CHAPTER III

RESULTS AND DISCUSSION

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The biodegradation tests were modified from two main sources. Firstly, the experimental set up to test the CO_2 evolution from the aerobic biodegradation of plastic material was modified from the ASTM standard test method D5209-91 (1992). Secondly, the measurement of PHA biodegradability in river water based on weight loss, changes in DOC, molecular weight, pH and microbial count by Doi *et al.* (1996) and also in the ASTM standard test method (D5209-91) were taken as references. Modifications by providing sterile conditions and additional control reactors; and other analysis such as scanning electron microscopy (SEM), infrared red (IR) spectroscopy and gas chromatography (GC) were made in order to obtain more specific and accurate results for the carbon balance analysis to verify the extent of the PHA biodegradation.

3.1 Preliminary studies

The number of microorganisms in the river water increased after the pretreatment (aeration & storage) of river water based on ASTM standard test method D5209-91 (Table 3.1). The microorganisms continued to grow at 4°C. This shows that Kayu Ara River water was a rich source of highly active microorganisms as inoculum for biodegradability test of PHA films. It was suggested in the ASTM standard test method that the supernatant of the microbial source provided 1% of inoculum to the synthetic mineral salt solutions in the test reactors. This would make up a final microbial population of 10^4 to 20 X 10^4 colony-forming units (CFU)/ml. With reference to Doi *et al.* (1996), however, the entire treated river water was used as an inoculum in this experiment. This was to provide a better simulation of the river

water, not only the microbial community, but also other impurities in their original concentration, which may also affect the rate of PHA biodegradation in the river water.

Table 3.1- Number of microorganisms in river water before and after pretreatment.

River water treatment	Number of microorganisms per ml			
	Fresh river water	After Aeration (4hr - 4.5hr)	After storage at 4°C for 2 days	After purging with scrubbed air (overnight)
Run 1 (Trial run)	8.0×10^5	9.0×10^5	2.0×10^6	5.5×10^5
Run 2	5.4×10^5	9.2×10^5	1.7×10^6	2.1×10^6

Run 1 was done for 10 days to test the feasibility of the modified Sturm-test (measure biodegradability based on CO_2 evolution) (Muller *et al.*, 1994) set up in the lab according to ASTM standard (D 5209-91) for determining the aerobic biodegradation of plastic materials (Figure 2.1). Some improvements have been designed and incorporated into Run 2. The air flow rate of 50-100ml/min controlled at the main air supply inlet before scrubbing and distributing to all reactors was too high as the air bubble released in every reactor exceeded 1 to 2 bubbles/s as recommended by ASTM standard (D 5209-91). High air supply might cause O_2-toxicity to the microorganisms (Table 3.4). The death of microorganisms might proceed with the lysis of cells, which reduced the pH of the river water (Brock *et al.*, 1994). A tubing-screw used to adjust the diameter of the tubing was fixed to the air-inlet tubing of every reactor for the finer adjustment of the airflow rate to 1-2 bubbles /s in Run 2.

Though there was an increase in the weight of PHA film by 1.7% after 10 days (Table 3.2), the PHB film which was used as a positive control has reduced in weight by 37.2%

(13.6mg). This shows that the experimental set up was feasible for degradation of test polymers.

Table 3.2– Weight loss of PHA & PHB in unstirred river water at 28°C

Experiment	Incubation period	Weight loss (%)	
		PHA	PHB
Run 1 (Trial run)	10	-1.7	37.2
Run 2	28	-5.8	64.5

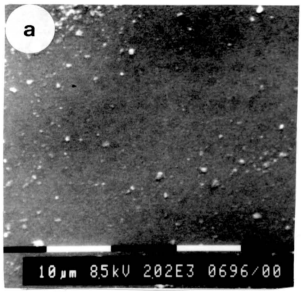
The PHA film changed from transparent to translucent after 10 days in trial run. The scanning electron micrograph (SEM, Figure 3.1) showed particle patches of various sizes attached to the smooth surface of the day 10 PHA film as compared to the original film surface. Further more, the PHA incubated in sterilised river water (Figure 3.1*c*) contained very few and very tiny particles as compared to the PHA incubated in untreated river water (Figure 3.1*b*). Hence, majority of the particle patches might mostly be microbial colonies or a resultant from microbial activity on the PHA surface. This is supported by Imam *et al.*'s (1999) finding that only after colonization of the plastic that biodegradation occurred significantly. To prevent the suspended particles in the river water from sticking to the PHA and contributing to the final weight of the PHA film, the river water for Run 3 was filtered aseptically through Whatman filter paper no. 1.

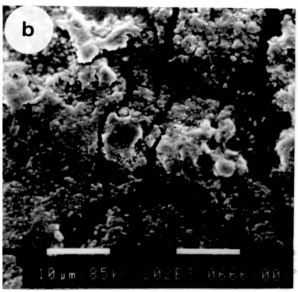
The weight of biomass attached to the film surface might be higher than the weight loss of degraded PHA. Therefore, the degradation might not be detected through weight loss. In order to remove the surface attachment of PHA & PHB films, sonication was introduced in

Run 2. The day 5 and day 10 PHA films of Run 2 had melted after sonication for 1 hr. Hence, sonication time was reduced to 30 minutes, and the water bath was changed every 15 minutes to keep the water temperature low. The day-15 PHA film surface attachment of Run 2 (Figure 3.2*d* & Figure 3.4*c*) was lesser as compared to the surface attachment on day-10 PHA film of trial run (Figure 3.2*c* & Figure 3.1*b*). This shows that sonication can partially remove the surface attachment. Difficulty in the separation of biomass from the polymer film was mentioned by Muller *et al.* (1994) in the application of Sturm-test in the lab as a biodegradability test method. The surface attachment could be microorganisms. In the period of 28 days, PHA in sterilised river water has a weight loss (10.6%) whereas PHA in untreated river water has a weight gain (5.8%). The microorganisms in the untreated river water could have attached to the film surface whereas in the sterilized river water, the dead microorganisms could not attach to the film surface.

The PHA film incubated in the river water changed from transparent originally to increasingly translucent with time (Figure 3.3). However, the control PHA film incubated in sterile river water was still transparent on day 28. The SEM showed that there was increasing surface attachment on the PHA film with time (Figure 3.4*a,c,d*) but no visible changes on surface morphology was observed until day 28 (Figure 3.4*e,f*). The cracks on the PHA film incubated for 21 days (Figure 3.4*d*) may be due to the shrinking of the microbial colonies on the film surface while drying that pulled and torn the film at the edge of the colony. The day 28 PHA film surface was highly blemished (Figure 3.2*e* & *f*). At higher magnification (503X), wave-like pattern in concave surface between less eroded surface was visible on the film (Figure 3.4*e*). A closer look at the concave surface revealed many irregular holes and pits between less eroded area (Figure 3.4*f*). The PHA incubated for 28 days in sterile condition (Figure 3.4*b*) had no detectable morphological effect on the exposed

polymer surface except for some mild lines of folding, which may be caused by handling. The SEM analysis of PHA film again showed that microorganisms play an important role in the degradation of PHA in river water.





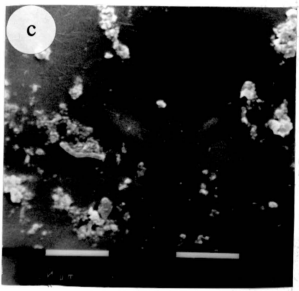


Figure 3.1 – Scanning electron micrographs (SEMs) of PHA film surfaces after incubation in unstirred river water at 28°C in trial run for *(a)* 0 days; *(b)* 10 days and *(c)* 10 days in sterile condition. *Note.* magnification 2.02E3 times = $2.02X10^3$ times.

