purified PHA was dissolved in chloroform and layered on the KRS-5 window. After evaporation of the chloroform, the PHA polymer film was subjected to FTIR analysis. The FTIR was carried out on a Bruker Instrument Spectrometer (Model Apex II, Spectrum one, Germany) in transmission mode over a range of 4,000 to 400 cm^{-1} and with a resolution of 4 cm^{-1}. One hundred scans were recorded for the sample.

Solution nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX300 (Switzerland) with ^{1}H at 400 MHz. The solvent chloroform-*d* and tetramethylsilane (TMS) were used as internal references for chemical shifts in ^{1}H-NMR.

3.3.4 Quantitation of PHA

One gram of cell dried weight from 1-L culture medium (Section 3.3.1) was resuspended using magnetic stirring bars overnight at room temperature. One mL of

concentrated H_2SO_4 was added. Hydrolysis digestion was used to determine PHB production which were weighed directly into the tubes and suspended in 1-mL of deionized water (HPLC grade) prior to acid addition. The closed tubes were heated for 120 min at 90°C without mixing. After cooling, the all digested supernatant was transferred and made up 100-800 mL, depending on viscosity suspension (dilution factor; the calculation was shown in Appendix B) using deionized water (HPLC grade) (modified from Hesselmann *et al.*,(1999)). The diluted supernatant was collected and quantified by HPLC (Agilent LC1200 Series, USA) equipped with UV 210 nm detector using Aminex-87H column (Bio-Rad, USA). The HPLC conditions were 25-µL injection volumes, 5 mM H_2SO_4 and 20% CH_3CN mobile phase was used to elute PHA with flow rate of 0.5 mL min^{-1}, and the column temperature was 55°C. Similarly that, the PHB concentration standard was determined by HPLC (Agilent LC1200 Series, USA) with PHB and poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) of natural origin and with 12wt% polyhydroxyvalerate (PHV) standard (Sigma Chemicals Co., St Louis, MO, USA) as the standards (Figure B-1, B-2 in Appendix B).

The PHB contents (percentage of PHB dry weight per cell dry weight) were calculated as the percentage of the ratio of the amount of PHB to the amount (dry weight) of cells (Wang *et al.*, 1997). Residual cell concentration was defined as cell concentration minus PHB concentration. The PHB synthesis rate was defined as grams of PHB synthesized per gram of residual cells per hour. The PHB yield coefficient on glycerol ($Y_{PHB/S}$) was calculated as the PHB produced per unit mass of glycerol consumed. Total parameters were shown on how to calculate in Appendix B-4.

3.3.5 Microbial community dynamic study

3.3.5.1 DNA extraction

Method 1: Genomic DNA was extracted from 5-mL liquid samples using benzyl chloride method (Zhu *et al.*, 1993). The procedure was as follows: 1 g of pellet harvested by centrifugation was used. To each sample, 5-mL extraction buffer (100 mM Tris-HCl, pH 9.0, 40 mM EDTA), 50-µl 20% SDS and 300-µl benzyl chlorideare added. The tube is vortexed and incubated at50°C for 1 hour. The tube was added 300-µl 3 M sodium acetate, pH 5.0 and was kept on ice for 30 minutes. After centrifugation at 12,000 g, 4°C for 15 minutes, the supernatant was collected, and DNA was precipitated to a fresh tube with isopropanol. The DNA of samples was purified using 500- µl phenol: chloroform (25:24 v v^{-1}) solution, supernatant was kept after centrifugation for 15 min and added

600-µl chloroform: isoamyl alcohol (24:1 v v^{-1}) solution. The supernatant was collected after centrifugation for 15 min, and added 600-µl isopropanol, incubated on ice for 1 hour. The pellets were washed with 800-µl ethanol (70%) and resuspended in 30-µl TE buffer (10 mM Tris, 1 mM EDTA: pH 7.4). The genomic DNAs were checked by 0.8% w v^{-1} agarose electrophoresis (Condition: 100V, 40 min).To eliminate contaminating genomic DNAs, 10 mg mL^{-1} RNase-Free DNase (Promega, USA) was added to each RNA extract before incubation at 37°Cfor 1 hour. Purified DNA samples were used for further molecular analyses.

