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Influence of solvent extract on properties of biomaterial PHA derived from *Novosphingobium* sp. THA_AIK7

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Abstract

Polyhydroxyalkanoates (PHA) production by *Novosphingobium* sp. THA_AIK7 was investigated in Mineral salt medium (MSM) supplemented with 2% (v/v) of crude glycerol as a carbon source. The polymer were extracted by sodium hypochlorite and chloroform. Both of extracted films was white, opaque and has a smooth surface. PHA film casting from sodium hypochlorite extraction had bigger pore size than chloroform casted film. The contact angle obtained from polystyrene tissue culture plate (TCP), sodium hypochlorite and chloroform casted film were 78, 81 and 80 degree, respectively. Decomposition temperature (Td), melting temperature (Tm) and crystallization temperature (Tc) were examined against PHB standard. Td of 285, 299 and 298˚C were acquired from PHB, sodium hypochlorite and chloroform casted film, respectively. Tm value of 170, 167 and 178˚C and Tc value of 66, 54 and 92˚C were acquired from sodium hypochlorite, chloroform and PHB, respectively. Biocompatibility study of PHA film was tested with Vero cell. Vero cells grown on tissue culture plate (TCP) presented typical fibroblast morphology. However, Vero cells were unable to adhere on sodium hypochlorite casted film and consequently cell lysis was observed. Vero cells could attach on chloroform casted film but could not develop normal fibroblast appearance and was incapable of proliferating on casted film. The results shown here indicated that the extraction methods were still not proper for biocompatible test. The finding of a suitable method for biomaterial preparation will be done in the next phase.

Keywords : *Biomaterial, Novosphingobium sp. THA_AIK7, Polyhydroxyalkanoates*

1. Introduction

Polyhydroxyalkanoates (PHA) are a family of biopolyesters in which currently approximately 150 different monomer units have been identified as constituents (Steinbüchel, 2001; Steinbüchel and Valentin, 1995), whereas homopolymer poly-beta-hydroxybutyrate (PHB) is the most abundant (Anderson and Dawes, 1990). PHA have become the substitute materials for conventional plastic in response to the harmful effect of the plastic waste problem (Cavalheiro et al., 2009). PHA have been promising in medical and pharmaceutical applications due to their biodegradability and biocompatibility with immunological inertness and slow degradation in human tissue (Verlinden et al., 2007; Akaraonye et al., 2010). PHA have been investigated for medical uses such as wound management, heart valves, vascular graft, orthopedy, drug delivery, and urology (Williams et al., 1999; Akaraonye et al., 2010). One important criterion for a biomedical product is endotoxin contamination. According to US Food and Drug Administration (FDA) regulations, the endotoxin content of medical devices should not exceed 20 USP endotoxin units per device (Williams et al., 1999). One characteristic of the species of the genus Sphingomonas is unlike common gram-negative bacteria. They contain glycosphingolipids (GSLs) as the cell envelope component instead of lipopolysaccharide (LPS) which is well known as endotoxin (Takeuchi et al., 2001; Kawahara et al., 2010). The PHA film produced from a gram-negative bacterium Novosphingobium sp. THA_AIK7 has been reported for endotoxin-free (Teeka et al., 2012). Sodium hypochlorite is commonly used for PHA extraction but it has been reported for 50% of PHA molecular weight reduction (Berger et al., 1989). Chloroform alone was chosen to test for PHA purification and the result showed that the 87% recovery could be attained after incubation at 35˚C for 48 h (Teeka, 2012). This article reports the properties of PHA film extracted by two different methods of sodium hypochlorite and chloroform. The feasibility study of PHA casted films for biomedical use was also investigated.

2. Materials and Methods

2.1 PHA film preparation

Novosphingobium sp. THA_AIK7 was grown in Mineral salt medium (MSM) supplemented with 2% (v/v) crude glycerin and 1 g/L monosodium glutamate at initial pH of 7 (modified from Ramsay et al., 1990). The aerobic condition was maintained at 30 ˚C and 150 rpm for 72 h. The bacterial cells were collected and PHA film was extracted by sodium hypochlorite and chloroform method. For sodium hypochlorite method, 5% sodium hypochlorite was added and incubated at 37˚C for 1 h. The polymer was washed twice and dissolved in chloroform. For chloroform method, fifteen milliliters of chloroform was directly added to bacterial cell and incubated at 40˚C for 3 days. Both methods, PHA film was casted after chloroform evaporation.

