CHAPTER FOUR

RESULTS AND DISCUSSION

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4.1 Studies of soil condition for biodegradation

4.1.1 Moisture content of soil

The percentage of moisture content in the garden soil under natural and enclosed environments were measured and calculated as mentioned in section 3.3.1 in the Materials and Methods.

Table 4 : Results of percentage moisture content in soil

Environment of soil	Day	Weight of soil before drying (g)	Weight of soil after drying (g)	% moisture content in the soil
Natural	0	17.09	14.00	18.08
Natural	90	18.50	15.10	18.38
Enclosed	0	73.81	58.47	20.78
Enclosed	90	71.75	56.01	21.94

The percentage of moisture content for both experiment remained approximately about 20 % at day 0 and day 90. From the result obtained, it showed that the water content in the soil do not change much during the duration of the experiment. Kimura *et al* reported in 1994 that moisture content of soil effect the biodegradation of PHB/HV and 60% maximum water holding capacity was found to be the fastest condition for

degradation followed by 40%, 25% and 100% of maximum water holding capacity during 32 days of incubation time. The moisture content for soil burial test is normally set at 20-30% because if the soil is too wet or too dry, the microbial activities will be affected and thus lowering the degradation rate (Seal, 1994). In this experiment, 20% of moisture content in the garden soil did support degradation of the PHA

4.1.2 pH of soil

The pH of the garden soil for the natural and enclosed environments were found to be 6.5 at day 0 and 6.0 at day 90.The pH of the soil did not change much during the whole period of the degradation experiment . The small decrease in pH could be due to the accumulation of hydroxyalkanoic acids in the soil due to the breaking down of PHA in the soil.

Studies conducted by Briese *et al*, 1994 found out that degradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by aerobic sewage sludge was maximal between pH 7 and 8.5 (light alkaline range). This agreed with the optimum pH for PHB depolymerases from *Alcaligenes faecalis* (pH 7.5) (Tanio *et al*, 1992) and *Pseudomonas lemoigne* (pH8.0) (Nakayama *et al*, 1985). From the same study, they also found that the pH values of the culture fluid decreased significantly due to accumulation of 3-hydroxybutyric and 3-hydroxyvaleric acids.

From the result obtained, the soil condition was found to be quite acidic but this condition did not mean that PHA could not be degraded. There could probably be some microbes which have the ability to degrade PHA in an acidic medium. Studies found that there are microbes which have the ability to degrade PHB in acidic condition such as the *Pseudomonas pickettii* (Shiraki *et al*, 1995).

4.2 The Analytical Studies of Biodegradability

4.2.1 Gross weight loss

In this biodegradation experiment, several films of PHA produced from *P.putida* using SPKO as the carbon substrate were prepared for degradation test in the natural environment. Test carried out in an enclosed environment was mainly to detect the carbon dioxide evolution. The natural environment involved in this experiment was garden soil and soil from the same study site was used for the enclosed environment .

The garden soil has a pH of 6.5 with a moisture content of about 20 % on the 0 day and remained approximately the same throughout the experiment due to the unchanged climate in the area of experiment. The natural and enclosed experiment were conducted as mentioned in Section 3.2.1 and 3.2.2. To determine the weight loss, the samples were removed at the specified time intervals mentioned in Table 5 below. Weight loss for

PHA in the enclosed environment was obtained once only that is at the end of the experiment, 90 days. The samples were washed with distilled water to remove any adhered soil, dried thoroughly in the dessicator before the residual weight was taken as mentioned in Section 3.4.1

Table 5: Results of gross weight loss

Day in soil (day) (natural environment)	Initial weight of sample (mg)	Final weight of sample (mg)	Weight loss (%)
0	22.2	22.2	0.00
11	22.2	20.9	5.86
45	25.6	22.9	10.55
90	30.8	27.3	11.36
(Enclosed environment)			
0	230.0	230.0	0.00
90	230.0	214.1	6.91

From data obtained, the weight loss in natural environment was found to increase with a corresponding increase in time. Graph of weight loss versus day was plotted (Figure 7). The percentage loss of weight for natural environment at 11 days, 45 days and 90 days were 5.86%, 10.55% and 11.36% respectively. For the enclosed environment, the PHA lost 6.91% of its initial weight after 90 days.

