APPENDIXES A

Appendix A: European Standards (EN)

Biodegradability in Europe: Regulation EN 13432

A standard is a technical publication that is used as a rule, guideline or definition. Essentially, it is a repeatable way of doing something, developed through consensus. Standards are created by bringing together all interested parties, including manufacturers, consumers and regulators of a particular material, product, process or service. Everyone benefits from standardization through increased product safety and quality as well as lower transactions costs and prices.

The European Standard EN13432 "Requirements for resins recoverable through composting and biodegradation - Testing scheme and evaluation criteria for the final acceptance of biological resins" resolves this problem by defining the characteristics that a material must have, in order to be defined as "compostable" or "biodegradable". This norm is a reference point for material manufacturers, public authorities, composters and also very useful to consumers. According to the European Standard EN13432, a compostable material must have the following characteristics:

- Biodegradability is determined by measuring the actual metabolic conversion of the compostable material into carbon dioxide. This property is quantitatively measured using the standard test method, EN14046 (which is also published as **ISO 14855**: biodegradability under controlled composting conditions). The acceptance level is 90%, which must be reached in less than 6 months.
- Disintegrability is the fragmentation and loss of visibility in the final compost (absence of visual contamination). This is measured with a composting test (**EN14045**). The test material is degraded, together with organic waste, for 3 months. After this time, the compost is sieved with a 2 mm sieve. The residues of test material with dimensions higher than 2 mm are considered as not having disintegrated. This fraction must be less than 10% of the initial mass. Absence of negative effects on the composting process. This is checked with a composting test. Low levels of heavy metals (below the predefined maximum values), and absence of negative effects on the quality of the compost (e.g. reduction of the agronomic value and presence of eco-toxicological effects on the growth of plants). A plant growth test (OECD; the Organization for Economic Co-operation and Development Test 2008, modified) is carried out on compost samples where the

degradation of the test material has taken place. There must be no difference from control compost. Other chemical-physical parameters that must not be different from those of the control compost after the degradation are the pH, salinity, volatile solids, N, P, Mg, K.

- Each of these points is necessary for the definition of compostability, but each point alone is not sufficient. For example, a biodegradable material is not necessarily compostable because it must also break up during one composting cycle. On the other hand, a material that breaks up over one composting cycle, into microscopic pieces that are not totally biodegradable is not compostable.

EN14045:2003 for title entitled "Packaging. Evaluation of the disintegration of packaging materials in practical oriented tests under defined composting conditions"

EN14046:2003or ISO 14855 for title entitled "Determination of the ultimate aerobic biodegradability of plastic materials under controlled composting conditions –Method by analysis of evolved carbon dioxide"

ASTM was determined to be the method recommended for analyzing bio-based content

ASTM D6866-05 (Plastics Standards)

ASTM D6866 is a widely used method in the bioplastics industry. This standard quantifies the biobased content relative to the material's total organic content and does not consider the inorganic carbon and other non-carbon containing substances present. It must be noted that ASTM D6866 only quantifies the biobased content of a material but results do not have any implication on the material's biodegradability.

The terms biobased and biodegradability may be related, but they are not synonymous nor are they interchangeable. If a material is biobased, it comes from plants or animals, but it does not necessarily follow that it is biodegradable. A material is biodegradable only if microbes in the environment can break it down and use it as a food source. Braskem, a leading Brazilian petrochemical company, is one of the many bioplastics companies that use ASTM D6866 to certify the biobased content of their products.

ISO 14040 and ISO 14044 are the Environmental Management Life Cycle Assessment Package to identify opportunities to improve the environmental performance of products at various points in the life cycle. The life cycle assessment study in the environmental management process consists of defining the scope and goal, inventory analysis, assessing the impact and interpreting the data. ISO 14040 and ISO 14044 Environmental

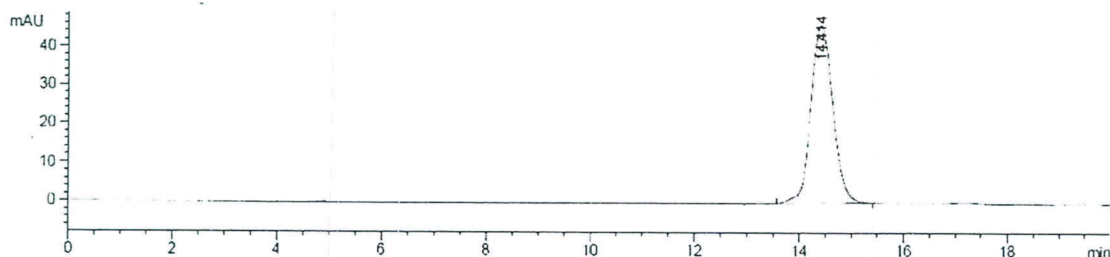
Management Life Cycle Assessment Package provide the principles, framework, requirements and guidelines to conduct a life cycle assessment study.

