

## Biosynthesis and Lipase-Catalysed Hydrolysis of 4-Hydroxybutyrate-Containing Polyhydroxyalkanoates from *Delftia acidovorans*

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### ABSTRACT

**Aims:** Polyhydroxyalkanoates (PHA) having various molar fractions of 4-hydroxybutyrate has been successfully synthesized by *Delftia acidovorans*.

**Methodology and results:** The monomer compositions of the PHA were varied by cultivating the bacterium in a mixture of 1,4-butanediol and sodium valerate,  $\gamma$ -butyrolactone and sodium valerate as well as 4-hydroxybutyric acid and sodium valerate, which resulted in the production of PHA terpolymers. Although the highest terpolymer content achieved was only 57 wt% of the dry cell weight, the 4HB molar fractions can be regulated from 2-50 mol% when culture conditions such as initial pH, inoculum concentration and aeration were varied. The *in vitro* degradation of [P(3HB-co-50 % 4HB)] synthesized by *D. acidovorans* were also studied by monitoring the erosion rate of the copolymer in aqueous solutions of lipases (Lipase A 'Amano' 12 and Newlase F). Results have shown that the types of lipases, concentration of lipase solution and pH of the buffer solution influenced the degradation rate of the PHA copolymer.

**Conclusion, significance and impact of the study:** The overall results have shown that *D. acidovorans* is a very promising strain for the production of 4HB containing PHAs with specific compositions which are very suitable to be tailor made into biodegradable and biocompatible materials for medical applications.

**Keywords:** polyhydroxyalkanoate, terpolymer, 4-hydroxybutyrate, 3-hydroxyvalerate, *Delftia acidovorans*

### INTRODUCTION

Polyhydroxyalkanoates (PHAs) are microbial polyesters with many potential applications. PHAs that contain 4-hydroxybutyrate (4HB) monomer are especially interesting as bioabsorbable materials for applications in medical devices (Martin and Williams 2003). Biocompatibility is an important factor that determines the applicability of any material in medical devices. Although PHA is generally accepted as a biocompatible material, the source of this polymer is mainly gram-negative bacteria, which contains endotoxins. Various extraction and purification methods have been reported to remove endotoxins from PHA (Zinn *et al.* 2007).

Among the various wild-type microorganisms that have the ability to synthesize PHA that contains 4HB, *Delftia acidovorans* has been shown to have the most efficient metabolic pathways for the production of poly(4-hydroxybutyrate) [P(4HB)] homopolymer and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] copolymer (Saito and Doi 1994). In previous studies (Siew *et al.* 2007; Siew *et al.* 2009; Ying *et al.* 2008) we have found that PHAs extracted from *D. acidovorans* were generally safe based on cytotoxicity, genotoxicity and implant tests. Therefore, *D. acidovorans* is a potential strain for the production of PHAs for medical applications. One of the greatest challenges in the biosynthesis of

PHAs for various applications is the ability to control the monomer compositions in a wide range. It is known that the properties of PHA are dependent on the type and molar ratios of different monomers. While 3HB and 3HV monomers contribute to the stiffness and strength of PHAs (Holmes 1985), 4HB monomer resulted in their flexibility and elasticity (Saito *et al.* 1996). In addition, 4HB monomers enabled the PHA to be hydrolyzed by lipases (Jaeger *et al.* 1995), which makes the PHA attractive as an absorbable biomaterial. The biosynthesis of P(3HB-co-4HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] with controlled composition by *D. acidovorans* have been reported. However, not much is known about the strategy to produce poly(3-hydroxybutyrate-co-4-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV-co-4HB)] terpolymer by *D. acidovorans* with precise molar fractions.

The discovery of the ability of *D. acidovorans* to produce P(3HB-co-4HB-co-3HV) dates back to 1992 (Kimura *et al.* 1992). However, 4HB was the major constituent monomer in the terpolymer chain (44-88 %) and the increase of 3HV monomers hampered the overall PHA accumulating ability of the strain. Recent developments of the production of P(3HB-co-3HV-co-4HB) terpolymer by other bacterial strains (Amirul *et al.* 2008; Chanprateep and Kulpreecha 2006; Ramachandran *et al.* 2011) have suggested the possibility of improving the production and compositions of the terpolymer by *D. acidovorans*.

