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Characterization and Molecular Study on Polyhydroxybutyrate Produced from *Rhodococcus equi*

A Dissertation

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SUMMARY

Many soil samples were collected from different places and screened for PHA production by Sudan Black B stain. Twenty isolates had a positive result for Suddan Black B stain. The results were confirmed by staining the viable colonies with the selective stain Nile Red; three isolates gave positive results. One isolate with a more pink fluorescent with Nile Red stain was selected for the present study. This isolate was characterized and identified by biochemical tests as *Rhodococcus* then the identification was confirmed by 16S rRNA and by blasting the sequence of 16S rRNA to NCBI database, the blast results showed 99% identity to *Rhodococcus equi*.

A one-stage cultivation method was utilized for studying PHA biosynthesis from *R. equi* using various carbon sources. The composition and the content of biopolymer were quantified by gas chromatography (GC). Besides, Crud Palm Kernel Oil (CPKO 1%) was the effective carbon for PHB production. Cell dry weight was 1.43 g/L and PHB content =38.07 wt%.

Nuclear Magnetic Resonance (NMR) analysis confirmed the chemical structure of extracted biopolymer as PHB homopolymer. Moreover the thermal properties of PHB were characterized to include the melting temperature (T_m) and the glass transition temperature (T_g) that reached to 173 °C and 2.79 °C respectively as determined by differential scanning calorimetry analysis (DSC), while the decomposition temperature (T_d) was 276 °C as determined by thermogravimetric analysis (TGA). The average molecular weight ($\overline{M}_w$) was 642 KD, weight number average mass ($\overline{M}_n$) was 373 KD and polydispersity ($\overline{M}_w/\overline{M}_n$) was 1.72 as determined by the gel permeation chromatography (GPC).

The phase contrast light microscope was used to viewing *R. equi* during PHB production which was characterized by a bright appearance that

was increased with time, while fluorescent microscope using Nile Blue A stain illustrated the bacterial cells under best production conditions with the bright orange fluorescent that indicated PHB accumulation inside cells.

Transmission Electron Microscope (TEM) images revealed the presence of PHB granules inside the bacterial cells with white, spherical, ovoid and elongated shape. These granules had different sizes and different numbers about (3-11) granules per cell.

The *PhaC* gene encoded the synthase enzyme which was considered as the key enzyme for PHA biosynthesis, G-D and G-1R primers were used for the amplification of *PhaC* gene by PCR with a size of 508 bp. The sequence of this gene blast to NCBI database and the results demonstrated 78% similarity to *PhaC* gene sequence of *Rhodococcus aetherivorans* (accession no. CP011341.1). On the other hand, real time PCR technique was used for the evaluation of *PhaC* gene expression level considering *gyrB* as a reference gene depending on log phase as a control. It was concluded that the expression level of *PhaC* gene at lag phase was ~ 1.16 folds, while at the stationary phase was ~2 folds. The PHA synthase enzyme for *R. equi* was also characterized with optimized production conditions and evaluated with activity 30.83 (U/mg protein).

Different formulations of PHB films were prepared to study *in vivo* degradation in soil for 6 weeks which included: PHB films, PHB-TiO_2 composite films, PHB nanofiber films, PHB-TiO_2 composite nanofiber films and P(3HB) films, PHB-TiO_2 composite films prepared from ultraviolet light (UV) treatment of PHB sheets. The results of degradation experiments revealed the significant decrease in molecular weight (Mw), number-average molecular

weight (Mn) and the polydispersity (Mw/Mn) for all films. The weight loss percentage was carried out as a function of degradation which increased with time, but nanofibers of PHB and its composites showed a faster degradation when compared to other films and completely degraded after 3 weeks.

The microbial populations in soil at the burying site were increased continuously at each week. Crack was the major physical changes of all films at each week of degradation experiment. SEM micrographs showed the surface morphology of different PHB film forms before and after biodegradation experiment, which took after 6 weeks. SEM micrographs illustrated various surface changes that included pores, cavities, grooves, incisions, slots and pointers for the growth of microorganisms that secrete PHA depolymerase enzyme which on the whole caused the degradation of all biopolymer films. Nanofibers of PHB and its composites in presence of TiO_2 were demonstrating more surface changes with the rupture of most of the nanofibers and ultimately droping the diameter from 500 nm to 400 nm for PHB nanofiber and from 550 nm to 450 nm for PHB-TiO_2 composite nanofiber films. UV treatment for the PHB sheets and used it in preparing the PHB films and their composite with TiO_2 caused the increasing of biodegradation in compare to the similar one in the absence of UV treatment.

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List of Abbreviations

BSA	Bovine serum albumin
CDW	Cell dry weight
$CDCl_3$	Deuterated chloroform

$CHCl_3$	Chloroform
CME	Caprylate methyl ester
CPKO	Crude palm kernel oil
(CPKO+S. V)	CPKO+ Sodium Valerate
CPO	Crude palm oil
C_T	Threshold cycle
DMF	Dimethylfomamide
DSC	Differential Scanning Calorimetry analysis
DTNB	1- 5, 5,-dithiobis-92-nitrobenzoic acid
GMOs	Genetically-modified organisms
GC	Gas Chromatography
(glucose+S.V.)	Glucose+Sodium Valerate
GPC	Gel Permeation Chromatography analysis
MM	Mineral medium
Mn	Number-average molecular weight
Mw	Molecular weight
Mw/Mn	Polydispersity
NMR	Nuclear Magnetic Resonance
NPCM	Non-PHA cell mass
OD	Optical density
OsO_4	Osmium tetroxide
PAO	Palm acid oil
PHA	Polyhydroxalkanoate
PhaA	ß-ketothiolase
PhaB	NADPH-dependent acetoacetyl-CoA reductase
PHB	Polyhydroxybutyrae
PhaC	PHA synthase enzyme
PFAD	Palm fatty acid distillate
PKAO	Kernel acid oil
PO	Palm olein
PS	Palm stearin
SDS	Sodium dodecyl sulfate
SEM	Scan electron microscope
TBE	Tris-borate-EDTA
TCA	Trichloroacetic acid
*T*d	Decomposition temperature
TEM	Transmission Electron Microscope
*T*g	Glass transition temperature
TGA	Thermogravimetric analysis
Tm	Melting temperature

TSB	Tryptone soya broth
WCO	Waste cooking oil

Chapter One

Introduction and Literature Review

1.1. Introduction

Polyhydroxyalkanoates (PHAs) are a microbial polyester that is completely biodegradable plastics and received a considerable interest from researchers because of their similar material properties to conventional plastics and complete biodegradability (Sudesh, 2013). Many types of PHA with different properties have been reported depending on the chemical structure that depended on the produced organisms and growth conditions. Over 150 different PHA monomers being reported, PHA with flexible thermal and mechanical properties have been developed (He *et al.,* 1999) utilize the renewable sources and have many applications in medicine, pharmacy, packages etc. (Bugnicourt *et al*., 2014).

However, the most common forms found in microbial cells are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). They can be made into plastic materials with properties that are similar to petrochemical plastics and can replace these materials in many applications (Chen, 2009).

PHB has many valuable properties such as moisture resistance, water insolubility and optical purity. This distinguishes PHB from other currently available biodegradable plastics which are either water soluble or moisture sensitive. PHB also shows a good oxygen impermeability (Lindsay, 1992; Holmes, 1998). PHB is the most widely recognized PHA and was firstly found in microbes in 1925 by Lemoigne (Dawes and Senior, 1973), it is a linear polyester of D(-)-3hydroxybutyric acid which aggregated as intracellular granules by a wide groups of gram positive and gram negative bacteria when concentrations of supplements such as nitrogen, phosphorous or oxygen are limiting growth (Dawes, 1988; Dio, 1990). At least 75 different genera of bacteria have been known to accumulate PHB (Aysel, *et al*., 2002), for example, *Ralstonia eutropha* (Chakraborty, *et al*., 2009), *Pseudomonas putida* (Castro *et al*., 2013) and *Bacillus subtilis* (Salhiyanarayanan *et al*., 2013).

The limited number of studies about the production of PHA from *Rhodococcus* sp., Hori and co-workers were identified the ability of *Rhodococcus aetherivorans* IAR1 to produce PHBV from toluene without supplying a secondary carbon source as the precursor (Hori *et al*., 2009). Gram positive bacteria are more interesting than gram negative for PHAs production because the lack of lipopolysaccharide (LPS) that caused immunogenic reactions which gave the gram positive bacteria a specific character for the production of PHAs using a wide range of biomedical applications (Valappil *et al*., 2007). PHB is used as a raw material in several medical applications because it degraded to 3HB which is considered as a natural constituent of mammalian blood (Zinn *et al*., 2001; Vroman and Tighzert, 2009).

PHAs, particularly poly 3-hydroxybutyrate (PHB), are biocompatible and biodegradable with an increased interest in medical applications and they are used in manufacture suture, cardiovascular patches, stents, articular cartilage repair devices, nerve and tendon repair devices, wound dressings and used in artificial organs (Chen and Wu, 2005). PHB can be used as microcapsules in therapy or as materials for the cell and tablet packaging, food packaging applications, development the scaffolding material in tissue engineering (Güven *et al*., 2008; Bugnicour *et al*., 2014) and have a role in the delivery system of drugs (Wang *et al*., 2008; Brigham and Sinskey, 2012).

The increase of the non degradable waste materials in the environments results from using the petroleum polymer promote many countries to developing specific programs to the production of ecofriendly degradable materials and used certain strategies for the elimination of the waste from environment, therefor, PHAs production and degradation have interest of many researchers.

The present study aims at the following:

1- Isolating and identifying the suitable bacterial isolates for PHB Production.

2- Optimizing the conditions for PHB production utilizing various carbon sources.

3- Extracting and characterizing the produced PHB which included the structural and thermal characterization.

4- Qauntitative analysis of synthase gene (*PhaC* gene) by PCR and qualitative analysis of *PhaC* gene by real time PCR.

5- Evaluating the PHB synthase enzyme.

6- Studying the degradation of PHB films in different forms.

1.2. Literature Review

1.2.1. Chemical structure of polyhydroxalkanoates (PHAs)

PHAs are biopolymer consisting from carbon, oxygen and hydrogen as shown in Figure (1-1). R-3-hydroxyalkanoic acid and 4-hydroxyalkanoic acid monomer unit are the major building blocks of PHA. PHA molecule is typically made up of 600 to 35,000 (*R*)-hydroxy fatty acid monomer units (Shah, *et al.* 2008). Each monomer unit harbors a side chain *R* group which is usually a saturated alkyl group (Figure 1.1) but can also take the form of unsaturated alkyl groups, branched alkyl groups, and substituted alkyl groups although these forms are less common (Khanna and Srivastava, 2005).

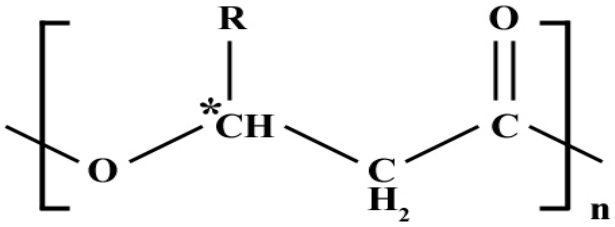
PHAs are divided into three groups depending on the number of carbon atoms within each monomer (Lu, *et al.* 2009):

A– PHAs having 3 to 5 carbon atoms are named short-chain length PHAs (SCL-PHA).

B – PHAs having 6 to14 carbon atoms are named medium-chain length (MCL-PHA).

C– PHAs having 15 or more carbon atoms are named long-chain length PHA (LCL-PHA).

About 150 different PHA monomers have been identified and this number keeps on expanding with the presenting of new types of PHA through the chemical or physical modification of naturally-occurring PHA, or through the creation of genetically-modified organisms (GMOs) to produce PHA with specialized functional groups (Zinn and Hany, 2005).



Poly(3-hydroxyalkanoate)

R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure (1-1): Polyhydroxyalkanoate (PHA) chemical structure. The nonmenclatur and carbon number for PHA compounds is determined by the functional alkyl *R* group. An asterisk denotes chiral center of PHA-building block (Khanna and Srivastava, 2005).

1.2.2. Properties of polyhydroxyalkanoates/ polyhydroxybutyrate

PHAs have been the subject of numerous studies because of its distinguished features that include: water insoluble and relatively resistant to hydrolytic degradation, good ultra-violet resistance but poor resistance to acids and bases, soluble in chloroform and other chlorinated hydrocarbons, biocompatible and hence suitable for medical applications, sinks in water, facilitating its anaerobic biodegradation in sediments, nontoxic, less 'sticky' than traditional polymers when melted (Bugnicourt *et al*., 2014). The PHAs and its properties in comparison with the major synthetic polymers are listed in Table (1-1).

The molecular weight of PHB differs according to organism, growth condition and extraction method, and it can vary from 50,000 to over a million. This polymer has important properties of thermoplasticity and biodegradability and has a considerable commercial interest (Dawes, 1988). PHB is a highly crystalline thermoplastic polymer with a relatively melting temperature (170-180 °C) and glass transition temperature (0-5 °C) (Ha and Cho, 2002).

The thermal properties of PHB can be proved by grafting suitable monomers onto PHB to increase thermal stability of PHB without need the conventional stabilizers or antioxidants used in stabilizing commodity thermoplastics (Yamaguchi and Arakawa, 2006). It can improve the thermal stability of PHB by grafting maleic acid (MA) to PHB by different methods resulting in the good improvement in the crystallization and degradation behaviors of PHB (Hong and Lin, 2010), while another study used a pomac extract (EP) to improve the thermal stability and mechanical properties of PHB (Persico *et al.*, 2014) and many other reports related to this field.

Table (1-1): comparison of PHAs biopolymers and synthetic polymers properties. (Doi *et al.*, 1995; Sudesh *et al.*, 2000; Aoyagi *et al.*, 2003; Averous, 2004; Ashby *et al.*, 2005; Tanadchangsaeng *et al.*, 2009).

Property	*Tm* (°C)	*Tg* (°C)	Crystallinity (%)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation at break (%)
Bio-based polymers:						
P(3HB)	175–180	4	70	3.5	43	8-3
UHMW-P(3HB)	182	4	80	097	175	104
P(3HB-*co*-10% 3HV)	162	-1	59	0.8	20	50
P(3HB-*co*-20% 3HV)	135	-2	58	1.2	32	100-50
P(3HB-*co*-16% 4HB)	152	-8	45	NA	26	444
P(3HB-*co*-10% 3HHx)	127	-1	34	0.52	21	400
P(3HB-*co*-14% 3H4MV)	136	-2	32	0.55	17	230
mcl-PHA	43	-4	NA	0.001	4.7	335
PLLA	152	58	1-0	2.05	NA	9
PBS	114	-28	41	0.25	45	470
PCL	65	-61	67	0.19	14	500
Synthetic polymers:						
PS	240	90	NA	3.2	36	2
PP	168	-20	60	1.3	36	350
PP HDPE	133	-8	NA	0.2	17	680
LDPE	123	-36	30	0.4	20	620
PVC	160-300	110	NA	3.2	46	60
PET	260	75	NA	2.5	56-70	73-100

(T_m): melting temperature.
(T_g): glass-transition temperature.
NA: not available.

Abbreviation: (3HB) 3-hydroxybutyrate; (UHMW) ultra-high-molecular-weight; (3HV) 3-hydroxyvalerate; (3HP) 3-hydroxyvalerate; (4HB) 4-hydroxybutyrate; (3HHx) 3-hydroxyhexanoate; (3H4MV) 3-hydroxy-4-methylvalerate; (PLLA) poly(L-lactic acid); PBS, polybutylene succinate; (PCL) polycaprolactone; (PS) polystyrene; (PP) polypropylene; (HDPE) high-density polyethylene; (LDPE) low-density polyethylene; (PVC) polyvinyl chloride; (PET) polyethylene terephthalate.

1.2.3. Extraction of polyhydroxyalkanoates

Several extraction methods have been developed which involved cell wall or cell membrane disruption. The extraction operations are preferably done with a low cost and the use of non-harmful and environmentally friendly chemicals. Solvent extraction was the oldest and most common method with high purity using several chlorinated hydrocarbon solvent like: chloroform, 1,2-dichloroethane, methylene chloride or some cyclic carbonates like propylene and ethylene carbonates. In some cases, a mixture of solvents like (chloroform/methanol) and (dichloromethane/ethanol) is used (Baptist, 1962). The use of solvents destroys the natural morphology of PHA granules that is useful in certain applications such as the production of strong fibers (Barham, 1990). Another problem connected with the use of solvents is that it creates hazards for the operators and for the environment (Gorenflo, 2001). The extraction with chemical solvents is efficient to remove endotoxins which appear with gram negative bacteria causing an inflammatory response in medical applications (Tan *et al.*, 2014).

Digestion methods involve chemical digestion and enzymatic digestion. The chemicals used are surfactants such as anionic sodium dodecyl sulfate (SDS), sodium hypochlorite, chelate-hydrogen peroxide treatment, and a new method by dissolving of non-PHA cell mass (NPCM) by protons in aqueous solution. Some time, the chelate is used with surfactants to form a complex with divalent cations of the outer membrane of gram negative bacteria causing the weakness of membrane and helping in disruption of cells.The new method suggested to use chloroform with sodium hypochlorite to reduce the polymer degradation. Another method used surfactant with sodium hypochlorite to reduce the cost (Jacquel *et al.*, 2008).

An enzymatic digestion method using several enzymes pancreatin, bromelian, trypsin purity 88–90% has characterized with good recovery but high costs of enzymes (Kapritchkoff *et al.*, 2006).

In mechanical disruption, there are methods used for PHA recovery without chemical treatment like bead mill disruption that require several passes, high pressure homogenization featuring with the poor disruption rate for low biomass levels, disruption by using ultrasonication that some time used also before chemical treatment with chemicals and centrifugation combined with the chemical also used for microbial PHA extraction (Jacquel *et al.*, 2008).

There are several developed methods in progress including supercritical CO_2 featured with a low cost and toxicity low recovery, the use of cell fragility featured with bacterial cells highly accumulated of granules, air classification with high purity and low recover, dissolved air flotation requires several consecutive flotation steps and a spontaneous liberation with a low recovery (Anis *et al.*, 2013).

1.2.4. Screening for polyhydroxyalkanoates production

Various methods have been suggested to detect the accumulation of PHA granules inside bacteria. The phenotypic methods depend on staining of viable colonies or cells after growing on mineral salt to allow the accumulation of PHA granules. Sudan black stain used for primary screening of bacterial cells for PHA accumulation and granules appeared as black with a pinkish background (Singh, 2014). Nile Blue A and Nile Red are alternative stains that have the ability to diffuse into microbial cytoplasm causing fluorescent of viable colonies or granules inside cells when illuminated with UV light or examined by fluorescent light microscope and the fluorescent intensity increased when the accumulated granules inside cells increased (Ostle and Holt, 1982; Amirul *et al*., 2009; Bhuwal *et al*., 2013).

The molecular detection method is used to confirm the results of phenotypic methods by designing primers of *PhaC* gene which encodes of PHA synthase and amplified by PCR. This method is potential to identifying microbes that produce PHA by the detecting synthase gene (Solaiman and Ashby, 2005).

The primers I-179L (forward) 5'-ACAGATCAACAAGTTCTACATCTT CGAC-3' and I-179R (reverse): 5'-GGTGTTGTCGTTGTTCCAGTAGAGGA TGTC-3' were used to detect the PHA positive bacterial isolate from different environment through detecting PHA synthase gene (Khan *et.al*, 2013, Cirstea *et al.*, 2012). Sheu and co-workers resulted that the following primers: (*phaC*F1) 5'-ATCAACAGGGATTTACTACAAGTCCCTTCCGGACT-3', (*phaC*F2) 5'-G TCCCGGTTGGGAATGGGCCAAAGGTCCGTTACCCGGCTGGCGCCCC-3', (*phaC*R4) 5'-AGGTAGTTGTTTCGACCCCGGAAACCAAACGGGATAG TTTGTCCA-3' were suitable for detecting *PhaC* gene in gram positive and gram negative bacteria (Sheu *et al.*, 2000).

1.2.5. Biosynthesis of polyhydroxyalkanoates/ polyhydroxybutyrate

PHAs are structurally simple macromolecules intracellularly accumulated inside bacteria under unbalanced environments such as nitrogen and phosphate oxygen or magnesium limitation with excess of carbon source and polymerizing soluble intermediates into insoluble molecules to energy storage molecules (Sudesh *et al.*, 2000).

Chen and co-workers (1991) revealed that PHA biosynthesis is a complex process controlled by several enzymes catalyzing different metabolic pathways. The PHA biosynthesis pathway requires three enzymatic activities [Figure (1-2)]: namely ß-ketothiolase, acetoacetyl-Coenzyme A (CoA) reductase and PHA synthase encoded by *phaA, phaB* and *phaC* respectively. The first step involves the condensation of two acetyl-CoA to form acetoacetyl-CoA catalyzed by the

enzyme ß-ketothiolase (PhaA). This is followed by a reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (PhaB). The last step is catalyzed by the enzyme PHA synthase (*PhaC*) and it involves polymerization of (*R*)-3-hydroxybutyryl-CoA monomer into PHB with a release of a free CoA molecule (Lee, 1996; Stubbe *et al*., 2005).

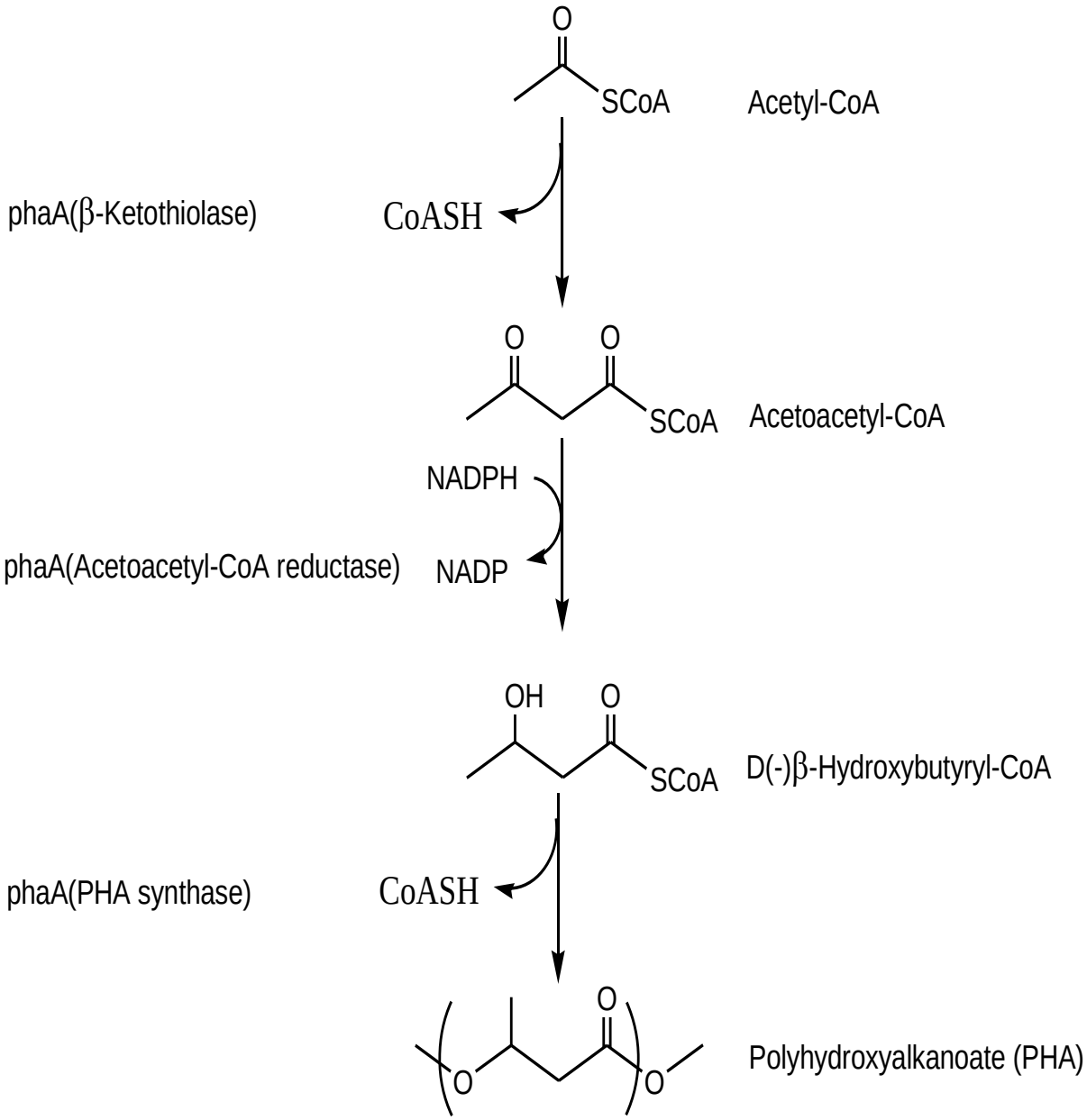


Figure (1-2): Pathway for PHA production by *A. eutrophu* (Kranz *et al*., 1997).

For the synthesis of PHAs composed of 3-hydroxyalkanoic acids of C6–C16, the hydroxyacyl-CoA precursors are derived from fatty acid metabolism (Figure 1-3). These precursors can be obtained either from ß-oxidation of alkanes, alkanols or alkanoic acids, mainly by an enantioselective enoyl-CoA hydratase (encoded by ***phaJ***) that produces the (R)-hydroxyacyl-CoA (Tsuge *et al*., 2003), or from fatty acid ***de novo*** biosynthesis using an (R)-3-hydroxyacylACP:CoA transacylase (encoded by ***phaG***) to produce the substrates for the PHA synthase from a nonrelated carbon source, such as carbohydrates (Matsumoto *et al*., 2001).

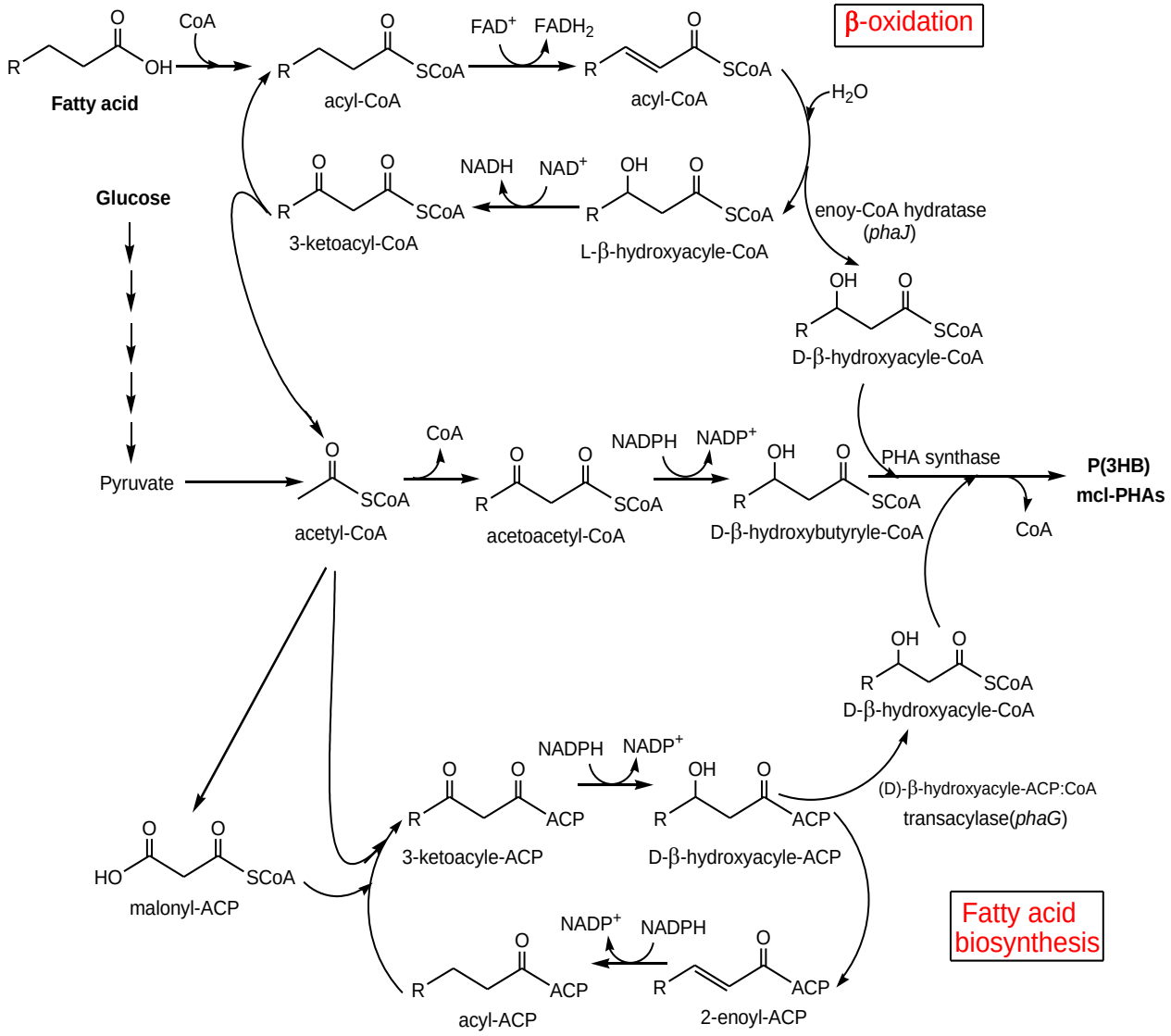


Figure (1-3): Metabolic pathways and genetics involved in the production of P(3HB) (Peña *et al*., 2014).

