CHAPTER 4

RESULTS AND DISCUSSION

4.1 Production of Poly(3-hydroxybutyrate) from Glycerol using Upflow Anaerobic Sludge Blanket (UASB) Bacterial Community

4.1.1Growth of glycerol-utilizing microorganisms

The 1-L batch experiment showed that the microbial consortium is capable of growing in the glycerol concentrations up to 50% (Figure 4.1). No growth was observed at glycerol concentrations of 60% and 70% (data was no shown). Growth characteristics at initial glycerol concentration ranging from 10% to 50% were similar (Figure 4.1), with 10 h lag phase, 20 h exponential phase and 90 h stationary phase before reaching the decline phase. The increase of the initial glycerol concentration results in a low density of biomass. During the cultivation, the growth phase occurred at 10 h (cell concentration of 4.220 ± 0.289 g L^{-1}) in medium without glycerol and gradually decreased as 2.032 ± 0.020, 2.064 ± 0.001 and 1.886 ± 0.062 g L^{-1}, using the initial glycerol as 10, 30 and 50 % v v^{-1}, respectively. Figure 4.1 showed that culture grew exponentially through 20 h incubation for all cases. The highest growth of mixed culture being 7.891 ± 0.003, 7.357 ± 0.028 and 6.639 ± 0.014 g L^{-1} were recorded on media containing 10, 30 and 50% by volume of glycerol concentrations, respectively, at120h incubation period while 9.378 ± 0.061 g L^{-1} of control. Among using at different glycerol concentrations, the bacterial growth was also the lowest of cell concentrations when cultivation on medium contained 50% v v^{-1} glycerol though it could be increased when reducing of glycerol concentration in medium. The results illustrated affecting on cell growth when increasing glycerol concentration.

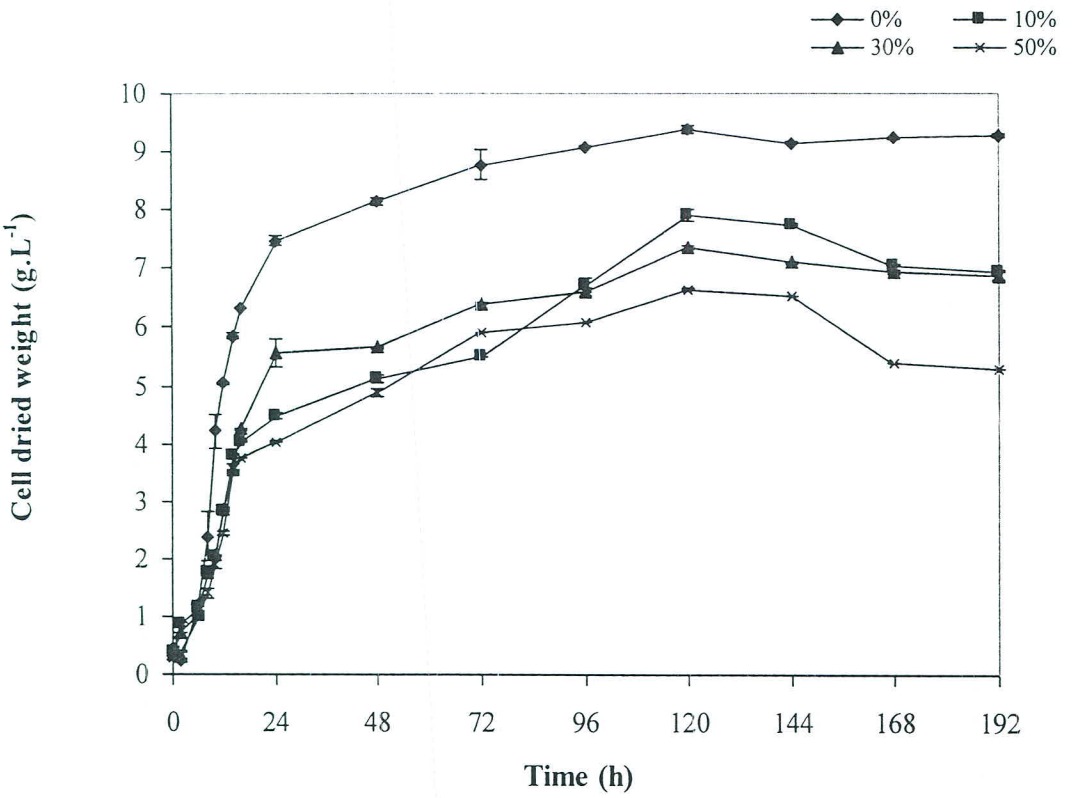

Figure 4.1 Growth of microbial seed represented with cell dried weight (CDW) per L culture medium on varied (% v v^{-1}) glycerol concentration including 10, 30, 50 and without glycerol as control. Cultivation was an agitation rate of 200 rpm at 30°C in 1-L flasks (n=4).

Figure 4.2 displayed that the microorganisms could utilize glycerol as a carbon source and an energy source during their growth. With respect to glycerol utilization, Figure 4.2 shows that, the glycerol utilization by mixed cultures increased along with increasing incubation period to reach the maximum after 120 h (5 days). From the beginning of the 10% glycerol feed, the aerobic pathway of glycerol utilization was fully activated as shown by the rapid glycerol utilization within 5 days, whereas the 30% and 50% showed low activity (Figure 4.2). The aerobic condition was described a new process for the production of PHA based on the aerobic enrichment of activated sludge to obtain mixed cultures able to store PHA at high rates and yields (Dionisi*et al.*, 2004).

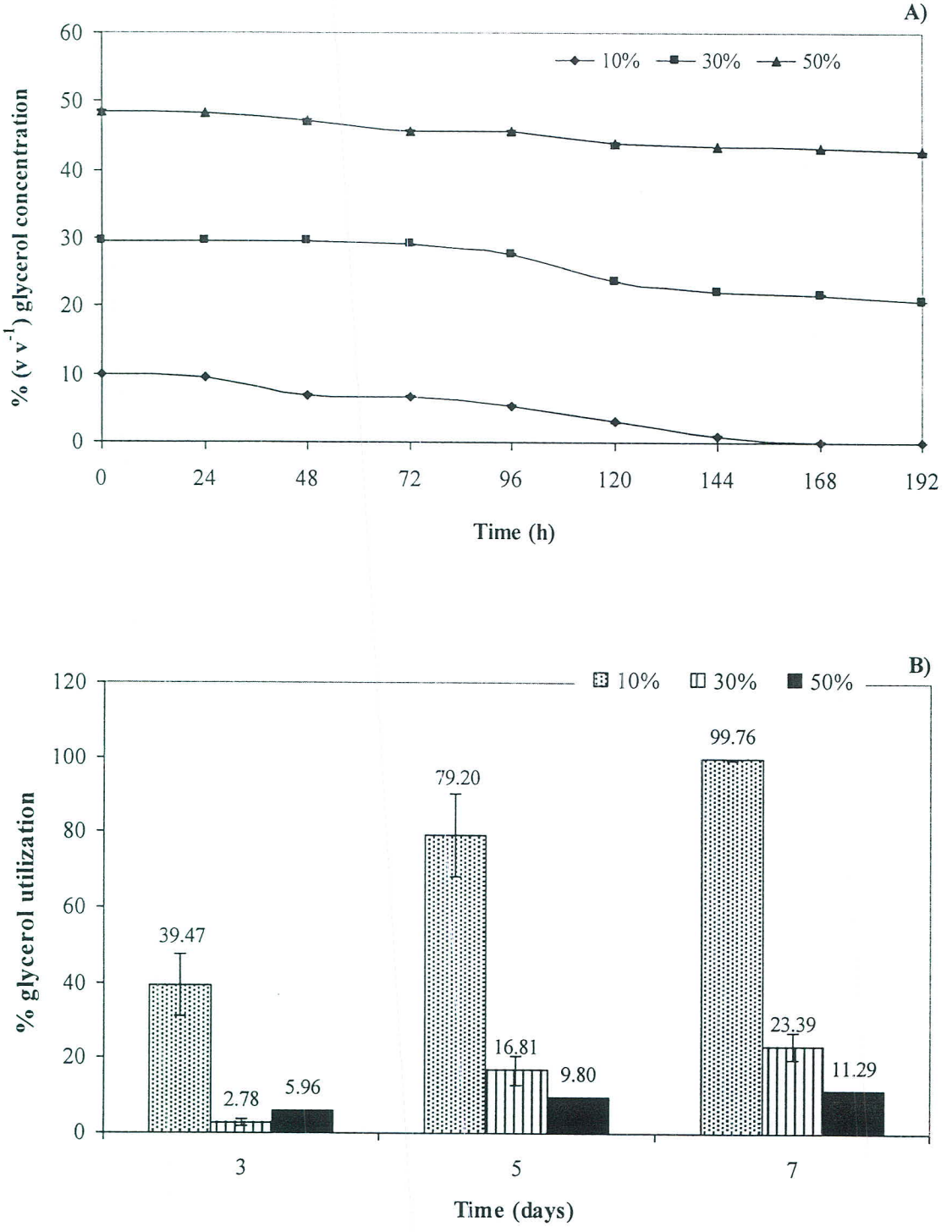

Figure 4.2 Glycerol concentrations versus times (A) and percentages of glycerol utilization (B) of mixed culture in 10, 30, and 50 (% v v^{-1}) glycerol concentration were cultivated during 168 h by incubation at 30°C using 1-L shaken flasks as a batch culture (n=3).

Approximate 39.47, 79.20and 99.76% of glycerol were utilized after 3, 5 and 7 days incubation period, respectively at the initial glycerol concentration of 10%, whereas

lower glycerol utilization appears at the initial glycerol concentrations of 30 and 50% (Figure 4.2). As the similarly direction of them, the percentage of glycerol utilization at 30%initial glycerol concentration within 3, 5 and 7 day periods were recorded as 2.78, 16.81 and 23.39% of glycerol utilization, while percentage of utilization as 5.96, 9.80 and 11.29 during 3, 5 and 7 days when grew on medium supplemented with 50% initial glycerol concentration as carbon source. Figure 4.1showed effect of used too high glycerol concentration that bacterial culture could not utilize and deplete until not only remaining in medium but also affecting on cell growth. Moreover, the results considered the effective condition of cell concentration (Figure 4.1) and substrate concentration found that at 10% initial glycerol concentration was the appropriate condition. These results corresponded with Ashby *et al.* (2005) suggested that high glycerol concentration caused an osmotic stress and subsequently low biomass yields (Ashby *et al.*, 2005). The result obviously suggested that it not only play a substrate role but also an inhibiting/toxic role at glycerol concentration above 10%. Therefore, at initial glycerol concentration of 10% v v^{-1} was used as the suitable substrate concentration for the next experiment.