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**Table 1:** Effect of sodium valerate concentration on the biosynthesis of P(3HB-co-3HV-co-4HB) by *D. acidovorans*<sup>a</sup>

Carbon source			Dry cell weight (g/L)	PHA content <sup>b</sup> (wt %)	PHA composition (mol %)		
1,4-BD (w/v)	Glu (w/v)	S.Val (w/v)			3HB	3HV	4HB
0.5	0.5	0.1	2.3	32	87	8	5
0.5	0.5	0.2	2.4	36	86	13	1
0.5	0.5	0.3	2.4	40	79	16	5
0.5	0.5	0.4	2.6	42	78	17	5
0.5	0.5	0.5	2.4	47	76	20	4
0.5	0.5	0.6	2.6	46	75	20	5
0.5	0.5	0.7	2.6	57	75	20	5
0.5	0.5	0.8	2.6	24	66	29	5
0.5	0.5	0.9	2.6	20	70	26	4
0.5	0.5	1.0	2.4	16	74	21	5

1,4-BD, 1,4-butanediol; Glu, glucose; S.Val, sodium valerate; 3HB, 3-hydroxybutyrate; 3-HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate.

<sup>a</sup> Incubated for 48h at 30 °C, initial pH 7.0.

<sup>b</sup> PHA content in dry cells.

As such, the main focus of this study was to evaluate the ability of *D. acidovorans* to produce P(3HB-co-3HV-co-4HB) with various monomer compositions. The concentrations of carbon sources were varied and different culture conditions were applied to study their effect on the incorporation of 3HV and 4HB monomers. Besides, P(3HB-co-50 mol%-4HB) copolymer which was successfully produced in our previous studies by *D. acidovorans* (Lee *et al.* 2004) was used to study the effect of two types of commercial lipases, their concentrations and pH on the *in vitro* degradation of the polymer.

## MATERIALS AND METHODS

### Biosynthesis of P(3HB-co-3HV-co-4HB) terpolymers

*D. acidovorans* JCM 10181 was cultivated and maintained as previously described (Lee *et al.* 2004). Two- step cultivation was practiced by using *D. acidovorans* for the biosynthesis of terpolymer. For the first stage of cultivation, 3 % (vol/vol) of fresh culture was inoculated into 100 mL NB medium (pH 7.0) with 1 % (wt/vol) glucose in a 500 mL flask. After 24 h, the cells were recovered by centrifugation at 4000 g, 16 °C for 10 min. To promote synthesis of PHA, the centrifuged cells were transferred into the second stage medium which consisted of a 100 mL nitrogen free medium (pH 7.0). Carbon sources such as glucose, 1,4-butanediol (1,4-BD), sodium 4-hydroxybutyrate (S4HB),  $\gamma$ -butyrolactone ( $\gamma$ -BL) and sodium valerate were autoclaved separately and added aseptically to the medium to promote synthesis of P(3HB-co-3HV-co-4HB) terpolymers.

### Preparation of sodium valerate and sodium 4-hydroxybutyrate

Sodium valerate was prepared by mixing valeric acid with

NaOH solution. 1 M of NaOH solution was prepared by dissolving 40 g of NaOH in 1 L of (95 %) ethanol. Then, approximately 102 mL of valeric acid was poured into the

NaOH solution. The sodium valerate precipitate that was formed was recovered and dried in an oven at 45 °C to constant weight. The sodium valerate was used as the 3HV precursor carbon source. The sodium salt of 4-hydroxybutyrate was produced by the reaction of  $\gamma$ -butyrolactone with NaOH solution. A total of 60 g NaOH was stirred in 1 L of absolute ethanol until complete dissolution. Approximately 120 mL  $\gamma$ -butyrolactone was poured into the NaOH solution. The precipitated sodium salt of 4-hydroxybutyrate was placed in 45 °C oven until constant weight. After the drying process, the S4HB was kept in an airtight bag for further use.

### Analytical procedures

At the end of second-stage cultivation (48 h), bacterial cells were collected by centrifugation and subjected to lyophilization. In order to determine PHA content and composition, approximately 25 mg of lyophilized cells was subjected to methanolysis with 2 mL of chloroform and 2 mL of methanolysis solution [85 % (vol/vol) methanol and 15 % (vol/vol) sulphuric acid] at 100 °C for 140 min. The resulting hydroxyacyl methyl esters were analyzed by gas chromatography according to well-established method (Braunegg *et al.* 1978).

