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Fermentation, biodegradation and tensile strength of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) synthesized by *Delftia acidovorans*

Wen-Chuan Hsieh [a,*], Yuki Wada [b], Chih-Pong Chang [c]

[a] *Department of Biological Science and Technology, I-Shou University, Yanchao, Kaohsiung 82445, Taiwan*
[b] *Department of Biological and Chemical Engineering, Gunma University, Gunma 376-8515, Japan*
[c] *Department of Textile Engineering, Chinese Culture University, Taipei 11192, Taiwan*

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ABSTRACT

This study discusses poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) that is produced by *Delftia acidovorans* bacteria in fed-batch cultures from various carbon sources. The experimentally determined optimal fed-batch culture conditions are as follows: pH: 7.0, incubation temperature: 26 °C, incubation time: 72 h, and concentration of carbon source: 10 g/L. The maximum 4HB unit composition of P(3HB-*co*-4HB) obtained using the fed-batch culture with a cell concentration of about 2.5 g/L was 94 mol% although the P(3HB-*co*-4HB) content remained low, at around 13 wt%. The extent of the enzymatic degradation of P(3HB-*co*-4HB) was determined by involved PHB depolymerase and lipase. The tensile strength of P(3HB-*co*-4HB) markedly decreased during 2 h of degradation by PHB depolymerase, whereas that of P(3HB-*co*-4HB) degraded by lipase for 50 h was at least 3 MPa.

1. Introduction

The biotechnology field has attracted considerable attention in recent years, because biotechnology can be exploited in the food sector, environmental protection, medical services and other sectors, it has become a competitive field of research in many countries (Amirul *et al.*, 2008; Hein *et al.*, 1997; Valentin and Dennis, 1997; William *et al.*, 1999). Since the 1980s, because of environmental pollution as well as the daily worsening of petroleum resources, the use of biotechnology to develop an alternate energy or material source has been a very important area of research (Hiramitsu *et al.*, 1993; Kang *et al.*, 1995; Kunioka *et al.*, 1988, 1989).

The development of biodegradable polymer is one area of biotechnology research, not only can biodegradable polymer be manufactured in an environmentally friendly way, but also it is degradable to carbon dioxide and water by microorganisms in the environment. Accordingly, such manufactured synthetic products do not need further processing after they are used. They are highly favored for both environmental protection and medical application (Kimura *et al.*, 1992; Saito and Doi, 1994; Saito et al., 1996).

Poly(hydroxyalkanoate)s [PHAs] are typically biodegradable, with good biocompatibility, making them attractive for use in tissue engineering in both in vitro and in vivo studies (Jiang *et al.*, 2004; Levenberg *et al.*, 2003; Siew *et al.*, 2007; Wang *et al.*, 2003, 2004). Since the product of decomposition is harmless to humans, using them in the human body has no detrimental side effect. Their biodegradation rates and mechanical properties can be modified for specific applications. They are particularly effective for implantation and can be easily manufactured in the desired forms. The major concern associated with polymer scaffolds is their low mechanical strength after enzymatic degradation and retention failure. Therefore, the mechanical strength and biodegradability of these biodegradable polymers must be improved.

Microorganism-synthesized polymers are generated using organic acids, saccharides and alcohols as starting materials. Currently, these microorganisms have synthesized polymers that have included P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-3HP), P(3HB-*co*-4HB) and others (Choi *et al.*, 1999, 2003; Kamiya *et al.*, 1989; Renner *et al.*, 1973; Senior and Dawes, 1971; Sombatmankhong *et al.*, 2007; Yokouchi *et al.*, 1973). The specificity of the matrices of microorganisms is such that different microorganisms and culture conditions of pH, temperature, microorganism concentration, carbon source and others, produce various biodegradable polymers (Doi *et al.*, 1990; Lee *et al.*, 2004; Müller and Hoffmann, 2006; Taidi *et al.*, 1994).