During the normal growth condition, ß-ketothiolase is inhibited by free CoA molecules coming out of the Krebs cycle. However, during the imbalance nutrient condition, the entry of acetyl-CoA into the Krebs cycle is restricted. The excess acetyl-CoA is channelled into PHB biosynthesis and resulted in the accumulation of PHAs (Ratledge and Kristiansen 2001; Verlinden *et al.*, 2007).

1.2.6. The key enzyme PHA synthase

PHA synthases are the key enzymes of PHA biosynthesis that catalyzed polymerization of 3-hydroxyacyl-CoA into PHAs with a concomitant release of CoA. According to its substrate specificity and subunit composition amino acid sequence, the PHA synthases are classified into four classes (Rehm and Steinbuchel, 1999; Nomura and Doi, 2006) as distinguished in Table (1-2). Generally, PHA synthases are bounded with other proteins to PHA granules surface during PHA accumulation (Haywood *et al*., 1989).

Class I and class II PHA synthases comprise enzymes consisting of only one type of subunit (*PhaC*) with molecular masses between 61 kDa and 73 kDa (Qi and Rehm, 2001). According to their *in vivo* and *in vitro* substrate specificity, class I PHA synthases (e.g. in *Ralstonia eutropha*) preferentially utilize CoA thioesters of various (*R*)-3hydroxy fatty acids comprising 3 to 5 carbon atoms, whereas class II PHA synthases (e.g. in *Pseudomonas aeruginosa*) preferentially utilize CoA thioester of various (*R*)-3-hydroxy fatty acids comprising 6 to 14 carbon atoms (Slater *et al.,* 1992; Amars and Rehm, 2003).

Class III PHA synthesis (e.g. in *Allochromatium vinosum*) comprise enzymes consisting of two different types of subunits: (i) the *PhaC* subunit (molecular mass of approx. 40 kDa) exhibiting amino acid sequence similarity of 21–28% to class I and II PHA synthases and (ii) the PhaE subunit (molecuar mass of approx. 40 kDa) with no similarity to PHA synthases. These PHA

synthases prefer CoA thioesters of (*R*)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms (Liebergesell *et al*., 1992; Yuan *et al*., 2001). Class IV PHA synthases (e.g. in *Bacillus megaterium*) resemble class III of PHA synthases, but PhaE is replaced by PhaR (molecular mass of approx. 20 kDa) (McCool and Cannon, 2001).

Table (1-2): PHA synthase classes as proposed by (Rehm, 2003).

Class	Subunits	Species	Substrate
I	PhaC ~60-73 kDa	*Cupriavidus necator*	$3HA_{SCL}$-CoA (~C3-C5) $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II	PhaC ~60-65 kDa	*Pseudomonas aeruginosa*	$3HA_{MCL}$-CoA (~ ≥C5)
III	PhaC ~40 kDa PhaE ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA ($3HA_{MCL}$-CoA [~C6-C8], 4HA-CoA,5HA-CoA)
IV	PhaC ~40 kDa PhaR ~22 kDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

1.2.7. The important renewable carbon sources for polyhydroxyalkanoates/ polyhydroxybutyrate production

Carbon source is the major factor which could reduce the cost of PHA production by selecting the cheap and feasible carbon source. Various renewable carbon sources are used for PHA biosynthesis. The substrate costs were approximately 28- 50% of the total production cost as reported by many previous studies (Lee and Choi, 1998; Braunegg *et al*., 2004).

The most common carbon sources for PHB biosynthesis are sugars such as glucose, fructose and sucrose (Borah *et al*., 2002). The previous study revealed the production of PHB using carbon dioxide (CO_2) as a carbon source by *Cupriavidus necator* (Mozumder *et al*., 2015), the methanol used as a carbon substrate for the accumulation of PHB by *Methylobacterium* (Braunegg, 1999), the molasses and glycerol have been utilized for the good production of PHB (Hiremath *et al.*, 2015).

Many scientists' efforts have been concentrated on using various industrial, agricultural, domestic and industrial effluent wastes as cheap carbon sources of microorganisms to accumulate PHAs to reduce the cost of production and overcoming the environment and pollution problems (Saharan *et al*., 2014). These include paper mill wastes (Bengtsson, 2008) dairy wastes (Bosco and Chiampo, 2010) distillery effluent oil mill wastes (Hassan *et al*., 2013). *Lactobacillus acidophilus* utilized agro-industrial wastes (date molasses) yielding 0.412g/50ml of PHB (Hamieh *et al.*, 2013). Adline and Praveen (2013) reported the ability of *Bacillus silvestris* to utilize the waste water yielding 0.021g/l of PHB.

Plant oils such as soybean oil, palm oil and corn oil are attractive carbon sources for PHA production as they are generally less expensive than most sugars. More cell biomass and PHA production are accumulating in the plant oil

culture (0.6-0.8 g of PHA per g of oil) as they contain a higher carbon content per weight contrasted with sugar (Akiyama *et al*., 2003).

Oil palm fruit is unique compared to other oil-producing crops as different types of oils could be extracted from the mesocarp and the kernel of the oil fruit, respectively. Crude palm oil (CPO) is the primary product obtained from the mesocarp while crude palm kernel oil (CPKO) is derived from the kernel. Further chemical and physical refining results in various palm oil products include, palm olein (PO), palm stearin (PS), refined, bleached and deodorized (RBD) palm oil, kernel olein, and kernel stearin as well as by-products such as palm kernel acid oil (PKAO), palm acid oil (PAO), and palm fatty acid distillate [PFAD] (Sudesh *et al*., 2011) (Figure**:** 1-4). CPKO is used as a sole of carbon by recombinant *C. necator* strain Re2160/pCB113 yielding 77(wt%) of P(3HB-co-3HHx) copolymer containing a high 3HHx monomer fraction (Wong *et al*, 2012).

Some bacteria secrete lipases when using plant oils as a source of carbon (DiRusso *et al*,. 1999). Surfactants are used to inhibit growth of bacteria or to stimulate their growth depending on the surfactants structure and bacterial species. Budde and co-workers evaluated the use of gum Arabic (GA) as an efficient emulsifier for growth of *Ralstonia eutropha* on plant oil to increase the production of PHB, but it does not consider the surfactant as an alternative of the carbon source for PHA biosynthesis (Budde *et al.*, 2011).

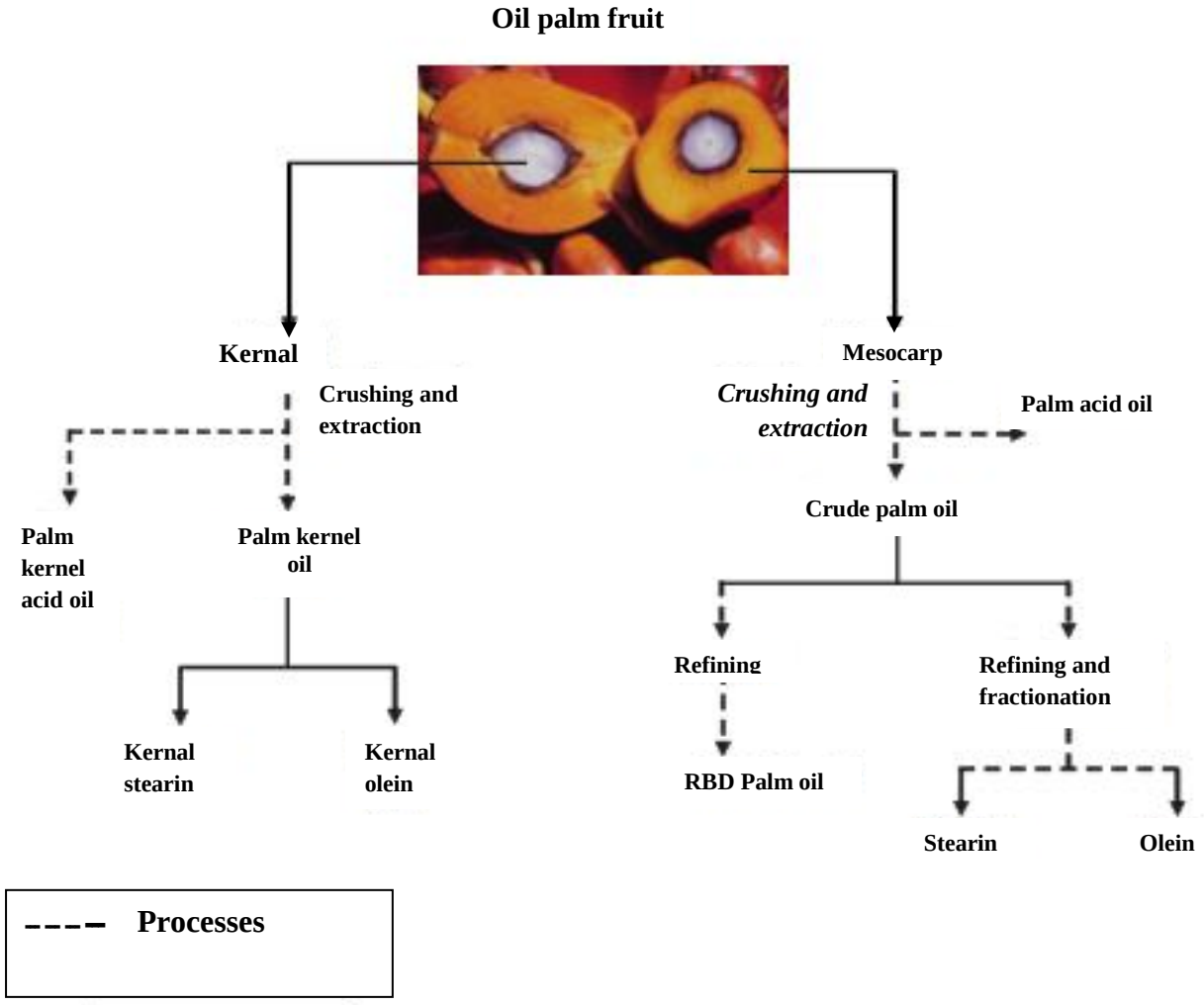


Figure (1-4): Cross-section of oil palm fruit with various oil fractions that extracting in a typical palm oil milling industry (Maycock, 1992).

1.2.8. The important polyhydroxybutyrate and other polyhydroxy‗ alkanoates producers

More than 250 species of prokaryotes are mainly bacteria having the ability for the biosynthesis and the accumulation of PHB and other PHAs under stressed environmental conditions with the availability of carbon sources as the energy sources. *Cupriavidus necator* (known as *Ralstonia eutropha* or *Alcaligenes eutrophus*) accumulated 80% of the cell dry weight and considered the most commonly used producer for PHAs. Besides, the mutant *C. necator* was used by Imperial Chemical Industries (ICI plc) for the production of PHBV

copolymer under the trade name of Biopol (Luzier, 1992). *Bacillus*, *Caryophanon*, *Microcystis*, *Corynebacterium*, *Clostridium*, *Microlunatus*, *Micrococcus, Nocardia*, *Rhodococcus*, *Staphylococcus*, *Streptomyces* are the main genera of gram positive bacteria which have the ability for PHAs productions (Lu *et al*., 2009). *Bacillus cereus* UW85 is able to produce PHB under the stressed environment but forming the spores; therefore, many attempts to evaluate nonsporing mutants of *Bacillus* can produce PHAs with overcome the spore forming (Labuzek and Radecka 2001). The genus *Rhodococcus* is a common aerobic Gram positive bacterium which has an important role in the bioremediation of polluted environments. Such bacteria can be used in various biotechnology applications as a result of their ability to degrade various organic compounds into less hazardous substances (Alvarez, 2010). Also, they have the ability to produce and accumulate triacylglycerol (TGA) by *Rhodococcus opacus* (Kurosawa *et al*., 2015) and glycogen by *Rhodococcus ruber* and *Rhodococcus equi* (Hernandez and Alvarez, 2010). There are few reports demonstrating the production of PHAs using several species of *Rhodococcus* such as *Rhodococcus fascians*, *Rhodococcus erythropolis* and *Rhodococcus opacus* (Alvarez *et al*., 1997; Fuchtenbusch and Steinbuchel, 1999). The bacterium *Rhodococcus rubber* also produces copolymer PHBV with high 3HVcontent from succinic acid as the carbon source (Williams *et al*., 1994).

Several gram negative bacteria were employed as efficient producers of PHAs. *Burkholderia*, *Azohydromonas*, *Cupriavidus* and *A. lata* (ATCC 29714) are the gram negative bacterial species that reported their ability to produce PHB in a range between 50% -88% of cell dry mass from various sugars including glucose, sucrose and fructose (Gome *et al*., 1996). The *pseudomonas* species are the major gram negatives bacteria for PHB production which had a

very high frequency (67%) in PHB production, and most of the strains with high productivity were placed in this group (Wang and Bakken, 1998). Thermophilic *Geobacillus* sp. AY 946034 strain was found to accumulate (PHB) using glucose as carbon source and the highest yield reached to 68.9% of Cell dry weight (Giedraityte and Kalediene, 2015).

Genetic engineering strategies depend on the optimization of the microbial metabolism towards biopolymer production. *Escherichia coli* strains have been genetically modified to produce PHB from glucose with a high molecular weight reaching to 10^7 which is suitable for processing to strong films (Park *et al.*, 2005, Kahar *et al*., 2005).

Various strategies have been developed to produce bioplastic from plants which are efficient to use direct carbon sources for PHA production because its ability to fix carbon dioxide sources through photosynthesis leading to lowering the cost by eliminating the carbon input cost. The cost of PHAs production from plants does not require feedstock and fermentation settings as in the case of bacteria which helps in reducing the cost of production (Yunus *et al.*, 2008). Acetyl-CoA is important for the PHAs biosynthesis, the availability of acetyl-CoA in the mitochondria, cytoplasm, plastid, and peroxisome of plant cell contribute the synthesis of PHAs in any of these organelles with the high availability storage space. PHB production from plants requires the genetic engineering of *phbA*, *phbB* and *phbC* genes of *Ralstonia eutropha* (Batool and Ilyas, 2014). Arabidopsis was the first plant that reported its ability to PHB production in cytoplasm through the expression of three genes to convert acetyl-CoA into bioplastic (Madison and Huisman, 1999). Saruul and co-workers (2002) reported the production of PHB by transgenic Alfalfa, the three genes of *Ralstonia eutropha* were introduced to plant by *Agrobacterium* and reported

that the PHB in the leaves of transgenic plants ranged from 0.025-1.8 g/kg dry weight.

Several experiments resulted in the production of PHB by transgenic yeast, Leaf and co-workers concluded that *Saccharomyces cerevisiae* INVSc1/PHA1 holding the PHB synthase genes of *Ralstonia eutropha* in its cytoplasm could produce PHB (Leaf *et al*., 1996), while Abuelhamd and co-workers observed that *Schizosaccharomyces pombe* Q01/PHB integrated with biosynthetic genes of PHB into the chromosomes could synthesize more amount of PHB homopolymer (Abuelhamd *et al.*, 2007).

1.2.9. The structure of polyhydroxyalkanoates granules and the models of formation

PHAs are accumulated intracellular granules stored in the cytoplasm as insoluble inclusion bodies. The size and number of granules differ according to different species, each cell has 3-13 granules with diameter range about 0.2-0.5 µm as observed in *Alcaligenes eutrophus* (Byron, 1994). In addition to essential enzymes for biosynthesis [Figure (1-5)], other proteins have a major role in the granules regulation and formation including PHA depolymerase (PhaZ), regulatory proteins (PhaR) and phasins (PhaP) (Luengo *et al*., 2003). PhaZ is responsible for PHA degradation, PhaR regulate the PHA synthesis and phasins production. Phasins have an effect on the number and size of granules (Jendrossek and Handrick, 2002*; Pötter* and *Steinbüchel*, 2005).

There are two models for the PHAs granule formation as described in Figure (1-6):

A – The micelle model, as in the figure, shows that the PHA synthase interacts with monomeric substrates in the cytoplasm. At first, the polymer synthesized and the chain is elongated from the synthase units. The produced hydrophobic chain of polymer interacts with hydrophilic synthase units form micelle-like

molecules causing aggregation of granules. The synthesis unit remains bound to granule surface; therefore, it become insoluble (Gerngross *et al*., 1994).

B – The budding model is the most supportive in the literature. The synthase binds to the membrane of cytoplasm biopolymer chain by hydrophobic interactions with growing into perplasm between phospholipid bilayer. This granule buds towards the cytoplasm surrounding by membrane, then is separated from the membrane to the cytoplasm and other proteins then is attached on PHA granules (Tian *et al*., 2005).

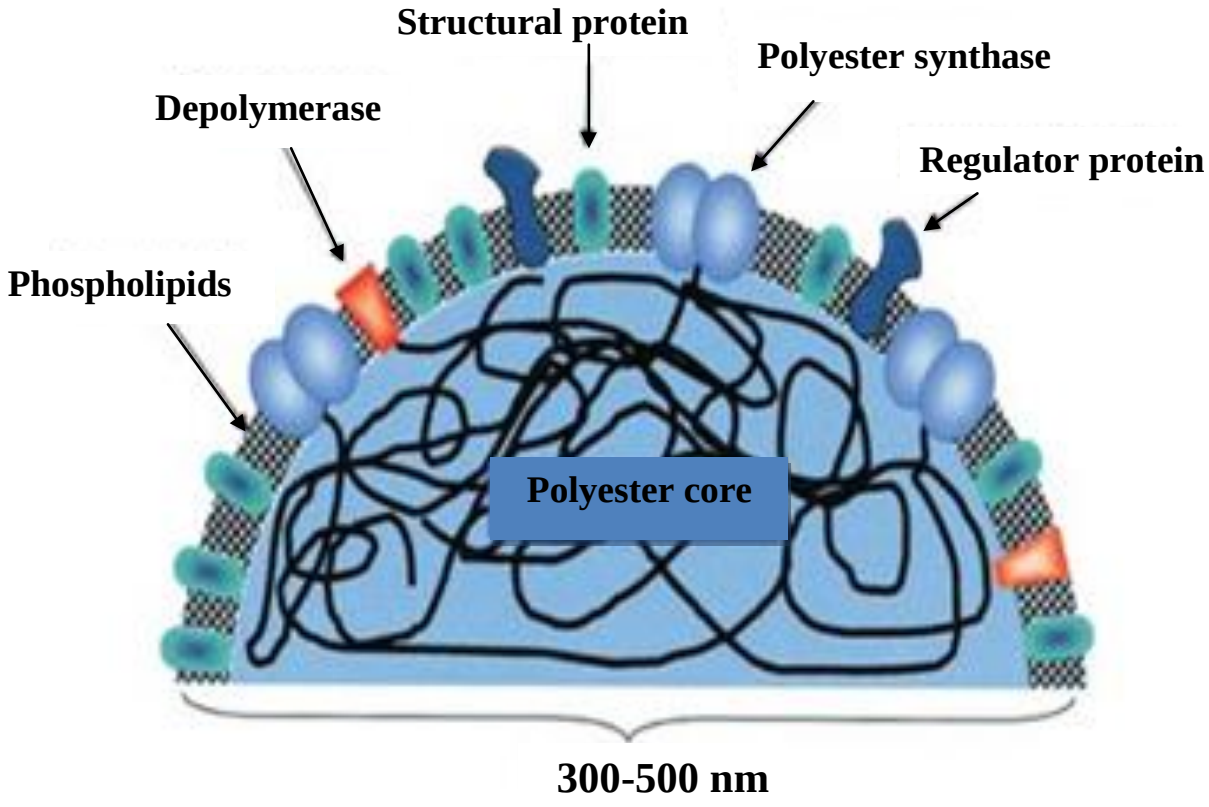


Figure (1-5): Schematic drawing of PHAgranules with related proteins (Grage *et al.*, 2009).

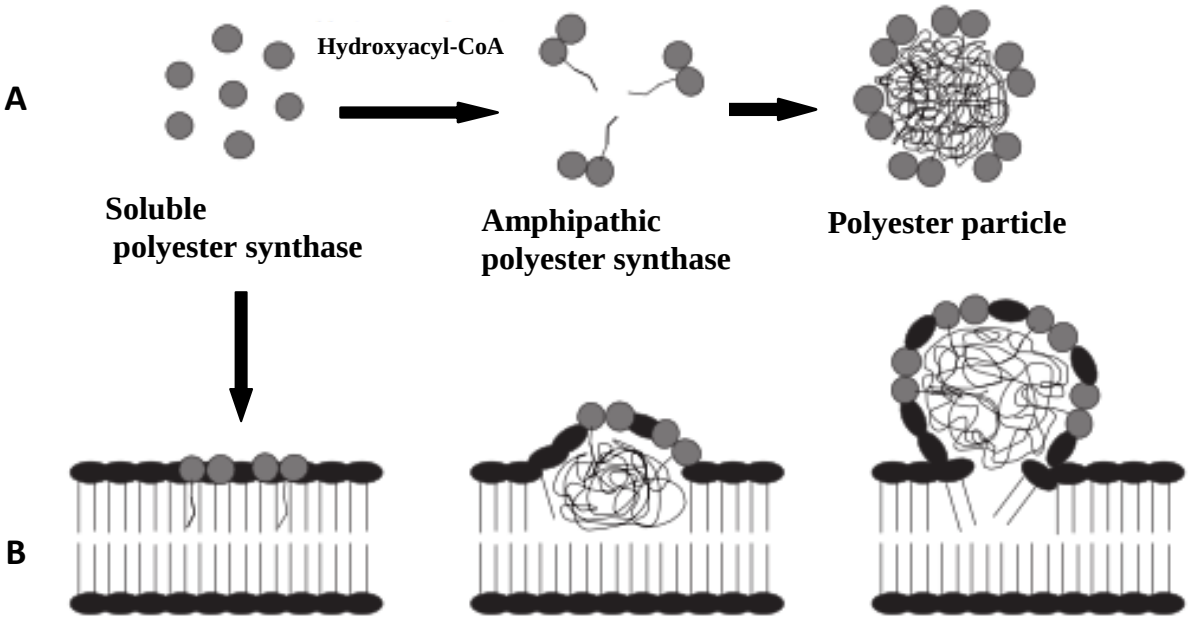


Figure (1-6): Models of PHA granule formation (A) micelle model (B) budding model (Tian *et al*., 2005).

1.2.10. Biodegradation of polyhydroxyalkanoates/ polyhydroxybutyrate

Biodegradation is a biological activity of living organisms to decomposite the complex structure of compounds to nontoxic products causing a lower molecular weight, the end results of biodegradation used as energy and nutrients for anabolism of none producing organisms (Braunegg *et al*., 1998).

The PHAs are degraded either under aerobic conditions producing CO_2, H_2O and methane as a synthetic polymer [Figure (1-7)] or under aerobic conditions producing CO_2 and H_2O (Jendrossek and Handrick, 2002). There are two ways of degradation **(I)** Degradation of PHAs in the cellular cytoplasm by intracellular depolymerase; therefore, it is named intracellular degradation, **(II)** Degradation by extracellular depolymerase in the surrounding environment; therefore, named extracellular degradation (Mergaert, *et al.*, 1992).

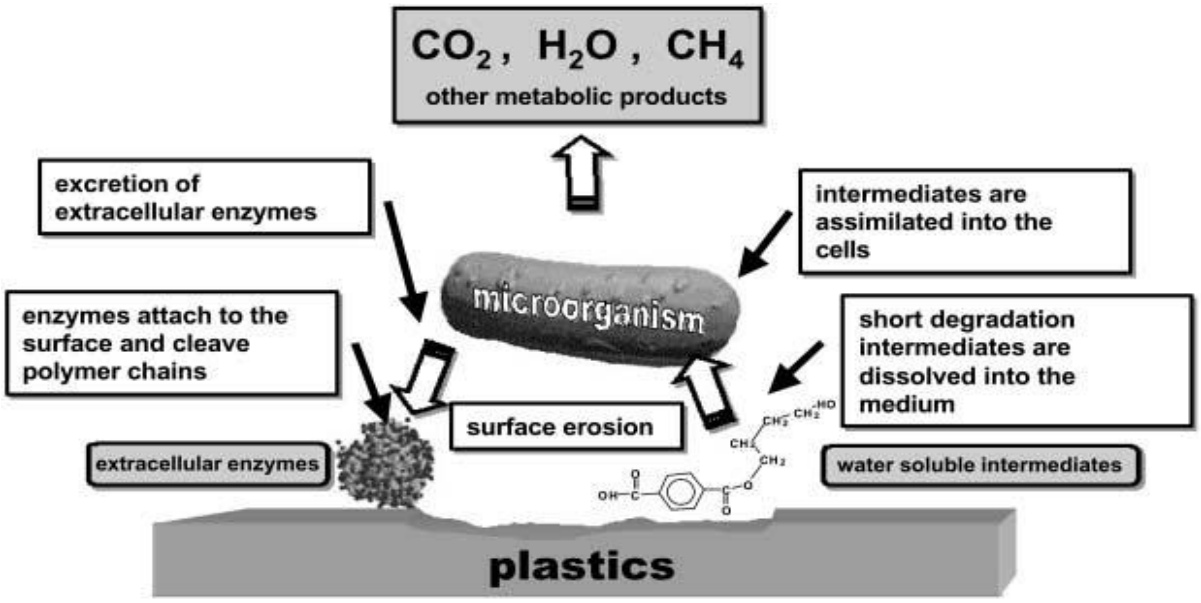


Figure (1-7): Schematic representing the enzymatic degradation of polymer by microorganisms (Mueller, 2006).

The biodegradatin is affected by many factors including microbial activity, polymer composition, temperature, moisture and molecular weight, PH, nutrient content, oxygen, crystanillity (Boopathy, 2000; Bernard, 2014). Also, the surface area of polymer effect on the degradation rate, as the less area will restrict the quantity of microbial population on the surface (Tokiwa *et al.*, 2009).

PHB is degraded to 3-hydroxybutyric acid by PHA depolymerase and oligomer hydrolase. The 3-hydroxybutyric acid is then oxidized by a dehydrogenase to acetylacetate. Once the ß-ketothiolase reacts upon the acetylacetate acetyl-CoA is generated and is utilized for cell regeneration (Doi and Fukuda, 1994; Kobayashi *et al.*, 2005).

Several bacteria and fungi have the ability to degrade the PHAs aerobically and anaerobically for example*: Pseudomonas, Actinomadura, penicillium Aspergillus sp., Microbispora, Saccharomonospor, Streptomyces,*

and Thermoactinomyces, Bacillus. Different environments involve soil, salt and fresh water, anaerobic sludge containing several microorganisms that could degrade PHA, but the soil is the most natural environment for PHA degradation (Tokiwa *et al.*, 2009; Boyandin *et al.*, 2012).

In vivo degradation of PHB film inside living organisms resulted nontoxic metabolites and produced 3-hydroxybutyrate that naturally exist in blood. For this purpose, it can be used in implant (Lee, 1996). Many studies observed the possibility of acceleration of the PHB degradation rate by the addition of polymers or plasticizers. On the one hand, amorphous or hydrophilic additives give rise to a higher water adsorption and accelerate hydrolysis (Freier *et al.*, 2002).

1.2.11. Polyhydroxyalkanoates/ polyhydroxybutyrate applications

PHAs have a wide range of potential applications because of its desired features such as biocompatibility means it has a nontoxic effect on living organisms and its degraded product is 3-hydroxybutyrate which is the natural constituent of blood, and biodegradability means that is degraded by living organisms to ecofriendly products (Volova *et al*., 2003; Braunegg *et al*., 2004).

PHB have many potential applications in medicine due to its biodegradability and biocompatibility. PHB and related PHAs have been used to develop devices including sutures, repair devices, repair patches, slings, cardiovascular patches, orthopedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone marrow scaffolds, and threads for wound dressings (Chen and Wu, 2005). PHB electrospinned into nanofibres have a role in repairing the human tissues such as nerves, cartilage, blood vessels, and skin (Barnes *et al*., 2007).

PHB is reliable for bone tissue engineering without any undesirable chronic inflammatory responses after the implantation period up to 12 months or structural breakdown *in vivo* during the implantation period. Bone is rapidly formed close to the material and subsequently becomes highly organized, with up to 80% of the implant surface lying in the direct apposition to the new bone (Doyle *et al*., 1991).

P(3HB) as scaffolds has been evaluated to augment the pulmonary artery for the regeneration of arterial tissue. These living vascular grafts engineered from autologous cells and biodegradable polymers functioned well in the pulmonary circulation as a pulmonary artery replacement (Shinoka *et al.*, 1998). PHA is used as a drug carrier, and it was reported that PHB released drug faster than other carriers (Bissery *et al*., 1985).

