

# รายงานวิจัยฉบับสมบูรณ์

## Final Research Report

Isolation of Polyhydroxyalkanoates Producing Bacteria and its  
Production Conditions Using Crude Glycerol from a Biodiesel Plant  
as a Substrate

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โครงการวิจัยนี้ได้รับทุนสนับสนุนจาก เงินงบประมาณแผ่นดิน

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Isolation of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) producing bacteria and its production conditions using crude glycerol from a biodiesel plant as a substrate

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**Kamontam Umsakul**

## บทคัดย่อ

การศึกษานี้สามารถคัดแยกแบคทีเรียแกรมลบ ที่ผลิตพอลิไฮดรอกซีอัลคาโนเอต *Pseudomonas mendocina* PSU (PM-PSU) ได้จากสิ่งแวดล้อมโดยใช้ของเหลือใช้จากไบโอดีเซลเป็น แหล่งคาร์บอนเดี่ยว นับเป็นการศึกษาครั้งแรกที่สามารถผลิตพอลิไฮดรอกซีอัลคาโนเอตจากของเสีย จากไบโอดีเซลโดยใช้ไอโซเลต PM-PSU แบคทีเรียชนิดนี้ถูกจัดอยู่ในกลุ่มที่มีการเจริญสัมพันธ์กับการ สะสมพอลิไฮดรอกซีอัลคาโนเอต เนื่องจากการสะสมพอลิเมอร์เกิดขึ้นพร้อมกับการเจริญ ของเหลือใช้ จากไบโอดีเซลช่วยส่งเสริมการเจริญ และถูกเปลี่ยนไปเป็นพอลิไฮดรอกซีอัลคาโนเอตได้อย่างมี ประสิทธิภาพที่ความเข้มข้นของกลีเซอรอลในของเหลือใช้จากไบโอดีเซลเท่ากับ 2 เปอร์เซ็นต์ปริมาตร โดยปริมาตรหรือ 20 กรัมต่อลิตร โดย PM-PSU เจริญได้น้ำหนักเซลล์แห้งสูงสุดเท่ากับ 3.65 กรัมต่อลิตร และผลิตพอลิไฮดรอกซีอัลคาโนเอตได้ถึง 76.89 เปอร์เซ็นต์โดยน้ำหนักเซลล์แห้งด้วยอัตราการผลิต เท่ากับ 0.072 กรัมต่อลิตรต่อชั่วโมง ความเข้มข้นที่เพิ่มขึ้นของกลีเซอรอลในของเหลือใช้จากไบโอดีเซล ที่มากกว่า 3 เปอร์เซ็นต์ปริมาตรโดยปริมาตรหรือ 30 กรัมต่อลิตรมีผลเชิงลบต่อการเจริญและการสะสม พอลิเมอร์ โดยให้น้ำหนักเซลล์แห้งและปริมาณพอลิเมอร์สูงสุดที่ผลิตเพียงแค่ 2.90 กรัมต่อลิตรของ น้ำหนักเซลล์แห้งและ 64.77 เปอร์เซ็นต์โดยน้ำหนักเซลล์แห้งของพอลิไฮดรอกซีอัลคาโนเอตผลิตได้จาก PM-PSU เมื่อเจริญใน 3 เปอร์เซ็นต์ปริมาตรโดยปริมาตรหรือ 30 กรัมต่อลิตรของกลีเซอรอลในของเหลือ ใช้จากไบโอดีเซล สำหรับการศึกษาล้างในโตรเจนที่เหมาะสมและภาวะที่เหมาะสมสำหรับการเลี้ยง เชื้อเพื่อให้ได้น้ำหนักเซลล์แห้งและปริมาณพอลิเมอร์สูงสุด พบว่า แอมโมเนียมซัลเฟตเป็นแหล่ง ในโตรเจนที่ดีที่สุด เป็นที่น่าสนใจว่า PM-PSU สามารถผลิตเอ็กโซพอลิแซ็กคาไรด์ในกลุ่มแอลจินेटที่ มีน้ำหนักโมเลกุลโดยเฉลี่ยประมาณ 665,000 ดาลตัน พร้อมกับการสะสมพอลิไฮดรอกซีอัลคาโนเอต และได้ปริมาณสูงสุดประมาณ 2.15 กรัมต่อลิตรเมื่อสิ้นสุดการเลี้ยง การศึกษายีนสำหรับสังเคราะห์พอลิไฮดรอกซีอัลคาโนเอตด้วยพีซีอาร์โดยใช้ไพรเมอร์ที่จำเพาะ พบว่า PM-PSU มียีนสำหรับการ สังเคราะห์ พอลิไฮดรอกซีอัลคาโนเอตสายสั้น ( $phbC_p$ ) และสายกลาง ( $phaC_p$ ) และสามารถผลิตมอนอ เมอร์สามชนิด ได้แก่ 3-ไฮดรอกซีบิวทิเรต 3-ไฮดรอกซีออกตาโนเอต และ 3-ไฮดรอกซีเดคานอโนเอต จากการวิเคราะห์ด้วยวิธีแก๊สโครมาโตกราฟีและแมสสเปกโตรเมตรี โปรตอนนิวเคลียร์แมกเนติก- เรโซแนนซ์และอัลตราเซนตริฟิวเจชัน เป็นที่น่าสนใจว่ายีนสำหรับสังเคราะห์พอลิ-3-ไฮดรอกซีบิว ทิเรต ไม่เคยมีรายงานมาก่อนใน สปีชีส์ *Pseudomonas mendocina* ดังนั้น การศึกษาครั้งนี้ นับเป็นรายงาน แรกที่พบการสะสมพอลิ-3-ไฮดรอกซีบิวทิเรตในสปีชีส์นี้ พอลิไฮดรอกซีอัลคาโนเอตที่ได้จากการศึกษา นี้ อาจจะสามารถประยุกต์ใช้ได้กับงานทางชีวการแพทย์ เช่น ตัวนำส่งยาเนื่องจากอัตราการย่อยสลายช้า กว่าเมื่อเทียบกับพอลิไฮดรอกซีอัลคาโนเอตสายยาว

**คำสำคัญ:** พอลิไฮดรอกซีอัลคาโนเอต พอลิ-3-ไฮดรอกซีบิวทิเรต ของเหลือใช้จากไบโอดีเซล

*Pseudomonas mendocina*

## ABSTRACT

A Gram negative PHA-producing bacterium *Pseudomonas mendocina* PSU (PM-PSU) was isolated from environmental sources using a biodiesel liquid waste (BLW), by-product from biodiesel production, as the sole carbon source. This is the first study to produce PHA from BLW by *P. mendocina* PSU isolate. *P. mendocina* PSU was classified as growth-associated PHA-producing bacteria because the accumulation of PHA and growth occurred simultaneously. The BLW efficiently supported cell growth and it was readily converted to PHA using 2.0 % (v/v) or 20 g/L of total glycerol in the BLW. The PM-PSU accumulated up to 3.65 g/L of DCW and 76.89%DCW of PHA with a productivity of 0.072 g/L/h. Further increases of the glycerol concentration in the BLW to above 3.0 % (v/v) or 30 g/L had an adverse effect on cell growth and PHA accumulation i.e. only 2.9 g/L of DCW and 64.77%DCW of PHA content were observed in PM-PSU when grown with 3.0 % (v/v) or 30 g/L of glycerol in the BLW. To achieve the highest biomass and PHA content in PM-PSU, the most suitable nitrogen source and optimal cultivation conditions were investigated. It was also of interest that, an exopolysaccharide, alginate (average molecular weight of  $6.65 \times 10^5$ Da) was produced from PM-PSU together with the accumulation of PHA and reached to about 2.15 g/L at the end of cultivation.

PCR amplification for the PHA synthase genes using specific primers was performed and found that two PHA synthase genes e.g. *phbC<sub>PS</sub>* encoding for short chain length (SCL)-PHA and *phaC<sub>PS</sub>* encoding for medium chain length (MCL)-PHA were amplified from PM-PSU and three PHA monomers e.g. 3-hydroxybutyrate (3HB), 3-hydroxyoctanoate (3HO), and 3-hydroxydecanoate (3HD) were detected by gas chromatography-mass spectrometry (GC-MS), proton nuclear magnetic resonance (<sup>1</sup>H-NMR) and ultracentrifugation analysis. It was again of interest that *phbC<sub>PS</sub>* had not been previously reported in a *Pseudomonas mendocina* spp. so this is the first report for identification of P(3HB) accumulation by this species. The physical and mechanical properties of the PHA produced including their molecular weight were also determined. It is possible that the produced PHA in this present study are promising for use in some biomedical applications such as drug carriers due to their lower rate of degradation in comparison to longer chain PHA.

**Key words:** Polyhydroxyalkanoates, poly-3-hydroxybutyrate, biodiesel liquid waste, *Bacillus aryabhatai* ST1C, *Pseudomonas mendocina*

### **Aims of this study:**

1. To screen for the microorganisms that are able to produce high amount of P(3HB-co-3HV) using the crude glycerol from the bio-diesel production plant.
2. To investigate the optimal conditions for the production of 3-hydroxybutyrate (3HB), 3 hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD)
3. To investigate PHA synthase and its substrate specificity

The main focus of this study has been on the possibility of using the crude glycerol as a substrate for the microbial production of polyhydroxyalkonates or PHAs, a natural fully biodegradable polymer with many potential uses as alternatives to petroleum derived polymers. The main by-product of the biodiesel production is crude glycerol. The crude glycerol used in this study had an average composition of glycerol (43.89 %), methanol (15.00 %), biodiesel (21.90 %), free fatty acids (14.40 %) and others (4.81 %) (the data obtained from the biodiesel production pilot plant, PSU) and the initial pH was roughly 9.0-9.5. One solution to reduce the PHA production costs is to find a new inexpensive carbon sources. This crude glycerol is a good candidate because of its low price. One liter of crude glycerol is only 5 baht and at the same time the waste disposal problems from excess crude glycerol would be minimized.

The glycerol content and fatty acid components of crude glycerol that can be used for PHA production were analyzed. According to the Thai Industrial Standards Institute (TISI) 336-2538 and AOCS Official Method Ca 5a-40 methods, the total glycerol content and the free fatty acid in crude glycerol are 43.15 % wt and 15.65 % wt, respectively. In addition, the fatty acid composition in crude glycerol was examined by GC analysis and it was found that there are three main fatty acid component e.g. palmitic acid, steric acid and oleic acid that accounted for about 93% of the total fatty acids .

Another reason why the crude glycerol is a good substrate for PHA production is because of its high carbon and nitrogen ratio that is a crucial criterion for PHA biosynthesis. When crude glycerol was analyzed by a CHNS-O analyzer (CHNS-O analyzer, CE Instruments Flash EA 1112 Series, Thermo Quest, Italy) the carbon and nitrogen content averages in the crude glycerol were 43.65 % and 0.15 %, respectively; therefore, the C/N ratio in the raw crude glycerol is about 290.

From the crude glycerol composition analysis above, it can be assumed that the glycerol and free fatty acid are the major carbon sources for microbial growth and PHA production.

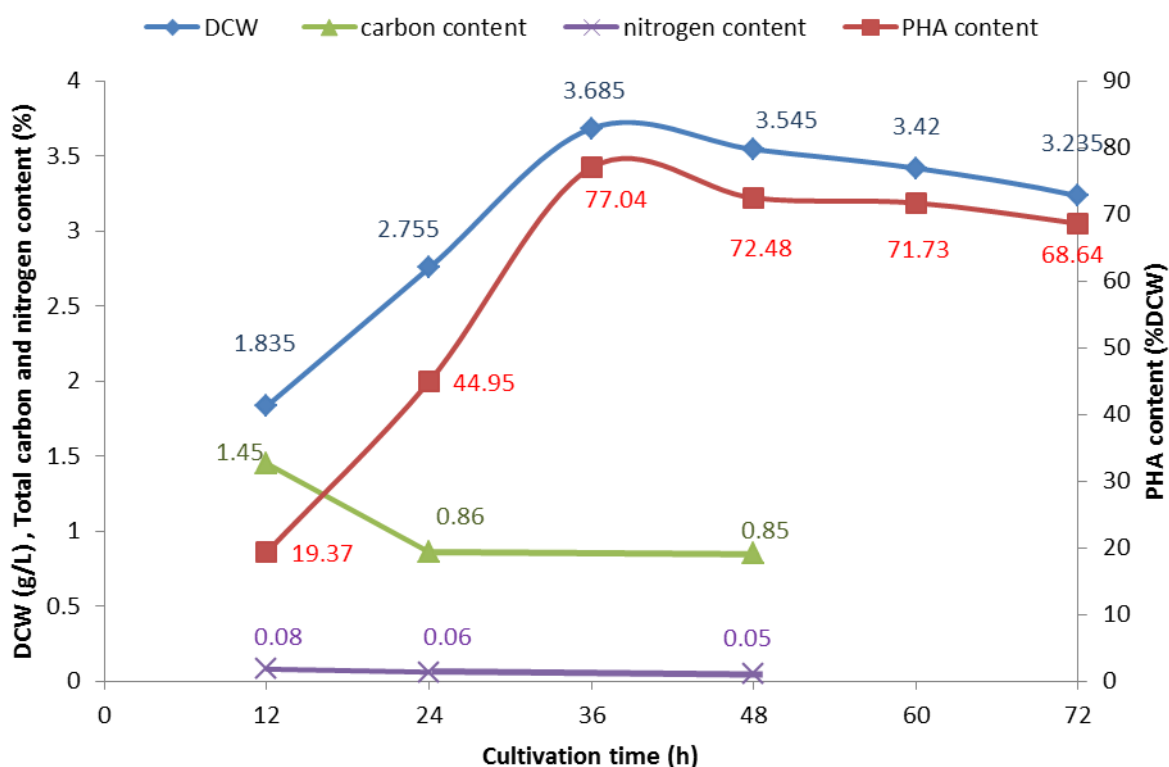
## **1. Microorganism**

The *Pseudomonas* sp. PSU was isolated from biodiesel contaminated wastewater. The microbial identification of this *Pseudomonas* sp. PSU based on biochemical characteristics was carried out using a biochemical test kit (Vitek 2 compact, bioMérieux VITEK®). The new isolated *Pseudomonas* sp. PSU can be identified as *Pseudomonas putida* with 98% probability. A full length 16S rDNA sequence of 1,378 bps was obtained by PCR. The BLASTX analysis revealed a 98.69% identity to the sequence of the 16S rRNA gene of *Pseudomonas mendocina* LMG 1223T (accession no.Z76664). The isolate was identified as a strain of *Pseudomonas mendocina* and then was deposited in GenBank under the code name *Pseudomonas mendocina* PSU (KC414580).

## **2. PHA production by *Pseudomonas* sp. PSU using crude glycerol**

The pre-grown inoculum was transferred to MSM containing 2.0% total glycerol in crude glycerol as a sole carbon source and 1.0 g/L of ammonium sulfate as a nitrogen source and then incubated at 37 °C with 200 rpm shaking for 72 h.

As shown in Figure 1, *Pseudomonas* sp. PSU produces PHA up to 77 % DCW with a 3.6 g/L of biomass concentration at 36 h of cultivation and PHA productivity of 0.20 g/L/h when cultivated in MSM containing 2.0% total glycerol in crude glycerol as a sole carbon source.