The fermentation of *A. eutrophus* and *C. acidovorans* using fructose, glucose and an organic acid as mixed carbon sources generates the biodegradable polymer (Kamiya *et al.*, 1989; Mitomo *et al.*, 2001). Since several microorganisms can use metabolize

* Corresponding author. Tel.: +886 7 6151100x7319; fax: +886 7 6155150.
E-mail address: wenchuan@isu.edu.tw (W.-C. Hsieh).

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carbohydrates, some special organic acids (such as butyric acid, 1,4-butanediol) are applied herein to reduce contamination.

In this work, the effect of the concentration of the carbon source, pH and incubation time on the biosynthesis of the P(3HB-*co*-4HB) polymer was investigated. The production of P(3HB-*co*-4HB) by *D. acidovorans* from the mixed carbon sources of *n*-butyric acid and 1,4-butanediol was evaluated. The weight loss and the tensile strength of P(3HB-*co*-4HB) upon degradation by the enzymes were also discussed.

2. Materials and methods

2.1. Microorganism and culture conditions

The microorganism, *D. acidovorans* (IFO13582), was used herein. Poly(hydroxyalkanoates) were synthesized by the two-step fermentation of *D. acidovorans*. The seed culture were initially grown on a reciprocal shaker in 500 mL Sakaguchi flasks at 26 °C in a nutrient-rich medium (pH 7.0, 1 L) that contained 10 g polypeptone, 10 g yeast extract, 5 g meat extract and 5 g $(NH_4)_2SO_4$. The cells were harvested after 48 h and rinsed with water. Under these culture conditions, no accumulation of polyesters in the cells was observed.

To promote polyester synthesis, approximately 2.5 g of the rinsed cells were transferred to inoculate in a 10 L fermenter that contained a mineral medium (pH 7.0) and different carbon sources. The volume of the culture was 5 L in all of the fed-batch experiments. The media in the second step were nutrient-free (pH 7.0, 1 L) and contained (per L) 7.16 g $Na_2HPO_4{\cdot}12H_2O$; 2.65 g KH_2PO_4; 0.2 g $MgSO_4{\cdot}7H_2O$; 0.1 g $(NH_4)_2SO_4$; 1 g $CaCl_2{\cdot}2H_2O$; 5 g citric acid; 1 mL microelement solution. The microelement solution contained the (per L) 0.217 g $CoCl_2{\cdot}6H_2O$; 0.0162 g $FeCl_3{\cdot}6H_2O$; 0.0078 g $CaCl_2{\cdot}2H_2O$; 0.118 g $NiCl_2{\cdot}6H_2O$; 0.135 g $CrCl_3{\cdot}6H_2O$; 0.156 g $CuSO_4{\cdot}5H_2O$ in an aqueous solution of 0.1 M HCl. The cells were cultivated in these media at 26 °C for 72 h, harvested by centrifugation (4000 rpm, 15 min), rinsed in water and methanol and finally vacuum-dried at room temperature. The composition of the medium has been presented elsewhere (Doi *et al.*, 1990; Hsieh *et al.*, 2006).

The polyester was extracted from the dried cells using hot chloroform, and was then purified by precipitation with *n*-heptane. The polymers were then vacuum-dried for 48 h. The polymer yield was determined using the following formula:

$$Y = \left(\frac{Wa}{Wd}\right) \times 100 \quad (1)$$

where *Y* represents the polymer content (wt%), *Wa* is the polymer weight (g) and *Wd* is the dried cell mass (g).

2.2. Analytical methods

The compositions of the poly(hydroxyalkanoate) samples were determined from the 500 MHz ^{1}H NMR spectra, which were recorded using a JEOL α-500 spectrometer. The ^{1}H NMR spectra were obtained at room temperature from a $CDCl_3$ solution of the poly(hydroxyalkanoate) (10 mg/mL) with a 4.7 μs pulse width, 5 s pulse repetition, a 5000 Hz spectral width, 16 K data points and 32 accumulations. The internal chemical shift standard was tetramethylsilane (Me_4Si, $\delta = 0$).