APPENDIXES B

Appendix B: Calculated of standard curve i.e. Poly [(R)-3-hydroxybutyric acid] (PHB) and poly(3-hydroxybutyrate-co-valerate) (PHBV), glycerol, glucose.

Appendix B-1: The purity of the standard Poly 3-hydroxybutyric acid (PHB) was purchased from Sigma-Aldrich Co. Ltd. is defined as 100%.

The standard PHB was freshly prepared as a stock solution at 20 g L^{-1} by dilution to various concentrations at 2, 4, 6, 8 and 10 g L^{-1} according to modified Hesselmann *et al.*, (1999). Then, 1-mL of concentrated H_2SO_4 was added for hydrolysis digestion. To determine recovery efficiency PHB was weighed directly into the tubes and suspended in 1-mL of dH_2O (HPLC grade) prior to acid addition. The closed tubes were heated for 90 min at 90°C without mixing. After cooling, the digest was quantitatively transferred into a volumetric flask (100-mL) using dH_2O. Two mL of the diluted digest were centrifuged (15 min, 11,000 x g) and 1-mL of the solution was used for HPLC (Hesselmann *et al.,* 1999). Subsequently, the solution was filtered with 0.45 μm diameter filter and mixed with dH_2O (HPLC grade). The separation was performed on an Aminex-87H300 x 7.8 column (Bio-Rad, USA) and then the compound was analyzed by UV detector at a wavelength of 210 nm. The mobile phase was 5 mM H_2SO_4 and 20% CH_3CN with a flow rate 0.5 $mLmin^{-1}$ and the column temperature was 55°C. Standard curve was used to analyze concentration of polyhydroxybutyrate (PHB) that extracted from mixed culture. HPLC peak of poly [(R)-3-hydroxybutyric acid] at 2 g L^{-1} and retention time of PHB is 14.4 min.



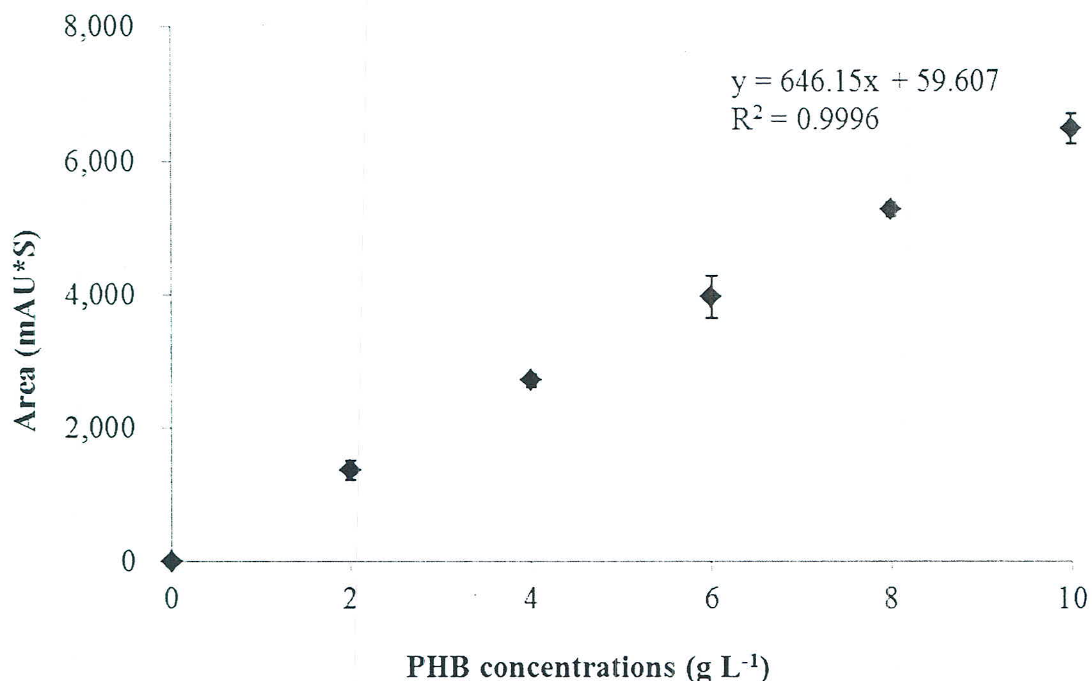


Figure B-1 Standard of Poly [(R)-3-hydroxybutyric acid] detect with HPLC method

Appendix B-2: The purity of the standard poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid; PHBV) of natural origin and with 12wt% polyhydroxyvalerate (PHV), and pure poly-3-hydroxybutyric acid of natural origin was purchased from Sigma-Aldrich Co. Ltd. USA.

