CHAPTER FOUR

RESULTS & DISCUSSIONS

4.1 Comparison Between Batch and Fed-Batch Cultivations of P. oleovorans

4.1.1 OA as the Carbon Substrate

In this study, OA (at 0.5% w/v) was the sole carbon source in both batch and fed-batch cultivations. OA was initially immiscible in the nitrogen limiting E2 medium, however salt formation was observed as fermentation proceeded. The whittish suspension of fine salt of OA distributed evenly in the medium. No clumping of OA salt was observed at agitation of 300 rpm and above. Thus, to avoid solid aggregation the stirrer speed was adjusted to 300 rpm until the dissolved oxygen concentration (% saturation) reached 40% whereupon the cascade mode was initiated. In cascade mode the % saturation was set at 40% and thus the agitation speed was adjusted automatically to meet the requirement. Meanwhile, the aeration was manually changed from time to time to accommodate dissolved oxygen demand. To avoid any foam formation during vigorous agitation antifoam was added. In the fed-batch cultivation about 4.0 g of OA was added at 18, 30 and 42 hours. The addition was initiated after the nitrogen content started depleting.

It was observed that as the fermentation proceeded the medium became acidic as indicated by the inflow of more basic (2M NaOH) solution compared to acidic (2M H_2SO_4) solution in order to maintain the pH at 7.0. To monitor the growth of *P. oleovorans* on OA under this condition,

optical density was measured at 660 nm (Table 2). Figure 4 shows the pattern of growth. The bacteria took about 4 hours of adaptation to the new environment before entering the exponential growth. The presence of lag phase was due to change of environment encountered by the bacteria upon being transferred from nutrient rich medium to mineral medium.

In the batch culture the exponential growth occurred between 4 and 12 hours after inoculation. After 12 hours the OD dropped dramatically. The oxygen demand also decreased after 12 hours. This corresponded to a drop in ammonium content (0.0087 mg/ml) of the medium at approximately 12 hours (Table 4). The probable reason for the drop in OD was loss of bacterial cells due to lysis. To reach maximum growth rate bacteria should obtain energy, essentially from adenosine triphosphate (ATP) and increase the rate of DNA synthesis and replication (Chesbro *et al.*,1990). Absence of ammonia retarded the biosynthesis of cellular protein and hence impaired the duplication process. The transport of LCFAs into cell requires *fadD* and *fadL* genes products (Nunn, 1986). Thus, during nitrogen limitation the uptake of fatty acids would be hindered. Ineffective agitation could also result in poor substrates uptake, as a consequences the cell would utilise its internal carbon and energy reserve (PHA granule). This might explain the low PHA yield (2.50±0.77% CDW) obtained.

The specific growth rate during batch cultivation was 0.3491 per hour. The cell yield obtained was 1.45±0.19 g/l. The PHA cellular content was

Table 2

Optical density (660nm) of *P. oleovorans* grown on OA in batch and fed-batch system.

TIME (Hour)	BATCH				FED-BATCH			
	OD (660nm)				OD (660nm)			
	R1	R2	R3	Average	R1	R2	R3	Average
0	0.3025	0.2742	0.2518	0.2762	0.3255	0.3275	0.3295	0.3275
4	0.2840	0.2855	0.2835	0.2843	0.8045	0.7865	0.7955	0.7897
8	2.1868	2.8365	2.3220	1.5975	2.6420	2.5655	2.6090	2.6055
12	4.8635	4.3880	4.6775	4.6430	3.8160	3.8605	3.8090	3.8285
24	3.1125	4.1050	4.3485	3.8553	6.3670	5.9505	5.6610	5.9928
48	2.7440	2.4190	3.5085	2.8905	6.9765	6.9750	6.9760	6.9758

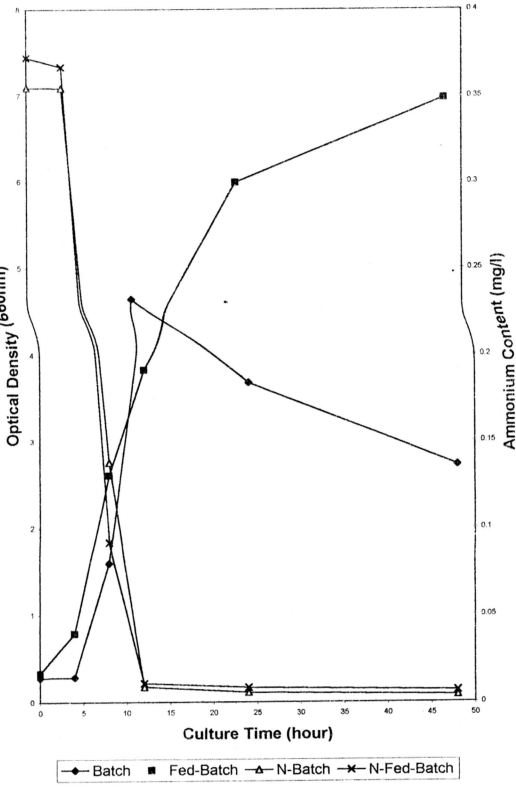

Figure 4

Optical density and ammonium content of cultures of *P. oleovorans* grown on OA.

2.50±0.77 % of CDW (Table 5) which is equal to a final PHA yield of 36.3 mg/l and overall PHA productivity was 0.76 mg/l/h. The total cell yield from OA ($Y_{x/s}$) was 322.2 mg/g and the PHA yield from OA ($Y_{p/s}$) was 8.05 mg/g.

4 g of OA was added intermittently (at 18, 30, 42 Hours) during fed-batch cultivation. It was noted that after each addition of OA during fed-batch cultivation the cellular oxygen requirement increased dramatically. The high demand for oxygen was indicated by the vigorous agitation to meet requirement of set % saturation. As noted in figure 4, the OD continued to increase after 12 hours of cultivation despite of the fact that ammonium was limiting. The growth pattern in fed-batch culture differed markedly from that of the batch culture.

Figure 4 shows that intermittent addition of oleic acid enhanced active growth for a longer period despite the limitation of nitrogen after 12 hours of fermentation. Under conditions of nitrogen limitation, many microbes derepress the synthesis of enzymes involved in the utilisation of organic nitrogen sources (Harder and Dijkhuizen, 1983). During the batch fermentation the cells entered stationary phase at approximately 12 hours. thus the availability of organic nitrogen from cell lysis and excess carbon sources explained an increase in OD despite the nitrogen limitation during the fed-batch cultivation. In the fed-batch culture cell density increased to 3.35±0.78 g/l and PHA yield to 32.72±0.30% CDW. Table 5 shows the comparison of cell and PHA yields between batch and fed-batch cultivation. A

significant increase in CDW as well as PHA content was observed in the fed-batch culture compared to the batch culture.

4.1.2 SPO as the Carbon Substrate

The same batch and fed-batch cultivation strategies were employed when SPO was used-as sole carbon source except that, during the fed-batch cultivation 50 ml of milky solution of SPO (50mg/ml) was added intermittently. This equivalent to 2.5 g of SPO added each time. Active metabolism was observed after each addition judging from vigorous agitation due to high cellular oxygen demand. Due to appearance of undissolved salts of fatty acids the growth was monitored by cell viability (CFU/ml). Oleic acid and palmitic acids are the dominant fatty acids present in SPO (Table 1).