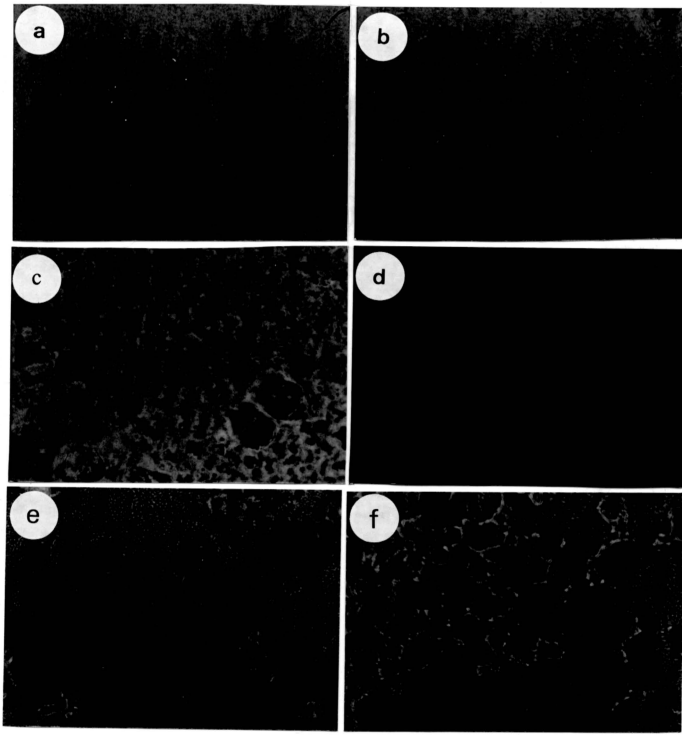


Figure 3.2 – Phase contrast micrographs of PHA film surfaces after incubation in unstirred river water at 28°C for *(a)* 0 days; *(b)* 10 days (in sterile river water); *(c)* 10 days; *(d)* 15 days and *(e)-(f)* 28 days. All magnifications are at 200 X except for *(e)* at 100 X. Samples *(b)-(c)* were from Run 1 and not sonicated wherease samples *(d)-(f)* were from Run 2 and sonicated.

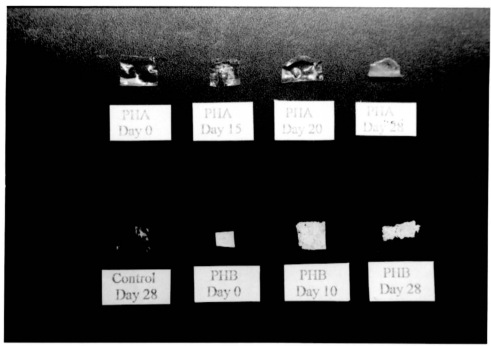


Figure 3.3 – PHA & PHB film surface morphology before and after incubation in unstirred river water at 28°C (Run 2). The Control PHA was incubated in sterile river water. The samples shown here were cut from the original films tested.

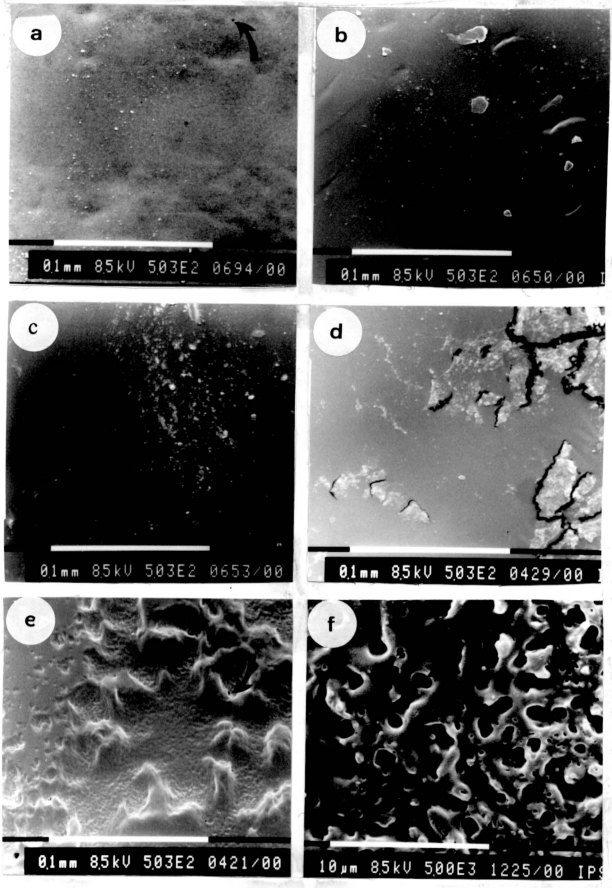


Figure 3.4 – SEMs of PHA film surfaces after incubation in unstirred river water at 28°C in Run 2 for *(a)* 0 day; *(b)* 28 days (in sterile river water); *(c)* 15 days; *(d)* 21

(e)-(f) **28 days. The arrows indicate the pitted holes.** *Note.* **magnification 5.03E2 times = $5.03X10^2$ times.**

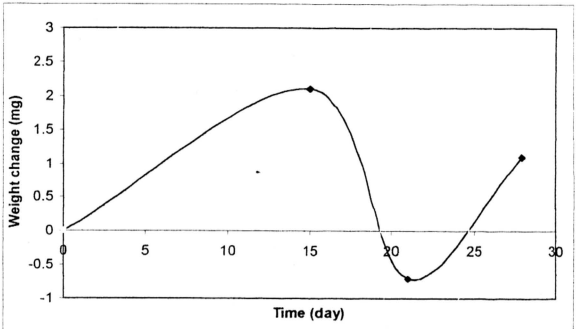


Figure 3.5 - PHA weight change in unstirred river water at 28°C.

The weight change curve of PHA (Figure 3.5) shows an inverse trend to that of microbial population in the water (Figure 3.6). On day 15, there was a reduction of number of microbes in the river water but a weight gain in PHA. The microbes from the river water might have attached to the PHA film. On day 21, there was an increase in the number of microorganisms but a weight loss in PHA. There might be degradation of PHA accompanied by release of soluble products from the PHA film, which caused a reduction in weight of PHA on day 21. The soluble product of PHA degradation might further be utilised by other microorganisms in the river water for growth. Hence, an increase in the microorganism in the river water was observed between day 15 to day 21. After day 21, there was a gradual weight gain in the PHA film, which was parallel to a gradual decrease in microbial population in the river water after day 21 (Figure 3.6). Though biodegradation could not be deduced from the weight

data, the cumulative CO_2 released from the reactors showed that there was degradation and 52.4%-100% of the degradative CO_2 came from biodegradation (Figure 3.7). Since there was no significant weight loss of PHA in 28 days, the period of biodegradation test was extended to about 3 months (86 days) in Run 3 to obtain the weight-loss-biodegradability and the biodegradability trend of PHA.

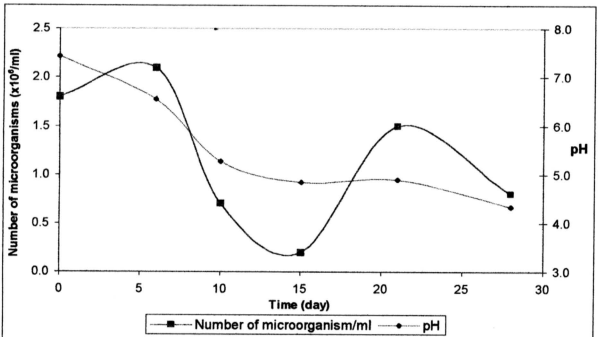


Figure 3.6- pH & number of microorganisms in unstirred river water at 28°C.

In order to obtain a carbon-balance equation for the degradation process of PHA (PHA+O_2 + biomass ⟶ residual PHA+ new biomass + intermediate products + CO_2), it has to be ensured that the CO_2 analysed must come purely from the degradation process of PHA. This can only be done by setting up control reactors to quantify the amount of CO_2 released from the non-degradative processes, including (i) CO_2 from microbial activity on carbon substrate other than PHA in the river water (since the river water $DOC_{initial}$ = 58.1 mg/l) and (ii) CO_2 from air supply (Table 3.3). At the same time, the major processes (biological/ physical &

chemical) that contributed to the degradation of PHA can be deduced. Hence, control reactor C3 was introduced in Run 2.

Table 3.3 – Test reactors and control reactors and the processes that release the CO_2 from the reactor

Experiment	Reactor	PHA/ PHB*	Microbe	Source of CO_2			
				Biodegradation *a*	Degradation (physical/ chemical) *b*	Microbial (from other C-sources) *c*	Air supply *d*
Run 1 (Trial run)	Test	+	+	+	+	+	+
	C1	-	+	-	-	+	+
	C2	+	-	-	+	-	+
	PHB positive control	+*	+	+	+	+	+
Run 2	Tests	+	+	+	+	+	+
	C1	-	+	-	-	+	+
	C2	+	-	-	+	-	+
	C3	-	-	-	-	-	+
	PHB positive control	+*	+	+	+	+	+

Note. The reactors labeled C... are controls. Every PHA and PHB film was enclosed in a polyester net. The controls without PHA or PHB were incubated with empty polyester net. For Run 2, (1) CO_2 due to biodegradation is CO_2 of (Test -C3) -(C1-C3)-(C2-C3); (2) CO_2 due to physical &/chemical degradation is CO_2 of (C2-C3); (3) CO_2 due to degradation (biological, physical &/ chemical) = (1)+(2).

