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Factors influencing the release of *Mitragyna speciosa* crude extracts from biodegradable P(3HB-*co*-4HB)

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ABSTRACT

Copolyesters of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) were produced by *Cupriavidus* sp. (USMAA2-4) (DSM 19379) from carbon sources of 1,4-butanediol and γ-butyrolactone. The composition of copolyesters produced varied from 0 to 45 mol% 4HB, depending on the combination of carbon sources supplied. The P(3HB-*co*-4HB) films containing *Mitragyna speciosa* crude extract were prepared with the ratio varying from 10 to 40% (w/w). The *in vitro* crude extract release of the films was studied in 0.1 M phosphate buffer (pH 7.4) at 37 °C. Although the release rate was slow, it was maintained at a constant rate. This suggests that the crude extract release was due to the polymer degradation because the amount of crude extract released was consistent. The amount of degradation was based on the films' dry weight loss, decrease in molecular weight and surface morphology changes. The degradation rate increased with the 4HB content. This showed that the polymer degradation is dependant on the molecular weight, crystallinity, thermal properties and water permeability. The different drug loading ratio which led to surface morphology changes also gave an effect on polymer degradation.

1. Introduction

In 1988, a random copolyester of R-3HB, R-4HB and P(3HB-*co*-4HB) was isolated from *Ralstonia eutropha* (Kunioka et al., 1988). *R. eutropha* accumulated P(3HB-*co*-4HB) from 4-hydroxybutyric acid or 1,4-butyrolactone under nitrogen-free conditions (Doi et al., 1989, 1990). It was found that P(3HB-*co*-4HB) copolymers with a high proportion of 4HB units (60–100 mol%) were produced by *R. eutropha* from 4-hydroxybutyric acid in the presence of some additives such as carbon source and also successfully produced pure P(4HB) homopolymer in this bacterium (Nakamura and Doi, 1992). The crystallinity and thermal properties such as melting temperature and glass transition temperature of P(3HB-*co*-4HB) decreased with the 4HB fraction. At levels of around 20–40 mol% 4HB, the P(3HB-*co*-4HB) copolymers behave like elastic rubbers (Kunioka et al., 1988; Saito and Doi, 1994).

Poly(3-hydroxyalkanoate-*co*-4-hydroxyalkanoate) (P(3HB-*co*-4HB)) has unique physicochemical properties such as thermoplasticity, biodegradability and biocompatibility that serves as a suitable candidates in medical and pharmaceutical applications such as drug delivery systems (Doi and Fukuda, 1994). Most PHA polymers would break down and release hydroxyl acids that are significantly acidic but less inflammatory than many currently used synthetic, absorbable polymers (Türesin et al., 2000).

Mitragyna speciosa Korth or 'Kratom' in Thailand and 'Biak-biak' in Malaysia belongs to the family of Rubiaceae, genus *Mitragyna* and species of *Speciosa*. It is indigenous to Southeast Asia, notably in Thailand and Malaysia (Jansen and Prast, 1988a). The leaves are consumed either in powder form or having them boiled in water. The boost in energy and strength is experienced within 5–20 min after consumption. People in Southern Thailand use the leaves as traditional medicine for common illnesses such as cough, diarrhea, muscle pain and hypertension. Thai and Malay natives or opium addicts use the leaves as an opium substitute when opium itself was unavailable (Matsumoto et al., 2004). Mitragynine, the main component extracted from *M. speciosa*, has a molecule formula of 9-methoxy-corynantheidine, $C_{23}H_2ON_2O_4$ with the molecular weight of 398.50. It has a melting point ranged from 102 to 106 °C and boiling point from 230 to 240 °C. It is soluble in chloroform, alcohol and acetic acid. It has an UV absorbance at 254 nm and is very stable (Jansen and Prast, 1988a,b).

