

CHAPTER II
BIOSYNTHESIS OF POLY- β -HYDROXYALKANOATES BY
***Hydrogenophaga* sp. ISOLATED FROM**
ENVIRONMENT DURING BATCH
FERMENTATION

2.1 Introduction

Synthesized plastic from petroleum industry has been widely used in daily. Because of non-degradable and accumulating for long time in environment, it causes the problems of air pollution, solid waste disposal and even global warming when it is destroyed by burning and the increasing demands of polymeric materials for our use. In addition, reserves of petroleum stock are limited and running out. The price of petroleum products are also increasing. Due to these results, many researchers have paid attention to investigating other materials to replace for synthesized plastic.

Polyhydroxyalkanoates (PHAs), a main kinds of biodegradable plastic and classified as polyester, was the first biomaterial discovered in *Bacillus megaterium* and characterized in 1925 (Lemoigne, 1926). The polyester may contain proportions of 3-hydroxyacids (Braunegg, Lefebvre, & Genser, 1998). The general formulae of the monomer unit is $-\text{[O-CH(R)-CH}_2\text{-CO]}-$. According to the size of the alkyl substituent (R) mechanical properties of PHAs differ (Vroman & Tighzert, 2009). Typically, PHAs can be divided into three groups by number of carbon atoms in their side chain-Short chain length (scl) PHAs are composed of 3-5 carbon atoms, while medium chain length (mcl) PHAs consist 6-15 carbon atoms and long chain length (lcl) comprise 15 and above carbon atoms (Anderson & Dawes, 1990; Steinbuchel & Valentin, 1995; Lee & Gilmore, 2005). The structure of PHAs depends on carbon sources supplying and microbial types. The majority of biosynthesis of PHAs is

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performed using various microorganisms mostly bacteria. They can produce PHAs from a number of substrates and accumulated in their cell as carbon source and energy reserve (Anderson & Dawes, 1990). In addition, depending on PHAs accumulation behaviour, microorganism can be categorised into two groups. The first group requires the limitation of some nutrient such as nitrogen or phosphate. The member of this group belong *Cupiavidus necator* (formerly from *Ralstonia eutropha*) and *Pseudomonas oleovorans* etc. Secondly, they do not depend on nutritional limitation and they can also accumulate PHAs during their growth such as *Alcaligenes latus* (Yezza et al., 2007), *Azotobacter vinelandii*, *P. putida*, *P. aeruginosa* (Fernandez, Rodriguez, Bassas, & Vinas, 2005), *Bacillus mycoides* (Thakur, Borah, Baruah, & Nigam, 2001) and recombinant *Escherichia coli*. However, Wang and Lee (1998) reported that PHAs in term of poly-3-hydroxybutyrate (PHB) can be produced with high productivity and high polymer content by *A. latus* via fed-batch fermentation.

Although, PHAs are interested and widely studied by many researcher for a long time but the PHAs production are limited by their production cost. A major problem to the commercialization of PHAs is their much higher production cost compared with petrochemical-based synthetic plastic materials or other biodegradable polymers (Luengo, Garcia, Sandoval, Naharro, & Olivera, 2003). Much attempt has been devoted to reduce the cost of PHAs by the isolation of better bacterial strains to produce PHAs from a cheap carbon source such as sweet sorghum (Kaewkannetra et al., 2008). These bacteria can be isolated from various sources such as water and soil environments using a simple method that can monitor the accumulation of PHAs. For example the conditions for bacterial PHB production can be met in soil, due to its heterogeneous nature. Nitrogen availability in soil varies with microsites. It may become a limiting factor for bacterial growth, especially in some nitrogen-poor (carbon-rich) sites (Wang & Bakken, 1998). According to these results, some PHA-producing strains such as *Bacillus*, *Arthrobacter*, *Aureobacterium*, *Corynebacterium*, *Curtobacterium*, *Pseudomonas*, *Micrococcus*, and *Acinetobacter* can be isolated from soil environments.

There are various simple methods for detecting intracellular PHA granules which are applied to the screening of PHA producers such as Sudan Black staining and Nile blue A staining (Ostle & Holt, 1982) and etc. The positive result showed as a

black color or fluorescent granules under microscope. Although they are feasible and easy methods, however they would take a rather time-consuming and labour intensive work to screen a large numbers of environmental isolates. Alternative staining methods have recently been developed for directly staining colonies or growing bacteria on plates containing Nile blue A or Nile red (Spiekermann, Rehm, Kalscheuer, Baumeister, & Steinbuchel et al, 1990). The dye can be directly diffused to microbial cytoplasm and resulting in fluorescent colonies that can be observed by UV illumination without the step of microscopic observation. Therefore, it would be stated that the colony-staining is a suitable method for screening large numbers of microbial strains.