The control reactors suggested in the above was applicable to deduce the amount of CO_2 from various sources. It was discovered that the scrubbed air still contained CO_2 and contributed to a major proportion of the outlet CO_2 (Figure 3.7). The reduction in the number of flasks of CO_2 scrubbing solutions from six 1 liter Erlenmeyer flasks as recommended by the ASTM standard D 5209-21 (1992) to 3 flasks as recommended by

Muller *et al.* (1994) was not suitable in this experiment. CO_2 from the air cannot be fully scrubbed even though the scrubber solution [$Ba(OH)_2$] and NaOH was not saturated yet. Either three additional Erlenmeyer flasks with 700ml of CO_2 scrubber solutions need to be added or the scrubber solutions could be transferred from the three Erlenmeyer flasks to three cylinder columns to increase the depth of the scrubber solutions for better scrubbing effect. Since the control reactors could account for the unscrubbed CO_2, it was not necessary to change the CO_2 scrubbing unit in Run 3.

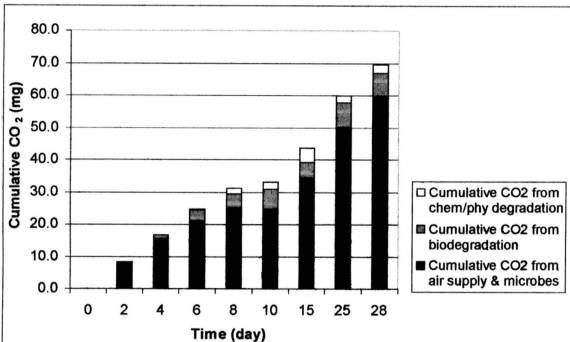


Figure 3.7 - Cumulative CO_2 released from degradation of PHA, air supply and microbes in unstirred river water at 28°C

A summary of the results for Run 1 & 2 is shown in Table 3.4. Theoretically, there should be zero or positive increase in cumulative CO_2 with time. However, the cumulative CO_2 from biodegradation and that from physical &/ chemical degradation reduced on certain days. This could be accounted from the higher rate of air supplied to the test reactors than that supplied to the control reactors. The air supplied to each reactor was adjusted to 1-2 bubble/s every

2-3 days. However, sometimes the aeration rate in the reactors would start to differ greatly within that period. Some reactors would maintain at the same aeration rate, some will be slower and a few would stop completely. This might be due to increasing resistance of outlet flow in various degrees from two main sources. Firstly, the wetting of the outlet air filter in different degree among the reactors created different resistance to the outlet airflow. Secondly, the formation of insoluble barium carbonate salts in the barium hydroxide solution of the CO_2 outlet trap would increase the viscosity and resistance of the solution to airflow. Since it was expected from Table 3.3 that there were more sources of CO_2 in the test reactors as compared to the control reactors, more barium carbonate salts would be formed in the CO_2 absorber of the test reactors. Higher resistance to airflow would be expected in the test reactors as compared to the control reactors. As a result, less CO_2 from air supply would be released from the test reactors than the control reactors. The CO_2 from degradation of PHA derived from the difference of CO_2 between the test and control reactors would give a lower value at certain days. Hence, the cumulative CO_2 for PHA degradation might be lower at certain days.

The monomer composition of PHA in Run 2 fluctuated in a zigzag manner (Figure 3.8). There was a big change in all the monomer composition on day 10 and day 15 which could not be accounted for. However, comparing the monomer composition of PHA incubated for 28 days with the unincubated PHA, there was a total disappearance of C8 and C16 monomers (Table 3.5). The mixing of the entire river water system in the reactors by the air bubble supplied at 1-2 bubbles/s was unsatisfactory to maintain a homogenous supply of air and nutrients to the microorganisms around the PHA and for the removal of biodegradation products from PHA. This may create a local difference of the air content, microorganisms

and nutrient around the PHA depending on the distance of PHA from the aeration outlet. Hence, the biodegradation rate of PHA in each of the 5 test reactors in Run 2 might be different which consequently gave fluctuated monomer composition curve for PHA in Figure 3.8. Therefore, stirring of river water was introduced in Run 3 to maintain the homogeneity of river water content around the PHA within the reactor.

Table 3.4 - Time-dependent changes of PHA weight, cumulative CO_2 released, pH and number of microorganisms in unstirred river water at 28°C

Experiment	Time (days)	Weight loss (%)	Cumulative CO_2 from biodegradation (mg)	Cumulative CO_2 from physical &/ chemical degradation (mg)	pH	Number of microorganism /ml
Run 1 (Trial run)	0	0	-	-	7.6	5.5×10^5
	10	-1.7	-	-	5.4	5.3×10^4
Run 2	0	-	-	-	7.4	1.82×10^6
	2	-	0.4	0.4	-	-
	4	-	1.3	0.0	-	-
	6	-	3.1	0.4	6.5	2.05×10^6
	8	-	4.4	1.8	-	-
	10	-	6.2	2.2	5.3	7.15×10^5
	15	-11.8	4.9	4.4	4.8	1.70×10^5
	18	-	-	-	-	-
	21	4.4	-	-	4.9	1.49×10^6
	25	-	7.5	2.2	-	-
	28	-5.8	7.0	2.6	4.3	8.37×10^5

Table 3.5- Monomer composition of PHA in unstirred river water at 28°C

Time (day)	PHA monomer composition (%w/w)				
	C8	C10	C12	C14	C16
0	44.2	24.8	9.6	14.2	7.3
28	0.0	25.0	29.4	45.6	0.0

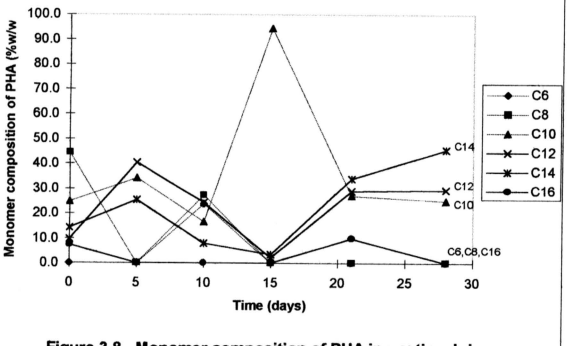


Figure 3.8 - Monomer composition of PHA in unstirred river water at 28°C

3.2 PHA INHERENT BIODEGRADABILITY STUDIES

From here onwards, the discussion will be based on Run 3.

3.2.1 CO_2 released

% CO_2 biodegradability of PHA is a measure of the biodegradability of PHA based on the comparison between the measured CO_2 value and the theoretical maximum CO_2 value released from the complete degradation and conversion of all carbon in PHA into CO_2 (Pirt, 1975). An accurate and precise value of %CO_2 biodegradability could not be obtained in this experiment. There was a reduction in the cumulative %CO_2 biodegradability and cumulative %CO_2 degradability of PHA with time (Figure 3.9). Logically, there should be an increase or at least a horizontal plateau. Since the values of %CO_2 biodegradability, %CO_2 degradability and %CO_2-chemical and physical degradability were obtained by the difference between the CO_2 released from the test reactors and the control reactors (Table 3.3 & Appendix 2), the difference in the amount of released CO_2 from each reactor may not only be due to the different reactions in the reactor, but also the difference in the amount of air supplied to each reactor. Therefore, any differences between the initial air supplied to the test and control reactors will also affect the amount of CO_2 released from these reactors. Hence the CO_2 value derived from the difference of released CO_2 between both types of reactors will increase the error of the result.

Even though Run 3 was designed to obtain a carbon balance for PHA biodegradation, an accurate and precise value for the CO_2 from the biodegradation process of PHA could not be obtained due to non-uniform rate of air supplied to the test reactors and control reactors.

This problem can be rectify by fixing a computerized automatic airflow adjuster in every inlet air pipe to ensure a constant air flow is maintained through out the experiment. Secondly, the dimension of the air pipe and the type of air filter used must be uniform through out the reactors.