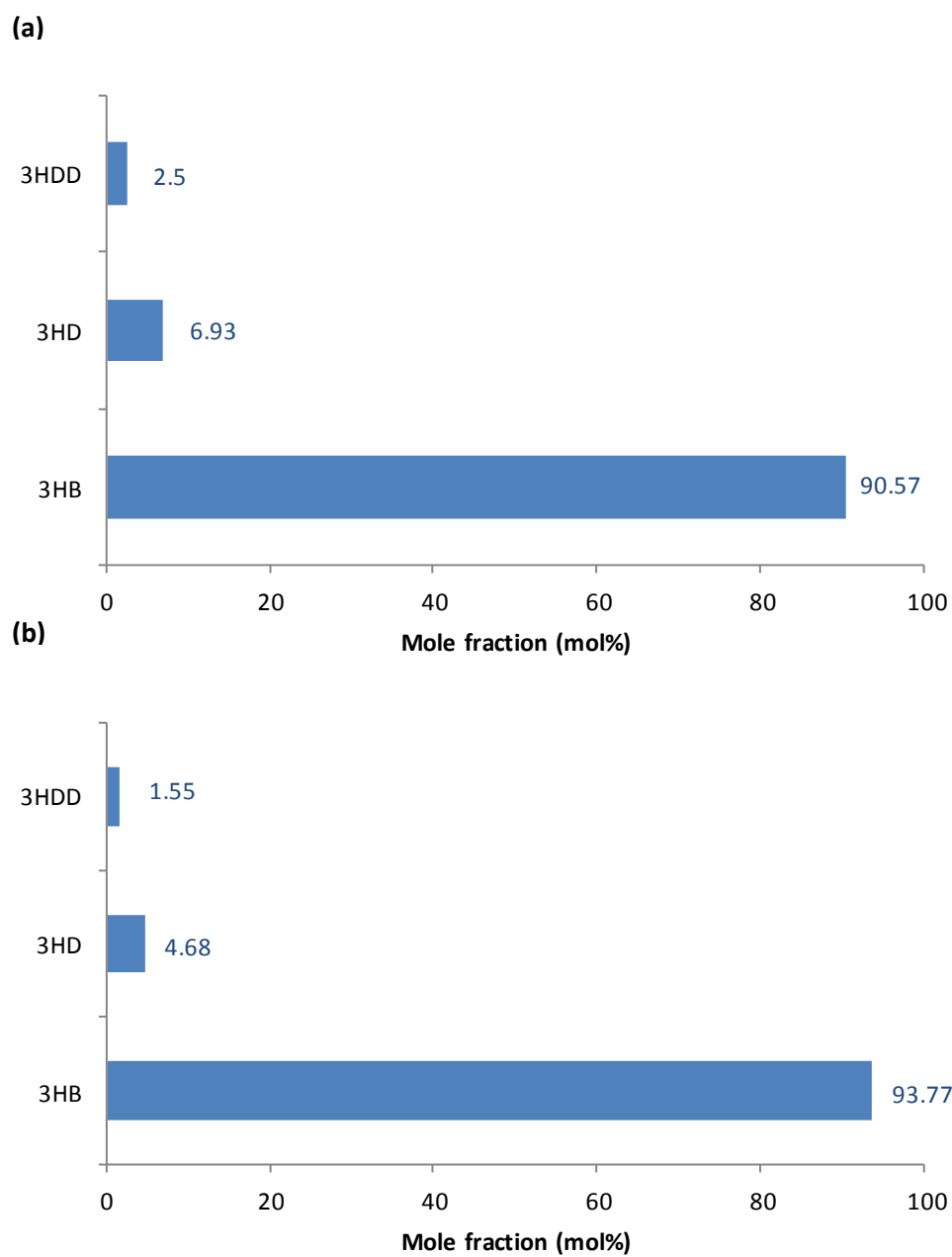


**Figure 1** Cell growth, PHA content and substrate utilization in *Pseudomonas* sp. PSU when cultivated in MSM containing 2.0% total glycerol in crude glycerol as a sole carbon source and 1.0 g/L ammonium sulfate as a nitrogen source for 72 h.

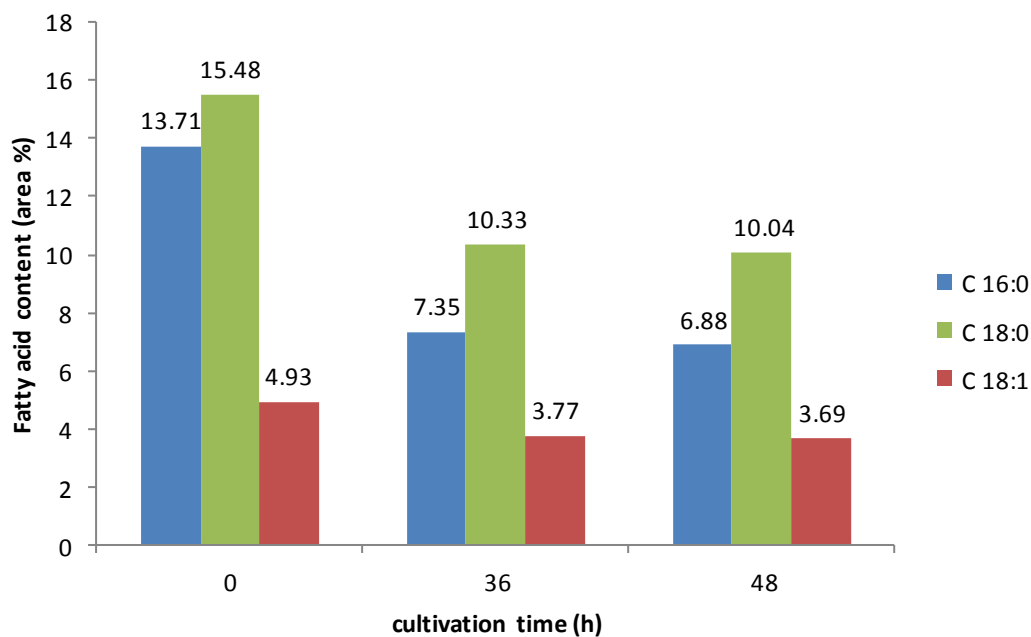
*Pseudomonas* sp. PSU was cultivated in MSM containing 2.0% total glycerol in crude glycerol as a sole carbon source, it can produce 3HB as a major component and 3HD and 3HDD as a minor component in PHA (Figure 2). It was of interest that when the incubation time increased from 36 h to 48 h, the mole fraction of 3HB tends to gradually increase whereas the mole fraction of 3HD and 3HDD tended to decrease. Therefore, it was of interest to determine if and when the 2 major carbon components i.e. glycerol and the fatty acids were being consumed.

To determine the glycerol and fatty acid utilization capability of *Pseudomonas* sp. PSU at 48 h, GC-MS analysis was carried out. Although the three main fatty acids e.g. palmitic acid, stearic acid and oleic acid were used by this strain and they were still present at 6.88%, 10.04% and 3.69%, respectively whereas the glycerol content decreased from 44.27 % to 0.88 % at 48 h (Figure 3 and 4).





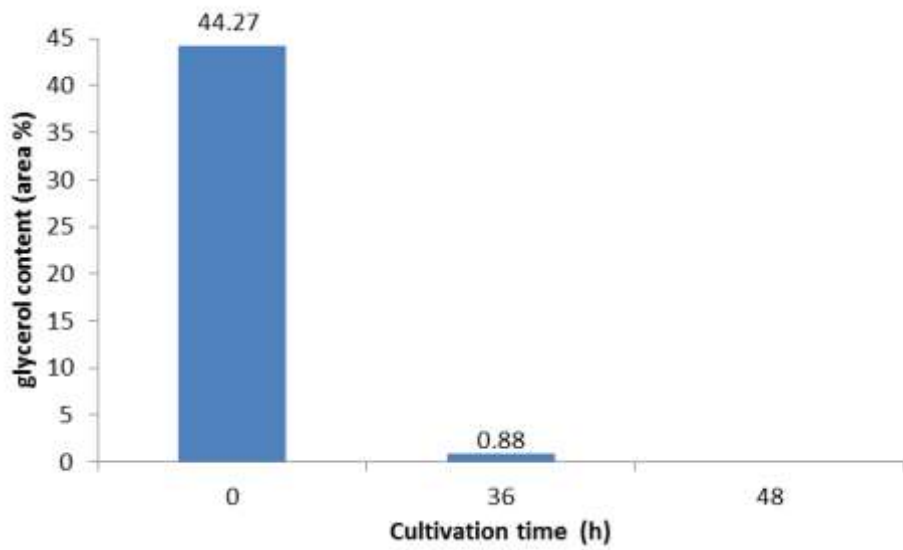
**Figure 2** Mole fractions of the monomer composition of PHA produced from *Pseudomonas* sp. PSU when cultivated in 2.0% total glycerol in crude glycerol as a sole carbon source for 36 h (a) and 48 h (b)



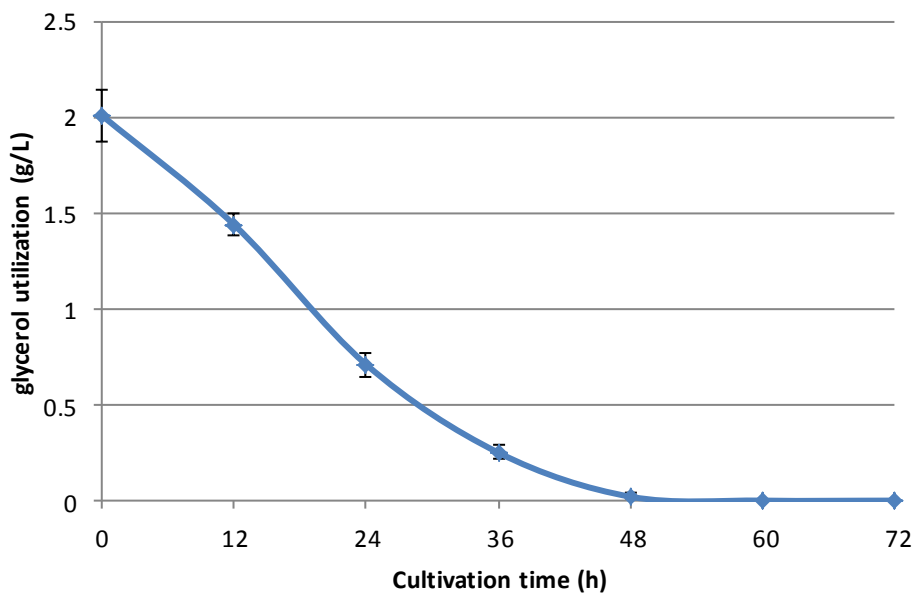
**Figure 3** Fatty acid content e.g. palmitic acid, stearic acid and oleic acid in crude glycerol after cultivation at 0, 36 and 48 h obtained by GC-MS analysis.

Glycerol was therefore the preferred substrate for growth of *Pseudomonas* sp. PSU and this was probably the reason for 90 mol% of the PHA was 3HB (Figure 2) with only a small amount of 3HD and 3HDD being produced. It may be that these small amounts were produced directly from the utilized fatty acids.

(a)



(b)

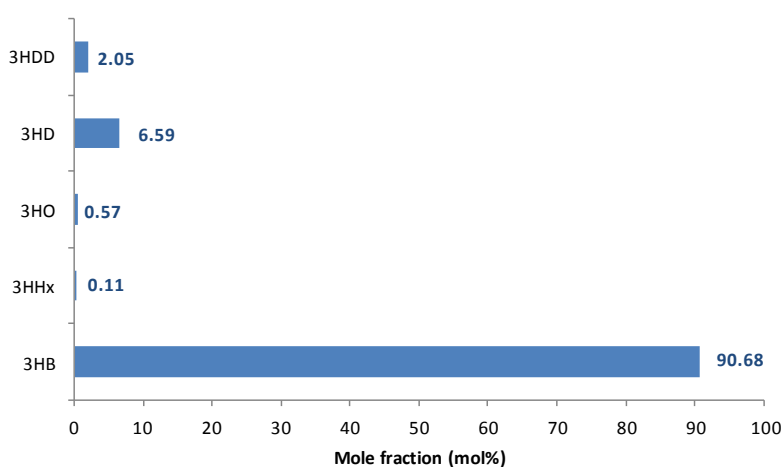


**Figure 4** Glycerol content in crude glycerol after cultivation at 0, 36 and 48 h obtained by GC-MS analysis (a) glycerol utilization profile using chemical method (b) (Bondioli and Della Bella, 2005)

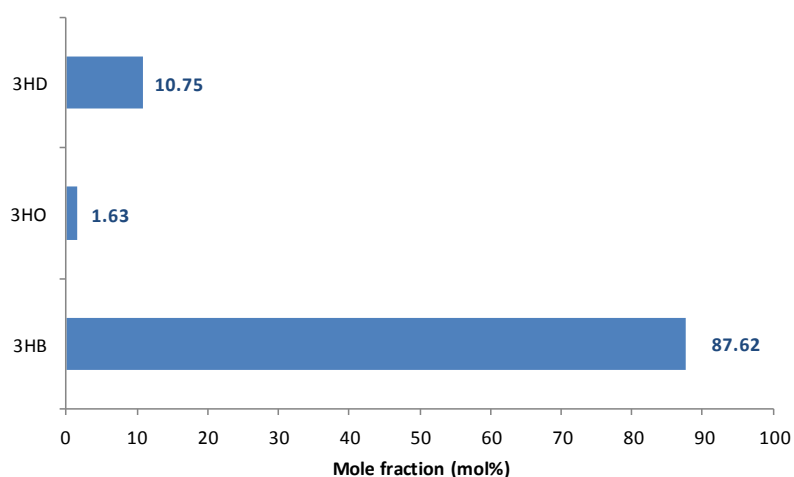
### 3. PHA production by *Pseudomonas* sp. PSU using mixed carbon source

The PHA production was then investigated using a mixed carbon source because it had been shown that the fatty acid composition of the PHA produced by other species of *Pseudomonas* was dependent on the types of substrates in the growth medium. However, the aim of this study is to use an inexpensive carbon source, so the crude glycerol was used as a main carbon source and then the fatty acid e.g. octanoic acid and decanoic acid were separately added to the crude glycerol to make a total concentration in the culture medium at 20 g/L.

(a)



(b)

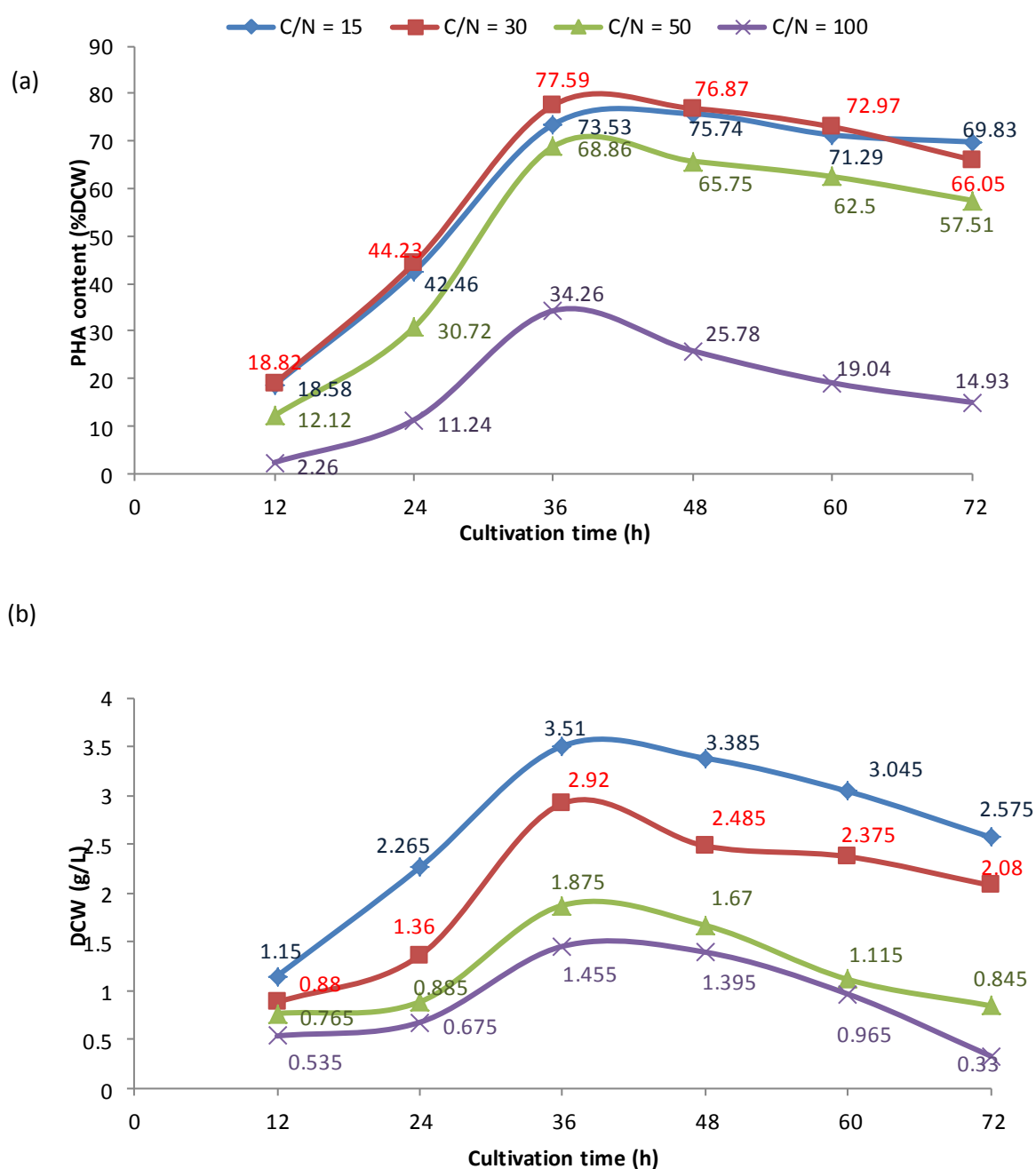


**Figure 5** Mole fraction of monomer composition in PHA produced from *Pseudomonas* sp. PSU when cultivated in a mixed carbon source of 1.5% of crude glycerol and 5.0 g/L of octanoic acid (a) or decanoic acid (b) at 48 h.