Thailand is an agro-industry country, fruitful with very cheap sugar and starch based agricultural raw materials and it locates in the tropical area, thus there are accepted that, this area has appropriate environments and temperature for expansion of microorganisms. This study aims to isolate sucrose utilising bacteria from agricultural crop plantation soil for production of PHAs via batch fermentation.

2.2 Materials and Methods

2.2.1 Collection, cultivation and isolation of soil microbes

Soil samples were collected from sugarcane plantation area in the North-eastern of Thailand. The samples then were preserved at 4 °C prior to use. Primary isolation of soil microbes was investigated by ten-fold serial dilution technique. A gram of soil sample was transferred to sterile nutrient broth (NB) and incubated at 30 °C for 24 h. Then, the culture was diluted in 0.9% normal saline with different dilutions (10^{-4} , 10^{-5} , 10^{-6} and 10^{-7}) and spread on sterile nutrient agar (NA). The plates were incubated at 30 °C for 24 h. Finally, the several single colonies of soil bacteria were picked and transferred to mineral salt agar. Screening for the soil bacteria PHA producers were carried out in mineral salt agar containing with 10 g/L sucrose as a sole carbon source. The culture flasks were incubated at 30 °C for 48 h. The PHAs producing capabilities of the microbes were confirmed using 0.3% Sudan Black B (w/v) in 70% (v/v) alcohol and Nile blue staining methods as described by Wang and Bakken (1998). The viable colonies were directly observed under

ultraviolet light (312 nm) and exposing fluorescence to detect the accumulation of lipid compounds including PHAs (Spiekermann et al., 1990).

2.2.2 Identification of PHAs-producing microbes

Pure isolates containing lipophilic inclusions were observed by microscopic technique. Their morphology and biochemical characters of the microbe were observed. The PHA-producing strains were identified by sequencing partial sequences of their 16S rDNA. The chromosomal DNA of the pure isolates was isolated by routine methods as previously described by Slater, Voige, and Dennis (1988) and Chien, Chen, Choi, Kung, and Wei (2007) and used for further identification. The partial sequencing of their 16S rDNA was amplified with bacterial universal primer PA (5'-AGAGTTTGATCCTGGCTCAG-3') and primer PH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards, Rogall, Bocker, Emde, and Bottger, 1989) and generated the product length of 1519 bps. The PCR product was then subjected to ABI PRISM310 (PE Biosystems) according to manufacturer's instruction. The nucleotide sequences of partial 16S rDNA were compared with the 16S rDNA sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI), USA, by using their World Wide Web site (<http://www.ncbi.nlm.nih.gov>), and the BLAST (Basic Local Alignment Search Tool) algorithm program and then deposited in the database under accession numbers AB3000163.

2.2.3 Batch fermentation of PHA in shake flasks

The selected strain was maintained in nutrient broth and incubated in orbital shaking at 200 rpm, 35 °C for 24 h prior use. Then, the 5% (v/v) inoculums of the preculture was used to inoculate in 250 mL Erlenmeyer flask containing 50 mL sterilized mineral salt medium compost of (g/L); 20 g sucrose, 1.5 g KH_2PO_4 , 3.57 g Na_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $(\text{NH}_4)_2\text{SO}_4$, 1 g and 1 mL trace element solution prepared following the method described by Grothe, Moo-Young, and Chisti (1990). The results obtained after fermentation were reported in terms of percentage on dry cell weight: DCW (% w/w), PHA productivity (g/L.h), PHA yield ($Y_{p/s}$) and biomass yield ($Y_{x/s}$).

2.2.4 Sucrose uptake

Sucrose uptakes in culture medium were monitored in terms of Total organic carbon (TOC) using TOC analyzer (TOC-5000, Shimadzu Corporation, Japan, with ASI-5000 auto injector).

2.2.5 Biomass measurement

Biomass concentration was measured off-line by optical density (OD) at 660 nm (1 OD 660 nm = 0.308 g of dry cell weight, DCW) on a Shimadzu UV-Visible spectrophotometer. Calculation has been made by plotting a calibration curve between the DCW and OD values.

2.2.6 Biomass recovery, extraction and determination of PHAs

After fermentation, biomass was collected by centrifugation ($\times 8,000$, 10 min). Then, supernatant was discarded; the pellet was washed for twice with distilled water and dried in hot air oven at 60 °C until constant weight. Intracellular PHA in biomass was measured using a modified method (Lee & Gilmore, 2005). In brief, a commercial bleach of sodium hypochlorite (NaClO, contained 6% active chlorine) was added to the wet biomass pellet and was mixed vigorously using vortex mixer. Then, the mixture was incubated at 37 °C for 1 h until non-PHA biomass dissolved and white flocculants lipid was precipitated. Hereafter, the PHA recovery was conducted. The mixture was re-centrifuged and washed with distilled water twice to remove the bleach solution which was contained of dissolved cell debris. The cells were washed twice with acetone to remove a low-molecular-weight lipid (Anderson & Dawes, 1990). The white cell pellet was resuspended with ethanol solution and then was re-centrifuged. The obtained white pellet was dried in hot air oven at 60 °C until reached a constant weight. All measurements were weighed in triplicates. It should be noted that in a large proportion of PHAs was extracted in Soxhlet apparatus and extraction with an excess of chloroform at 100 °C for 3 h. The mixture was filtered to remove cell debris and precipitates in cold methanol or casted onto cleaning glass Petri disk. The PHA product was dried in hot air oven at 60 °C over night. The PHA was characterized by nuclear magnetic resonance (NMR) analysis to confirm the structure of PHA meanwhile thermal properties were measured by differential scanning calorimetry (DSC) and thermal degradation of PHA using thermal gravimetric analysis (TGA).