Meaning;	12 g PHV prepared from	100 g PHBV
	2 g PHV prepared from	(100/12) x 2 = 16.67 g PHBV

Therefore, preparing 2 g L^{-1} PHV would 16.67 g PHBV dissolved in 1-L dH_2O (HPLC grade). Consequently, the standard solution was subjected to HPLC by defined at this concentration equally 100% by weight of PHV. The standard of poly(3-hydroxyvaleric acid) was freshly prepared as a stock solution at 2 g L^{-1} by dilution to various concentrations at 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 gL^{-1}. Then, 1-mL of concentrated H_2SO_4 was added for hydrolysis digestion. To determine recovery efficiency PHV was weighed directly into the tubes and suspended in 1-mL of dH_2O (HPLC grade) prior to acid addition. The closed tubes were heated for 90 min at 90°C without mixing.

After cooling, the digest was quantitatively transferred into volumetric flask (100-mL) using dH_2O. Two mL of the diluted digest were centrifuged (15 min, 11,000 X g) and 1-mL of the solution was used for HPLC (Hesselmann *et al.*, 1999). Subsequently, the solution was filtered with 0.45 μm diameter filter and mixed with dH_2O (HPLC grade). The separation was performed on an Aminex-87H300 x 7.8 column (Bio-Rad, USA) and then the compound was analyzed by UV detector at a wavelength of 210 nm. The mobile phase was 5 mM H_2SO_4 and 20% CH_3CN with a flow rate 0.5 $mLmin^{-1}$ and the column temperature was 55°C.The standard curve was used to analyze the concentration of polyhydroxybutyrate (PHB) extracted from the mixed culture. HPLC peak of poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid; PHBV) at 0.4 g L^{-1} and retention time of PHB is 14.4 min and PHV is 6.1.

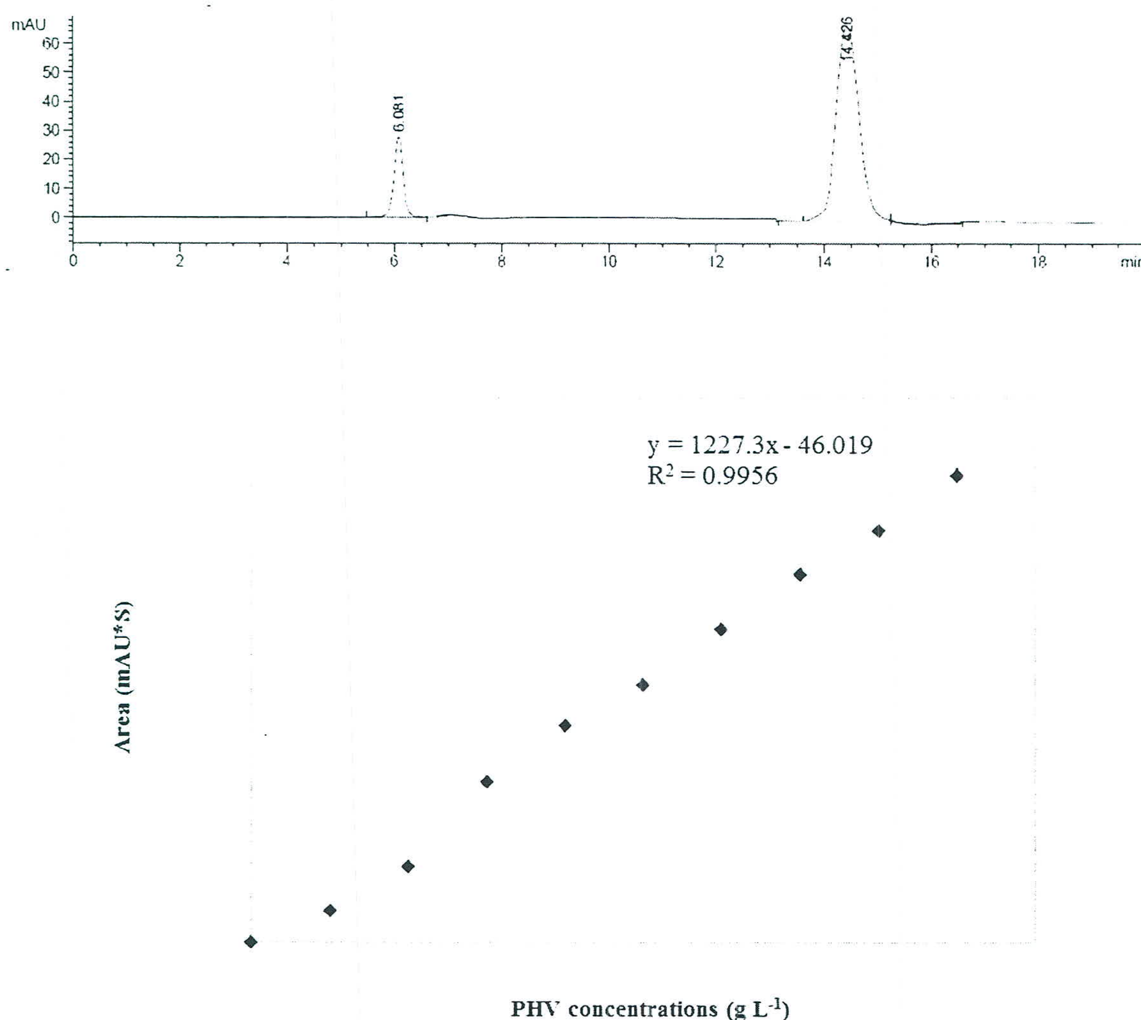


Figure B-2 Poly(3-hydroxyvaleric acid) standard curve for calculation of concentration