Figure 5 shows that SPO supported growth to $11.3X10^9$ CFU/ml in batch culivation at 12 hours and then viable cells declined to $8.9X10^9$ $CFUml^{-1}$ at 48 hours (Table 3, Figure 5). SPO existed in the form of suspended salts of fatty acids. Insufficient mixing may limit the cellular uptake. Hence, poor utilisation of SPO could result in poor cell and PHA yield. The final cell and PHA yield obtained from batch cultivation on SPO were 1.54±0.03 g/l and 3.4±0.76 % CDW, respectively (Table 5). The specific growth rate (μ) was 0.2164 per hour. The total cell yield from SPO ($Y_{x/s}$) was 308.0 mg/g and PHA yield from SPO ($Y_{p/s}$) was 10.47 mg/g. The PHA yield was 52.4 mg/l and overall PHA productivity was 1.1 mg/l/h.

Table 3

Viable cell count of *P.oleovorans* grown on SPO in batch and fed-batch system.

TIME (Hour)	BATCH				FED-BATCH			
	CFU/ml X 10^9				CFU/ml X 10^9			
	R1	R2	R3	Average	R1	R2	R3	Average
0	1.4	1.4	1.5	1.4	1.6	1.3	1.6	1.5
4	1.4	1.3	3.2	2.0	2.9	2.3	2.4	2.5
8	5.0	5.2	6.5	5.6	4.3	6.5	5.0	5.3
12	9.8	12.0	12.0	11.3	8.5	8.9	12.3	9.9
24	8.5	9.9	9.6	9.3	12.6	9.2	14.6	12.1
48	9.5	8.5	8.7	8.9	21.6	22.6	18.2	20.8

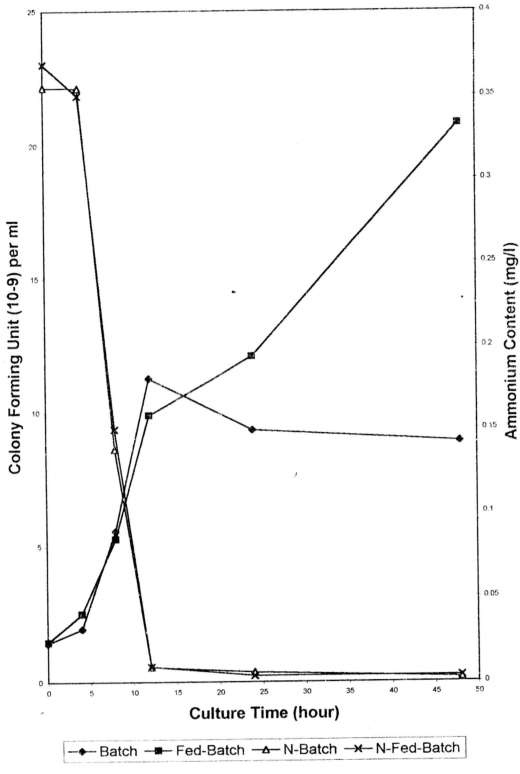

Figure 5

Viable cell count and ammonium content of cultures of *P. oleovorans* grown on SPO.

Table 4

Data on the change of Ammonium content of the cultures during 48 hours of cultivation in batch and fed-batch system when *P. oleovorans* cultivated on OA or SPO.

***Average of triplicate**

TIME (hour)	OA		SPO	
	Ammonium content (mg/l)		Ammonium content (mg/l)	
	Batch	Fed-batch	Batch	Fed-batch
0	0.3549	0.3722	0.3549	0.3685
4	0.3546	0.3668	0.3545	0.3502
8	0.1378	0.0920	0.1378	0.1499
12	0.0087	0.0108	0.0089	0.0089
24	0.0056	0.0083	0.0056	0.0063
48	0.0042	0.0067	0.0024	0.0035

Table 5

Comparison of biomass and PHA content obtained from batch and fed-batch system when *P. oleovorans* cultivated on OA or SPO.

[a]Cell Dry Weight (CDW)±Standard deviation (SD) (n=3)
[b]Amount of Polymer, % CDW±SD (n=3)

Substrate 0.5% w/v	BATCH		FED-BATCH	
	Biomass Yield[a] (g/l)	PHA Content[b] (% CDW)	Biomass Yield[a] (g/l)	PHA Content[b] (% CDW)
OA	1.45 ± 0.19	2.50 ± 0.77	3.35 ± 0.78	32.72 ± 0.30
SPO	1.54 ± 0.03	3.40 ± 0.76	1.9 ± 0.66	14.46 ± 1.18

In fed-batch cultivation the viable cell count increased exponentially to $20.8X10^9$ $CFUml^{-1}$. Availability of supplemented carbon substrate enabled the cells to maintain the energy requirement for growth and PHA accumulation under nitrogen limitation. The condition resulted in a cell yield of 1.9±0.66 g/l and PHA content of 14.46±1.18% CDW (Table 5).

The cell yield and PHA content observed were inferior compared to those obtained from fed-batch cultivation with addition of OA (Table 5). This might be due to insufficient amount of each addition of SPO (2.5 g) compared to OA (4.0 g). Addition of SPO had resulted in appearance of solid suspension which sometimes clumped together. Uneven distribution of SPO resulted in less efficient mass transfer and thus affected the final cell yield and PHA content. Though the cell and PHA yield were not as large as those obtained when grown fed-batch on OA, a significant increase in the accumulation of PHA was observed in the fed-batch system when compared to the batch system (Table 5). This suggested that during carbon supplementation, in fed-batch some of the carbon substrate was used for PHA synthesis but not for biomass generation.

4.2 Analysis of PHA from P.oleovorans

Palm olein is composed predominantly of unsaturated oleic acid (42%) and saturated palmitic acid (40%) (Table 1). The composition of the MCL_{PHA} is controlled by the specificity of the PHA-synthesizing system,

the structures of the fatty acids and the degradation pathway for long-chain fatty acids. (Eggink *et al.*,1993). Reports have shown that 3-hydroxyacyl-coA intermediates araising from degradation of fatty acids in β-oxidation serve as precursors for PHA biosynthesis in *Pseudomonas putida* (Eggink *et al.*,1995, Lageveen *et al.*,1988). Due to enhanced enzyme specificity of PHA synthase for 3-hydroxyhexanoyl-coA, 3-hydroxyoctanoyl-coA and 3-hydroxydecanoyl-coA, MCL_{PHA} generally contain a high concentration of C_6, C_8 and C_{10} monomers. Tan *et al.*, 1998, investigated the possible incorporation of 3-hydroxybutyryl groups into PHA produced by *P. oleovorans*. It was found that sodium butyrate could support limited growth but not PHA accumulation. It is known that SCL_{PHA} is not accumulated by *P.oleovorans*, as it is synthesised through different metabolic route from that of MCL_{PHA}.

4.2.1 IR Analysis

In the present study the extracted polymer was sticky in character. The infrared spectrum of the polymers produced by *P.oleovorans* grown on SPO and OA were indentical and showed strong carbonyl peaks of an ester (Williams and Fleming, 1989) at 1741.55 and 1742.12 cm-l, respectively (Figure 6).