Gel permeation chromatography (GPC) was performed using an HLC-802A high-performance liquid chromatograph (Tosoh) at 38 °C, equipped with a series of four columns of TSK-gel and an RI-8 differential refractometer. Chloroform was used as the eluent at a flow rate of 0.1 mL/min, with 1 mL of sample solution at a concentration of 1.0 g/L. A calibration curve was plotted using polystyrene standards with a narrow polydispersity, and the apparent molecular weights were then calculated.

PHB depolymerases that were purified from *Ralstonia pickettii* T1 (Shirakawa *et al.*, 1986) (which mainly degrades the P(3HB) component) and lipase (which degrades the P(4HB) component) from *Rhizopus delemar* (Mukai *et al.*, 1993) were used herein. The enzymatic degradation of the P(3HB-*co*-4HB) film was proceeded at 37 °C in a 0.1 M phosphate buffer (pH 7.4). The P(3HB-*co*-4HB) films (initial weight, about 8 mg; film dimensions, 4 mm × 20 mm; film thickness, 0.1 mm) were placed in small test tubes with 4.0 mL of the buffer. The reactions were induced by adding PHB depolymerase (2 μg) or (1 mL) aqueous lipase (100 μg). The reaction solutions were incubated in a reciprocal shaker and shaken approximately 70 times/min. The film was removed after a particular period, washed in methanol and water, and then completely dried. The degree of enzymatic degradation was expressed as the decrease in weight as a percentage of the initial weight of the sample.

The mechanical properties of the degraded films were measured at room temperature using a tensile tester (Tensilon, Toyo Baldwin, Japan) with a crosshead speed of 2 mm/min. The films were harvested following enzymatic degradation.

3. Results and discussion

3.1. Effect of cultivation time on biosynthesis of P(3HB-co-4HB)

The microbial poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] was synthesized by fermentation with *Delftia acidovorans*. To evaluate the effect of the cultivation time on the biosynthesis of the P(3HB-*co*-4HB) polymer, biosynthesis was performed by two-step fermentation (at pH 7.0) with 10 g/L of 1,4-butanediol as the sole carbon source at 26 °C. Fig. 1 plots the relationship between the 4HB fraction and the polymer content for various incubation times in two-step fermentation. When the incubation time was 24 h, the lowest of the 4HB fraction of P(3HB-*co*-4HB) copolymer and the content were 28 mol% and 2 wt%, respectively, suggesting that the incubation time did not suffice to accumulate the polyester in the bacterial cells. However, at an incubation time of 96 h, the 4HB fraction of the copolymer and the content became 79 mol% and 8 wt%, respectively, indicating that the microorganism consumes the polymers that accumulated in the bacterial cells as the carbon sources became deficient in the culture solution at an incubation time of 96 h.

3.2. Effect of pH on biosynthesis of the P(3HB-co-4HB)

To evaluate the effect of changes in pH during the biosynthesis of the P(3HB-*co*-4HB) polymer, biosynthesis was conducted using

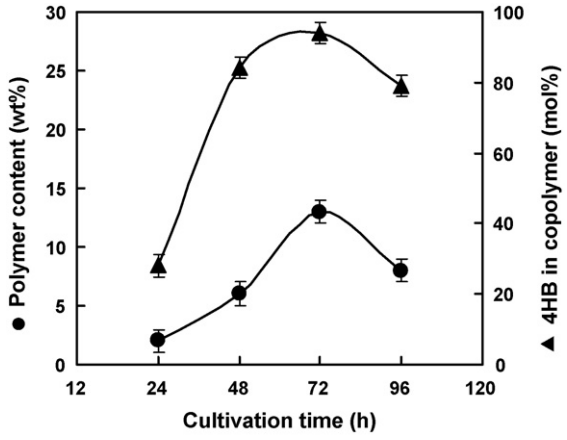

Fig. 1. Relationship between the 4HB fraction and the polymer content for various incubation times in two-step fermentation.