4.1.2 PHB production

The PHA biosynthesized in the experiment was identified the PHA type as polyhydroxybutyrate (PHB). Figure 4.3 presented the PHB production in different glycerol concentrations. Result indicated that maximal PHB biopolyester accumulation for all cases was observed after 120 h incubation period, which was corresponding to the stationary growth phase (Figure 4.1). The production in their in 30% and 50% glycerol was 3.330 ± 0.012 and 0.724 ± 0.005 g PHB L^{-1}, respectively and increased to 3.953 ± 0.100 g PHB L^{-1} in 10% glycerol. While cultures showed the highest growth in the presence of control (Figure 4.1) but in case of production showed the least in both of PHB production 0.080 ± 0.000 g PHB L^{-1} (Figure 4.3) and percentage of PHB content 0.86 ± 0.01 (Figure 4.4). Thus, it's possibility of bacterial seeds which composed of PHA-bacterial strains (Table 4.2). The results could be explained by the bacteria culture growing on the medium without glycerol as the carbon source, but the remaining yeast extract and tryptone (Section 4.2.1), affecting on the bacteria could growth whereas the PHB production occurred in short period and gradually decreased until rarely disappear (Figure 4.3).

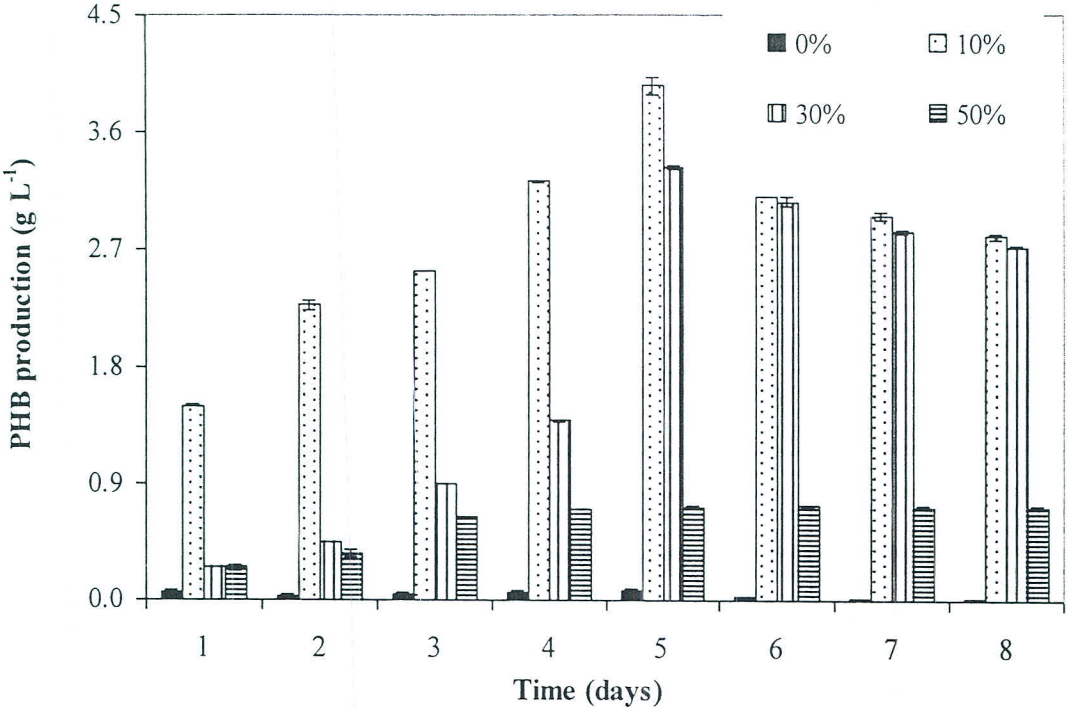

Figure 4.3 Effect of various glycerol concentrations on PHB production using 1-L shaken flasks as a batch culture. The cultivations were an agitation rate of 200 rpm, at 30°C for 8 days (n=4).

The highest percentage of PHB content and productivity were 50.09 ± 1.25% and 0.008 g L^{-1} h^{-1} (Table 4.1), respectively for 5 days cultivated on a medium supplemented with 10% v v^{-1} glycerol. 30% glycerol gave the highest PHB content (%) with productivity being 45.27 ± 0.33 % (Figure 4.4) and 0.007 g L^{-1} h^{-1}(Table 4.1), respectively.

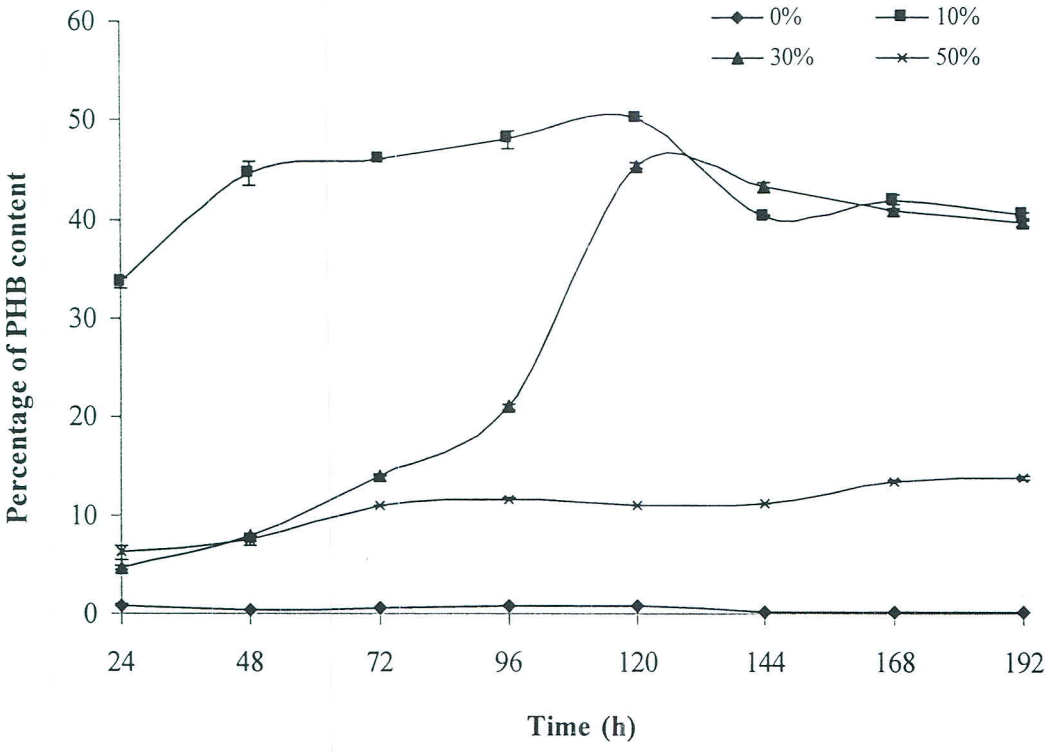

Figure 4.4 Percentage of PHB content was grown the different glycerol concentrations as carbon source during 192 h incubation at 30°C using shaken flasks at an agitation rate of 200 rpm as a batch culture (n = 4).

The maximum of PHB productivity and the PHB content (%) of cultures were found during 120 h (Figures 4.3 and 4.4) and dropped as 0.004 g L^{-1} h^{-1} and 40.30 ± 0.27% within 192 h. 10% v v^{-1} initial glycerol concentrations were utilized as 79.20 % of initial glycerol concentrations (Figure 4.2) after 120 h, which was possible to affect the time of PHB content with culture due to glycerol was also exhausted from the medium. The PHB accumulated usually when grown in the presence of excess carbon and/or under limitation of a nutrient such as O, N, P and S (Sudesh *et al.*, 2000; Kessler *et al.*, 2001). The results similarly that the decreasing of PHB productivity and the PHB content (%) of cultures since 120 h to 192 h were 0.007 to 0.004 g L^{-1} h^{-1} and 45.27 ± 0.33 to 39.52 ± 0.30 % (Figure 4.4), respectively when using 30% v v^{-1} initial glycerol concentrations due to this glycerol concentration above 10% was not the suitable concentration on PHB production (Figure 4.3).

Since the PHB had accumulated intracellularly as reserves of carbon and energy by a wide variety of bacteria (Anderson *et al.*, 1990) and then the large of cell dry weight should be correlated to the PHB contained in cells. On the contrary, both PHB production

and the PHB content (%) in medium without glycerol found the least amount (Figures 4.3 and 4.4) in spite of having obtained the greatest cell dried weight (Figure 4.1). From results, the possibility maybe occurred from qualified PHB determination. That mean possibly happened to inability of PHB determination method whereas quantitative PHA production method according by Hesselmann *et al.* (1999) reported that depolymerization was achieved by hydrolytic digestion. By the digestion of commercial PHB and PHB/PHV required 120 min before the recoveries were 100%, since complete dissolution of the particles required about 100 min. From mention, it could be claimed the effective of PHB determination. Even though, we tried to do this method in PHB and PHV standard which purchased from Sigma Chemicals Co., St Louis, MO, USA (Appendix B). The recovery of standards was more than 95% (Figures B-1 and B-2; Appendix B). Therefore, it could be used this method without loss PHB accumulation in cells. The less PHB products were observed in medium supplemented with either 30% or 50% throughout experiments (Figure 4.3). Moreover, the percentage of PHB contents of them was less than in others, especially 50% of initial glycerol concentration (Figure 4.4). From the foregoing results revealed the important of glycerol concentration that had been evolved to produce PHB. These results were in agreement with those of Mahishi *et al.* (2003) that reported that glycerol as a carbon source also supported maximal PHB accumulation by PHB up to 60% of cell dry weight accumulated in the recombinant *E. coli* cells after 30 h incubation time at 37°C.