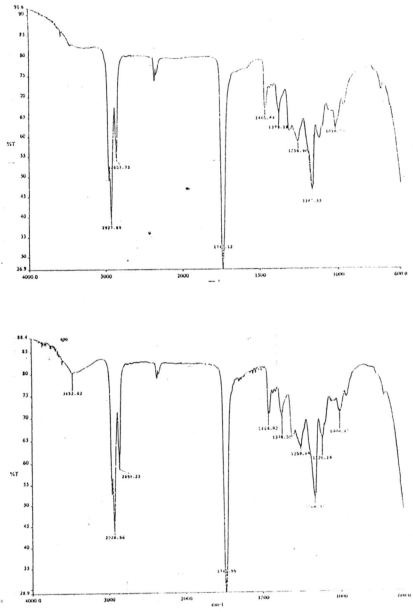

Figure 6

IR spectrum of PHA extracted from *P. oleovorans* grown on (A) OA and (B) SPO.

4.2.2 ^{1}H NMR Analysis

Figures 7 and 8 show the ^{1}H NMR spectra of PHA from OA and SPO respectively. The signal designated '***a***' was due to methylene protons (CH_2), '***b***' was due to methine proton (CH), and '***c***' was due to the first methylene protons appearing on the side chain. Signals at 0.8342-0.9013 ppm (Figure 7) and 0.7926-0.8598 ppm (Figure 8) were due to methyl protons '***e***'. High intensity signals at 1.2351 ppm (Figure 7) and 1.1868 ppm (Figure 8) were due to overlapping of methylene protons present on the side chain. In figure 8, when grown on OA, additional resonances appeared in the PHA. These resonances, indicated by arrows, suggested the presence of unsaturation in some of the monomers. The unsaturation was confirmed to occur in OA-derived PHA when analysed on ^{13}C NMR. Such resonances were not obvious in the PHA produced from SPO (Figure 8).

4.2.3 ^{13}C NMR Analysis

The presence of signals at 120–140 ppm of the ^{13}C NMR of OA-derived PHA (Figure 9) suggested the appearance of carbon with double bond (CH=CH). This is not observed in the ^{13}C NMR spectrum of SPO-derived PHA (Figure 10). Signals at 169.522 ppm (OA) and 169.572 ppm (SPO) were due to overlapping signals of carbonyl carbon (-CH=O) on

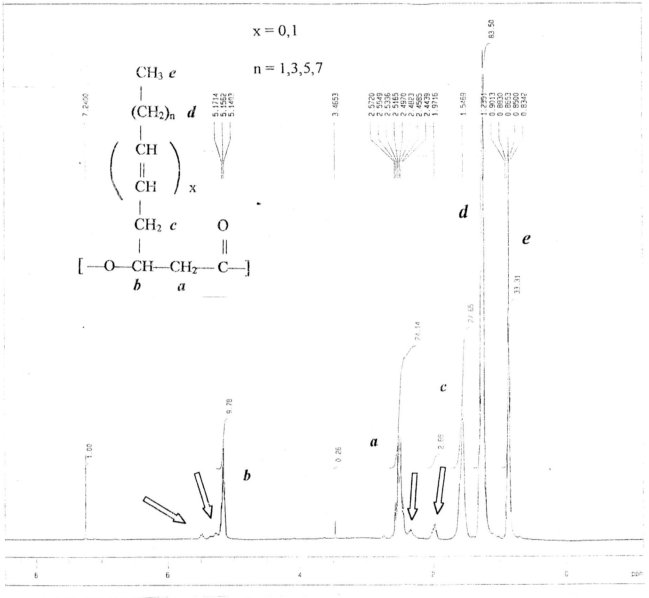

Figure 7

The ^{1}H NMR spectrum of PHA extracted from *P. oleovorans* grown on OA.

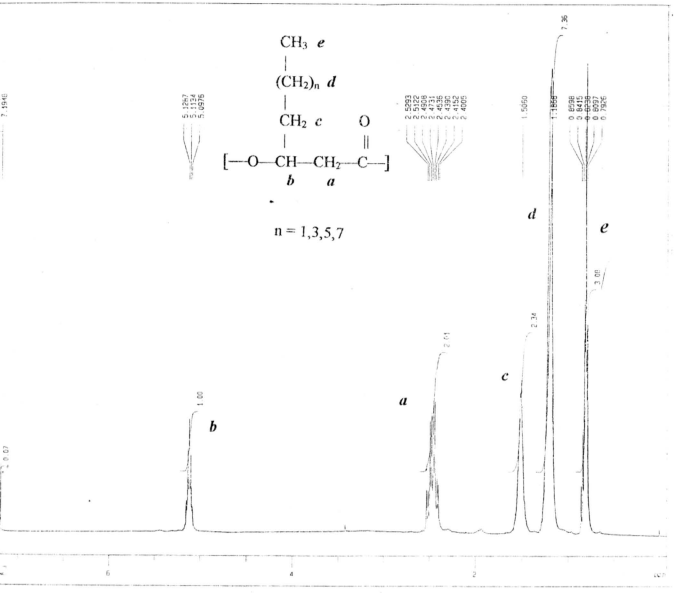

Figure 8

The ^{1}H NMR spectrum of PHA extracted from *P. oleovorans* grown on SPO.

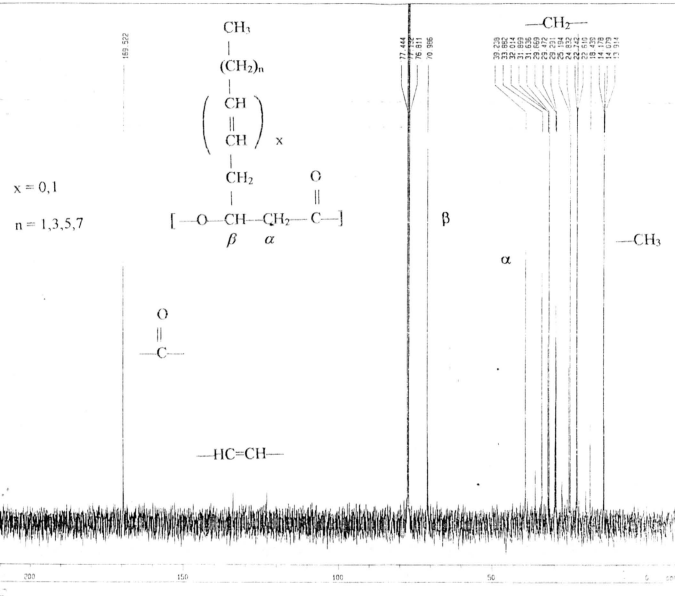

Figure 9

The ^{13}C NMR spectrum of PHA extracted from *P. oleovorans* grown on OA.