2.2.7 Thermal properties of extracted PHAs

The extracted PHA was analyzed in both differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA) compared to the standard of biopolymer in term of poly- β -hydroxybutyrate (PHB) (Sigma-Aldrich, USA). It was also remarked that the PHB is mostly found in PHAs. The DSC was performed using 10 mg samples under N₂ atmosphere, using the following temperature program: heating temperature (-20 to 200 °C) (10°C/min), cooling temperature (200 to 20 °C) (10°C/min) and a second heating (-20 to 200 °C) (10°C/min) (Xu, Luo, Wu, Xu, & Chen, 2006). The phase transition temperatures were reported as glass transition temperature (T_g) and melting temperature (T_m). Thermal gravimetric analysis (TGA) was performed using a TGA instrument (Mettler-Toledo, TGA/SDTA 851, USA) calibrated with indium. The temperature was ramped at a heating rate of 10 °C/min under nitrogen, to a temperature well above the degradation temperature of the polymers (Modi, Koelling, & Vodovotz, 2011).

2.3 Results and discussion

2.3.1 Isolation of soil microbes and PHAs observation

Accumulation of PHA granules in the microbial cells was preliminary analyzed by Sudan black B method. Among 72 isolates, 33 isolates can grow on sucrose agar medium and were further observed for PHA accumulated in their cells under microscopic technique. In this case the pure bacterial strain of *Alcaligenes eutrophus* TISTR 1095 was used as a reference positive strain for PHAs accumulation. In Figure 2.1, the results revealed that, only 3 isolated strains coded as V22, V29 and V33 showed positive results after staining by Sudan black B and small black colour represented PHAs granules in microbial cell. The Isolate strain V33 clearly showed in strong black colour than others. Hence, only the strain V33 was further used. Then, the PHAs producing microbes was confirmed by staining method using Nile blue A. The results are shown in Figure 2.2, when the 0.5 µg/ mL Nile blue A was directly added into mineral salt medium and incubated for 3 days. The culture plate contained of the isolated V33 and two reference strains (PHAs-positive strain of *A. eutrophus* TISTR 1095 and PHAs-negative strain of *E. coli*) were observed PHAs intact in bacterial colony under UV light at 312 nm. Colonies from the portion plate of

A. eutrophus TISTR 1095 and the isolate V33 clearly exhibited a very strong fluorescence caused by the presence of the PHAs in their cell which can be blinded by Nile blue A. In addition, the isolate V33 was defined as Gram negative bacteria having rod-shaped cell and yellow pigment (See Figure 2.2 right). This observation is in agreement with previous studied of Ostle and Holt (1982) who reported that Nile blue A could diffuse to only PHAs granules while other inclusion bodies (such as glycogen and polyphosphate) do not stained with this dye. However, under the same agar plate, *E. coli* showed in a weak for fluorescence light because this microorganism, which originally had no PHAs accumulation capability (Miyake, Miyamoto, Schnackenberg, Kurane, & Asada, 2000).