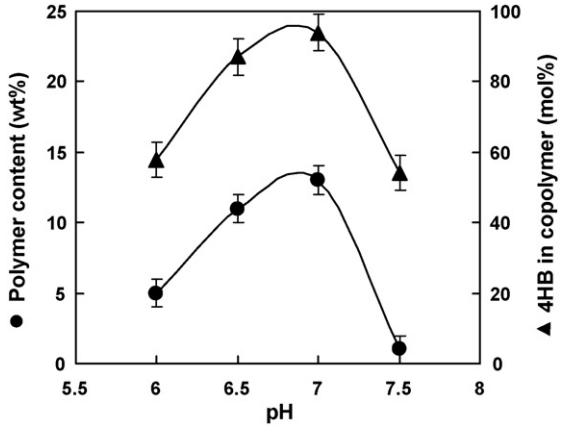

Fig. 2. Relationship between the 4HB mole fractions and polymer contents at various pH values in two-step fermentation.

two-step fermentation with 10 g/L of 1,4-butanediol as the sole carbon source at 26 °C for 72 h. Fig. 2 plots the relationship between the 4HB mole fractions and polymer contents at various pH values in two-step fermentation. The highest 4HB fraction and copolymer content in the pH 7.0 culture solution that contained 10 g/L of 1,4-butanediol were approximately 94 mol% and 13 wt%, respectively. As the pH value in the culture solution increased above 7.0, the 4HB fraction and the polymer content declined significantly to arround 54 mol% and 1 wt%. The 4HB fractions at pH 6.5 and 6.0 were 87 mol% and 58 mol%, respectively, and the corresponding polymer contents were 11 wt% and 5 wt%.

3.3. Effect of concentration of carbon source on biosynthesis of P(3HB-co-4HB)

To evaluate the effect of the carbon source concentration on the biosynthesis of P(3HB-*co*-4HB) polymer, the biosynthesis was performed using two-step fermentation (pH 7.0) at 26 °C for 72 h. Table 1 presents the results of the copolyester generated by *D. acidovorans* for various concentrations of the carbon source in the two-step fermentation for 72 h at 26 °C. *D. acidovorans* yielded the P(3HB-*co*-4HB) copolyester from *n*-butyric and 1,4-butanediol as the mixed carbon sources. The 4HB fraction and polymer content of P(3HB-*co*-4HB) were determined from changes in the mixed carbon source concentrations of 5 g/L, 10 g/L and 15 g/L. P(3HB-*co*-4HB) was produced from 10 g/L of the mixed carbon sources, and the polyester content in the dried cells was as high as 13 wt%, while the 4HB fraction of the polymer was 65 mol%. The 4HB fraction of the polymer was 65 mol% when 5 g/L of the mixed carbon source was used, yielding an outcome similar to that with 10 g/L of the mixed carbon sources, but the polymer content was lower, at around 9 wt%. When the polymer was produced by biosynthesis from 15 g/L of the mixed carbon sources, both the 4HB fraction and polymer content were at their lowest values of 16 mol% and 4 wt%, respectively.

Table 2
Production of P(3HB-*co*-4HB) copolymer by *D. acidovorans* from various carbon sources (10 g/L) for 72 h at 26 °C using two-step fermentation.

Carbon source (10 g/L)	Dry cell weight (g/L)	Polyester content (wt%)	PHA composition[a] (mol%)	
			3HB	4HB
Glucose	1.8	6	100	0
Saccharose	1.5	2	100	0
Fructose	1.5	1	100	0
Glutaric acid	2.1	3	100	0
n-Butyric acid	2.6	14	100	0
4-Hydroxybutyric acid	2.1	2	5	95
1,4-Butanediol	2.5	13	6	94
r-Butyrolactone	2.2	5	88	12

[a] Determined by ^{1}H NMR.