### Enzymatic degradation of P(3HB-co-50 mol%-4HB)

Lipase A "Amano" 12 from *Aspergillus niger* and Newlase F from *Rhizopus niveus* (both from Amano Enzymes Inc, Japan) were used in this study. P(3HB-co-50 mol%-4HB) was biosynthesized as described previously by Lee and coworkers with the addition of glucose and 1,4-butanediol in the second stage medium as carbon sources. Lipase solutions of Lipase A and Newlase F with 4 different concentrations (5 U, 50 U, 500 U and 5000 U) each were prepared by dissolving the appropriate amount of lipase powder in 0.1 M phosphate buffer according to the information supplied by Amano Enzymes Inc, Japan. Two sets of experiments were conducted whereby phosphate buffer of pH 5 and pH 7 were used to dissolve each of the lipases. 14 pieces of 1 cm<sup>2</sup> cast films of P(3HB-co-50

mol%-4HB) (initial weight of about 0.5 mg  $\pm$  0.1 mg) were added into each flask with 100 mL phosphate buffer. All flasks were incubated in a rotating shaker (CERTOMAT R) at 200 rpm and 37 °C. 2 sample films were removed every 24 h from each flask, washed with distilled water and dried in incubator (37 °C) for 24 h before being weighed. The weight loss of the films was recorded throughout a period of 7 days.

## RESULTS AND DISCUSSION

### Effect of sodium valerate concentration on P(3HB-co-3HV-co-4HB) terpolymer content and composition

Sodium valerate was used as the precursor for 3HV monomer in the biosynthesis of P(3HB-co-3HV-co-4HB) terpolymer in this study. It was not possible to use sodium propionate as 3HV precursors since it displayed toxicity to the bacterial cells and contributed to subsequent reduction of terpolymer production (results not shown). Previous reports have revealed that the addition of glucose during cultivation significantly boosted the PHA accumulating capacity of bacteria as it induces certain enzymes that are involved in the generation of acetyl-CoA (Taidi *et al.* 1994; Lee *et al.* 2004). Therefore, the combination of 1,4-BD, glucose and sodium valerate was used to study the effect of sodium valerate concentration on the biosynthesis of P(3HB-co-3HV-co-4HB). **Table 1** shows the effect of sodium valerate concentration on P(3HB-co-3HV-co-4HB) terpolymer content and composition synthesized by *D. acidovorans*. The combination of three carbon sources resulted in terpolymer containing 8-29 mol% 3HV monomers, whereas the 3HB and 4HB monomers were almost unaffected and remained at approximately 80 mol% and 5 mol% respectively regardless of the concentrations of sodium valerate. Although a highest terpolymer content of 57 wt% was achieved, the addition of glucose had a negative impact on the study.

The addition of glucose in the medium has rendered the cells to favor the accumulation of 3HB, whereby the 3HB monomer composition constantly maintained at a much higher percentage if compared to the other two monomers. The pathway to biosynthesize 3HB by glucose (TCA cycle) was clearly preferred by the bacterium to the extent that the increase in the amount of 3HV precursors has little effect on the monomer composition of 3HV in the resulting terpolymer. Addition of glucose into the second-stage medium has significantly enhanced 3HB monomer composition. A similar observation was made by Madden *et al.* (2000) when glucose, propionic acid and  $\gamma$ -BL were fed into the medium. Besides, the accumulation of 4HB monomer was only around 5 mol%. Since it was concluded that the addition of glucose will be suitable only for the production of P(3HB-co-3HV-co-4HB) terpolymer with low 3HV and 4HB monomer compositions, glucose should be eliminated from the list of carbon sources for the production of P(3HB-co-3HV-co-4HB) terpolymer with wider range of compositions.