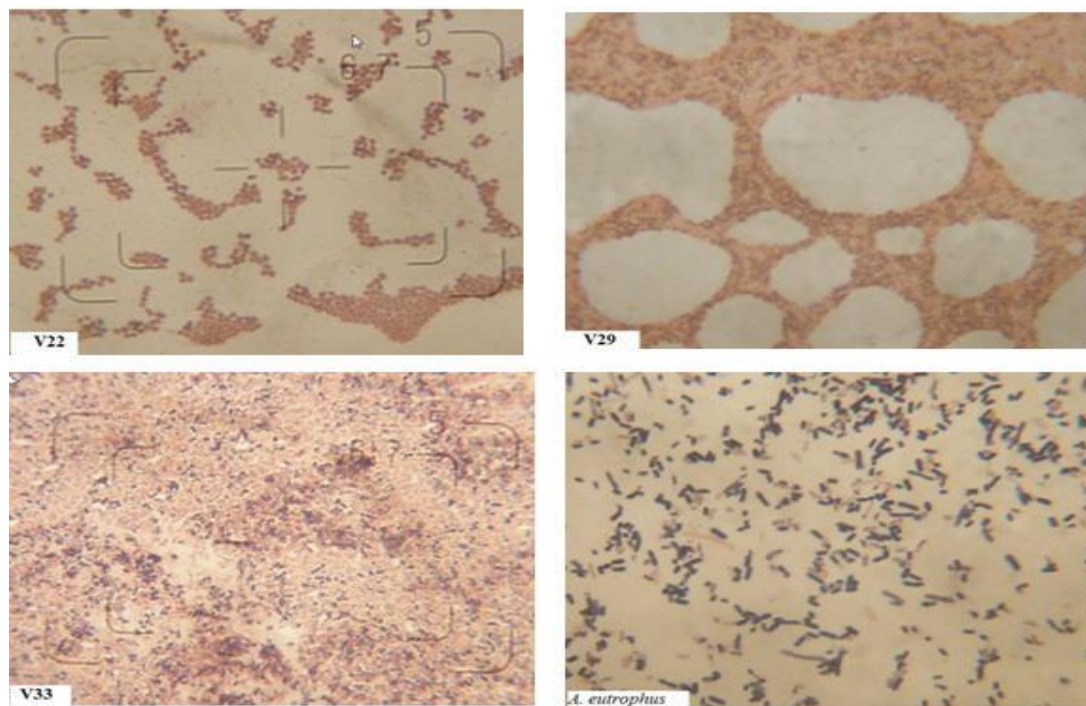


Figure 2.1 Micrographs of bacteria containing PHA inclusions

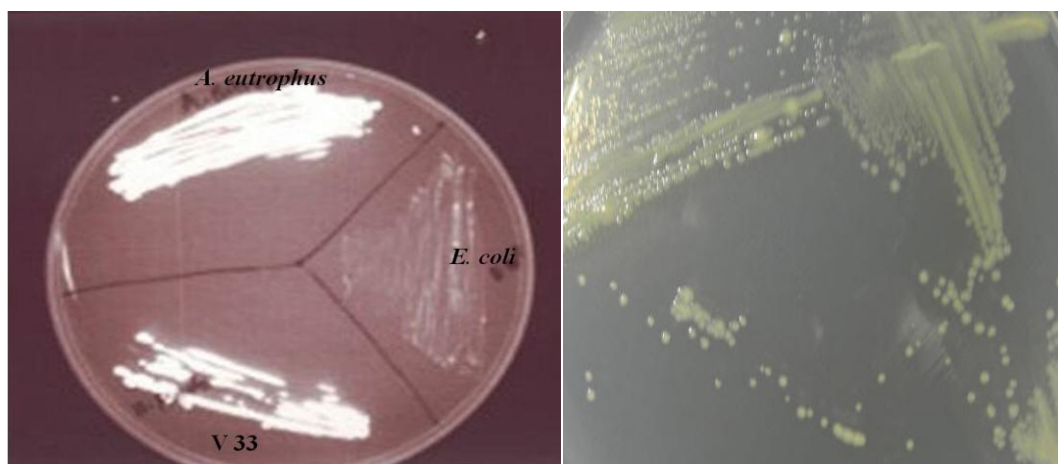


Figure 2.2 (Left) *A. eutrophus*, the strain V33 and *E. coli*; (right) a yellow bright colony of the isolate V33

2.3.2 Identification of the isolated strain

The isolated V33 was identified using the sequences of partial 16S rDNA technique and compared to those available in the public databases. It was found that V33 was closely related to that bacterial strain of *Hydrogenophaga* sp. (99% identify). The phylogeny based on these partial 16S rDNA sequences and related *Hydrogenophaga* sp. is shown in Figure 2.3. Previous studies have reported that *Hydrogenophaga* sp. was isolated from soil, mud, and water by enrichment for hydrogen bacteria. The strain was gram-negative, rod-shaped cell and yellow pigmented hydrogen-oxidizing bacteria (Willem, Busse, Goor, Pot, Falsen, Janzent, et al., 1989). They can grow and produce PHAs from various substrates such as glucose, galactose, xylose, arabinose (Choi, Song, & Yoon, 1995) whey lactose (Koller, Bona, Chiellini, Fernandes, Horvat, Kutschera, et al., 2008), fructose (Mahmoudi, Sharifzadeh-Baei, Najafpour, Tabandeh, & Eisazadeh, 2010) and recently in sucrose.