3.4. Cultivation of Delftia acidovorans using various carbon sources

Table 2 presents the results of the production of polyester by *D. acidovorans* with numerous carbon substrates at 26 °C. The P(3HB) homopolymer was generated by *D. acidovorans* with glucose, saccharose, fructose, glutaric acid or *n*-butyric acid as the only carbon source. The P(3HB-*co*-4HB) copolymer was produced from 4-hydroxybutyric acid, 1,4-butanediol or gamma-butyrolactone, and the polyester contents in the dried cells were as high as 14 wt% when 1,4-butanediol was used. *n*-Butyric acid and 1,4-butanediol were used as the mixed carbon sources to increase the polymer contents of P(3HB-*co*-4HB) synthesized by *D. acidovorans.* P(3HB-*co*-4HB) with a wide composition was synthesized by *D. acidovorans* from the mixed carbon sources of *n*-butyric acid and 1,4-butanediol, which are relatively cheaper than the other chemicals. These results suggest that *D. acidovorans* can synthesize P(3HB-*co*-4HB) with a wide range of compositions from a mixed carbon source of *n*-butyric acid and 1,4-butanediol in the feed.

The samples of P(3HB-*co*-4HB) copolymer whose enzymatic degradation and tensile strength were evaluated, were produced from *n*-butyric acid and 1,4-butanediol by *D. acidovorans* in two-step fermentation at 26 °C for 72 h. Table 3 presents results concerning the production of P(3HB-*co*-4HB) copolymer.

3.5. Enzymatic degradation of P(3HB-co-4HB)

Fig. 3 presents the enzymatic degradation of P(3HB-*co*-4HB) films in an aqueous PHB depolymerase from *R. pickettii* T1. The copolymers with lower crystallinity (4HB: 10.9% and 20.0%) degraded faster than P(3HB), indicating preferential degradation in the amorphous regions. The rate of degradation of the copolymer of P(3HB-*co*-51.5 and 75.6% 4HB) was slower than that of P(3HB). To evaluate effectively the tensile strength of the degraded copolymer, the degradation time was maintained below 5 h.

Fig. 4 presents the enzymatic degradation of P(3HB-*co*-4HB) films in aqueous lipase from *R. delmer*. This lipase is known selectively to degrade the 4HB component and an inverse behavior to that in Fig. 3 is clearly expected. P(3HB) exhibited no weight loss

Table 1
Production of P(3HB-*co*-4HB) copolymer by *D. acidovorans* from various concentrations of carbon source in two-step fermentation for 72 h at 26 °C.

Carbon sources (g/L)		Dry cell weight (g/L)	Polyester Content (wt%)	PHA composition[a] (mol%)		Molecular weight[b]	
Butyric acid	1,4-Butanediol			3HB	4HB	$Mn \times 10^{-4}$	*Mw*/*Mn*
2	3	2.2	9	35	65	7.1	2.6
4	6	2.5	13	35	65	9.1	3.1
6	9	2.4	4	84	16	7.5	5.8

[a] Determined by ^{1}H NMR.
[b] Determined by GPC.

Table 3
Production of P(3HB-*co*-4HB) copolymer by *D. acidovorans* from butyric acid and 1,4-butanediol in two-step fermentation for 72 h at 26 °C.

Sample no.	Carbon sources (g/L)		4HB fraction[a] (mol%)	Molecular weight[b]		Melting point[c] (°C)
	Butyric acid	1,4-Butanediol		$Mn \times 10^{-4}$	Mw/Mn	
1	10	0	0	9.6	3.1	172
2	8	2	10.9	8.5	2.8	169
3	6	4	20.0	6.9	2.5	157
4	4	6	51.5	7.2	3.2	43
5	2	8	75.6	5.4	3.0	46

[a] Determined by ^{1}H NMR.
[b] Determined by GPC.
[c] Determined by DSC.

even after 24 h of treatment. P(3HB-*co*-51.5% and 75.6% 4HB) exhibited rapid weight loss, to 40% after 3 h of treatment. The weight loss rate of other samples of intermediate composition declined as the 4HB content decreased, gradually leveling off at over 50 h. Unlike that of PHB depolymerase (Fig. 3), the rate of erosion rate by lipase barely depended on the crystallinity of the samples, suggesting that a low 4HB copolymer content can be exploited in tissue engineering.