PHA granule binding protein PhaP was used in a receptor-mediated drug-specific delivery system (Figure 1-8). The system consists of PHA nanoparticles, PhaP, and ligands fused to PhaP. The PHA nanoparticles were used to package most hydrophobic drugs,PhaP fused with ligands produced by over expression of their corresponding genes in *Pichiapastoris* or *Escherichia coli* was able to attach to hydrophobic PHA nanoparticle. At the end, the ligands were able to pull the PhaP–PHA nanoparticles to the targeted cells with receptors recognized by the ligands. This study found that the receptor-mediated drug-specific delivery system ligand–PhaP–PHA nanoparticles was taken up by macrophages, hepatocellular Carcinoma Cell BEL7402 *in vitro*, and hepatocellular carcinoma cells *in vivo*, respectively (Wang *et al*., 2008).

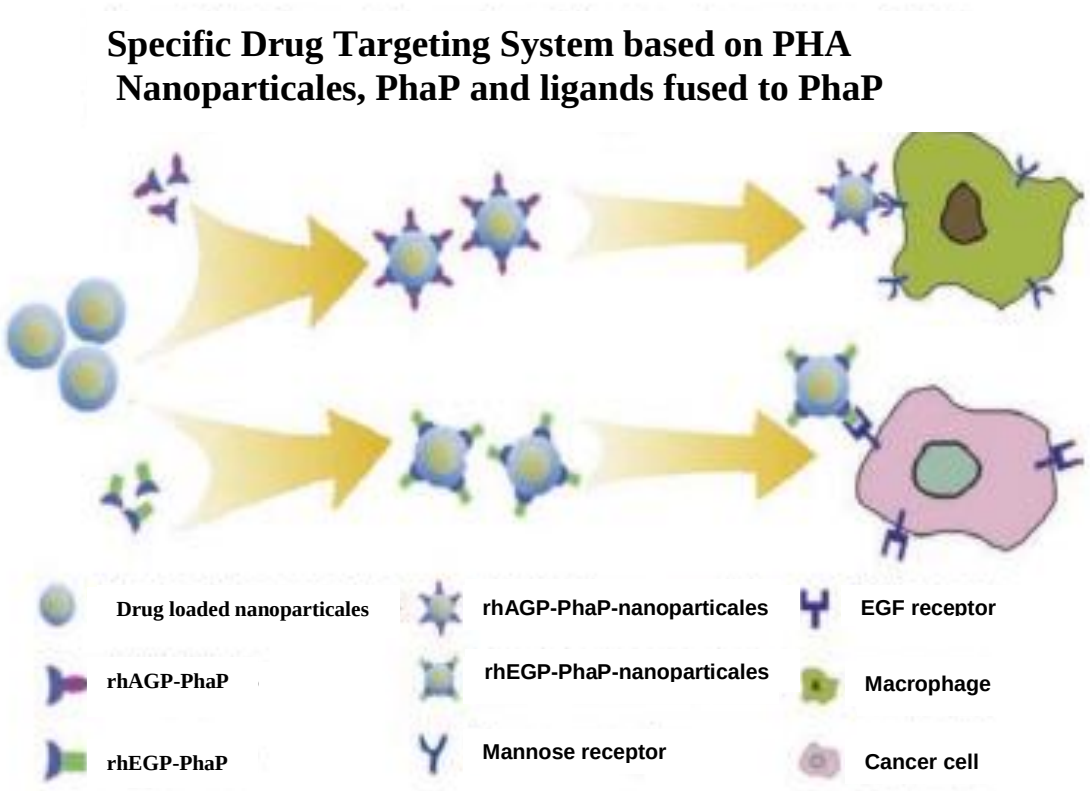


Figure (1-8): PHA and phasing-based specific drug delivery systems (Wang *et al*., 2008).

PHB microspheres were used as carriers for the anti-tumor drug rubomycin and they inhibited proliferative activity of Ehrlich's carcinoma in mice (Shishatskaya *et al*., 2008). The nano- sized PHA has the ability to penetrate deeply into the target body tissues and adsorption by the cells (Chen and Wu, 2005). PHA has cosmetic applications as cosmetic oil-blotting film returned to its ability to oil absorption in addition to nontoxic and biodegradable properties (Sudesh *et al*., 2007).

PHAs and PHB copolymers which could degrade anaerobicaly have important agricultural applications including encapsulation of seeds, encapsulation of fertilizers, biodegradable plastic films for crop protection and biodegradable containers for hothouse facilities, coating of herbicides and insecticides (Verlinden *et al.*, 2007; Yogesh *et al*., 2012).

PHAs have also been processed into toners for printing applications and adhesives for coating applications (Madison and Huisman 1999). They can also be used for packaging applications for deep drawing articles in the food industry, for example, bottles, laminated foils, fishnets, flowerpots, sanitary goods, fast foods, disposable cups, agricultural foils and fibers in textiles (Bugnicourt *et al*., 2014).

The piezoelectric nature of PHAs allowed them to be used in the following applications: pressure sensors for keyboards, stretch and acceleration measuring instruments, material testing, shockwave sensors, lighters, gas lighters; acoustics: microphone, ultrasonic detectors, sound pressure measuring instruments; oscillators: headphones, loudspeakers, for ultrasonic therapy and atomization of liquids (Philip *et al*., 2007).

Chapter Two

Materials and Method

2.1. Materials

2.1.1. Equipments and apparatus

The following equipments and apparatus were used during this study:

Equipments	Company
Autoclave	(Hirayama, Japan)
Incubator	(Memmert, Germany)
Distillator	(Chemopharm Sdn Bhd , Malaysia)
pH- meter	(Mettler-Toledo, China)
Millipore filter	(Sartorius, Germany)
Vortex	(Stuart, England)
Shaker incubator	(Rexmed, Taiwan)
Laminar air flow hood	(ESCO, Singapore)
Hot plate	(IKA, Germany)
Sensitive balance	(Sartorius, Germany)
Centrifuge	(KUBOTA, Japan)
Spectrophotometer	(Hitachi, Japan)
Centrifuge	(Hettich, Germany)
Rotary evaporator	(Eyela, Japan)
Desiccator	(Japson, India)
Electronic oven	(Memmert, Germany)
Lyophilizer	(Labconco Corporation, USA)
Nuclear Magnetic Resonance (NMR)	(Bruker Biospin Corporation, USA)
Thermogravimetric (TGA)	(Perkin Elmer, USA)
Differential Scanning Calorimetry (DSC)	(Perkin Elmer, USA)
Gas chromatography (GC)	(Agilent, United States)
Gel Permeation Chromatography (GPC)	(Labnet, USA)
Thermo Cycler (PCR)	(Bio-Rad CFX96, Singapore)
RT-PCR system	(ACTGene, USA)
Nanodrop	(Olympus, Japan)
Light microscope	(Olympus, Japan)
Phase contrast light microscope	(Olympus, Japan)
Fluorescent microscope	(Phillips CM12/STEM, Germany)
Transmission electron microscope (TEM)	(Philip CM 12, Germany)
Sorvall MT500 Ultramicrotome	(Boeckeler, USA)
Digital camera	(Nikon, Japan)

UV-transilluminator	(Fuence, Japan)
Electrosprayer	(Fence, Japan)

2.1.2. Chemicals and media-ready to use

Materials	Company
Agar, nutrient broth, Luria broth, tryptone soya broth, Safranin, Crystal violet, Carbolfuchsin, methylene blue, glycerol, mannitol, maltose, Phenolaphthalein phosphate agar,	(Himedia, india)
NaOH, NaCl, KH_2PO_4, Na_2HPO_4, NH_4Cl, $MgSO_4.7H_2O$, lead citrate, uranyl acetate, iodine, Sodium valerate, urea, Glucose	(BDH, England)
Ethidium bromide, Agarose Tolouidine blue, Nitrate broth, Deuterated chloroform, chloroform, Caprylate methyl ester (CME), 1- 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB), Trichloroacetic acid (TCA), Hecameg, Christensen's urea agar, Tributyrin agar	(Sigma, USA)
Phenol red broth base	(Acumedia, USA)
Urea, Tributyrin	(Fluka, Switzerland)
Tris-acetate-EDTA, 50X (TAE), McDowell-Trump fixative	(Thermo Fischer Scientific, USA)
Osmium tetraoxide, resin	(PELLA, USA)
Methanol, ethanol, acetone, hexane, sulfuric acid,	(QREC, Malaysia)
Nile blue A,	(Sigma, India)
Nile red	(Across organics, USA)
Sudan Black B	(Sd-fine-chem, India)
Dimethylfomamide (DMF)	(Fisher Scientific, UK)
TiO_2 nanoparticals	(Degussa, Germany)

2.1.3. Medium and reagents

2.1.3.1. Minimal salt medium (MM) (Doi *et al.*, 1995)

This medium is composed of the following components:

KH_2PO_4 …….. 2.80 (g/L)

Na_2HPO_4 ……. 3.32 (g/L)

$MgSO_4.7H_2O$ …… 0.25 (g/L)

NH_4Cl …… 0.50 (g/L)

Trace elements ……. 1.00 ml/L

The pH was adjusted to 7

The composition of trace elements solution was (Kahar *et al.*, 2004):

$CoCl_2.6H_2O$…….. 0.22 (g/L)

$FeCl_3$…… 9.7 (g/L)

$CaCl_2$…….. 7.8 (g/L)

$NiCl_2.6H_2O$……. 0.12 (g/L)

$CrCl_3.6H_2O$…….. 0.11 (g/L)

$CuSO_4.5H_2O$…….. 0.16 (g/L)

2.1.3.2. Phenol red broth base for carbohydrate fermentation

The phenol red broth that contained phenol red as an indicator was used for the fermentation test of carbohydrates (mannitol and maltose). It was done by Preparing 10 % of mannitol and maltose in distilled water separately and sterilizing by filteration. Then, 10 ml of each carbohydrate was added to 100 ml of medium aseptically and used for the fermentation test.

2.1.3.3. The Christensen's urea medium

It was prepared according to the instructions provided by the manufacturing companies and heated to 50 °C, then added 50 ml/L of 40 % urea solution that sterilized by filtration. Mixed well and distributed in sterilized tubes.

2.1.3.4. Tributyrin agar

It was prepared according to the instructions provided by the manufacturing companies and sterilized by an autoclave, then heated to 80 °C and 10 g/L neutral tributyrin was added and mixed well and poured into plates.

2.1.3.5. Nile Red stain

The stain solution was prepared by dissolving 0.25 mg Nile Red per ml dimethylsulfoxide (DMSO) and added to the sterilized medium to give a final concentration of 0.5 µg dye/ml medium.

2.1.3.6. Sudan Black B stain (Smibert and krieg, 1981)

The stain was prepared by dissolving 0.3 g of the stain in 100 ml of 70% ethanol.

2.1.3.7. Normal saline (0.85 %)

It was prepared by dissolving 0.85 gm of NaCl in 100 ml of distilled water and sterilized by an autoclave at 121 °C for 15 minutes.

2.1.3.8. Nile Blue A solution (1%)

It was prepared by dissolving 1 gm of Nile Blue A in 100 ml of 95 % ethanol and filtered to remove non-dissolved particles. The solution was kept in an amber bottle and sealed tightly to avoid evaporation of ethanol.

2.1.3.9. Acetic acid solution (8 %)

It was prepared by mixing 8 ml of acetic acid with 92 ml of distilled water and stored in universal bottle.

2.1.3.10. Nitrate test reagent (Atlas *et al.*, 1995)

The nitrate reagent involved two reagents:

Reagent A: 0.8 g of sulfanilic acid was added to 5N acetic acid up to 100 ml.

Reagent B: 0.5 g of dimethyle- α-naphthylamine was added to 5N acetic acid up to 100 ml.

2.1.3.11. Oxidase reagent (Colline and Lyne, 1987)

It was prepared by dissolving 1g of tetramethyl-p-phynylene diamine dihydrochloride in 100ml distilled water.

2.1.3.12. Ethidium bromide (Green and Sambrook, 2012)

This dye was prepared at a concentration of 10 mg/ml by dissolving 1 mg in 100 ml of distilled water and kept at 4 °C.

2.1.3.13. McDowell-Trump fixative (McDowell and Trump, 1976)

This fixative was prepared as follows:

0.2 M buffer …….. 50 ml
37% formaldehyde …….. 11 ml
25% glutaradehyde …….. 4 ml
Distilled water ……. 100 ml

2.1.3.14. Phosphate buffer saline (0.1 M, pH 7.2): (Green and Sambrook, 2012)

NaH_2PO_4. H_2O …….. 3.1 g

Na_2HPO_4 (anhydrous) …….. 10.9 g

This buffer was prepared in 1 liter of distilled water and stored for up to 1 a month in 4 °C.

2.1.3.15. Preparation of 5, 5ʾ-dithiobis-(2-nitrobenzoic acid): (DTNB) reaction solution

DTNB solution 20 mg/ml was prepared as stock by well mixing 20 mg of DTNB with 1 ml of absolute ethanol, then mixed 200 µl of DTNB stock

solution and 7.44 ml of 0.5 M potassium phosphate buffer (pH 7.8) in Falcon tube, finally pre-wrapped with aluminium foil.

2.1.3.16. *PhaC* assay buffer

PhaC assay buffer was prepared by mixing 150 mM potassium phosphate buffer (pH 7.2), 0.2% (w/v) glycerol and 0.05% (w/v) Hecameg [Methyl-6-O-(N-heptylcarbomyl)-**α**-D-glucopyranoside] and kept at 4 °C.

2.1.3.17. Substrate 3-hydroxybutyrate-CoA (3HB-CoA)

The formula weight of 3HB-CoA is equivalent to 853.6 g/L, which makes up 1M concentration. 25 mg substrate was dissolved in 10 ml solution to obtain a stock solution with concentration 2.9 mM. The volume of 3HB-CoA to be added relies on the final concentration of this substrate compound for one reaction (0.6 mM).

2.1.4. Kits

Kits	Company
DNeasy kit	(QIAGEN, USA)
Easy-spin™ (DNA free) total RNA extraction kit	(iNtRON Biotechnology, Germany)
IQ™ SYBR® Green Supermix kit	(Bio-RAD, USA)
iScript™ Reverse transcription supermix kit	(Bio-RAD, USA)
Coomassie Plus ™ (Bradford) assay kit	(Thermo scientific, USA)

2.1.5. Strains

Cupriavidus necator H_{16} ATCC 17699 (known as *Ralstonia eutropha*) and mutant *Ralstonia eutropha* $PHB^{-}4$ were used as a positive control and as a negative control respectively during the study of PHB production by *R. equi*.

2.2. Methods

2.2.1. Sterilization methods

The media and substrates were sterilized by autoclaving at 121 °C (15 Ib/in^2) for 15 minutes, while the sensitive materials were sterilized by membrane filtration using Millipore filter paper (0.22 µm).

2.2.2. Collection of soil samples

Samples of soil were collected from a different fertile land in Iraq and Malaysia. Each sample was kept separately in the sterile plastic bags and transferred to the laboratory.

2.2.3. Isolation of bacteria

To obtain the bacteria from soil samples, 1gm of soil sample was added in 99 ml of sterile distilled water and stirred. The mixture was filtered by Whatman filter paper to remove the debris. After a serial dilution of each sample (10^{-3}-10^{-10}), transfer 0.1 ml of each dilution to nutrient agar plates medium and spread then incubate at 30 °C for 3 days. The pure colonies of each sample were picked and subcultured for several times and kept for screening of PHA production.

2.2.4. Maintenance of the pure isolates

The pure isolates were maintained on a nutrient agar plate and in glycerol stocks. These isolates were sub-cultured monthly to maintain the cell viability. Glycerol stocks were used for a long term storage. The pure culture of bacteria was inoculated into 50 ml of a nutrient broth in 250 ml shaker flask and incubated at 30 °C for 24 hours. Then, 12.5 ml of sterilized glycerol 99% was added into the culture and stirred for 15 minutes to resuspend the bacterial cells.

Finally, pipette aseptically 1 ml of the suspension into Eppendorf tubes and kept at -20 °C.

2.2.5. Screening of polyhydroxybutyrate producing bacteria by Sudan Black B (Singh and Parmar, 2011)

The bacterial isolates were grown on mineral medium (MM) media at 30 °C for 3 days, a thin smear on a glass slide was prepared and air dried. Then stained with Sudan Black B solution and dried for 5-10 minutes. The slide was washed with distilled water, then drops of safranin were added as counter stain for 10 seconds, wash with distilled water and air dried. Finally the slide was examined under oil immersion microscope for PHA granules.

2.2.6. Screening of polyhydroxybutyrate producing bacteria by Nile Red (Spiekermann *et al.*, 1999)

The isolates were qualitatively tested for PHB production by culturing the colonies on MM plate supplemented with Nile Red at concentration 0.5 µg/ml. The agar plates were examined by UV light (312 nm) after a suitable period of incubation at 30 °C in comparison with *Cupriavidus necator* H_{16} as a positive control and *Ralstonia eutropha* PHB^{-4} as a negative control.

2.2.7. Growth curve measurement

The growth curve was established by inoculation three loops full of the bacterial culture from the tryptone soy agar (TSA) plate to a 50 ml flask of tryptone soy broth (TSB) and left in a shaker incubator at 30 °C, 200 rpm and approximately 1ml of bacterial broth was withdrawn aseptically each 3 hours. Each time has triplicates and the absorbance was read at each time at 600 nm using a spectrophotometer.

2.2.8. Identification of bacteria

2.2.8.1. Morphological and physiological characterization

The PHB positive isolate was grown on TSA at 30 °C for 3 days. Phase contrast light microscopy (Nikon Labophot-2 with the software ViS VER. 2.90) was used to study the morphology of bacterial cells and to ensure the purity of the cultures from contamination. The bacterial colonies were also captured by a digital camera.

2.2.8.2. Biochemical tests

2.2.8.2.1. Gram Stain (Atlas *et al*., 1995)

Smear of a fresh bacterial cell was heat fixed on a glass slide. The heat fixed bacterial cells were flooded with crystal violet stain for 1 minute, then the stain was rinsed with distilled water. A drop of iodine solution was added onto the smear and allowed to stand 1 minute before it was rinsed with water, decolorizing of the stain by 95% ethanol for 10 seconds, safranin was applied as a counter stain for 30 seconds. Then, the slide was rinsed with distilled water to remove the excessive dye. The gram positive bacteria will remain blue-violet in appearance.

2.2.8.2.2. Motility test (Colline and Lyne, 2004)

Semisolid (less agar powder added) rich medium was used for the motility test. It was heated and approximately 5 ml was put into each tube and autoclaved for sterilization, the tubes were allowed to cool in vertical position. Fresh culture was picked and stabbed into the center of the medium with an inoculating needle to a depth of 1/2 inch. The inoculated tubes were incubated at 30 °C for 48 hours. For a negative result, the bacterial growth would be visible along the stab line and the surrounding medium remained clear.

2.2.8.2.3. Oxidase test (Colline and lyne, 1987)

2-3 drops of tetramethyl-p-phynylene diamine dihydrochloride reagent were added on Whatman No.1filter paper, a loop full of the bacterial colonies was smeared onto filter paper. The color immediately changed to purple color for oxidase-positive bacteria.

2.2.8.2.4. Phosphatase test (Colline and Lyne, 2004)

The Phenolaphthalein phosphate agar was inoculated with bacterial colonies and incubated for 24 hours at 30 °C, then the colonies were exposed to ammonia vapor. The change of colony color to the bright pink- red color was the positive result.

2.2.8.2.5. Urease test (Atlas *et al*., 1995)

The Christensen's urea medium was prepared in the slant tubes and inoculated with bacteria by loop. After incubation for 24 hours at 30 °C, the change of medium color to pink was the positive result of this test.

2.2.8.2.6. Lipase test (Brown, 2005)

This test was used to detect the ability of bacteria for production of lipase exoenzyme. The tributyrin agar plates were inoculated by bacterial cells and incubated at 30 °C for 24 hours. The bacterial positive result will exhibit a clear zone around growth.

2.2.8.2.7. Nitrate reductase tests (Atlas *et al*., 1995)

The nitrate broth tubes were inoculated with the bacterial isolate and incubated for 24 hours at 30 °C. After incubation time, 2-3 drops of nitrate reagent **A** (sulfuric acid) were added, then added 2-3 drops of nitrate reagent **B** (naphthylamine). The change of color to red was the positive result.

2.2.8.2.8. Acid fast stain (Colline and Lyne, 2004)

Smear of fresh bacterial culture was heat fixed and covered by carbolfuchsin dye for 5-10 minutes with heating and add more stain when dried, then washed with distilled water. Then, it was decolorized by acid alcohol for 10-20 seconds, then washed with distilled water. The counter stains (methylene blue) was added for 30 seconds, then wash with distilled water. The cells with a positive result appeared red when examined by oil immersion.

2.2.8.2.9. Spore formation (Brown, 2005)

Smear of fresh bacterial isolate was fixed by heating, then saturated with malachite green stain with heating and more stain was added as required. After 3-5 minutes, left slide cooled and washed with water, then added safranin as a counter stain. After 1-2 minutes, it was washed with water, dried and examined by oil immersion lens. The spores had a bright green appearance.

2.2.8.2.10. Carbohydrates fermentation test (Brown, 2005)

The phenol red broth base that contained phenol red as indicator was used for the fermentation test of carbohydrates (mannitol and maltose). The broth distributed in the tubes contained inverted Durham tubes and then inoculated with bacteria and incubated for 24 hours at 37 °C. The change of phenol red broth color to yellow with gas bubbles inside the Durham tube was the positive result of this test.

2.2.8.2.11. Oxygen requirement (Brown, 2005)

This test was done by preparing sterilized nutrient agar tubes and inoculated with fresh bacterial isolate by a stab inoculation method using sterilized needle, then the tubes were incubated at 30 °C for 2 days. After incubation time, the growth position was noted, the aerobic bacteria grew on the top of agar.

2.2.8.2.12. Growth at different temperature

The nutrient agar plates were inoculated with the bacterial isolated of the present study, then incubated at 4, 30, 37 and 55 °C for 24-48 hours. The heavy growth was the positive result (Cruickshank *et al*., 1975).

2.2.8.3. API Coryne. Test

The strip of the commercial Api Coryne- kit (bio Merieux-vitek, Hazelwood, MO) was used for the identification of the selected isolate of this study. It consists of 20 tubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of carbohydrates. The pure culture was first streaked on NR agar plates and incubated for 24 hours at 30 °C. Few of pure colonies were resuspended into 0.85 % sterile saline solution to prepare a suspension with a turbidity equal or greater than McFarland Standard 6, then 0.5 ml of suspension was pipette into ampoule of the API GP medium. The first eleven tests of the strip inoculated by suspension and the last nine tests were inoculated by API GP medium containing the suspension followed by incubation at 30 °C for 24. A positive test results show changes in the medium color. Reading of the kit resulted in seven digit numerical codes, which were compared to an analytical profile index. The results were recorded and analyzed according to standard method by referring to the apiwebTM stand alone V 3.0 software.

2.2.8.4. Molecular identification of the isolate using 16S rRNA

The DNA of the positive isolate was extracted using the DNeasy kit by following the instructions of the kit. The DNA was used as a template for PCR using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (Frank *et al*., 2008). The PCR reaction that used to isolate and amplification 16S rRNA gene from bacterial cells was carried out with the following conditions: 2 minutes at 95 °C, 30 seconds at 62 °C, 5

minutes at 72 °C. Electrophoresis of PCR products were run horizontally using Agarose gels (0.7%) in Tris-borate-EDTA (TBE1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells on gel. Generally, gel was run for 2-3hours at100 V for 1 hour and immersion it in ethidium bromide for 15 minutes, destained in distilled water for 10 minutes then visualized PCR products by U.V illumination at 302 nm.

The similarity and identity of the sequence obtained was compared to other sequences in the Gene Bank database using nucleotide- nucleotide Blast command (Altschul *et al.,* 1997) in the National Center for Biotechnology Information (NCBI).

2.2.9. Quantitative study of polyhydroxyalkanoate biosynthesis

2.2.9.1. Carbon sources

Carbon sources such as glucose, glycerol, olive oil, sodium gluconate, palm acid oil (PAO), palm oil, waste cooking oil (WCO), crude palm kernel oil (CPKO), CPKO+ sodium valerate (CPKO+SV), glucose+sodium valerate (glucose+SV) were screened at a concentration of 1% (v/v) or (w/v) to promote the biosynthesis of PHA. Sodium valerate was used as a precursor of 3HV in a combination with some substrates at (1:1). A Stock solution of sodium valerate was prepared from valeric acide. 40 g of NaOH was dissolved in 1 L of (95%) absolute ethanol and stirred. Then 102 ml of valeric acid was added slowly into the NaOH solution. The salt precipitate of valeric acid was then recovered and dried completely in the oven at 45 °C until a constant weight was achieved and sterilized by autoclaving at 121 °C for 15 minutes under pressure and kept for use.

2.2.9.2. One-stage cultivation method

The biosynthesis was carried out using a one-stage cultivation method in Erlenmeyer flasks. The bacterial isolate that gives the intensive fluorescent with

Nile Red stain was used for the biosynthesis of PHA. The preculture was prepared by inoculating three loops full of fresh bacterial cells into 50 ml TSB medium and incubated at 30 °C on a rotary shaker at a speed of 200 rpm for 24 hours. Approximately, 3% (v/v) of the bacterial broth was used to inoculum the flask contain 50 ml MM medium with adding the substrates separately in sterile conditions and incubated for 48 hours at 30 °C under aerobic conditions in a shaker incubator at 200 rpm.

2.2.9.3. Harvesting and lyophilisation of the cells

The bacterial cells were harvested by centrifugation at 8000 RPM for 10 minutes at 4 °C using Kubota High Speed Refrigerated Centrifuge 6500 with AG-100A rotor. Collected cells were washed with distilled water, but in the state of oil substrates the cells were washed with hexane to remove the residual oil, then washed with water to remove the remaining hexane. The harvested cells were transferred to clean weighted bijoux bottles with 1 ml of distilled water and frozen at -20 °C for 24 hours before lyophilisation, then subjected to freeze drying for 2 days using a Labconco Freezone 4.5 L bench top freeze dryer.

2.2.9.4. Measurement of cell dry weight (CDW)

The pre-weighted bijoux bottles which contained the harvested cells were weighed after lyophilisation to measure the CDW. The CDW was calculated as in the following equation:

$$\text{CDW (g/L)} = \frac{(\textbf{Final weight of bijoux bottle} - \textbf{Initial weight of bijoux bottle})}{\textbf{50 ml}} \times \textbf{1000}$$

2.2.9.5. Determination of polyhydroxyalkanoate content and composition by gas chromatography (GC) (Kato *et al.*, 1996)

2.2.9.5.1. Preparation of methanolysis solution

Methanolysis solution was prepared inside the fume hood. This solution consisted from methanol and concentrated sulfuric acid with ratio 85: 15 (v/v). It was prepared by pouring 85 ml of chilled methanol into a beaker which was kept on ice to lower the temperature during the exothermic reaction then followed by drop-wise sulfuric acid slowly. The solution was stirred by a magnetic stirrer, kept in a Scott bottle and stored in the chiller at 4 °C for a further use.

2.2.9.5.2. Preparation of caprylate methyl ester (CME)

The internal standard (CME solution) was prepared in the fume hood by pouring 0.2 ml of caprylate methyl ester into volumetric flask, then topped up to 100ml with analytical chloroform to make the ratio of CME: chloroform (1:500). The solution was mixed, the flask was sealed and wrapped with an aluminium foil and stored in a chiller at 4 °C.

2.2.9.5.3. Methanolysis

Approximately, 15-25 mg of lyophilised cells was weighed and transferred to a screw-capped methanolysis tube. Then, 2 ml of chloroform and 2 ml of methanolysis solution was added into cells. The tubes were placed in the heating block for 140 minutes at 100 °C with gentle tapping every half an hour to homogenize the mixture. After heating, the mixture was cooled to room temperature, 1 ml of distilled water was added and vortexed for 1 minute to induce the separation of two layers. The top layer consisted of water and cell debris. The lower layer consisted of chloroform and methyl ester was recovered into new tubes containing anhydrous sodium sulfate to remove any traces of water. 0.5 ml of chloroform with a methyl ester layer was then mixed with 0.5

ml of caprylate methyl ester (CME) as an internal standard to the ratio of 1:1 in a clean GC vial and subjected to GC analysis (Braunegg *et al*., 1978).