The work presented here primarily aims to construct the release of *M. speciosa* crude extract from solvent cast P(3HB-*co*-4HB) films. The effects of P(3HB-*co*-4HB) composition, molecular weight, crys-

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tallinity, thermal properties, water uptake ability, various drug loading level and surface morphology were studied to produce a predictable *in vitro M. speciosa* crude extract release.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Cupriavidus sp. (USMAA2-4) (DSM 19379) used in this study was isolated from a soil sample collected in Sg. Pinang, Penang, Malaysia. Biosynthesis of P(3HB-*co*-4HB) was carried out in 2-step batch cultivation process (Amirul et al., 2004). For the first step, the microorganisms were grown in nutrient broth, which consists of 10 g/l peptone, 10 g/l yeast extract, 5 g/l Lab Lemco and 5 g/l NaCl for 20–24 h at 200 rpm with room temperature. The cells were then harvested by centrifugation at 7500 × *g* for 10 min at 19 °C, washed with sterile distilled water and transferred into nitrogen-free medium. At second-step, cells were transferred into mineral medium, which consists of 5.8 g/l K_2HPO_4, 3.7 g/l KH_2PO_4, 10 ml/l $MgSO_4{\cdot}7H_2O$ (0.1 M) and 1 ml/l trace element and culture for 48 h. Filter-sterilized carbon sources such as 1,4-butanediol and γ-butyrolactone with respective concentration was added into the medium to promote PHA synthesis. Cells were then harvested by centrifugation at 7500 × *g* for 10 min at 19 °C. The collected cells were then frozen at −20 °C. Dried cells were obtained by freeze drying for 1–3 days.

About 1.0 g freeze-dried cells were refluxed in 200 ml chloroform for 4 h. The extract was filtered to remove cell debris, and the chloroform was concentrated to a volume of about 15 ml using a rotary evaporator. The concentrated solution was then added drop-wise to 150 ml of rapidly stirred methanol to precipitate the dissolved PHA. The precipitated PHA was recovered by filtration using a 0.45 μm PTFE membrane. Copolymer collected, dried at room temperature and copolymer samples were prepared by solvent casting techniques.

2.2. Mitragyna speciosa crude extracts preparation

The *M. speciosa* Korth leaves collected from Gelugor (Penang, Malaysia) were dried at 50 °C for 12 h or until a constant weight was obtained. Then, it was grind into powder and collected in a condensed thimble. Methanol (500 ml) was used as the extraction solvent in a heating mantle Soxhlet for 3–4 h or until no changes of the solvent colour. The methanol was then dried up with a rotary evaporator until 5–10 ml of the extract solutions was left and was then dried overnight at room temperature (27 ± 1 °C) (Jansen and Prast, 1988a,b). Samples were analysed with GCMS (Hewlett Packard 6890 GC equipped with auto samples, quadrupole mass spectrometer and chemstation data system) to confirm the peak of Mitragynine compound. The GCMS monitoring mode was set at selective ion monitoring (SIM); $m/z = 214$ for mitragynine).

2.3. Film preparation with various drugs loading level

The solvent cast P(3HB-*co*-4HB) films were prepared by dissolving 1 g of the copolymer into 10 ml of chloroform. As for the solvent casting of P(3HB-*co*-4HB) loaded with *M. speciosa* crude extract, films were fabricated with 10, 20, 30 and 40% (w/w) of the crude extract. Both copolymers and crude extracts were dissolved with 10 ml of chloroform and were poured into glass Petri dishes (5 cm in diameter). The surface of the cast films was scanned with the scanning electron microscope (Leo Supra 50 VP Field Mission SEM, Carl-Ziess SMT, Oberkochen, Germany) (Zhao et al., 2003).

2.4. Polymer composition

The copolymer composition was determined by gas chromatography (GC) (Shimadzu GC-14B with Shimadzu-C-RGA Chromatopac) analysis (Braunegg et al., 1978). Approximately 20 mg of lyophilized cells or extracted pure polymer were subjected to methanolysis in the presence of chloroform, methanol and sulphuric acid (85:15% (v/v)) and the reaction mixture was incubated at 100 °C for 140 min. Then, it was left to cool to room temperature. Distilled water was then added and the mixture was vortexed for 5 min. The chloroform layer was transferred into a clean vial with Na_2SO_4 anhydrous (to absorb the excessive water). Sample (2 μl) was injected into the GC.