3.6. Tensile strength of degraded P(3HB-co-4HB) films

The tensile strength of biodegraded polymer is an important factor in tissue engineering. Accordingly, the tensile strength of biodegraded P(3HB-*co*-4HB) polymers was examined. Fig. 5 plots the tensile strength of P(3HB) and P(3HB-*co*-4HB) films degraded by PHB depolymerase and lipase at 37 °C. The tensile strength of P(3HB) (○) degraded by PHB depolymerase was lower than that of P(3HB) (●) degraded by lipase (Fig. 5(a)). Since PHB depolymerase degrades mainly the P(3HB) component (Shirakawa *et al.*, 1986), the tensile strength of P(3HB) using PHB depolymerase slowly declined to 14 MPa in 24 h of degradation. However, the change in tensile strength change of P(3HB) after 24 h of degradation by lipase was undetectable.

In Fig. 5(b), enzymatic degradation using PHB depolymerase substantially reduced the tensile strength of the 10.9 mol% (◆) and 20.0 mol% (■) 4HB copolymers. The size of the amorphous region

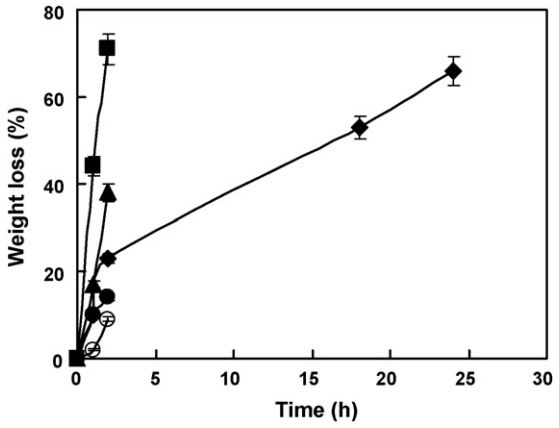

Fig. 3. Weight loss of degraded P(3HB) and P(3HB-*co*-4HB) by PHB depolymerase from *R. pickettii* T1 at 37 °C (◆: P(3HB); ■: 10.9 mol% 4HB; ▲: 20.0 mol% 4HB; ○: 51.5 mol% 4HB; ●: 75.6 mol% 4HB).

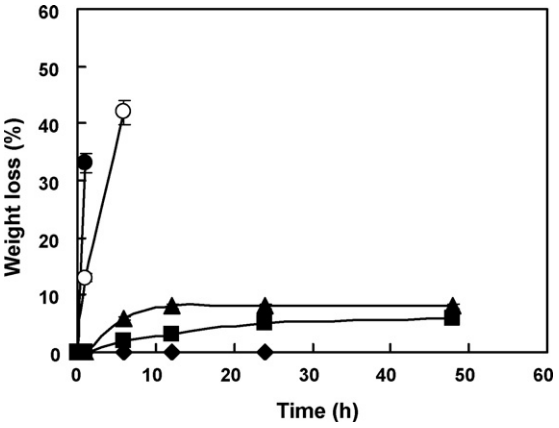

Fig. 4. Weight loss of degraded P(3HB) and P(3HB-*co*-4HB) by Lipase from *R. delmer* at 37 °C (◆: P(3HB); ■: 10.9 mol% 4HB; ▲: 20.0 mol% 4HB; ○: 51.5 mol% 4HB; ●: 75.6 mol% 4HB).