### Production of P(3HB-co-3HV-co-4HB) terpolymer with a wide range of monomer composition

The next attempt to synthesize P(3HB-co-3HV-co-4HB) with a wide range of 4HB and 3HV monomers was by studying the effect of combinations of two carbon sources which are responsible for the generation of each of the respective monomers. *D. acidovorans* was cultured together with the combination of three different types of 4HB structurally related carbon sources (1,4-BD,  $\gamma$ -BL or 4HBA) and sodium valerate to observe the resulting compositions and amount of terpolymer produced. The removal of glucose from the list of carbon feedstock successfully limited the channeling of excess acetyl-CoA that was generated mainly from glucose to the formation of 3HB monomers. The combination of 1,4-BD or 4HBA with sodium valerate enabled the control of 3HV and 4HB monomer compositions when the concentrations of both the monomer precursors was adjusted accordingly, as shown in **Table 2**.

**Table 2:** Biosynthesis of P(3HB-co-3HV-co-4HB) containing different 3HV and 4HB compositions from different carbon sources in various concentrations<sup>a</sup>

Carbon source		Dry cell weight (g/L)	PHA content <sup>b</sup> (wt%)	PHA composition (mol%)		
1,4-BD (vol/vol)	S. Val (wt/vol)			3HB	3HV	4HB
1.0	0	2.6	23	16	0	84
0.8	0.2	2.9	24	39	27	24
0.6	0.4	2.5	33	58	35	7
0.4	0.6	2.4	50	49	43	8
0.2	0.8	2.5	28	34	64	2
0	1.0	2.5	22	12	88	0
$\gamma$ -BL (vol/vol)		S.Val (wt/vol)				
1.0	0	2.2	6	46	0	54
0.8	0.2	2.5	31	39	54	7
0.6	0.4	2.5	23	37	55	8
0.4	0.6	2.4	44	47	45	8
0.2	0.8	2.6	48	42	52	6
0	1.0	2.5	22	12	88	0
S4HB (vol/vol)		S.Val (wt/vol)				
1.0	0	2.5	25	4	0	96
0.8	0.2	2.5	22	25	35	40
0.6	0.4	2.6	28	36	51	13
0.4	0.6	2.6	43	23	71	6
0.2	0.8	2.5	36	10	86	4
0	1.0	2.5	22	12	88	0

1,4-BD, 1,4-butanediol;  $\gamma$ -BL,  $\gamma$ -butyrolactone; S4HB, Sodium 4-hydroxybutyrate; S.Val, sodium valerate; 3HB, 3-hydroxybutyrate; 3-HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate.

<sup>a</sup> Incubated for 48 h at 30 °C, initial pH 7.0.

<sup>b</sup> PHA content in dry cells.

The combination of 1,4- BD with sodium valerate resulted in the biosynthesis of P(3HB-co-3HV-co-4HB) containing the widest range of 3HV monomer composition (27-88

mol%) as well as the highest amount of PHA content (50 wt%). The combination of 4HBA with sodium valerate also produced a comparable trend in 3HV monomer composition with the highest 4HB monomer composition of 40 % in the terpolymer. However, the monomer composition of 3HV remained constant at around 50 mol% when  $\gamma$ -BL was used together with sodium valerate as carbon sources. The observation may result from the poorer ability of  $\gamma$ -BL to be incorporated as 4HB monomers (54 mol% of 4HB when used as sole carbon source) if compared to 1,4-butanediol (84 mol% of 4HB when used as sole carbon source) or 4HBA (96 mol% of 4HB when used as sole carbon source). This suggested that  $\gamma$ -butyrolactone was not a suitable carbon source to be used for the production of P(3HB-co-3HV-co-4HB) with controlled compositions. According to the results obtained in this study, the increase in the composition of 3HV simultaneously suppressed the accumulation of 4HB monomers, whereby the monomer composition of 4HB was seen to decrease drastically together with the accumulation of 3HV monomers. The presence of sodium valerate has a very negative impact on the accumulation of 4HB.

The 4HB monomer compositions dropped drastically even when only 0.2% of sodium valerate was used. Fortunately, only a low amount of sodium valerate was needed to obtain a significant accumulation of 3HV monomers. Therefore, the amount of sodium valerate to be used in the subsequent experiment was adjusted to minimize its 4HB inhibiting effect in order to produce a terpolymer with a higher composition of 4HB monomers. Although the combination of 4HBA and sodium valerate has the advantage of generating a terpolymer with a higher 4HB or 3HV composition (40 and 86% respectively), the availability and lower pricing of 1,4-BD in the market might make it a better choice as one of the raw material for the mass production of the terpolymer. The choice of carbon sources to use will also depend on the application of the final product.