2.5. Degradation

The degradation study was carried out according to the method reported by Mao et al. (2005). One milligram of each P(3HB-*co*-4HB) film with 0.2-mm thickness cut into 3 cm^2 squares was used and degradation was monitored using 0.1 M phosphate buffer (pH 7.4) in shaking condition at 100 rpm at 37 °C. The changes in the weight of each film were taken on a weekly basis with analytical balance (Mettler Toledo MX5). The excess water on the film surface was blotted with a filter paper and then placed in a desiccator chamber with the temperature of 50 °C and at 65% humidity for at least 30 min or until it reached a constant weight. Molecular weight of each film were checked and recorded.

2.6. Water uptake

The initial weight of each composition of P(3HB-*co*-4HB) was recorded. The water absorption rate for each film was tested in sterile 0.1 M phosphate buffer (pH 7.4) at 37 °C. The changes in weight of each film were taken weekly with analytical balance (Mettler Toledo MX5). Before the weight of the film was taken, the excess water on the film surface was blotted with filter papers and the weight was taken for three repetitive times on a weekly basis (Wen et al., 2003).

2.7. P(3HB-co-4HB) molecular weight

All copolymer films' molecular weight data were obtained at 35 °C by using gas permeation chromatography (GPC) [Waters Model 600E GPC system and Water 410 (Differential Refractometer) detector with a PL gel 5 μ MIXED C column]. Chloroform was used as the eluent at a flow rate of 0.8 ml/min and a sample concentration of 1 mg/ml was used. Polystyrene standards with a low polydispersity index were used to make a calibration curve as reported previously (Nakamura and Doi, 1992).

2.8. P(3HB-co-4HB) thermal properties

The copolymer thermal properties were determined by the differential scanning calorimeter (DSC) (Pyris DSC, PerkinElmer) equipped with a cooling accessory. Polymer samples (1 mg) were encapsulated in aluminium pans and heated at 20 °C/min from −50 to 200 °C. The melting temperature and enthalpy of fusion were determined from the DSC endotherms. The samples were maintained at 200 °C for 1 min and then rapidly quenched to −50 °C. They were again heated from −50 to 200 °C at the heating rate of 20 °C/min. The glass transition temperature was taken as the midpoint of the heat capacity change.

2.9. P(3HB-co-4HB) with various drug loading

In order to determine the degradation of the film during the studies, the surface was observed under a scanning electron microscope (SEM) (Leo Supra 50 VP Field Mission SEM, Carl-Ziess SMT). These films were initially lyophilized and then mounted onto aluminium stubs, coated with gold spray in a sputtering device for 1.5 min at 15 mA and examined under a SEM (Zhao et al., 2003).

2.10. In vitro drug release

The drug release was done accordingly to the method reported (Mao et al., 2005). These formulated films with 0.2-mm thickness were cut into 3 cm^2 squares. The dry weight of each film was recorded and placed into individual vial containing 5 ml of filter sterilized (0.2 μm filter) 0.1 M phosphate buffer, pH 7.4. The samples were then incubated at 37 °C under shaking condition at 100 rpm. The rate of drug release was then taken at various sampling intervals for 6 months. At each sampling point, the buffer was replaced with a fresh solution. At the end of the study, these films were removed from the buffer solution and dried overnight at 37 °C with 65% humidity, where the final weight was recorded (Türesin et al., 2000). A standard curve was also constructed by using pure mitragynine standard samples of known concentration (50–500 μg/ml, $R = 0.9994$) and quinine with concentration of 25 μg/ml as reference samples. The concentration of mitragynine released from the film was assayed by GC–MS set at selective ion monitoring (SIM) mode at $m/z = 214$ for mitragynine.