There are many factors that could contribute to the loss of the PHA weight besides microbial activities. PHA-degrading microorganisms have been isolated from soil which are *Pseudomonas lemoigne, Acidovorax delafieldii and Variovorax paradoxus* (Mukai *et al,*1994). Fungi was also found to be involved in the biodegradation process. Fungi constitute an important part of microbial populations participating in degradation process (Matavulj *et al*, 1992). Studies investigated by Kunioka *et al* in 1989 found that fungi in the soil played an important role in biodegradation of PHB-copolymers. The PHA degradation could also be due to the macroorganisms activities such as ants, crickets and snails where they can consume the plastic as food (Wool, 1994). The activity of microbes in the enclosed environment might have been slower compared to the natural environment resulting in a slower rate of biodegradation as seen in the percentage of gross weight loss. In the natural environment the moisture content, pH and sunlight are more natural to the microbes and these factors could encourage their activities.

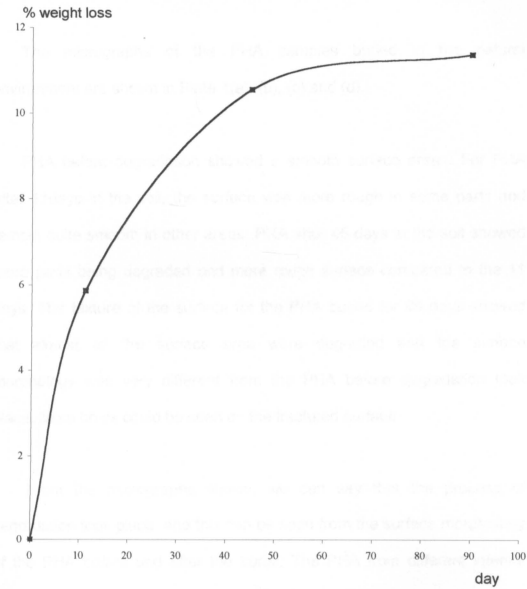
Before the PHA is ultimately converted to carbon dioxide and water, the PHA will undergo steps of mineralization whereby the PHA will be converted to intermediates. The intermediates can be either natural or toxic to the organisms in the soil and if they are toxic, the breaking down of the

polymer could be suppressed thus percentage of weight loss is also decreased.

Kimura *et al* 1994 showed that the biodegradation of plastics proceeds actively under different soil conditions according to their properties, because the microorganisms responsible for the degradation are different and they have their own optimal growth conditions in the soil . Different soil will then give different rate of PHA biodegradation.

The OECD test guideline recommends a greater biodegradability than 60% gross weight loss after 28 days in soil as a passing level for ready biodegradable substance (OECD, 1981). Even though the PHA studied did not pass this criteria, degradation of the PHA did occurred but only at a lower rate and can be classified as inherently biodegradable.

Figure 7: Percentage of weight loss of PHA in natural environment



4.2.2 Surface morphology of PHA

4.2.2.1 Phase contrast microscopy

The micrographs of the PHA samples buried in the natural environment are shown in Plate 1(a), (b), (c) and (d).

PHA before degradation showed a smooth surface area . For PHA after 11days in the soil, the surface was more rough in some parts and remain quite smooth in other areas. PHA after 45 days in the soil showed more parts being degraded and more rough surface compared to the 11 days. The texture of the surface for the PHA buried for 90 days showed that almost all the surface area were degraded and the surface morphology was very different from the PHA before degradation took place. More holes could be seen on the fractured surface.

From the micrographs shown, we can say that the process of degradation took place and this can be seen from the surface morphology of the PHA before and after the burial. The PHA from different interval times of burial also showed a different surface morphology from each other meaning that some extent of physical degradation had occurred in the soil.

4.2.2.2 Electron microscopy

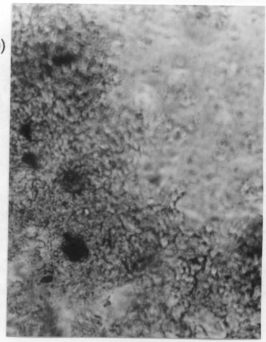
The electron microscope study was carried out for the PHA samples buried in both natural and enclosed environments. The micrograph of the specimen before the degradation process (0 day) showed a smooth surface area and we can observed it as shown in Plate 2 .After burying for 11 days there seemed to show some changes on the surface area. The surface was more rough and the polymer was seen to be degraded due to the existence of holes on the surface itself. From the observation of the surface , there were cracks on the polymer film and eroded exposed area. At 45 and 90 days, the surface appeared more uneven with bigger holes.