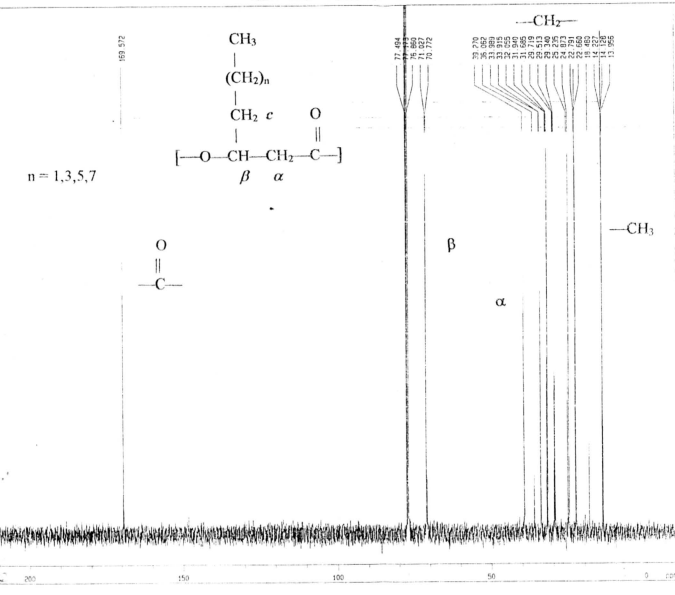

Figure 10

The ^{13}C NMR spectrum of PHA extracted from *P. oleovorans* grown on SPO.

different monomers. The carbonyl resonances of different saturated monomers were not resolved in distinct peaks and appeared at the same chemical shift. Signal at 70.986 ppm (OA) and 71.027 ppm (SPO) were due to methine carbon designated β and signals at 39.238 ppm (OA) and 39.270 ppm (SPO) were due to main chain methylene carbon designated α. Carbon bearing side chain methylene protons on the side chains resolved at different chemical shifts and located in the region 18.0 ppm to 35.0 ppm. These different methylene carbons resolved at distinct peaks and showed chemical shift comparable to those described by Gross *et al.*, (1989) and Tan *et al.*, (1997). The terminal methyl carbon resolved further upfield which is 13.0 to 14.0 ppm. The chemical shift of the methyl carbon of the longest pendant group appeared downfield. Thus data from the NMR analysis concluded that the PHA derived from SPO and OA were a MCL_{PHA}.

4.2.4 GC Analysis

GC analysis revealed the composition of the PHA. Figure 16 and 17 show the GC spectrum obtained from OA and SPO-derived PHA, respectively. The retention time of monomers from OA and SPO-derived PHA corresponded to those 3-hyhydroxyalkanoic acid methyl ester standards (C_8=15.937 min, C_{10}=20.435 min, C_{12}=24.548, C_{14}=28.333 min, C_{16}=32.917) observed in figure 11. The presence of additional peaks at retention times approximately 7.0 and 10.4 mins (adjacent to peak of internal standard) (Figure 16 and 17) suggested that those were C_4 and C_6 respectively. These

were deduced from the equation of regression line in figure 12. The concentration of the peaks were determined as stated in section 3.12.3.2. Table 6 shows that C_8 was the dominant monomer obtained in both OA and SPO-derived PHA. This confirmed the enhanced ability of the PHA synthase to polymerise 3-hydroxyoctanoyl-coA compared to others. The carbon number of the monomer differed by two carbon atoms. It reflected the substrates which comprised of even carbon number fatty acids.

It also suggested that intermediates of β-oxidation served as precursors of PHA biosynthesis due the presence of even carbon number of shorter chain length monomers. 3-hydroxybutyrate was also detected in both OA and SPO-derived PHA produced by *P. oleovorans*. The R_t of C_4 detected in the PHA (Figure 16 and 17) corresponded to the R_t of the 3-hydroxybutyric acid methyl ester standard (Figure 20B) and C_4 detected in the isolate FLP1 (Figure 20A). The accumulation of trace amount of PHB from *Pseudomonas oleovorans* has also been reported (Gross, *et al.*,1989 and Preusting *et al.*, 1990).

The appearance of C_4 monomer was rather interesting as it is known that the strain produces only a MCL_{PHA}. The possible effect of SPO and OA on the incorporation of C_4 is worth examining. However, one should not eliminate possible contamination caused by the presence of complex PHB (c-PHB) which is a low molecular weight PHB present in the plasma membrane (Reusch, 1995).

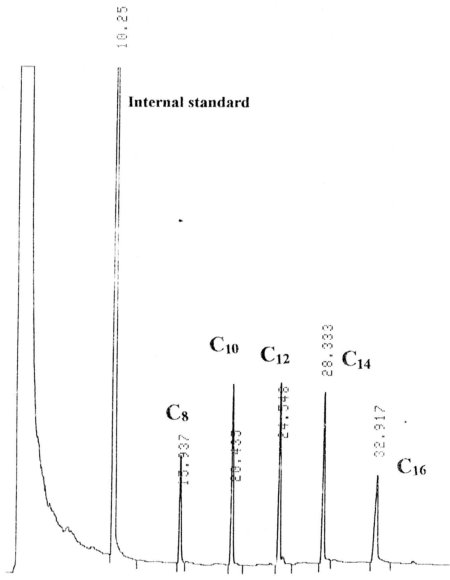

Figure 11

The GC chromatogram of polyhydroxyalkanoic acid methyl ester standards. Benzoic acid methyl ester is the internal standard (0.1% v/v).

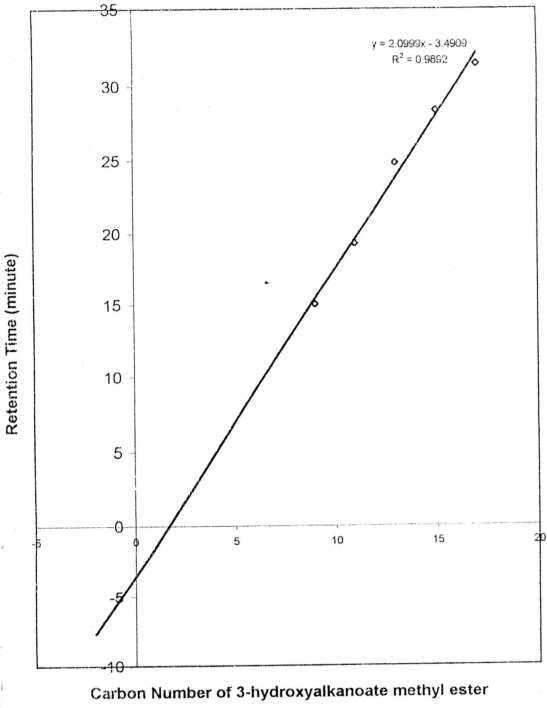

Figure 12

Standard graph for retention time vs carbon number of 3-hydroxyalkanoate methyl ester.