2.2.9.5.4. Gas Chromatography analysis

The GC machine was used to quantify the PHA content and composition. The resulting methyl ester was analyzed by GC according to an internal standard method (Braunegg *et al.,* 1978). The injection process was programmed and analyzed automatically with 2.0 µl injection volume. The GC analysis took about 28 minutes and the program was set as follows:

Injector

Carrier gas : Nitrogen

Injection temperature : 270 °C

Pressure : 84.2 kPa

Purge flow : 14.0 ml/min

Column

Initial temperature :70 °C

Final temperature : 280 °C

Flame ionization detector

Temperature : 280 °C

Hydrogen flow rate : 40 ml/min

Compress air flow rate : 400 ml/min

2.2.9.5.5. Estimation of polyhydroxyalkanoate content

The monomers of PHA were identified depending on the peaks at a certain retention time in GC chromatogram content in weight % of the CDW

and the monomer composition in mol% was estimated as in the following formula:

P(3HB) homopolymer

$$\text{PHA content (wt\%)} = \frac{K \times (A3HB \times K3HB)}{ACME \times B} \times 100\%$$

P(3HB-co-3HV) copolymer

$$\text{PHA content (wt\%)} = \frac{K \times [(A3HB \times K3HB)] + (A3HV \times K3HV)]}{ACME \times B} \times 100\%$$

$$\text{Monomer composition of 3HB (mol\%)} = \frac{(A3HB \times K3HB)}{(A3HB \times K3HB) + (A3HV \times K3HV)} \times 100\%$$

$$\text{Monomer composition of 3HV (mol\%)} = \frac{(A3HV \times K3HV)}{(A3HB \times K3HB) + (A3HV \times K3HV)} \times 100\%$$

K = GC factor = 12.8

K_{3HB}=3HB factor= 1

K_{3HV}= 3HV factor = 0.53

$_{A3HB =}$ Area of 3HBmonomer peak

A_{3HV} = Area of 3HVmonomer peak

A_{CME} = Area of internal standard (CME) peak

B = Weight of lyophilised that used for methanolysis in the unit of mg

While the total P(3HB) homopolymer and P(3HB-co-3HV) copolymer were calculated as follows:

Total PHA (g/L) = PHA content (wt %) × CDW (g/L)

2.2.10. Extraction of polymer (Sudesh *et al.*, 2004)

PHB granules accumulated inside the cells were extracted and recovered by blending lyophilized cells for 5 days in chloroform with a ratio of cells to chloroform (1 g: 100 ml). The digested cells were separated by filtration using Whatman ® No. 1 filter paper to remove the cell debris and to collect the chloroform extract. After filtration, the solution was concentrated to about 20 ml by using Eyela N-1000 rotary evaporator. The concentrated polymer solution was precipitated by drop wise addition into vigorously stirred chilled methanol to form a white material as a precipitate. The precipitated and purified polymer was later collected, air-dried and used for further characterization work.

2.2.11. Characterization study of polymer

2.2.11.1. Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR and ^{13}C NMR analyses were recorded using a Bruker Advance 500 NMR (Bruker Biospin Corporation, USA) spectrometer operating at 500 MHz, 25 ˚C by dissolving polymer sample in deuterated chloroform ($CDCl_3$) at a concentration of 25 mg/ml using Tetramethysilane (Me_4Si) as an internal chemical shift reference.

2.2.11.2. Differential Scanning Calorimetry analysis (DSC)

The thermal properties of extracted polymer were measured using instrument Pyris 1 equipped with cooling accessory. The instrument was equilibrated at room temperature prior to cooling at -40 °C at heating rate 5 °C /min. 5 mg of polymer was encapsulated in an aluminium pan and maintained at about -40 °C 1 minute followed by heating to 200 °C at heating rate 5 °C/min. The second cooling and heating was performed under the same manner except for the heating which started from -40 °C to 200 °C. The glass transition temperature (T_g), and melting temperature (T_m) were determined from the DSC thermogram.

2.2.11.3. Thermogravimetric analysis (TGA)

The thermal stability of PHB polymer was determined using the instrument STA 6000 (Perkin Elmer, USA). Approximately 10 mg of polymer sample was loaded with an aluminium pan and heated from about 30 °C to 920 °C at a heating rate of 10 °C/min under nitrogen atmosphere. This analysis was used to measure the decomposition temperature (T_d) which defined as a temperature for 5% weight loss of the polymer sample.

2.2.11.4. Gel Permeation Chromatography analysis (GPC)

The molecular weights (M_w), number average molecular weight (M_n) and polydispersity (M_w/M_n) of the purified polymer were determined by an Agilent 1200 gel permeation chromatography (GPC) system connected to a refractive index detector with Shodex K-806 columns. Polymers were dissolved in HPLC grade chloroform at a concentration of 1 mg/ml and filtered through 0.45 µm PTFE membrane. The eluent was chloroform with a flow rate of 0.8 ml/min at 40 °C. Universal calibration was generated by using a narrow polydispersity polystyrene standard (Agilent, United States).

2.2.12. Qualitative analysis of polyhydroxybutyrate granules

2.2.12.1. Phase contrast light microscope

Routinely phase contrast light microscopy (Nikon Labophot-2 with the software ViS VER. 2.90) was used as a qualitative method to determine the presence of PHB granules and the purity of the culture morphology of bacterial cells. The isolate was grown in MM medium in the presence of best carbon source (CPKO) and the formation of PHB granules was checked at different times (12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs).

2.2.12.2. Fluorescent Microscope

The bacterial cells cultured in MM broth using CPKO as best substrate were smeared, heat fixed on a glass slide and flooded by the Nile Blue A solution 1% and incubated at 55 °C for 10 minutes, then washed slide with 8% of acetic acid solution for 1 minute to remove unbound stain. The slide was rinsed with tap water and covered with a glass cover slip and observed under oil immersion using 100 x magnification fluorescent microscope fitted with cellSens® Version 1.4 Software.

2.2.12.3. Transmission Electron Microscope (TEM)

2.2.12.3.1. Preparation and fixation of cells

The pure cultured cells were cultivated under favoring conditions for the accumulation of PHB. The cells were harvested with centrifuge at 1000 RPM, 4 °C for 10 minutes. The cells were prefixed with McDowell-Trump fixative which is prepared in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 24 hours. The cells were centrifuged and resuspended in the same phosphate buffer twice to ensure the complete removal of any trace amount of fixative. The cells were post-fixed in 1% (v/v) osmium tetroxide (OsO_4) at room temperature for 1 hour. The post-fixed cells were washed twice with distilled water. After the fixation steps, the centrifuge tube containing the post-fixed cells were placed in the water bath at 45 °C for about 10 minutes. 2% (w/v) agar solution was prepared by dissolving the agar in boiling water and then poured into test tube while the agar solution was still molten and placed in the same water bath. After both, the agar solution and the post-fixed cells, had equilibrated at 45 °C, a small drop of agar was transferred to the tube of cells with resuspended the cell in agar, immediately, the agar with cells were poured on a glass microscope slide and allowed to solidify. The solidified agar containing the cells was cut into small cubes, approximately1 mm^3 size by a sharp blade.

2.2.12.3.2. Dehydration

A series of dehydration using 50, 75, 95 and 100% ethanol and 100% acetone was done in the following order:

50% ethanol ……. 15 minutes

75% ethanol ……. 15 minutes

95% ethanol ……... 15 minutes (2x)

100% ethanol …… 30 minutes (2x)

100% acetone …… 10 minutes (2x)

2.2.12.3. 3. Embedding

The agar cubes containing cells were infiltrated with a resin using a mixture of acetone: Spurr's resin was mixed in the ratio of 1:1 in a rotator at 2 rpm for 15-30 minutes. The mixture was replaced with fresh Spurr's resin and left overnight in the rotator. This step was repeated every 24 hours for 3 subsequent days. The samples were embedded in resin and placed in an oven at 60 °C for 48 hours.

2.2.12.3.4. Sectioning and staining

The resin blocks were first grinded and smoothen using a sand paper. The resin blocks roughly trimming using a razor blade under microscope at 10x magnification, then followed by a fine trimming using a glass knife. Sorvall MT500 Ultramicrotome was used for sectioning. A knife boat was subsequently prepared to collect the semi-thin sections. The knife-boat was filled with distilled water and placed under the microscope. The knife-boat and resin block were adjusted to a parallel. The distance of the knife-boat with the block was adjusted carefully until the cutting edge touches the surface of the block. The semi-thin sectioning was carried out to obtain semi-thin sections with 1 µm of

thickness. Semithin sections were picked by eyelash tool and stained with Tolouidine Blue and observed under a microscope with 100x magnification to confirm the presence of the bacterial cells on the thin sections before proceeding to ultra- thin sectioning. All steps were repeated with a new knife-boat. The ultra thin sections (90 nm of thickness) were obtained and placed onto a copper grid. Finally, they were stained with uranyl acetate and then with lead citrate solution for 15 minutes respectively. Both solutions act as dyes to provide clearer images during the observation under TEM.

2.2.13. Detection and sequencing PHA synthase gene (*PhaC*)

The DNA of the selected bacteria was extracted by following the instruments for DNeasy kit and used as a template to detect *PhaC* gene using the universal primers G-D primer as forward (5'-GTGCCGCC(GC)(CT)(AG)(GC)ATCAACAAGT-3'), and G-1R primer as reverse (5'-GTTCCAG(AT)ACAG(GC)A(GT)(AG)TCGAA-3') (Romo *et al.*, 2007). The PCR components and setting were listed in Table (2-1) and Table (2-2) using Veriti thermocycler. The detection of PCR products was carried out by electrophoresis as described previously (2.2.8.4.), part of PCR product was sent for sequencing at first base laboratory and analyzed by blasting the result in NCBI (National Center for Biotechnology Information) to confirm the result.

Table (2-1): PCR components.

Components	Amount (µl)
Water nuclease free	14.5
5X PCR buffer	5
dNTP mix, 10mM each	0.5
Forward primer, 10 µM	1.25
Reverse primer, 10µM	1.25
DNA template	1
Thermostable DNA polymerase (2 U/µl)	0.25
Additive	1.25
TOTAL	**25**

Table (2-2): Thermal cycling protocol.

Cycle steps	Temp. (°C)	Time	Number of cycles
Initial denaturation	98	2 min	1
Denaturation	98	10 s	30
Annealing	60	30 s	
Extension	72	30 s	
Final extension	72	10 min	1

2.2.14. Gene expression level analysis

2.2.14.1. RNA extraction

A single colony of *R. equi* was inoculated in 10 ml LB and incubated overnight. Time points for RNA extraction were determined from the growth curve obtained previously using easy-spin ™ [DNA free] total extraction kit depending on the log phase as a control in this study. The concentration and purity of RNA were then measured using NanoDrop Spectrophotometer. The RNA samples at three time points (lag phase, log phase, stationary phase) were subjected to electrophoresis (2.2.8.4).

2.2.14.2. Conversion of RNA to cDNA

The cDNA was prepared from RNA using iScript™ Reverse transcription supermix for RT-qPCR. The reaction contained: 10µl of RNA, 8 µl iScript™ Reverse transcription supermix and 22µl of nuclease free distilled water. The PCR reaction was performed according to the following conditions:

95 °C for 5 minutes, 62 °C for 30 seconds, 75 °C for 5 minutes, finally cooled the reaction at 4 C for 1 minute. The electrophoresis of cDNA samples at all time points was performed and visualized as mentioned above.

2.2.14.3. Relative Quantitative real-time PCR (qRT-PCR)

Real time-PCR was used to study the level of expression for *PhaC* gene (target gene) and *gyrB* gene (*gyrB* gene used as reference gene) using Bio-Rad CFX 96 real time PCR system with IQ SYBR® Green supermix (Bio-Rad) kit. The final volume of each real time PCR reaction was 10 µl containing: 2 µl of nuclease free distilled water, 0.5 µl of 10 Mm of each forward and reverse primers for target and reference gene as listed in Table (2-3), 5 µl of IQ SYBR® Green supermix and 2 µl of cDNA.

The experiment was performed in triplicate as the following protocol: 95 °C for 3 min, 95 °C for 15 sec, 60 °C for 1 min and followed with cooling at 40 °C for 30 sec then melting from 55 °C to 95 °C followed with cooling at 40 °C for 30 sec with an increase of 0.5°C in each cycle and collected the data during the cycles of amplification for analysis.

2.2.14.4. Standard curves for qRT-PCR assay

The standard curves for target gene (*PhaC* gene) and reference gene (*gyrB* gene) were generated to determine the PCR efficiency. Serial dilutions were performed using the same primers in Table (2-3). The data were collected and analyzed using Bio-Rad CFX 96 Manager™ Software. The analysis of the data depended on the threshold cycle (C_T) and efficiency of PCR amplification.

Table (2-3): Primers for *PhaC* gene and *gyrB* gene.

Genes	Primers
gyrB	**Forward Primer:** 5'-GTCGAGCAGGGTCAAGTGTA-3'
	Reverse Primer: 5'-AGCTCCTTGGCGTTCATCT-3'
PhaC	**Forward Primer:** 5'-CCTTCTCGATTACAGTGATCCG-3'
	Reverse Primer: 5'-AACGTCTGCCCCATGTTC-3'

2.2.15. PHA synthase assay (Yuan *et al.*, 2001)

2.2.15.1. Bovine serum albumin (BSA) standard curve for protein determination (Bradford, 1976)

The linear working range of BSA was determined to be within 125-1000 µg/ml. 50 µl of each BSA and sample was placed into labelled tubes, then 1 ml of dye reagent (Coomassie Plus ^{TM} protein assay reagent) was added and kept for 10 minutes at room temperature. Blank was prepared by mixing 50 µl water with 1 ml of the reagent dye. The absorbance was measured at 595 nm. The absorbance value was plotted over the concentration. The resulted standard curve was used to determine the protein concentration of protein in the sample as in Appendix (1).

2.2.15.2. Determination of PHA synthase activity

All prepared solutions and samples including *PhaC* assay buffer, DTNB stock solution, 3HB-CoA substrate, protein sample and trichloroacetic acid (TCA) were kept cold throughout the reaction procedure. Two sets of Eppendorf tubes were prepared for all except 0 sec tube and labelled accordingly (0, 20, 40, 60, 90, 120 sec). 50 µl TCA was added into all the tubes except 120 sec tube. 20 µl of *PhaC* assay buffer was added only into 0 sec tube. The appropriate volume of substrate (21 µl), buffer and enzyme/protein was added into 120 sec tube, to make up a total of 100 µl reaction volume. The 120 sec tube was vortexed and incubated at 30 °C. Upon 2 minutes of incubation, 20 µl of sample was removed at 20, 40, 60, 90 and 120 sec. At the final 2 minutes, 50 µL TCA was added into 120 sec tube. All the tubes were incubated at room temperature for 1 minute and centrifuged at 12,600 rpm for 1 minute. About 65 µl supernatant from each tube except 0 sec was transferred into a new set of Eppendorf tubes. 385 µl DTNB reaction solution was added into all tube and only 380 µl reaction solution was added to 0 sec tube. The solution was mixed well and 450 µL of the solution was transferred into a cuvette and the

absorbance was measured at 412 nm. Blank was performed using 450 µL of 0.5 M potassium phosphate buffer (pH 7.8). The concentration of CoA released was determined spectrosphotometrically (Gerngross *et al.,* 1994) from the following formula:

$$\textbf{Concentration of CoA released} = \frac{\mathbf{A412}}{\mathbf{13600}} \times \frac{\mathbf{70}}{\mathbf{20}} \times \frac{\mathbf{450}}{\mathbf{65}}$$

The recorded CoA concentration was used to determine the synthesis enzyme activity (Takase *et al.,* 2004). A linear graph was plotted using the values of CoA concentration (µmol) versus time. The m value (slope) was then used to calculate the PHA synthase activity (U/mg), whereby activity was defined by:

$$\textbf{Activity} = \frac{\textbf{m value of equation (U)}}{\textbf{Total protein (mg)}}$$

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1.0 µmol CoA/min.

2.2.16. Biodegradation studies in soil

Different formulations of PHB films were prepared for degradation study:

2.2.16.1. Preparation of PHB films and PHB-TiO_2 composite films (Sridewi *et al.,* 2006)

The conventional solvent-cast technique was used for PHB films and PHB-TiO2 composite films preparation. PHB films were prepared by dissolving 0.3 mg of the extracted polymer in 30 ml of chloroform in a Schott bottle with stirring magnetically for 30 minutes, then poured into a glass petri dish (9 cm in diameter) as the casting surface. The petri dishes were covered with puncture aluminium sheets and left in a dark place for 24 hours at room temperature (30

°C) for complete evaporation of chloroform. The commercial TiO_2 powder (Degussa, P25) was used for composite preparation which prepared in a manner resembling the conventional solvent-casting method, 0.3 gm of PHB and 0.18 gm TiO_2 powder were dissolved in chloroform.

2.2.16.2. Preparation of PHB nanofiber and PHB-TiO_2 composite nanofiber films (Sudesh, 2013)

PHB nanofiber was prepared by an electrospinning technique using (Esprayer ES-2000) with 4 % (w/v) of PHB in a mixed solvent of $CHCl_3$/DMF at a ratio (8:2), then run electrospinning with voltage 15 Kv and extrusion rate 40 µl/min, mixed the solution by a magnetic stirrer for 2 days at room temperature to homogenize the solution. Before electrospinning running, the solution was heat- stirred at 55 °C for 2 hours. The solution was loaded in a glass syringe equipped with stainless steel needle having 0.5 mm diameter. The maximum loading of the glass syringe was 1 ml. The distance from the needle tip to the collector was fixed at 20 cm. The copper collecting plate was covered with insulating material, leaving a circular opening (5 cm diameter) for deposition of the resultant fiber. For PHB-TiO_2 composite nanofiber films were prepared in the same manner of electrospinning with adding 0.12 g TiO_2 powder.

2.2.16.3. Preparation of PHB films and PHB-TiO_2 composite films after Ultraviolet light (UV) treatment

Another PHB films were prepared by the conventional solvent-cast technique as mentioned previously and placed under UV light source 30 W with distance 5 cm. After treatment, these sheets were used as a source for PHB in the preparation of PHB films and PHB-TiO_2 composite films by following the method stated in (2.2.16.1).

However, all Schott bottles used for preparing the nanocomposite films in the conventional solvent-cast technique and the electrospinning technique were wrapped with aluminium foil and kept in a dark place until use because TiO_2 is photosensitive. Also, all the cast films were aged for one week before subjecting to degradation to reach equilibrium crystallinity.

2.2.16.4. Biodegradation experiment designs

The site chosen to study the degradation was the fertile garden (University Science of Malaysia, USM). The films were cut into pieces measuring 1cm × 1cm and placed inside none degradable mesh bags with (8 cm × 4 cm) measurement. Each bag was divided into a small pouch (1.5 cm × 1.5 cm) measurement. All film samples were prepared in triplicate with weighing and placed in each pouch, then closed by sew using a none degradable thread. The mesh bags were fixed on metal mesh and buried in soil for 6 weeks at 10 cm under the soil.

2.2.16.5. Determination of molecular mass of film samples

The molecular weight of the film samples was determined before and after the degradation experiment as mentioned previously in (2.2.11.4).

2.2.16.6. Measurement of biodegradation percentage by weight loss

Each week, one mesh bag that contained all film samples was taken out and washed by sterile distilled water to remove the particles of soil, then left at room temperature for 24 hours. The dried film samples were placed in a desiccator for an hour to get the constant weight. The weight of films samples was recorded to determine the degradation percentage through the measurement of the weight loss percentage by using the following formula (Yew *et al.*, 2006):

$$\textbf{Degradation \%} = [(W_1 - W_2) / W_1] \times 100$$

Where W_1 is the initial weight of the film and W_2 is the weight of the film after degradation.

2.2.16.7. Quantitative microbial counting

The standard spread plate technique was used to measure the microbial growth in the soil of the buried and near from samples at each week. The medium that used was TSA, 1 gm of soil was dissolved in 99 ml of normal saline 0.85% with gentle mixing by a vortex followed by a serial dilution, then spread 0.1 ml of each dilution onto TSA plate in triplicate for each dilution. The plates were incubated at 30 °C for 24 hours, then counted the plates with 30 – 300 colonies to measure the CFU in term $\log_{10}$ for each ml ($\log_{10}$ CFU/ml), the calculation beginning from the first week until the sixth week.

2.2.16.8. Microscopic observation of surface changes after degradation

The changes of surface morphology of the film samples were checked at each week by Olympus S240 stereo microscope fitted with a JVC K-F55B color video camera. For more detail about the morphological changes of the films surface after degradation time, the samples were cut to 5 mm x5 mm, fixed on aluminium stumps, coated with gold for 15 sec, and viewed under scan electron microscope (SEM) Carl-Ziess SMT, Oberkochen at an acceleration voltage of 5 kV.

2.2.17. Statistical and Data Analysis

The experimental data for PHB biosynthesis was analyzed by SPSS software in combination with one way anova. The experimental data for degradation was analyzed as factorial experiments with completely randomized design and the least significant difference (L.S.D.) at level 0.05 was used to show the results significantly (Al-Rawi and Khalf Allah, 2000).

Chapter three

Results and Discussion

3.1. Primary screening of polyhydroxybutyrate production

Fifty soil samples were collected from different fertile lands to obtain the pure isolates, The Sudan Black staining method was utilized for the analysis of PHB accumulation for bacterial isolates. Only twenty isolates have been reported to have positive results of Sudan Black B.

The Nile Red staining of viable colonies was used as a direct and a rapid tool for direct detection of PHB accumulation within the bacteria cells (Amirul *et al*., 2009; Van and Thi, 2012). Three isolates had a positive result, but only one of these isolate had visible and more fluorescent with Nile Red stain isolated from fertile soil sampled at Gunung Lang, Perak, Malaysia and used as a reference positive strain in the present study. A pink fluorescent under UV illumination was obtained as a result of the binding between stain and polymeric granules (Berlanga *et al.*, 2006). The production of pink fluorescent can discriminate the isolate that produced PHB when compared to both the PHB-positive control (*Cupriavidus necator* H16) and PHB-negative control (*R. eutropha* PHB^{-}4) as shown in Figure (3-1).

(A) Isolate **(B) Positive control** **(C) Negative control**

Figure (3-1): Fluorescent Nile Red staining of *R. equi* cells accumulating PHB. The bacterial cells were grown in MM medium containing Nile Red stain with concentration 0.5 µg/ml for 2 days at 30 °C.

3.2. The growth curve study of polyhydroxybutyrate isolate

The bacterial isolate of the current study was allowed to grow on TSB shaking flask at 30 °C for 48 hours and the optical density (OD) was checked each 3 hours at 600 nm. The growth curve was plotted by taking time on x-axis and OD on y-axis and obtained the curve as in Figure (3-2). It is clear that, the OD was increased with increasing time in which it was highest after 24 h and declined gradually but still high even after 48 h.

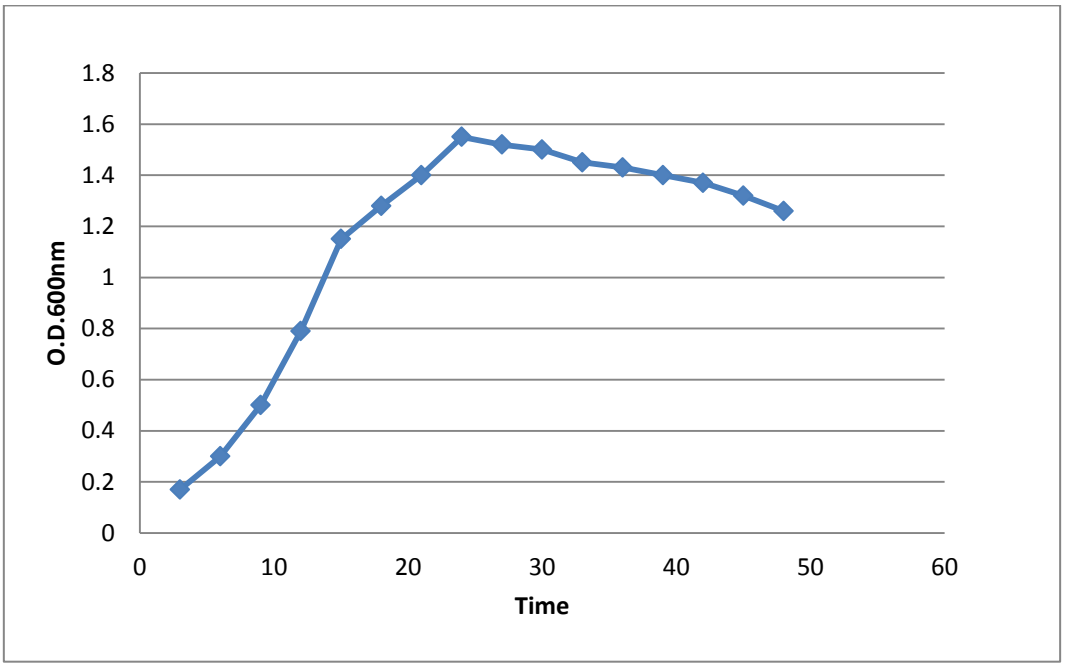


Figure (3-2): Growth curve of bacterial isolate produced PHB.

3.3. Identification of isolate

The morphological and physiological characters were used to identify the PHB isolate that has been grown cultured on TSA at 30 °C for 2 days. The morphological characters of colonies are listed in Table (3-1) and Figure (3-3). The isolate was rod/coccus Gram positive, non-motile and none spore forming. The cells appeared as single and pairs, 1.90–1.98 µm in length and 1.72–2.66 µm in width at the optimum temperature (30 °C) for the growth. The

biochemical tests showed that the isolate was aerobic, positive results for catalase test, urease test, nitrate reductase test, acid fast stain, fermentation of mannitol and maltose sugar as shown in Table (3-2). According to the morphological, physiological, and biochemical tests, the isolate was identified as *Rhodococcus* sp. (Balows *et al*., 1991; Silva *et al.*, 2010). The table (3-3) showed the results of API Coryne. kit that identified the isolate also as *Rhodococcus* sp. with accuracy 99%. The identification was confirmed by16S rRNA analysis (Frank *et al*., 2008). The genomic DNA was extracted and used as a template to amplify the 16S rRNA gene by PCR, then the products of PCR were subjected to agarose gel. The size of PCR fragment was approximately 1477bp as shown in Figure (3-4) and Figure (3-5) exhibited the sequence of 16S rRNA gene.

Table (3-1): Morphological characters of colonies.

Cultural characteristics	Observations
Colony configration	Circular
Colony margin	Smooth
Colony elevation	Raised
Diameter	1-3mm
Color	Beige

Table (3-2): Morphological, physiological and biochemical characters of bacterial isolate. (+): Positive. (–): Negative.

Characters	Results
Rod-cocci	(+)
Cell in single	(+)
Cell in pairs	(+)
Cell motile	(–)
Cell length	1.90-1.98μm
Cell width	1.72-2.66μm
Spore formation	(–)
Urease	(+)
Nitrate reductase	(+)
Lipase	(+)
Phosphatase	(+)
Gram stain	(+)
Acid fast stain	(+)
Oxidase	(–)
Mannitol	(+)
Maltose	(+)
Growth at 4 °C	(–)
Growth at 30 °C	(+)
Growth at 37 °C	(–)
Growth at 55 °C	(–)
Oxygen requirement for growth	(+)

Table (3-3): List of reading table for API Coryne. Strip with real results.

Tests	Negative results	Positive results	Real results
NIT	colorless very pale pink	dark pink red	positive (dark pink)
PYZ	colorless very pale brown very pale orange	brown orange	positive (brown)
PYRA	colorless beige - pale purple pale orange	orange	negative (colorless)
PAL	colorless beige - pale purple pale orange	purple	positive (purple)
ßGUR	colorless pale grey pale beige	blue	negative (colorless)
ßGAL	colorless beige - pale purple pale orange	purple	negative (colorless)
∝GLU	colorless beige - pale purple pale green	purple	positive (purple)
ßNAG	colorless beige - pale purple pale brown pale grey	brown	negative (colorless)
ESC	colorless grey	black	positive (black)
URE	yellow orange	red pink	positive (red pink)
GEL	no diffusion of black pigment	diffusion of black pigment	negative(no diffusion of black pigment)
0 GLU RIB XYL MAN MAL LAC SAC GLYG	red orange	yellow yellow - orange	negative (red) negative (red) negative (red) negative (red) negative (red) positive (yellow) negative (red) negative (red) negative (red)
CAT	no bubbles	bubbles	positive (bubbles)

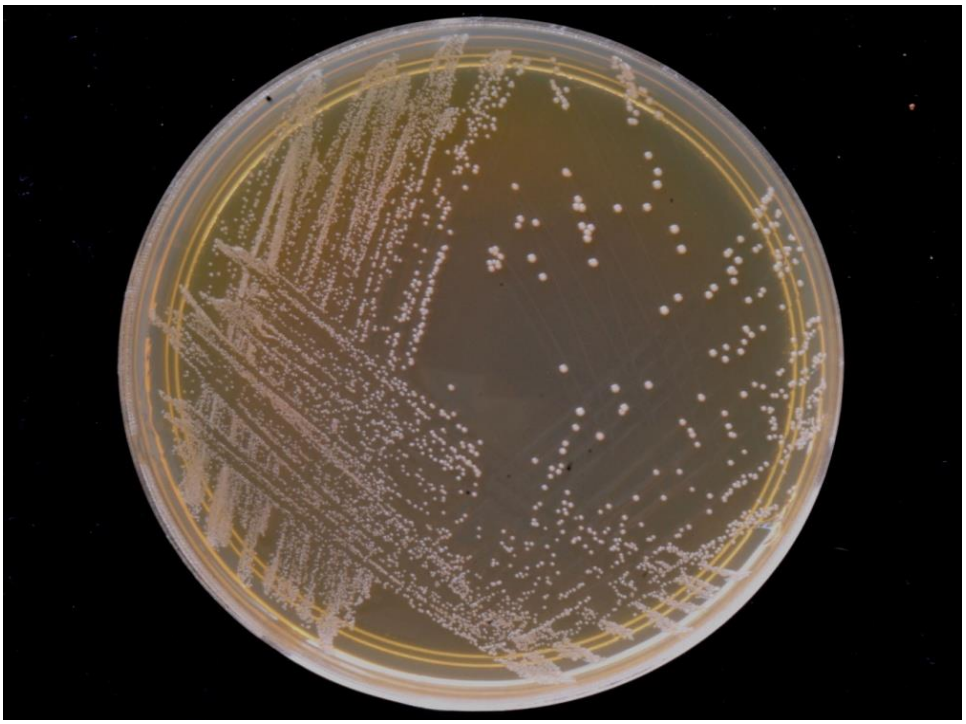


Figure (3-3): Bacterial colonies after growth on TSA at 30 °C for 2 days.