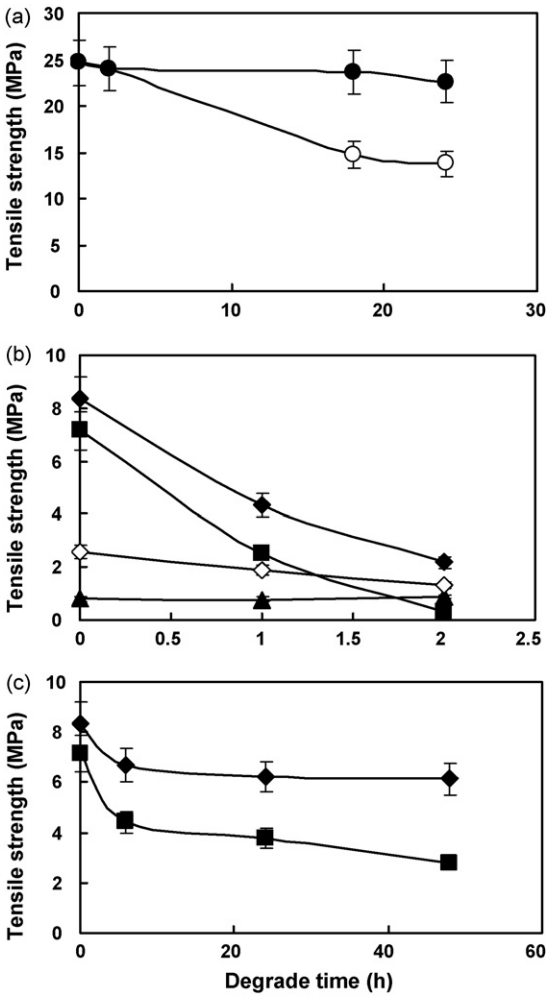

Fig. 5. Tensile strength of P(3HB) and P(3HB-*co*-4HB) films degraded by PHB depolymerase (a (○) and b) and lipase (a (●) and c) at 37 °C (○, ●: P(3HB); ◆: 10.9 mol% 4HB; ■: 20 mol% 4HB; ▲: 51.5 mol% 4HB; ◇: 75.6 mol% 4HB).

of copolymer increased when the copolymer was mixed with a low 4HB component (4HB: 10.9 mol% and 20.0 mol%) rather than P(3HB) homopolymer, suggesting that the cause of the drop in tensile strength is the rapid degradation, while lipase degrades mainly the 4HB component of P(3HB-*co*-4HB) (Mukai *et al*., 1993). As the amount of 4HB component increased, the degradation of the 51.5 mol% and 75.6 mol% 4HB copolymers rapidly declined. Therefore, the tensile strength of P(3HB-*co*-4HB) degraded by the lipase substantially decreased (data not shown). The tensile strengths of the 10.9 mol% (◆) and 20.0 mol% (■) 4HB copolymers degraded by lipase were at least 3 MPa at 50 h, as presented in Fig. 5(c), suggesting that P(3HB-*co*-4HB) materials with low 4HB content are effective in tissue engineering applications, with a favorable enzymatic selection and biodegradability.

4. Conclusion

To maximize the polymer content from two-step fermentation by *D. acidovorans*, this study investigated the fermentation conditions by varying the concentration of the carbon sources, pH value, incubation temperature and incubation. The experimentally determined optimal conditions are as follows.

pH	7.0
Incubation temperature (°C)	26
Incubation time (h)	72
Concentration of carbon source (g/L)	10

The highest polymer content was approximately 13% of dry cell weight.

P(3HB-*co*-4HB) was biosynthesized by *D. acidovorans* using a sole carbon source of 1,4-butanediol or a mixed carbon source of *n*-butyric acid and 1,4-butanediol. The 4HB component content in P(3HB-*co*-4HB) increased with the 1,4-butanediol content in the mixed carbon source. The maximum 4HB unit composition of P(3HB-*co*-4HB) obtained upon synthesis by *D. acidovorans* was 95 mol%.

Although the enzymatic degradability of P(3HB-*co*-4HB) declined as the 4HB component content increased, lipase slowly degraded the P(3HB-*co*-4HB) material with the low 4HB component content, indicating that 4HB component content in P(3HB-*co*-4HB) can be controlled for tissue engineering or environmental applications, based on enzymatic selection and biodegradability considerations.

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