Once again the major component of the produced PHA was 3HB (90.68 mol%), but there was little change in the amount of 3HD (6.59 mol%) and 3HDD (2.05 mol%) when cultivated in 1.5% of crude glycerol and 5.0 g/L of octanoic acid (Compare Figure 2a and 5a). Whereas when it was cultivated in 1.5% of crude glycerol and 5.0 g/L of decanoic acid, the PHAs produced consisted of 3HB (87.62 mol%), increased 3HD (10.75 mol%) and 3HO (1.63 mol%) (Figure 5b). Therefore, for *Pseudomonas* sp. PSU, the presence of longer chain fatty acids as co-substrates with glycerol for growth had little effect on the HA composition but there was a higher reduction in cell growth and PHA production with a mixed carbon sources than in crude glycerol alone. This may due to the reduced amount of glycerol.

#### **4. Carbon to Nitrogen ratio**

The suitable carbon to nitrogen ratio (C/N) was examined by varying the C/N ratio from 15 to 100. As showed in Figure 6, the maximum PHA content of 77.59 % DCW (PHA productivity of 0.23 g/L/h) was obtained when *Pseudomonas* sp. PSU was cultivated in a C/N of 30 at 36 h of cultivation. On the other hand, at a C/N of 15 the highest DCW of 3.51 g/L was received.



**Figure 6** PHA production (a) and cell growth (b) and in *Pseudomonas* sp. PSU when cultivated in crude glycerol with various C/N from 15 to 100 for 72 h.

However, the C/N ratio had a strong effect on the cell growth because the maximum cell growth was obtained at C/N of 15 whereas the PHA reached the highest content at C/N of 30. These may due to the effect of crude glycerol concentration that can inhibit the microbial growth.

Comparison of cell growth and PHA production among different strain of *Pseudomonas mendocina* were shown in Table 1, the result showed that our new isolated strain can produce the highest PHA of 77.59 % DCW with maximum PHA productivity of 0.23 g/L/h but the biomass is slightly less than *P. mendocina* 0806 that could produce 3.6 g/L of DCW. Therefore, this *Pseudomonas mendocina* PSU is an effective microorganism for PHA production and this is the first report of the production of PHA from crude glycerol-biodiesel waste product by *Pseudomonas mendocina* spp.

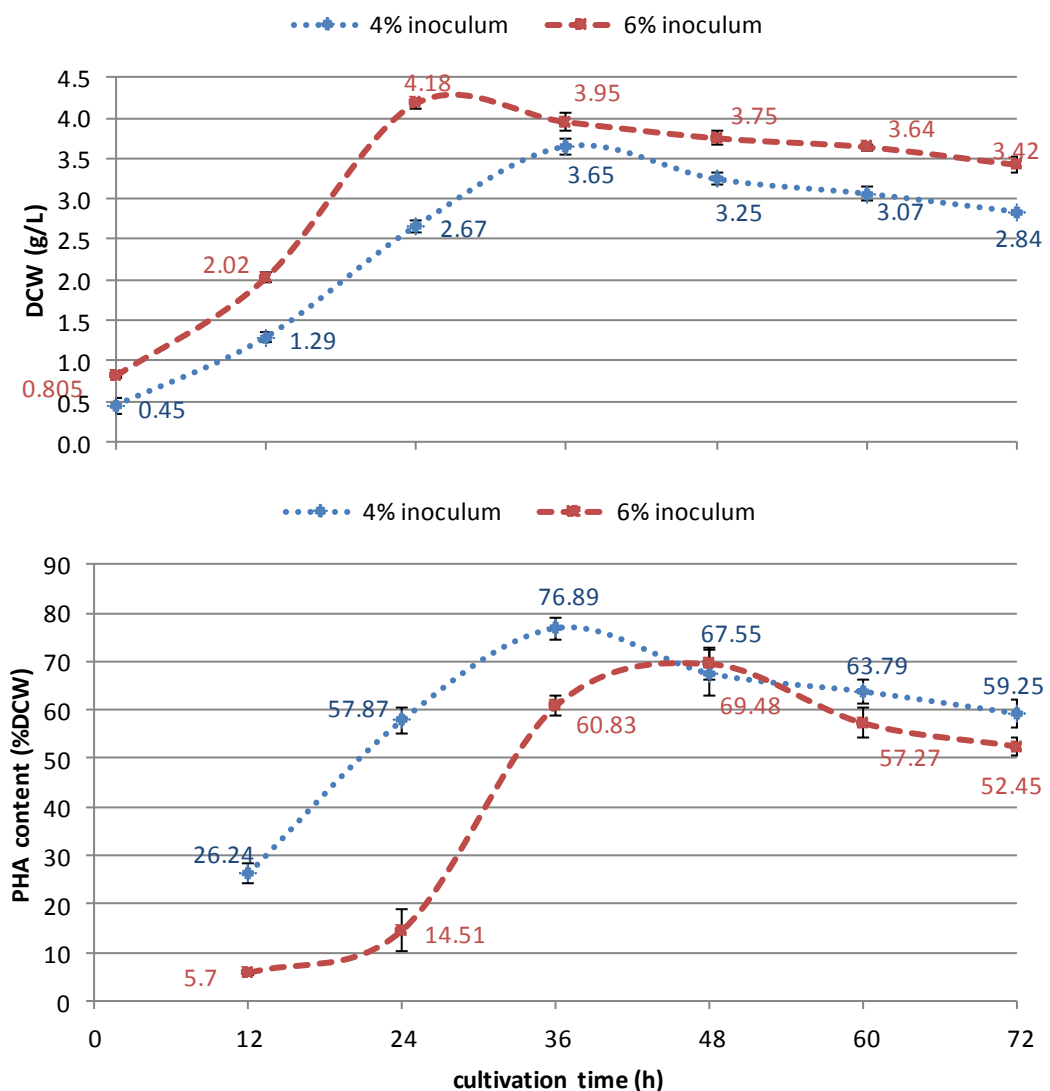
**Table 1** Cell growth and PHA production among different strain of *Pseudomonas mendocina*

<b>Strain</b>	<b>Carbon source</b>	<b>Biomass (g/L)</b>	<b>PHA content (% DCW)</b>	<b>Productivity (g/L/h)</b>	<b>Main monomer in PHA produced</b>	<b>References</b>
<i>P.mendocina</i> 0806	glucose	3.6	45	-	3HHx,3HO and 3HD	Tian et al.2000
<i>P.mendocina</i> NK-01	glucose	1.73	20.23	-	3HO and 3HD	Guo et al.2011
<i>P.mendocina</i> CH50	sodium octanoate	0.87	31.38	0.03	3HO	Rai et al. 2011
<i>P.mendocina</i> PSU	crude glycerol	3.51	77.59	0.23	3HB,3HD and 3HDD	This study



## 5. Effect of inoculum on PHA production

To study the effect of % inoculum of seed culture on biomass concentration and PHA production, the percentages of *Pseudomonas mendocina* PSU inoculums of 4% and 6% v/v were used. Figure 7 clearly showed that at 6% inoculum, cells grew better than at 4% inoculum and reached the maximum growth of 4.18 g/L before 24h, however, it had an adverse effect on PHA production because at 6% inoculum PHA content was less (69.48%DCW) and produced much later than at 4% inoculum (76.89%DCW).

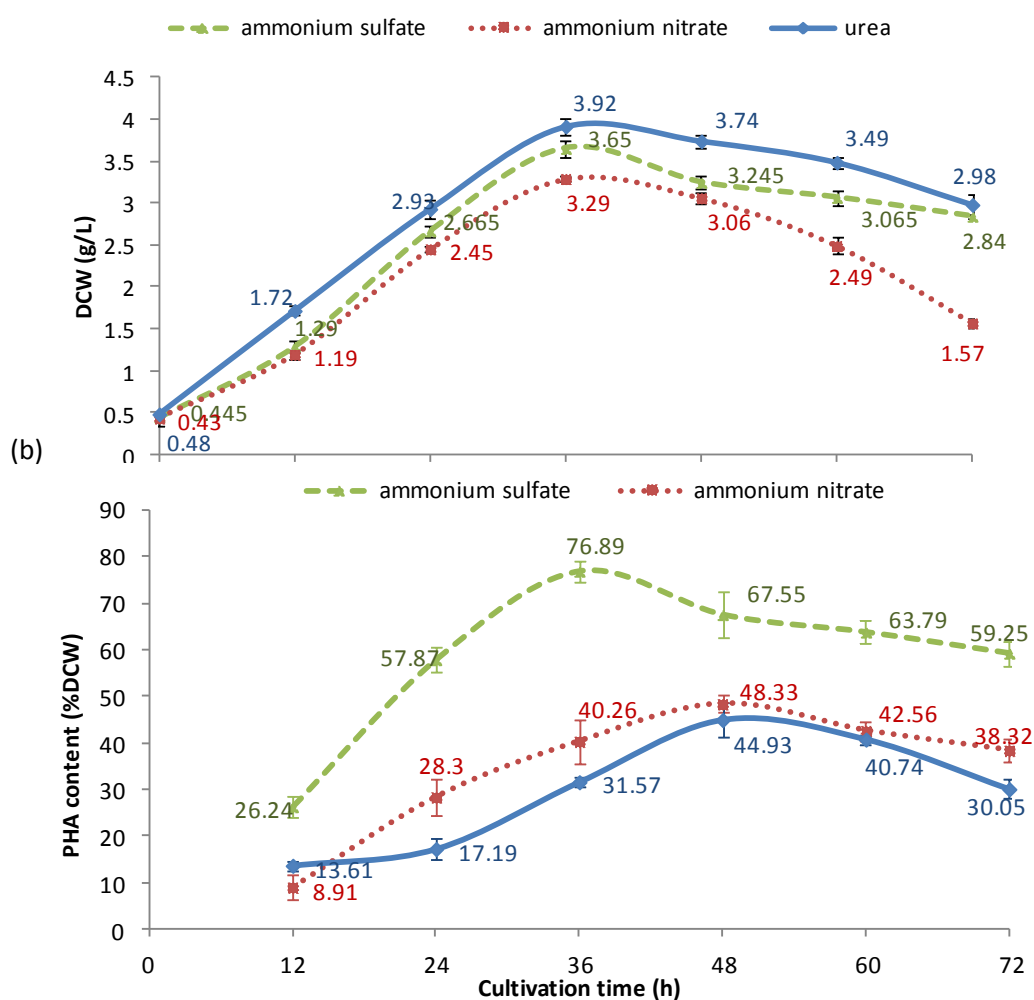


**Figure 7** Effect of the percentages of inoculum on cell growth and PHA production by *Pseudomonas mendocina* PSU when using 2.0% crude glycerol as a sole carbon source

## 7. Effect of nitrogen source

The best nitrogen source was investigated. Nitrogen source e.g. ammonium sulfate, ammonium nitrate and urea with an equal molar of nitrogen content were supplemented in MSM containing 2.0% crude glycerol as a sole carbon source. Figure 8 showed that the maximum cell growth (3.92 g/L) obtained when using urea was slightly higher than that of ammonium sulfate (3.65 g/L) and ammonium nitrate (3.29 g/L). On the other hands, the production of PHA was the highest when using ammonium sulfate at 36 h followed those of ammonium nitrate and urea (Figure 8).

(a)



**Figure 8** Comparison of cell growth (a) and PHA production (b) by *Pseudomonas mendocina* PSU among three different nitrogen source e.g. ammonium sulfate, ammonium nitrate and urea when using 2.0% crude glycerol as a sole carbon source and 4% inoculum

## 8. Optimum conditions for PHA production

To obtain an optimal conditions in shake flask cultivation, Response Surface Methodology (RSM) was carried out using the Box-Behnken design (BBD) which contained 17 experimental runs for optimizing the three individual parameters e.g. ratio of carbon to nitrogen, C/N (20,40,60), pH (6.0,7.0,8.0), and temperature (30, 35, 40°C) and then cell growth and PHA content were determined.

According to RSM statistical model, it can generate optimal conditions for the highest response values of cell growth and PHA content (Table 2) and then the experiments were verified under the optimal conditions at C/N of 40, pH 7.0 and 35 °C.

**Table 2** Predicted and experimental values of the response at optimal conditions

	C/N	pH	Temperature (°C)	DCW (g/L)	PHA content (%DCW)
Optimal conditions	40.29	7.11	34.38	3.61	77.78
Modified condition	40	7.0	35	3.58±0.09 (n=5)	77.27±0.83 (n=5)

The biomass concentration and PHA production under optimal condition and modified condition are compared in Table 2. The results showed that the DCW and PHA content are almost similar.

## 9. Substrate specificity of PHA synthase

According to the previous experiment that *Pseudomonas mendocina* PSU is able to produce two PHA synthase enzyme e.g. SCL-PHA synthase and MCL-PHA synthase.

Therefore, to initially investigate the substrate specificity of each PHA synthase, various carbon sources are supplied and then the PHA content and monomer compositions are determined by GC and GC-MS.

### Experimental design (Shake flask cultivation)

Carbon source : condition 1-4 (see below)

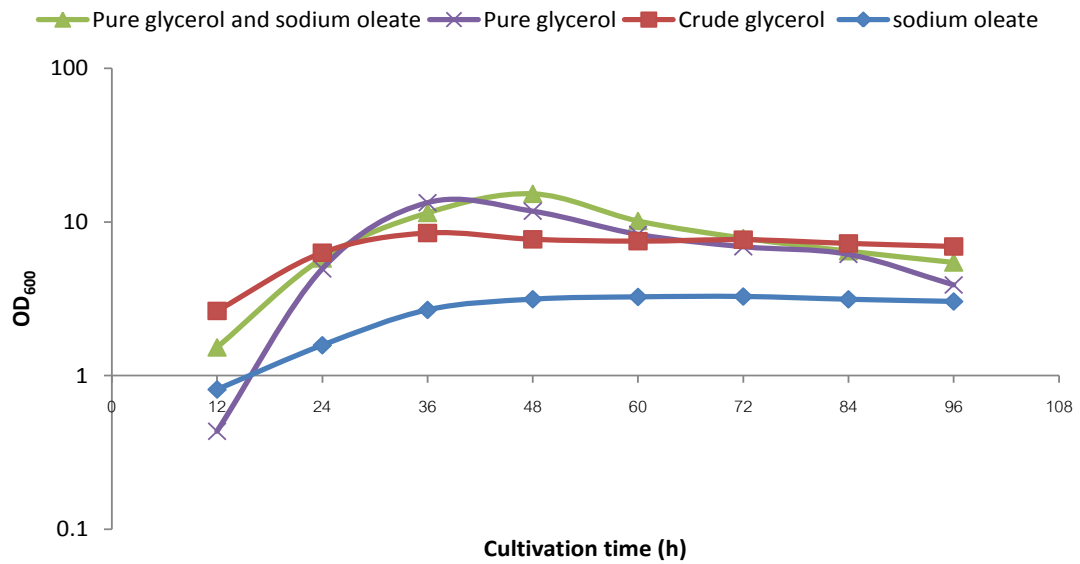
Nitrogen Source : 0,1 % (w/v) ammonium sulphate

Condition 1 : 1,0% (v/v) Pure glycerol + 1,0% (w/v) sodium oleate (C18)