Appendix B-3: Glucose and glycerol calibration curves were determined by HPLC, using an Aminex HPX-87H column from Bio-Rad, USA. HPLC (Agilent LC1200 Series, USA) equipped with refractive index detector (RI). Each solution was injected into HPLC column (Aminex HPX-87H column, 300 x 7.8 mm) and eluted by 5 mM H_2SO_4 with a flow rate 0.60 mL min^{-1} (Gao *et al.*, 2006; Koller *et al.*, 2005).

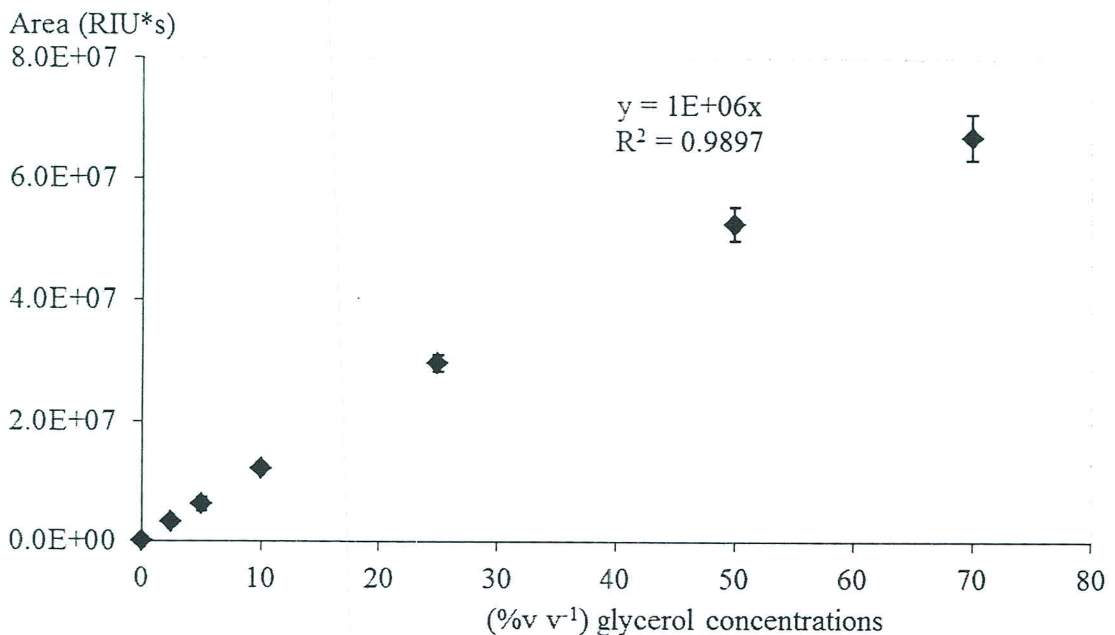
Purified commercial – glycerol grade (Qrec, purity 99.5%, New Zealand) standard curve was used to determine the concentration of using glycerol from culture supernatant in various conditions. The standard 99.5% pure commercial glycerol standard was freshly prepared in various concentrations at 0, 2.5, 5, 10, 25, 50 and 70 v v^{-1}. The glycerol concentrations were analyzed using HPLC. The concentrations of glycerol consumption were calculated by comparing the data with the glycerol standard curve (between 0 and 70 v v^{-1}).

From purified commercial – glycerol grade (Qrec, purity 99.5%, New Zealand)
Meaning 100-mL solution contained purified glycerol 99.5-mL. For example at 10% v v^{-1}, glycerol would be dissolved in 10.05-mL and adding dH_2O until 100-mL.