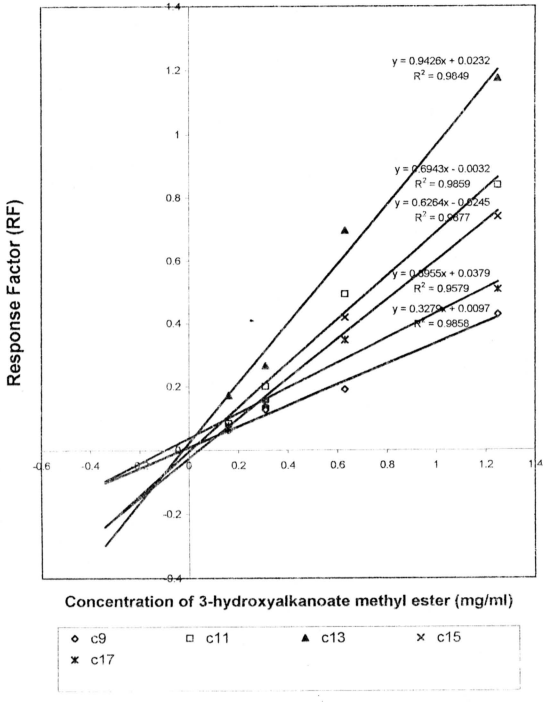

Figure 13

Standard graph for response factor vs concentration of methyl esters of 3-hydroxyalkanoates (C8,C10,C12,C14,C16).

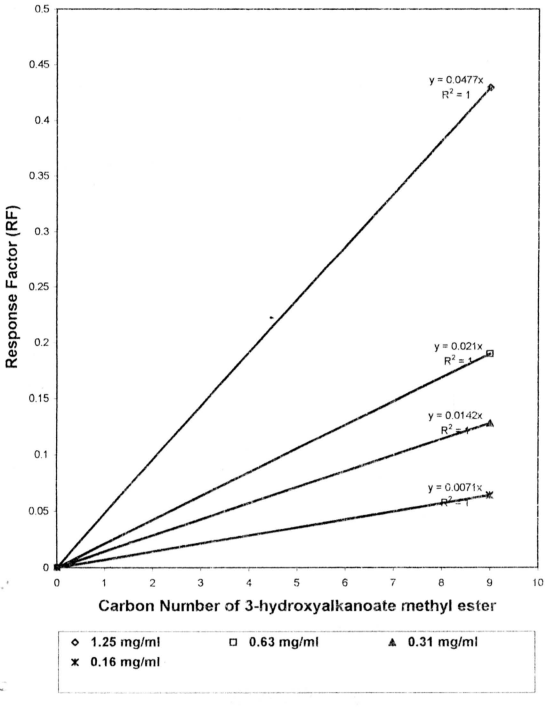

Figure 14

Standard graph for response factor vs carbon number of 3-hydroxyalkanoate methyl ester of different concentrations.

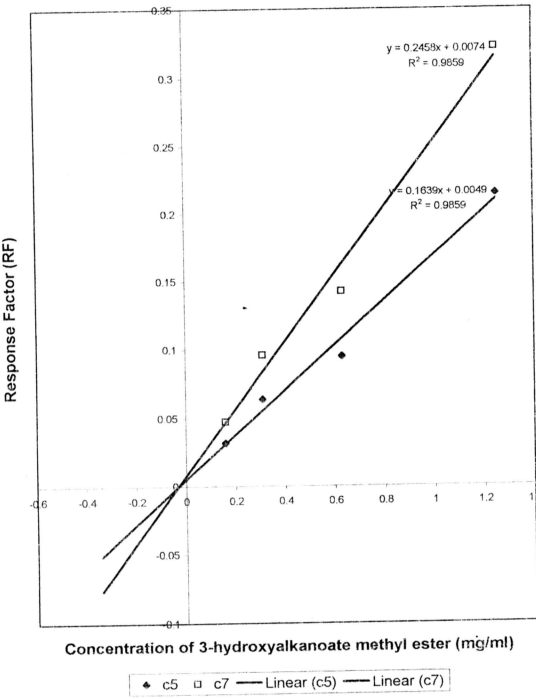

Figure 15

Standard graph for response factor vs concentration of methyl esters of 3-hydroxyalkanoates (C4,C6).

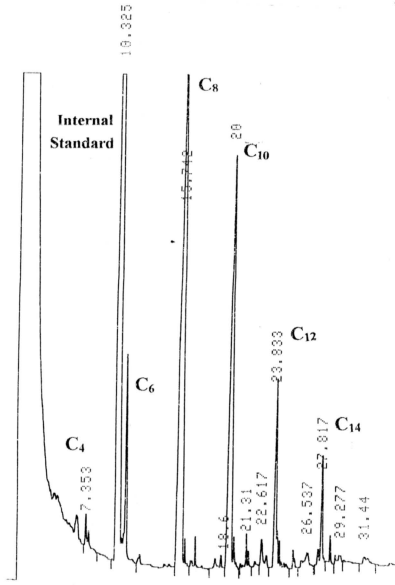

Figure 16

The GC chromatogram of the PHA composition accumulated by *P. oleovorans* grown on OA.

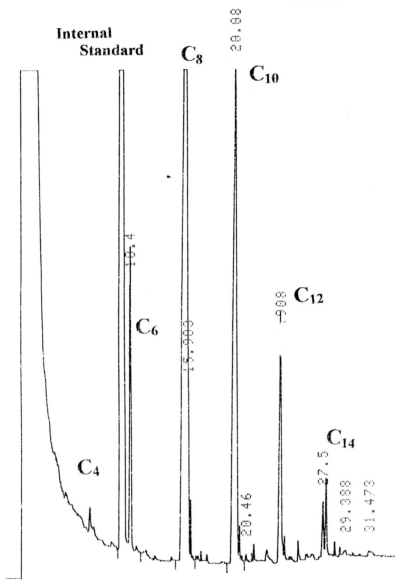

Figure 17

The GC chromatogram of the PHA composition accumulated by *P. oleovorans* grown on SPO.

Table 6

Monomer composition of PHA isolated from *P. oleovorans* cultivated from OA or SPO. Results show the average mole % (n=2) as determined by gas chromatography of the β-hydroxyalkanoate methyl esters obtained by acid hydrolysis of the PHA polymers.

Substrate	Relative amount of monomers in purified PHA (mol %)					
	C4	C6	C8	C10	C12	C14
OA	2.5	10.5	55.1	13.7	3.4	4.4
SPO	2.5	10.6	57.6	14.8	3.1	2.6

The incorporation of C_4 could be explained by cometabolism, however Tan *et al.*, (1998) reported that C_4 was not detected from PHA isolated from *P. oleovorans* fed with sodium butyrate and sodium octanoic. At this point, it would be premature to conclude that *P. oleovorans* had accumulated C_4 monomer from OA and SPO, in addition to the other medium chain length monomers.

4.3 Isolation and Screening of Isolates from POME

POME ponds have high content of oil (triacylglycerols), diacylglycerols, monoacylglycerols and fatty acids (Redzwan *et al.*, 1997). The environment offers possibility of isolating oil-utilising PHA producers. As POME is rich in lipids, it could be habitated by bacteria which are capable of utilising oil and its degradative products for growth and metabolism including PHA production. Redzwan *et al.*, (1997) reported that about 48% of lipid-positive isolates from POME contained PHA. Therefore, POME was selected as site to screen for oil-utilising PHA producers.

Serially diluted samples from each pond were spread on solid medium (Appendix A.4). Single colonies were purified and later stained with Sudan Black B as the first-line screening process. Sudan Black B stained lipidic granules inside cells and their presence was an indication that the bacteria were able to accumulate lipidic granules including PHA. This

assumption was based on work by Williamson and Wilkinson (1958), where 89% of lipidic granule inside *Bacillus megaterium* was PHA.