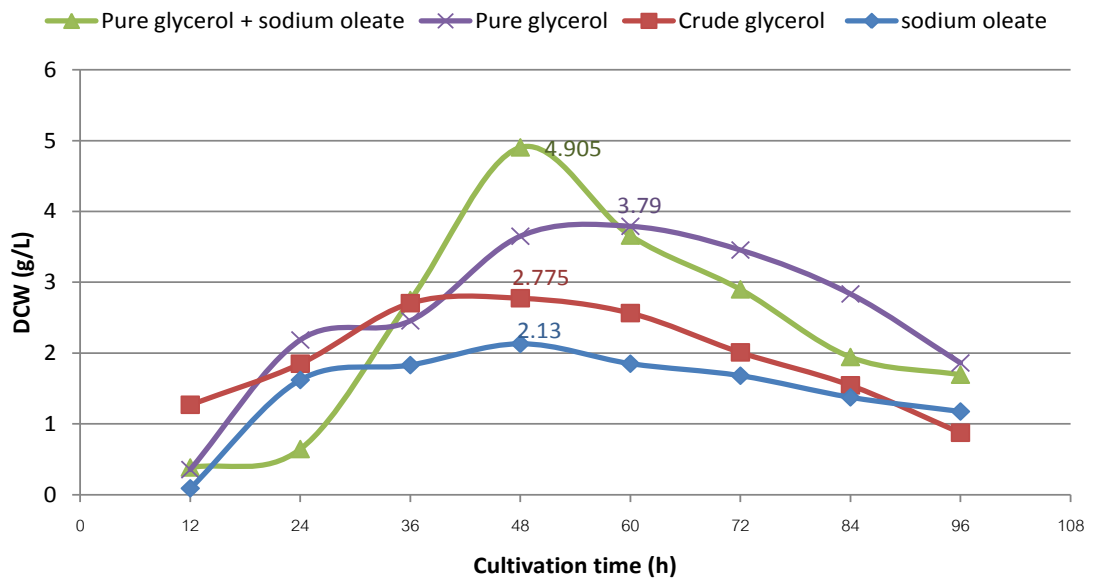
Condition 2 : 2,0% (v/v) Pure glycerol

Condition 3 : 2,0% (v/v) Crude glycerol

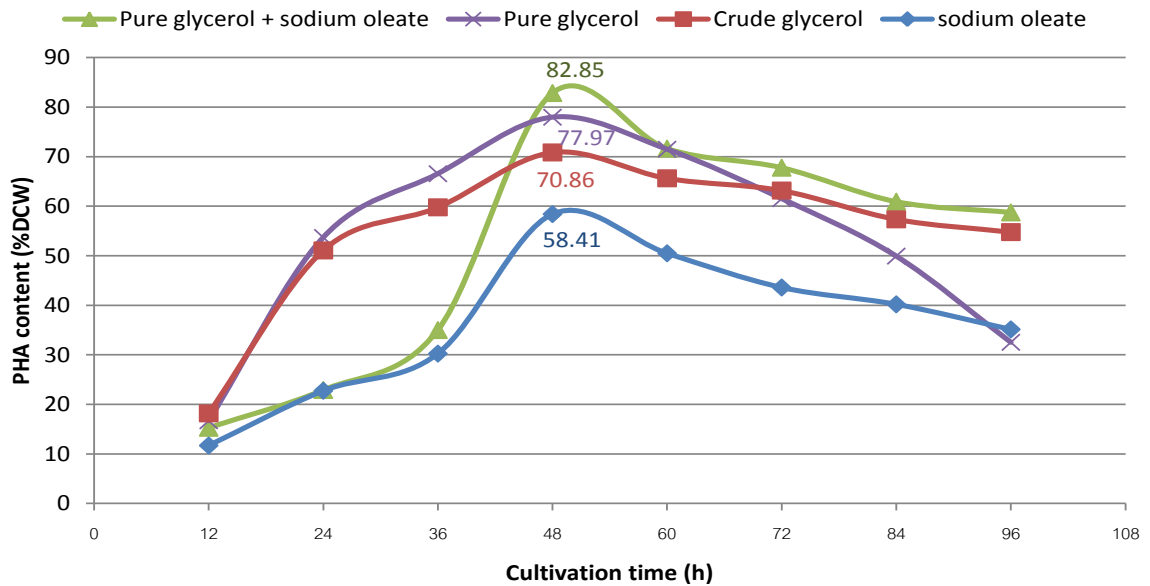
Condition 4 : 2,0% (w/v) Sodium oleate (C18)



**Figure 9** Cell growth profile (Optical density measurement at 600 nm) when *Pseudomonas mendocina* PSU cultivated in MSM supplemented with various carbon sources



**Figure 10** Cell growth profile (Dry cell weight) when *Pseudomonas mendocina* PSU cultivated in MSM supplemented with various carbon sources



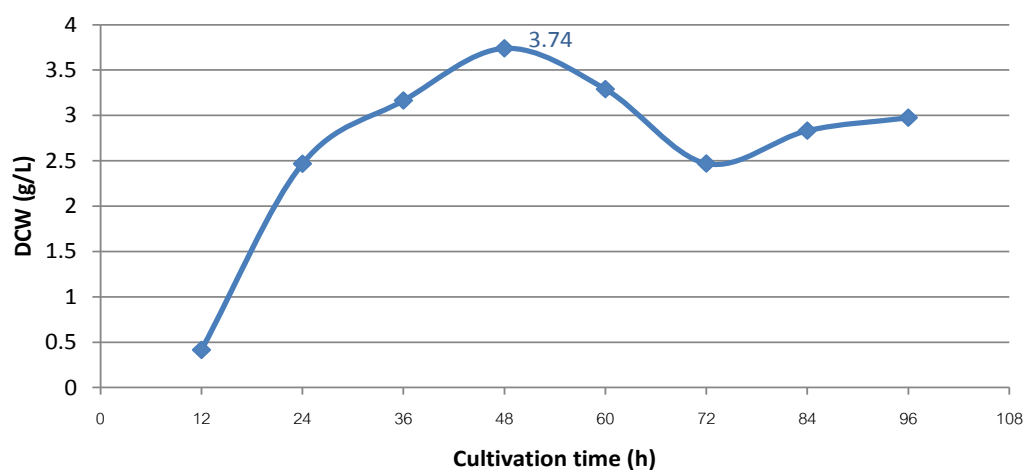
**Figure 11** PHA production when *Pseudomonas mendocina* PSU cultivated in MSM supplemented with various carbon sources

Up to 4.9 g/L and 83 %DCW of biomass and PHA content were produced when *Pseudomonas mendocina* PSU was cultivated in MSM supplemented with a mixture of pure glycerol and sodium oleate (C18) followed by pure glycerol, crude glycerol and sodium oleate (Figure 9-11).

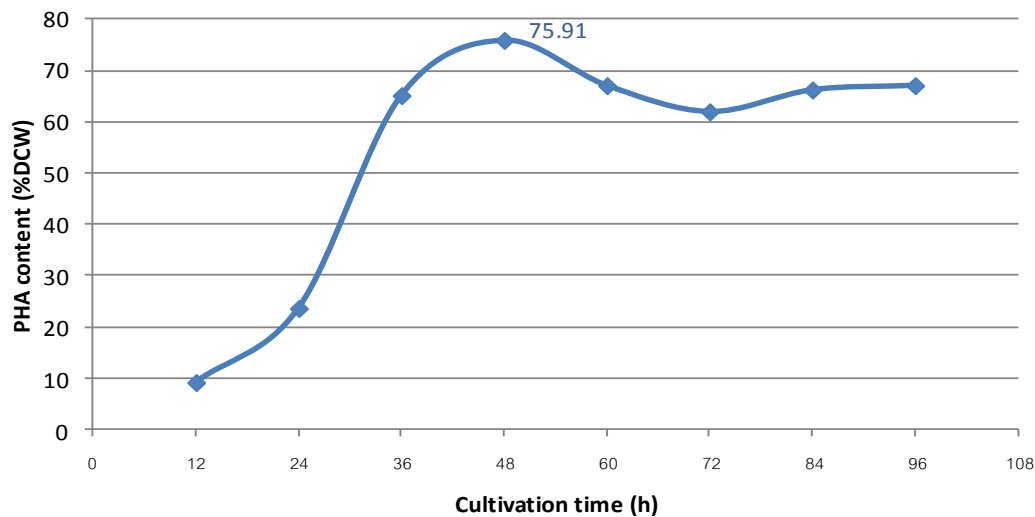
From these result it can be suggested that *P. mendocina* PSU can readily utilize glycerol and fatty acid and then convert to PHA through different PHA biosynthetic pathway. However, pure glycerol is likely to be easily metabolized comparing with waste product e.g. crude glycerol and longer chain fatty acid e.g. sodium oleate as indicated by the higher biomass and PHA production.

However, the further analysis of monomer composition and molar fraction of PHA using GC-MS are required in order to prove that this *P. mendocina* PSU prefer to use glycerol before fatty acid.

2-step feeding carbon source was carried out by mean of the first 48 h of cultivation, *Pseudomonas mendocina* PSU was supplied with 1.0 %(v/v) pure glycerol as sole carbon source and then 1.0 %(w/v) sodium oleate was fed at 48 h.

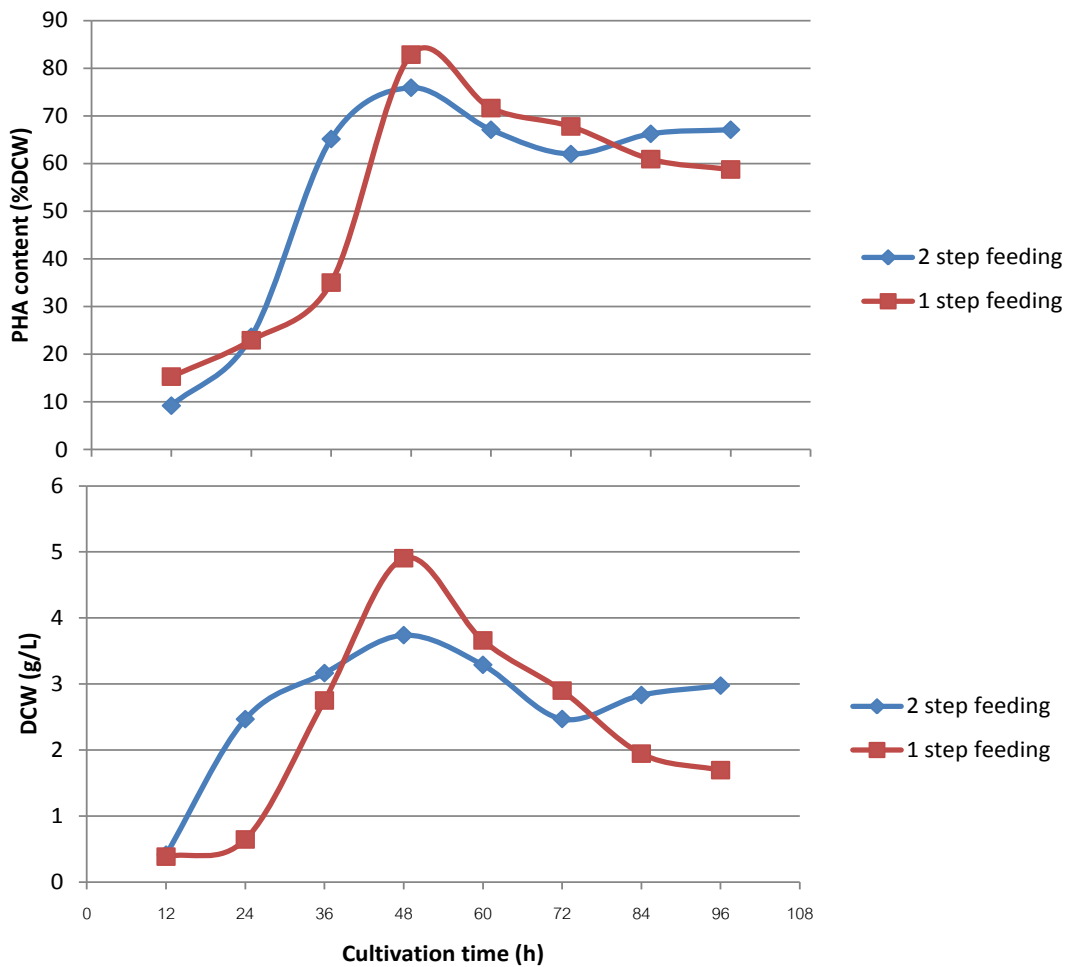


**Figure 12** Cell growth when *Pseudomonas mendocina* PSU cultivated in MSM supplemented with 2-step feeding carbon source e.g. pure glycerol followed by sodium oleate



**Figure 13** PHA production when *Pseudomonas mendocina* PSU cultivated in MSM supplemented with 2-step feeding carbon source e.g. pure glycerol followed by sodium oleate

At the beginning of cultivation until 48 h, *Pseudomonas mendocina* PSU was fed with pure glycerol. Cell density and PHA content increase accordingly to the cultivation time and reach 3.7 g/L and 76%DCW, respectively. When the culture was fed with fatty acid e.g. sodium oleate at 48 h, biomass concentration and PHA content were likely to decrease; however, after 72 h cell growth and PHA production started to gradually increase. This result suggested that after feeding with sodium oleate, *P. mendocina* PSU would take quite some time (from 48h to 72h) for adaptation itself to utilize a new complex carbon source as indicated by slight reduction of growth and degradation of PHA (Figure 12-13).



**Figure 14** Comparison of cell growth and PHA production from *Pseudomonas mendocina* PSU between one-step and two -step feeding of pure glycerol and sodium oleate

Figure 14 showed that the highest biomass concentration and PHA content were obtained in one-step feeding at 48 h and then after this time point, DCW and PHA accumulation start to continuously decrease whereas under two-step feeding, both cell growth and PHA production could be maintained at relatively high amount. These may be due to under two-step feeding, cells are sufficient feeding with carbon sources while under one-step feeding, cells would rapidly utilize carbon source for their growth and PHA storage after nitrogen is depleted at 36 h (from previous experiment).



## 10. Detection of the PHA synthase gene in PM-PSU

It was of interest that there are some organisms that produce SCL-MCL copolymers of PHA. This situation seems to be very rare in nature in comparison to the organisms able to produce only SCL-PHA or MCL-PHA (Rai et al. 2011; Kim et al. 2007). The examples of organism with an ability to produce mixed polymers include mainly *Pseudomonas* sp. e.g. *Pseudomonas* sp. 61–3 (Matsusaki et al. 1998; Hokamura et al. 2015), *Pseudomonas* sp. USM 4-55 (Sudesh et al. 2004; Tan et al. 2010), *Pseudomonas* sp. LDC-5 (Sujatha et al. 2007), *Pseudomonas stutzeri* 1317 (Chen et al. 2004), and *Pseudomonas extremaustralis* (Catone et al. 2014). This ability might require two separate systems for the accumulation of the monomers i.e. the presence of two different PHA synthases such as the SCL-PHA and MCL-PHA synthases. Molecular studies have shown that these strains possessed two PHA synthetic pathway, one responsible for P(3HB) production and the other for MCL-PHA accumulation each requiring its own specific PHA synthase (Matsusaki et al. 1998; Tan et al. 2010; Catone et al. 2014; Hokamura et al. 2015).

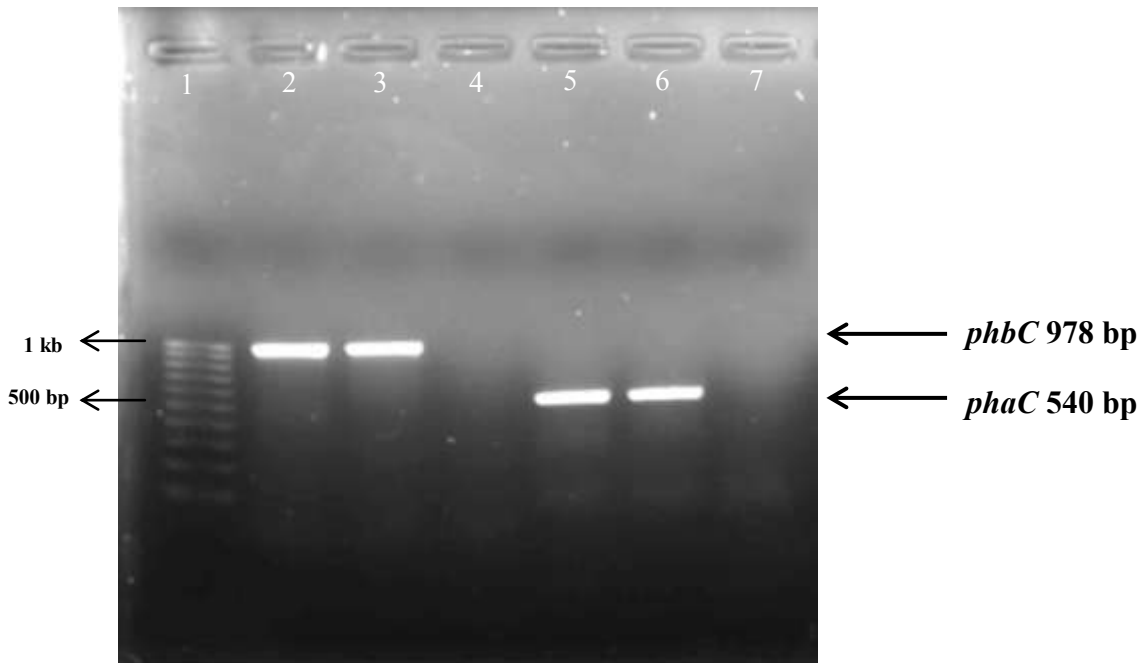
The *phbC<sub>Ps</sub>* gene encoding for the SCL-PHA synthase and *phaC<sub>Ps</sub>* gene encoding for MCL-PHA synthase in PM-PSU were amplified by PCR using two sets of primers as presented in **Table 4**. These primers were designed based on two highly conserved sequences deduced from the multiple alignment analysis of the *Pseudomonas* spp. *phbC* (Lee et al. 2011) and *phaC* (Solaiman et al. 2000; Sujatha et al. 2007) genes.