Other studies have found that microbes degrade PHAs through extracellular enzymes that bind tightly to the polymer films in a non-uniform manner, roughening the surface (Molitoris *et al*, 1996). These changes can be observed by electron microscope. This finding could support the reason of the surface being rough and eroded. The polymer buried for 45 and 90 days also showed similar surface morphology except that the 90 days sample seemed to have more holes on its surface indicating greater extent of degradation. This result supports the decrease in pH which could be due to the accumulation of polyhydroxyalkanoic acids resulted from the degradation of the PHA.

Plate 3 shows that in enclosed environment the polymer film was also degraded and the surface morphology did change even though the experiment was not carried out in the field. The surface appeared uneven with many holes compared to the surface of the polymer before the biodegradation process (0 day). Such morphological changes on the PHA film indicate that the polymer did degrade in the garden soil especially on the surface area. Doi *et al* 1990a reported that degradation occurred on the surface of PHA films and that the thickness of the films decreased with time .

(a)

(b)



(c)

(d)

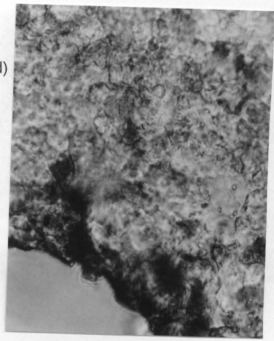
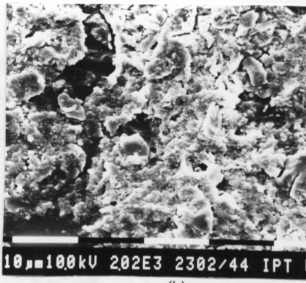
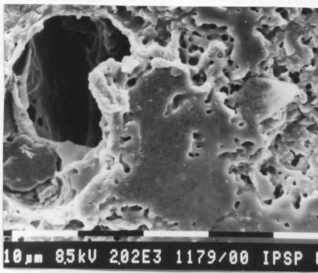


Plate 1: Surface of PHA buried in the soil of natural environment viewed under phase contrast microscope at day 0(a), 11(b), 45(c) and 90(d) at 200 X magnification.



(a) (b)



(c) (d)

Plate 2: Surface of PHA buried in the soil of natural environment scanned under EM at day 0 (a), 11(b), 45 (c) and 90 (d) at 2000 X magnification.

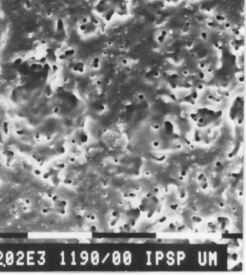


Plate 3: Surface of PHA buried in the enclosed environment scanned under EM at day 90 at 2000 X mgnification.

4.2.3 Carbon dioxide evolution

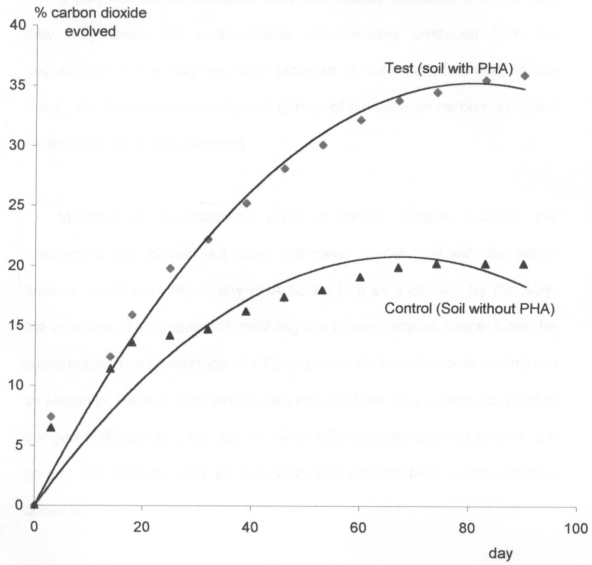
Carbon dioxide evolution test was carried out in the enclosed environment because it would be difficult to monitor the carbon dioxide evolution from the natural environment. The method of trapping the carbon dioxide is mentioned on Section 3.4.3 under Materials and Methods. The percentage of carbon dioxide evolved was calculated using the formula given in section 3.4.3.