2.3.3 Batch fermentation of sucrose medium

The *Hydrogenophaga* sp. was cultured in sucrose medium and its growth was monitored. In Figure 2.4, it was found that the exponential phase appeared after 6 h until 36 h then the cell reached to stationary phase. The PHAs were produced and accumulated in its granules after 30 h and maximum PHAs production was obtained at 2.41 g/L within 42 h of batch fermentation. However, PHAs

concentration was decreased after 48 h because this strain had an intracellular depolymerase enzyme and can utilise PHAs for its growth (Yoon & Choi, 1999). According to the results obtained, we could observe PHAs accumulated by the *Hydrogenophaga* sp. by using Sudan black B staining during different fermentation times (See Figure 2.5). The results indicated that, PHAs was accumulated during mid-log phase (30 h) to stationary phase (48 h) due to carbon and nitrogen appeared to imbalance condition in fermentation media. However, microbial cell containing PHAs showed a strong black colour at 42 h of fermentation. In case of the sucrose uptake in culture medium showed at 14.73 g within 48 h. The highest PHAs inclusions in microbial cell was 68.15% ($g_{\text{PHA}}/g_{\text{DCW}}$) giving a maximum PHAs yield ($Y_{\text{P/S}}$) of 0.17 ($g_{\text{PHA}}/g_{\text{Sucrose}}$) and a volumetric productivity for PHAs amounted to 0.057 $g_{\text{PHA}}/L.h.$

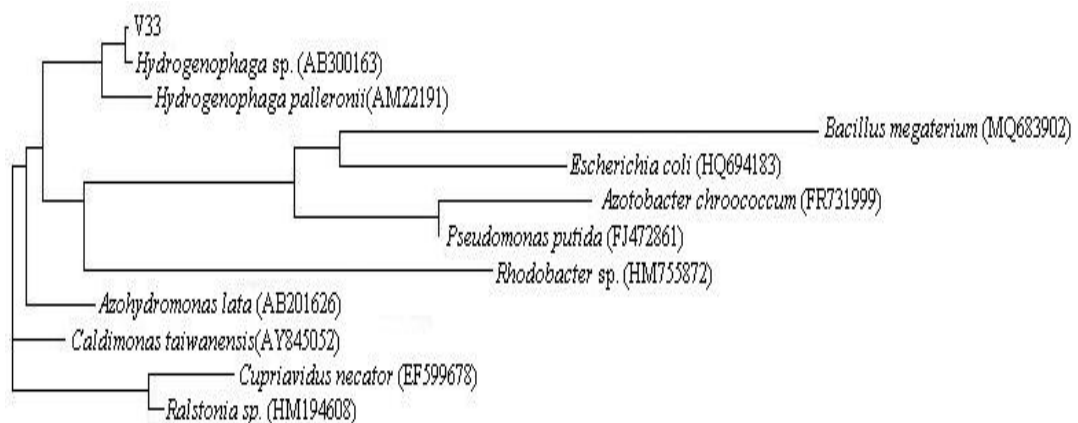


Figure 2.3 Phylogenetic analysis of 16S rDNA sequences of the isolate V33 and other bacteria

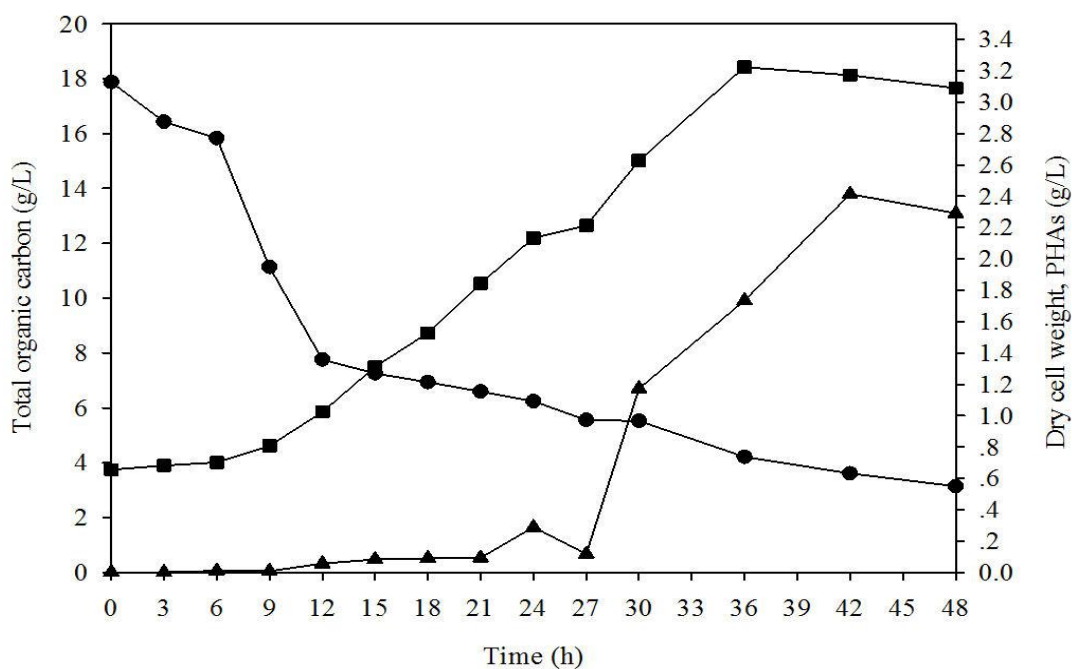


Figure 2.4 Profiles of TOC, DCW and PHAs as functions of time during batch fermentation (●—Total organic carbon (TOC), ■—Dry cell weight (DCW) ▲—PHAs)