**Table 4** PCR primers used for PHA synthase genes amplification

Primers	Sequence 5' to 3'	PCR product (bp)	References
<i>phaC<sub>Ba</sub></i>	(F) 5'-GATGTGTATTTGCTTGACTGGGG-3' (R) 5'-AGCCAATCGCCGATTGAAGGATA-3'	~760	Berekaa, 2012
<i>phbC<sub>Ps</sub></i>	(F) 5'-GCCTTCTACCTGCTGAATTCGG-3' (R) 5'-GGTGTGGCGCAGATACCA-3'	978	Solaiman et al. 2000
<i>phaC<sub>Ps</sub></i>	(F) 5'-ACAGATCAACAAGTTCTACATCTTCGAC-3' (R) 5'-GGTGTGTCGTTGTTCCAGTAGAGGATGTC-3'	540	Lee et al. 2011

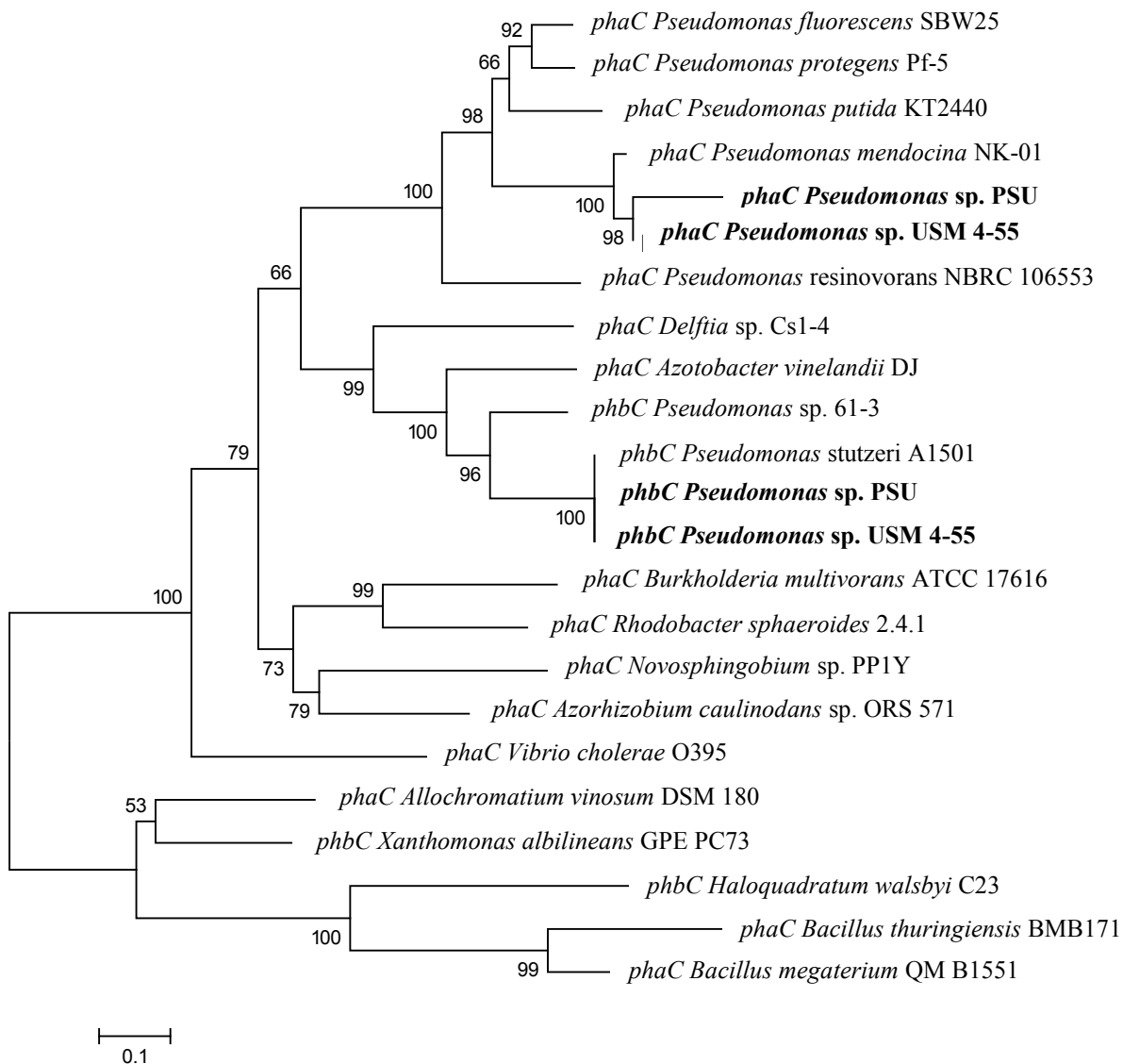
As expected, an approximately 978 bp and 540 bp of the DNA fragments were amplified from PM-PSU as shown in **Figure 15**. Therefore, it was presumed that this bacterium had two types of PHA synthase genes. The sequences of *phbC<sub>Ps</sub>* and *phaC<sub>Ps</sub>* of PM-PSU were further compared with the sequence of PHA synthase genes among

*Pseudomonas* species reported in GenBank (NCBI databases) and they exhibited 100% and 99% identity to the *phbC* and *phaC* of *Pseudomonas* sp. USM 4-55, respectively.



**Figure 15** PCR analysis of the SCL-PHA synthase gene (*phbC<sub>PS</sub>*) and MCL-PHA synthase gene (*phaC<sub>PS</sub>*) in *Pseudomonas* sp. 100 bp ladder (lane 1, left) lane 2-4 primers for *phbC* amplification were used (Lee et al. 2011) whereas lane 5 -7 primers for *phaC* amplification were used (Solaiman et al. 2000; Sujatha et al. 2007) lane 2 and lane 5: PM-PSU against positive control e.g. *Pseudomonas* sp. USM 4-55 (lane 3 and lane 6) and a negative control e.g. *Burkholderia* sp. USM (lane 4 and lane 7).

In addition, in order to study the relationship of the PHA synthase genes among different bacteria, a phylogenetic tree was generated from the 21 bacteria possessing the different PHA synthases and this is presented in **Figure 16**. It shows that *phbC<sub>PS</sub>* of PM-PSU was located in the same group as the *phbC* of *Pseudomonas* sp. USM 4-55 and *Pseudomonas stutzeri* A1501 with a strong bootstrap support of 100% whereas the *phaC<sub>PS</sub>* of this strain showed the closest relationship to *phbC* in *Pseudomonas* sp. USM 4-55 with a 98% bootstrap. Therefore, the PHA synthase genes of PM-PSU appear to be similar to the PHA synthase genes of *Pseudomonas* sp. USM 4-55.



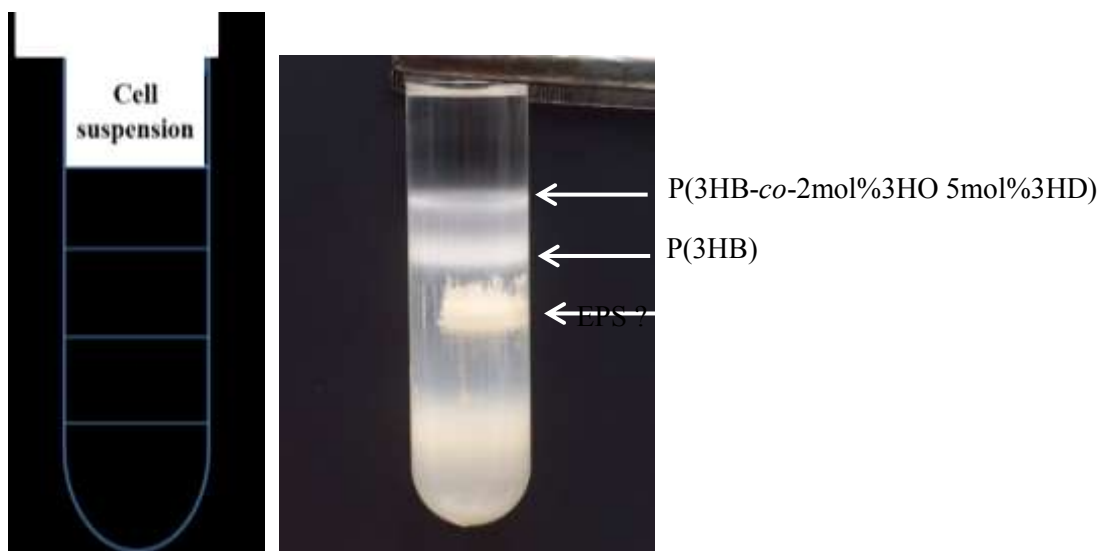
**Figure 16** Phylogenetic tree of *phbC* and *phaC* among 21 bacteria generated using the neighbor-joining method. Numbers above the branches indicate the number of times (%) the species grouped together in 1,000 bootstrap replicates. Bar 0.1 (evolutionary distance)

The *phbC* locus of *Pseudomonas sp.* USM 4-55 was clustered in the *phbBAC* operon which may be a unique property of the *Pseudomonas* species that can produce both SCL- and MCL-PHA whereas in the earliest operon model investigated, *Cupriavidus necator*, its operon was arranged as *phbCAB* (Peoples and Sinskey, 1989). Hence, the genetic organization in PM-PSU was presumably arranged in *phbBAC* operon that was similar to the

arrangement in *Pseudomonas* sp. USM 4-55; however, the further studies are needed to confirm this assumption.

### 11. Monomer compositions of the PHA produced from PM-PSU

According to the presence of the SCL-PHA gene (*phbC<sub>PS</sub>*) and the MCL-PHA gene (*phaC<sub>PS</sub>*) in PM-PSU, this bacterium can produce two types of PHA e.g. SCL-PHA and MCL-PHA, so a sucrose density gradient ultracentrifugation was carried out to demonstrate the presence of two different granules (Chee et al. 2010). This method was applied by Matsumoto et al. (2002) and Loo and Sudesh (2007) to separate different types of PHA granules on the basic of the PHA density that depended on the type of the monomer components.



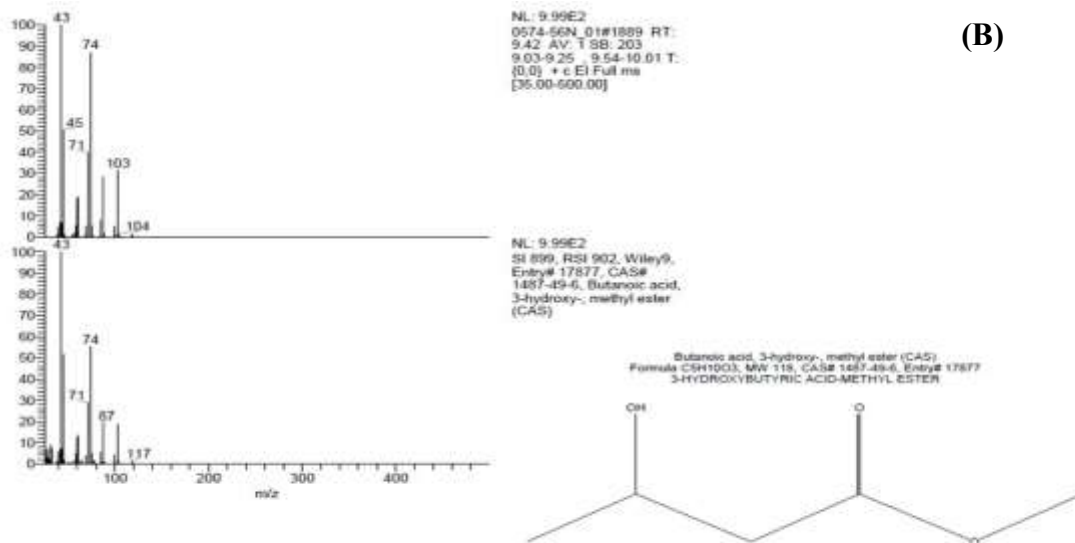
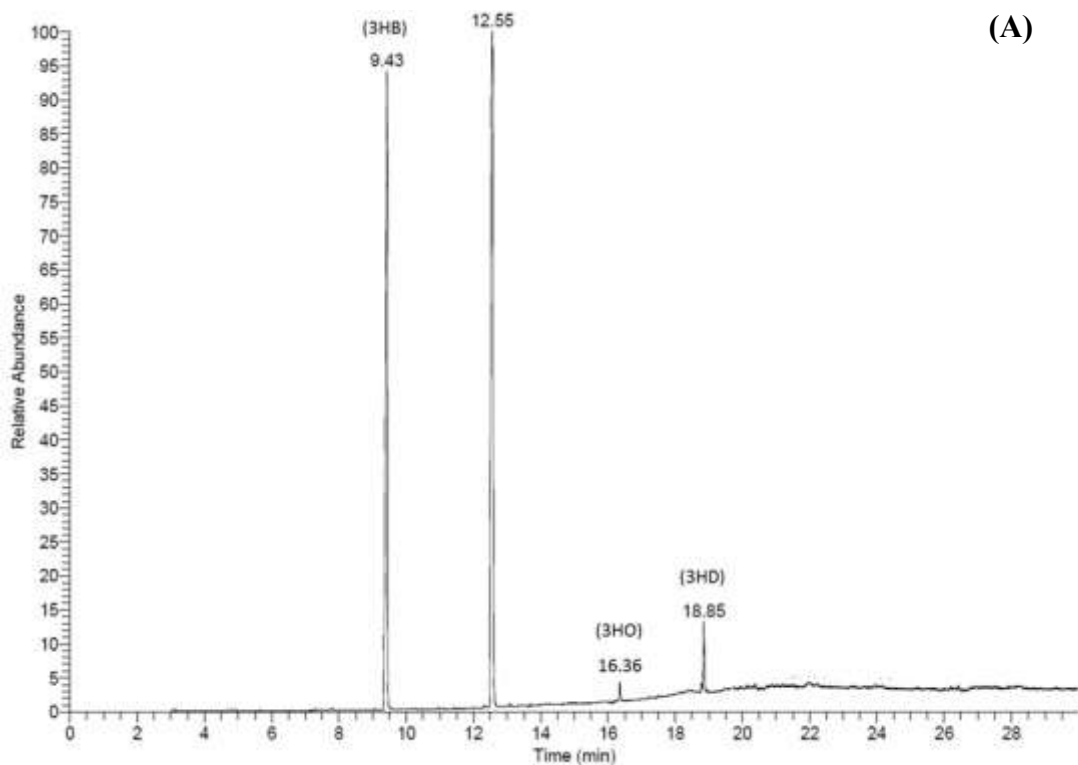
**Figure 17** Ultracentrifugation tube with a discontinuous sucrose gradient (four different sucrose concentrations: 1.0, 1.33, 1.67 and 2.0 M) (left) and after ultracentrifugation at 4°C, 280,000 x g for 2 h of the cell suspension obtained from a culture of PM-PSU grown with BLW for 36 h (right)

**Figure 17** shows the ultracentrifugation tube prepared with a discontinuous sucrose gradient, the result clearly confirmed that PM-PSU accumulated two types of PHA granule as seen by the presence of the two fractioning bands. SCL-PHA e.g. P(3HB) granule was concentrated in the band between 1.67 and 2.0 M sucrose solutions whereas another band

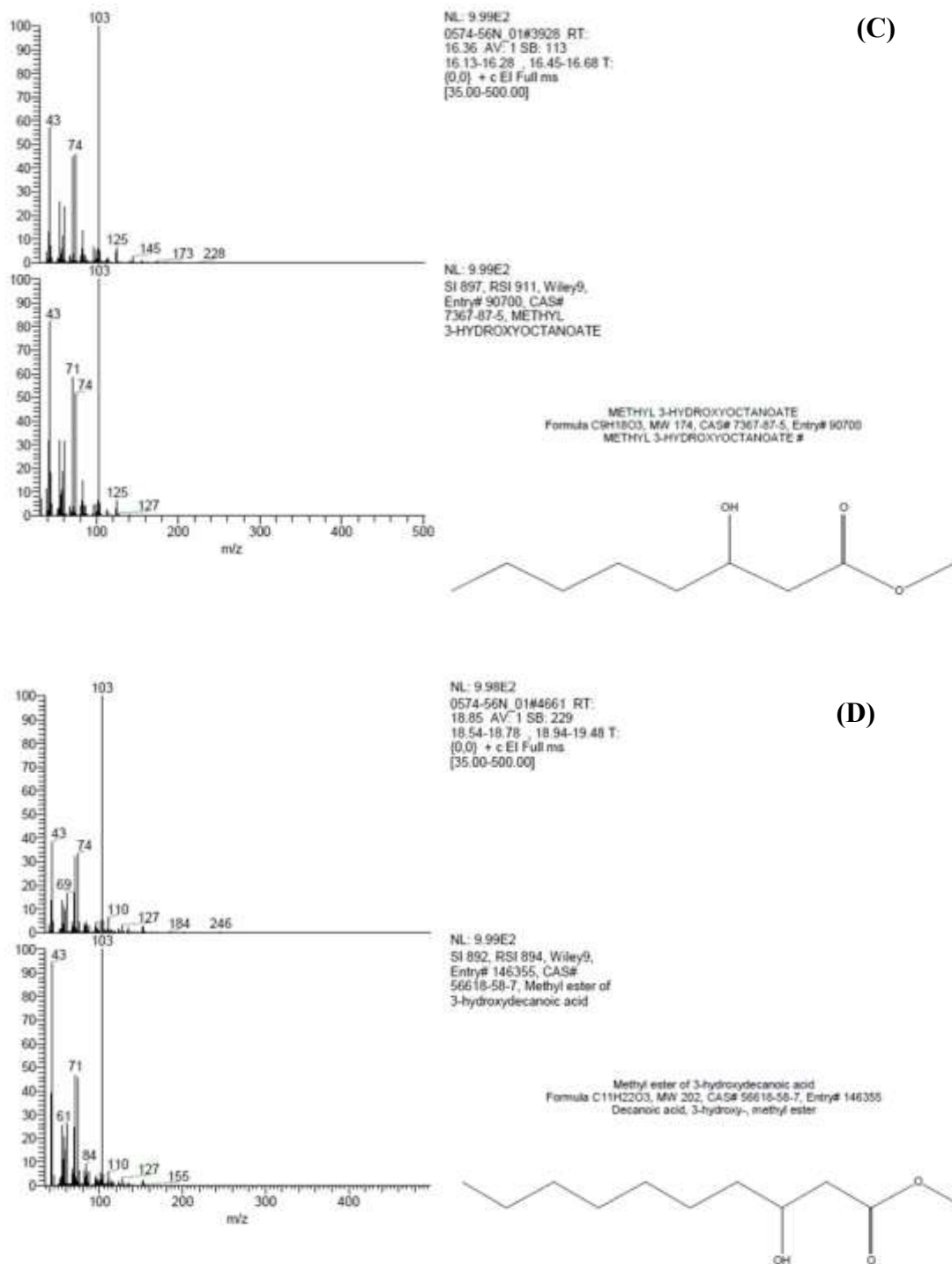
containing copolymer SCL- and MCL-PHA granule e.g. P(3HB-*co*-2mol%3HO5mol%3HD) existed at the layer above. This can now explain why the SCL-PHA monomer contained not only the shorter side chain but also had been packed compactly in the PHA granules while the MCL-PHA monomer possesses longer side chains and exhibits steric effects that cause the polymer chain to be arranged loosely and resulted in their different densities (Chee et al. 2010; Loo and Sudesh, 2007).