The carbon dioxide evolution measurement indicated the biodegradation of the test material. The polymer tested was degraded by bacterially induced surface erosion to water soluble products, which then can be metabolized to carbon dioxide or biomass. Biomass itself eventually will be degraded to carbon dioxide. However, over usual time scale of biodegradation test, complete mineralization to carbon dioxide generally will not be observed. (Starnecker *et al*, 1996). CO_2 evolution measurements in Biometer flasks is used as a standard FDA procedure for biodegradability testing, applicable and highly predicture of packaging material biodegradability in soil (Yabannavar *et al*, 1993). The aim of this investigation was to develop improved testing of the biodegradability of polymers by means of establishing carbon balances.

Table 6 : Percentage of CO_2 evolved in the enclosed environment

Day	% CO_2 evolved for control (soil without PHA)	% CO_2 evolved for test (soil with PHA)	% CO_2 evolved from PHA only (Test – Control)
0	0.0	0.0	0.0
3	6.5	7.4	0.9
14	11.4	12.4	1.0
18	13.6	15.9	2.3
25	14.2	19.8	5.6
32	14.7	22.2	7.5
39	16.2	25.2	9.0
46	17.4	28.1	10.7
53	18.0	30.1	12.1
60	19.1	32.2	13.1
67	19.9	33.8	13.9
74	20.2	34.5	14.3
83	20.2	35.5	15.3
90	20.2	35.9	15.7

Figure 8: Percentage of carbon dioxide evolved from the enclosed environment



From the experiment carried out, the CO_2 evolved was from the activity of the microbes in the garden soil and also from the breaking down of the polymer. Some microbes may not directly consume the PHA but they may utilize the water-soluble intermediates produced from the degradation of the polymer, thus increase in their activities and as the result , the CO_2 will increase too. A portion of the polymer carbon is utilized for the build up of new biomass.

In order to minimize the error of carbon dioxide evolved, the experiment was carried out using the same garden soil with the same amount of soil but without any PHA buried in it as a control. By this way, the activities of microbes not involving the biodegradation process can be substracted. The percentage of CO_2 evolution for both test and control are as shown in Table 6. This experiment has not level to a plateau as seen in the graph (Figure 8) , but the 15.7% of CO_2 evolved after 90 days in the garden soil already gave an indication that degradability of the polymer occurred.

4.2.4 Infrared Spectrophotometry

Infrared spectrophotometry (IR) is an analysis conducted to find out the functional group comprising a molecule and the overall configuration of the atoms as well. IR involves the twisting, bending, rotating and vibrational motions of atoms in a molecule. Many useful correlations have been found in the mid-infrared region. This region is divided into "group frequency" region and "fingerprint region" which falls between 4000-1300 cm^{-1} and 1300-650 cm^{-1} respectively. Upon interaction with infrared radiation, portions of the incident radiation are absorbed at particular wavelengths. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum.

For this test, the main purpose is to see whether the chemical composition of the PHA change before and after the degradation in the natural environment. Figures 9 (a), (b), (c), (d) are spectra of the PHA subjected to different period of degradation in soil. Three strong absorption bands can be observed in the region just below $3000cm^{-1}$. These bands are due to C-H stretching modes of the CH_3 (methyl) and the CH_2 (methylene) groups of the PHA molecules. The other significant absorption in the region above $1600cm^{-1}$ is the band at around 1745 cm^{-1} due to the presence of a C = O (carbonyl) of the ester group. The carbonyl group is the strongest band in the spectrum and easily recognised in all cases.

The CH_3 and CH_2 group has two stretching vibrational mode. For CH_3 the asymmetric stretching mode is at 2960 $cm^{-1}$and and the symmetric mode at 2870 cm^{-1}. For CH_2, these bands occur at 2930 cm^{-1} and 2850 cm^{-1}.

The CH_3 group has two bending vibrational mode which is 1450 cm^{-1} and 1375 cm^{-1} having characteristic of asymmetric and symmetric respectively. The scissoring vibration (one of the bending modes) of CH_2 gives a band near 1465 cm^{-1} which overlaps with the asymmetric band near 1450 cm^{-1}.