Furthermore, the effect of various glycerol concentrations was analyzed by the agitated flask cultures (500-mL in 1-L Erlenmeyer flasks) in medium supplemented with glycerol (Section 3.1.2, Chapter 3). Results for culture cell dry weight, PHB products, percentage of PHB contents, PHB productivity and PHB yield were shown in Table 4.1 (Seeing the calculated in Appendix B-4). The cultivations were an agitation rate of 200 pm at 30°C for 120 h incubation period.

Table 4.1 Effect of different glycerol concentrations on biomass and PHB accumulation in shaken flasks as a batch culture and incubated at 30°C for 120 h. Means from four replicated determinations of a representative experiment was shown.

Glycerol condition ($g\ L^{-1}$)	CDW ($g\ L^{-1}$)	PHB production[a] ($g\ PHB\ L^{-1}$)	% PHB content[b]	Glycerol utilization ($g\ L^{-1}$)	PHB productivity ($g\ PHB\ L^{-1}\ h^{-1}$)	$Y_{PHB/S}$[c]
0 (Control)	9.38 ± 0.06	0.080 ± 0.000	0.86 ± 0.01	0.00 ± 0.00	0.0001	-
126.1 (10%)	7.89 ± 0.00	3.953 ± 0.100	50.09 ± 1.25	97.97 ± 13.71	0.0080	0.040
378.3 (30%)	7.36 ± 0.03	3.330 ± 0.012	45.27 ± 0.33	159.60 ± 49.61	0.0070	0.022
630.5 (50%)	6.64 ± 0.01	0.725 ± 0.005	10.91 ± 0.05	62.86 ± 3.89	0.0010	0.012

[a] PHB was determined by high pressure liquid chromatography

[b] The amount of PHB is given as an average weight percentage of the CDW.

[c] PHB yield ($Y_{PHB/S}$) was calculated on the basis of glycerol utilization.

- (no detection)

All case conditions found that bacterial cultures grew more, especially without glycerol (control) that represented to cell dried weight so supported the formula medium used in research. Nevertheless, aim of research respected to investigate PHB accumulation in cells then many parameters were PHB production, percentage of PHB content and PHB yield ($Y_{PHB/S}$) would be determined. In both 10% and 30% v v^{-1} glycerol the bacterial cultures grew more and contained more polymer. Cell and PHB concentrations in medium supplemented with 10% v v^{-1} glycerol obtained in 120 h were 7.891 ± 0.003 g L^{-1} and 3.953 ± 0.100 g PHB L^{-1}, respectively, resulting in a PHB productivity of 0.008 g PHB L^{-1} h^{-1}. According to 30% v v^{-1} glycerol, the PHB production and productivity produced were recorded being 3.330 ± 0.012 g PHB L^{-1} and 0.007 g PHB L^{-1} h^{-1}, respectively. In addition, accumulation independent-culture continued until, at 50% v v^{-1} glycerol, 6.639 ± 0.014 g L^{-1} CDW and 0.725 ± 0.005 g L^{-1} of PHB had accumulated, which corresponded to the PHB productivity of 0.001 g PHB L^{-1} h^{-1}. However, in case of control (without adding glycerol) found the highest CDW (9.378 ± 0.061 g L^{-1}) but PHB

production and PHB productivity were the least in theirs. By PHB accumulation in cells and PHB content was 0.080 ± 0.000 g PHB L^{-1} and 0.855 ± 0.011 %, respectively. The results were used as an explanation to explain the important of glycerol in fermentation.

Then, this observation raised the possibility that glycerol concentrations (more than 10% by volume) in PHB production might affect PHB accumulation indirectly, by negatively affecting cell growth and PHB contents. Furthermore, a parameter of PHB yield ($Y_{PHB/S}$) calculated on the ratio of PHB production and glycerol utilization (the calculation is shown in Appendix B). The highest PHB yield ($Y_{PHB/S}$ = 0.041) showed when grew on 10% v v^{-1}glycerol. On the contrary, the PHB yields decreased as 0.022 and 0.012 in 30 and 50 (% v v^{-1}) glycerol, respectively. These results suggested that PHB productivity and PHB content (%) can be increased by applying 10% v v^{-1} glycerol. After examining the effect of glycerol concentration in flask cultures was chosen as the best strategy since it allowed the greatest enhancement of PHB production. Therefore, the appropriated concentration was 10% glycerol (Figures 4.3, 4.4 and Table 4.1).

4.1.3 Microbial Community Dynamics

Because of bacterial seeds comprised of many bacteria in granule such as bacteria capable of converting glycerol to PHB and unrelated bacteria to PHB production. The mixed culture was changed bacterial structure within community, depending on surrounding into system i.e. substrate concentration and/or type, culture time, feast-famine regime etc. Bacterial culture was analyzed and identified for determining dominant and stability bacteria in each period. The specific objectives were to i) stability in the mixed culture within process setup, and ii) preliminarily characterize the extent of microbial diversity of the consortium within different conditions. So, the study was investigated bacterial culture from the sludge for example stability of bacteria community during on cultivation which they were determined using DGGE technique, and identified the bacterial species using 16S rDNA sequencing.

The functional performance and microbial community structure were analyzed in batch operated under variable conditions. In general, these systems relied on the highest PHB production within 5 days (Figure 4.3), with respected to the time dependent chemical conversions in a cycle so all conditions were analyzed during the fifth day's period. For the population analysis, samples were taken after the batch had stabilized for at least 20 days (Figure 4.5).The microbial population dynamics monitored by DGGE for total bacterial community profile was shown in Figures 4.5. There were shifts in total bacterial

communities' composition at different time intervals, and the shifts in community composition occurred in different patterns in various conditions (Figure 4.5).

DGGE analysis is used to evaluate the effects of glycerol concentration on microbial communities (Muyzer *et al.,* 1992 and Nakatsu, 2000). 200 bp fragment of 16s rDNA was amplified with 338GC_F and 518_R primers in PCR reaction. The PCR products were run in DGGE to investigate the bacterial community shift over 20 day period of each glycerol concentration, including 10, 30 and 50% v v^{-1}. The changes of bacterial populations were obvious in each concentration and every 5 incubation days for 20 days, while the change after 20 day cultivation of theirs was not significantly established (Figure 4.5). The results indicated that glycerol concentration influenced the growth of bacteria.

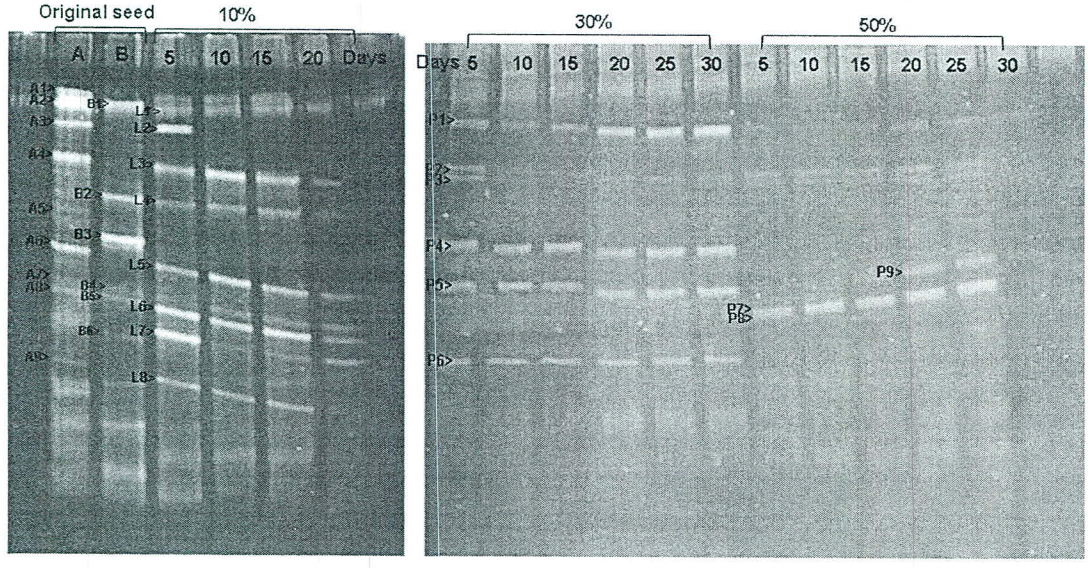

Figure 4.5 DGGE profiles of the amplified 16S rRNA gene fragments from original granule seeds (Lane A) while inoculated in a medium without glycerol for 5 days (Lane B). Mixed culture cultivated on broth using 10% v v^{-1} glycerol as carbon source during on 5, 10, 15 and 20 days (Left side). DGGE separation patterns of 16S rRNA genes amplified from bacterial cultures communities were grown on medium with 30 and 50% v v^{-1} glycerol concentrations as carbon source (Right side).

DGGE profiles of original granule seed for 0 and 5 days in medium without glycerol showed in Lanes A and B (Figure 4.5). Differences among bacteria communities in Lanes A and B illustrated shifting bacteria structure after cultivation in the medium. At 10, 30 and 50% v v^{-1} glycerol concentrations in bacterial cultures, DGGE profiles were

almost the same at all-time points; however the intensities of each dominant band were difference between each time point (Figure 4.5). Interestingly, DGGE profile of 10% v v^{-1} glycerol from the bacterial cultures showed the highest amount of dominant bands for 15 days (Figure 4.5). The dominant bands were represented and sequencing for identified bacteria strains (Table 4.3).

Although the cultivations were not freshly inoculated using the sludge, the microbial community shift still occurred within all conditions. Difference of original granule seed and after cultivated in each condition illustrated in Figure 4.5. Also Lane A showed a different community, interestingly already with a larger fraction of strain conversely after cultivation on medium without glycerol (Lane B) displayed a lower bacterial populations. Different of bacteria communities between Lane A and Lane B illustrated shifting bacteria structure after cultivation in medium. Likewise, adding glycerol as carbon source into medium found that bacteria patterns in Figure 4.5 were displayed significantly both the amount of dominant bacteria and stability of bacteria in each time. During the 5^{th} days in all conditions (Figure 4.5), the highest amount of bacteria isolates appeared at 10% glycerol whereas other concentrations less than especially 50% concentration.