**Table 3:** Effect of initial pH on the biosynthesis of P(3HB-co-3HV-co-4HB) by using 0.1% (wt/vol) sodium valerate and 0.9% (vol/vol) 1,4-butanediol<sup>a</sup>

pH	Dry cell weight (g/L)	PHA content <sup>b</sup> (wt%)	PHA composition (mol%)		
			3HB	3HV	4HB
5.0	2.0	10	32	29	39
5.5	2.1	11	38	25	37
6.0	2.3	21	42	23	35
6.5	2.4	25	50	20	30
7.0	2.5	31	32	35	33
7.5	2.4	29	20	40	40
8.0	2.6	28	10	43	47
8.5	2.6	26	4	46	50

3HB, 3-hydroxybutyrate; 3-HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate.

<sup>a</sup> Incubated for 48 h at 30 °C, initial pH 7.0.

<sup>b</sup> PHA content in dry cells.

### Effect of initial pH on the P(3HB-co-3HV-co-4HB) terpolymer content and composition

Besides regulating the concentrations of carbon sources to control the monomer compositions of PHA, other culture conditions will also determine the end result of the biosynthesis. pH is an important feature that one has to take into consideration especially when the end products are directly affected by pH variation. It was previously reported that the monomer composition of P(3HB-co-4HB) could be regulated by varying the initial pH of the culture medium (Lee *et al.* 2004). Therefore, this parameter was applied in this study to investigate the effect of pH on P(3HB-co-3HV-co-4HB) monomer composition. Here we report, by changing the initial pH of the second-stage medium, significant effect was observed on the PHA composition of the terpolymer produced. A mixture of 0.1 % (wt/vol) sodium valerate and 0.9 % (wt/vol) 1,4-BD were used as carbon sources. The PHA content and monomer composition were analyzed and listed in **Table 3**. Results indicated that pH acted as a balance to level the monomer composition between 3HB versus 3HV and 4HB. For instance, at a slightly acidic pH of 6.5, 3HB accumulation was at the optimum point (50 mol%), and this subsequently brought about the lowest monomer composition of 4HB (30 mol%) and 3HV (20 mol%). Most probably, the production of acetyl-CoA was affected when the initial pH of the culture medium was changed. In contrast, at an alkaline pH of 8.5, 3HB mol% was much lowered (4 mol%), and the monomer composition of 4HB and 3HV increased to 50 and 46 mol% respectively. A pH below 6 resulted in a much lowered PHA accumulation. This phenomenon showed that the PHA accumulating process is very sensitive to pH change and a pH of 6 and above is favorable for PHA production by *D. acidovorans*. The fact that it is possible to control the monomer composition of the terpolymer produced without much affecting the total PHA content by the manipulation of culture pH was interesting. A neutral pH balances the weight of each monomer whereas at an alkaline pH, the accumulation of 3HB was much lowered and therefore a terpolymer with improved composition of both 3HV and 4HB has been achieved. In the year 2007, Loo and Sudesh reported that when sodium valerate was used as sole carbon source for PHA production in *D. acidovorans*, pH had little effect on the 3HV monomer composition of the PHA produced. However, in this study it was found that when 1,4-BD was added together as carbon feed stock, the molar fractions of the terpolymer produced can be controlled by pH changes.

### Effect of inoculum concentration on the P(3HB-co-3HV-co-4HB) terpolymer content and composition

Nutrient limitation is one of the key factors that are responsible in stimulating PHA producers to stock up on the biopolymer. Therefore, two-stage cultivation was applied on *D. acidovorans* to biosynthesize P(3HB-co-3HV-co-4HB). Recent study had shown that inoculum concentrations for the first stage culture would have an

effect on the PHA polymerization process in the bacterial cells. The concentrations of inoculum that was inoculated into the first stage culture medium would directly affect the time for the cells to reach stationary phase; therefore cultures with different inoculum concentrations were harvested at different period before being inoculated into the nitrogen-free second stage medium (Lee *et al.* 2004).