The band emerging at region 3570 cm^{-1} to 3450 cm^{-1} is detected as the stretching absorption of O-H that is bonded intramolecularly. The precise position of the band depends on the atom to which the hydroxyl group is attached. Table 7 shows the position of bands for all the major structural bonding type found in the PHA studied.

If there is any structural modifications closer to the absorbing center, it will affect the energy associated with the absorption, and lead to a shift of the absorption band to higher or lower frequencies. However, these frequency shifts have been found to lie within defined limits and the numerical value often provides valuable information on the structural environment of the associated group (Furniss *et al*, 1989).

Since the functional group remained before and after biodegradation process being carried out, the chemical composition of the PHA did not change significantly even after degradation. There could also be other possibility. The PHA from the IR measurement was from the bulk PHA and the surface that has already being broken down and degraded to smaller molecules could have been soluble in the solvent and were lost in the washing and precipitation process. So, it will always show the same spectra since the bulk PHA is the part that is being measured. The IR results appear to support the assumption where the microbes consume the PHA by not preferentially feeding on certain functional groups.

Table 7 : Characteristic of IR absorptions detected in PHA studied before and after degradation in garden soil of natural environment (with reference to Furniss et al, 1989)

Groups/vibration	Range (cm^{-1}) (wave number)	Comments *
1)Alkanes and alkyl groups		
C-H stretching		
CH_3	2972-9253	(s) asymmetric
CH_2	2936-2916	(s) asymmetric
CH_3	2882-2862	(s) symmetric
CH_2	2863-2843	(s) symmetric
C-H bending		
CH_3	1470-1430	(m) asymmetric
CH_2	1485-1445	(m) overlaps above
$CH_3 - CH_2$	1380-1370	(m) symmetric
2)Esters		
C=O stretching		
Saturated, acyclic	1750-1735	(s)
3) Alcohols		
O-H stretching		
Intramolecular H-bonded	3570-3450	(v) sharp

* Abbreviation used to express intensity of absorptions : (s), strong; (m), medium and (v), variable.

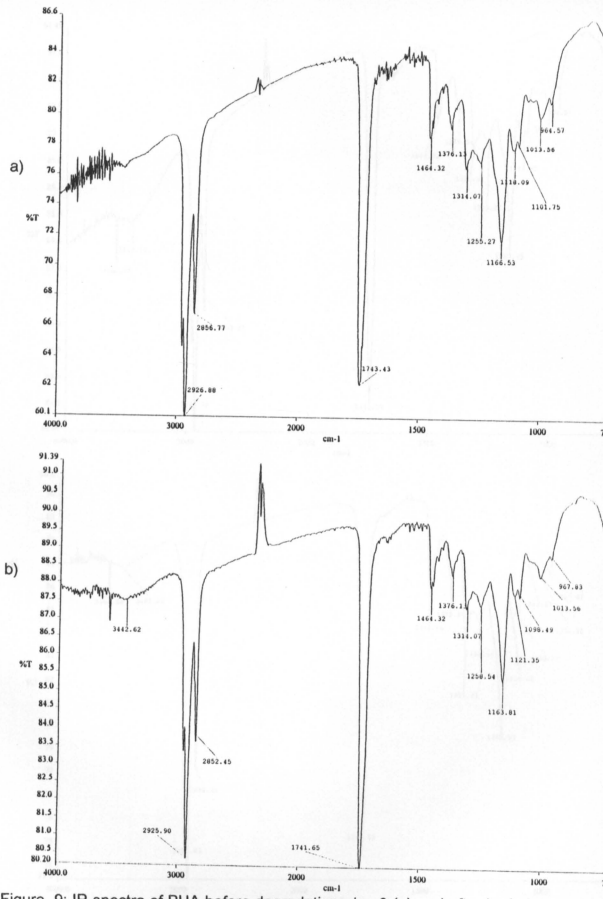


Figure 9: IR spectra of PHA before degradation, day 0 (a) and after buried for 11days (b) in the natural environment.