The fifth day variations in each concentration were evaluated by comparison of DGGE profiles from similar time-points within the same individuals. To clarify, results were explained within similarly condition and compared with another condition under same time-points after that would conclude into perspective. Initially, the variable bacteria communities under cultivation on 10% glycerol region of 16S rDNA could be successfully amplified from all 8 samples in the first 5 days and started to change substantially in the next 5 days until to 20^{th} days. Stability of community changes in the microbial structure were observed after 10 days (disappearance of L2 in Figure 4.5). When comparing to the different presence of bands from original seed were obviously either existence or disappearance of some bands occurred at least such as disappearance of A5 after grew on 10% glycerol on 5 days. Even as, both of 30 and 50% glycerol found a losing of bands in line with by significantly. Disappearance of 2 bands inside 30% and a gradually happening on some bands after 15^{th} days in 50% glycerol could refer to adaptive bacteria in microbial structure although inappropriate to usability in the further study. These results supported favorable of microbial populations to medium supplemented with 10% glycerol as carbon source.

However, the stability or adaptability of this microbial community under variational surrounding was interesting as main issue because for many mixed microorganism could live and acclimatize as long as supporting or adding nutrient between cultivation, causing maintenance of mixed culture during on fermentation by feast and famine periods in other words sequencing batch reactors (SBR) were used to storing bacteria with a feast–famine regime (Reis *et al.*, 2003). Although, this study showed existing of mixed culture at least 20th days when adding substrate together with inoculum at the only first time.

4.1.4 Scaling up into 10-L reactor

The experiments in the 1-L shaken flasks found the suitable medium supplemented with 10% v v^{-1} glycerol according to Table 4.1.Subsequently, this condition was cultivated into the 10-L fermentor tank, according to Section 4.2.1. The analysis methods were used similarly in the 1-L reactor. In this study, the samples were kept on the fifth days for determination of cell concentrations, glycerol utilization, percentage of PHB contents and the microbial populations. The results showed in Table 4.2 which the PHB productivity and the PHB content (%) were 0.009 g PHB L^{-1} h^{-1} and 51.13 ± 1.70 %, respectively. Consequently, the cultures were analyzed with the DGGE method according to Chapter 3, section 3.3.5.2. The bacteria pattern was similarly in 1-L flasks (data not shown). The dominant bands from 1-L flasks were represented and sequencing for identified bacteria strains (Table 4.3).

Table 4.2 Effect of reactor size between 1-L reactor and after scaling up into 10-L reactor on biomass and PHB accumulation under cultivation on medium supplemented with 10% v v^{-1} glycerol concentration and incubated at 30°C for 120 h. Means from duplicated determinations of a representative experiment were shown.

Reactor capacities	CDW (g L^{-1})	PHB production[a] (g PHB L^{-1})	% PHB content[b]	Glycerol utilization (g L^{-1})	PHB productivity (g PHB L^{-1} h^{-1})	$Y_{PHB/S}$[c]
1-L	7.89 ± 0.00	3.95 ± 0.10	50.09 ± 1.25	97.97 ± 13.71	0.008	0.040
10-L	6.42 ± 0.10	3.28 ± 0.16	51.13 ± 1.70	112.08 ± 0.52	0.009	0.029

[a] PHB was determined by high pressure liquid chromatography

[b] The amount of PHB is given as an average weight percentage of the CDW.

[c] PHB yield ($Y_{PHB/S}$) was calculated on the basis of glycerol utilization.

Table 4.3 Nucleotide sequences of 16S rRNA gene fragments obtained from the DGGE bands of the amplified samples of bacterial communities from the original granule seeds and after cultivated in each condition

Band[a]	Partial 16S rDNA sequence			Putative division
	Accession number	Closest relative	Similarity (%)	
A1	GU003086	Uncultured bacterium	98	Activated sludge
A2	FJ968473	*Bacteroidales* bacterium	98	Wastewater treatment plan
A3	EU252503	*Dysgonomonas* sp.	88	-
A4	CU917641	*Bacteroidetes* bacterium	98	Mesophilic anaerobic digester
A5	GQ377472	Bacterium enrichment culture	93	Methanogenic phenol-degrading enrichment culture
A6	EU545403	*Citrobacter freundii*	98	-
A7	CU914837	Uncultured bacterium	96	Enriched seawater polluted by crude oil
A8	FJ238518	*Klebsiella* sp.	94	-
A9	GQ418065	Uncultured *Klebsiella* sp.	94	Biological degreasing systems
B1	GQ167196	Uncultured bacterium	98	Hydrogen fermentor
B2	GQ916639	*Bacillus* sp.	98	-
B3	GQ868433	Uncultured *Clostridium* sp.	98	-
B4	FJ837445	Uncultured bacterium	98	-
B5	GQ167179	Uncultured bacterium	98	Hydrogen fermentor
B6	GQ167182	Uncultured bacterium	98	Hydrogen fermentor

Table 4.3 Nucleotide sequences of 16S rRNA gene fragments obtained from the DGGE bands of the amplified samples of bacterial communities from the original granule seeds and after cultivated in each condition (CONT')

Band[a]	Partial 16S rDNA sequence			Putative division
	Accession number	Closest relative	Similarity (%)	
L1	GU003086	Uncultured bacterium clone	98	Activated sludge
L2	EU252503	*Dysgonomonas* sp.	98	-
L3	AY554420	*Bacteroidetes* bacterium	93	-
L4	GU011953	*Bacillus cereus*	98	-
L5	EU545403	*Citrobacter freundii*	98	-
L6	GQ898826	Uncultured bacterium	98	-
L7	FJ238518	*Klebsiella* sp.	94	-
L8	FJ876436	*Clostridium* sp.	98	Wastewater treatment plants
P1	GU003086	Uncultured bacterium clone	98	Activated sludge
P2	FJ968473	*Bacteroidales* bacterium	98	Wastewater treatment plants
P3	GQ916639	*Bacillus* sp.	98	-
P4	EU545403	*Citrobacter freundii*	98	-
P5	CU914837	Uncultured bacterium	96	Enriched seawater polluted by crude oil
P6	FJ876436	*Clostridium* sp.	98	Wastewater treatment plants
P7	CU914837	Uncultured bacterium	96	-
P8	FJ238518	*Klebsiella* sp.	94	-
P9	FJ609663	Bacterium enrichment culture	98	-

[a] DEEG bands correspond to band on DGGE profile in Figure 4.5

Among the original seeds and the three different concentrations trials, slight differences were seen in the changes of bacteria populations with time (Figure 4.5). Sequencing of the cutting bands revealed that the main bacteria in sludge belong to genus of *Bacillus*, *Bacteroides*, *Citrobacter*, *Clostridium*, *Dysgonomonas* and *Klebsiella* (Table 4.3). Interestingly, the one genera *Clostridium* sp. was only found in the 10 and 30% concentration in Line L8 and P6, respectively. Even though, the bacteria seed was cultivated on medium without glycerol on 5 days found that *Bacillus* sp. and uncultured *Clostridium* sp.

These results were in agreement with another study that reported the genera of *Bacillus*, *Citrobacter*, *Clostridium* and *Klebsiella*, corresponding to PHA production strains. One of dominant cultured bacteria indicative of microbial diversity in functional effluent treatment plants were *Citrobacter* strains (Rani *et al.*, 2008). Rehman *et al.* (2007) screened the different contaminated environments for polyhydroxyalkanoates-producing bacterial strains that one of PHA-producing strains showed resemblance to *Citrobacter*. Then, Misra *et al.* (2006) reported about many bacterial groups which could produce PHB and certain copolymers include *Clostridium*. Additionally, Zhang *et al.* (1994) described *Klebsiella* strains could produce PHB by using sugarcane molasses as carbon source. Ciesielski *et al.* (2010) studied the characterization of mixed population responsible for the conversion of crude glycerol into PHA by the cultivation-dependent and -independent methods. Molecular analysis revealed that mixed populations consisted of microorganisms affiliated with four bacterial lineages: alpha, gamma- *Proteobacteria*, *Actinobacteria* and *Bacteroides*. On the other hand, *Dysgonomonas* strain could not found in publish papers, correlated to PHA accumulation in cells at the present.

Although, unlike the fermentation study (Figure 4.5), no obvious PHB-producing isolates in the growth was noted in the batch composting process. This phenomenon was intriguing, although the explanation was not clear. Although the molecular analyses revealed the presence of tolerant species even during the periods of highest concentration (up to 50% glycerol), molecular approach did not give any information about the abundance of these species. Nevertheless, it could explain suitable condition and during time of optimum PHA production (Figure 4.4).

4.1.5 Identification of the biopolyesters

The structures of extracted biopolyesters were investigated by FTIR and ^{1}H-NMR analyses. Figure 4.6a represents the obtained ^{1}H NMR spectrum of the polyester in $CDCl_3$

solution. In ^{1}H NMR spectrum of the biopolyester, a double resonance at 1.274 ppm and a multiplet resonance at 2.520 ppm are corresponding to methyl protons ($-CH_3$) and methylene protons ($-CH_2$), respectively, while a multiplet resonance at 5.260 ppm indicates the methine proton (-CH) attached to the asymmetric carbon. The IR spectrum of the biopolyesters is shown in Figure 4.6b. There was a strong adsorption band at 1279 cm^{-1}, which is characteristic for ester bonding. Other adsorption bands at 1379, 1455, 2940, and 1653 cm^{-1} for CH_3, $-CH_2$, -CH, and C=O groups respectively are given in Figure 4.6. The extracted biopolyester was later identified as poly(3-hydroxybutyrate) when compared to the poly(3-hydroxybutyrate) by HPLC.