The bottom layer of P(3HB) showed another discrete band. This layer was assumed to be exopolysaccharide (EPS), produced from PM-PSU, due to its high molecular mass which can penetrate through the 2M sucrose by ultracentrifugation. However, to clarify whether the two polymer chains were being accumulated in the same cell or in different cells, freeze-fracture electron microscopy was applied. Previous studies have shown that the granules of the two different PHA polymers have two different morphologies e.g. a needle-type structure for P(3HB) homopolymer and a mushroom-type structure for the P(3HA) copolymer were observed in *Pseudomonas* sp. 61-3 which indicating that this bacterium also synthesized and stored both P(3HB) and P(3HB-*co*-3HA) granules simultaneously as separate granules in the same cell (Fukui et al. 1998). Therefore, PM-PSU, which showed similar characteristics to *Pseudomonas* sp. 61-3 was assumed to have separate PHA granule inside the cell.

Moreover, gas chromatography-mass spectrometry (GC-MS) and proton nuclear magnetic resonance ( $^1\text{H-NMR}$ ) were then analyzed to determine the monomer compositions and the structure of the produced PHA. The analytical results showed that PM-PSU produced three types of PHA monomers e.g. 3-hydroxybutyrate (3HB), 3-hydroxyoctanoate (3HO), and 3-hydroxydecanoate (3HD) by comparing their mass spectrogram of the produced PHA from PM-PSU with that of the standards in the GC-MS analysis (**Figure 18**).



**Figure 18** The monomeric composition of PHA produced from PM-PSU grown with BLW as determined by GC-MS analysis. (A) GC-MS chromatogram (B) 3-hydroxybutyryl (3HB); RT=9.43 Benzoic acid was used as an internal standard; RT=12.55 (RT stand for retention time)

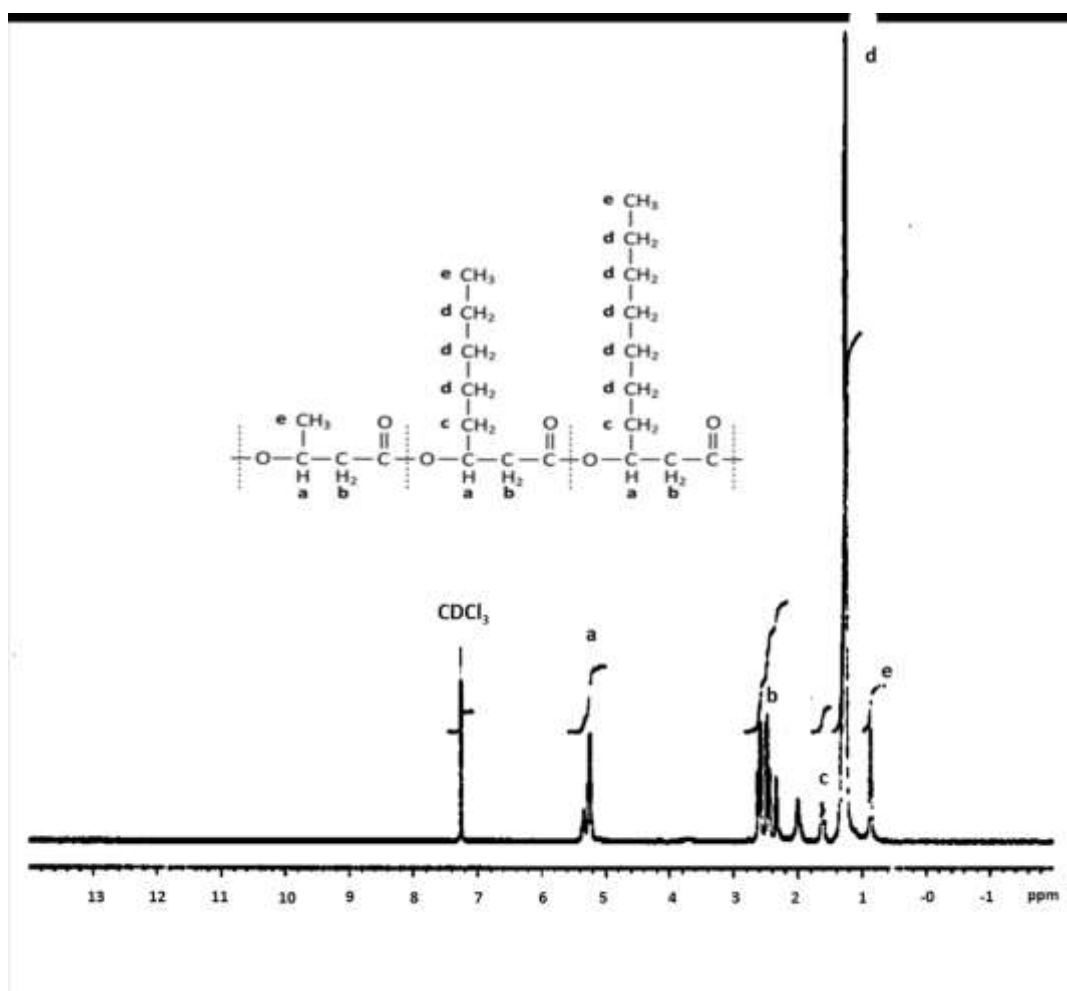


**Figure 18** (cont.) (C) 3-hydroxyoctanoate (3HO);  $R_T=16.36$  (D) 3-hydroxydecanoate (3HD);  $R_T=18.85$ . Benzoic acid was used as an internal standard;  $R_T=12.55$  ( $R_T$  stand for retention time)

An analysis of the polymer structure of the type of PHA produced from PM-PSU was then performed using  $^1\text{H-NMR}$ . The resulting spectrum exhibited a typical chemical shift pattern for the already published spectra for this polyester (Shahid et al. 2013). At 5.2 ppm

(a), the spectrum showed a specific signal of the methine protons attached to an ester function. The polymer structure was in addition verified by the absence of a signal at 4.1 ppm that was characteristic of the CH groups attached to an alcohol function. The methylene protons of C2 had a set of signals between 2.5 and 2.6 ppm (b). The terminal methyl group was shown at 0.9 ppm (e). The methylene protons of the C4 gave a signal at 1.6 ppm (c), whereas all other methylene hydrogens of the saturated side chains generated a signal at 1.3 (d) (Figure 19).

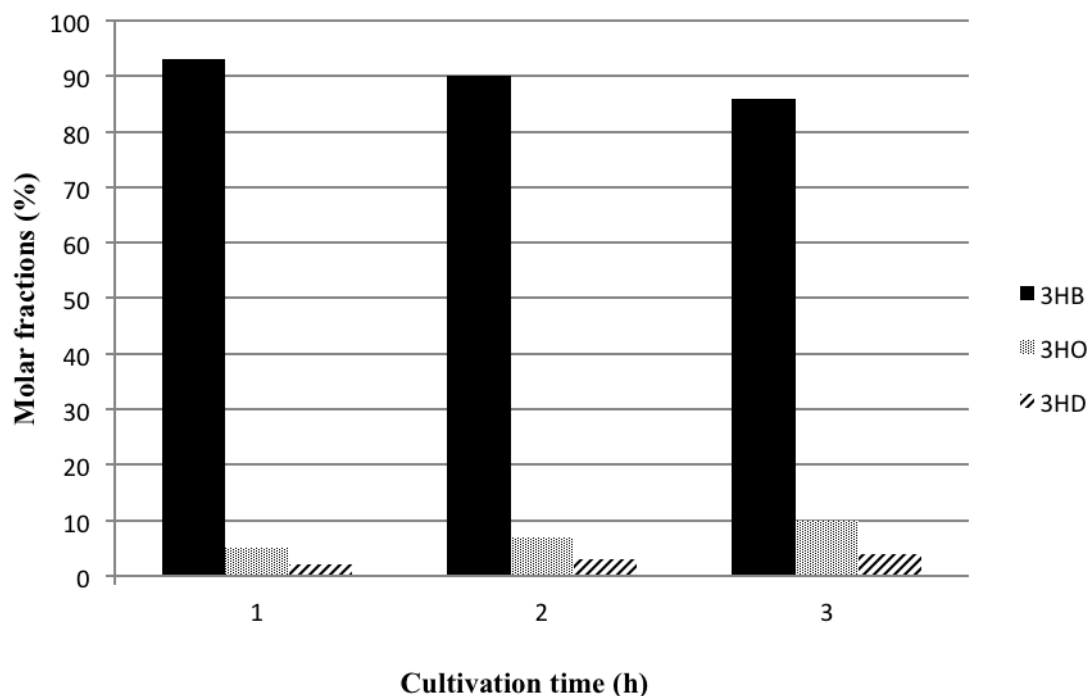
The results obtained from the GC-MS and  $^1\text{H-NMR}$  were in agreement with the result obtained from the sucrose density gradient ultracentrifugation, therefore, it can be concluded that PM-PSU produced three different types of PHA monomers e.g. 3HB, 3HO, and 3HD.



**Figure 19**  $^1\text{H-NMR}$  spectrum of PHA produced from PM-PSU when grown with BLW and its schematic structure

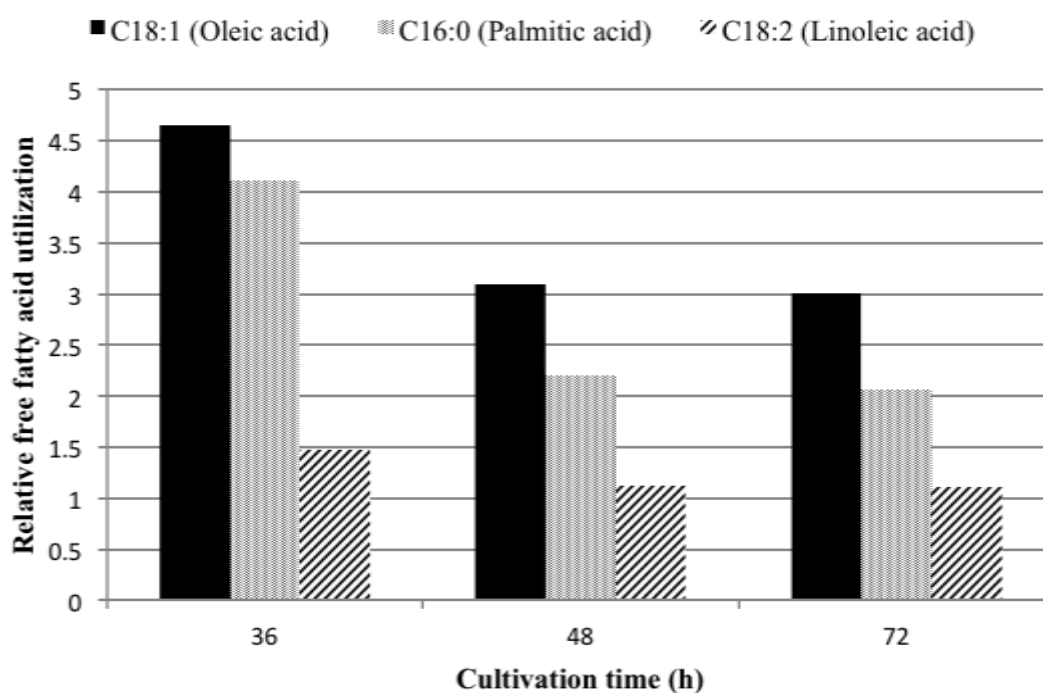


It was also of it interest that, the amount of the monomer components in the produced PHA by PM-PSU did vary slightly during the cultivation time as the proportion of 3HB decreased whereas the 3HO and 3HD increased with time (**Figure 20**).



**Figure 20** Monomer compositions in the produced PHA by PM-PSU when grown with 20 g/L of total glycerol in BLW that had a C/N of 40 at 35°C and an initial pH of 7.0 at different cultivation times. The data was obtained by GC-MS analysis.

For the change in the monomer compositions during the cultivation time it can be suggested that PM-PSU may prefer to utilize glycerol prior to any FFA and/or FAME as indicated by the highest 3HB accumulation at an initial stage of growth and PHA biosynthesis; however, after 48 h the glycerol carbon supply was completely depleted in the culture medium, then the FFA and/or FAME started to metabolize and accumulate more MCL-PHA e.g. 3HO and 3HD. This result can be explained by the ability of PM-PSU to utilize only 30% of the FFA/FAME (Acid value method, the French standard NF T60-204, 1985) at 48 h when virtually all growth had ceased. Then, after the depletion of glycerol, the consumption of FFA/FAME gradually increased as indicated by the reduction of the three major free fatty acids in the BLW e.g. oleic acid, palmitic acid and linoleic acid in the culture medium measured by GC analysis (**Figure 21**).



**Figure 21** Utilization of three major free fatty acid components in the BLW by PM-PSU along with the cultivation measured by gas chromatography analysis

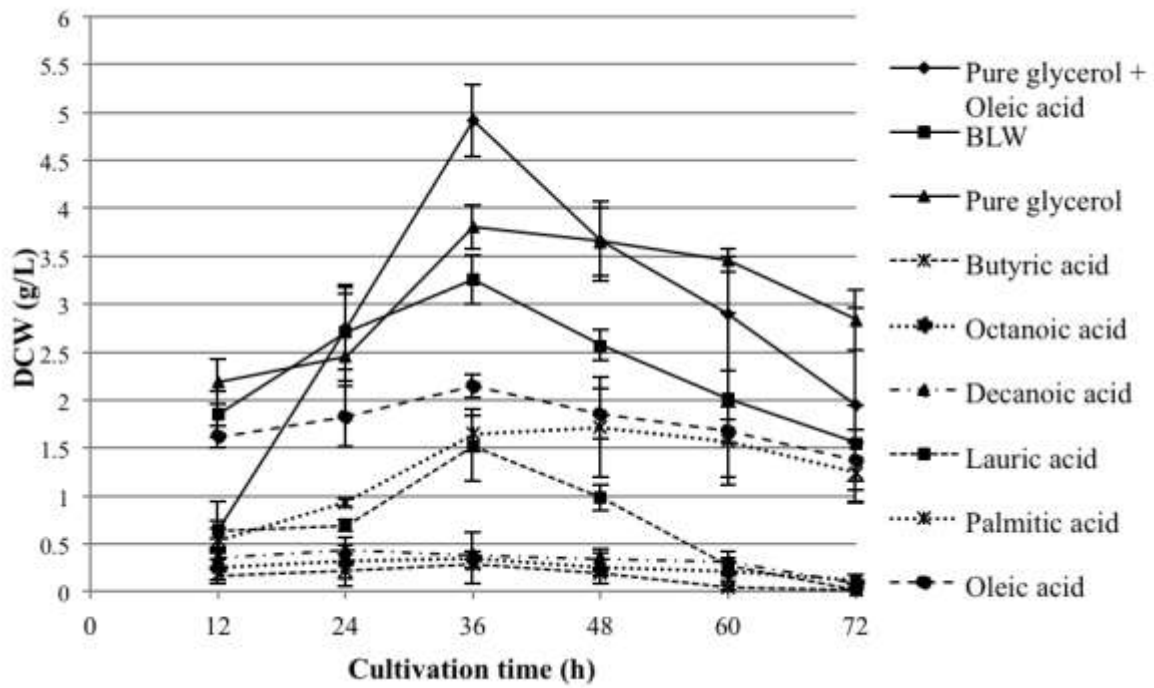
In this study, six fatty acids e.g. butyric acid, octanoic acid, decanoic acid, lauric acid, palmitic acid, and oleic acid were supplemented into MSM to observe the bacterial growth and PHA production compared with PG, BLW and a mixture of PG and oleic acid. As expected, both the obtained DCW and PHA content were very low compared with the use of PG. The highest cell growth and PHA production among fatty acids was with oleic acid but the produced DCW and PHA content were only half of that produced with PG. However approximately 4.92 g/L with a PHA content of 83% DCW was obtained when PM-PSU was grown with a mixture of PG and oleic acid followed by PG, BLW, oleic acid and palmitic acid, respectively (**Figure 22**). These results indicated that the short-chain fatty acids e.g. butyric acid and medium-chain fatty acid e.g. octanoic acid, decanoic acid and lauric acid produced much lower cell growth. Perhaps the shorter *n*-alkyl chain fatty acids were more toxic to bacterial cell due to their lower *pKa* values. The smaller *pKa* values could increase the proton concentration and electrical conductivity (Harris, 2003). In response to the accumulation of protons, free energy was released and protons were expelled from the cells to maintain a normal proton gradient. This excessive energy demand may have resulted in the reduction of microbial activity and growth hence there was a lowering of the yield for PHA

production (Axe and Bailey, 1995; Loo and Sudesh, 2007) whereas with oleic acid the presence of the double bond might act as an alternative electron acceptor (Voet et al. 2004).