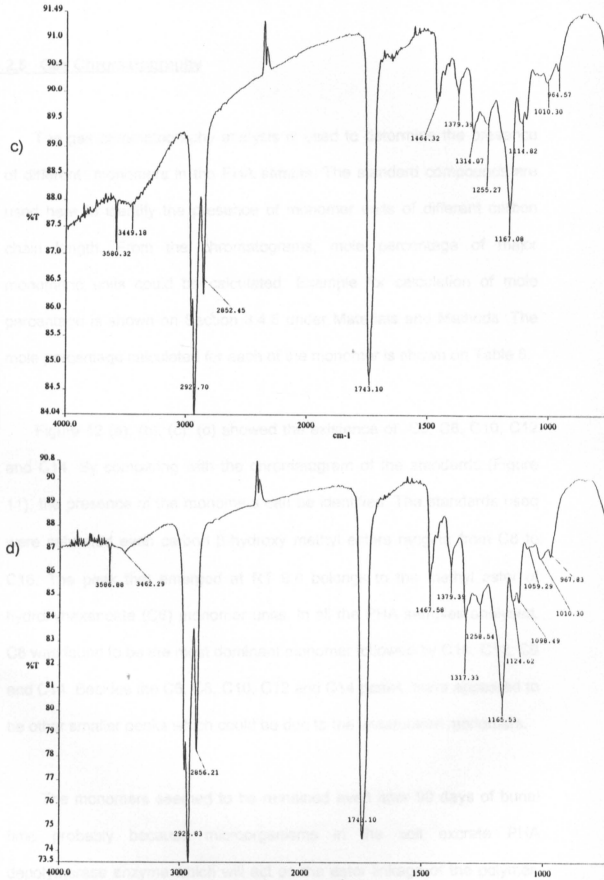


Figure 9: IR spectra of PHA buried in the natural environment for 45 days (c) and 90 days (d).

4.2.5 Gas Chromatography

The gas chromatography analysis is used to determine the presence of different monomers in the PHA sample. The standard compounds are used here to identify the presence of monomer units of different carbon chain length. From the chromatograms, mole percentage of major monomeric units could be calculated. Example for calculation of mole percentage is shown on Section 3.4.5 under Materials and Methods. The mole percentage calculated for each of the monomer is shown on Table 8.

Figure 12 (a), (b), (c), (d) showed the existence of C6, C8, C10, C12 and C14. By comparing with the chromatogram of the standards (Figure 11), the presence of the monomers can be identified. The standards used were saturated even carbon β-hydroxy methyl esters ranging from C8 to C16. The peak that emerged at RT 9.8 belongs to the methyl ester of hydroxyhexanoate (C6) monomer units. In all the PHA samples analysed, C8 was found to be the most dominant monomer followed by C10, C12, C6 and C14. Besides the C6, C8, C10, C12 and C14 peaks, there appeared to be other smaller peaks which could be due to the unsaturated monomers.

The monomers seemed to be remained even after 90 days of burial time probably because microorganisms in the soil excrete PHA depolymerase enzyme which will act on the ester linkage of the polymer.

Some microorganisms such as bacteria and fungi, excrete extracellular P(3HB) depolymerase that hydrolyze poly(3-hydroxybutyrate) and its copolymer into oligomers and monomers in the vicinity of the cells, and the resulting products are absorbed and utilized as nutrient (Doi, 1990). The microbes do not actually choose any specific monomers to feed but they will attack the ester linkage. That is the reason why the monomers remained even after degradation.

Foster *et al*, 1995 showed that the extracellular depolymerase enzyme from *Pseudomonas maculicola* can degraded PHAs with relatively long alkyl substituents at the 3 position: poly-3-hydroxyoctanoate (PHO), poly-3-hydroxynonanoate (PHN) and their copolymers (P[HO-co-HN]) and poly-3-hydroxyundecanoate (PHU). However, the enzyme was unable to degrade PHAs with shorter alkyl groups including PHB.