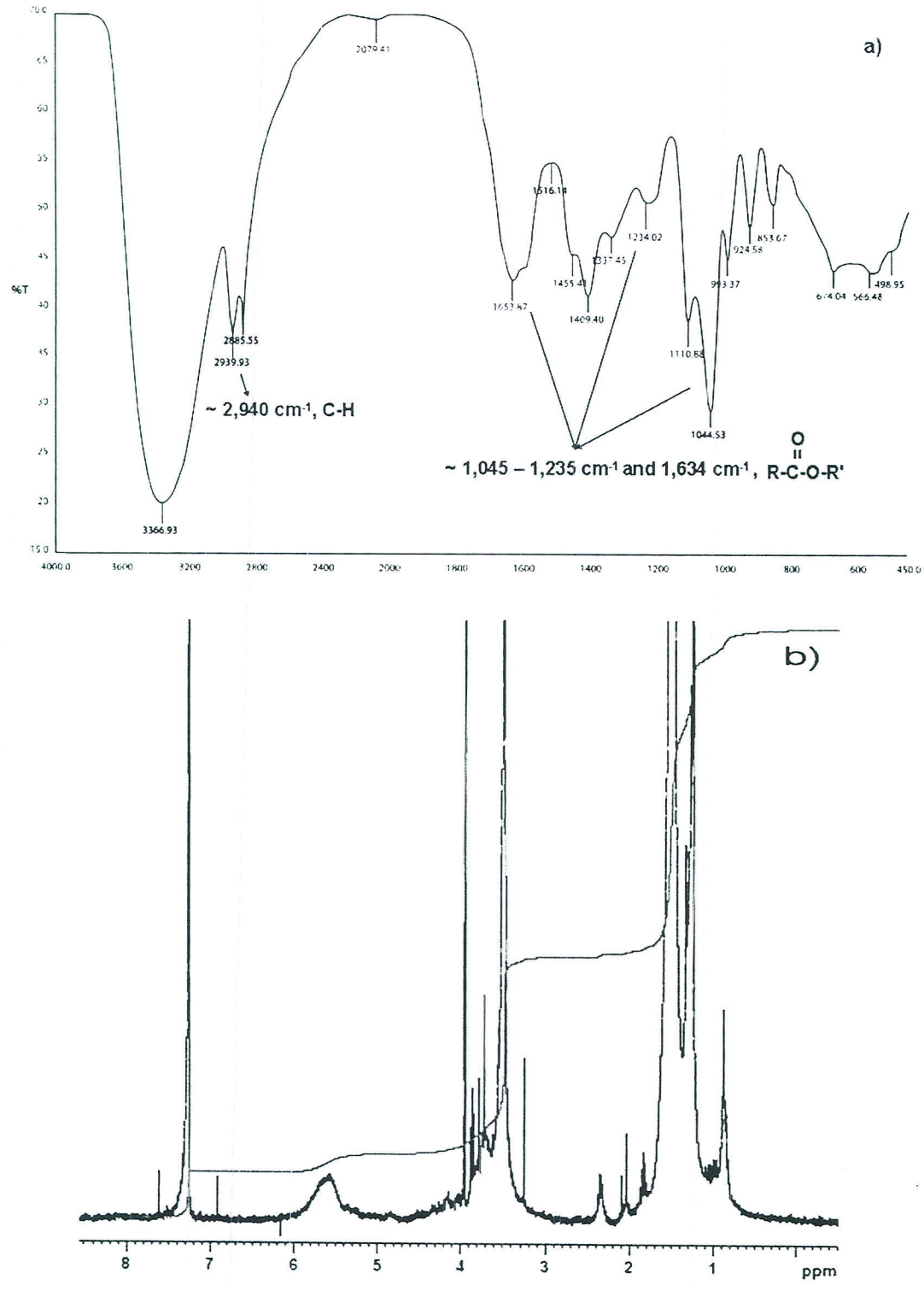

Figure 4.6 Fourier-transformed infrared (FTIR) spectra (a) and ^{1}H-NMR spectra (b) of partially purified PHA extracted from mixed culture when using 10% v v^{-1} glycerol concentration as C-sources and incubating for 72 h at 30˚C, 200 rpm on a rotary shaker.

4.2 Influence of Co-substrate and Nitrogen Source on Polyhydroxybutyrate Production from Glycerol

4.2.1 Influence of nitrogen sources on PHB production

The sole nitrogen source in medium usually used a complex nitrogen sources (Section 3.3.6) showed influence on the production of PHB (Figure 4.7). The maximum percentage of PHB contents in medium using $(NH_4)_2SO_4$ (*A*), yeast extract (*Y*) and tryptone (*T*) as the sole nitrogen source was 69.50 ± 2.29, 33.91 ± 0.57 and 52.73 ± 1.99 % at concentration 3, 1.2 and 0.6 g L^{-1}, respectively. These concentrations were similarly used in normal medium condition. Biosynthetic PHA are reported to occur in many bacteria for carbon and energy storage if a carbon source is provided in excess and if growth is impaired due to lack of an essential nutrient, especially when nitrogen is limited (Ramsay *et al.,* 1992; Suzuki *et al.,* 1986). It is known that ammonium salts are a good source of assimilable nitrogen for bacteria growth, fermentation media are often supplemented with inexpensive inorganic nitrogen form, such as ammonium sulphate (Beaulieu *et al.,* 1995). The presence of inorganic chemicals such as ammonia or ammonium salts as a source of nitrogen is an important requirement during the growth phase in order to maximize the concentration of biomass responsible for accumulation of PHB.

The presence of excess nitrogen had a negative effect on the maximum PHB content reached in batch experiments for PHB accumulation. The PHB productions lower the presence of sole nitrogen source. In the experiments with single ammonium addition, the rate of PHB production increased more than in the ammonium starvation experiment (Figure 4.7). Generally the trend from these experiments seems to be that ammonium addition leads to higher PHB productions and PHB contents than a shortage of ammonium (Beaulieu *et al.,* 1995), which is in agreement with our observations.

Our experiments show that the presence of excess nitrogen had a negative effect on the maximum PHB content reached in batch experiments for PHB accumulation. The PHB productions were lower the sole nitrogen source was present. In the experiments with single ammonium addition, the rate of PHB production increased more than in the ammonium starvation experiment (Figure 4.7). Generally the trend from these experiments seems to be that ammonium addition leads to higher PHB productions and PHB contents than a shortage of ammonium (Beaulieu *et al.,* 1995), which is in agreement with our observations.

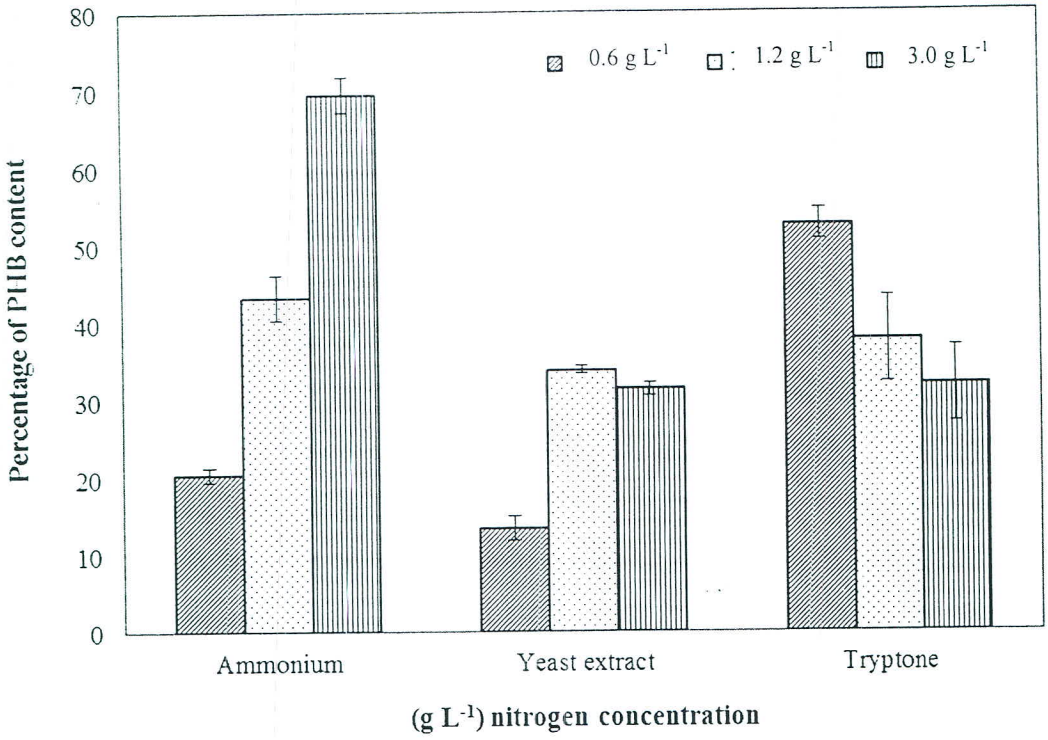

Figure 4.7 Effect of nitrogen sources at different concentrations on PHB content (%) for 120 h at 30°C, 200 rpm in the shaken flasks (n=2).

4.2.2 Effect of various glucose concentrations supplementation on PHB content

A set of experiments was designed to investigate the effects of glucose concentrations on cell growth (Figure 4.8) and PHB production in batch cultures of mixed culture using 10 % v v^{-1} glycerol and $(NH_4)_2SO_4$ as carbon and nitrogen sources, respectively.