Density of glycerol grade = 1.261 g mL^{-3}

Thus, = 1.261 x 10 = 12.61 g

So, at 10% v$v^{-1}$of glycerol solution in 1-L medium = 126.1 g L^{-1}



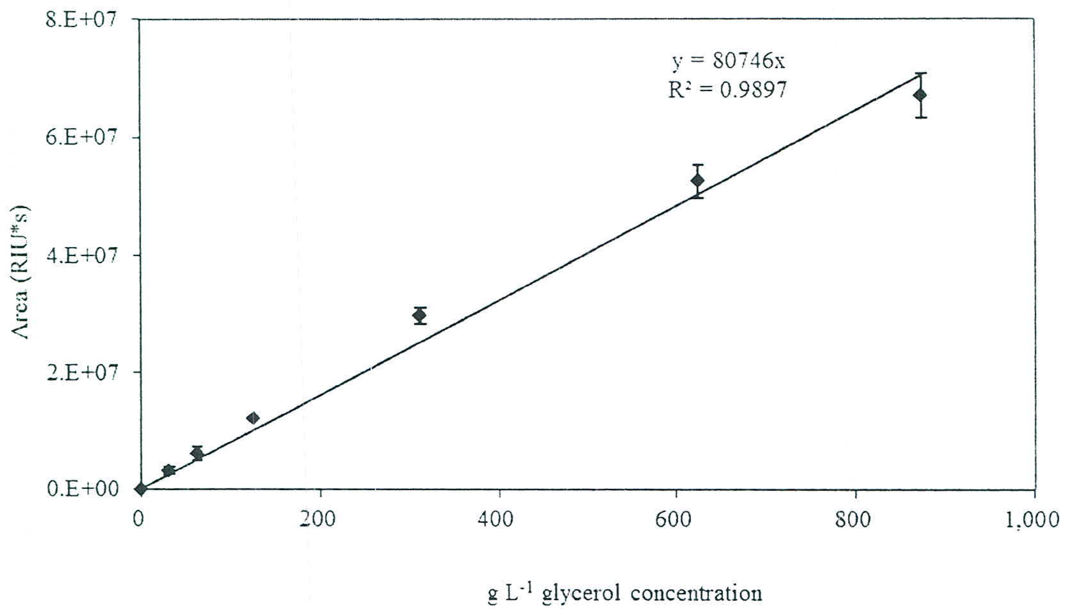


Figure B-3 Standard of purified commercial – glycerol grade (Qrec, purity 99.5%, New Zealand) detect with HPLC method

In case of pure glucose standard (BDH, England) was freshly prepared in various concentrations at 4, 8, 12, 16, and 20 g L^{-1}. The glucose concentrations were analyzed using HPLC. The concentrations of glucose consumption were calculated by comparing the data with the glucose standard (between 0 and 20 g L^{-1}).

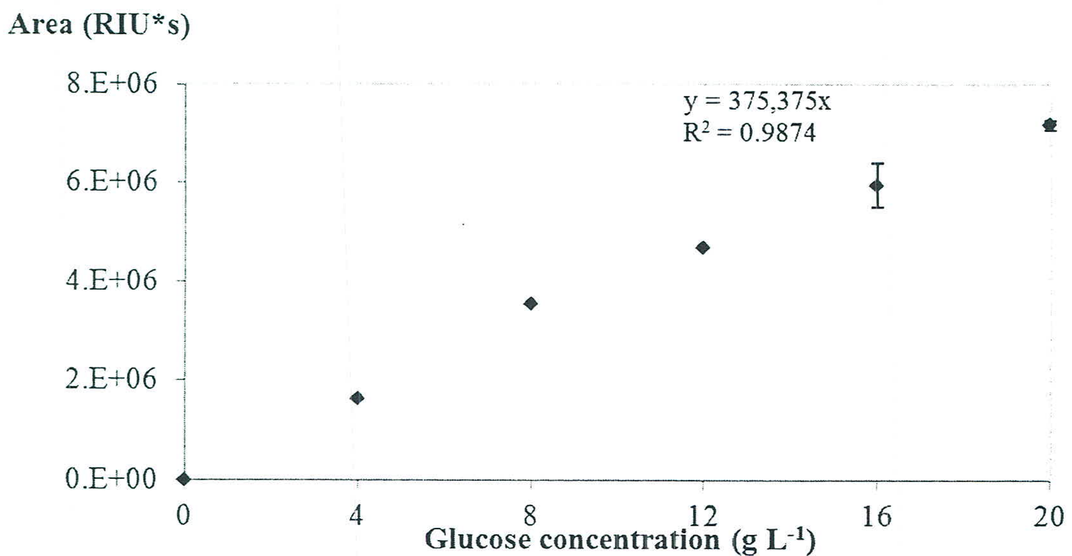


Figure B-4 Glucose standard curve for calculation of glucose concentration.

Example The supernatant of mixed culture was cultivated in a medium supplemented with 10% v v^{-1} glycerol (126.1 g L^{-1}) and 1 % wv^{-1} (10 g L^{-1}) glucose under 72 h, an agitation rate of 200 rpm at 30°C. Poly [(R)-3-hydroxybutyric acid] (PHB) and poly [(R)-3-hydroxyvaleric acid] (PHV), glycerol, glucose were measured by HPLC in 1-mL culture broth samples that had been centrifuged at 10,000 x g for 10 min. The supernatant was first filtered through a non-sterile 0.45 μm nylon membrane filter to remove cells and solids, and was then frozen. The glycerol concentrations were analyzed using HPLC (Agilent LC1200 Series, USA) follow as previous method.

1. The value of Poly [(R)-3-hydroxybutyric acid] (PHB) can be determined from the following equation:

At retention time = 14.4 min

If 1 g CDW after hydrolysis digestion is suspended and then made up with deionized water depending on viscosity suspension for instant made up 1.5 L (dilution factor = 1.5 L).