Study showed that the enzymatic degradation of poly-3-hydroxyalkanoates by PHA depolymerase from *Alcaligenes faecalis* was stongly dependent upon the composition of the polyesters and markedly decreased with an increase in the side-chain length of the 3-hydroxyalkanoate monomeric units (Kanesawa *et al*, 1994). In 1994 Doi *et al* found that the number of enzymes capable of hydrolyzing the respective PHA sample decreases as the carbon number of the monomeric units increased. From the studies, it showed that the increase of carbon number

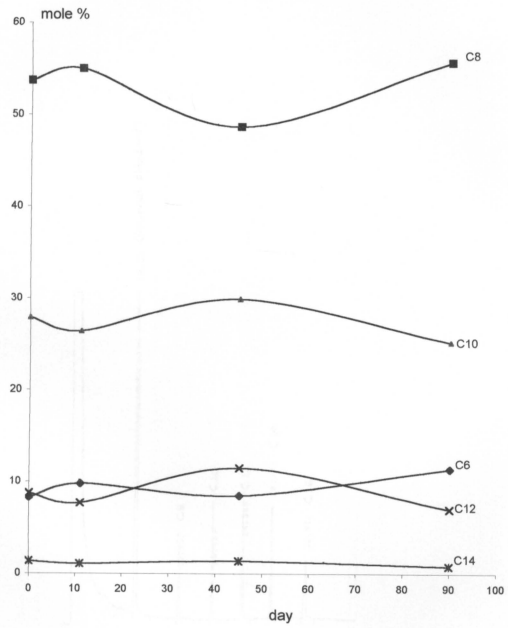
in the side chain will give a decrease in the biodegradation rate of the polymer.

Looking at Table 8, it appears that the longer monomer units (C10-C14) were more readily broken down compared to the shorter monomers. The longer monomers might be more easily attacked by enzymes due to their longer side chains. This may explain the lower proportion of longer monomers (C10, C12, C14) in the 90 days PHA sample compared to the original 0 day PHA sample.

Table 8 : Mole percentage of monomers in the PHA after different burial periods in the natural environment

Mole % / Time (day)	C6	C8	C10	C12	C14
0	7.4	53.0	29.0	8.9	1.5
11	9.8	55.0	26.4	7.7	1.1
45	8.5	48.7	29.9	11.5	1.4
90	11.4	55.7	25.2	7.0	0.8

Figure 10 : Monomer content of the PHA after various periods of burial



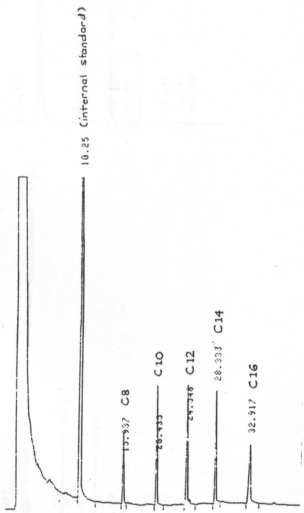


Figure 11: Gas chromatogram of standards: methyl ester of fatty acids.

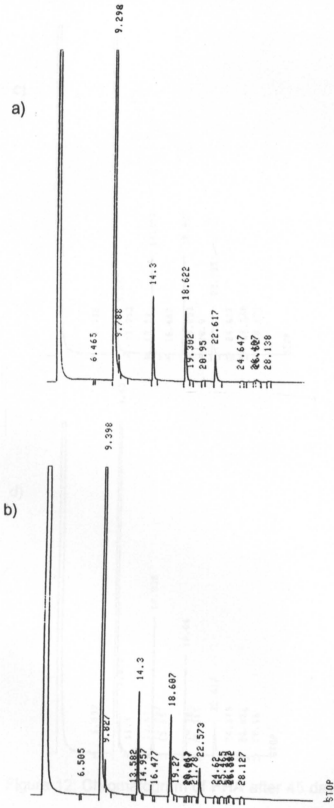


Figure 12: Chromatogram of PHA at day 0 (a) and after 11days buried in the soil of natural environment (b)

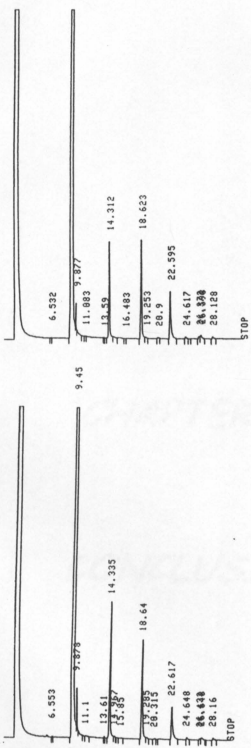


Figure 12: Chromatogram of PHA after 45 days (c) and 90 days (d) buried in the soil of natural environment