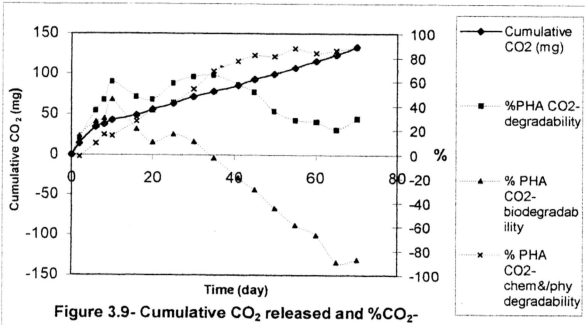


Figure 3.9- Cumulative CO_2 released and $\%CO_2$-degradability, $\%CO_2$ biodegradability and $\%CO_2$-physical and chemical degradability of PHA in stirred river water at 28°C.

In order to minimize the error from derived value, the direct measured value of the CO_2 released from the test reactors (cumulative CO_2 in Figure 3.9) would be sufficient to show the trend of PHA degradation. The rate of CO_2 released was high during the first 6 days and slowed down to a constant rate, which was maintained until day 70. Similar trend was observed in PHA weight loss (Figure 3.14) and pH reduction (Figure 3.11). Therefore, the degradation of PHA was accompanied by the release of CO_2 and acidic products. The CO_2 released during the constant rate period (from day 10 to day 70) would not only originate

from the initial air supplied, but also from the PHA degradation since there was a continuous reduction of PHA weight during this period.

The cumulative CO_2 released from 4 reactors with different content of PHA film and microorganisms was shown in Figure 3.10. Comparing the cumulative CO_2 released from the control reactor (PHA+, Microbe (M)-) and the test reactor (PHA+, M+), it seems that chemical and physical reactions were the major degradation process of PHA as compared to biodegradation. However, the weight loss of PHA in sterile river water by day 86, which could be due to chemical and physical reactions was only 31.1% of the original PHA weight as compared to 71.3% weight loss of PHA in unsterilized river water, which could be due to biological, chemical and physical degradation. Hence, microorganisms played a significant role in degrading the PHA in river water. The excess CO_2 in the control reactor might be due to higher air-supply rate to the control reactor as compared to the test reactors. Doi (1995) has shown that no degradation of P(3HB) takes place in sea water without microorganisms. A test on the biodegradability of a binary blend of poly ((*R*)-3-hydroxybutyric acid) and poly ((*R,S*)-lactic acid) showed that the rate of enzymatic hydrolysis was much faster than the rate of non-enzymatic hydrolysis (Koyama & Doi, 1995).

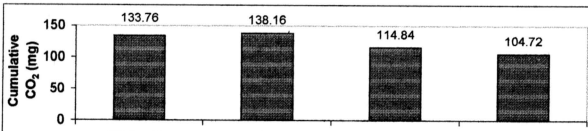


Figure 3.10- Cumulative CO_2 released within 70 days from stirred river water at 28°C which differs in the presence of PHA & microorganisms.

3.2.2 Viable cell count and pH

The microorganism growth pattern was sigmoidal (Figure 3.11), which is typical of a batch culture. There was a drop in microbe count during the first 6 days, which was most probably due to the death of the microorganisms and adjustment of some of the microorganisms to the biodegradability test set up which was quite different from the natural river environment. The microbial count was still very high (4.5×10^5/ml) on day 6. There is a high possibility that PHA-degrading-microorganisms were present in the river water. PHA-degrading microorganisms are abundant in the natural water and highly diverse. 52 bacteria, 9 streptomycetes and 3 moulds were isolated by Mergaert *et al.* (1992) from the freshwater in the university pond and found to be PHB-degraders. There was a 33.6% (3.7mg) weight loss of PHA during the first 6 days of incubation. Microbial degradation may play a significant role in the PHA weight losses that can be further verified in the later part of the discussion. The reduction in pH from pH 7.48 to pH 5.23 could be due to lysis of microorganisms and the release of hydroxyalkanoic acids from PHA degradation (Jendrossek *et al.*, 1996). This is further supported by the fact that on the final day, pH of the test reactor was so much lower (pH 4.69) as compared to the pH of the control reactor without microbes (pH 6.75).

The exponential growth phase extended from day 6 to day 20, stationary phase from day 20 to day 30 and death phase from day 30 to day 86. During these three phases, the PHA weight loss continued to increase at a polynomial pattern (Figure 3.14) and the pH reduced slowly from pH 5.23 (on day 6) to pH 4.69 (on day 86). This shows that there was a continuous degradation of PHA since day 0 till day 86. The death of the microorganisms was expected in a closed system because all the acidic products of PHA degradation and

microbial metabolism would result in the acidity of the surrounding medium. This would inhibit the activity of PHA depolymerases, which was reported so far to work best around pH 7-8 (Jendrossek *et al.*, 1996). The viability of the microorganisms would also be affected. Since the microbial activities would be lower during the death phase (day 30 to day 86), the continued weight loss of PHA during this period might be lower also and could be contributed by physical and chemical reactions.

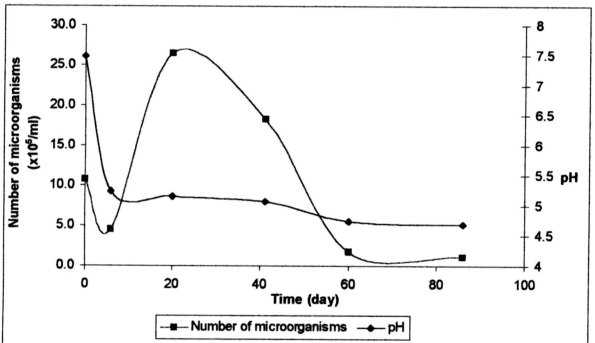


Figure 3.11 -Number of microorgnaisms and pH in stirred river water at 28°C

The lab test in this experiment was designed as a start to provide a better-controlled and monitored environment to test the inherent biodegradability of PHA. However, in the complex ecosystem, the growth of the microorganisms and PHA degradation may be lower or higher depending on the environmental conditions such temperature, moisture, aerobicity, organic materials (Kimura *et al.*, 1994; Mergaert *et al.*, 1995), ionic strength,

inorganic nutrients, toxic chemicals and competition among microorganisms. Further more, these environmental factors also fluctuate with time. Therefore, it should be born in mind that the degradation of PHA in the field cannot be predicted from lab experiments. Results obtained from laboratory set-up of PHB biodegradability test using pure cultures and enzymes were contrary to those obtained from the natural environment in compost soils (Doi, 1990; Briese *et al.*, 1994; Kanesawa *et al.*, 1994; Mergaert *et al.*, 1994)

Any result obtained is accurate to the extent of the experimental conditions within the test period. There were several cuts in electricity supply, which stopped the aeration and stirrer for 4 hrs to 60 hrs. Air leakage also occurred due to disconnection of the air tubing. In order to ensure that there was still sufficient number of viable cells to continue with the biodegradation process, viable cell count was done after these incidents. There was contamination in all the control reactors containing the sterile river water (Table 3.6). This might be due to the wetting of the outlet air filter and reverse airflow when there was air leakage and a stop in air supply. Contamination might have also occurred to the test reactors but due to the high acidity in the test reactors, the microbial growth could have been inhibited. The experimental set up that is recommended by ASTM standard (D5209-91, 1992), from which this experiment was derived, is in a non-sterile condition where no sterilization of air is required. This might be a better simulation of the field environment that is open to the influx and out flow of microorganisms. However, a sterile control was set up in this experiment so that the microbial count can be recorded to deduce the carbon balance. Due to contamination, an accurate carbon balance cannot be obtained but the analysis of inherent biodegradability of PHA is not affected.

Table 3.6 – pH and number of microorganisms in stirred river water in the test reactors and control reactors on day 0 and day 86 at 28°C

Reactor	PHA	Microbe (M)	pH_i (Day 0)	pH_f (Day 86)	Number of microorganisms /ml (Day 0)	Number of microorganisms /ml (Day 86)
Test 1 to Test 5	+	+	7.48	4.69	1.07×10^6	1.10×10^5
C1	-	+	7.44	5.38	1.85×10^6	2.83×10^5
C2	+	-	7.52	6.75	0	$>3.00 \times 10^6$
C3	-	-	7.52	6.55	0	1.75×10^5

Note. For Test 1 to Test 5 reactors, pH_i and number of microorganisms/ml on day 0 were an average value from 5 test reactors; pH_f and number of microorganisms/ml on day 86 were the value obtained from Test 5 reactor only. C1, C2 and C3 are control reactors varied in the presence of PHA and the treatment of sterilization of the river water.