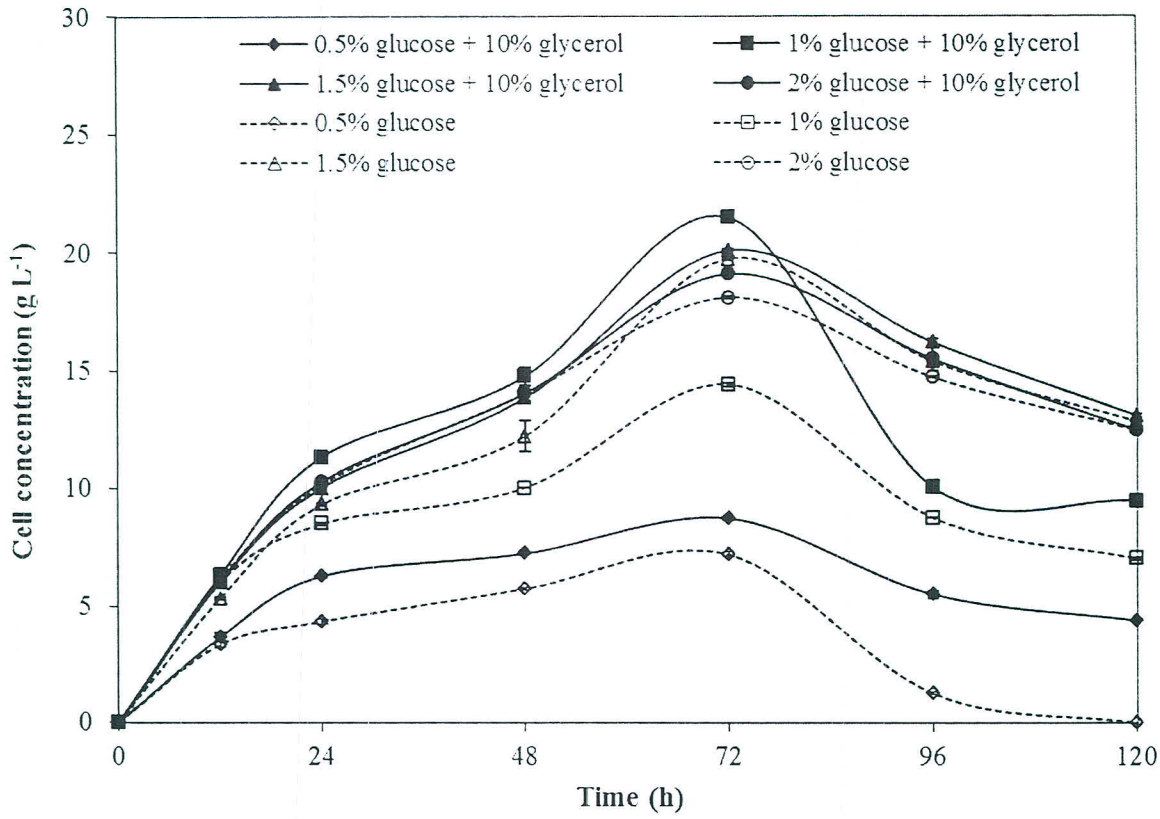

Figure 4.8 Biomass in the culture media containing 10% v v^{-1} glycerol supplemented with (% w v^{-1}) various glucose concentrations (n=2).

Figure 4.8 showed the highest cell concentration in the medium supplemented with 1 % w v^{-1} glucose as 21.48 ± 0.13 g L^{-1}, while it was 14.40 ± 0.09 g L^{-1} when cultivated in the same glucose concentration without glycerol at 72 h. In case more adding 1 % w v^{-1} glucose, the cell concentrations were gradually dropped 20.08 ± 0.05 and 19.08 ± 0.01 g L^{-1} when adding 1.5 and 2.0 w v^{-1} glucose, respectively at 72 h. The cell concentrations increased along with culture time until 72 h and then gradually dropped to 120 h (Figure 4.7). The results were considerable in medium using glycerol by comparing with glucose. The total concentrations were examined by without glycerol. Nevertheless, the results showed the cells drop below the control that the glycerol contained in medium, depending on each glucose concentration (Figure 4.9).

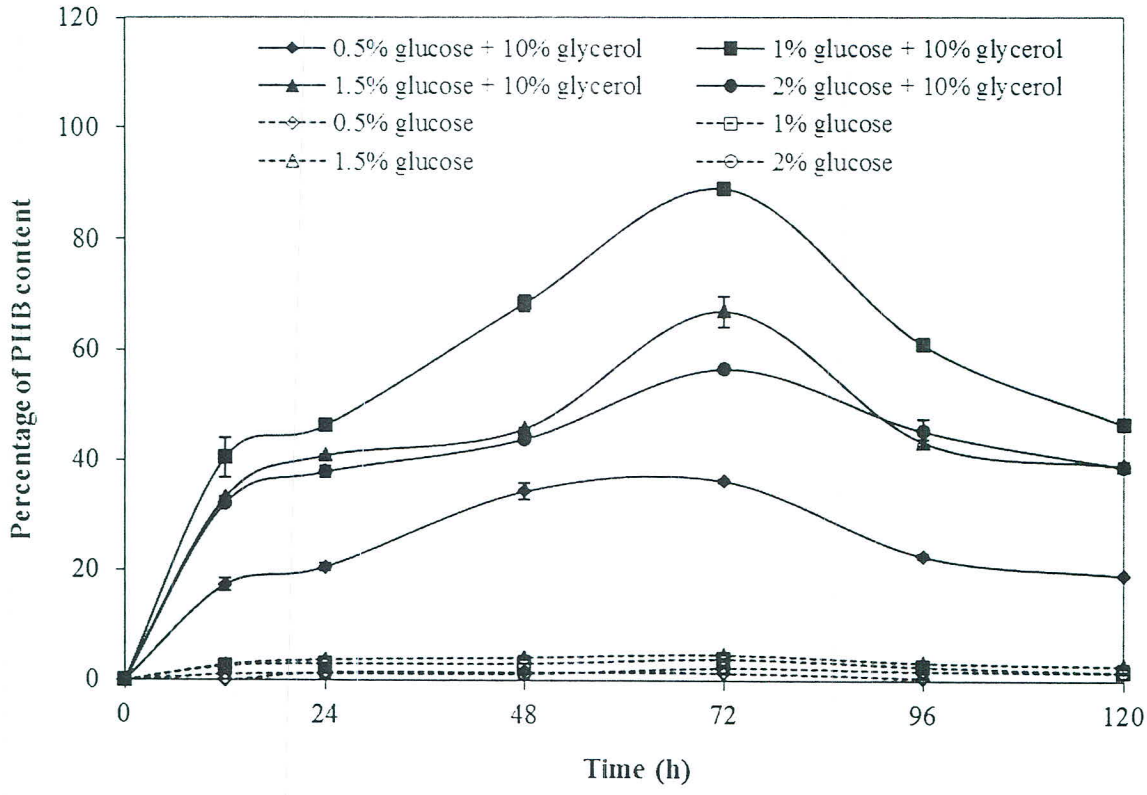

Figure 4.9 Percentage of PHB contents in the culture media containing 10 % v v^{-1} glycerol supplemented with (% w v^{-1}) various glucose concentrations (n=2).

Results showed that glycerol supplemented with all glucose concentrations supported the PHB contents (Figure 4.9). The maximum percentage of PHB content was 89.01 ± 0.13 % when cultivated on medium supplemented with 1 % w v^{-1} glucose for 72 h. The PHB content of 1 % w v^{-1} glucose without glycerol was 3.93 ± 0.03%after 72 h. This can be explained the efficiency of using glucose for promoting PHB production and using a shorter time by however, it did not substitute for glycerol because glycerol is not only a cheaper substrate, but also has higher PHB contents (Figure 4.9).

Some researchers reported that glucose could support growth but without PHA any being produced (Ramsay *et al.,* 1992). Similar observations were found by other scientists. Belfars *et al.* (1995) reported that glucose and ammonium ions were inhibitory at certain levels, which affect the specific growth rate and PHB production. Inhibition by ammonium ions Heinzle and Lafferty (1980) and substrate inhibition by carbon source (Lee *et al.*, 1991) on PHB production have been reported. Moreover, the effect of the high glucose concentration (more than 1 % w v^{-1} glucose), shown in Figure 4.9, showed that there was 89.03 ± 0.13 % PHB produced by using 1 % w v^{-1} glucose as co-substrate, while less than

66.97 ± 2.73 % PHB was produced when glucose was in the range of 1.5 to 2.0 % w v^{-1}. This could be due to the carbon source (glucose) limitation which was also favorable for the PHB accumulation in microorganisms. Shang *et al.* (2003) reported that limitation of carbon source may cause a slow – down in the tricarboxylic acid cycle, and a decrease in the free coenzyme – A concentration, which will result in a rapid accumulation of PHB in cells. These suggestions could refer to capable of mixed culture to biosynthesis PHB products from glycerol. However, the key of substrate of this study was glycerol which adding glucose supported as co-substrate.

The results illustrated the 10 % v v^{-1} glycerol accompanied by 1 % w v^{-1} glucose concentration as carbon source for producing PHB from cultures. Shang *et al.* (2003) found that the highest PHB productivity was obtained with glucose at 0.9 % w v^{-1} glucose. One of the researches conducted by Hori *et al.* (2002), *Bacillus megaterium* PHB content in the cell reached a maximum level after growth with glucose. Moreover, numerous studies carried out on the feeding of glucose (Kim *et al.*, 1994; Ryu *et al.*, 1999), glucose and an organic acid (Choiand Lee 1999; Kim *et al.*, 1994; Shang *et al.*, 2003), and an organic acid or fatty acid to achieve a high cell density and high productivity. After testing several glucose feeding strategies, Kim *et al.* (1994) pointed out that the maintenance of glucose from 1.0 to 2.5% w v^{-1} glucose was significantly for high productivities.

The added glucose was the co-substrate, which probably is not a good solution; however, the results indicated many advantages from using glucose, including increased cell production and PHB accumulated in cells or reduced incubation time. The results found that the usability of substrates (both glycerol and glucose) could save energy expenses (72 h) and the high PHB production. Interestingly, the adding glucose in media obviously shorten the PHB production time (from 5 to 3 days). Properties of glucose being monosaccharide were readily utilized by bacteria, hence their growth and subsequent production of PHA was higher. Table 4.4 compared to percentage of PHB contents and PHB productivity between 72 and 120 h in each condition.

Table 4.4 Effect of different glucose concentrations which were supplemented in mineral medium containing 3 g L^{-1} $(NH_4)_2SO_4$ as nitrogen source on biomass and PHB accumulation in shaken flasks as the batch culture and incubated at 30°C for 72 and 120 h, respectively. Means from duplicated determinations of a representative experiment was shown.