2.2 PHA film surface study

The dehydrated PHA films were cut into square shapes (0.5 x 0.5 cm) and mounted on aluminum stump, coated with gold in JEOLJFC-1600 auto fine coater for 30 min at 15mA and examined under the scanning electron microscope JEOLJSM-6510 (Zhang et al., 2000).

2.3 Wettability of PHA film study

Wettability of PHA film was examined by measuring contact angles of water with a sessile drop method using Contact Angle Meter (Kyowa DM-CE2). The drop of approximately 3 µL was gently placed down on the surface of the PHA films and polystyrene tissue culture plate as a control (Zhang et al., 2000).

2.4 Thermal property study

2.4.1 Thermogravimetric analysis (TGA)

TGA measurements were conducted on a TA Instrument TGA 2950 Thermogravimetric analyzer. Samples of approximately 15 mg were used for each measurement. The samples were heated at a rate of 10˚C/min from room temperature to 400˚C in a nitrogen atmosphere (Guo et al., 2013). Poly (R)-3-hydroxybutyric acid (PHB) (Sigma-Aldrich, #363502–10G) was used as standard polymer.

2.4.2 Differential scanning calorimetry (DSC)

Melting temperature (Tm) and Crystallization temperature (Tc) were analyzed by Differential scanning calorimetry (DSC) with a Perkin Elmer DSC7. Sample was heated from -10˚C to 200 ˚C at 10˚C/min, held for 2 min to ensure melting, then cooled to -10˚C at 10˚C/min. Melting temperature (Tm) and crystallization temperature (Tc) thermogram of the polymer was recorded from the first heating scan's data (Zheng et al., 2005).

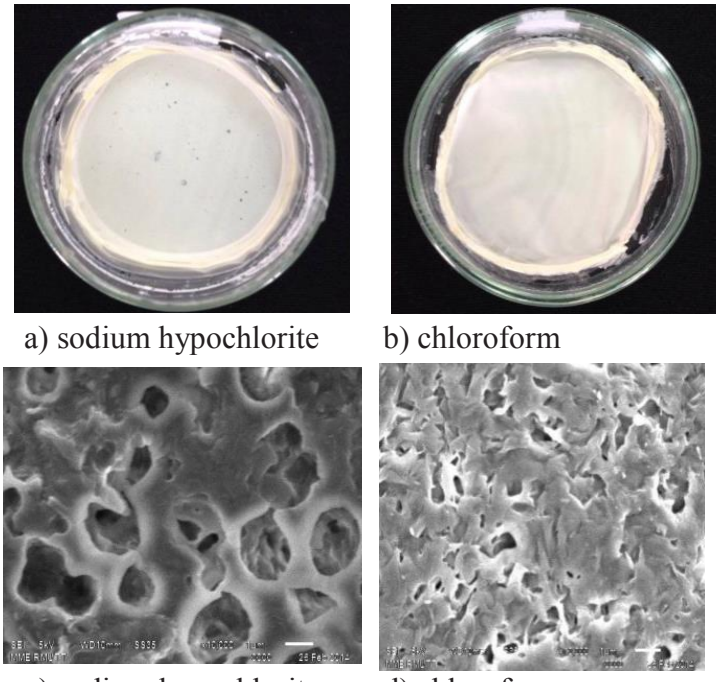
2.5 Biocompatibility study

PHA films were cut and placed in 96-well tissue culture plate (TCP). They were soaked with 70% alcohol for 30 min and washed twice with PBS buffer. Each treatment was seed with Vero cell ATCC CCL-81™ of 6 x 10^5 cells/well. All specimens were incubated in 5% CO_2 incubator at 37˚C. The viability and morphology of Vero cell were observed after 24 h. Vero cell grown on tissue culture plate was used as control to show the normal fibroblast appearance.