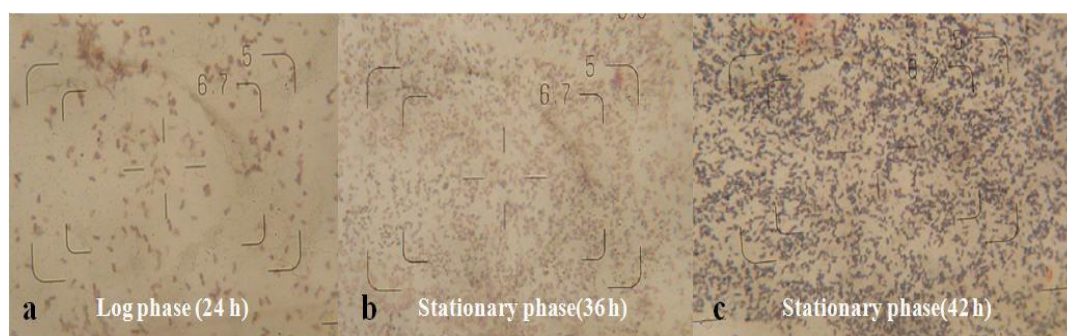


Figure 2.5 PHA inclusions in *Hydrogenophaga* sp. cell after staining with Sudan black B under different culture times. a: Log phase, 24 h; b: Stationary phase, 36 h; c: Stationary phase, 42 h

2.3.4 Characterisation of PHAs

One gram of PHAs produced from *Hydrogenophaga* sp. was extracted by hot chloroform and then was casted on clean Petri disk and oven-dried in

circulating air at 50 °C for 2 h (Pachekoski, Agnelli, & Belem, 2009) and finally a thin PHAs film was obtained and illustrated in Figure 2.6. The PHAs film was further characterized using $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ techniques. The spectrum of $^1\text{H-NMR}$ obtained was as shown in Figure 2.7a. Three groups of signals peak at 1.25, 2.52 and 5.23 ppm were found corresponding to CH_3 , CH_2 and CH , respectively. Meanwhile, the $^{13}\text{C-NMR}$ of PHA showed the chemical shifts of 19.72 (CH_3), 40.75 (CH_2), 67.58 (CH), and 169.10 (CO), respectively (See Figure 2.7b). The results obtained in both techniques are in good agreement with previous works (Yoon & Choi, 1999; Pachekoski et al., 2009; Jan, Roblot, Courtois, & Courtois, 1996) and identified the PHA extracted from *Hydrogenophaga* sp. as polyhydroxybutyrate (PHB) or polyhydroxybutyric acid.

For the DSC and TGA results of the T_m (173.31 °C) and the enthalpy of PHAs fusion (87.73 J/g) are shown in Figure 2.8. They showed in similarly matched with the data obtained both from standard PHB (176.29 °C and 86.49 J/g) and from other works (Yezza et al., 2007; Pachekoski et al., 2009). All data were summarised in Table 2.1. However, it seems that the T_m of PHAs was slightly lower than the value from the standard PHB. It may improve a widen PHAs processing window and make the process become easier. In case of the T_g values, they were closely matched between the standard PHB and the PHAs. High melting enthalpy was obtained from both cases. This can be explained that because of a high crystalline structure calculated to be 59.24% and 60.09% found in the standard PHB and PHAs. Properties of a high T_m and a high crystalline nature indicate in a high degree of polymerization. Hence, the PHB could be suitable for commercial utilisation (Luengo et al., 2003; Jan et al., 1996; Redzwan, Gan, & Tan, 1997). For thermogravimetric analyses of the PHAs produced by *Hydrogenophaga* sp. compared to the standard PHB are shown in Figure 2.9. The PHAs gave a rapid thermal degradation between 210 and 280 °C while the standard PHB represented at between 255 and 293 °C. It is indicated that biopolymeric material obtained can possibly be further used in a large scale processing of bioplastic (Koller et al., 2008).



Figure 2.6 A casted-film PHAs obtained from *Hydrogenophaga* sp.