3. Results and discussion

3.1. P(3HB-co-4HB) biosynthesis, composition and water uptake

Biosynthesis of P(3HB-*co*-4HB) was carried out using a two-step cultivation process. The two-step cultivation is widely used to produce PHAs because nutrient depletion, such as nitrogen, oxygen, and other essential elements in excess carbon is favourable for the accumulation of PHAs. Therefore, 2-step cultivation is applied, whereby cell biomass from nutrient broth is transferred to a second-step in which the medium is usually nitrogen-limited or nitrogen-free (Sudesh et al., 2000). When pre-cultured *Cupriavidus* sp. USMAA2-4 cells were transferred to nutrient broth, a period of 24 h was required for maximum growth of the strain (13.5 g/l) and further cultivation led to a stationary growth phase (results not shown). The cells were then harvested and transferred into mineral medium containing various combinations of 1,4-butanediol and γ-butyrolactone.

Table 1 shows the effect of 1,4-butanediol and γ-butyrolactone on the production of P(3HB-*co*-4HB) by *Cupriavidus* sp. (USMAA2-4). The content of PHA produce was in the range of 27–54 wt% when different concentration of 1,4-butanediol and γ-butyrolactone was used. The composition of 4HB increased almost linearly with γ-butyrolactone (Fig. 1). In other words, the 3HB content increased when the amount of 1,4-butanediol fed increased. The results obtained were similar to the results reported by Doi et al. (1989) in which the 3HB content increased when the amount of 1,4-butanediol fed increased.

The degradation rate was increased from 1 to 31% when the 4HB content increased from 0 to 45 mol%. This shows that the degradation process was dependent on the polymer composition. Incorporation of 4HB into the 3HB chain alters crystallinity, thermal properties, solubility, water uptake and surface morphology that would eventually influence the biodegradation rate of the polymer.

Table 1
Effects of carbon sources concentration on the P(3HB-*co*-4HB) biosynthesis; percentage of polymer degradation in phosphate buffer and percentage of water penetration in 6 months study

1,4-Butanediol (g/l)	γ-Butyrolactone (g/l)	Cell dry weight (g/l)	PHA content (wt%)[a]	PHA composition (mol%)[a]		PHA yield (g/l)	Degradation(%) ($n = 3$) (mean ± S.D.)[b]	Percentage of water penetration ($n = 3$) (mean ± S.D.)[c]
				3HB	4HB			
1.75	0	12.3	54	100	0	6.6	1 ± 0.34	0.02 ± 0.01
1.40	0.35	9.6	46	89	11	4.4	19 ± 0.63	0.21 ± 0.07
1.05	0.70	8.4	41	78	22	3.4	21 ± 0.82	0.32 ± 0.11
0.70	1.05	6.5	35	70	30	2.3	24 ± 0.97	0.45 ± 0.13
0.35	1.40	5.6	27	55	45	1.5	31 ± 1.04	0.53 ± 0.14

[a] Calculated from GC analysis.
[b] Gravimetry.
[c] Gravimetry.

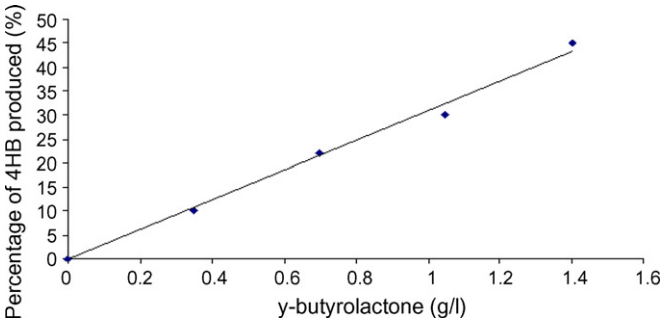

Fig. 1. The 4HB produced vs. the amount of γ-butyrolactone added.