The PHB concentration (g L^{-1}) = [(Area (RIU*s) - 59.607)/ 646.15] x dilution factor

= [(8,295.65-59.607)/646.15] x 1.5

= 19.119 g L^{-1}

2. The value of Poly [(R)-3-hydroxyvaleric acid] (PHV) can be determined from the following equation:

The PHV concentration (g L^{-1}) = (Area (RIU*s) + 46.019)/ 1227.3

Nevertheless, it cannot occur at retention time 6.2 min.

3. The value of glycerol (g L^{-1}) can be determined from the following equation:

The glycerol concentration (g L^{-1}) = (Area (RIU*s)/ 80746

= 5,229,796.40/ 80746

= 64.768 g L^{-1}

The glycerol consumption (g L^{-1}) at t time was glycerol concentration at time t and initial glycerol concentration at time zero (t_0). Therefore at t time;

Glycerol consumption (t) = Initial glycerol (t_0) – glycerol (t);

Glycerol consumption on 120 h = 9,940,141.37/80746 - 5,229,796.40/80746

= 123.104 - 64.768 g L^{-1}

= 58.335 g L^{-1}

In other words;

Residual glycerol (t) = Initial glycerol (t_0) – glycerol consumption (t)

= 123.104 - 58.335 g L^{-1}

= 64.768 g L^{-1}

4. The value of glucose (g L^{-1}) can be determined from the following equation:

The glucose concentration (g L^{-1}) = (Area (RIU*s)/ 375,375

= 8,933.925/ 375,375

= 0.024 g L^{-1}

Appendix B-4: Parameter calculations

PHB content (% by weight) was defined as the ratio of PHB concentration to cell concentration (Wang and Lee 1997). The percentage of PHB content in batch culture is described by simple equations:

$$\text{PHB content (\% by weight)} = \frac{\text{PHB dry weight per L}}{\text{Cell dry weight per L}} \times 100$$

Cell concentration (CDW; g L^{-1}) = Total of cell dry weight per L

Where cell dry weight per L is the total of cell concentration, t is the cultivation time, and $t_0$is the cultivation at time zero. Therefore, to the calculation of residual CDW is to determine initial of cell concentration minus the appearance of cell concentration during the time indicated.

At t time; Residual CDW (g L^{-1})= CDW (t_0) – CDW (t)

or alternatively:

PHB productivity or PHB synthesis rate (g PHB L^{-1} h^{-1})for which the PHB synthesis rate was defined as grams of PHB synthesized per gram of residual cells per hour according to Wang and Lee 1997 as follows:

$$\text{PHB productivity(g PHBL}^{-1}\text{ h}^{-1}) = \frac{\text{PHB dry weight/ Residual CDW}}{\text{Period of } t \text{ hour}}$$

By measuring the ratio of grams of PHB synthesized per gram of residual cells during a certain time *t* period to at hour *t* time can be calculated.

$$\text{PHB yield} = \frac{\text{PHB production (g L}^{-1})}{\text{Total of cell dried weight (g L}^{-1})}$$

The PHB yield coefficient relative to substrate utilization ($Y_{PHB/S}$) was calculated according to Ramadan *et al.* (1985), which is defined as the increase in PHB mass per glycerol consumed once the cultures have reached the stationary phase.

Therefore, $$\text{PHB yield } (Y_{PHB/S}) = \frac{\text{PHB production (g L}^{-1})}{\text{Glycerol utilization (g L}^{-1})}$$

APPENDIXES C

Appendix C: Tabulation of raw data

Table C-1 Growth of mixed culture in medium containing 0, 10, 30 and 50% v v^{-1}glycerol as carbon source at 30°C, 200 rpm (n = 3)