3.2.3 Dissolved organic carbon (DOC) in river water

Dissolved organic carbon (DOC) is defined as the organic material that passes a 0.45-μm filter and is not lost by evaporation during the analytical procedure used for the measurement (Minear & Keith, 1984). In this experiment, DOC is a measure of the total biomass and intermediate products of PHA degradation. These measurements are required to deduce the carbon balance for PHA degradation: PHA + O_2 → residual PHA + intermediate products + new biomass + CO_2. The residue of the filtration represents the residual PHA particles suspended in river water. DOC from the 0.45-μm filtrate represents both the new biomass and intermediate products. The biomass was further removed by filtration with a 0.22μm filter in order to derive the DOC of the intermediate products and then back calculate to deduce the DOC of the new biomass.

Theoretically, samples without microorganisms should have a lower DOC than samples with microorganisms. However, half of the river water samples containing microorganisms showed a lower DOC than the same samples in which microorganism were filtered off by a 0.22-μm filter (Figure 3.12). This may be due to the extended storage period of the filtered samples before analysis which could have allowed the microorganisms that could survive in acidic medium and low temperature to utilize the dissolved organic compound in the water sample. This may explain the lower DOC in the river water samples containing microorganisms as compared to the DOC of the same sample without microorganisms.

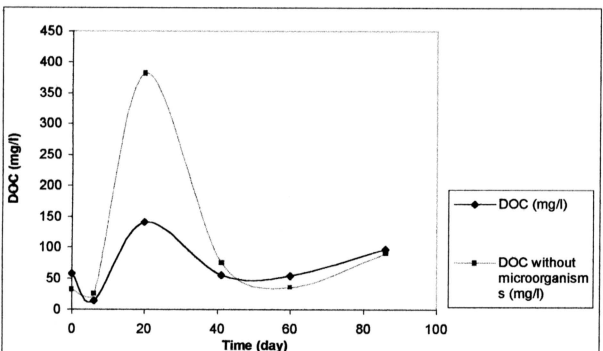


Figure 3.12 - DOC of stirred river water during the biodegradation of PHA at 28°C.

The river water was acidified and stored below 4°C. It was planned to be analyzed within 28 days as recommended by APHA (1989). However, the TOC-analyzer in the author's

department broke down during the period of the experiment and was not repaired by the end of the experimental period. Hence the storage time of the samples was extended from 28 days to between 52 days and 139 days before analysis by a commercial lab (ALS Technichem).

The DOC curve for the river water samples without microorganisms (Figure 3.12) has a similar trend with the curve for the number of microorganisms per ml in the river water (Figure 3.11). Therefore, other than the dissolved organic compound that was originally present in the natural water of River Kayu Ara, the microbial metabolic products could also contributed to the DOC of the river water. The biodegradability tests of various PHA samples in natural water from river Arakawa showed that about 1mgC/l (Abe & Doi, 1996) to 2mgC/l (Doi *et al.*, 1996) of the DOC were attributed to the water-soluble intermediate products. The initial DOC of the river water in which the microorganisms was filtered off was high (32.2mgC/l). These dissolved organic compounds in the river water might be utilized by the microbes faster than the released soluble degradation products of PHA, which resulted in a reduction of DOC to 25.3mg/l by day 6.

3.2.4 GC analysis of river water

Since the standard monomers of PHA available in the author's lab are in the form of methyl ester of 3-hydroxyalkanoic acids, which are hydrophobic and volatile, GC is suitable for the analysis of monomers in the river water sample. The soluble products of PHA degradation in the river water and those accumulated in the microorganisms were further hydrolyzed into monomeric units and methylated to form methyl ester of 3-hydroxyalkanoic acids and extracted with chloroform to be analyzed with GC.

There were some PHA monomers, i.e. C6, C10, C12 and C14 in the original river water (Figure 3.13), which shows that the river water might support the PHA-degrading microorganisms that utilize the PHA monomers as carbon substrates. The concentration of all monomers except C8 increased within the first 6 days. These monomers might come from the biodegradation of the PHA film, which was accompanied by PHA weight loss and pH reduction of river water. C8 could not be found in the river water until day 86 even though there was a complete removal of C8 from PHA film within the first 6 days indicated by GC analysis of the PHA film. This could be accounted by the high affinity and metabolism of the C8 monomer by the C8-metabolizing microorganisms in the river water, which rapidly metabolized the C8 monomers into other chemical compounds. Mergaert *et al.* (1995) reported that the intermediates from the biodegradation of P(3HB) and P(3HB-*co*-3HV) in the fresh water pond and sea water were rapidly metabolized by the degrading microbes too.

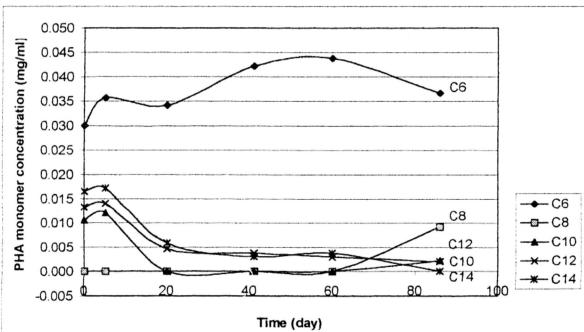


Figure 3.13 - PHA monomer composition in river water within 86 days at 28°C.

The rapid decrease of the monomer concentrations between day 6 and day 20 could be due to the higher rate of monomer metabolism by the microorganisms (since this was the growth phase period of the microorganisms) than the rate of monomer released from the biodegradation of PHA. The monomer concentration was quite stable between day 20 to day 60. The PHA monomer utilization rate (monomer output) and the PHA degradation rate (monomer input) were both declining during this period. This could be attributed to the reduction in the number of microorganisms. During this period, the rate of PHA weight loss also declined (Figure3.14).

The PHA film was completely disintegrated on day 86. Even though there were no visible PHA particle in the water, the residue recovered by filtration with Whatman filter paper no. 1 showed the presence of weight. When the PHA film had totally disintegrated into tiny suspended particles, the heightened increase in surface area of the PHA could promote high rate of hydrolysis of the remaining monomers (C10, C12 and C14) in PHA and diffusion of the hydrolysis products into the river water. Right now, the pathway of PHA hydrolysis was still unclear. Since there was a decrease in C12 and C14 monomers in the water between day 60 to day 80, these monomers could have been further hydrolyzed into smaller units by other microorganisms and /or metabolized

3.2.5 PHA dry weight

There is a trend of increase in the %weight loss of PHA film in a parabolic manner from day 0 to day 86 (Figure 3.14). All the weight was a direct measure of the dried PHA film except for the day 86 value which was the weight of the suspended PHA particles in the river water, recovered by filtration with Whatman paper no.1 because the PHA film was

completely disintegrated on day 86. The degradation of PHA occurred throughout the experimental period. Evaluation of the of P(3HB-*co*-14%3HV) biodegradability in river water at 25°C for 28 days based on biological oxygen demand (BOD) by Doi *et al.* (1996) also showed similar trend. The low pH and reduced number of microorganisms with time might reduce the activity and amount of the PHA depolymerases, which in turn reduce the biodegradation rate of PHA.

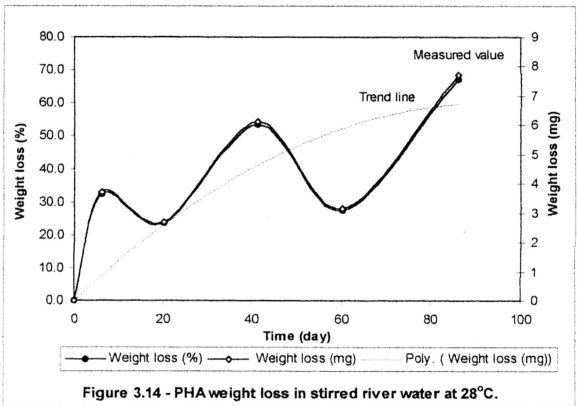


Figure 3.14 - PHA weight loss in stirred river water at 28°C.

Note: The trendline was a 2nd order polynomial drawn with a computer program.

The unevenness of the measured weight loss curve might be due to the differences in aeration and stirrer rate among the reactors, which in turn affected the degradation rate of PHA films in the reactors. Among the available stirrers, the stirring speed of a few stirrers could not be adjusted. This limit has to be tolerated in the experiment in order to provide a homogenous environment within each reactor.