Method 2: Genomic DNA was isolated as follows: 20 g sample of sludge samples were washed with 40-mL 1x phosphate buffer saline (0.1 M; pH 8.0, Sigma), pelleted by centrifugation for 1 min, sonificated, suspended in the proteinase K buffer (100 mM Tris-HCl; 10 mM EDTA; pH 8.0) and added 200-µL 10% (w v^{-1}) SDS and a further incubated at 65°C for 1 h. DNA was purified using Genomic DNA Mini Kit (A&A Biotechnology, Poland) and stored 20°C until DNA PCR amplification. The quality of the DNA extracts was routinely checked by using 1% w v^{-1} agarose electrophoresis (Condition: 80V, 30 min).

3.3.5.2 DGGE analysis

After DNA extraction, 16S rDNA fragments were amplified by PCR that pair primers for DGGE comprised of 518_R (5' ATT ACC GCG GCT GCT GG 3') and 338GC_F (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CA 3'). The PCR conditions were as follows: 94°C for 3 min followed by amplification in which the following conditions were used: denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation for 2 min at 72°C with an additional 3 min at 72°C for each cycle for 25 cycles. Presence of PCR products was confirmed by electrophoresis in 0.8% (w v^{-1}) agarose gel stained with ethidium bromide and visualized under short-wavelength UV light.

DGGE was performed with a D-CODE Universal Mutation System (Bio-Rad, USA). The PCR samples (30 µL) were applied directly to 8% polyacrylamidegels (37.5:1 acrylamide:bisacrylamide) in a 1xTAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.5) with a denaturing gradient ranging from 40to 60%. Denaturation of 100% corresponds to 7 M urea and 40% formamide. The gradient gel was cast with agradient delivery system (Bio-Rad, USA). Electrophoresis was run at a constant voltage of 130 V at

60°C. After 5 h of electrophoresis, the gel was stained with ethidium bromide for 20 min. and viewed under an ultraviolet transilluminator.

3.3.5.3 DNA sequencing

The interesting DGGE bands were cut from DGGE gel and sequenced to identify the bacterial species. Briefly, the cut DGGE bands were put in an eppendorf tube containing 30-μL steriled water for DNA extraction. Then, the DNA solution was amplified by PCR as explained Section3.3.5.2by using 338GC_F and 518_R primers without GC clamps. The PCR product was purified using Gel clean up kit (Eppendorf, U.S.A.). Then, the purified PCR product was ligated through pGEM®-T Easy Vector (Promega, U.S.A.) of which the reaction was described as below:

Reaction Component	1x Reaction (μL)
The purified PCR product (150 μg DNA)	3
pGEM®-T Easy Vector (30 ng μL^{-1})	1
T_4 DNA ligase (3 units μL^{-1})	1
Ligase buffer	5
Ligase reaction	10

The ligase reaction was incubated at 4°C, overnight. The ligase product was transformed to the competent *E.coli* DH5α cell by electroporation technique. The transformed solution was incubated at 37°C for 1 h, shaking at 250 rpm. Then, the transformed solution was spread on LB agar containing 100 μg mL^{-1} amplicilin, 30 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-galprepared in dimethyl formamide; DMF) and 30 μg mL^{-1}Isopropyl β-D-thiogalactopyranoside (IPTG in sterile distilled water).

White colonies were selected to check the insert fragment. The white colonies were grown in the LB broth containing 100 μg mL^{-1}amplicilin at 37°C, overnight. Then, the plasmid was extracted by Miniprep kit (Eppendorf, USA) and cut by *Eco* RI restriction enzyme (Toyobo, Japan) to confirm the inserted fragment. The restriction digestion condition (total volume of 10-μL) was as described below;

Reaction Component	1x Reaction (μL)
Plasmid (pGEM®-T Easy)	2.0
*Eco*RI enzyme	0.5
Buffer	1.0
Sterilized water	6.5

The inserted fragment was examined by running it in 1% agarose gel electrophoresis. The correct DNA insert fragment was about 200 base pairs. The plasmids having the correct DNA insert fragment was sent for sequencing at Macrogen, Korea. The obtained nucleotide sequences were compared with the sequences from GenBank and EMBL databases using the BLAST online service (www.ncbi.nlm.nih.gov/blast).