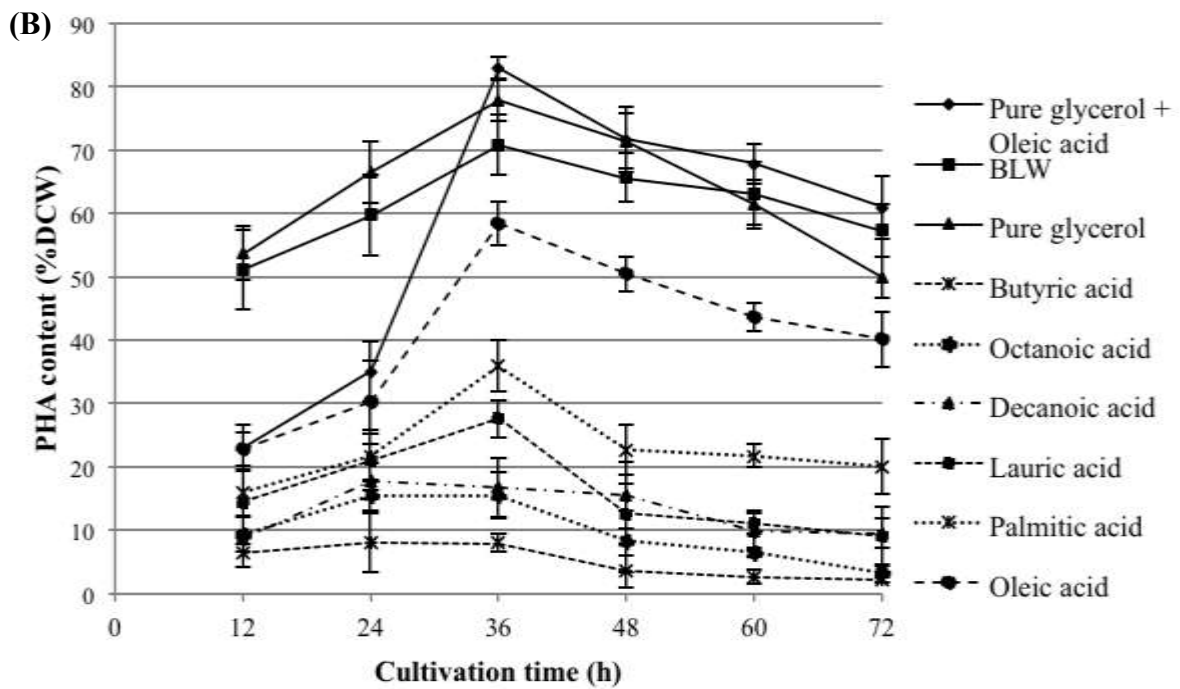
In addition this experiment clearly demonstrated that different carbon sources produced different percentages of molar fractions and their molar fractions also varied during the cultivation period. Butyric acid, octanoic acid and decanoic acid resulted in the accumulation of homopolymers of 3HB, 3HO and 3HD, respectively whereas PHA copolymers were produced when the cells were grown with lauric acid, palmitic acid and oleic acid and also PG (**Figure 23**). This may be due to the PHA synthases of PM-PSU having a broad range of substrate specificity that was related to the different carbon sources while the variations of the PHA monomers in the PHA during cultivation was presumably dependent on the different rates of carbon utilization by the bacteria; however, 3HB was normally the most abundant when the cells were grown with different carbon sources. From these results it seems possible that the production of a variety of PHA monomers with different molar fractions of monomers from various carbon substrates could be achieved by PM-PSU and allow for different physical properties that could broaden their applications.

According to the production of different molar fractions of the PHA monomers at different stages of cultivation when PM-PSU was grown with various carbon sources such as PG, some fatty acids and BLW, it was suggested that the substrate specificity of the PHA synthase and its metabolic pathway plays important roles for the accumulation of the PHA monomer produced. Moreover, to investigate the substrate specificity of each of the PHA synthases e.g. *phbC*, *phaC1* and *phaC2* in PM-PSU, genes cloning and characterization were performed and the activity of each PHA synthase gene was preliminarily examined under different carbon substrates.

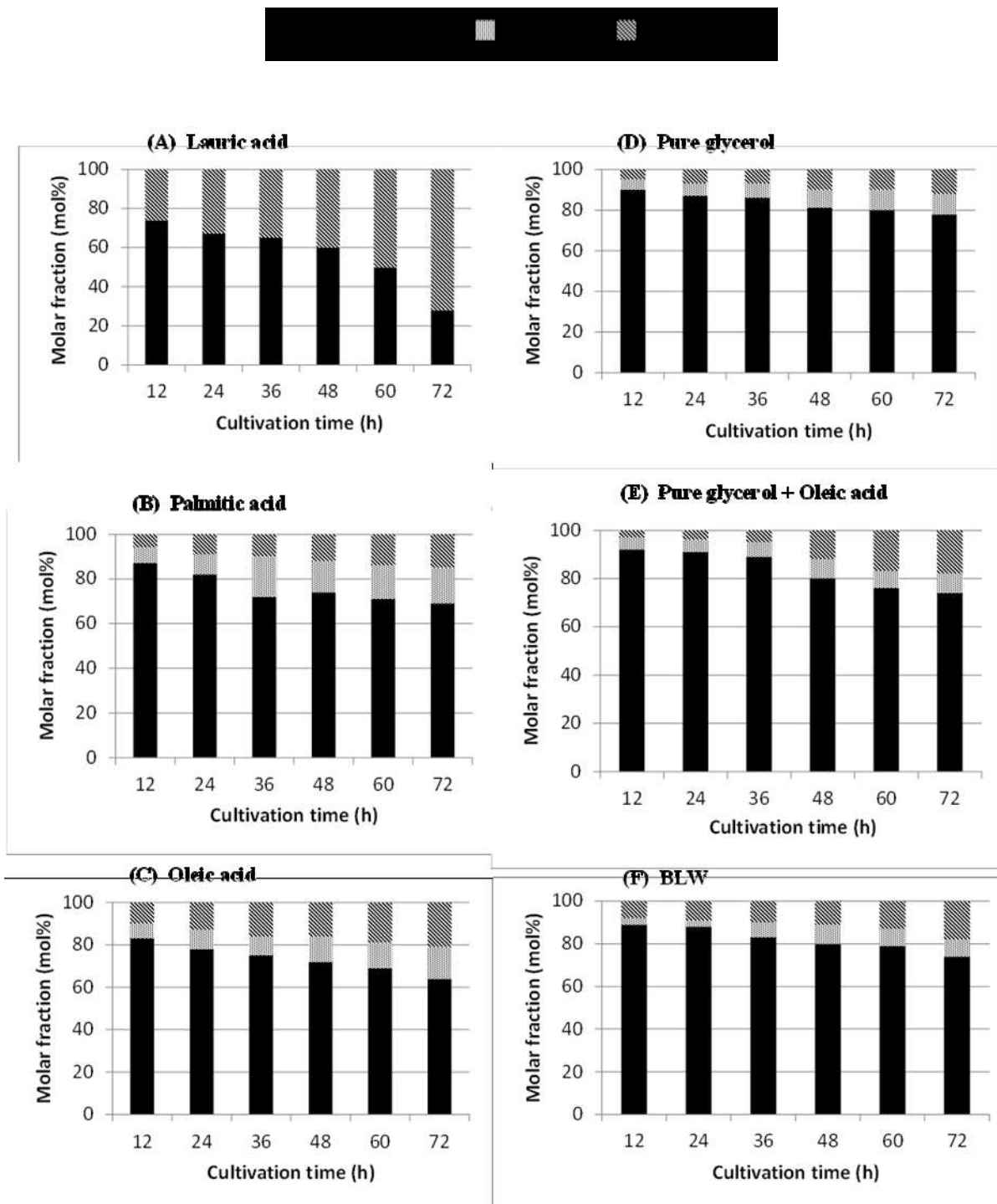
In this study, PM-PSU can utilize various carbon substrates present in the BLW through different metabolic pathways; however, glycerol was the substrate most preferred by this strain. Briefly, glycerol was converted to GAP followed by pyruvate and acetyl-CoA, a precursor for P(3HB) biosynthesis through glycolysis and the pyruvate decarboxylation process as described previously.



(A)



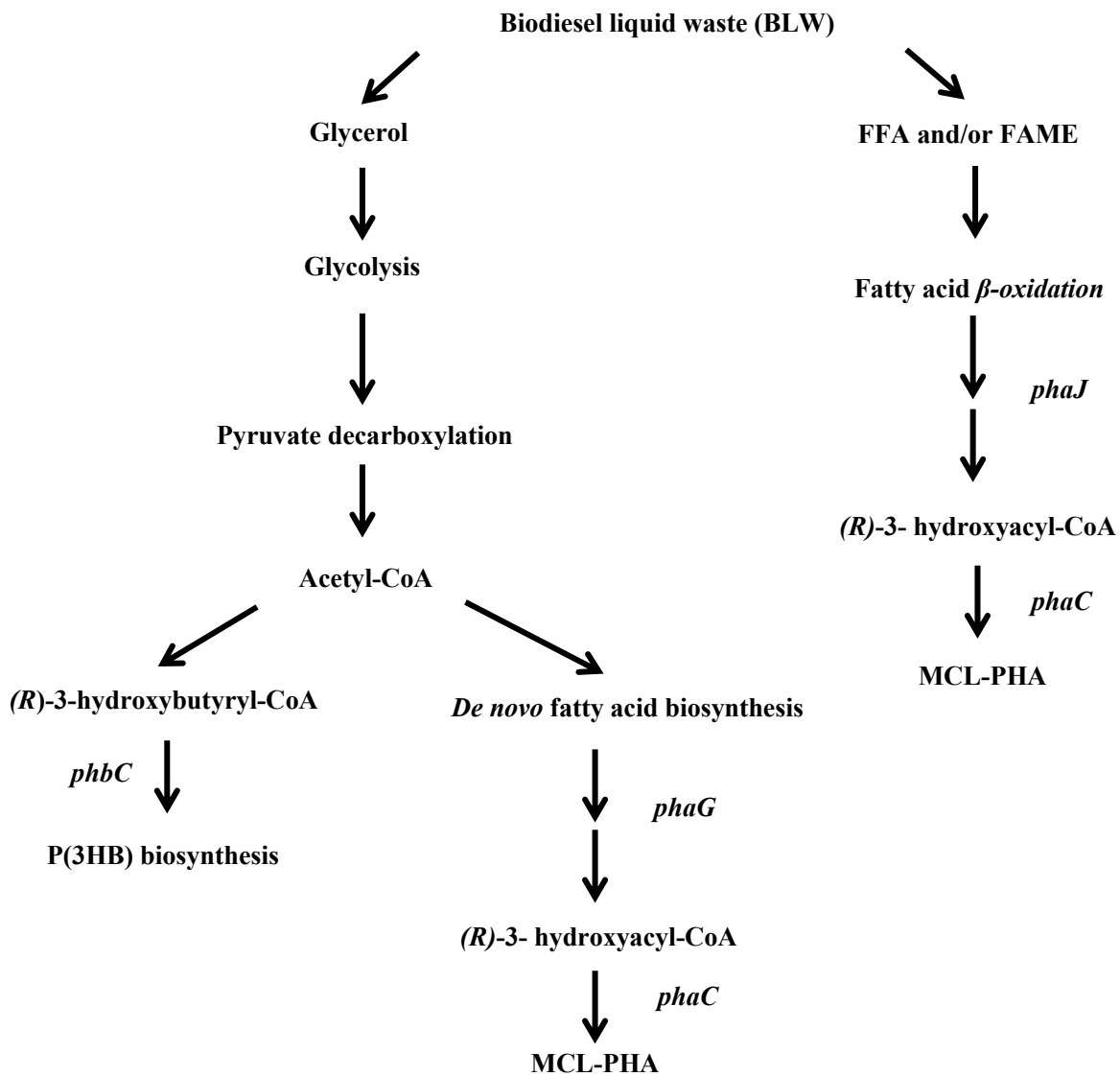
**Figure 22** Cell growth (A) and PHA content (B) of PM-PSU cultivated in MSM supplemented with 20 g/L of various carbon sources at 35°C and an initial pH of 7.0 with a shaking speed of 200 rpm. Each data point is a mean value of three independent experiments and the vertical bar represents the standard deviation



**Figure 23** Molar fractions and monomer compositions of the produced PHA from PM-PSU during cultivation with (A) Lauric acid (B) Palmitic acid (C) Oleic acid (D) Pure glycerol (PG) (E) PG + Oleic acid (F) BLW

Not only is P(3HB) accumulated but this bacterium could produce MCL-PHAs e.g. 3HO and 3HD through *de novo* fatty acid biosynthesis from glycerol due to the presence of *phaC<sub>Ps</sub>* that encoding for MCL-PHA synthase. A structurally unrelated carbon source, glycerol, provided acetyl-CoA that was then converted to malonyl-CoA and then malonyl-ACP to enter the *de novo* fatty acid biosynthetic pathway and eventually generated (*R*)-3-hydroxyacyl-ACP intermediate that was converted to the (*R*)-3-hydroxyacyl-CoA substrate for MCL-PHA polymerization by the function of the *phaG* gene encoding for 3-hydroxyacyl acyl carrier protein (ACP)-CoA transferase (Matsumoto et al. 2001; Lu et al. 2009). In addition, the BLW used in this study also contained FFA and/or FAME that was also used as another carbon source for MCL-PHA biosynthesis through the fatty acid  $\beta$ -oxidation pathway. The intermediates from the  $\beta$ -oxidation cycle can be converted to the (*R*)-3-hydroxyacyl-CoA, precursor for MCL-PHA synthesis, by the action of the *phaJ* gene encoding for the (*R*)-specific enoyl-coenzyme A (CoA) hydratase, epimerase, and ketoacyl-CoA reductase activity (Madison and Huisman, 1999; Fiedler et al. 2002; Lu et al. 2009). Therefore, in this study, the change in the molar fractions during the cultivation when PM-PSU was grown with BLW strongly indicated that the availability of the carbon supply, the specificity of the PHA synthase and its specific metabolic pathways directly influenced the monomer percentages of the PHA produced (Kek et al. 2010). The proposed PHA biosynthesis through different metabolic pathways by PM-PSU when grown with the BLW is presented in **Figure 24**.

PHA produced by PM-PSU that consisted of 3HB, 3HO, and 3HD was expected to show a wide range of physical and mechanical properties that may provide similar properties to petroleum-based plastics such as LDPE (Nomura et al. 2008; Chen, 2010).



**Figure 24** The proposed PHA biosynthesis through different metabolic pathways by PM-PSU when grown with the BLW containing glycerol and FFA and/or FAME

## 12. Alginate production and its molecular weight

Extracellular polymeric substance such as exopolysaccharides (EPS), form a complex mixture of high MW polymers produced by this bacterium perhaps to protect the cells from harsh external environments as well as to provide an available energy and carbon source when the carbon substrate was limited (Wang and Yu, 2007; Saharan et al. 2014). The amount and types of EPS produced can vary extensively depending on many factors such as the bacterial strain, cultivation time, substrate, and growth state (Sheng and Yu, 2006).