**Table 4** shows the effect of inoculum concentration on the ability of PHA accumulation by the bacterium and the monomer compositions of the terpolymer. Significant impact can be observed when the inoculum concentrations were merely increased from 1 to 3 %. Although the total PHA content had increased to 31% at 3 % of inoculum concentration, the 3HB monomer composition has clearly decreased from 57 % to 32 %. 4HB on the other hand was positively affected with increase of monomer composition from 11 to 33%. Interestingly, the monomer composition of 3HV was not affected by the concentration of inoculum. Generally, PHA producing bacteria can be divided into non-growth and growth associated PHA producers. *D. acidovorans* is a type of non-growth associated PHA producer; therefore two-stage cultivation is favorable for its production of PHA. Although much work has been done on PHA accumulation by two-stage cultivation, the question of the effect on the physiological state of cells when transferred into second-stage medium is still unanswered. Cultures started with different inoculum concentrations may need different time to reach stationary phase. It should be noted that all cultures were transferred into second-stage when they have reached the stationary phase. Therefore, a culture started from a higher inoculum concentration will have a younger cell population that has only multiplied several times before being transferred. These young cells have a better ability to incorporate 4HB monomer. It resulted in the P(3HB-co-3HV-co-4HB) terpolymer having a high molar fraction ( 40 mol%) of 4HB monomer in the second stage. On the other hand, old cell populations are able to accumulate a high 3HB monomer composition in the terpolymer. The role of  $\beta$ -ketothiolase enzymes in the production of P(3HB) has been demonstrated recently (Lindenkamp *et al.* 2010) and there may be a difference in the concentration of these enzymes in younger and older cells. It is however curious that the age of cells has no effect on the overall accumulation of 3HV monomer by the strain.

#### Effect of aeration on the P(3HB-co-3HV-co-4HB) terpolymer content and composition

Among the other factors which have significant impact on the biosynthesis of PHA, oxygen limitation was shown to affect PHA production under nitrogen limited conditions (Pozo *et al.* 2002). We have attempted to control the monomer compositions of P(3HB-co-3HV-co-4HB) produced by *D. acidovorans* by regulating the oxygen transfer rate into the culture medium. The effect of aeration on the biosynthesis of P(3HB-co-3HV-co-4HB) terpolymer has been tested by varying the agitation speed, as in **Table 5**. The culture volume was kept

**Table 4:** Effect of different inoculum concentration on the biosynthesis of P(3HB-co-3HV-co-4HB) by using 0.1% (wt/vol) sodium valerate and 0.9% (vol/vol) 1,4-butanediol<sup>a</sup>

Inoculum concentration % (vol/vol)	Dry cell weight (g/L)	PHA content <sup>b</sup> (wt%)	PHA composition (mol%)		
			3HB	3HV	4HB
1	2.4	20	57	32	11
2	2.5	25	46	34	20
3	2.5	31	32	35	33
4	2.6	29	32	34	34
5	2.6	26	31	33	36
6	2.7	24	30	33	37
7	2.8	23	29	35	36
8	2.8	24	30	32	38
9	2.9	23	29	41	40
10	3.0	20	28	32	40

3HB, 3-hydroxybutyrate; 3-HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate.

<sup>a</sup> Incubated for 48 h at 30 °C, initial pH 7.0.

<sup>b</sup> PHA content in dry cells.

**Table 5:** Effect of agitation speed on the biosynthesis of P(3HB-co-3HV-co-4HB) by using 0.1% (wt/vol) sodium valerate and 0.9% (vol/vol) 1,4-butanediol<sup>a</sup>