Condition	CDW ($g\ L^{-1}$)	PHB production[a] ($g\ L^{-1}$)	Residual CDW[b] ($g\ L^{-1}$)	% PHB content[c]	PHB productivity ($g\ L^{-1}\ h^{-1}$)
72 h (% w v^{-1} glucose)					
0.5 glucose + glycerol	8.74 ± 0.05	3.17 ± 0.05	5.57 ± 0.00	36.29 ± 0.33	0.008
0.5 glucose	7.22 ± 0.10	0.10 ± 0.01	7.12 ± 0.11	1.42 ± 0.12	0.000
1.0 glucose + glycerol	21.48 ± 0.13	19.12 ± 0.08	2.36 ± 0.04	89.01 ± 0.13	0.112
1.0 glucose	14.40 ± 0.09	0.57 ± 0.01	13.84 ± 0.08	3.93 ± 0.03	0.001
1.5 glucose + glycerol	20.08 ± 0.05	13.45 ± 0.58	6.63 ± 0.53	66.97 ± 2.73	0.028
1.5 glucose	19.72 ± 0.08	0.93 ± 0.01	18.78 ± 0.07	4.74 ± 0.04	0.001
2.0 glucose + glycerol	19.08 ± 0.01	10.80 ± 0.06	8.28 ± 0.06	56.59 ± 0.32	0.018
2.0 glucose	18.07 ± 0.07	0.43 ± 0.01	17.65 ± 0.06	2.37 ± 0.04	0.000
120 h (% w v^{-1} glucose)					
0.5 glucose + glycerol	4.38 ± 0.05	0.84 ± 0.00	3.55 ± 0.05	19.13 ± 0.12	0.002
0.5 glucose	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.000
1.0 glucose + glycerol	9.49 ± 0.20	4.42 ± 0.07	5.07 ± 0.13	46.56 ± 0.26	0.007
1.0 glucose	7.04 ± 0.05	0.12 ± 0.00	6.93 ± 0.05	1.65 ± 0.02	0.000
1.5 glucose + glycerol	13.08 ± 0.07	5.11 ± 0.07	7.97 ± 0.14	39.04 ± 0.77	0.005
1.5 glucose	12.83 ± 0.03	0.36 ± 0.03	12.48 ± 0.00	2.79 ± 0.25	0.000
2.0 glucose + glycerol	12.46 ± 0.01	4.84 ± 0.06	7.62 ± 0.05	38.86 ± 0.42	0.005
2.0 glucose	12.46 ± 0.04	0.20 ± 0.00	12.26 ± 0.04	1.63 ± 0.03	0.000

[a] PHB was determined by high pressure liquid chromatography

[b] Residual CDW was calculated by subtracting the weight of PHB.

[c] The amount of PHB is given as an average weight percentage of the CDW.

Glycerol was used as 10% v v^{-1} or 126.1 gL^{-1} or 1.37 M

Table 4.4 shows different PHB productivity during on 72 h and 120 h when combined glycerol and glucose, while using glucose as the carbon source found that the

PHB productivity in each concentration was below. It was possible to use the suitable glucose concentration which it was monosaccharide and readily utilized by bacteria, hence their growth and PHB accumulated in cells was rapidly. The optimized condition was 1 % w v^{-1} glucose and 10 % v v^{-1} glycerol and verified with the highest PHB productivity of 0.112 g PHB L^{-1} h^{-1} after 72 h (the calculation was shown in Appendix B). The Table 4.4 showed the highest of PHB content and PHB productivity was 89.01 ± 0.13 % of dry cell weight and 0.112 g PHB L^{-1} h^{-1}, respectively under cultivation on medium supplementation with 10 % v v^{-1} glycerol and 1 % w v^{-1} glucose as co-substrate and $(NH_4)_2SO_4$ in 1-L the shaken flasks. Then, this condition was selected and applied into 10-L fermenter tank (Figure 3.3, Chapter 3) under a simple process likely the batch system according to section 3.3.6.2. The PHB productions were represented effective of PHB accumulated in cells i.e. PHB content (%), PHB productivity and cell concentration.

4.2.3 Optimal PHB production in 10-L reactor

In this study, the 10-L reactor was studied under cultivation conditions similar to the 1-L flasks. In addition, the bacterial cultures from the reactor could be rapidly accumulated PHB in cells within 72 h as shown in Table 4.5.

Table 4.5 Effects of different reactor capacities of between 1-L and 10-L on biomass and PHB accumulation at 30°C during on 72 h and 120 h (n=2).

Reactors	CDW (g L^{-1})	PHB production[a] (g PHB L^{-1})	Residual CDW[b] (g L^{-1})	% PHB content[c]	PHB productivity (g PHB L^{-1} h^{-1})
at 72 h					
1-L	21.48 ± 0.13	19.12 ± 0.08	2.36 ± 0.04	89.01 ± 0.13	0.112
10-L	20.61 ± 0.29	17.10 ± 0.01	3.50 ± 0.30	83.01 ± 1.20	0.068
at 120 h					
1-L	9.49 ± 0.20	4.42 ± 0.07	5.07 ± 0.13	46.56 ± 0.26	0.007
10-L	9.95 ± 0.33	3.57 ± 0.05	6.38 ± 0.29	35.89 ± 0.73	0.005

[a] PHB was determined by high pressure liquid chromatography

[b]Residual CDW was calculated by subtracting the weight of PHB.

[c]The amount of PHB is given as an average weight percentage of the CDW.

Table 4.5 displays the decrease in PHB productivity although the optimum time when compared to the flasks. At 72 h of 10-L reactor, the PHB productivity was 0.068 g PHB L^{-1} h^{-1}, and then the percentage of PHB contents were approximately 83.01 ± 1.20 %,

whereas the PHB productivity and the percentage of PHB contents in 1-L were 0.112 g PHB L^{-1} h^{-1} and 89.01 ± 0.13 %, respectively.

Furthermore, the bacteria community structures cultivated at the optimized conditions in the 10-L fermentor tank for 0, 3 and 5 days, comparing with the bacteria populations were inoculated on prior modified medium were shown in Figure 4.9.

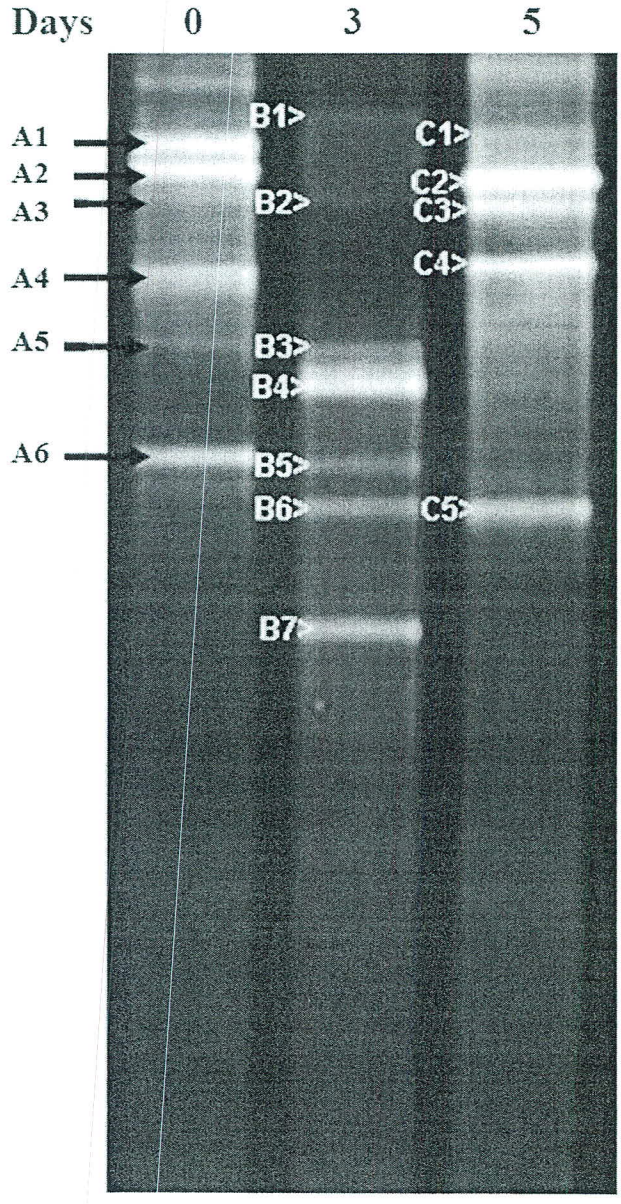

Figure 4.10 The DGGE analysis of bacterial 16S rRNA gene PCR products amplified from the mixed microbial communities. The mixed cultures cultivated in glycerol and glucose as co-carbon sources and ammonium sulphate as the sole nitrogen source within 10-L reactor. Lane numbers refer to time series during 0, 3 and 5 days, respectively.

The analysis of the PCR-DGGE sequencing indicates that the microbial community of the culture was significantly different during the time of process on PHB accumulation in the 10-L reactor. The isolation of DNA was amplified with GC-338_F and 518_R primers in PCR reaction as described in Method 3.3.5.Figure 4.10 illustrated changes of bacterial community pattern at different of cultivation time of 10-L reactor that the bacterial populations represented by the changes in relative abundance of DNA bands from the 0-3rddays to the 3rd and 5th days. The DGGE profiles were almost the same at all time points; however the intensities of each dominant band were difference between each time point (Figure 4.10), for example; the similar bacteria were fragment A4 and C4, fragment A6 and B5 or fragment B6 and C5. Then, the dominant bands in each were sequenced (Table 4.6). Fragment A4 and C4 which are the dominant bands, showed high sequence similarity to *Bacteroidetes bacterium* (98%). Fragment A6 and B5 were matched closely to *Citrobacter freundii* (98%). Fragment B6 and C5 corresponded to *Klebsiella* sp. (97%).