Table 2.1 The summarised data from differential scanning calorimeter (DSC) analysis of PHAs samples

| Samples | T _g (°C) | T _m (°C) | Δ H (J/g) | Crystallinity (%) |
|------------------------------------|---------------------|---------------------|-----------|-------------------|
| Standard PHB | 2.81 | 176.29 | 86.49 | 59.24 |
| PHA from <i>Hydrogenophaga</i> sp. | 2.32 | 173.31 | 87.73 | 60.09 |

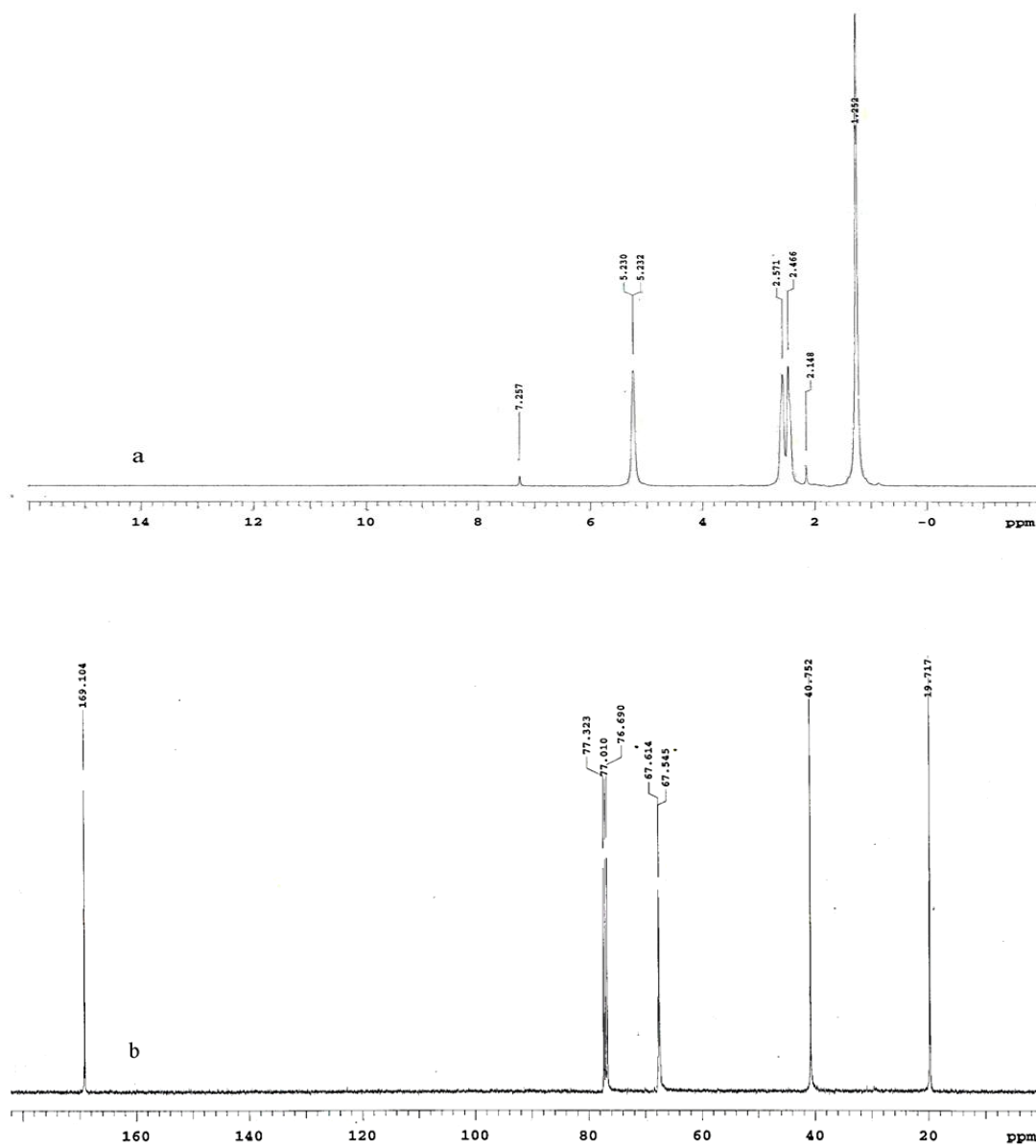


Figure 2.7 a: ^1H -NMR spectrum; b: ^{13}C -NMR spectrum of the extracted PHAs

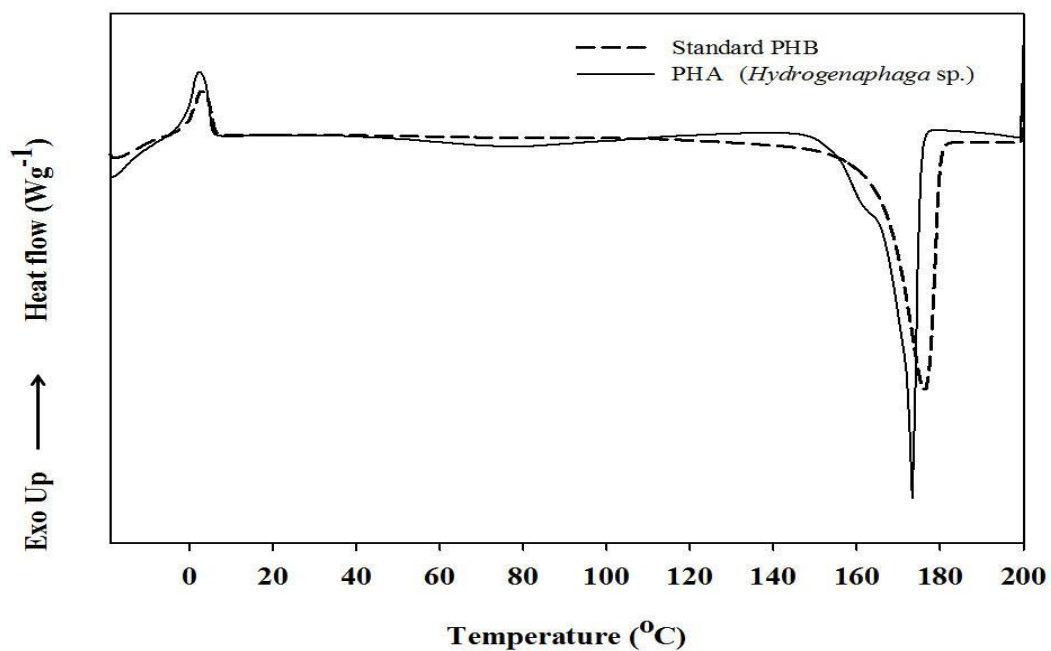


Figure 2.8 The DSC thermogram of the standard PHB and PHAs from *Hydrogenophaga* sp.

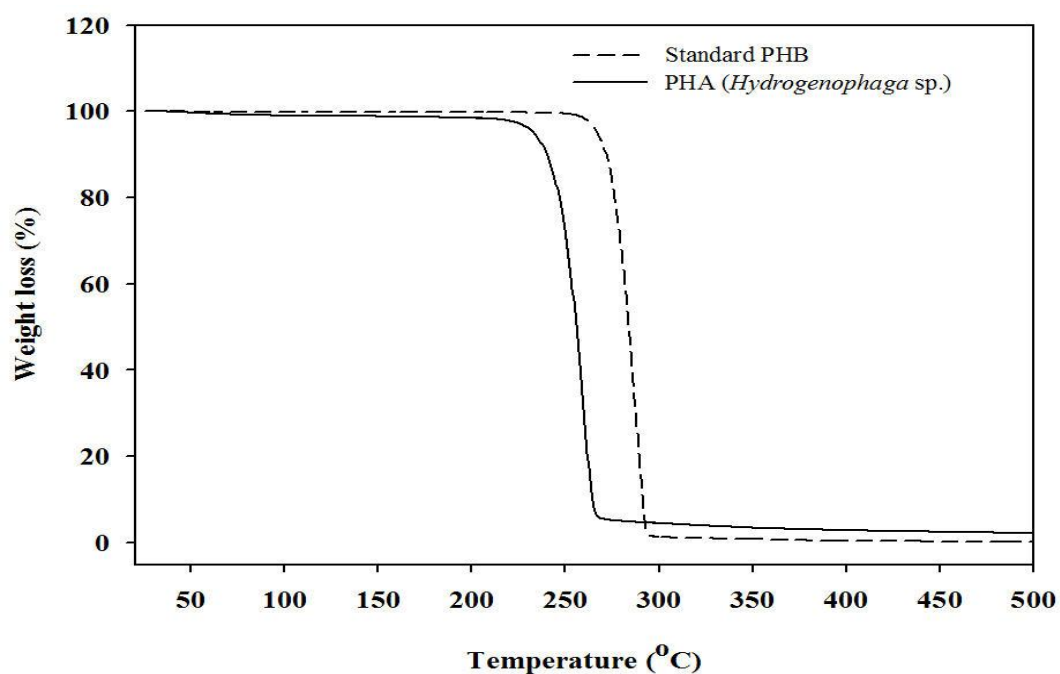


Figure 2.9 Thermogravimetric analysis of the standard PHB and PHAs from *Hydrogenophaga* sp.

2.4 Conclusion

An isolated soil bacteria identified as *Hydrogenophaga* sp. showed successfully for utilising several refined sugars especially sucrose. It has proven in its potential to produce PHAs in a high content (68.15% w/w) when sucrose medium was used as a sole carbon source and giving maximum PHAs yield ($Y_{P/S}$) of 0.17 ($\text{g}_{\text{PHA}}/\text{g}_{\text{Sucrose}}$) and a productivity of 0.057 $\text{g}_{\text{PHAs}}/\text{L.h}$. Further study will be fulfilled on the possibility of *Hydrogenophaga* sp. to use directly mixed sugars obtained from agricultural crops such as sugar cane and sweet sorghum as sole carbon sources for production of PHAs by using media optimisation technique via other fermentations of fed-batch, repeated batch, and repeated fed batch processes.

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