The phylogenetic tree was drawn from 16S rRNA sequence of the bacterial isolate for the present study as in Figure (3-6) and the blast analysis revealed a 99% identity to the complete sequence of 16S rRNA gene of *R. equi* strain DSM20307 (accession no. NR041910.1), followed by 98% identity to the complete sequence of 16S rRNA of *R. opacus* B4 strain B4 (accession no. NR074632.1), and followed by 98% identity to a partial sequence of 16S rRNA of *R. wratslaviensis* strain NCIMB 13082 (accession no. NR026524.1). The fourth closest identity was 97% identity to the complete sequence of 16S rRNA *Nocardia farcinia* IFM 10152 strain IFM (accession no. NR074702.1) as in Table (3-3).

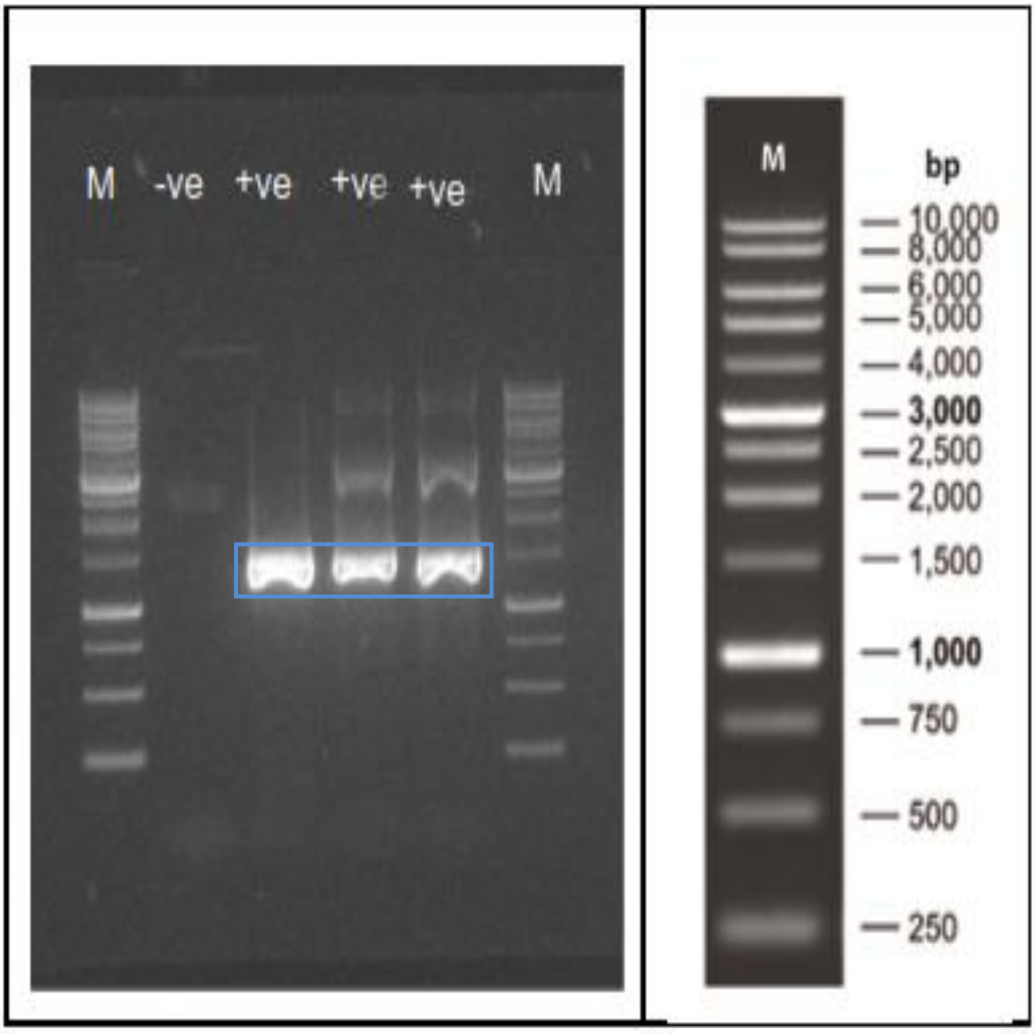


Figure (3-4): Electrophoresis of PCR products for 16S rRNA with size 1477bp in comparison with marker.
(+ve): duplicates for PCR products.

GGCTTCGGGTGTTACCGACTTTCATGACGTGACGGGCGGTGTGTACA
AGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTA
GCGACTCCGACTTCACGGGGTCGAGTTGCAGACCCCGATCCGAACTG
AGACCGGCTTTAAGGGATTCGCTCCACCTCACGGTATCGCAGCCCTC
TGTACCGGCCATTGTAGCATGTGTGAAGCCCTGGACATAAGGGGCAT
GATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAGT
CTCCTGCGAGTCCCCACCATTACGTGCTGGCAACACAGGACAAGGGT
TGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGAC
GACAGCCATGCACCACCTGTACACCGACCACAAGGGGGGCCGTATC
TCTACGGCTTTCCGGTGTATGTCAAACCCAGGTAAGGTTCTTCGCGTT
GCATCGAATTAATCCACATGCTCCGCCGCTTGTGCGGGCCCCCGTCA
ATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCGC
TTAATGCGTTAGCTACGGCACGGATCCCGTGGAAGGAAACCCACAC
CTAGCGCCCACCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTG
TTCGCTACCCACGCTTTCGCTCCTCAGCGTCAGTTACTGCCCAGAGA
CTCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGC
TACACCAGGAATTCCAGTCTCCCCTGCAGTACTCAAGTCTGCCCGTA
TCGCCCGCACGCTCGGGGTTGAGCCCCAAGTTTTCACGGACGACGCG
ACAAACCGCCTACGAGCTCTTTACGCCCAGTAATTCCGGACAACGCT
CGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTGGCCGGTGC
TTCTTCTGCAGGTACCGTCACTTGCGCTTCGTCCCTGCTGAAAGAGGT
TTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGG
CTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC
TGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCGCCCTCTCAGGCCGG
CTACCCGTCGTCGCCTTGGTAGGCCATTACCCCACCAACAAGCTGAT
AGGCCGCGGGCCCATCCTGCACCAGTAAACCTTTCCAACCCCCACCA
TGCAGTGGAGGCTCATATCCGGTATTAGACCCAGTTTCCCAGGCTTA
TCCCAGAGTGCAGGGCAGATCACCCACGTGTTACTCACCCGTTCGCC
GCTCGTGTACCCCGAAGGGCCTTACCGCTCGACTTGCATGTGTTAAG
CACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTCT

Figure (3-5): 16S rRNA sequence with size 1477 bp.

Table (3-4): Top 4 hits blast results of 16S rRNA sequences for PHB producing isolate.

Description	Max score	Total score	Query cover	Ident.	Accession
Rhodococcus equi strain DSM 20307 16S ribosomal RNA gene, complete sequence	2540	2540	98	99	NR041910.1
Rhodococcus opacus B4 strain B4 16S ribosomal RNA, complete sequence	2500	2500	100	99	NR074632.1
Nocardia farcinia IFM 10152 strain IFM 10152 16S ribosomal RNA complete sequence	2488	2488	100	97	NR074702.1
Rhodococcus wratislaviensis strain NCIMB 13082 16S ribosomal RNA gene, partial sequence	2488	2488	98	98	NR026524.1

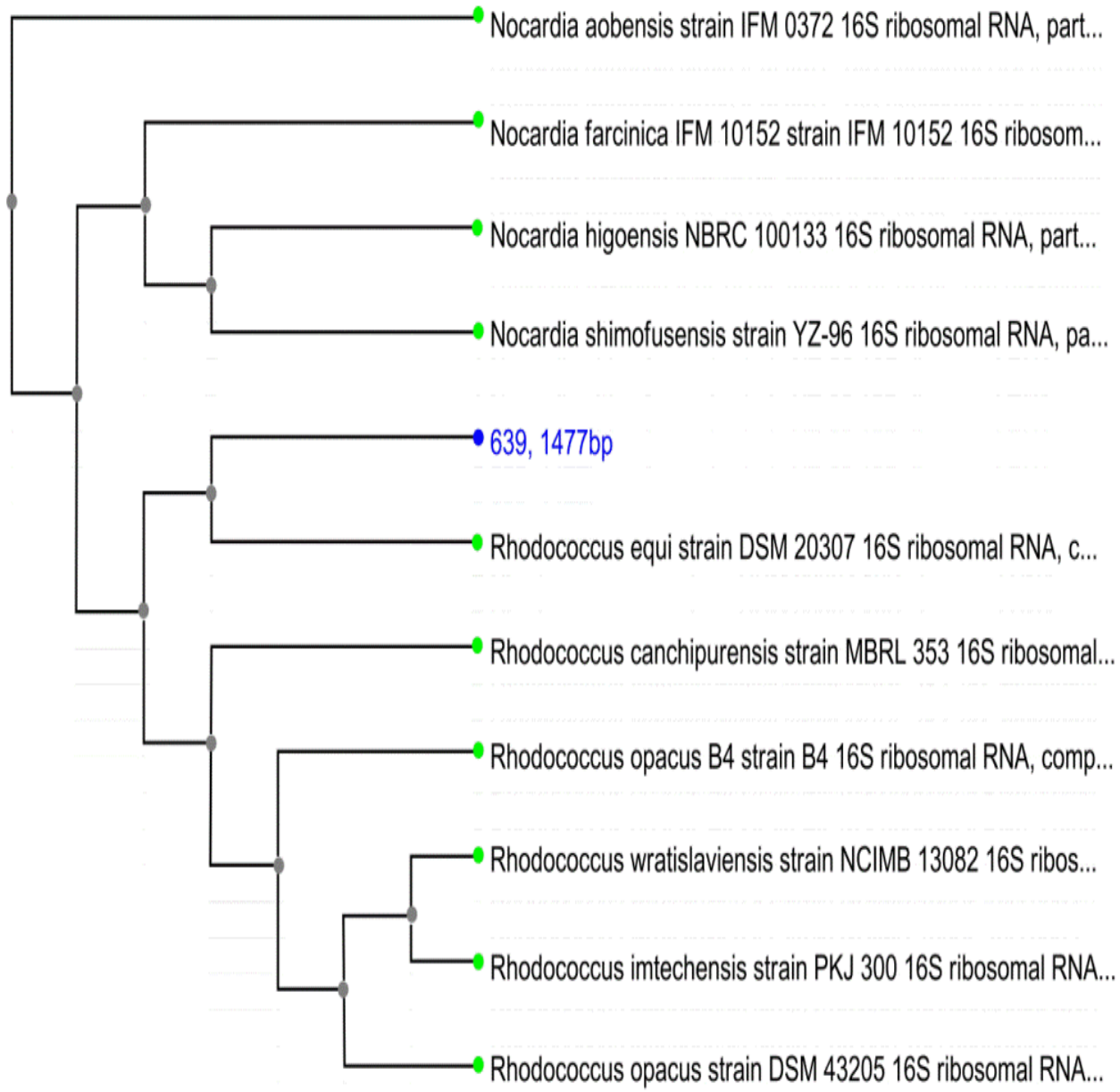


Figure (3-6): Phylogenetic tree based on 16S rRNA showing the similarity relationships between the isolate of the present study (1477bp) and related species. It is appeared closest to *R. equi* strain DSM2030 16S rRNA (accession no. 041910.1).

3.4. Biosynthesis of polyhydroxyalkanoates by *Rhodococcus equi* using different carbon sources

Various carbon source substrates were chosen to study their effects on the bacterial growth, PHB accumulation and the total PHB content using *R. equi* followed isolation and identification. The composition and the content of biopolymers were quantified by the use of GC and the results obtained are summarized in Table (3-4) and represented in Figure (3-7). The highest bacterial growth (CDW = 1.63 g/L) was obtained with significant effect when glycerol was used as the carbon source, followed by sodium gluconate (CDW=1.45g/L) and CPKO (CDW= 1.43 g/L) with significant effect. While the highest PHB accumulation (PHB content =38.07 wt%) was seen significant effect when CPKO was used followed by glucose (PHB content =4.11 wt%) and the highest total PHB was obtained by CPKO (0.55g/L) with a significant effect.

The most important factor in the PHB biosynthesis is the reduction of the production cost and therefore, the carbon source substrates have to be inexpensive and commercially available (Sudesh and Iwata, 2008). It could be concluded in the present study that *R. equi* gave the maximum production with a significant effect for the CDW, PHB accumulation and total PHB together when CPKO was used as a carbon source compared to other carbon sources used as seen in Figure (3-7) means that CPKO was the best carbon for PHB biosynthesis by *R. equi*, which is rich in the saturated and unsaturated fatty acids for the synthesis (Bhubalan *et al*., 2010; Sudesh, 2013). CPKO was used as a potential carbon source with different calculated values for PHB accumulation by *Ralstonia eutropha* (Jain *et al*., 2013). Moreover, Wong and co-workers have used CPKO as a carbon source for the production of P(3HB-*co*-3HHx) copolymer containing a high 3HHx monomer proportion using recombinant *C. necator* (Wong *et al*., 2012). The results reported by Pieper and

Steinbiichel (1992) for the production of PHB using *Rhodococcus rubber* in the presence of fructose as a carbon were close to the ones reported in this study.

Table (3-5): Biosynthesis of PHAs from different carbon sources using a one-stage cultivation of the MM medium by *R. equi* at 30 °C for 2 days.

Substrates	CDW (g/L)	PHA content (wt%)	Total PHA (g/L)	PHA composition (mol%)	
				3HB	3HV
Glucose	0.60± 0.06	4.11±0.70	0.023 ±0.004	100	0
Glucose+ SV	0.90±0.02	2.23±1.15	0.02±0.01	98	2
CPKO	1.43±0.24	38.07±0.69	0.55±0.09	100	0
CPKO+ SV	0.56±0.04	3.98±1.09	0.02±0.01	73	27
Glycerol	1.63±0.60	2.43±0.11	0.04±0.01	100	0
Sodium gluconate	1.45±0.05	0.32±0.06	0.01±0.001	100	0
Olive oil	0.87±0.58	2.06±0.20	0.02±0.01	100	0
PAO	0.43±0.04	4.57±0.68	0.02±0.004	100	0
Palm oil	0.14±0.03	1.77±0.37	0.002±0.001	100	0
(WCO)	0.33±0.04	0.98±0.15	0.003±0.001	100	0

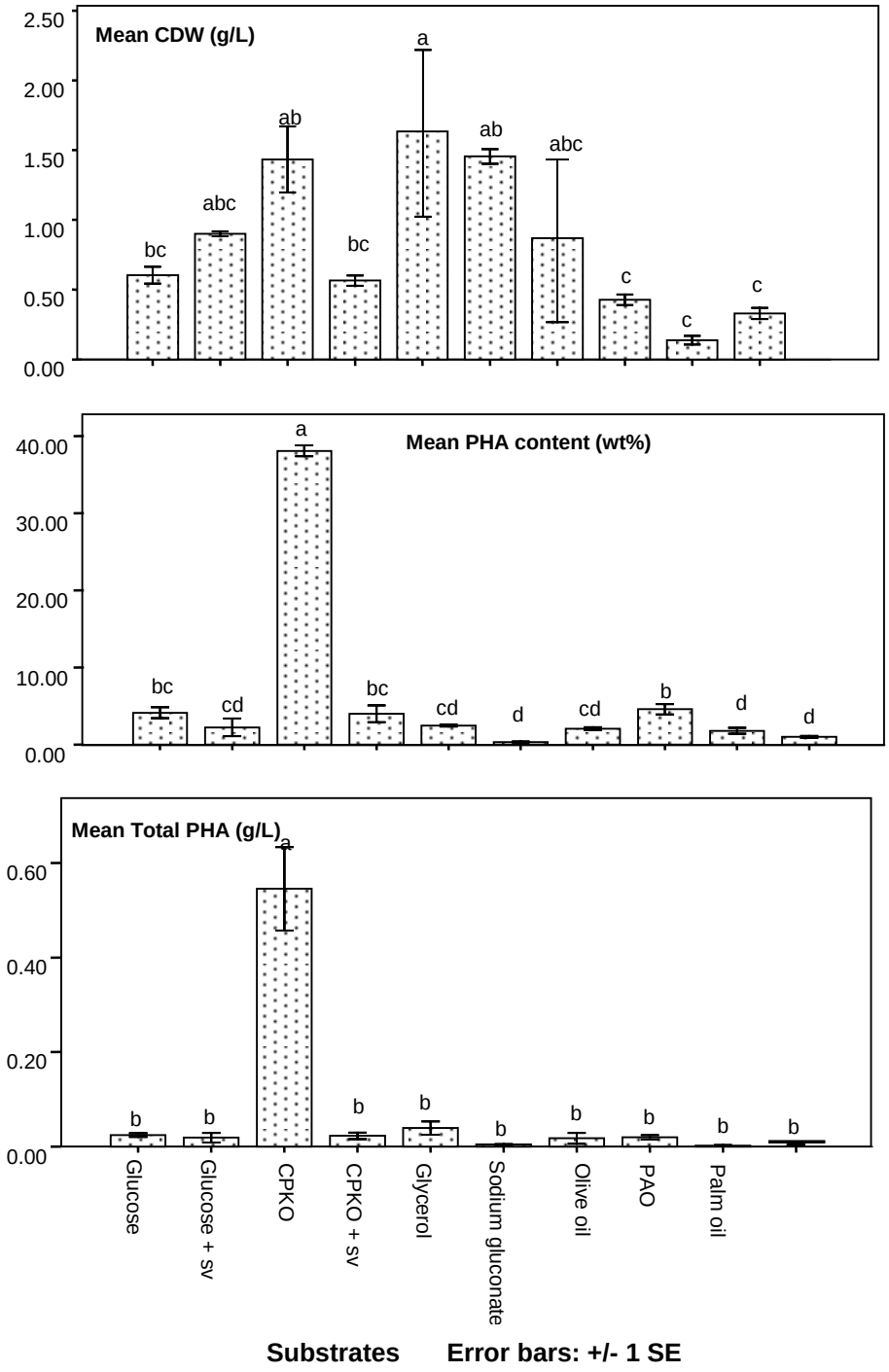


Figure (3-7): Effect of different carbon sources on CDW, PHA content and total PHA of *R. equi* with the significant and none significant effect. Data shown are means from triplicates. Means in the same column with different superscripts are significantly different (Duncan test, $p < 0.05$).

3.5. Chemical structure

The pure extracted PHB polymer that appeared whitish as in Figure (3-8) was used to study the chemical structure by NMR analysis to determine the quality of PHB structural composition. The ^{1}H NMR spectra obtained from PHB sample produced from *R. equi* in presence CPKO 1% as carbon source was shown in Figure (3-9). Both peaks observed in the spectra coincide, corresponding to the different types of carbon atoms in the PHB structure. The spectrum shows a doublet at 1.30 ppm, which is attributed to the methyl group coupled to one proton. The doublet of quadruplet at 2.57 ppm was attributed to the methylene group adjacent to an asymmetric carbon atom bearing a single atom. The multiplet at 5.27 ppm was a characteristic of methylene group. Two other signals were observed, a broad one at 1.30 ppm which was due to water and another one at 7.28 ppm attributed to the solvent used, i.e. chloroform. Identical observations were earlier reported by (Bonthrone *et al.*, 1992; Jan *et al*., 1996).

The ^{13}C NMR analysis also confirmed the structure of PHB polymer extracted by *R. equi* [Figure (3-10)]. The four peaks were assigned to the methyl (CH3) at 19.65 ppm, methylene (CH2) at 40.66 ppm, methane (CH) at 67.77 ppm and carbonyl (C=O) at 169.162 ppm. This chemical shift signals of ^{13}C NMR spectrum of recovered PHB from *R. equi* was identical to the results of the commercial PHB (Fabiane *et al*., 2007) as shown in Table (3-5).

Table (3-6): Chemical shift signals obtained the ^{13}C NMR spectra for PHB sample and commercial PHB, compared to the results by (Fabiane *et al.*, 2007).

C atom	PHB sample	PHB (Fabiane *et al.*, 2007)
CH3	19.65	19.65
CH2	40.66	40.66
CH	67.77	67.77
C=O	169.162	169.03

Figure (3-8): PHB polymer extracted from *R. equi.*

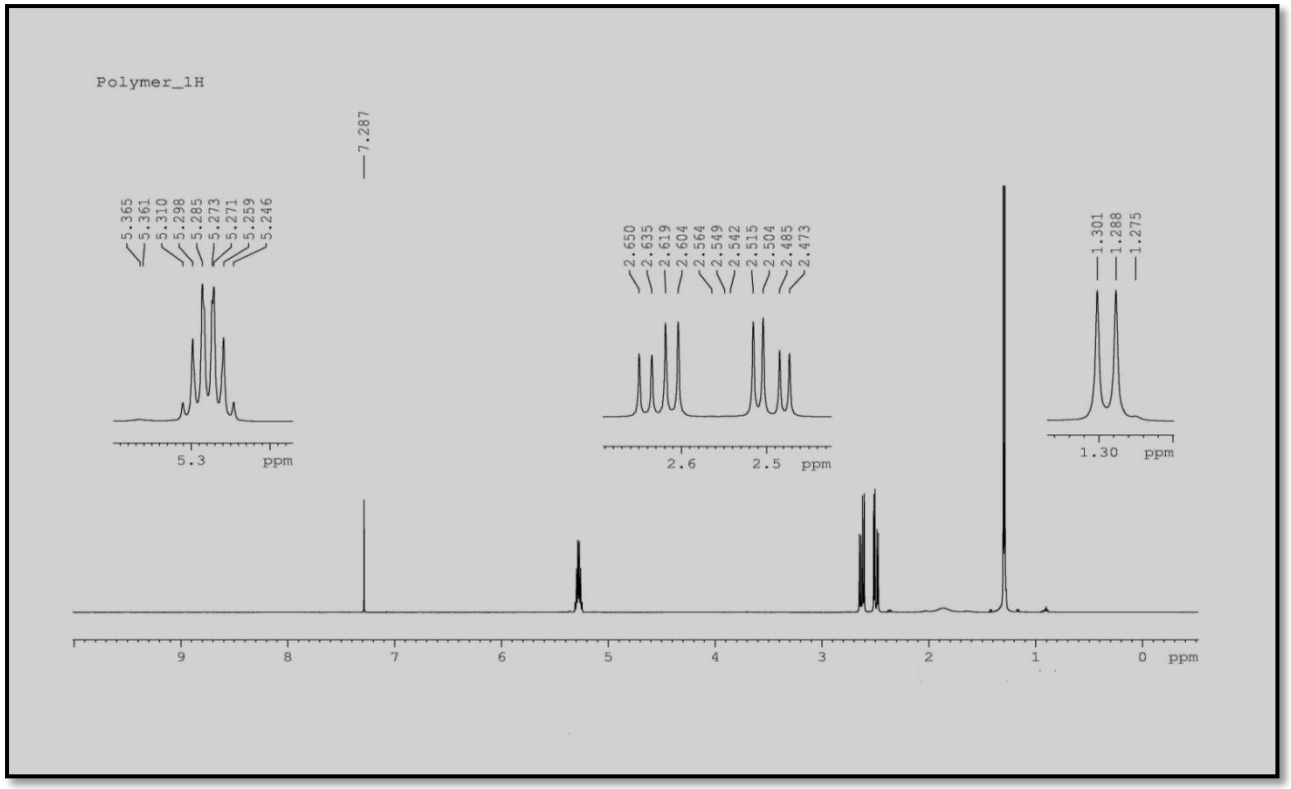


Figure (3-9): ^{1}H NMR spectrum of PHB polymer produced by *R. equi* using CPKO as a carbon source.

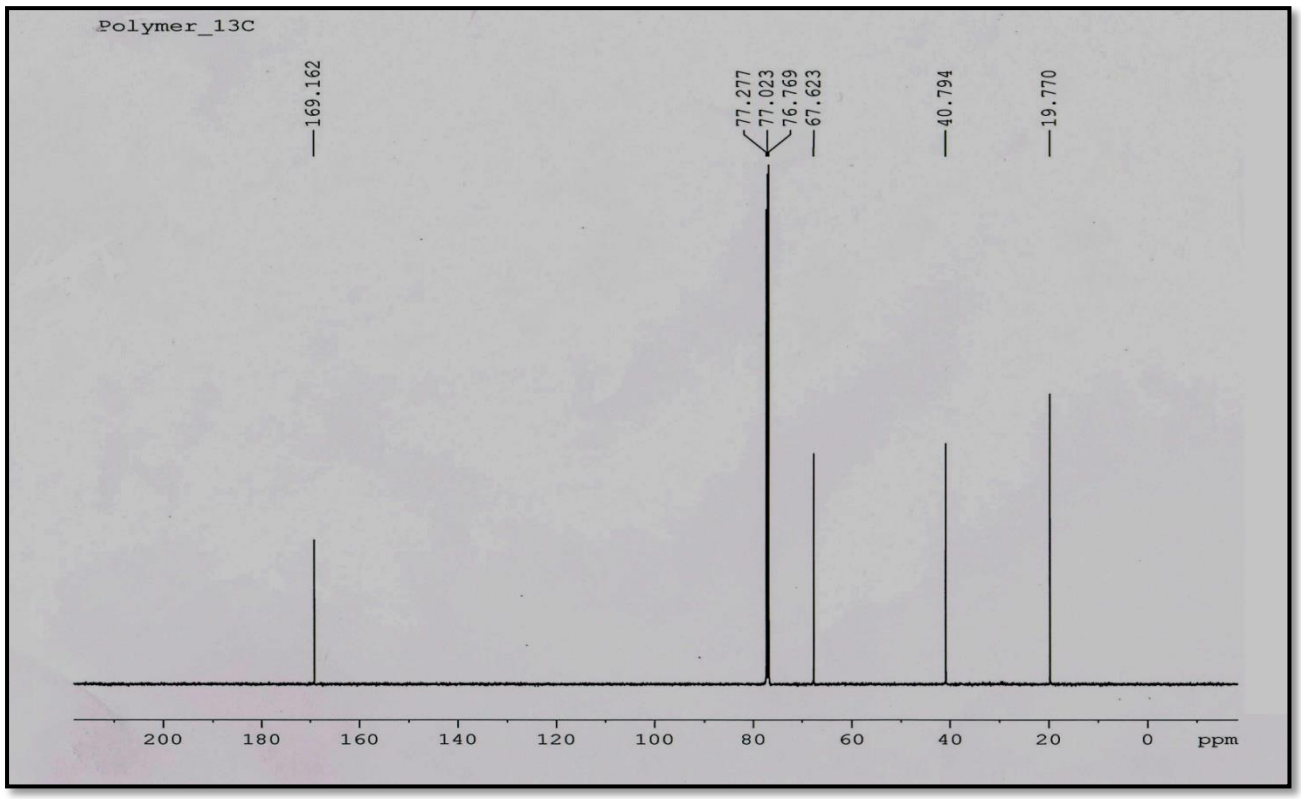


Figure (3-10): ^{13}C NMR spectrum of PHB polymer produced by *R. equi* using CPKO as a carbon source.

3.6. Thermal properties

Figure (3-11) and Figure (3-12) show the thermal properties of extracted PHB investigated by (DSC) and (TGA). The results are listed in Table (3-6) showed that the melting temperature (T_m) of the extracted PHB using CPKO as a carbon source was 173 °C. These results were supported by the publications of Erceg and co-workers (2005) and Marjadi and Dharaiya (2014). The glass transition temperature (T_g) was in the range mentioned by a previous study (Candola *et al*., 1988) which was equal to 2.79 °C. The decomposition temperature (T_d) was approximate to 276 °C. These values were close to the results of PHB produced by *Bacillus sphaericus* NII 0838 (Sindhu *et al*., 2011) that is mainly associated with the ester cleavage of the PHB component by ß-elimination reaction (Choi *et al*., 2003). The thermal properties of PHB in the present study were in good agreement with the results of PHB produced from *Cupriavidus necator* cells when used cooking oil as a carbon source (Martino *et al.*, 2014).

Table (3-7): Thermal properties of PHB produced by CPKO 1% as carbon source.