In this study 45 pure isolates obtained from three different POME ponds were subjected to Sudan Black B staining (Appendix B.1) The lipidic granules were viewed under light microscope. Out of 45, only 10 isolates were stained positively with Sudan Black B (Plate 2). The negatively-stained isolates were considered to be unable to produce PHA (Plate1).

The potential PHA producers were subjected to a two-stage cultivation. In the first stage isolates were grown in nutrient rich medium (Appendix A.2) for biomass production and then transfered into nitrogen limiting E2 medium (Appendix A.3) containing palm olein as sole carbon source, to stimulate PHA accumulation. None of the isolates was able to grow on palm oil as carbon substrate after even 86 hours. This was indicated by change in turbidity and colour of the cultures. Thus, saponified palm olein was used as carbon source. All the isolates grew on saponified palm olein after 48 hours of cultivation.

The cells were smeared on glass slide, heat-fixed and stained with Nile Blue A (Appendix B.2). The stain was employed as it was more conclusive for detecting PHA ganules in cells. All isolates were able to grow on SPO, however only one isolate, EX4.13 was stained positively with Nile Blue A (Plate 2). This suggested hat EX4.13 was able to grow and

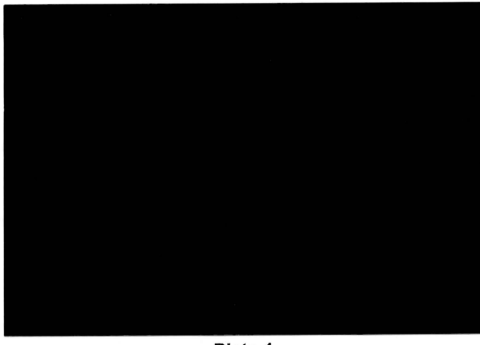

Plate 1

Isolate grown at 48 hours negatively-stained with Sudan Black B (400X magnification)

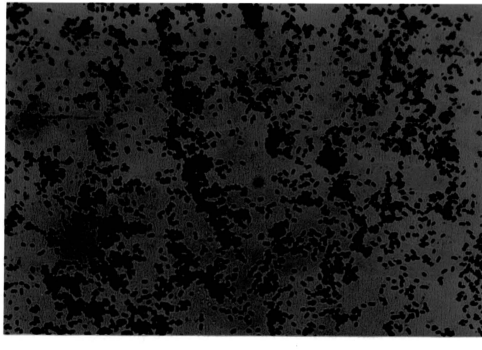

Plate 2

Isolate grown at 48 hours positively-stained with Sudan Black B (400X magnification)

accumulate PHA from SPO under unbalanced condition. This isolation procedure did produce a large number of bacterial isolates which are capable of utilising fatty acids for growth but not for PHA accumulation. Furthermore, none of the isolated bacteria could utilise palm oil directly.

The result was suprisingly poor when considering that the site of sampling was an oil rich environment. One possible cause may be the isolation procedure. Diluted samples were spread on agar containing complex substrates but not palm oil. Thus, the bacteria which grew on the agar plates had not been selected to use palm oil as carbon source. Furthermore, the several 'isolate-purification' steps in which single colonies were repeatedly streaked on nutrient agar might have contributed to the isolates losing their ability to utilise palm oil. Thirdly, during the cultivation the accumulation of PHA was supposedly triggerd by depletion of nitrogen.

However, It had been reported that certain types of nutrient limitation in the media may also affect the isolation of PHA producing strains. Senior *et al.*, (1972) reported that accumulation of PHA in *Azotobacter beijerinckii* was triggered by oxygen limitation rather than by nitrogen limitation. Thus, possibly some of the PHA producers had been left out due to unsuitable environment for PHA accumulation. Lastly, some of the PHA producers might not have been detected due to assimilation of PHA during sporulation. Formation of endospores reduced the PHA content in *Bacillus cereus* (Kominek and Halvorson, 1965). It was suggested that E2 medium

was a potential contributing factor for sporulation. E2 medium was formulated to stimulate PHA accumulation in *P. oleovorans*, and certain elements in the composition may also encourage sporulation. Halvorson, (1957) prepared sporulation medium for rapid and simultaneous sporulation in asporogenous bacteria. All minerals found in the sporulation medium were also present in E2 medium.

4.4 Isolation by Enrichment

Due to unsuccessful isolation of bacteria capable of utilising palm oil for growth and PHA accumulation, we proceed to carry out isolation by enrichment technique. A quantity of POME (2.5% v/v) was added to a mineral medium (Appendix A.5) containing palm oil as sole carbon source. After a month of cultivation a loopful of the culture was spread on nutrient agar plate. Seven pure isolates were obtained and purified on nutrient agar. Each purified isolate was subjected to a two-stage cultivation. In the second stage, E2 medium containing palm olein (PO) (1% w/v) was used. Nitrogen limiting E2 medium was used despite the possible loss of PHA due to sporulation in *Bacillus* sp. because nitrogen limitation triggered high PHA accumulation compared to the other nutrient limitation (Steinbuchel and Schlegel, 1989).

Out of the seven isolates, two were able to grow at 48 hours. Growth was indicated by an increase in turbidity of the culture and a change

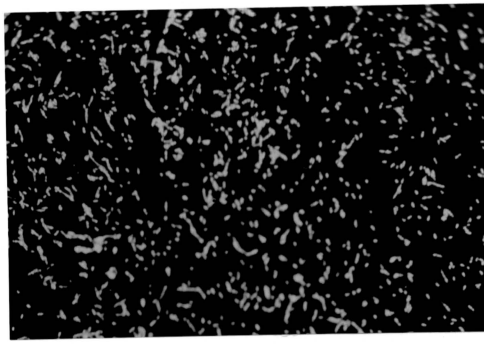

Plate 3

Isolate grown at 48 hours positively-stained with Nile Blue A (400X magnification). PHA granules appear fluorescent orange

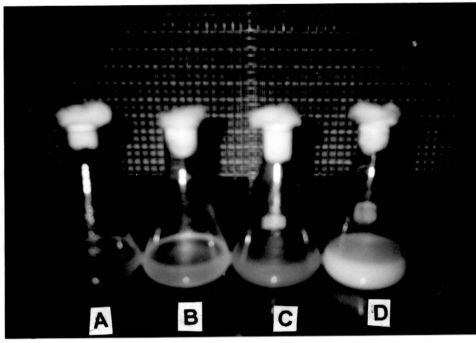

Plate 4

Observation of growth based on changed in turbidity and colour of cultural broth at 48 hours of cultivation in E2 medium (1% w/v PO).
(A) Absence of bacteria. (B) Presence of *P. oleovorans*. (C) Presence of FLP1. (D) Presence of FLP2.

in colour (Plate 4). The remaining five isolates showed no significant growth even at 5 days of cultivation in E2 medium. The ability to utilise PO directly suggested that both isolates produced lipase, which split the triglycerides into their fatty acids which were then utilised for metabolism. The remaining isolates would probably be able to assimilate the degradative products provided by the activity of PO utilising bacteria in the natural POME environment. However, by themselves they would be unable to use PO directly due probably to the absence of lipase. The two isolates, named FLP1 and FLP2, both stained positively with Nile Blue A (Plate 3) indicating that they accumulated PHA. The polymer from the isolates was extracted by solvent extraction. The resulting purified polymer was a whittish brittle material.