A maximum PHA weight loss of 71.3% was reached within 86 days. However, the film in sterilized river water only reached a weight loss of 27.2% within 86 days. The PHB incubated in the unsterilized river water also have a higher weight loss (100% weight loss in 86 days) as compared to the one incubated in sterile river water (12.8% weight loss in 86 days). Since both the PHA and PHB films incubated in unsterilized river water have much higher weight losses as compared to their respective films incubated in sterile river water, biodegradation appears to be a significant cause of PHA and PHB degradation. Chemical and physical hydrolysis of PHA and PHB in the water might occur at a slower rate than biodegradation.

3.2.6 Scanning electron micrograph (SEM)

Figures 3.15 to 3.17 show the scanning electron micrographs (SEMs) of PHA film surfaces before and after incubation in stirred river water at 28°C. The unincubated PHA film surface was smooth and homogenous with occasional tiny pits (Figure 3.15*a* & Figure 3.17*a*). Tiny pits were also found initially in PHB and unsaturated cross-linked derivatives of poly(3-hydroxyoctanoate) (PHO) (Molitoris *et al.*, 1996). After incubation for 6 days, irregular pits and blemishing effect between less eroded area was visible on the film surface (Figure 3.15*b*). In comparison, there were very little blemishing effect and no or very few pits on the PHA films incubated in unstirred river water for more than 6 days (Figure 3.4*c* & *d*). This shows that the mechanical force of the water current played an important role in speeding up the degradation of PHA film. The water current may physically erode the PHA film surface and also carry the water-soluble products from PHA biodegradation.

Prolonged incubation increased the blemish, which expanded to a larger area of the film surface by day 41(Figure 3.15*c*) and the entire film surface by day 60 (Figure 3.15*d*). The blemish can be seen as a coarse translucent surface on the PHA film (Figure 3.16*a*). Tiny pits

were also visible. The degradation might start from the film surface by forming surface lesions. From these lesions, degradation proceeded side ways to the adjacent PHA layers as well as inward through these lesions. As the hole became larger and converged with the surrounding holes, a resultant pattern visible on day 41 (Figure 3.17*c*) and day 60 (Figure 3.17*d*) can be found on PHA film. Patches of blemished upper layer varying in sizes and shapes were found protruding from the newly exposed inner PHA film layer, which was smoother than the upper layer. A closer view of the holes (Figure 3.17*b*) on the day 6 film surface indicates the presence of tiny pits on the inner wall of the hole. Degradation may proceed from these tiny pit side ways and inwards, forming wider and deeper holes as found on day 41 (Figure 3.17*c*) and day 60 (Figure 3.17*d*) PHA films. As the holes and pits converged in all directions, three-dimensional "cross-linked tunnel" might be formed, as shown in the SEM of the freeze-fractured PHB and cross-linked derivatives of PHO cross-section area (Molitoris, 1996). Visible tunnel-like hole can be seen on the PHA film (Control C,2) incubated in sterile river water for 86 days (Figure 3.17*e*).

The PHA film incubated in non-sterile river water was not visible any more by day 86. However, the sterile-incubated PHA film still existed on day 86, with reduced thickness and a large hole was visible (Figure 3.16*b*). This shows that the biodegradation rate of PHA in the presence of microorganisms is higher than the degradation rate due to physical and chemical action only. In the SEMs of PHB and cross-linked derivatives of PHO film, rod-shaped bacteria were found attached to the film surface, and even more around the ridges and within the pits (Molitoris *et al.*, 1996). This observation was possible as the samples were chemically fixed after incubation in pure bacteria culture fluid. The sonication of PHA films in this experiment with the purpose of removing attachments on the film to obtain the weight of the film only might have made difficult the detection of bacteria on the PHA film.

The sterile river water in which Control 2 was incubated was found to be contaminated on day 86. Hence, it cannot be concluded that the degradation observed on Control 2 was due to physical and chemical degradation only. However, the PHA control sample film (in Run 2) incubated for 28 days in stagnant river water which was not contaminated at the end of the period showed a weight reduction of 10.6% (2.2mg). This shows that PHA may be degraded by non-biological reactions. In one study (Mergaert *et al.*, 1992), no weight loss was found in the PHB after 3 months' incubation in sterile solutions. However, the study of chemical hydrolysis of bacterial PHB showed significant weight loss at pH 10 (Marchessault *et al.* 1994) and some reduction in molecular weight at pH 7.4 (Doi *et al.*, 1990). Therefore, the degradation of PHA in sterile condition can be due to chemical reactions from the unknown chemical substances present in the river water. It is concluded that biological, physical and chemical reactions may degrade the PHA film.

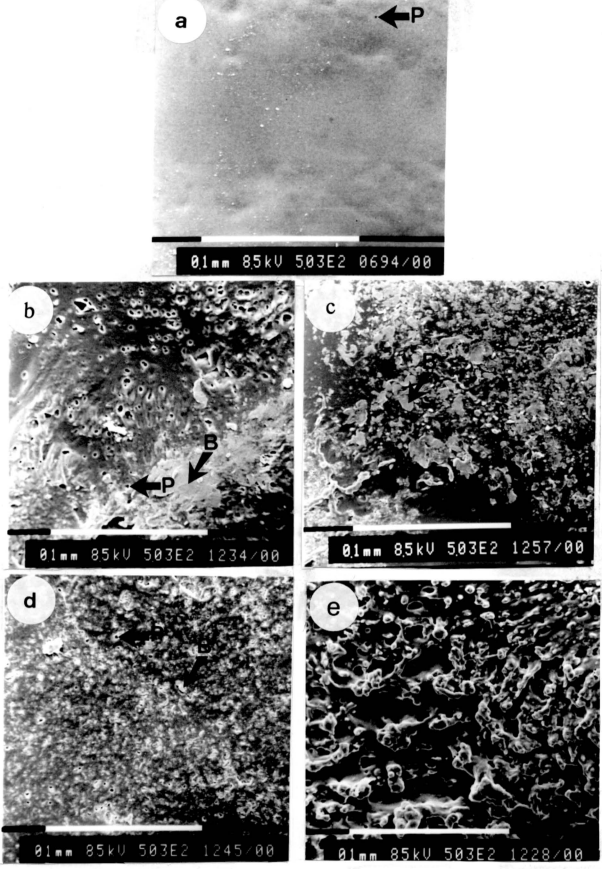


Figure 3.15 – SEMs of PHA film surfaces incubated in stirred river water at 28°C for *(a)* 0 days; *(b)* 6 days; *(c)* 41 days; *(d)* 60 days & *(e)* 86 days (in sterile river water). The PHA film incubated in non-sterile river water was completely disintegrated. B indicates blemished area; P indicates pit(s). Magnification 5.03E2 = 5.03 X 10^2.

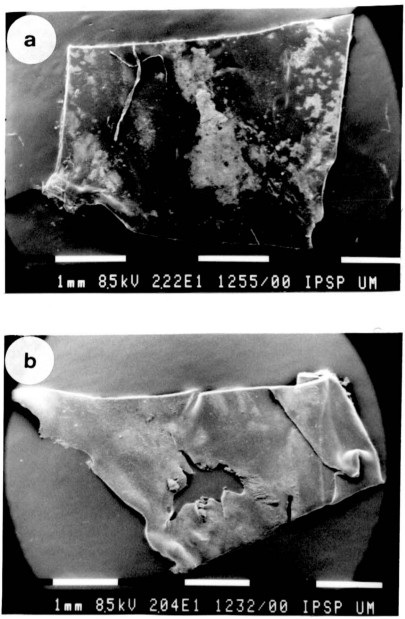


Figure 3.16 – SEMs of PHA film surfaces incubated in stirred river water at 28°C for *(a)* 41 days and *(b)* 86 days (in sterile river water). The film incubated in non-sterile river water was completely disintegrated on day 86. The sample was cut from a larger piece of PHA film reserved for other analysis. Magnification 2.22E1 = 2.22 X 10^1.