Term	Value
T_m	173 °C
T_g	2.79 °C
T_d	276 °C

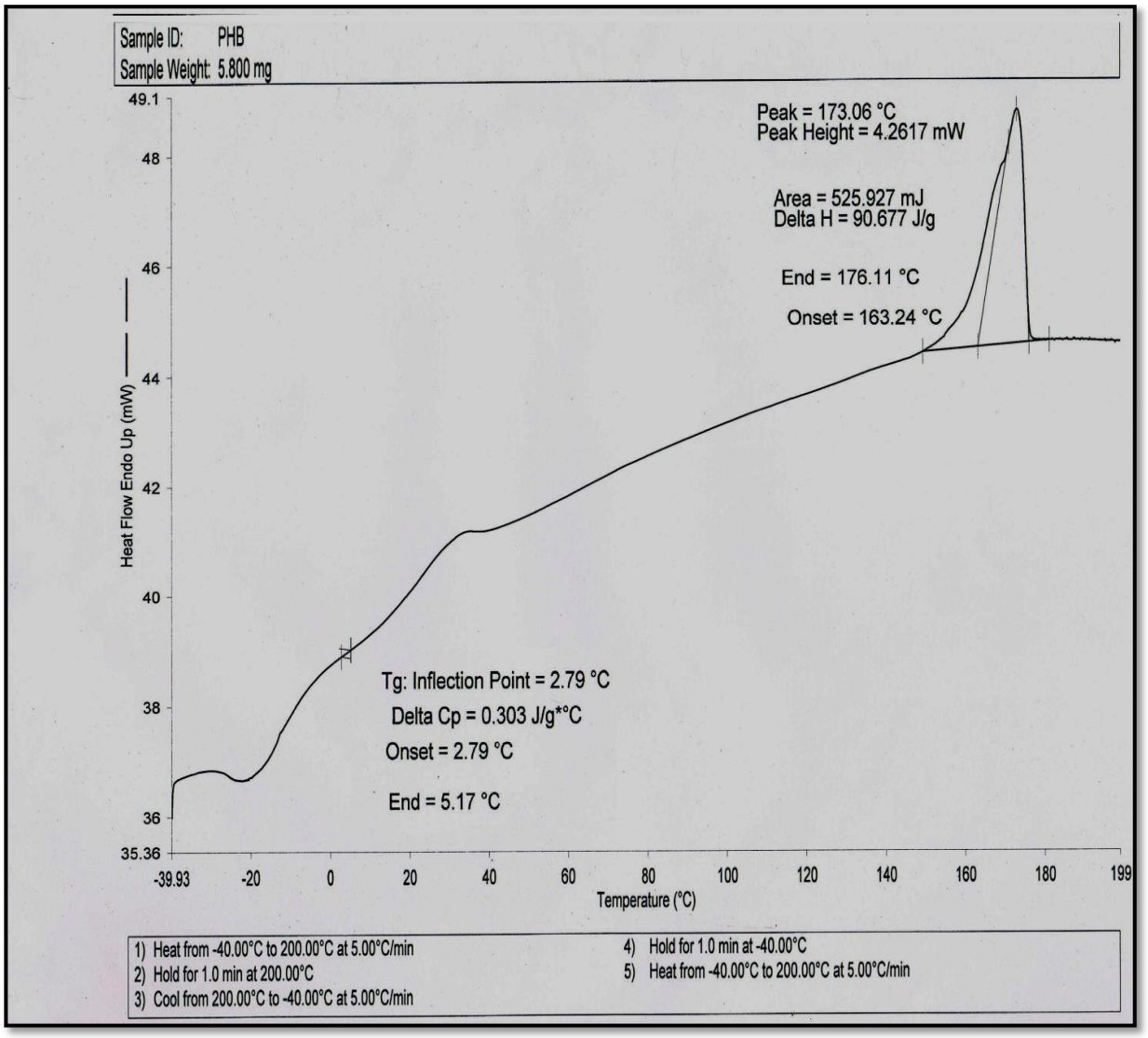


Figure (3-11): Melting temperature (T_m) and glass transition tempareture (T_g) of PHB produced by *R. equi* using CPKO as a carbon source.

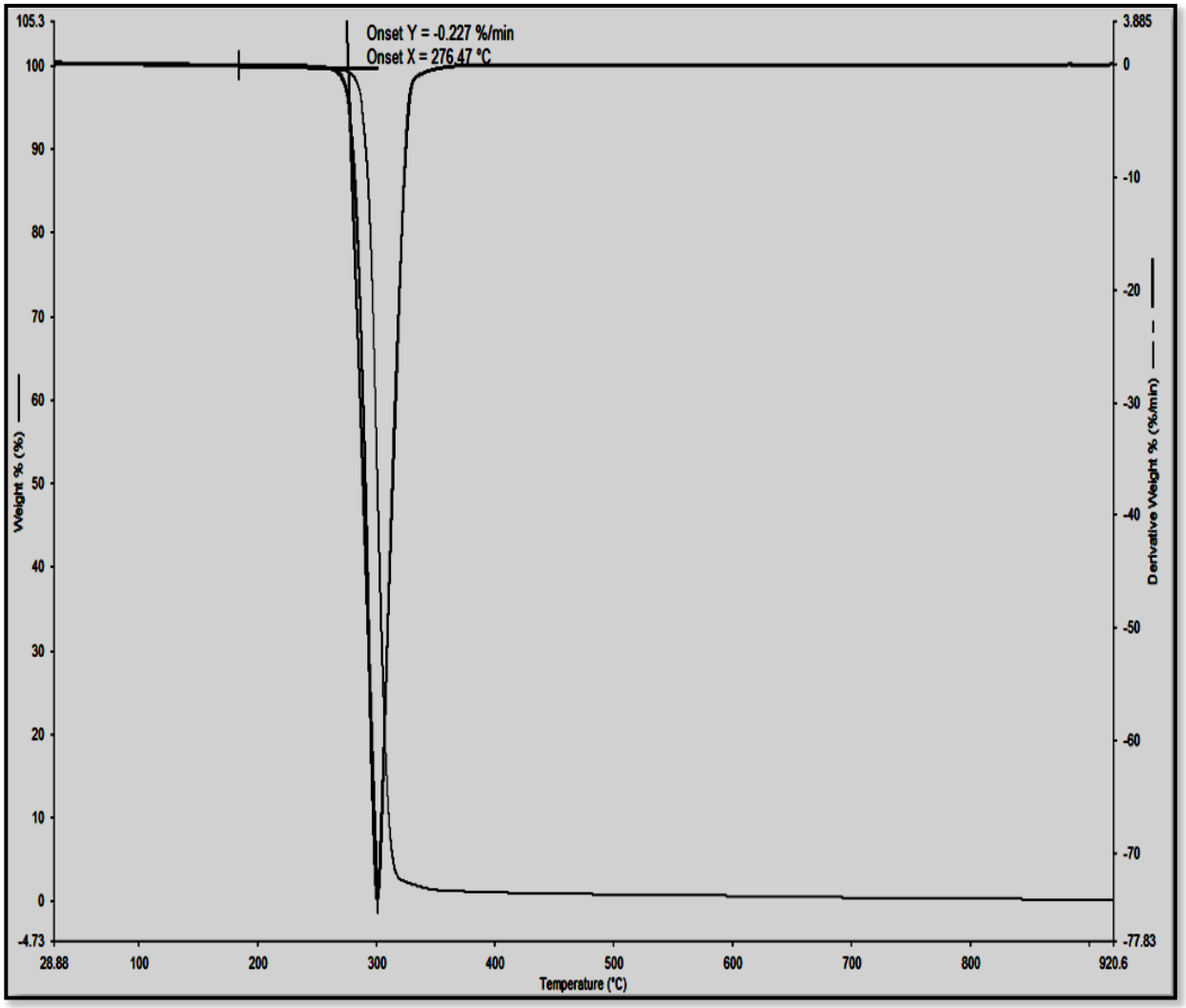


Figure (3-12): Decomposition temperature (T_d) of PHB produced by *R. equi* using CPKO as a carbon source.

3.7. Molecular Mass

The gel permeation chromatography (GPC) was used for estimating the molecular mass of PHB sample, since molecular mass is an important factor to determine physical properties of polymers (Anderson and Dawes, 1990). GPC was used for the determination of molecular weight ($\overline{M}_w$), weight number average mass ($\overline{M}_n$) and polydispersity ($\overline{M}_w/\overline{M}_n$) of polymer. The molecular mass of PHB recovered from *R. equi* cells was 642 KDa, weight number average mass was 373 KDa and polydispersity was 1.72.The results of GPC for

PHB of *R. equi* were listed in Table (3-7) in comparing with the results of other studies.

Table (3-8): Molecular mass of the extracted PHB in comparison with the results of other reports: weight average ($\overline{M}_w$), number average ($\overline{M}_n$) and polydispersity ($\overline{M}_w/\overline{M}_n$) determined by GPC.

Polymer	Producing bacteria	($\overline{M}_w$) (KDa)	($\overline{M}_n$) (KDa)	($\overline{M}_w/\overline{M}_n$)	References
PHB sample	*R. equi*	642	373	1.72	This study
PHB (soy)	*Cupriavidis necator*	790	348.8	2.26	(Fabiane *et al.*, 2007)
PHB (molasses and corn steep liquor)	*Bacillus megaterium* ATCC 6748	3900	2651	1.47	(Chaijamru and Udpuay, 2008)
PHB (pulp industry waste)	*Bacillus subtilis* MSBN17	640	380	1.68	(Salhiyanarayanan *et al.*, 2013)
PHB-co-PHAV(bagasse extract)	*Halomonas campisalis*	1394	838.5	1.66	(Kulkarni *et al.*, 2015)

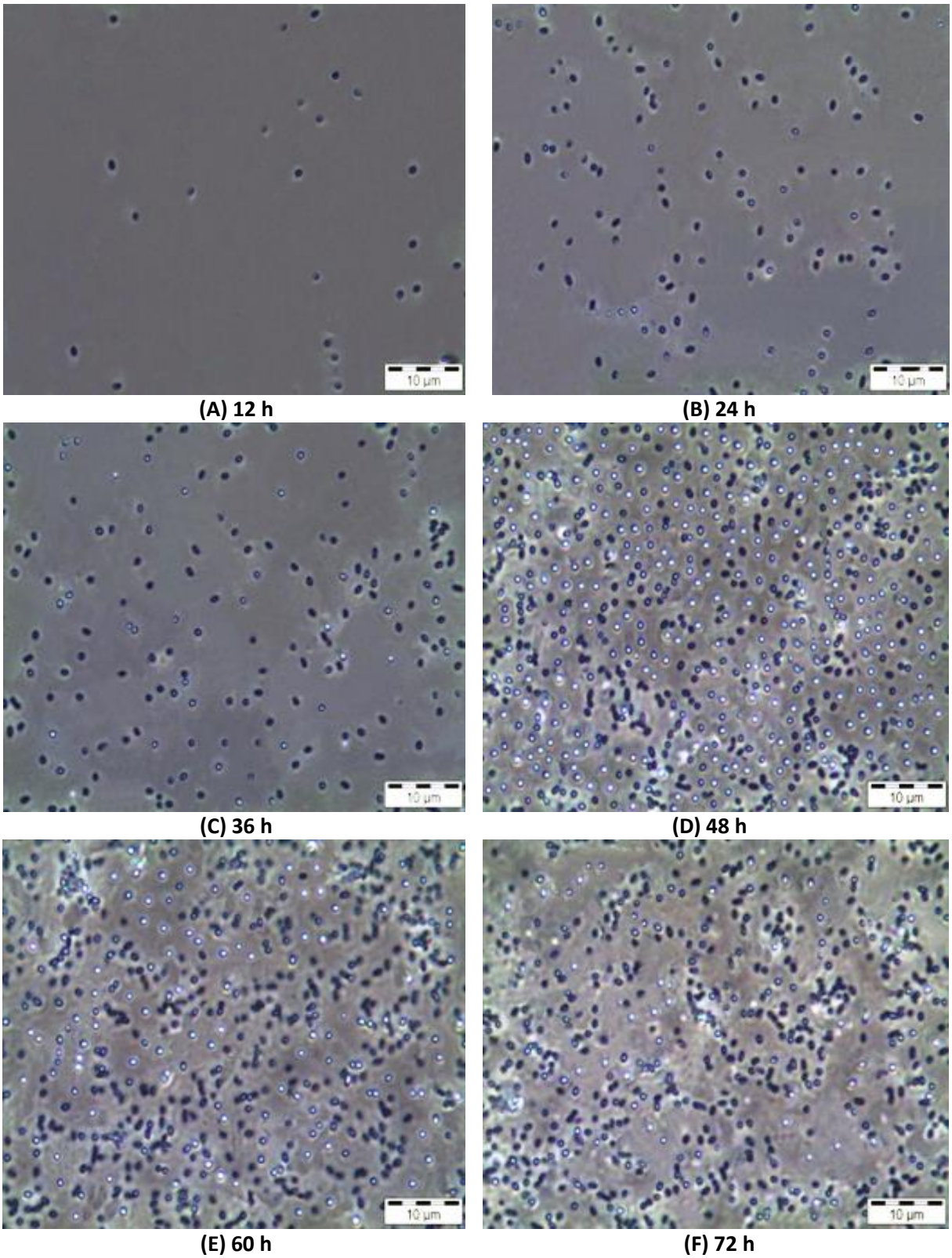
The molecular mass of PHB in the Table (3-7) guides us to the conclusion that the molecular mass of PHB must be affected adversely by microbes, carbon sources in addition to the methods of extraction and the environmental conditions. This results were in close to the results of PHB polymer from marine *Bacillus subtilis* MSBN17 (Sathiyanarayanan *et al.*, 2013), and molecular weight was within the range less than 10 $\times 10^5$ Da that qualifies it to be used in commercial applications (Dio, 1990). It has been reported in the another study that the molecular weight of PHB polymer was

affected by PHA synthase and the high activity of PHA synthase lower the molecular weight of biopolymer (Rehm, 2003).

3.8. Microscopic observations of bacterial morphology with polyhydroxybutyrate granules

The bacterium isolate was cultured in MM broth flasks with shaking at 30 °C for different times using CPKO as the good carbon source. The PHB granules aggregated within the bacterial cells had a bright appearance under phase contrast light microscope. The number of cells with PHB granules has been increased with increasing time as shown in Figure (3-13) and was higher after 48 h.

By fluorescent microscope using Nile Blue A staining for bacterial cells with the preferable PHB conditions, the cells illustrated with bright orange fluorescent indicate the PHB accumulation inside cells as in Figure (3-14). Also, the TEM micrograph confirmed the PHB accumulation. It was found that most of PHB granules were spherical, ovoid and elongated in shape in which number and size of the granules within the cells are highly dependent on the bacterial species, growth and environmental conditions (Uillaguamán *et al.*, 2005). The TEM showed that the cells having white granules with different sizes that ranged from small to large and different number of granules (3–11) for each cell as shown in Figure (3-15). This could be due to the effect of concentration of synthase that leads to the formation of several small granules and as a result shorter PHB chains were produced (Bhubalan *et al*., 2011).



(A) 12 h (B) 24 h

(C) 36 h (D) 48 h

(E) 60 h (F) 72 h

Figure (3-13): Observation of *R. equi* under phase contrast light microscope (×1000) during production of PHB in MM medium supplemented with CPKO (1%) at 30 °C.

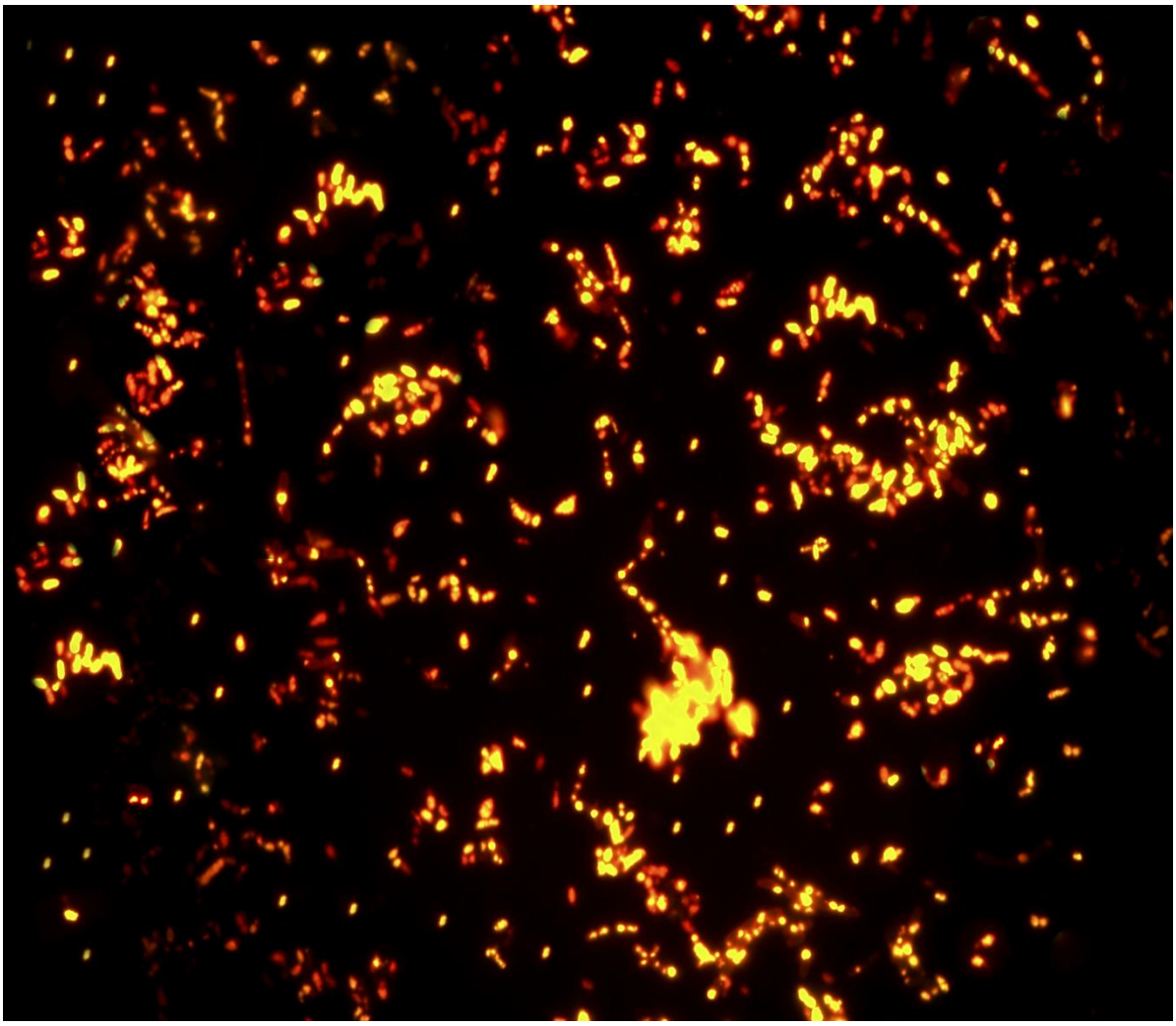
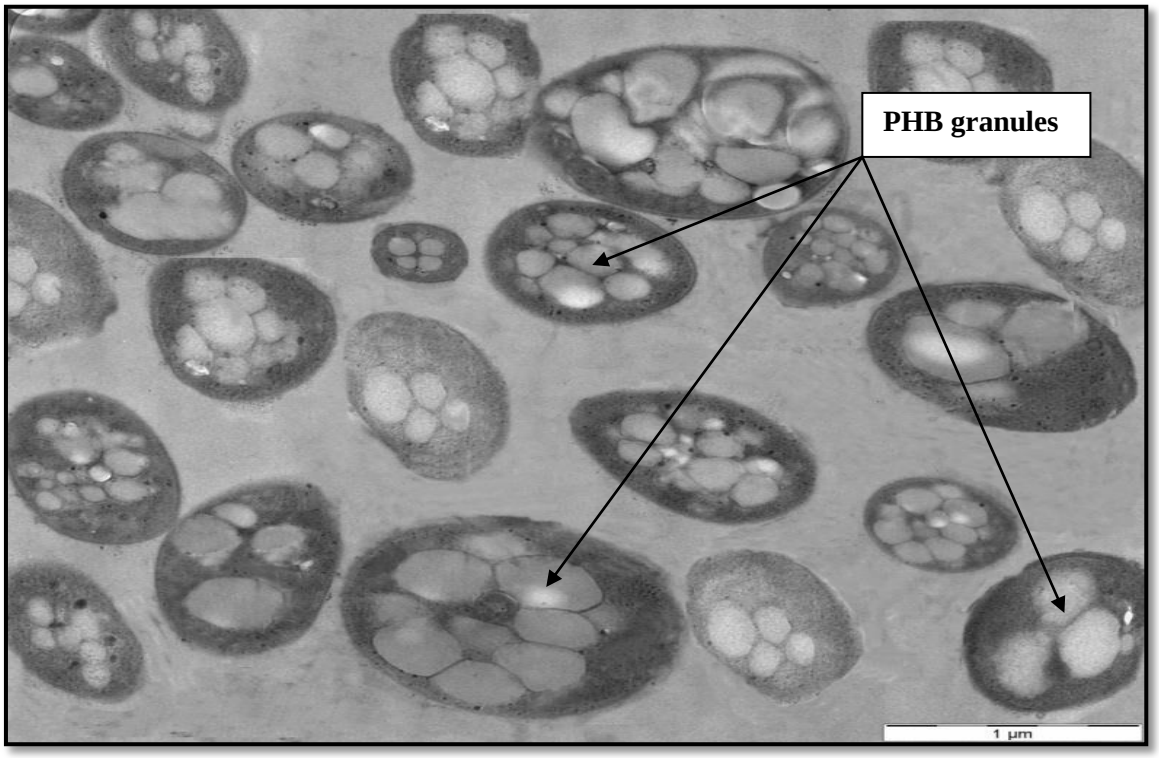
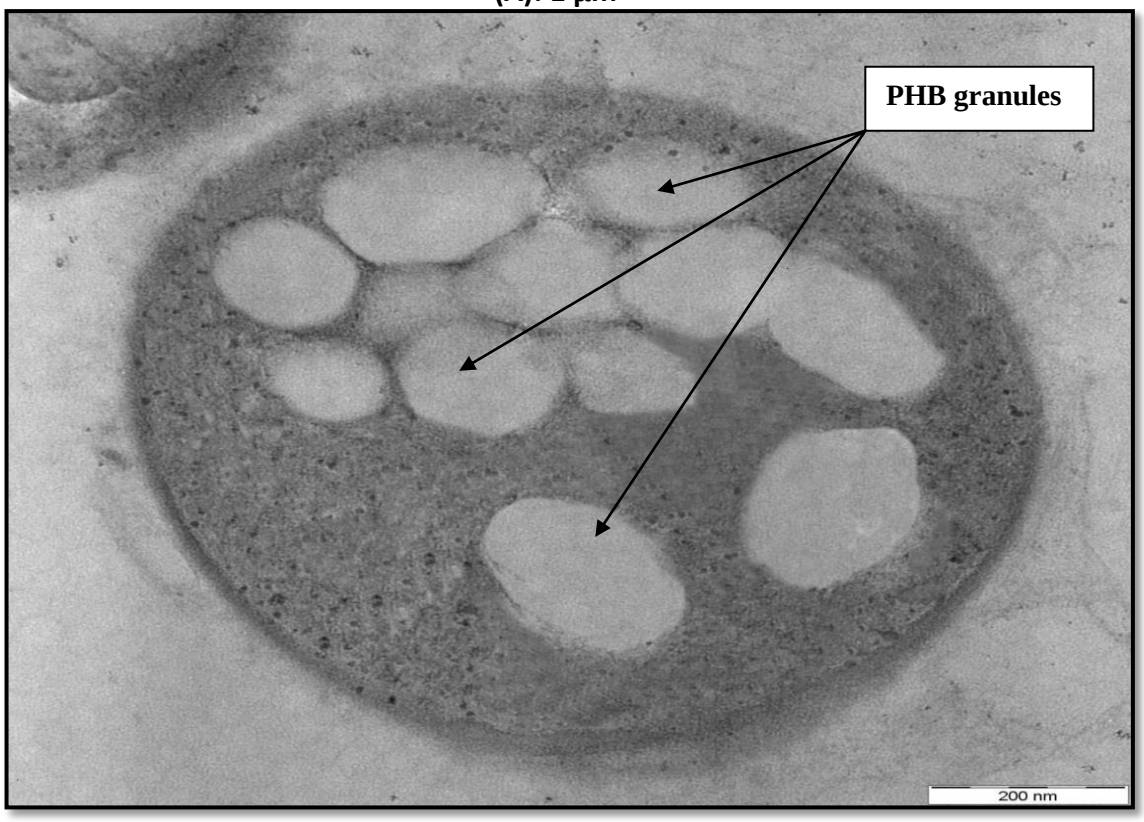


Figure (3-14): Observation of *R. equi* with PHB after growth in MM medium fed with CPKO 1 % under a fluorescent microscope using Nile blue A stain at 1000x magnification.



(A): 1 µm



(B): 200 nm

Figure (3-15): Observation of PHB granules inside *R. equi* under TEM after growth in MM medium fed with CPKO 1% at 30 °C for 2 days.

3.9. Amplification and sequencing of *phaC* gene

The *PhaC* gene was encoded to the PHA synthase enzyme which is the key enzyme of PHAs biosynthesis. The detection of *PhaC* gene reported the capacity to express the PHA synthase enzyme and the ability to accumulate and produce of PHA.

The electrophoresis of PCR product for *PhaC* gene as in Figure (3-16) demonstrated that the primers could be specifically used for the amplification of DNA fragment from *PhaC* gene. The duplicated bands in Figure (3-16) showed the *PhaC* gene with size approximately 508 bp.

The partial sequence of *PhaC* gene as in Figure (3-17) was blast to NCBI. The blast results presented in Table (3-8) showed that the *PhaC* gene of bacterial isolate of the present study was 78% similar to *PhaC* gene sequence of *Rhodococcus aetherivorans* (accession no. CP011341.1). This result established the presence of *PhaC* gene in *R. equi* using the primers which were used for cloning in other studies (Zhang *et al*., 2001). Also, the amplification of *PhaC* gene by PCR can be used as a simple and rapid method for screening the bacterial isolates to confirm the PHAs accumulation and production (Sheu *et al*., 2000).

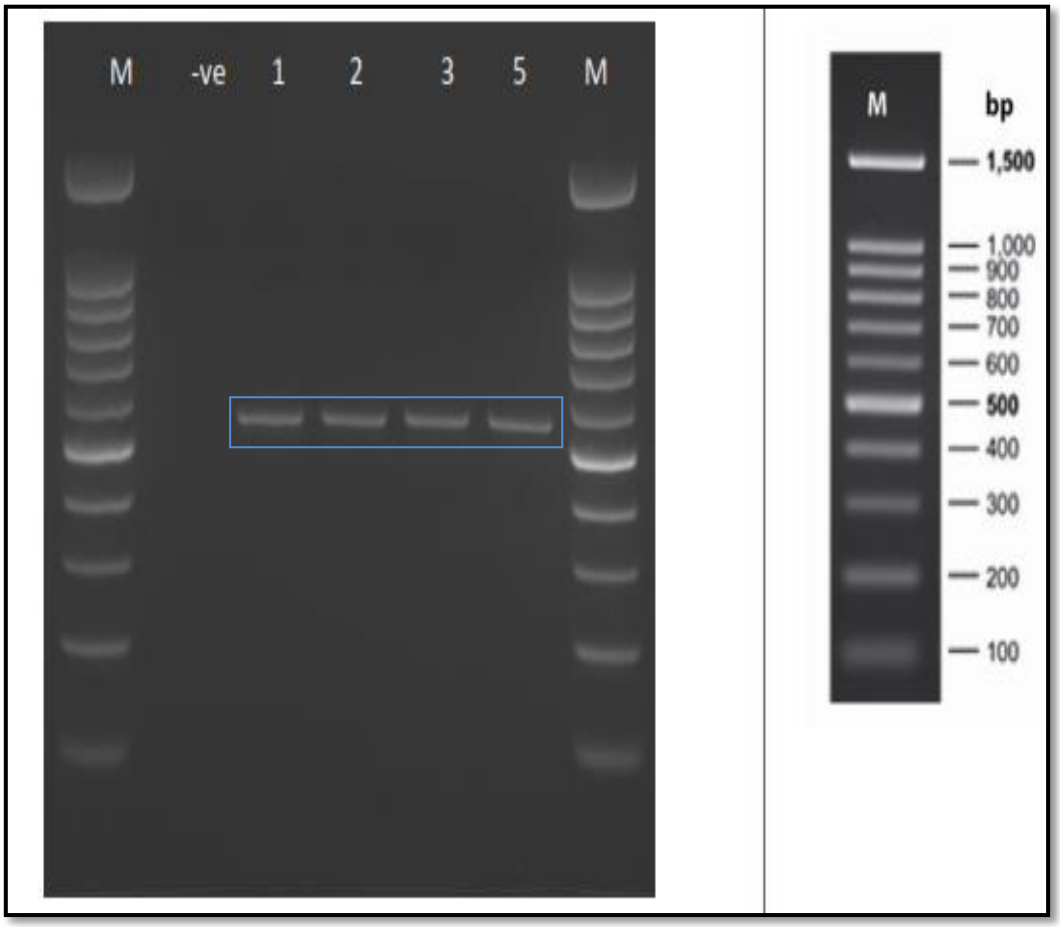


Figure (3-16): Gel electrophoresis of PCR product for *PhaC* gene.