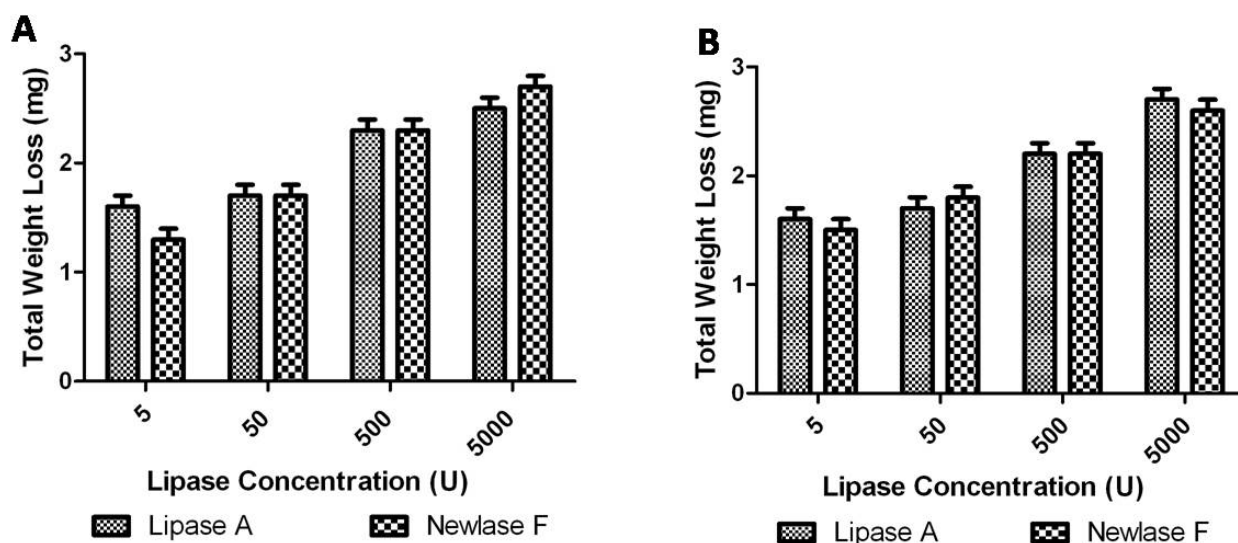
Agitation speed (rpm)	Dry cell weight (g/L)	PHA content <sup>b</sup> (wt%)	PHA composition (mol%)		
			3HB	3HV	4HB
50	2.1	17	3	50	47
100	2.2	28	21	48	41
150	2.5	31	32	35	33
200	2.2	30	40	33	27
250	2.2	31	54	30	16

3HB, 3-hydroxybutyrate; 3-HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate.

<sup>a</sup> Incubated for 48 h at 30 °C, initial pH 7.0.

<sup>b</sup> PHA content in dry cells.

constant at 100 mL in 500 mL conical flasks. The increase of agitation speed was expected to increase the oxygen transfer rate which further enhances the amount of dissolved oxygen in each culture flask. The influence of agitation speed on PHA content was most intense from the speed of 50 to 150rpm whereby the PHA content was seen to increase from 17 to 31 wt% with the increasing agitation speed. Further increase in the agitation speed did not have any profound effect on the PHA content. Instead, the PHA content was kept constant at 31 wt%. In this study, the molar fraction of 3HB increased sharply from 3 to 54 % when the aeration rate was increased. This sudden increase in accumulation of 3HB monomer was responsible for the increase of PHA content. At first glance the 3HV and 4HB monomer might seem to decrease with the increase of agitation speed. However, the net effect on the accumulation of both monomers was not much. The increase of 3HB monomer content was responsible in bringing down the relative compositions of 3HV and 4HB monomers. It was observed that a limitation in oxygen transfer rate (lowered agitation speed) greatly improved the molar fractions of 3HV and 4HB. It is



**Figure 1:** Total weight loss of P(3HB-co-50 mol%-4HB) films after 7 days of degradation in aqueous solutions of lipases at (A) pH 5 and (B) pH7.

interesting to note that the increase of agitation speed improves the composition of 4HB monomer in the production of P(3HB-co-4HB) by *D. acidovorans* (Lee *et al.* 2004). From the results obtained in this study it was observed that during the production of P(3HB-co-3HV-co-4HB) terpolymer by this strain, the increase in agitation speed aids in the incorporation of 3HB monomer instead. Therefore, by reducing the aeration rate to the culture medium, terpolymer with higher 3HV and 4HB monomers can be achieved. Another interesting observation was done by Ling *et al.* (2011) while investigating the effect of dissolved oxygen on the production of poly(3-hydroxybutyrate-co-3-hydroxy-4-methylvalerate) by *Chromobacterium* sp. USM2. It was found that the overall production of the copolymer was improved with the decrease of dissolved oxygen. The increase of 3HB monomer is the sole factor for the increase in PHA content, whereas there was negative effect on the incorporation of 3-hydroxy-4-methylvalerate monomer. In 1997, Hein and co-workers reported that supplementation of oxygen to culture medium was not favorable for the accumulation of P(4HB) homopolymer. 3HB monomer was incorporated when culture volume was reduced (increase in oxygen transfer). The molar fraction of 3HV and 4HB does not solely depend on the concentrations of 4HB and 3HV precursors. Our results have shown that by controlling the oxygen transfer rate it is possible to control the monomer compositions of the resulted terpolymer.