4.5 Biomass, PHA Content and Monomer Composition of the Isolates

Table 7 shows that when grown on PO for 48 hours, FLP1 gave a CDW of 4.2 g/l of which 50.0% was PHA. In comparison, FLP2 produced 2.0 g/l CDW comprising 12.6% PHA under the same conditions. Due to the promising results, the superior isolate (FLP1) was seen as a good PHA producer. It was grown in other palm oil related carbon substrate and the CDW and %PHA was calculated as in Table 7.

Table 7

Biomass yield, PHA content and monomer composition obtained from isolate FLP1 and FLP2 cultured in different carbon sources.

[a]Cell Dry Weight (CDW) (n=3)
[b]Amount of Polymer, % CDW (n=3),
[c]3-hydroxybutyric acid

Isolate	Carbon Substrate 1% w/v	Biomass Yield[a] (g/l)	PHA Content[b] (% CDW)	Composition of PHA (mol %)
				3HB[c]
FLP 2	Palm Olein	2.0	8.6	100
FLP 1	Crude Palm Oil	4.2	50.0	100
	Palm Kernel Oil	3.9	43.6	100
	Palm Olein	5.1	43.1	100
	Palm Stearin	4.3	57.4	100
	Oleic Acid	3.1	54.9	100

The PHA accumulated by FLP1 grown with different carbon sources was analysed with NMR. Table 8 shows that all of the extracted polymers comprised of four distinct signals when analysed in 100 MHz ^{13}C NMR (Figure 18). This suggested that only four distinct types of carbon atoms were present in the structure of the polymer. Each of the signals was due to the corresponding carbon as shown. The chemical shifts of these signals are identical to those reported by Doi *et al.*, (1986), where δCH_3 was at 19.79 ppm, δCH_2 was at 40.82 ppm, δCH was at 67.64 and $\delta C=O$ was at 169.16 ppm. They concluded that the polymer extracted from *Bacillus megaterium* was characterised as PHB.

The polymers were then analysed with 400 MHz ^{1}H NMR (Figure 19). The chemical shifts of the signals are summarised in Table 9. The spectrum is also identical to that obtained by Doi *et al.*, (1986), where doublet resonance at 1.274 ppm was assignable to methyl protons (CH_3), multiplet resonance at 5.26 ppm was assigned to methine proton (CH) and doublet of doublet and a quartet was due to the methylene protons (CH_2). Thus, it was concluded that the PHA was PHB. It was further confirmed with the appearance of only one peak which corresponded to standard 3-hydroxybutyric acid methyl ester when the sample was run on GC (Figure 20A).

Based on the results FLP1 was only able to accumulate SCL_{PHA} suggesting that the PHB was synthesised from condensation of two acetyl-

Table 8

Chemical shift assignments for ^{13}C resonance of PHB from isolates FLP1 and FLP2 grown on different carbon sources.

[a]Chemical shift (δ) are in ppm downfield from Me_4Si

Isolate	Substrate 1% w/v	$\delta^{13}C^a$			
		CH_3	CH_2	CH	C=O
FLP2	Palm Olein	19.896	40.932	67.744	169.267
FLP1	Crude Palm Oil	19.945	40.973	67.794	169.317
	Palm Kernel Oil	19.706	40.973	67.563	169.094
	Palm Olein	19.756	40.784	67.613	169.136
	Palm Stearin	19.706	40.743	67.563	169.086
	Oleic Acid	19.756	40.784	67.613	169.144

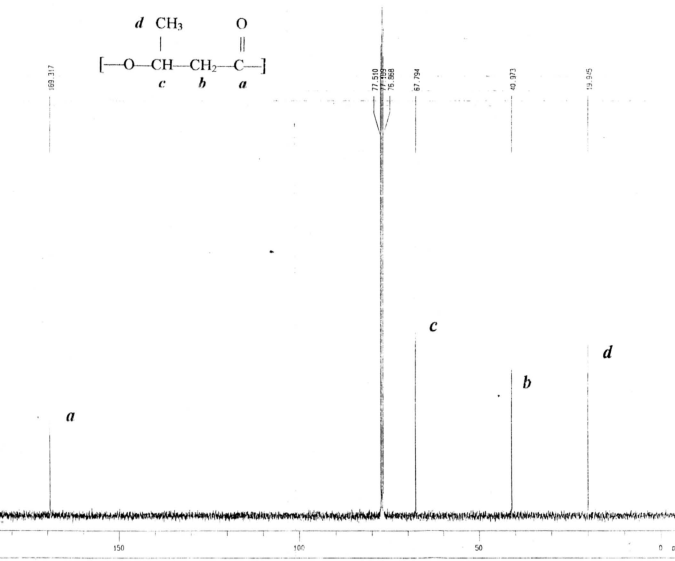

Figure 18

The ^{13}C NMR spectrum of PHA extracted from isolate FPL1 (*P. cepacia*) grown on palm olein.

Table 9

Chemical shift assignments for ^1H resonance of PHB from isolates FLP1 and FLP2 grown on different carbon sources.
[a]Chemical shifts (δ) are in ppm downfield from Me_4Si

Isolate	Substrate 1%w/v	δ^1H^a		
		CH_3 doublet	CH_2 doublet of doublet and quartet	CH multiplet
FLP2	Palm Olein	1.1844-1.2118	2.3767-2.5665	5.1641-5.2123
FLP1	Crude Palm Oil	1.1972-1.2125	2.3767-2.5671	5.1488-5.2288
	Palm Kernel Oil	1.2406-1.2558	2.4201-2.6104	5.1916-5.2715
	Palm Olein	1.270-1.2418	2.4219-2.6117	5.1928-5.2733
	Palm Stearin	1.2730-1.2889	2.4531-2.6435	5.2411-5.2893
	Oleic Acid	1.1978-2.2180	2.3785-2.5683	5.1494-5.2294

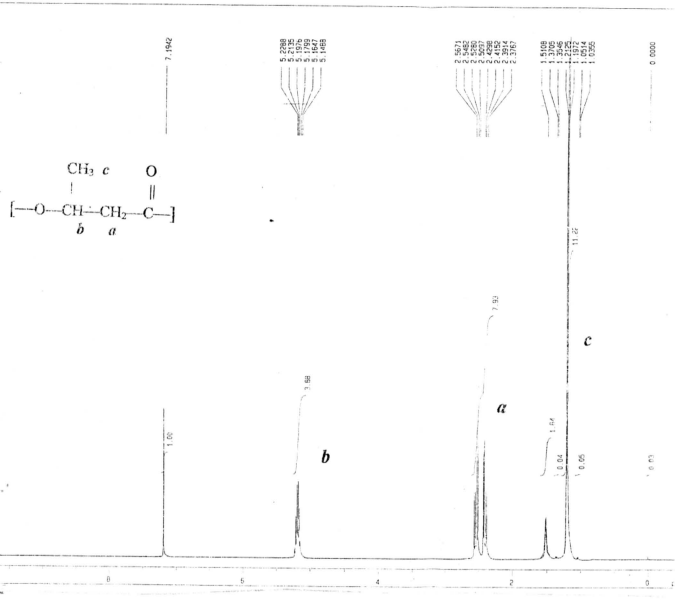

Figure 19

The ^{1}H NMR spectrum of PHA extracted from isolate FLP1 (*P. cepacia*) grown on palm olein.