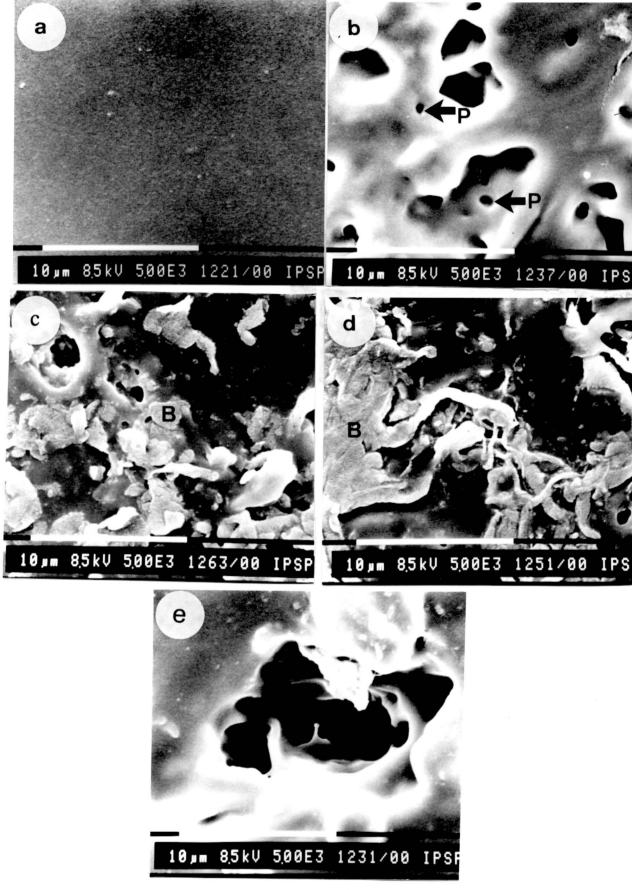


Figure 3.17 – SEMs of PHA film surfaces in stirred river water at 28°C for *(a)* 0 days; *(b)* 6 days; *(c)* 41 days; *(d)* 60 days; *(e)* 86 days (in sterile river water). The PHA film incubated in non-sterile river water was completely disintegrated on day 86. B indicates blemished area and P indicates pit(s). Magnification 5.00E3 = 5.00 X 10^3.

3.2.7 Infrared (IR) spectrometry analysis

All PHA samples yielded almost identical IR spectra regardless of the time the PHA sample was exposed to biodegradation (Figure 3.18*a-f*). The chemical groups represented by the IR peaks were similar to those chemical groups reported in the chemical structure of PHA (Figure 1.2 & Sudesh, 1994). These include the **O-H** stretching absorption at 3444-3451cm^{-1} region, the high intensity of asymmetric and symmetric stretching absorption of **–CH_3-** and **–CH_2** around 2972-2843cm^{-1} region which indicates the presence of a long aliphatic side-chain (Smet *et al.*, 1983) and the 1750-1735cm^{-1} region which indicates the presence of **–C=O** of the ester group (Sudesh, 1994).

Further evidence of the long aliphatic side chain could be derived from the "finger-print region" (1600 to 600cm^{-1}) which is unique for a particular structure. The absorption band around 1466cm^{-1} indicates the scissoring vibration of **-CH_2-**, the band around 1450cm^{-1} indicates the asymmetric vibration of **-CH_3**, the band around 1375 cm^{-1} indicates the **C-CH_3** group (Furniss *et al.*, 1989), either **–C=O** or **C-C** stretching vibration band is around 1300-1100cm^{-1} (Wade, 1987), and the most typical skeletal group, **–$(CH_2)_n$-**, where $n \geq 4$ shows a weak band near 725cm^{-1} (Tosi and Ciampelli, 1973).

The biodegradation process did not involve in the removal of the aliphatic side chain. It was reviewed by Jendrossek *et al.* (1996) that the PHA depolymerases mainly hydrolyse the ester linkages of PHA into monomers, dimers and oligomers depending on the type of PHA depolymerases. Hence, the aliphatic side chains (R) were still intact to the (**~O-CH(R)CH_2CO~**) backbone of PHA. The monomers, dimers and oligomers would still contain carbonyl group (**C=O**) and hydroxyl group (**-OH**) before and after hydrolysis.

Furthermore, the purification of PHA before IR analysis could have removed the hydrolysed units, which were relatively short and more readily soluble in the solvent. Hence, the IR spectra of the PHA samples were almost identical regardless of the exposure time of the PHA sample to biodegradation.

Two additional absorption bands (indicated by arrows) were found in the IR spectrum of the PHA sample degraded for 86 days in sterilized river water (Figure 3.18*f*). The absorption band at 804.39 cm^{-1} can be due to the out of plane bending absorption of carbon double bond (**R_2C=CHR**) (Streiwieser & Heathcock, 1976). New carbon double bond could be formed due to chemical oxidation of the PHA in the sterilized river water for an extended incubation period. The absorption band at 2359.77 cm^{-1} is due to CO_2 in air.

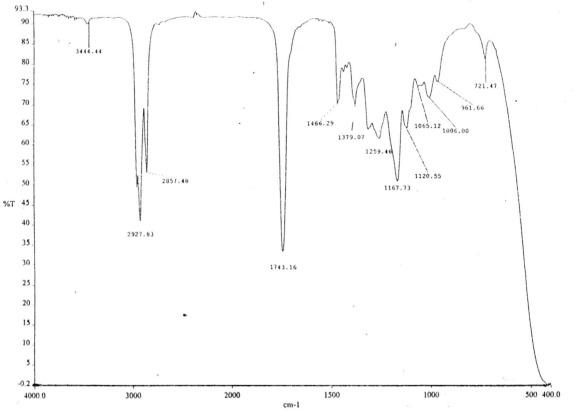


Figure 3.18 – *(a)* IR-absorption spectrum of unincubated PHA.

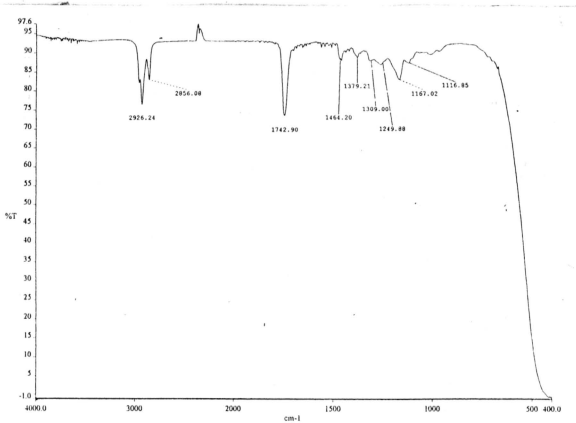


Figure 3.18 – *(b)* IR absorption spectrum of PHA incubated in stirred river water at 28°C for 6 days.

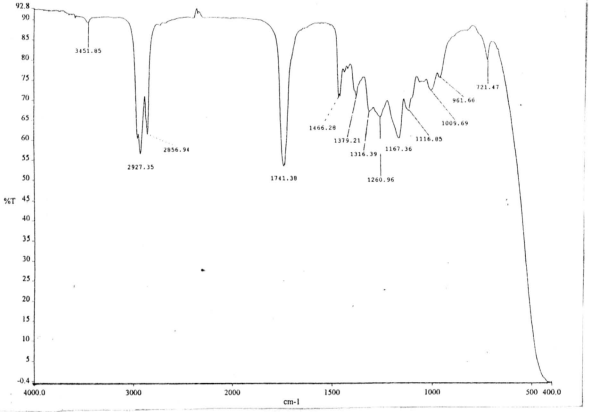


Figure 3.18 – *(c)* IR absorption spectrum of PHA incubated in stirred river water at 28°C for 20 days.

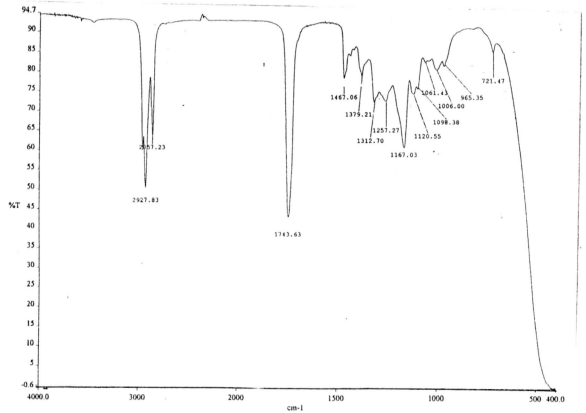


Figure 3.18 – *(d)* IR absorption spectrum of PHA incubated in stirred river water at 28°C for 41 days.

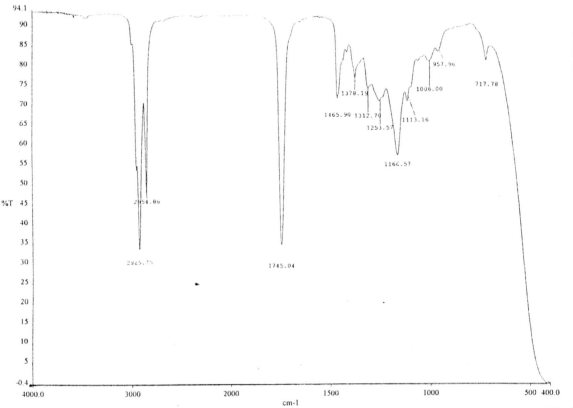


Figure 3.18 – *(e)* IR absorption spectrum of PHA incubated in stirred river water at 28°C for 60 days.