#### Enzymatic degradation of P(3HB-co-50 mol%-4HB)

4HB homopolymer and PHA copolymers with various 4HB monomer composition has been shown to be readily degradable by eukaryotic lipases (Vigneswari *et al.* 2009; Mukai *et al.* 1993). Besides being well proven with the ability to produce 4HB-containing PHA with controlled

compositions, the copolymer P(3HB-co-50 mol%-4HB) produced by *D. acidovorans* was also shown to be biocompatible (Siew *et al.* 2007). As such, a study was conducted to evaluate factors that affect the rate of *in vitro* biodegradation of P(3HB-co-50 mol%-4HB) film in the presence of eukaryotic lipases. The effect of pH and lipase concentrations on the erosion rate of polymer cast films were investigated by using lipases from *Aspergillus niger* (Lipase A "Amano" 12) and *Rhizopus niveus* (Newlase F). The reason for choosing these lipases as subject for study is because they are both proven to be able to degrade 4HB homopolymer (Mukai *et al.* 1993), but their ability to degrade P(3HB-co-4HB) copolymer was not much studied. Results collected in this study shows promising data of P(3HB-co-50 mol%-4HB) degradation by both kinds of lipases. Figure 1 show the total weight loss of polymer films after 7 days of lipases degradation in various enzyme concentrations in pH 5 and pH 7 respectively. For both lipases, maximum weight losses of polymer films after seven days were about 50 % of the original mass on day one. Studies have shown that lipases targeted specifically on 4HB monomers of PHA (Jaeger *et al.* 1995). It is of interest to note that at pH 5, total weight loss caused by the lipase type of Newlase F was higher than Lipase A. While at pH 7, Lipase A exerted more degradation on the polymer films than Newlase F. Although both types of lipases have the same active pH range of 5 to 8, Lipase A was shown to be more chemically reactive at pH 7, while Newlase F was more reactive at pH 5. This observation suggested that the copolymer is easily degradable by different types of lipases in a wide pH range and the maximum hydrolysis rate is achieved when the surrounding conditions support the optimum activity of the enzyme present. Both lipases have maintained their activity throughout the experiment and after seven days it can be observed that films incubated in enzyme solutions with the highest enzyme

concentration (5000 U) have resulted in the greatest weight loss if compared to the other lower concentrations of enzyme. The results obtained in this study have implied that P(3HB-co-50 mol%-4HB) copolymer produced by *D. acidovorans* may be developed as a suitable material for the manufacturing of sutures and scaffolds for biomedical applications, since it is readily degraded by eukaryotic lipases at and around the physiological pH. Further *in vivo* studies are necessary to determine the presence of sufficient concentrations of animal lipases with the ability to degrade this copolymer.

## CONCLUSION

This study has demonstrated the possibility to control the monomer compositions of P(3HB-co-3HV-co-4HB) produced by *D. acidovorans* through the balancing of physical and chemical parameters involved in the cultivation process. Since the strain was capable of producing high monomer compositions of 3HV and 4HB, these advantages were successfully combined to produce controlled compositions of P(3HB-co-3HV-co-4HB) terpolymer. In addition, the combination of 1,4-butanediol and sodium valerate as carbon sources was found to be most suitable for the mass production of controlled molar fractions of the terpolymer by this bacterial strain. P(3HB-co-50% mol 4HB) copolymer produced by *D. acidovorans* was also shown to be easily degradable by lipases with 50% weight loss after 7 days. Higher concentrations of lipase resulted in higher levels of biopolymer degradation. However, the rate of degradation was influenced by factors such as type of lipase, concentration of lipase solution and pH. The overall results have shown that *D. acidovorans* is a very promising strain for the production of 4HB containing PHAs with specific compositions which are very suitable to be tailor made into biodegradable and biocompatible materials for medical applications.

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