The water uptake by the copolymer in 0.1 M phosphate buffer (pH 7.4) at 37 °C without shaking was generally higher for lower molecular weight copolymer (higher 4HB fraction). This is because, P(3HB-*co*-4HB) has a hydrophobic group C–H bonds and are essentially water impermeable (Heller, 1987). Water penetrates into the polymer bulk and hydrolyses in the medium through chain scissions of the ester linkages in the copolymer backbone. For surface eroded polymers, water inhibition rate usually exceeds that of backbone hydrolysis (Tamada and Langer, 1993). When 4HB content increased, degradation also increased. Thus, water penetration will also increase. As shown in the results, the percentage of water absorbed increased from 0.02 to 0.53% when the 4HB content increased in the copolymer.

Polymer with higher molecular weight (lower 4HB content) had greater number of intra-molecular bonds and more open network structure was formed. Higher intra-molecular bond will cause more rigid structure and higher crystallinity that lowering the equilibrium water uptake and degradation rate (Young and Lovell, 1991). Therefore, networks formed from P(3HB-*co*-4HB) with higher 4HB fraction allowed more water penetration and increased hydrolysis process. The polyesters are hydrolytically cleaved into shorter chain of alcohols and acids, accelerate the polymer degradation (autocatalysis) and thus increased drug mobility's and release rates.

3.2. P(3HB-co-4HB) molecular weight

Table 2 shows that the number-average molecular weight (M_n) of P(3HB-*co*-4HB) decreased when the 4HB composition increased. This is because copolymer with lower 4HB content has longer chain length and this enhance the formation of physical entanglements among polymer chains and provide a relatively tighter network structure. When 4HB content increased, smaller number of intra-molecular bonds and less open network structure was formed.

After the drug release experiment, the M_n declines and this suggests that degradation process would immediately decrease the copolymer M_n. The results also showed that lower molecular weight of copolymer (higher 4HB content) degraded faster than higher molecular weight copolymer (lower 4HB content) and therefore increased the drug-released rate. Amorphousness increases and this led to higher degradation.

Films prepared from lower molecular weight polymer were porous with lower bulk density and higher specific area. But high molecular weight polymer will have longer chain length and enhance the formation of physical entanglements among polymer chains and provide a relatively tighter network structure. Less water could penetrate into the film and thus slows degradation (Lindardt, 1988). It is expected that these open and loose network structure within the films enables a drug to leach out easily and increases the drug release rate (Rosen et al., 1983).

3.3. P(3HB-co-4HB) crystallinity and thermal properties

As shown in Table 2, the enthalpy of melting (ΔH_m) and glass transition temperature (T_g) decreased when the 4HB content increased. The ΔH_m value is the indicator of crystallinity. Copolymer with lower ΔH_m value indicates lower crystallinity degree (Young and Lovell, 1991; Chu, 1985; Li et al., 1990a,b). As the esters groups in crystalline regions are resistant to hydrolysis, the rate of chain cleavage increases with decreasing degree of crystallinity (Heller, 1985). Thereby, P(3HB-*co*-4HB) with higher 4HB content would reduce crystallization and decrease in crystallinity and increase degradation rate. The T_g value is lower because 4HB content is higher. As reported, degradation will lower the T_g value and increase polymer chain motions, creating more free volume. More water was allowed to penetrate and thereby increasing degradation process (Park, 1994). As a result, the drug release rate was increased when 4HB content increased.

As for the melting temperature (T_m), they were in the range of 152–163 °C. No significant decreased values were observed. This shows that the copolymer have multiple melting behaviour (Mitomo et al., 2001). The 4HB component could hardly co-crystallize in the 3HB crystal lattice significantly, showing that the sequence of 4HB component is mostly excluded from the P(3HB) lattice crystal. In this case, the copolymer mostly shows the less-depressed value of T_m as a result of exclusion of the 4HB unit (Nakamura and Doi, 1992). As reported, only one crystalline form of the P(3HB) lattice was observed from the X-ray diffraction patterns of P(3HB-*co*-4HB) copolymers with compositions of 0–29 mol% 4HB (Saito and Doi, 1994). Thereby, the results showed in Table 2 suggests that the rates of crystal growth reduced with the 4HB fraction suggesting that the 4HB units are excluded from P(3HB) crystalline phase.