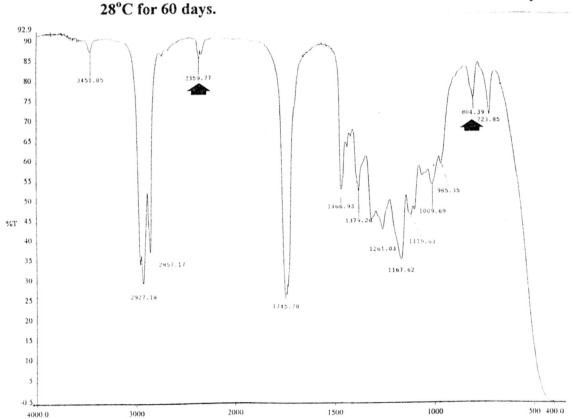


Figure 3.18 – *(f)* IR absorption spectrum of PHA incubated in stirred sterile river water at 28°C for 86 days.

3.2.8 Molecular weight by gas permeation chromatography (GPC)

It has been planned to measure the molecular weight of the PHA films by GPC in order to analyze the mechanism of PHA degradation. Due to the break down of the GPC instrument, the PHA sample (minimum 0.3mg) reserved initially for GPC analysis were insufficient for analysis by viscometry method, which requires a minimum of 100mg PHA sample (Jabatan Kimia, n.d.). Therefore molecular weight analysis could not be carried out.

3.2.9 Monomer composition of PHA by gas chromatography (GC)

The mole % and % w/w of the PHA monomers were both indicative of the composition of monomers in the PHA film. The mole % unit is more appropriate than the % w/w unit for the analysis of PHA biodegradation. It was reviewed by Jendrossek *et al.* (1996) that PHA degradation resulted in the removal of monomers, dimers and oligomers. Mole % would indicate the relative number of monomers in the PHA sample, which would change in accordance with the number of monomers removed during biodegradation. On the other hand, w/w % indicates the relative weight of each monomer in the PHA. W/w% is not a direct indication of the relative number of monomers in the PHA samples. However, the %w/w unit can be used to compare the monomer composition of PHA in this experiment with other research that reported the PHA monomer composition in %w/w. The detailed calculations of %w/w and mole% were shown in Appendix 5.

In both the PHA in this experiment and that of earlier work by Tan *et al.* (1997), C8 was the major monomer in PHA, followed by C10, C12 and C14 (Table 3.7). However, the % composition of each monomer differed between the two experiments although the substrates

used were both SPKO. These may be due to batch differences of the substrates. The influence of substrate composition on the PHA monomer composition has been shown by Tan *et al.* (1997) earlier. A colleague in the author's laboratory also obtained results of PHA monomer composition (not shown) which was very close to the author's finding. He has used the same batch of SPKO for the production of PHA by *Pseudomonas putida* PGA1, which was cultivated with similar condition except that the nitrogen source ($NaNH_4HPO_4.H_2O$) in E2 medium (production medium) was being replaced by yeast extract. There is also little difference between the value indicated by mole% and %w/w.

Table 3.7 – Monomer compositions of the original PHA film

Substrate	Unit	Relative amount of monomers in PHA				
		C6	C8	C10	C12	C14
SPKO	mole %	0.0	54.4	24.0	14.6	7.0
SPKO	%w/w	0.0	48.1	25.0	17.4	9.5
SPKO (Tan *et al.*, 1997)	%w/w	8.7	82.9	4.5	1.4	1.3

The C8 monomers were completely removed from the PHA film within 6 days (Figure 3.19) whereas they were still present in the control PHA (52.7 mole %) after 28 days in the sterile river water. There is a high possibility that PHA-degrading microorganisms were present in the river water and they could produce extracellular PHA depolymerases, which have higher affinity for C8 monomers. Abe *et al.* (1995) reported that the side-chain length of hydroxyalkanoate unit could highly affect the accessibility of the PHA ester bonds to the active site of PHA depolymerase. Hence, the rate of enzymatic degradation markedly decreased with an increase in the side-chain length of the 3-hydroxyalkanoate monomeric

units (Kanesawa *et al.*, 1994). Consequently, C8, having the shortest side chain will be hydrolysed at the highest rate, followed by C10, C12 and C14 as shown in Figure 3.19.

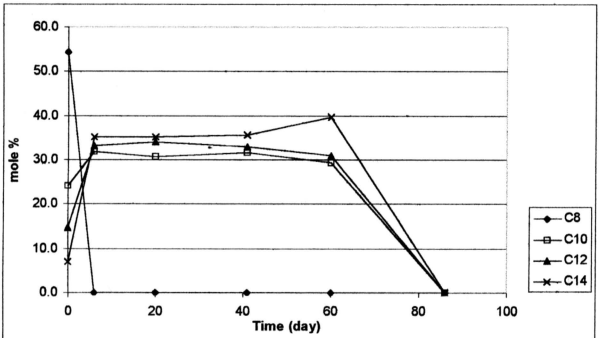


Figure 3.19 - Monomer composition of PHA film during the 86 days' period of biodegradability test in stirred river water at 28°C.

A few researchers reported that the enzymatic degradation of PHA films occurred on the film surface only because the enzymes do not penetrate the bulk of the polymer. The PHA tested includes the polymer blends of bacterial poly[*(R)*-3-hydroxybutyrate] and poly[(*R,S*)-3-hydroxybutyrate] (Abe *et al.*, 1995), a blend of bacterial poly[*(R)*--3-hydroxybutyric acid] and poly[(*R,S*)-lactic acid] (P[*(R)*-3HB]-P[(*R,S*)-LA])(Koyama & Doi, 1995), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (Doi, 1995). Koyama & Doi (1995) further showed that degradation through out the entire P[*(R)*--3HB]/P[(*R,S*)-LA] film only occurred by chemical hydrolysis in a phosphate buffered solution due to water permeability of the film matrix. However biodegradation seemed to occur in the entire PHA film in this experiment.

This is possible only if the entire PHA film is accessibility to PHA depolymerase. Marchessault *et al.* (1994) mentioned that crystallinity of the PHB film would affect the local mobility of the polymer chains in water and thereby influence enzyme accessibility into the entire polymer film. The local mobility of polymer chains increase with a decrease in crystallinity. This is further supported by the finding that the rate of enzymatic degradation for P[*(R)*-3HB] chains in an amorphous state was about 20 times higher than the rate in a crystalline state (Abe *et al.*, 1995b). Since the PHA film in this experiment is completely amorphous (Tan *et al.*, 1997), there is a high possibility that the PHA polymer chains have high local mobility under the test conditions. Furthermore, as far as has been reported, the molecular masses of PHA depolymerases are relatively small (below 100kDa), and most depolymerases consist of only one polypeptide (Jendrossek *et al.*, 1996). Therefore, this PHA could be highly accessible to PHA depolymerases in the river water for biodegradation process throughout the film. Another way to verify that PHA degradation occurred throughout the PHA film is by the change in the molecular weight (Mergaert *et al.*, 1995). However, the molecular weight could not be measured due to the breakdown of the gel permeation chromatograph instrument as explained in Section 3.2.7.

The loss of C8 monomers on day 6 consisted 48%w/w of PHA. This was comparable to the weight loss of PHA on day 6, which is 33.6%, which should be higher if the weight due to surface attachments is excluded.

The composition of C10, C12 and C14 monomers in the PHA film was almost constant from day 6 till day 40, while the PHA film continued to reduce in weight. These three monomers may be degraded at a similar rate or most probably at a rate depending on the

side-chain length. C10 and C12 monomers in the bulk PHA matrix could have been hydrolyzed but remained in the matrix.

From day 40 onwards, C10 & C12 compositions of PHA were reduced, showing a higher hydrolysis rate of C10, followed by C12. As the degradation proceeded, the surface area exposed to the river water also increased due to formation of "interconnected tunnels" within the PHA as explained in Section 3.2.6. Hence, the hydrolyzed units trapped within the film will diffuse out of the film in an increasing rate as the degradation proceeded.

In short, the rate of PHA hydrolysis depends on the chain length of the monomers. The shorter chain monomers appeared to be preferentially hydrolyzed compared to the longer chain monomers. Hence, C8 was completely hydrolyzed first, followed by C10, C12 and C14 in succession.