3. Results

3.1 PHA polymer characterization Scanning Electron Microscope (SEM)

Both of extracted films was white, opaque, not brittle and had a smooth surface. The surface morphology of the extracted films under SEM showed that PHA film casting from sodium hypochlorite had more number and bigger pore size than film derived from chloroform method (Figure 1). The higher pore size of PHA casted film may be affected from sodium hypochlorite digestion which can reduce about 50% of molecular weight (Berger et al., 1989).



a) sodium hypochlorite b) chloroform

c) sodium hypochlorite d) chloroform

Figure 1. Characterization of PHA film (a and b) and film surface under SEM (c and d) from sodium hypochlorite and chloroform extraction

Wettability

The contact angle obtained from polystyrene tissue culture plate (TCP), sodium hypochlorite and chloroform casted film were 78, 81 and 80 degree, respectively. The extraction method showed no effect on wettability of casted film. Yuan and Lee (2013) concluded that the contact angle which is lower than 90 degree has hydrophilic property. Therefore, each sample is a hydrophilic material which is proper for cell adhesion (Zhang *et al*., 2000).

Thermal property

Decomposition temperature (Td), melting temperature (Tm) and crystallization temperature (Tc) were examined against PHB standard. Td of 285, 299 and 298°C were acquired from PHB, sodium hypochlorite and chloroform casted film, respectively. The higher Td of PHA casted films than PHB standard may indicate THA_AIK7 PHA polymer contain more than one monomer or a longer chain polymer. Therefore, they need higher temperature to break the bond.

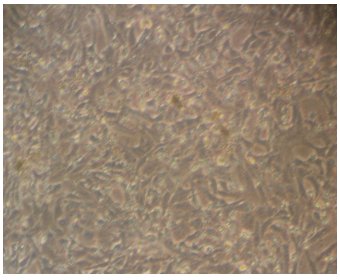
Table 1. Thermal properties of PHA film

	PHB (Standard)	Extraction method	
		Sodium hypochlorite	Chloroform
Td (˚C)	285	299	298
Tc (˚C)	92	66	54
Tm (˚C)	178	170	167

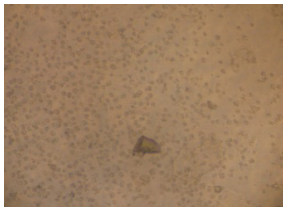
Tm and Tc showed the similar manner which casted film had lower temperature than standard PHB. Tm value of 170, 167 and 178˚C and Tc value of 66, 54 and 92˚C were acquired from sodium hypochlorite, chloroform and PHB, respectively (Table 1). The lower Tc of THA_AIK7 PHA than PHB showed the low crystallization degree of polymer (Zhao *et al*., 2003). Lu *et al.* (2011) mentioned that PHB has high degree of crystallinity. After blended with 4-hydroxybutyrate (4-HB), Tm and Tc value of P(3HB-co-4HB) copolymer was lower than pure PHB. It may suggest that THA_AIK7 PHA polymer contain another kind of monomer in their chain.

3.2 Biocompatibility

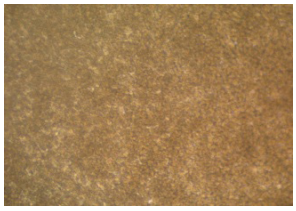
Biocompatibility study of PHA film was tested with Vero cell, a monkey kidney cell line, as an animal testing model. Vero cells grown on tissue culture plate (TCP) presented a typical fibroblast morphology (Figure 2a). However, Vero cells were unable to adhere on sodium hypochlorite casted film and consequently cell lysis was observed after 24 h (Figure 2b). It may cause from the toxicity of sodium hypochlorite, which still remain on PHA film, to animal cell. For chloroform casted film, Vero could attach on surface but they were unable to develop a normal fibroblast appearance. Accordingly, no cell proliferation on casted film was observed (Figure 2c).



a) Tissue culture plate (TCP)



b) sodium hypochlorite



c) chloroform

Figure 2. Characterization of Vero cell grown on different surface material

4. Conclusion

This present study shows that the properties of PHA film extracted from sodium hypochlorite and chloroform had different surface morphology and thermal properties. Biocompatibility was preliminary tested with Vero cell. Cell proliferation was not observed on both films. The results indicated that the extraction or the sterilization method of PHA film were still not proper. The finding of a suitable method for biomaterial preparation will be done in the next phase.

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