Table 4.6 Nucleotide sequences of 16S rRNA gene fragments obtained from the DGGE bands of the amplified samples of bacterial populations in 10-L bioreactor, cultivating on the mineral medium supplementation with 10 % v v^{-1} glycerol and 1% w v^{-1} glucose as substrates and 3 g L^{-1} ammonium sulphate as nitrogen sources during 0, 3 and 5 days, respectively

Band[a]	Partial 16S rDNA sequence			Related field
	Accession number	Closest relative	Similarity (%)	
A1	GU003086	Uncultured bacterium	98	Activated sludge
A2	FJ968473	*Bacteroidetes* bacterium	98	Wastewater treatment plan
A4	CU917641	*Bacteroidetes* bacterium	98	Mesophilic anaerobic digester
A6	EU545403	*Citrobacter freundii*	98	-
B4	GQ916639	*Bacillus* sp.	98	-
B5	EU545403	*Citrobacter freundii*	98	-
B6	EF031070	*Klebsiella* sp.	97	-
B7	FJ876436	*Clostridium* sp.	98	Wastewater treatment plants
C2	CU917641	*Bacteroidetes* bacterium	98	Mesophilic anaerobic digester
C3	EU252503	*Dysgonomonas* sp.	88	-
C4	CU917641	*Bacteroidetes* bacterium	98	Mesophilic anaerobic digester
C5	EF031070	*Klebsiella* sp.	97	-

[a] DEEG bands correspond to band on DGGE profile in Figure 4.9

Table 4.6 show that the main bacterial communities belonging to genus of *Bacillus*, *Bacteroidetes*, *Citrobacter*, *Clostridium*, *Dysgonomonas* and *Klebsiella* which their bacteria were found in the shaken flasks (Table 4.3). From Table 4.5 showed the highest PHA content of 83.01 ± 1.20 % in 10-L bioreactor for 72 h and then according to Table 4.6 found the dominant bands were affiliated to *Bacillus* sp., *Citrobacter freundii*, *Klebsiella*

sp. and *Clostridium* sp. which related to PHA-producing strains from previous reports. The PHB was first identified and isolated from the genus *Bacillus* (Lemoigne, 1926). Then, Macrae and Wilkinson (1958), reported that PHB served as an intracellular carbon and energy reserve in *Bacillus cereus* and *Bacillus megaterium*. Slepecky and Law (1969) further determined the role of the polymer in sporulation in *B. megaterium*. Then, Rehman *et al.*(2007) screened the different contaminated environments for polyhydroxyalkanoates-producing bacterial strains that one of PHA-producing strains showed resemblance to *Citrobacter*. Also, Zhang *et al.* (1994) described *Klebsiella* strains could produce PHB by using sugarcane molasses as carbon source. Although, reports of *Clostridium* sp of PHB productions are still rare (Valappil *et al.*, 2007).

4.3 Determining Microbial Dynamics of Polyhydroxyalkanoates – Producing Consortium in Waste Glycerol using RISA Technique

4.3.1 Dynamics of microbial community

Numerous discrete RISA bands, resulting from differences between the intergenic spacer region lengths of different bacterial species, were apparent. Each band represented at a unique ribotype (i.e. RNA complement of a cell by analogy with phenotype or genotype). Three different band patterns regarding the operation time were observed. The results suggested that microbial compositions in the community were changed with respect to the cultivation time (Figure 4.11).

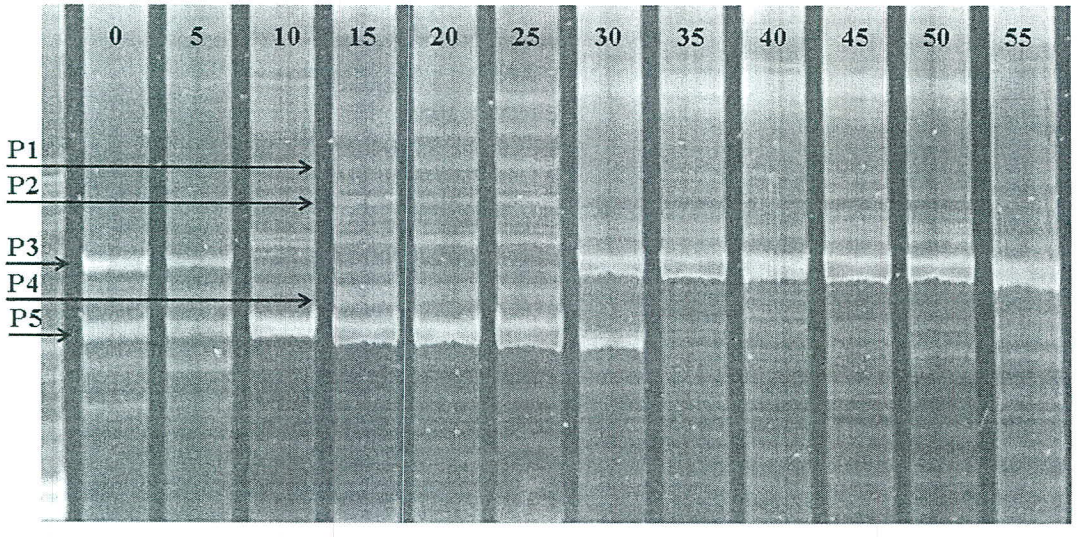

Figure 4.11 The RISA band patterns of the microbial samples collected from the reactor every 5 days

The original seeds exhibited rich varieties of bacterial species, with members represented by the dominant bands. The band pattern was changed after 5 days cultivation. Major changes in the microbial structure were observed after 10–day cultivation, related to the maximal percentage content of PHA and reached 47.6% of cell dry weight according to previous study (Ciesielski *et al.*, 2010). After 25-day cultivation, less RISA bands were observed. The RISA pattern was converted to a binary matrix, using presence-absence data and checked with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis with bootstrap confidence testing (1,000 resembling) for validation results, which interpreted values of distance between samples. The similarity among the isolates was estimated by UPGMA indices. The program illustrated cluster of samples that confirmed the trend of progressive community transition in the reactor (Figure 4.12).

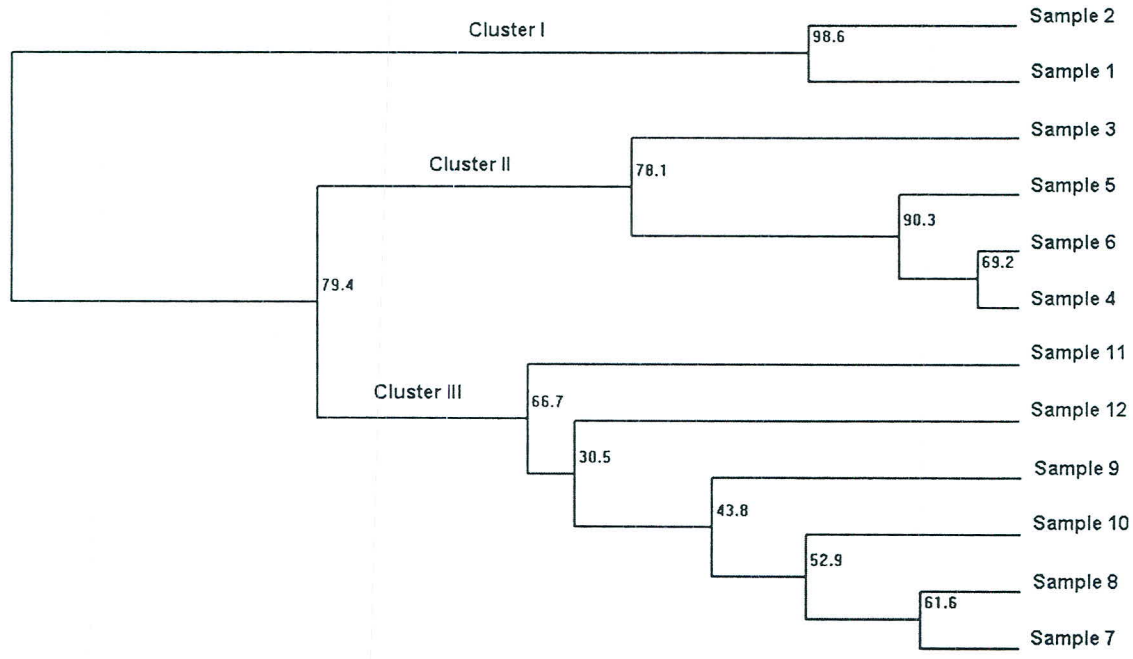

Figure 4.12 Phylogenetic tree generated by the neighbor-joining method showing the phylogenetic relationships among bacteria isolated from biomass utilizing the glycerol

Result of phylogenetic analysis based on the 16S rDNA sequences from evolutionary distances by neighbor-joining was shown in Figure 4.12.The closeness of bacteria community in each sample with respect to the cultivation time could be separated into two groups, including Group I was samples in range of 0 – 5 incubation days (Cluster I) whereas Group II comprised of two clusters being Cluster II and Cluster III. The Cluster

II represented cultivation bacteria culture between 10^{th} – 25^{th} days. The Cluster III was noticed in the last period of fermentation (between the 30^{th} and the 55^{th} days).

4.3.2 Identification of polyhydroxyalkanoate-producing bacteria

Dominant RISA bands, P1, P2, P3, P4 and P5 (Figure 4.11) were cut and performed 16S rRNA gene sequencing. The sequences showed similarities greater than 91% with sequences obtained from the NCBI GenBank database (Table 4.7). Two sequences were closely related to *Bacillus* sp. (P4 and P5), *Azoarcus* sp. (P1), *Flavobacterium columnare* (P2) and *Thauere* sp. (P3). The fingerprints produced by RISA have been shown to be highly reproducible and sufficient to classify both closely and distantly related species and subspecies. *Bacillus* strains vividly appeared during 0-30 day cultivation but high intensity during 10 – 30 day cultivation, in which the highest percentage content of PHA was observed, *Bacillus* sp. was previously reported its capability of PHB accumulation (Molnar *et al.*, 2008; Tajima *et al.*, 2003; Valappil *et al.*, 2007). *Thaurea* (P3), *Azoarcus* sp. (P1) and *Flavobacterium columnare* (P2) were not directly associated with the PHB accumulation, but were cultivable in the experimental conditions (Ciesielski *et al.*, 2009).

Table 4.7 Comparative analysis of nucleotide sequences of intergenic spacer region fragments obtained from the RISA bands

Phylotype	Closest relative	Accession number	% identity
P 1	*Azoarcus* sp. BH72	AM406670	91%
	Thauera sp. MZ1T	CP001281	85%
	Azoarcus aromaticum	CR555306	83%
P 2	*Flavobacterium columnare*	GU080023	100%
	Flavobacterium columnare	GU080021	100%
	Flavobacterium columnare	GU080022	100%
P 3	*Thauera* sp. MZ1T	CP001281	91%
	Uncultured bacterium clone 70105	AY484727	91%
	Azoarcus sp. BH72	AM406670	89%
P 4	*Bacillus cereus* strain HY-4	EU915688	99%
	Bacillus cereus strain HY-1	EU915686	99%
	Bacillus cereus biovar anthracis str. CI	CP001746	99%
P 5	*Bacillus pseudofirmus* OF4	CP001878	100%