It has become increasingly clear that alginate is one of the many EPS polymers that can be produced by a number of *Pseudomonas* species e.g. *P. aeruginosa* (Boyd and Chakrabarty, 1995; Pham et al. 2004; Remminghorst and Rehm, 2006b) and *P. mendocina* (Müller and Alegre, 2007). Alginate production probably plays a fundamentally important role in the ecological success of these microorganisms that often grow under environmental stress and/or starvation conditions (Rehm and Valla, 1997; Remminghorst and Rehm, 2006a; Hay et al. 2013). For example, Rehm and Valla (1997) found that under unbalanced growth conditions such as nitrogen or oxygen limitation, in addition to the intracellular accumulation of PHA, some bacteria, mostly from the genus *Pseudomonas* produced alginate.

During the observations made during PHA production in this study, it was noticed that in the cell-free culture medium there was obviously a high viscosity. This could be due to some secondary metabolites such as EPS being produced. Therefore, the produced EPS was extracted and its monomer composition was determined and compared with a commercial standard alginate (Sigma-Aldrich, USA) using liquid chromatography-mass spectrometry (LC-MS) analysis as described by Chaki et al. (2006).

According to the electrospray ionization mass spectrometry (ESI-MS) data, the major product ions at  $m/z$  177.1 stood for 1-deoxy- $\beta$ -D-mannuronic acid and/or 1-deoxy- $\alpha$ -L-guluronic acid. After these products were hydrolyzed under acid and a high temperature, 1-deoxy- $\beta$ -D-mannuronic acid and/or 1-deoxy- $\alpha$ -L-guluronic acid were converted to D-gluconic acid and/or L-gluconic acid as shown by the ions at  $m/z$  195.1 (**Table 5 and Figure 25**). However, the ions at  $m/z$  217.0 stood for acetylated 1-deoxy-4-enoyl- $\beta$ -D-mannuronic acid and/or acetylated 1-deoxy-4-enoyl- $\alpha$ -L-guluronic acid and were only found in the alginate produced from PM-PSU.



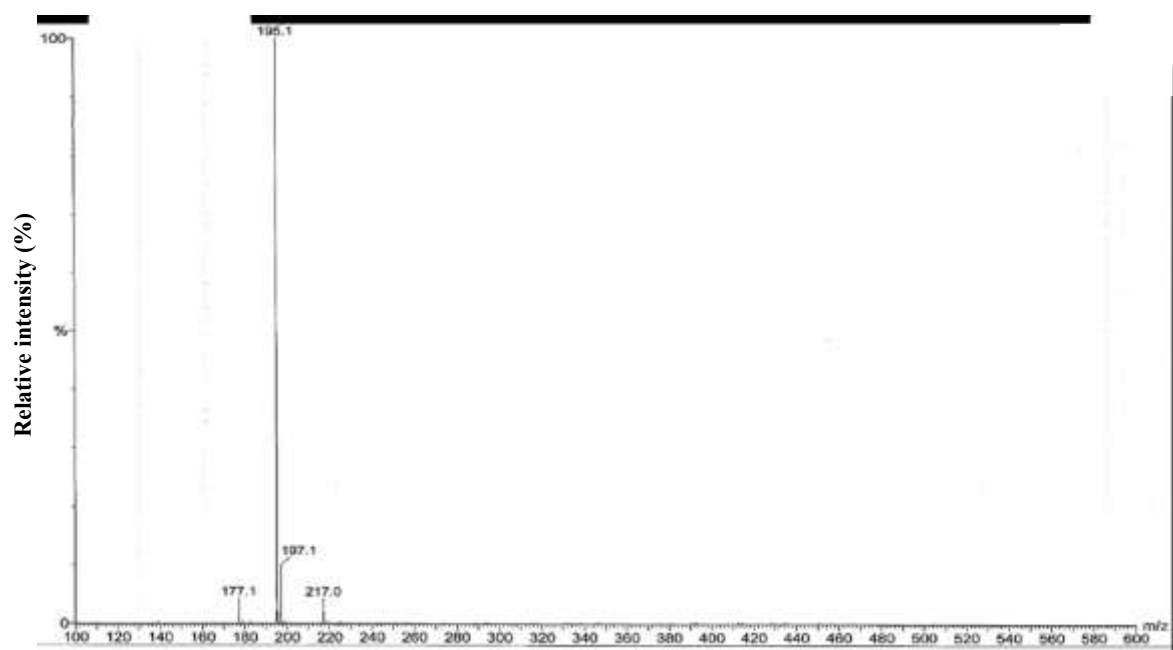
This result confirmed that the produced EPS from PM-PSU was alginate, in addition, acetylation of alginate at the hydroxyl groups at position C-2 and/or C-3 of the mannuronic acid residue had occurred and this is a normal occurrence in alginate producing-bacteria (Remminghorst and Rehm, 2006a, Hay et al. 2013). A similar result was reported by Müller and Alegre (2007), and the alginate synthesized by *P. mendocina* showed a similar acetylation pattern as that previously obtained from other *Pseudomonas* spp. and *Azotobacter vinelandi*.

**Table 5** The ESI-MS data of an acid hydrolyzed alginate sample from PM-PSU using commercial alginate as the standard in the negative ion mode

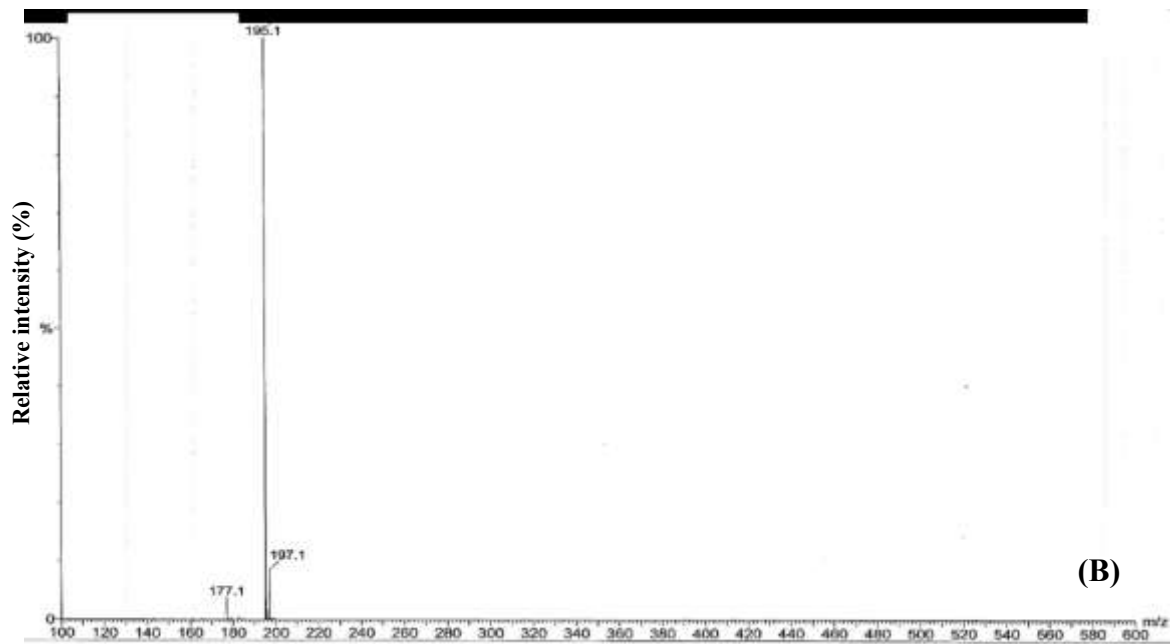
$m/z$ *		Products
Standard	Sample	
195.1	195.1	D-gluconic acid; L-gluconic acid
177.1	177.1	1-deoxy- $\beta$ -D-mannuronic acid; 1-deoxy- $\alpha$ -L-guluronic acid
ND	217	Acetylated 1-deoxy-4-enoyl- $\beta$ -D-mannuronic acid; Acetylated 1-deoxy-4-enoyl- $\alpha$ -L-guluronic acid

$m/z$ \* : mass-to-charge ratio

ND: not detected



(A)



(B)

**Figure 25** LC-MS analysis of the produced exopolysaccharide (EPS), alginate, from PM-PSU (A) and commercial standard alginate (B)

Moreover, the MW of the alginate produced from a commercial standard alginate and PM-PSU were determined by GPC analysis using pullulan as a standard. **Table 6** shows the average MW of the alginate produced from various bacteria and commercial. The average MW of the alginate produced from various bacteria and commercial. The average MW of the alginate produced from PM-PSU and commercial standard alginate (Sigma-Aldrich, USA) in this study were in the range of between 654,000-676,000 Da and 684,000 Da, respectively. In general, the MW of alginate varies considerably from 150 to 1700 KDa, depending on the source and extraction methods used (Moe et al. 1995).

**Table 6** Molecular weight of the alginate produced from various sources

Alginate producer	Molecular weight (Da)	References
<i>Azotobacter vinelandii</i>	270000-610000	Launey et al. 1986
<i>Azotobacter vinalandii</i>	154600-730000	Clementi et al. 1998
Alginate (Kimica)	59000-255000	Kakita and Kamishima, 2008
Alginate (FMC Biopolymer )	263000-541000	Fu et al. 2010 and 2011
Alginate (Sigma-Aldrich)	684000	This study
<i>Pseudomonas mendocina</i> PSU	654000-676000	This study

Alginate is a biomaterial that has found many applications in the biomedical and pharmaceutical industry due to its favorable properties including biocompatibility and ease of gelation. Bacterial alginate has been highly purified and has a high quality compared with alginate from seaweed. At present alginate hydrogels have been particularly attractive for wound healing, drug delivery, and tissue engineering because these gels showed similar structures to the extracellular matrices in tissues (Lee and Mooney, 2012) and are active in stimulating immune cells to secrete cytokines (Otterlei et al. 1991). In addition, alginate is applied in an immobilization matrix in both the food and non-food industry, for use in water treatment by increasing the flocculation process, and use as a reactive dye for printing in the paper and textile industry (Sabra and Deckwer, 2004).

## Conclusions

The newly isolated Gram negative PHA-producing bacteria e.g. PM-PSU were successfully isolated from environmental sources using a BLW, by-product from biodiesel production obtained from the biodiesel ethyl ester pilot plant, Faculty of Engineering, Prince of Songkla University, Thailand as the sole carbon source by a normal enrichment culture technique.

This is the first study to produce PHA from BLW by a *Pseudomonas mendocina* species and in addition, the production of SCL-PHA e.g. P(3HB) was presumably discovered in a *P. mendocina* spp. The accumulation of PHA was primarily observed by phase contrast microscopy and Nile red staining method. The colonies that produced strong fluorescence using the fluorescence microscope seemed to be perhaps the best candidates for PHA production. In this study, PM-PSU could efficiently convert BLW to PHA, up to 77%DCW of PHA with approximately 3.6 g/L of DCW but also accumulated an exopolysaccharide (EPS), alginate.

Due to BLW having a high carbon but low nitrogen content with glycerol and FFA and/or FAME as the major components, the microbial growth and PHA accumulation were supported and enhanced. Briefly, glycerol was converted to acetyl-CoA, the main P(3HB) precursor, through glycolysis and pyruvate decarboxylation process whereas the production of MCL-PHA occurred by *de novo* fatty acid synthesis from glycerol. In contrast, FFA and/or fatty FAME would be directly converted to (*R*)-3-hydroxyacyl-CoA, MCL-PHA intermediate, through the  $\beta$ -oxidation pathway. In addition to the carbon supply and the

metabolic pathways, the type of PHA produced primarily depended on the substrate specificity of the PHA synthase genes in each bacterium. PM-PSU possessed two types of PHA synthase genes e.g. *phbC<sub>Ps</sub>* and *phaC<sub>Ps</sub>* encoding for SCL- and MCL-PHA synthase that were responsible for the accumulation of SCL-PHA and MCL-PHA e.g. 3HO and 3HD, respectively.

In general, the waste from biodiesel production was contaminated with some impurities such as sodium and methanol from the transesterification process that decreased the growth and PHA yield because of osmoregulation; however, very low amounts of sodium (less than 0.5 %wt) and methanol were detected in the BLW used, thus the cell growth and PHA accumulation were not affected by high sodium ions and methanol in this study but when tested at 3.0 wt% of NaCl, there was a significant effect on the biomass and PHA content, and only 1.96 g/L of DCW and a PHA content of 9.26%DCW were obtained. The total glycerol concentration in the BLW seemed to strongly influence both microbial growth and PHA accumulation by those two bacteria. An increase of glycerol concentration decreased DCW and PHA content; however, at 2.0% (v/v) or 20 g/L of total glycerol in the BLW produced the highest cell growth and PHA production in most bacteria including PM-PSU. At 2.0% (v/v) total glycerol in BLW, the maximum 3.65 g/L of DCW and 76.89% DCW were obtained from PM-PSU. Any further increase in glycerol concentration in the BLW significantly decreased both DCW and PHA content. This can be explained in that at the higher glycerol concentrations there was a significant increase in osmotic stress that modified the cellular metabolism such as a decrease of enzyme efficiency and lower amount of biomass that also decreased the PHA yield. Although PM-PSU has growth-associated PHA production, so PHA accumulation and growth occurred simultaneously, some other parameters such as the type of nitrogen supply and the amount of nitrogen as indicated by the effects of the C/N in the culture medium, initial pH and incubation temperature also affected cell growth and PHA accumulation.

It was of interest that under starvation conditions, an alginate was produced by PM-PSU together with PHA production and reached 2.15 g/L at the end of cultivation. Due to its high quality and high purification of bacterial alginate, there could be several uses in the pharmaceutical industry and medical applications such as use as a hydrogel.

In conclusion, the present study has focused on the cost effective production of PHA by the use of inexpensive substrate e.g. BLW, a by product from biodiesel production, as the

sole carbon source for the growth of the newly isolated bacterial strain PM-PSU that efficiently converted the BLW to PHA as indicated by the production of a relatively high biomass and PHA content with about 45% of the growth substrate converted and at the same time this would help to eliminate or at least reduce a disposal problem associated with the excess BLW. In addition, under starvation conditions, not only PHA but alginate was produced by PM-PSU. These two products are promising for use in many medical and industrial applications.

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## FUTURE RESEARCH NEEDS

The current advances in fermentation, the development of PHA-producing bacterial strains such as the design of a mutant strain that can grow with a very low cost complex substrates and /or in waste materials and having the versatility to produce PHAs with a wide chemical variety mainly by modifications of the monomer composition and molecular mass by recombinant DNA technology, and in addition, the purification technology that would allow for the commercial production of PHAs. This present study has indicated some further prospects to achieve those goals as follows:

1. Genetic organization of PHA biosynthetic operons in PM-PSU was proposed to arrange the genes as *phbBAC*, that was a unique property of the *Pseudomonas* species that can produce two types of PHA granules. This will be further investigated by molecular biology techniques, for example, genome walking, is a method for determining the DNA sequence of unknown genomic regions flanking a region of known DNA sequence and ideal for identifying gene promoter regions where only the coding region (Shapter and Waters, 2014), for obtaining a better understanding in the regulation of the genes involved in PHA biosynthesis, in addition, it will be very useful for genetic manipulation or genetic engineering and/or improvement and fulfillment of the PHA biosynthetic pathways in PM-PSU.

2. In this study, the major monomer composition of the produced PHA by PM-PSU, is SCL-PHA i.e. P(3HB) that produces a brittle and stiff polymer; however, to modulate the molar fractions and type of the PHA accumulated e.g. an introduction of MCL-PHA into P(3HB) chain can be performed by genetically modifying the bacterial strains.

In addition, the composites and blending of PHAs with high MW or low MW materials have recently been attractive to many research groups because it is more economical and can produce a hybrid of PHA with desired properties.

3. The biosynthesis of PHA and alginate are directly related by competition for acetyl-CoA (Guo et al. 2011a and 2012). “*Alg8*” which is one of the alginate genes involved in alginate biosynthesis was disrupted with an insertion of one more copy of the PHA synthase gene “*phbC*” from PM-PSU that was found to increase the PHA content of the DCW by about 10% and reduce by about 60% alginate production. However, the biosynthesis of

alginate involves a cluster of genes (Hay et al. 2013), so only a modification to a single gene may not be sufficient to completely inhibit the alginate production by PM-PSU. However, the insertion of a PHA synthase gene may influence or have an effect on the PHA and/or alginate biosynthetic pathway; therefore, in a future experiment, the disruption of the alginate gene alone without any insertion will be further studied to determine the real function of this gene in alginate and PHA production in the hope that the biosynthesis of these two useful compounds can be efficiently controlled.

4. The chloroform extraction method used in this study is not an environmentally friendly method for PHA extraction/purification of its granule and also is a costly process. Therefore, perhaps the next generation of PHA extraction methods is to finding of a new biologically PHA granule extraction process.