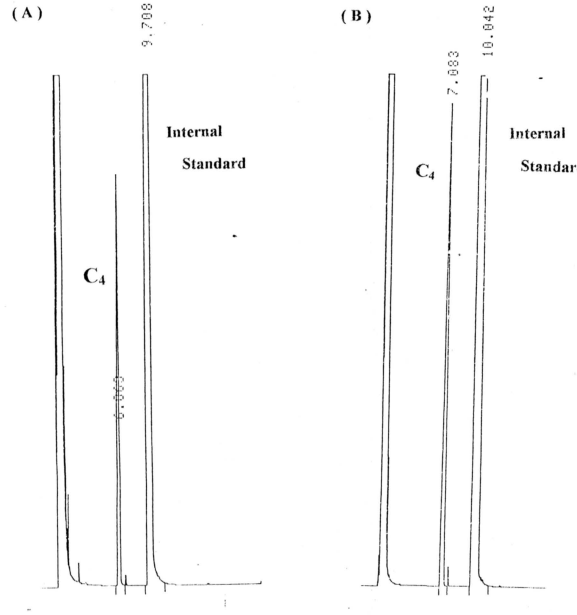

Figure 20

The GC chromatogram of the (A) PHA extracted from isolate FLP1 (*P. cepacia*) grown on palm olein and (B) standard 3-hydroxybutyric acid methyl ester.

coA moities (Figure 2). Absence of MCL_{PHA} suggested that the isolate did not synthesise PHA from intermediates of either β-oxidation or *de novo* synthesis of fatty acids as proposed to occur in most rRNA homology group 1 *Pseudomonas* sp. (Huijbert, *et al.*, 1992). In the isolate, the saturated fatty acids transported into the cells were degraded into its corresponding CoA derivative and these fatty acyl-coA was then shorthened by two carbon atoms successively into acetyl-coA in β-oxidation cycle. The unsaturated fatty acids would have been converted into their acyl-coA derivatives before the formation of acetyl-coA. In the excess of nitrogen, acetyl-coA was diverted into fatty acids synthesis or tricarboxylic acid (TCA) cycle for generation of energy and formation of aspartate, glutamate and other amino acids.

The citrate synthase reaction resulted in the liberation of free coASH. Under such growth condition intracellular concentration of acetyl-coA would be low and that of free coASH would be high. Free coASH was known to inhibit β-ketothiolase, a key enzyme in PHB synthesis. Under nitrogen limitation, acetyl-coA was mostly diverted into the synthesis of PHB as storage. Besides, in an excess of carbon, NADH accumulates and inhibits citrate synthase thus increases the level of acetyl-coA available for condensation (Oeding and Schlegel,1973 and Senior *et al.*, 1972).

Thus, this physiological condition explained that PHA accumulation was triggered by nitrogen limitation and was not growth related. PHA started to accumulate at the end of exponential growth when nitrogen

was limiting. As this was not mostly the case because *Alcaligenus latus* accumulated polymer during growth (Hrabak, 1992), a future study should include investigation of whether PHA produced by the isolate, FLP1, is growth related.

The isolate FLP1 is a gram negative cocci and was tentatively identified as *Burkholderia cepacia,* (BIOLOG, 80% similarity to the standard strain) (Figure 21). *B. cepacia* is a nutritionally versatile bacterium and also known as *Pseudomonas cepacia.* Ramsay *et al.*, (1989) had evaluated the possibility of producing PHB from *P. cepacia* and reported that the bacterium accumulated large quantities of high molecular weight PHB, indicating that it might be a suitable alternative to *A. eutrophus* for commercial PHA production. As the isolate FLP1 had only 80% similarity to the standard strain of *B. cepacia*, it would be prudent for the moment to identify it as *Pseudomonas* sp. (*Pseudomonas* being the former name for *Burkholderia*).

```
MICROLOG (TM) 3 RELEASE 3.30

Date          : 01/01/80
Hour          : 24
Plate Type    : GN
Media Type    : TSA/BUHI
Plate #       : 1
Strain Name   : 7
Strain #      : 2
Other Info    : 3
Input Mode    : Reader  : BIOLOG MICROSTATION
Data Base     : MicroLog GN

POSITIVE/NEGATIVE DATA
 XXX  = percent change in optical density versus A1 control well
<XXX> = positive, (XXX) = borderline, XXX = negative
-XXX- = percent change negative
 XXX* = data negative or borderline, "*" ID choice positive > 90% of time
 XXX* = data positive or borderline, "*" ID choice positive < 10% of time

      1     2     3     4     5     6     7     8     9    10    11    12
  +-----------------------------------------------------------------------
A |    0 <  13>    14     0 <396> <362>     2 <256> <426> <502> <429> { 52}
B |    7 <525> <441> <250> <273> <581> <512>    0    24  { 32+ <503> <463>
C | < 84>    24  { 49}  { 23} <586> <497> <559> <435>    27  <438> <593> <295>
D |  { 70} <594> <619> <120> <553> <610> <562> <225> <601>  { 57} <523>  { 35}
E | <479>  { 11}  { 63+  { 38}  { 44} <573> <363> <197> <577> <608> <404> <505>
F | <403>  { 47+ <101>  { 48} <323> <423> <175> <639> <539> <563>    26  { 32+
G | <639> <616>  { 38+ <141>  <49> <539> <508> <163> <357> <294> <198> <568>
H | <616> <307>    12     <4> <394> <187> <302>    5  <269> <281> <205> <555>

 BIO-NUMBER : 0136-3743-4367-7776-5177-5374-6777-6757

 SPECIES IDENTIFICATION     : BURKHOLDERIA CEPACIA

   CLOSEST SPECIES                                  SIM.   DIST.  AVG.   MAX
=> 11  BURKHOLDERIA CEPACIA                        0.804  2.848  1.375  3.713
   21  PSEUDOMONAS PICKETTII                       0.006  4.481  0.063  0.119
   31  BURKHOLDERIA GLADIOLI                       0.000  7.147  0.750  4.963
   41  PSEUDOMONAS GLATHEI                         0.000  7.192  0.438  1.894
   51  BURKHOLDERIA CARYOPHYLLI                    0.000  7.912  0.500  1.675
   61  PSEUDOMONAS PHENAZINIUM ------              0.000 10.241  0.281  0.366
   71  PSEUDOMONAS LOCOVENENANS                    0.000 10.659  0.094  2.300
   81  PSEUDOMONAS FLUORESCENS TYPE A              0.000 11.375  0.813  2.794
   91  PSEUDOMONAS MARGINALIS                      0.000 11.691  0.172  1.563
  101  PSEUDOMONAS CORRUGATA                       0.000 11.732  0.656  2.581
   other :                                          ----- -----  -----  -----
```

Figure 21

Data from BIOLOG shows the isolate FLP1 resembles *Burkholderia cepacia* (*Pseudomonas cepacia*) at 80% similarity.