3.4. P(3HB-co-4HB) various drug loading and surface morphology

Fig. 2 shows that the polymer pores were close to each other before drug loading. After drug loading, porous structure was formed because *M. speciosa* crude extract were entrapped within the polymer pores. The higher the drug loading the coarser the copolymer films formed because more crude extracts were

Table 2
Thermal properties and number average molecular weight of P(3 B-*co*-4 HB)

P(3HB-*co*-4HB)	ΔH_m (J/g)[a]	T_m (°C)[a]	T_g (°C)[a]	(M_n) ($\times 10^3$ Da)[b]		(M_n) decreased (%)
				Before release	After release	
P(3HB)	54	163	8	291	264	9.3
P(3HB-*co*-11%4HB)	41	160	−4	202	149	26.2
P(3HB-*co*-22%4HB)	37	152	−9	169	106	37.3
P(3HB-*co*-30%4HB)	19	160	−16	120	65	45.8
P(3HB-*co*-45%4HB)	16	161	−17	76	35	53.9

[a] Determined by DSC analysis.
[b] Calculated from GPC analysis.

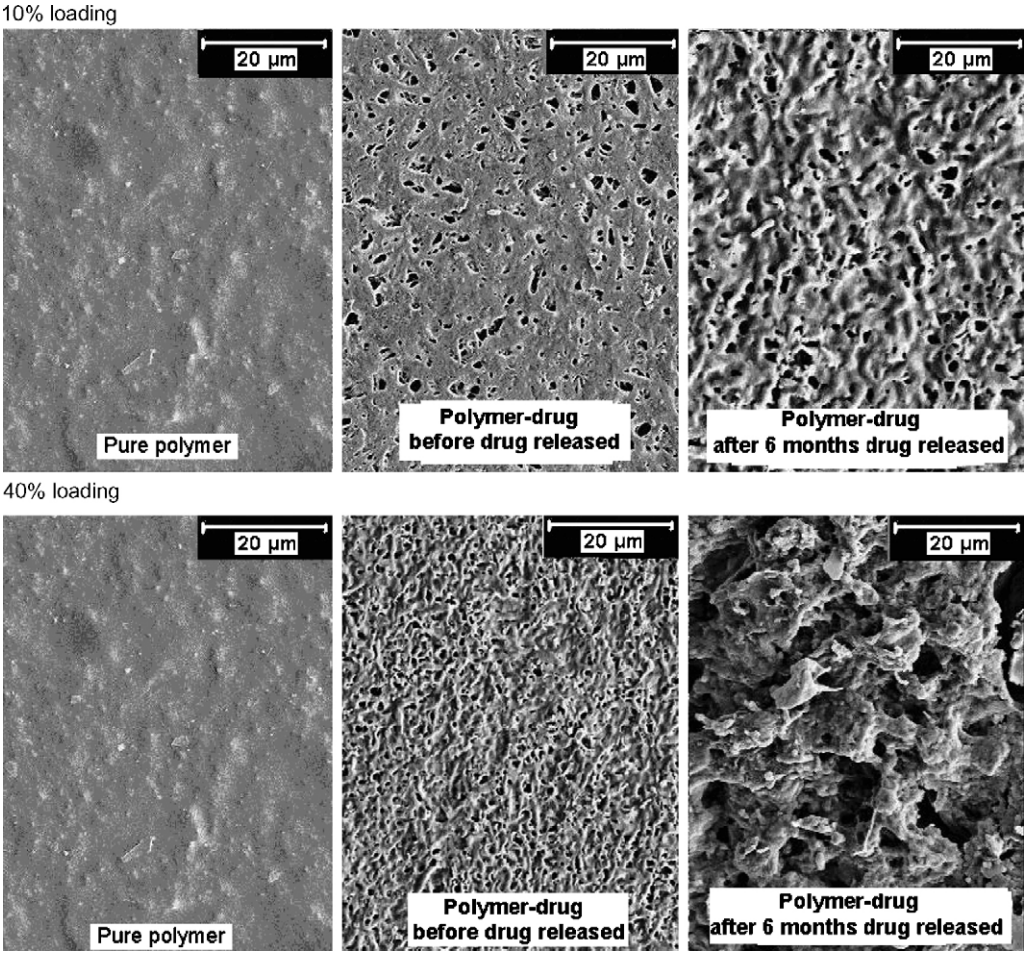

Fig. 2. SEM of P(3HB-*co*-4HB) with low (10%) and high (40%) drug loading.