Time (hours)	Culture turbidity (OD_{600})			
	0%	10%	30%	50%
0	0.007 ± 0.002	0.006 ± 0.002	0.045 ± 0.010	0.008 ± 0.001
2	0.044 ± 0.029	0.015 ± 0.002	0.058 ± 0.003	0.017 ± 0.004
3	0.478 ± 0.006	0.059 ± 0.001	0.083 ± 0.007	0.061 ± 0.007
6	0.572 ± 0.019	0.068 ± 0.000	0.124 ± 0.011	0.076 ± 0.013
7	0.702 ± 0.029	0.117 ± 0.002	0.236 ± 0.003	0.094 ± 0.020
8	0.766 ± 0.009	0.273 ± 0.016	0.264 ± 0.031	0.171 ± 0.099
9	0.907 ± 0.019	0.661 ± 0.024	0.631 ± 0.003	0.395 ± 0.194
10	1.203 ± 0.221	1.002 ± 0.066	0.713 ± 0.023	0.722 ± 0.194
12	1.311 ± 0.063	1.245 ± 0.060	0.939 ± 0.027	1.038 ± 0.179
14	1.385 ± 0.062	1.323 ± 0.075	1.166 ± 0.055	1.279 ± 0.095
16	1.572 ± 0.036	1.554 ± 0.130	1.407 ± 0.085	1.418 ± 0.108
24	1.690 ± 0.038	1.771 ± 0.071	1.532 ± 0.092	1.613 ± 0.154
48	1.874 ± 0.054	2.281 ± 0.005	1.723 ± 0.053	1.798 ± 0.093
72	1.988 ± 0.115	2.287 ± 0.000	1.834 ± 0.103	1.842 ± 0.112
96	2.018 ± 0.001	2.362 ± 0.006	1.965 ± 0.068	1.881 ± 0.074
120	2.028 ± 0.004	2.363 ± 0.025	1.978 ± 0.071	1.886 ± 0.054
144	1.980 ± 0.019	2.024 ± 0.036	1.614 ± 0.046	1.157 ± 0.056
168	1.823 ± 0.027	1.994 ± 0.027	1.462 ± 0.035	1.146 ± 0.051
192	1.602 ± 0.003	1.852 ± 0.029	1.333 ± 0.049	1.025 ± 0.020
216	1.524 ± 0.014	1.747 ± 0.031	1.094 ± 0.032	0.998 ± 0.018
240	0.927 ± 0.025	1.058 ± 0.039	0.847 ± 0.021	0.995 ± 0.001
264	0.747 ± 0.014	0.857 ± 0.084	0.830 ± 0.005	0.975 ± 0.019
288	0.454 ± 0.014	0.655 ± 0.042	0.667 ± 0.001	0.891 ± 0.047

Table C-2 Cell dried weight (CDW) of mixed culture in medium containing 10, 30 and 50% v v^{-1} glycerol as carbon source without glycerol as control at 30°C, 200 rpm (Figure 4.1)

Time (hours)	Cell dried weight (g L^{-1})			
	0% (Control)	10%	30%	50%
0	0.450 ± 0.023	0.861 ± 0.036	0.332 ± 0.013	0.264 ± 0.032
2	0.255 ± 0.038	1.117 ± 0.054	0.732 ± 0.001	0.391 ± 0.008
6	1.190 ± 0.092	1.758 ± 0.038	1.028 ± 0.009	0.980 ± 0.012
8	2.388 ± 0.426	2.032 ± 0.020	1.767 ± 0.029	1.400 ± 0.092
10	4.220 ± 0.289	2.833 ± 0.027	2.064 ± 0.001	1.886 ± 0.062
12	5.040 ± 0.023	3.770 ± 0.035	2.866 ± 0.004	2.449 ± 0.034
14	5.855 ± 0.040	4.038 ± 0.017	3.550 ± 0.092	3.528 ± 0.009
16	6.325 ± 0.006	4.473 ± 0.055	4.248 ± 0.011	3.742 ± 0.010
24	7.474 ± 0.100	5.116 ± 0.057	5.571 ± 0.229	4.025 ± 0.017
48	8.155 ± 0.075	5.494 ± 0.004	5.656 ± 0.016	4.878 ± 0.066
72	8.765 ± 0.260	6.712 ± 0.115	6.404 ± 0.024	5.911 ± 0.005
96	9.087 ± 0.010	7.903 ± 0.089	6.609 ± 0.066	6.086 ± 0.003
120	9.378 ± 0.061	7.737 ± 0.016	7.357 ± 0.028	6.639 ± 0.014
144	9.150 ± 0.023	7.059 ± 0.007	7.103 ± 0.009	6.524 ± 0.005
168	9.232 ± 0.014	6.948 ± 0.011	6.945 ± 0.033	5.393 ± 0.006
192	9.290 ± 0.035	0.861 ± 0.036	6.887 ± 0.084	5.282 ± 0.005

Table C-3 Percentage of glycerol concentration by volume of mixed culture in medium containing 10, 30 and 50% vv^{-1}glycerol as carbon source versus interval periods at 30°C, 200 rpm (Figure 4.2)

Time (hours)	% vv^{-1} glycerol concentration		
	10%	30%	50%
0	9.92 ± 0.05	29.62 ± 0.02	48.63 ± 0.03
24	9.55 ± 0.02	29.52 ± 0.05	48.33 ± 0.02
48	6.94 ± 0.01	29.52 ± 0.04	47.14 ± 0.21
72	6.71 ± 0.03	29.01 ± 0.09	45.73 ± 0.15
96	5.39 ± 0.09	27.67 ± 0.02	45.60 ± 0.34
120	3.02 ± 0.05	23.62 ± 0.05	43.86 ± 0.18
144	0.98 ± 0.09	22.07 ± 0.01	43.40 ± 0.04
168	0.05 ± 0.06	21.72 ± 0.02	43.14 ± 0.05
192	3.55 ± 0.02	20.64 ± 0.01	42.70 ± 0.03

Table C-4 Polyhydroxyalkanoate productions of mixed culture in medium containing 10, 30 and 50%vv^{-1}glycerol as carbon source and without glycerol as control at 30°C, 200 rpm (Figure 4.3)