TGTAATCGGCCCGGGTCGTAGCTTCGTGGATGGTCTGTGCGCCAAGG
CCGGACCGTCTTCCTCATCTCGTACCGGAACCCGGACGAGTCGATGA
GCGACATCGGCTTCGACGACTACCTCGTCGACGGGGTGTCGGCCGCG
ATGGACGTCGCCCTGGAGATCACGGGGGCGCCGACGATCGACCTCG
TCGGGCTGTGCCTCGGCGGTGCGATGGCGTCGATCACGGCCGGCTAC
CTGGCCGCCGTCGACGATCCCCGGATGGGCACGCTGACGCTGGCGA
ATACCCTTCTCGATTACAGTGATCCGGGCGAGCTGGGTCTGCTGACC
GATCCCGAGACGCTCGCCAAGGTCGACCTCGTGATGCAGCGGTCCG
GCTATCTGGACGGCGCGAACATGGGGCAGACGTTCGACCTGCTGCG
CGCCAACGACCTGATCTTCCGGTACTGGGTGAGCCGCTGGATGCTCG
GTGAGCCGCCGCAGGCGTTCGACCTCCTGTTCTGGAAAAAAA

Figure (3-17): Partial sequence of *PhaC* gene.

Table (3-9): Blast result of *PhaC* gene.

Description	Max score	Total score	Query cover	E value	Ident.	Accession
Rhodococcus aetherivorans strain IcdP1, complete genome	104	104	33%	1e-18	78%	CP011341.1
Rhodococcus aetherivorans I24 PHA synthase (*phaC*1) gene, complete cds	104	104	33%	1e-18	78%	HQ130734.1
R.ruber ORF1, phbCRr, ORF3 and ORF4	93.5	93.5	33%	3e-15	77%	X66407.1

3.10. Relative quantitative real time PCR

The RNA extraction was performed at three time points for the bacterial growth of *R. equi*, then viewed by a gel electrophoresis. Two bands were obtained indicated as 23S and 16S RNA as in Figure (3-18 a). The RNA at 3 time points was converted to cDNA and viewed by a gel electrophoresis, the cDNA appeared as smear as shown in Figure (3-19 b).

To study the expression level of *PhaC* gene, the conditions of the conventional PCR were optimized for the amplification of *PhaC* gene (target gene) and gryB (reference gene) and the same conditions were used for the real time PCR. The results of *PhaC* and *gyrB* genes after amplification were viewed by a gel electrophoresis as in Figure (3-19).

On the other hand, the standard curves of *PhaC* gene and *gyrB* gene as in Appendix (2) and Appendix (3) were established to measure the efficiency of amplification (E) for the target gene and the reference gene which were affected by many factors such as primer design, components of PCR reaction, amplicons

lengths, inhibitor presence and reaction conditions (Wong & Medrano, 2005), the efficiency (E) was calculated using the following formula $E = 10^{-1/slope}$. The real time PCR was chosen to evaluate the expression level of *PhaC* gene because it was the most sensitive method for quantitative analysis of certain gene (Muller *et al*., 2002).

The principle of relative expression calculation is based on the basic equation of Pfaffl method (Pfaffl, 2001; Yuan *et al*., 2006):

$$\textbf{Relative ratio (target/reference)} = \frac{E_{target}^{\Delta CTtarget}}{E_{ref}^{\Delta CTreferenc}}$$

Where as $\Delta CT(target) = CT_{(control)} - CT_{(sample)}$

$$\Delta CT(reference) = CT_{(control)} - CT_{(sample)}$$

This equation was used to calculate the expression level ratio of *PhaC* gene at growth phases depending on log phase as a control. It was concluded that the expression level of *PhaC* gene at lag phase ~1.16 folds, while at the stationary phase ~2 folds as shown in Figure (3-20).

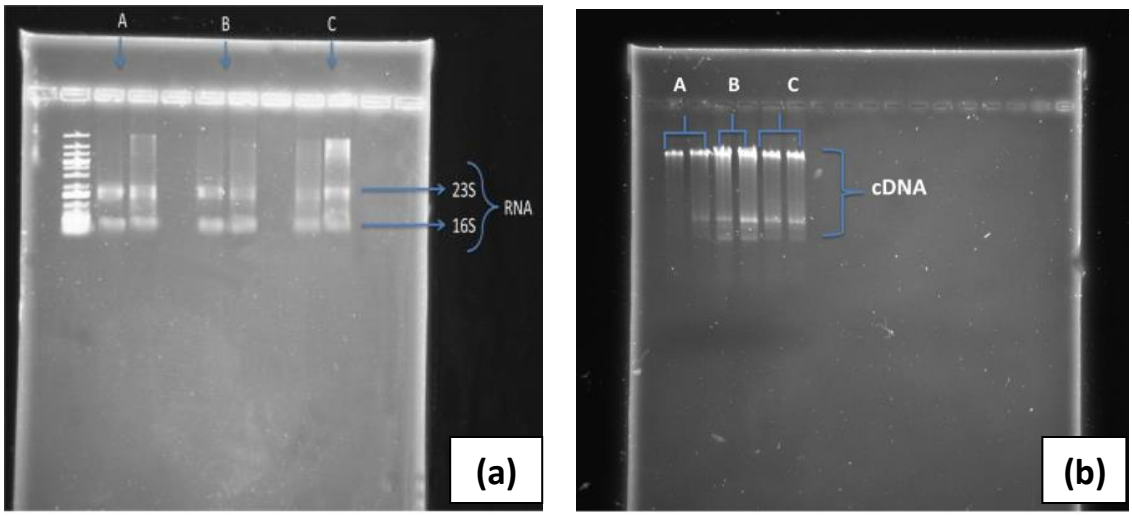


Figure (3-18): (a) Electrophoresis of RNA extracts at different time point with ladder marker 1kb showed 23S and 16S RNA. A- duplicate of RNA extract at lag phase B- duplicate of RNA at log phase C- duplicate of RNA extract at stationary phase (b) electrophoresis of RNA extracts after conversion to cDNA at lag phase (A), log phase (B) and stationary phase (C).

Figure (3-19): Electrophoresis of PCR results for the target gene and the reference gene.

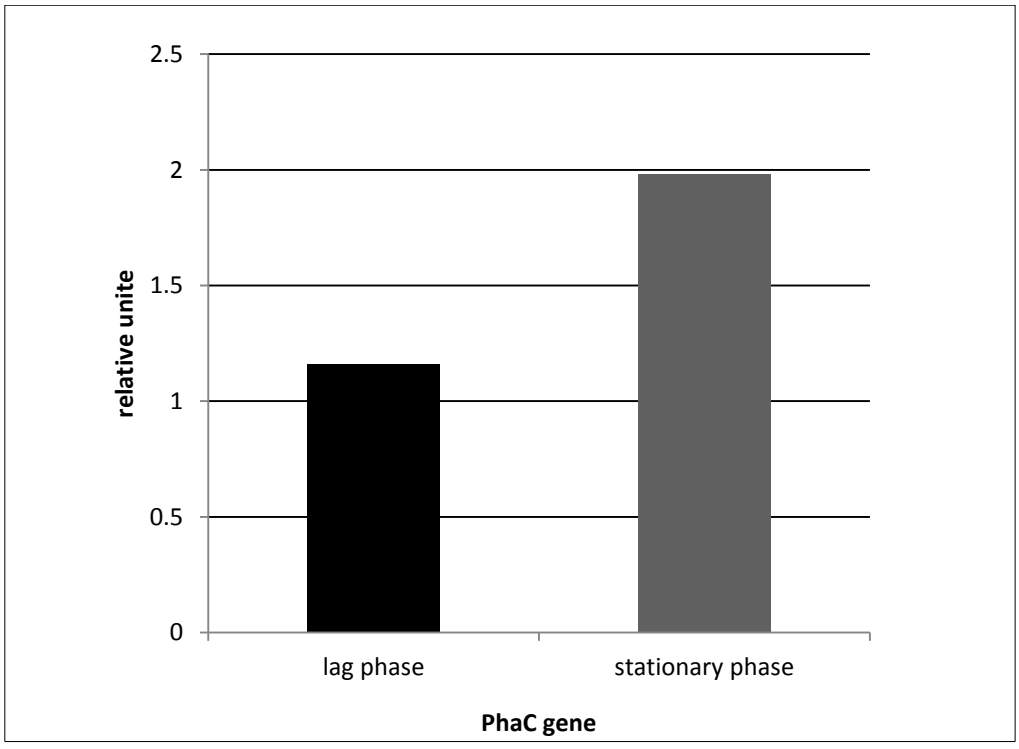


Figure (3-20): Relative expression ratio of *PhaC* gene.

3.11. Enzymatic activity of PHA synthase from *Rhodococcus equi*

To indicate the PHA synthase activity *in vitro*, the crude extract of *R. equi* cells were used to determine the synthase activity using 3-hydroxbutyrate-CoA as substrate during polymerization as in Figure (3-21). One unit of enzyme activity (U) was defined as the amount of enzyme required to catalyze the transformation of 1 µmol substrate per minute. The bacterial isolate *R. equi* having PHA synthase activity reached to 30.83 (U/mg protein) after the cultivation in MM medium for 48 hours at 30 °C. This result was close to the result recorded for another species of *Rhodococcus* (*R. Rubber*) reported with PHA synthase activity 34 (U/mg) (Pieper and *Steinbüchel*, 1992).

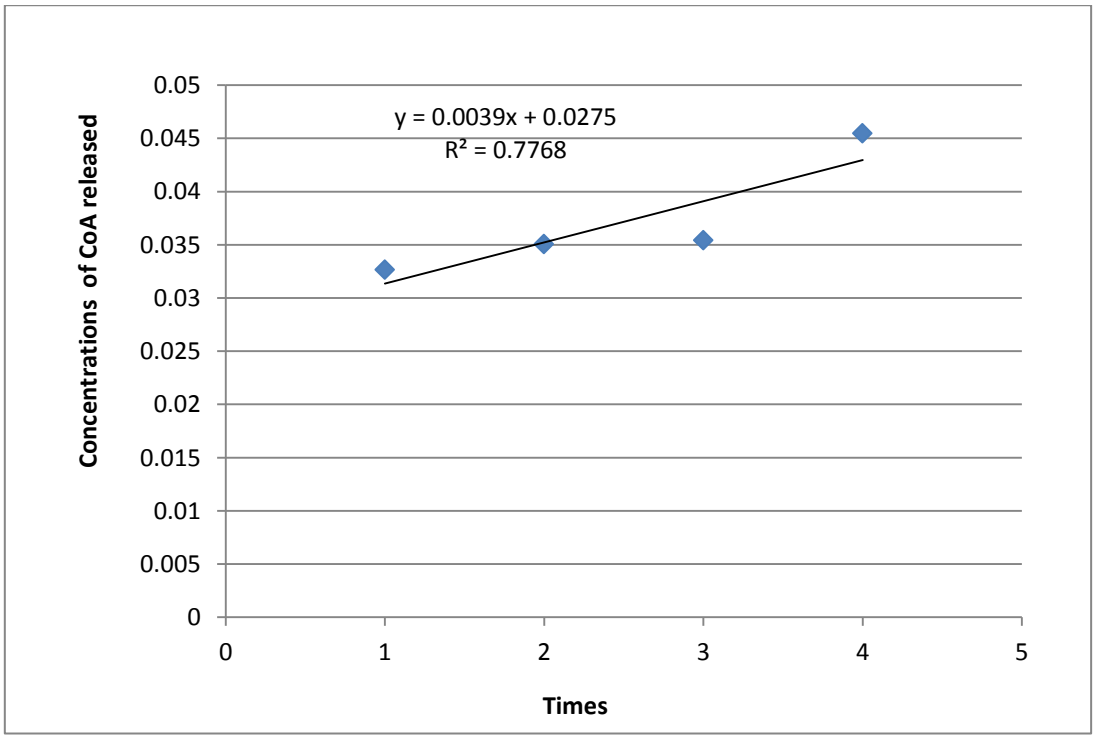


Figure (3-21): A linear graph of the CoA concentration (µmol) versus time that used to measure them value (slope) to calculate the PHA synthase activity (U/mg) for *R. equi* cultivated in 50 ml MM medium supplemented with 1% CPKO at 30 °C, 200 rpm in 250 ml flask for 48 hours.

3.12. Biodegradability of polyhydroxybutyrate films

The biodegradation was used to evaluate the degradation and disintegration by the enzymatic activity of living organisms, including bacteria, fungi and converted to CO_2 and H_2O under aerobic conditions and to CO_2, H_2O and methane under anaerobic conditions (Avella *et al*., 2005). Several factors that have effects on the biodegradation of polymer involved the environmental factors such as PH, nutrient supply, temperature, humidity and the microbial number and activity. Other factors related to the nature and composition of polymer films include crystanillity, surface area and the additive (Abou-Zeid *et al*., 2001: Boyandin *et al*., 2012).

As mentioned previously, all PHB films were prepared by the casting method, but the nanofiber films were prepared by electrospinning as in Figure (3-22) that shows the vertical set up of electrospinning.

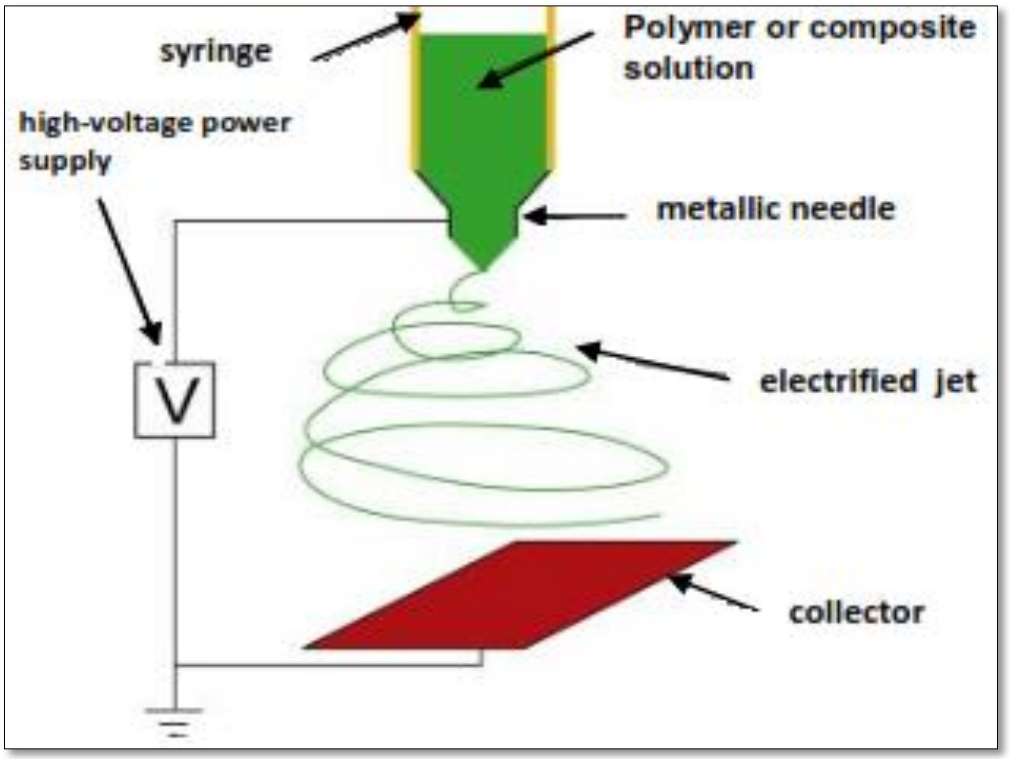


Figure (3-22): Vertical electrospinning of polymer solvent for preparing nanofibers (Li and Xia, 2004).

After the incubation time of degradation, the changes in molecular weight (Mw), the number-average molecular weight (Mn) and the polydispersity (Mw/Mn) for all polymeric films were measured by GPC. The degradation of all films was associated with a significant decrease in MW, Mn and Mw/Mn ratio. PHB films showed a more significant decreases in Mw and Mn, while, PHB nanofiber and PHB-TiO_2 composite nanofiber films showed a more significant decreases in Mw/Mn ratio. The results obtained were recorded in Table (3-9).

Table (3-10): Effect of soil degradation on the different type of PHB films after 6 weeks.

Treatment/ Films type	Before treatment			After treatment		
	MW (KDa)	Mn (KDa)	Mw/Mn	Mw (KDa)	Mn (KDa)	MW/Mn
PHB	642±0.45	373±0.02	1.72±0.13	396±0.19	275±0.06	1.54±0.11
PHB-TiO_2 composite	674±0.33	370±0.07	1.82±0.07	609±0.56	352±0.12	1.73±0.1
PHB nanofiber	554±0.02	421±0.16	1.79±0.15	538±0.81	301±0.07	1.32±0.03
PHB-TiO_2 composite nanofiber	624±0.14	389±0.09	1.78±0.46	495±0.71	350±0.01	1.41±0.15
PHB composite (UV treatment)	606±0.02	339±0.02	1.79±0.01	476±0.12	306±0.02	1.56±0.53
PHB-TiO_2 composite (UV treatment)	597±0.06	335±0.02	1.78±0.03	435±0.15	314±0.02	1.39±0.04

∗ All data were the standard deviation of three parallel studies.
∗L.S.D. at level (0.05) for interaction before and after degradation time for all films types for: Mw= 12.301, Mn= 10.312, Mw/Mn= 0.042.

On the other hand, the degradation percentage was estimated through the weight loss which increased with the incubation time of degradation for all polymeric films. The PHB nanofiber and PHB-TiO_2 composite nanofiber films showed the highest degradation percentage compared to other films. The complete degradation of nanofiber films was achieved after 3 weeks as noted in Figure (3-23). This remarkable degradation of nanofibers could be due to the high surface area, porosity and three dimensional structures which allowed more microorganisms to attach to the polymeric films (Gupta *et al*., 2005). The PHB film prepared from PHB sheets that has been treated by ultraviolet light (UV) and untreated PHB sample showed a weight loss of ~ 68 and 62%, respectively. While, PHB-TiO_2 composite films and the one prepared from PHB sheets treated by ultraviolet light (UV)showed a low weight loss (~ 51 and 56%, respectively) that could be due to the antibacterial activity of TiO_2 which could reduce the microbial growth and then lowering the degradation percentage (Ahmad and Sardar, 2013; Verdier *et al*., 2014).

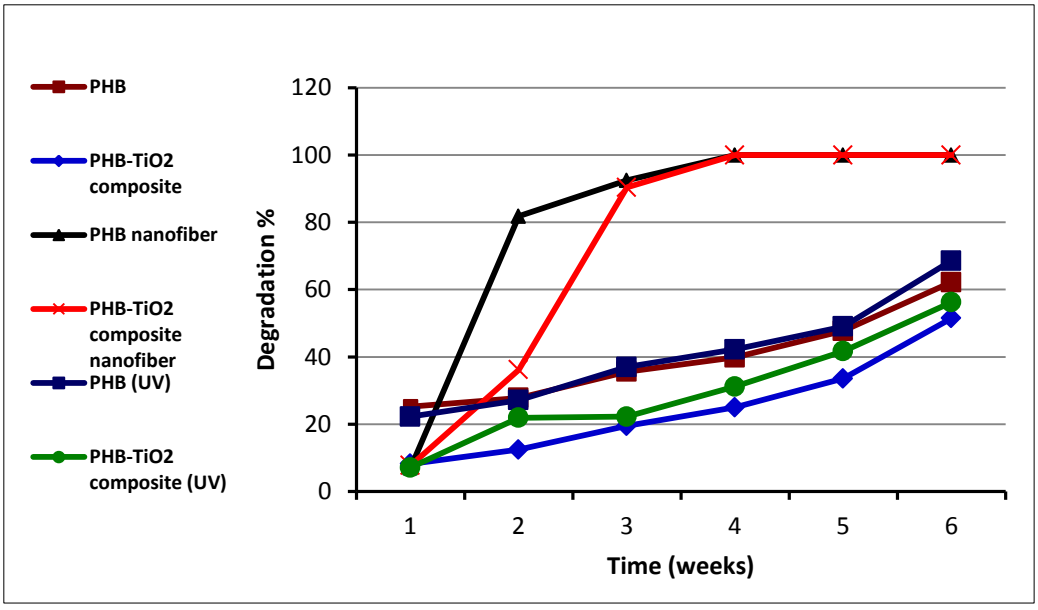


Figure (3-23): Degradation percentage of PHB films up to 6 weeks.

The number of microorganisms in soil at the buried site was expressed as $\log_{10}$ CFU/ml. It was concluded from Figure (3-24) that the microbial population increased with the incubation time that led to an increase in polymer degradation as a result of microbial activity. The soil microbes can exert depolymerase enzyme that can hydrolyze PHB polymer and consumed the metabolic degradation products as nutrients for its growth (Doi, 1990; Kumaravel *et al*., 2010).

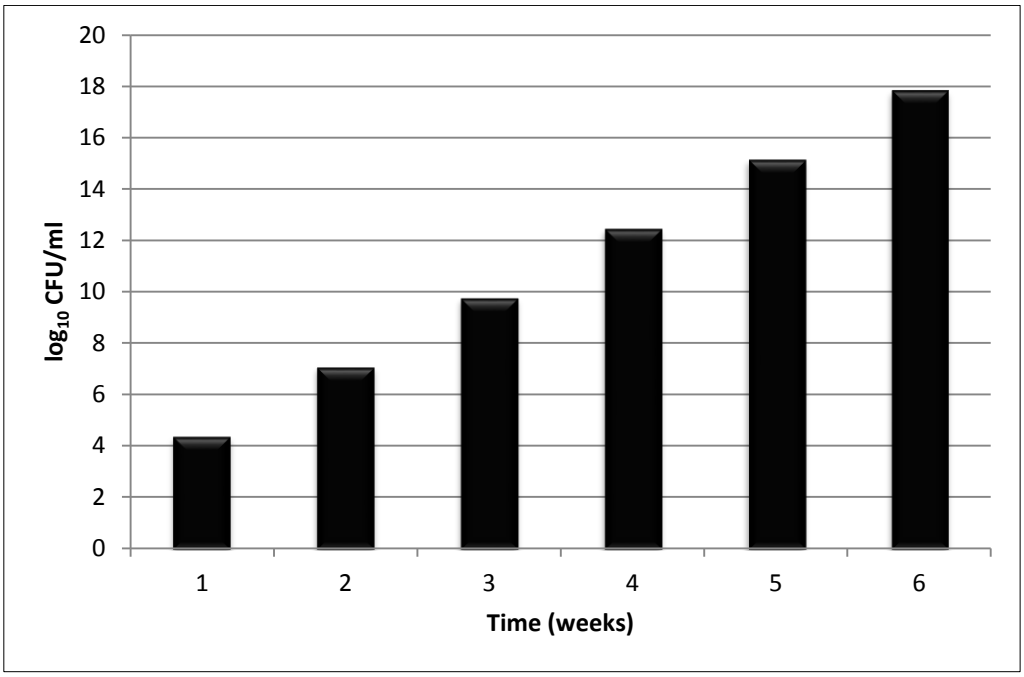


Figure (3-24): Microbial number in soil at the buried site for different PHB films as $\log_{10}$ CFU/ml. The soil samples were inoculated into TSA plates and incubated at 30 °C for 24 hours.

The physical changes in surface morphology of polymeric films were detected by the use of a light microscope as shown in Figures (3-25). Such changes include cracks, holes, gradual loss of parts and color changes. PHB nanofiber and PHB-TiO_2 composite nanofiber films were found to be completely degraded after three weeks. It was noted that there was a correlation between the weight loss percentage and the physical changes in surface morphology [Figures: (3-23) and (3-25)] and such correlation increases week

after week. The degradation of PHB nanofiber and PHB-TiO_2 composite nanofiber was complete after three weeks in which weight loss was 100%. But, the incorporation of TiO_2 in the films leads to reduction in biodegradation compared to the PHB films possibly because the inhibition of microbial growth by TiO_2 (Haghi *et al*., 2012; Verdier *et al*., 2014). The PHB and its composite films prepared from PHB sheets treated by ultraviolet light (UV) were found to have more cracks compared to the corresponding ones where prepared from the PHB sheets without UV treatment. Clearly, UV treatment accelerated the degradation which is consistent with the results reported by a previous study for the biodegradation of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (Shangguan et al., 2006).

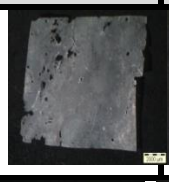
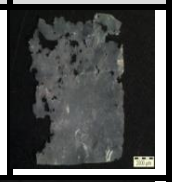
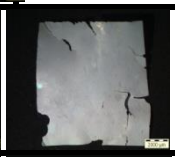
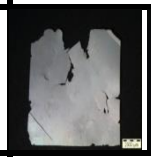
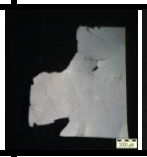
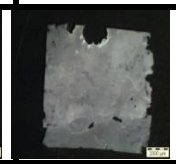
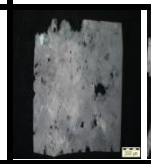
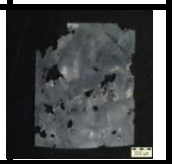
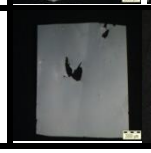
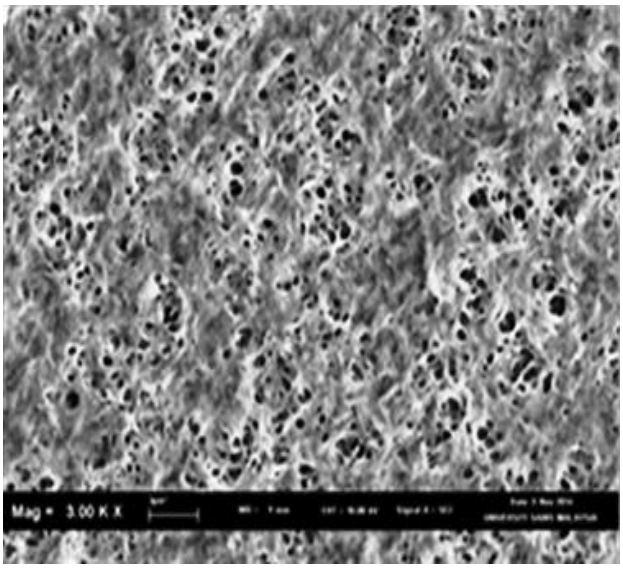
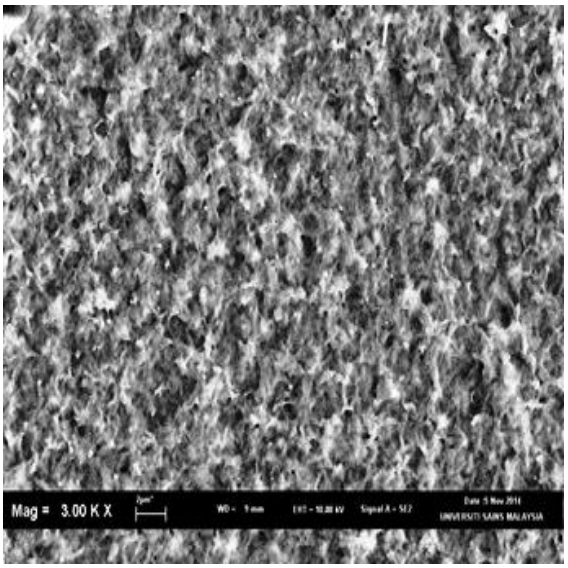
Weeks/ Films	1	2	3	4	5	6
PHB film						
PHB-TiO_2 composite film						
PHB nanofiber film						
PHB-TiO_2 composite nanofiber film						
PHB composite film (UV treatment)						
PHB-TiO_2 composite (UV treatment)						

Figure (3-25): Physical changes in polymeric films due to soil degradation (2000μm).

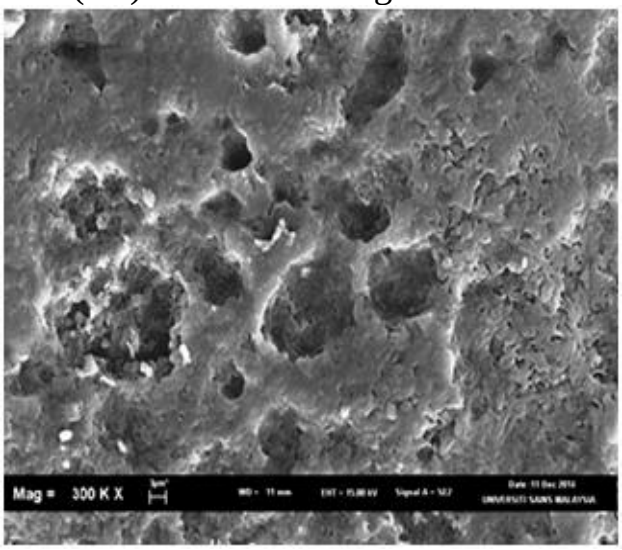
The SEM micrographs showed various changes had taken place within the surface of polymer films after a degradation period, the nonuniform surfaces were noted. The PHB and PHB-TiO_2 composite films were shown in Figure (3-26) confirmed various changes that had taken place within the surface of polymer films. Pores, cavity, grooves, incisions, slots on the surface in addition to inanimate spherical objects were noted after degradation. All the previous changes were the indications of degradation of polymeric film samples.