entrapped within the polymer pores. This resulted in higher initial release by dissolution because channels are formed as solid drug is dissolved and released (Ravivarapu et al., 2006; Pouton and Akhtar, 1996).

The drug was homogenously dispersed within the polymer matrix. Thus, they were dependant on the water penetration and polymer degradation in order to release from the copolymer films. Copolymer with higher drugs loading created more open pores on the films. This allowed more water to penetrate and dissolve the drugs. Drugs were released creating open water-filled channels that favour immediate release by self-diffusion. Such behaviour may result from a dramatic decrease in the acid-catalysed, self-accelerated ester bond cleavage that has been established as a prevalent mechanism for copolymer degradation and consequent drug release via surface erosion (Vert et al., 1997). Thereby, after the drug release experiment more uniform pores can be seen on the film surfaces.

3.5. Drug release

The total release of *M. speciosa* crude extract from P(3HB-*co*-4HB) films with various copolymer compositions and drug loading level in 6 months are presented in Table 3. The percentage of crude extract released increased with the 4HB content and the drug loading level especially during the initial drug release. This can be attributed to the incompletely incorporated drugs at or near the polymer surface were released upon contact with the buffered media through dissolution and diffusion. As reported during early stages, only little erosion occurred and the rate of drug release is typically by simple dissolution and diffusion (Nelson et al., 1987). Later, when a portion of the polymer slab erodes, crude extract escapes and dissolves in the buffer solution. When the drug loading level increased, clusters may form in the matrix. These clusters will be released and form water-filled channels. This channel will favour immediate release by self-diffusion through drug-filled pores in the

Table 3
Percentage of *Mitragyna speciosa* crude extracts released from P(3HB-*co*-4HB) with various composition and drug loadings in 6 months study

Drug loading ($n = 6$)	P(3HB-*co*-4HB) ($n = 6$) (mean ± S.D.)				
	0% 4HB	11% 4HB	22% 4HB	30% 4HB	45% 4HB
10% loading	2.8 ± 0.93	12.5 ± 4.60	16.0 ± 5.79	17.7 ± 6.71	19.5 ± 7.37
20% loading	3.8 ± 1.05	17.1 ± 6.35	18.5 ± 6.77	23.3 ± 8.45	27.2 ± 10.56
30% loading	4.2 ± 1.30	23.5 ± 8.45	26.6 ± 10.33	34.1 ± 12.60	45.2 ± 16.28
40% loading	5.0 ± 1.53	30.2 ± 11.06	32.4 ± 12.15	47.7 ± 17.80	53.6 ± 19.46

Calculated from GC–MS analysis.

polymer and therefore, increase the drug release rate (Nelson et al., 1987). At later stage, the drug release was slowed down. Water penetrates into the polymer bulk and hydrolyzes in the medium through chain scissions of the ester linkages of the copolymer backbone, releasing the entrapped drugs into the solvent (Zhang et al., 2003).

4. Conclusion

P(3HB-*co*-4HB) with various fraction of 4HB was successfully produced from the combination of two carbon sources at different concentration. *M. speciosa* crude extract can also be prepared with a simple extraction method. It is possible to get the release pattern of *M. speciosa* crude extract from chloroform cast P(3HB-*co*-4HB) films. Although the release rate was slow, it is still released at a constant rate. Factors such as the P(3HB-*co*-4HB) composition, molecular weight, crystallinity, thermal properties, water uptake ability, drug loading ratio and surface morphology did play an important role leading to polymer degradation. The results also indicate that the crude extract was released via polymer degradation.

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