Time (days)	PHB production (g L^{-1})			
	0%	10%	30%	50%
1	0.064 ± 0.006	1.503 ± 0.005	0.257 ± 0.002	0.250 ± 0.027
2	0.034 ± 0.012	2.278 ± 0.037	0.452 ± 0.002	0.363 ± 0.034
3	0.052 ± 0.003	2.530 ± 0.001	0.898 ± 0.003	0.648 ± 0.000
4	0.066 ± 0.009	3.219 ± 0.002	1.393 ± 0.006	0.705 ± 0.005
5	0.080 ± 0.005	3.953 ± 0.100	3.330 ± 0.012	0.724 ± 0.005
6	0.024 ± 0.004	3.109 ± 0.002	3.065 ± 0.041	0.729 ± 0.003
7	0.017 ± 0.001	2.953 ± 0.035	2.836 ± 0.019	0.727 ± 0.004
8	0.013 ± 0.001	2.800 ± 0.023	2.721 ± 0.013	0.727 ± 0.008

Table C-5 Percentage of PHB content was grown at different glycerol concentrations as carbon source during 168 hours incubation at 30°C using shaken flasks at an agitation rate of 200 rpm as a batch culture (Figure 4.4)

Time	% PHB content at different glycerol concentrations			
(hours)	0%	10%	30%	50%
24	0.856 ± 0.087	33.613 ± 0.519	4.623 ± 0.235	6.202 ± 0.700
48	0.417 ± 0.010	44.544 ± 1.217	7.984 ± 0.009	7.426 ± 0.597
72	0.594 ± 0.014	46.048 ± 0.011	14.023 ± 0.107	10.959 ± 0.012
96	0.728 ± 0.010	47.973 ± 0.857	21.073 ± 0.305	11.589 ± 0.075
120	0.855 ± 0.011	50.058 ± 0.252	45.266 ± 0.330	10.906 ± 0.046
144	0.259 ± 0.004	40.180 ± 0.103	43.147 ± 0.523	11.169 ± 0.033
168	0.179 ± 0.001	41.831 ± 0.535	40.840 ± 0.076	13.475 ± 0.091
192	0.136 ± 0.002	40.304 ± 0.272	39.516 ± 0.295	13.765 ± 0.166

Kinetics of polyhydroxyalkanoate-producing bacteria information

Table C-6 Effects of nitrogen sources contained in medium, including ammonium sulphate (A), yeast extract (Y) and tryptone (T) which were supplemented in addition to medium containing 10 % vv^{-1} glycerol on growth of mixed cultures (Figure 4.8)

Time (hours)	Cell concentration (gL^{-1})						
	A	Y	T	A + Y	A + T	Y + T	A+Y+T
0	0.671 ± 0.019	0.528 ± 0.016	0.390 ± 0.030	0.427 ± 0.032	0.338 ± 0.022	0.397 ± 0.002	0.362 ± 0.019
24	7.450 ± 0.098	6.690 ± 0.040	5.179 ± 0.004	6.424 ± 0.040	5.179 ± 0.004	5.153 ± 0.001	4.416 ± 0.086
72	9.180 ± 0.008	8.375 ± 0.151	6.678 ± 0.227	7.863 ± 0.074	6.373 ± 0.018	6.258± 0.028	5.495 ± 0.009
120	10.896 ± 0.071	10.805 ± 0.214	8.014 ± 0.021	9.500 ± 0.079	8.054 ± 0.048	7.978 ± 0.029	7.891 ± 0.003
168	8.670 ± 0.335	6.360 ± 0.004	4.484 ± 0.013	5.744 ± 0.012	4.188 ± 0.010	4.153 ± 0.040	7.031 ± 0.037

Table C-7 Effects of nitrogen sources contained in medium, including ammonium sulphate (A), yeast extract (Y) and tryptone (T) which were supplemented in addition to medium containing 10 % vv^{-1} glycerol on percentage of polyhydroxybutyrate (PHB) content of mixed cultures (Figure 4.8)

Time (hours)	Percentage of PHB content						
	A	Y	T	A + Y	A + T	Y + T	A+Y+T
0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
24	27.556 ± 0.632	30.662 ± 1.014	35.831 ± 0.050	31.019 ± 0.076	38.052 ± 0.375	38.075 ± 0.126	34.292 ± 1.021
72	59.042 ± 0.932	51.293 ± 0.020	48.557 ± 1.123	39.392 ± 0.403	49.580 ± 0.323	49.249 ± 1.180	46.033 ± 0.088
120	68.383 ± 1.294	62.755 ± 1.342	61.877± 0.057	59.187 ± 0.362	56.759 ± 0.309	52.561 ± 0.237	50.093 ± 1.249
168	48.191 ± 1.262	36.151 ± 0.295	32.163± 0.353	38.110 ± 0.217	29.552 ± 0.193	29.755 ± 0.272	42.006 ± 0.297