3.3.5.4 RISA analysis

The 16S rDNA fragments were amplified by PCR with pair primers. The bacterial intergenic spacer region (ISR) located between the small and the large subunit of rDNA was amplified with primers 1 (5'-TGC GGC TGG ATC CCC TCC TT-3') and 2 (5'-CCG GGT TTC CCC ATT CGG-3') described previously by Normand *et al.* (1996). The PCR conditions were as follows: 95°C for 10 min followed by amplification in which the following conditions were used: denaturation at 94°C for 30 s, annealing at 42°C for 30s and elongation for 1 min at 72°C with an additional 5 min at 72°C for each cycle for 35 cycles. Presence of PCR products was confirmed by electrophoresis in 1% (w v^{-1}) agarose gel containing with ethidium bromide and visualized under short-wavelength UV light.

RISA was performed with a D-CODE Universal Mutation System (Bio-Rad, USA). The PCR samples (30 µL) were applied directly to 5% polyacrylamidegels (29:1 acrylamide: bisacrylamide) in a 1xTBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA; pH 8.0). Electrophoresis was carried out at 60V for 100 min in 1xTBE buffer (89 mM Tris-base, 89 mM Boric acid, 2 mM EDTA; pH 8.0). Sizes of PCR products were estimated using 1 Kb molecular weight marker (Promega, USA). After approximately 2.5 h electrophoresis, the gel stained with SYBR Gold (Invitrogen) and viewed under an ultraviolet transiluminator and recorded with a CCD camera (Gel Logic 200, Eastman Kodak Company, USA). Bands were detected automatically from digital images of the gel using KODAK 1D 3.6 Image Analysis Software (Eastman Kodak Company, USA).

3.3.6 Optimum conditions of cell growth and polyhydroxybutyrate production

3.3.6.1 Influence cosubstrate and nitrogen sources on PHA production

Influencing conditions of polyhydroxybutyrate production were investigated after knowing of suitable conditions. According to the previous method (Section 3.1.2), the cultivation of bacterial granules for carbon and nitrogen source, additional carbon or/and deducting nitrogen sources were supplemented as follows:

1) Carbon source: 5, 10, 15 and 20 g L^{-1} glucose

2) Nitrogen source: 1.4 g L^{-1} $(NH_4)_2SO_4$

3) Carbon and nitrogen source: 10 g L^{-1} yeast extract and 5 g L^{-1} tryptone

After additional carbon and/or deducting nitrogen source were supplemented, the bacterial culture was placed on a rotary shaker at 200 rpm, at 30°C. Culture medium was collected on each day until to 7 days. The main criteria used to select so called, the optimum conditions were the increase of percentage of PHB content or short PHB production time or reducing costs for each bacteria. Furthermore, the conditions that contained high percentage of PHB content or short PHB production time or reducing cost for either carbon or nitrogen source were mixed.

3.3.6.2 Scaling up into a 10-L fermentor

The productive medium was selected by the optimized condition of results according to Section 3.3.6.1 containing the best of carbon and nitrogen sources which was applied into a 10-L reactor (MDL, Marubishi Bioengineering Co. Ltd., Tokyo, Japan). The growth and PHB production under the batch cultivation was also studied and the media and cultivation conditions were similar to in the shaking flasks. The batch cultivation was initiated as batch with an initial working volume of 0.14-L (2% v v^{-1} inoculum). After 10 h, at a biomass concentration of 1.03 ± 0.07 g L^{-1}, 6-L of media was introduced in the bioreactor. Reactor description and condition of each experiment was shown in Table 3.2. Fermentation was continued for 120 h at 30°C and the dissolved O_2 concentration maintained at 30% of air saturation by adjusting the agitation speed and aeration rate. Consequently, the bacteria cultures were grown in the reactor as batch cultivation.

Table 3.2 Operational parameters in 10-L experiment

Condition	Simple reactor
Working volume	7.0 ± 0.5 L
Temperature	30 ± 4°C
pH	7.0 ± 0.2
Stirrer	200 ± 10 rpm

During the incubation, cell suspension was indicated for a period within 5 days, centrifuged and the supernatant was analyzed for using HPLC.