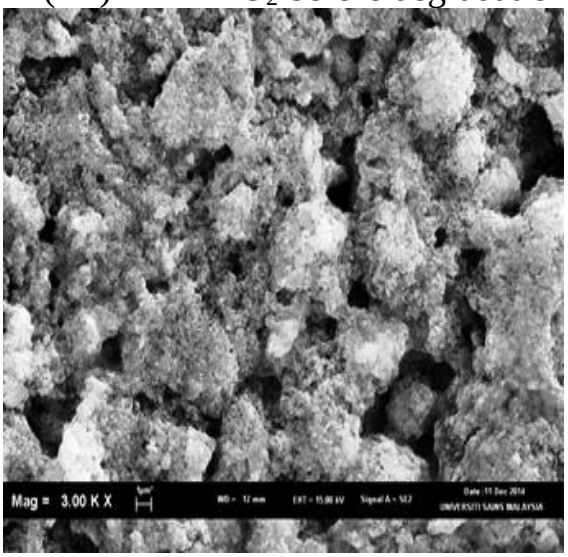
(A1) PHB before degradation



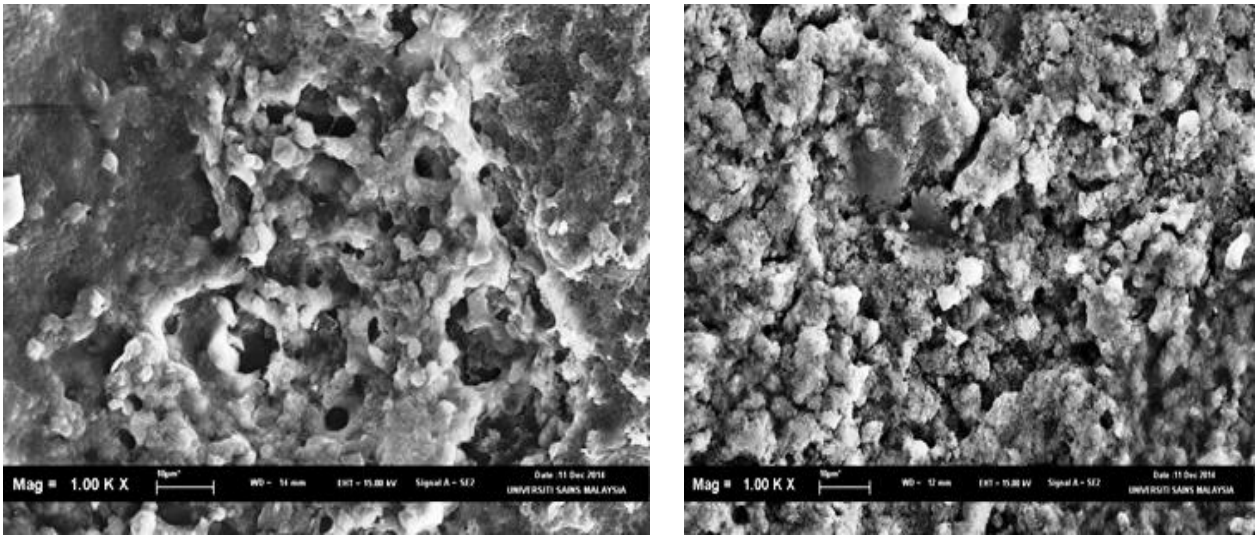
(B1) PHB-TiO_2 before degradation



(A2) PHB after degradation



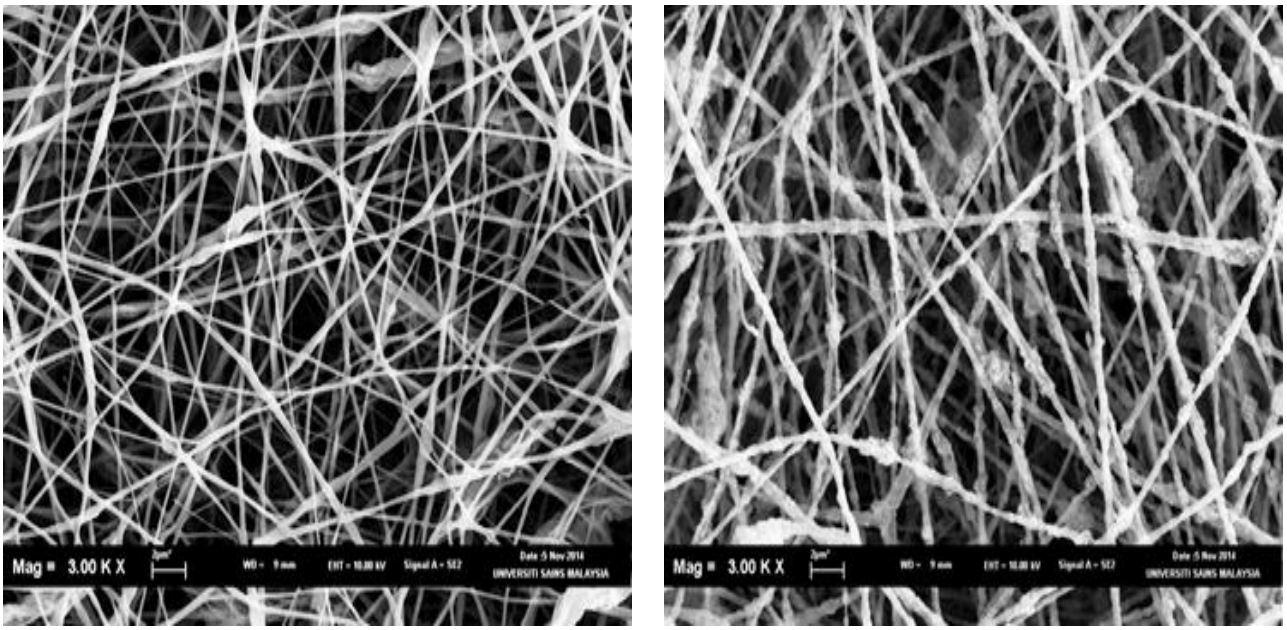
(B2) PHB-TiO_2 after degradation



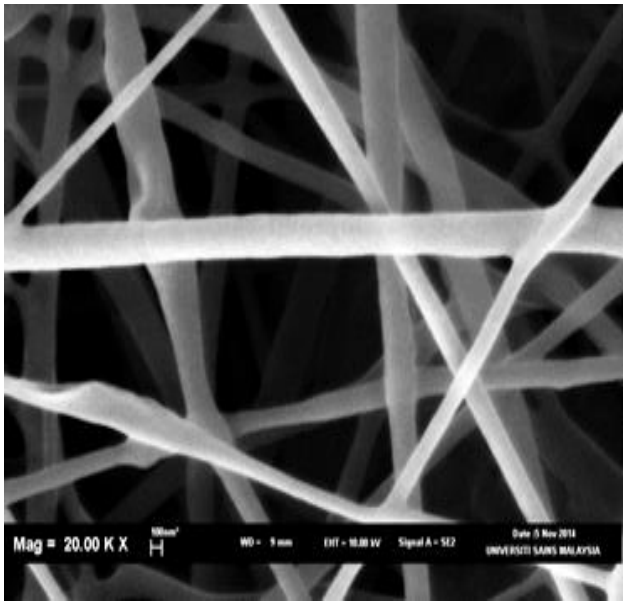
(A3) PHB after degradation (B3) PHB-TiO_2 after degradation

Figure (3-26): SEM micrographs for PHB and PHB-TiO_2 composite films before and after degradation.

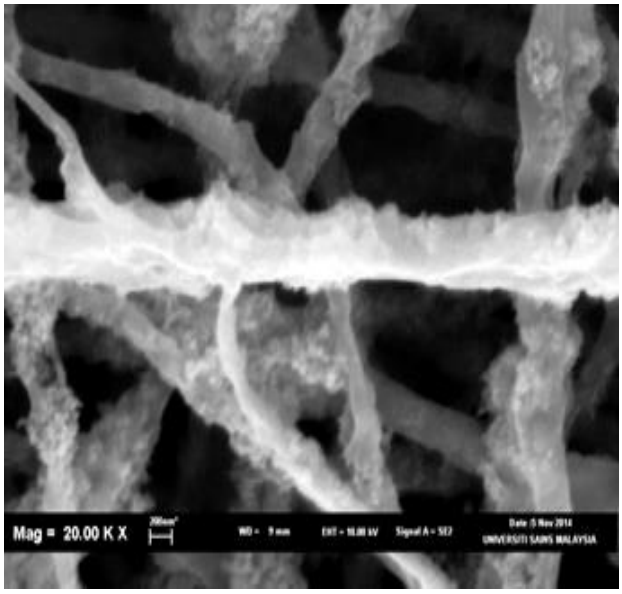
Figure (3-27) showed the surface morphology for PHB nanofiber (~500 nm diameter) and PHB-TiO_2 composite nanofiber films (~550 nm diameter) before degradation.



(A1) PHB before degradation (B1) PHB-TiO_2 nanofiber before degradation



(A2) PHB before degradation

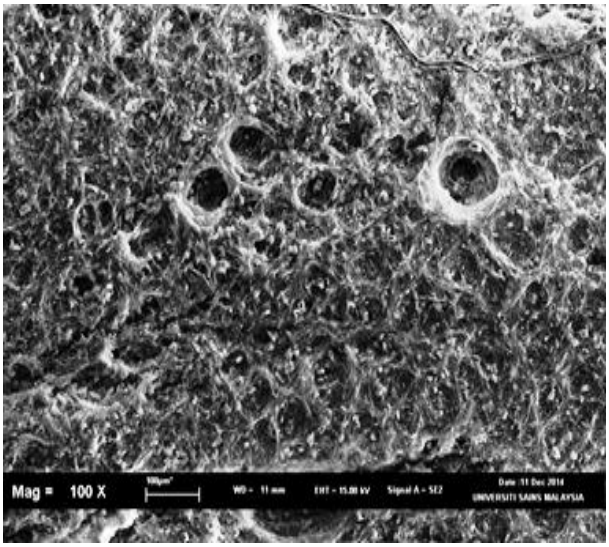


(B2) PHB-TiO_2 nanofiber before degradation

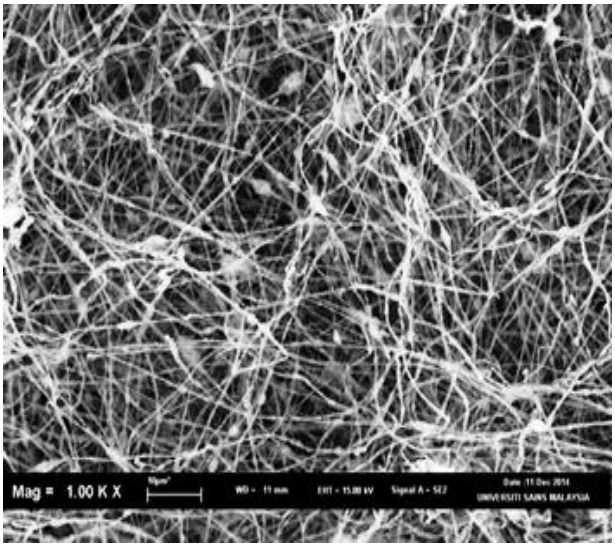
Figure (3-27): SEM micrographs for PHB and PHB-TiO_2 nanofiber films before degradation.

Figure (3-28) showed the nanofibers films surface after degradation. Clearly, it has indicated the non-rank surfaces with pores, cavities, grooves, extended objects like hyphae of fungi, spherical objects like bacteria, visible ruptures of most nanofibers. Also, it was clear that the nanofibers diameters decreased after degradation to be about 400 nm for PHB nanofiber films and around 480 nm for PHB-TiO_2 composite nanofiber films that means that the nanofibers became thinner after degradation compared to the corresponding ones before degradation.

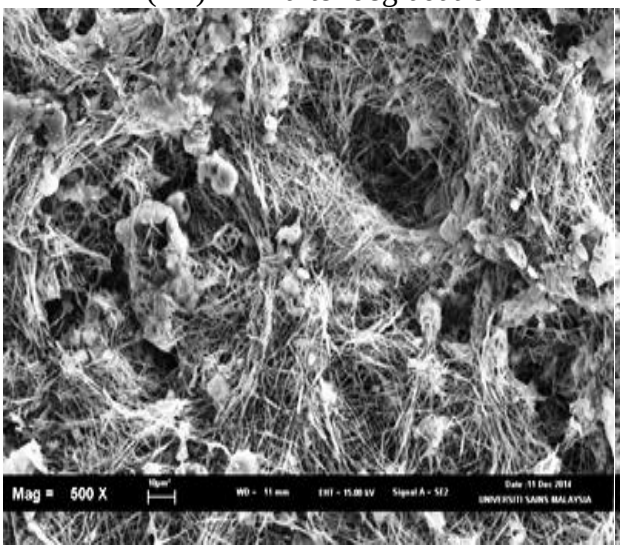
The three dimensional structures and high surface area of nanofibers accelerated the degradation of PHB nanofibers. While, PHB-TiO_2 composite nanofibers was faster in degradation compared to other samples, but was less than PHB nanofibers. Clearly, the addition of TiO_2 nanoparticles leads to the reduction in the antibacterial activity. Similar results for the surface morphology changes was reported for poly(hydroxybutyrate-co-hydroxyvalerate), PHBV and their composite (Buzarovska *et al*., 2009).



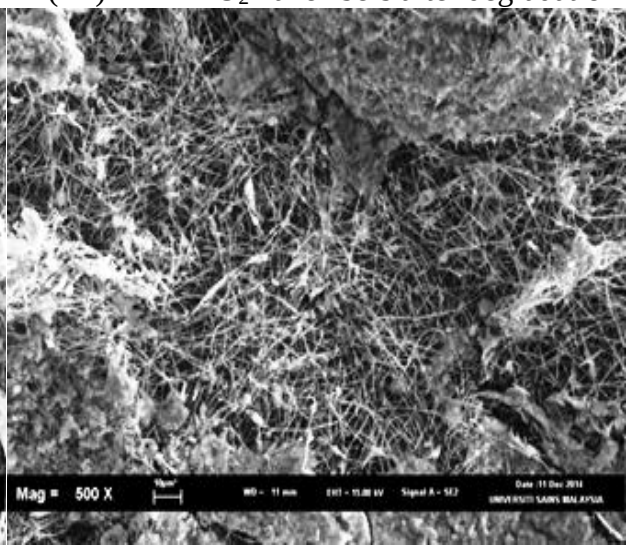
(A1) PHB after degradation



(B1) PHB-TiO_2 nanofibers after degradation

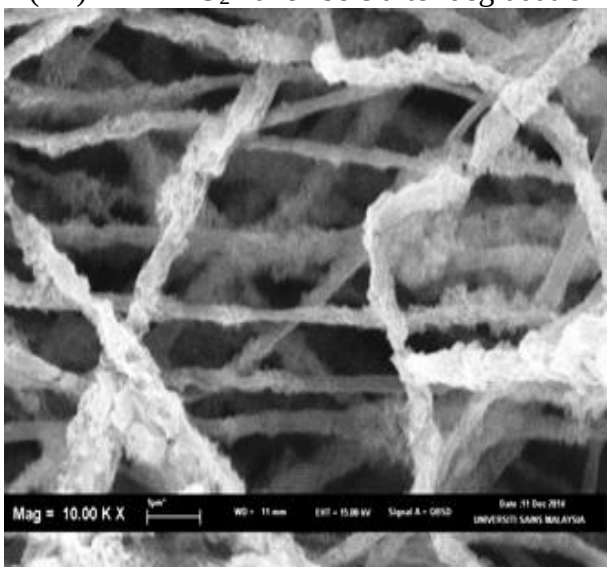


(A2) PHB after degradation



(B2) PHB-TiO_2 nanofibers after degradation

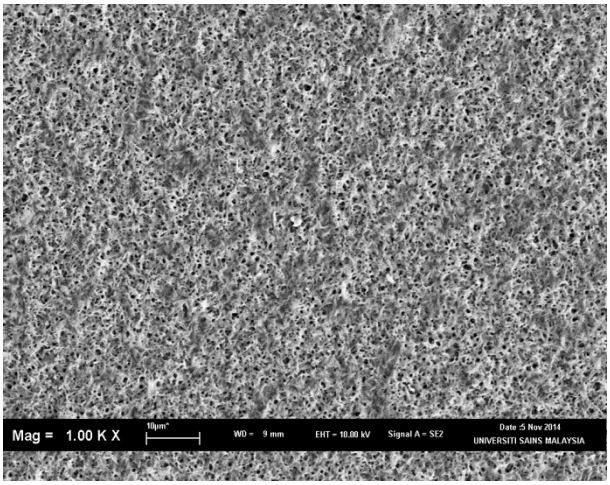
(A3) PHB after degradation



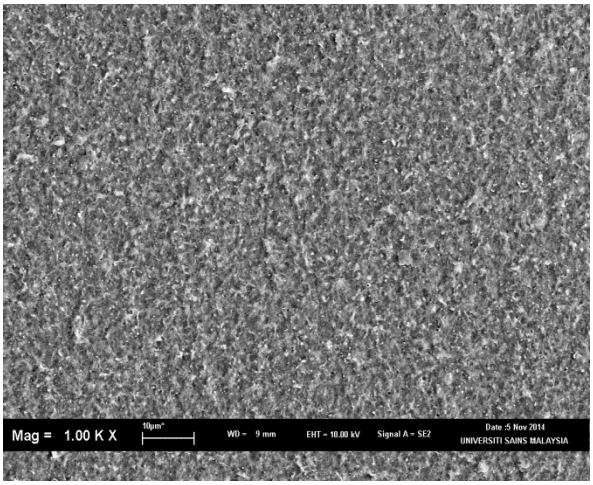
(B3) PHB-TiO_2 nanofibers after degradation

Figure (3-28): SEM micrographs for PHB and PHB-TiO_2 nanofibers after degradation.

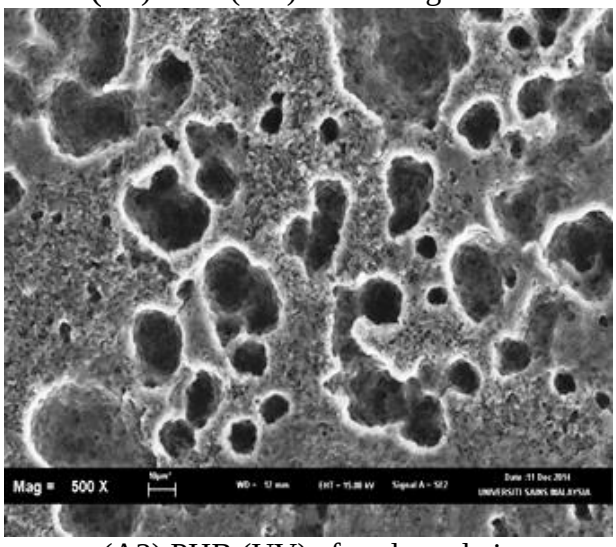
Using of UV light is responsible for initiation of polymer degradation (Shangguan *et al*., 2006). Figure (3-29) showed the SEM micrographs for the PHB film and its composite that have been prepared from UV treated PHB sheets. Clearly, more obvious physical changes have taken place within the polymeric surface compared to the ones involved no UV treatment. Such changes are clear signs of degradation of polymer film samples. Another study reported that TiO_2 accelerated Poly(hydroxybutyrate-*co*-hydroxyvalerate) degradation during UV treatment (Buzarovska *et al*., 2009). The present study proved that the polymeric films become more susceptible to biodegradation with UV treatment, previous study reported the same results during improvement the biodegradation of PHB film by UV radiation (Kessler *et al*., 2014).



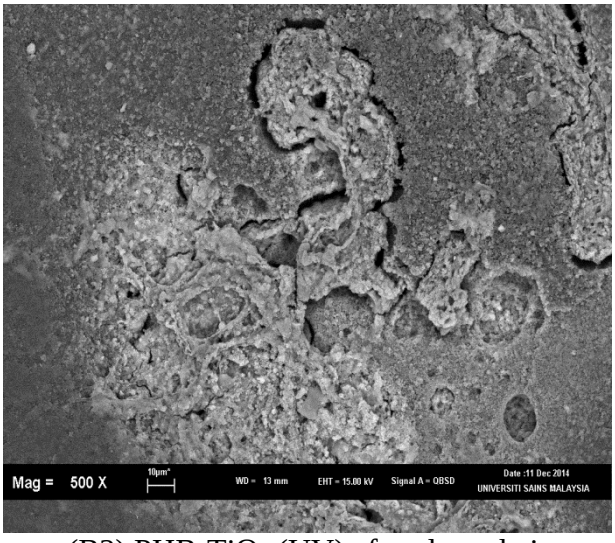
(A1) PHB (UV) before degradation



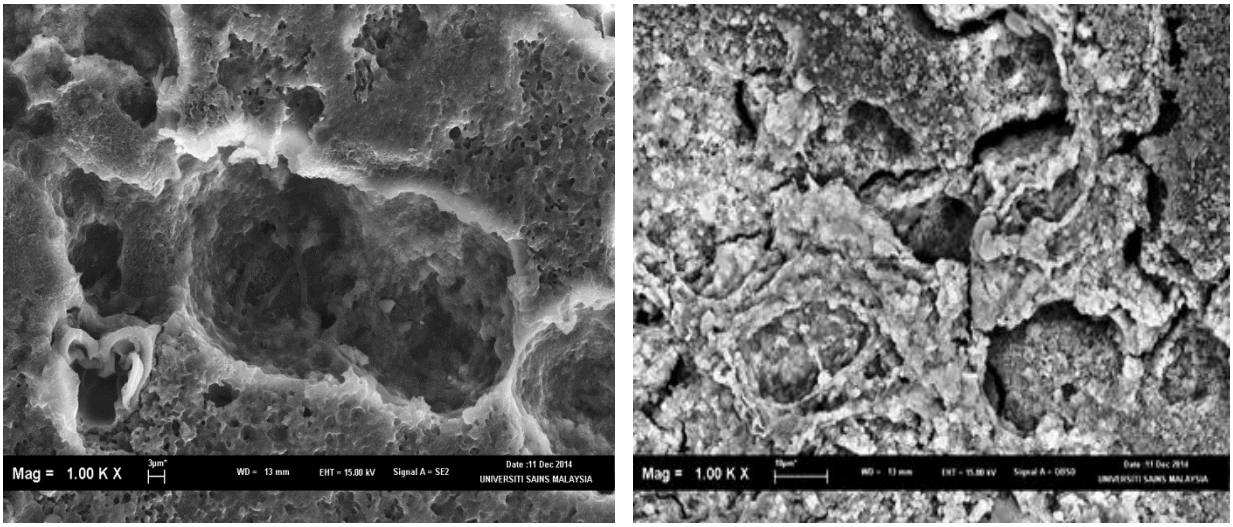
(B1) PHB-TiO_2 (UV) before degradation



(A2) PHB (UV) after degradation



(B2) PHB-TiO_2 (UV) after degradation



(A3) PHB (UV) after degradation (B3) PHB-TiO_2 (UV) after degradation

Figure (3-29): SEM micrographs for PHB and PHB-TiO_2 films that prepared from UV treated PHB sheets before and after degradation.

Conclusions and Recommendations

Conclusions

The researcher has come into the following conclusions:

1- The present study revealed the ability of gram positive bacteria (*R. equi*) for the production of polyhydroxybutyrate (PHB).

2- Crud Palm Kernel Oil (CPKO) is the effective carbon source for production of PHB.

3- The occurrence of *PhaC* gene encoded of PHA synthase enzyme.

4- The expression level of *PhaC* gene at lag phase was ~1.16 folds, while at the stationary phase was ~2 folds.

5- PHA synthase enzyme activity for *R. equi* under optimized conditions for the production was 30.83 (U/g protein).

6- The PHB films in different formulations degraded in soil without using any chemicals that can help to overcome the environmental pollution and using it for any applications related to degradation.

7- The PHB nanofiber films and their composite in the presence of TiO_2 were degraded faster than other PHB film samples.

8- The UV treatment of the polymeric sheets that used for preparing PHB films and their composite in the presence of TiO_2 was assisting in acceleration of the biodegradation.

Recommendations

In the light of the present study, the researcher has come into the following recommendations:

1- Studying the production of PHAs from others gram positive bacteria because of the lack of LPS which makes its biopolymer more suitable in medical applications.

2- Using other inexpensive carbon sources for PHAs production by fermentation to reduce the cost of production.

3- Cloning of *PhaC* gene to plant to get a large production amount and to other microorganisms.

4- Studying encapsulation of plant fertilizers with PHB nanofiber and its composites because this type of PHB film degrades rapidly due to having the largest surface area.

5- Improving the properties of PHB according to applications by incorporating it to nanoparticales or other materials.

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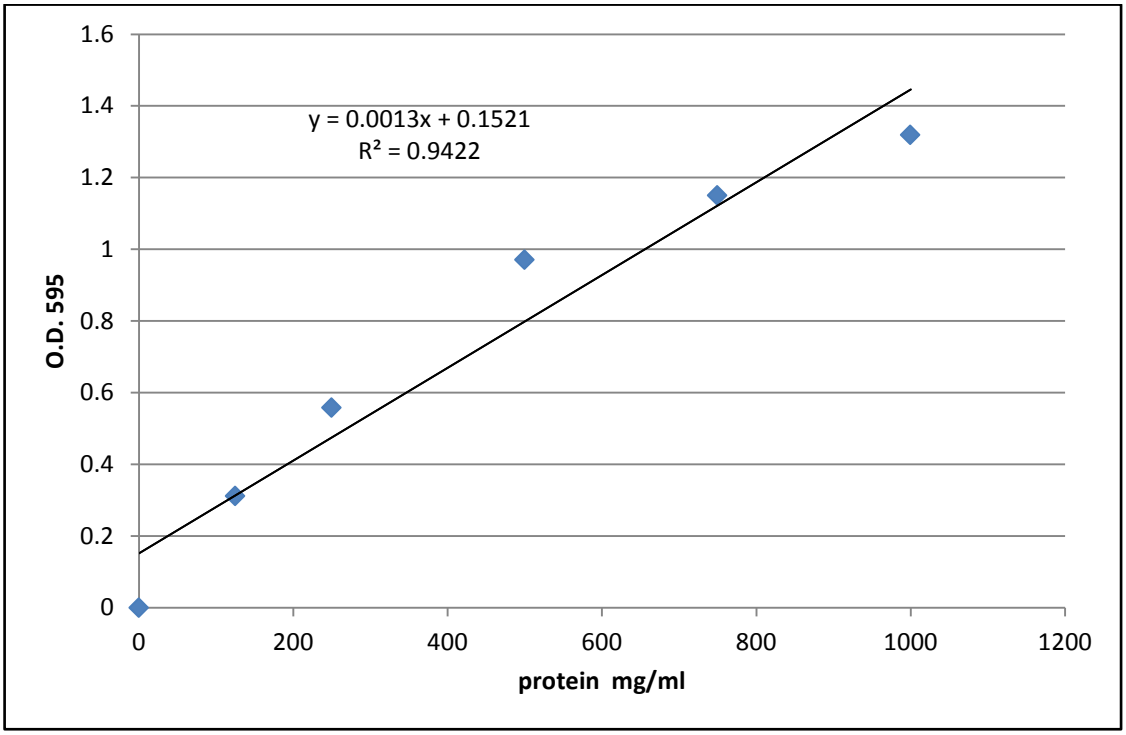
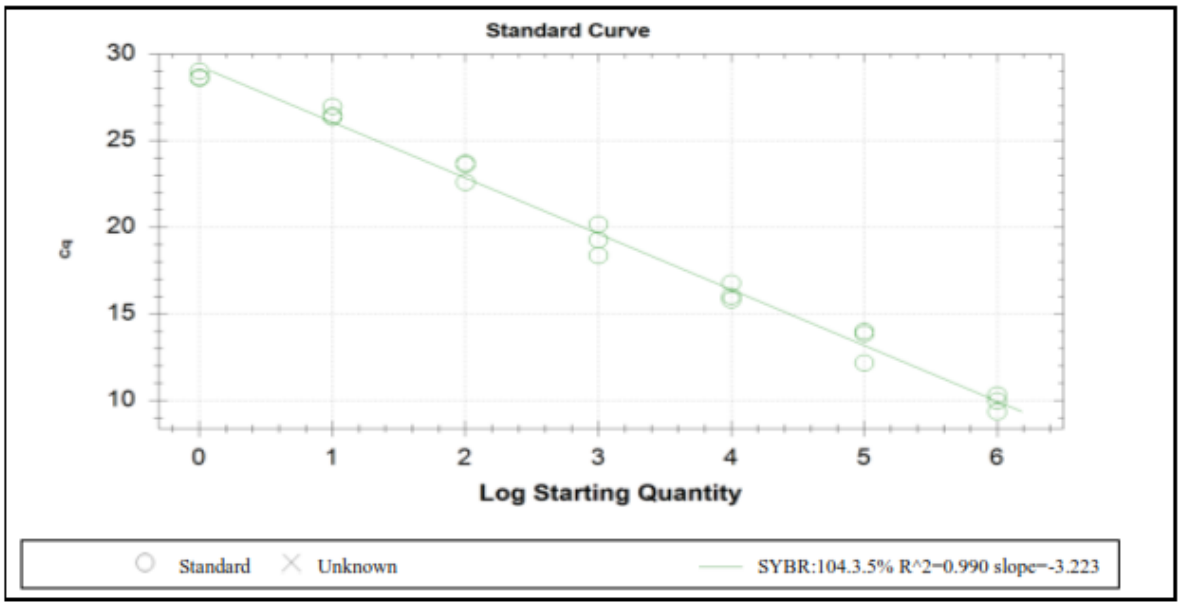
Appendices

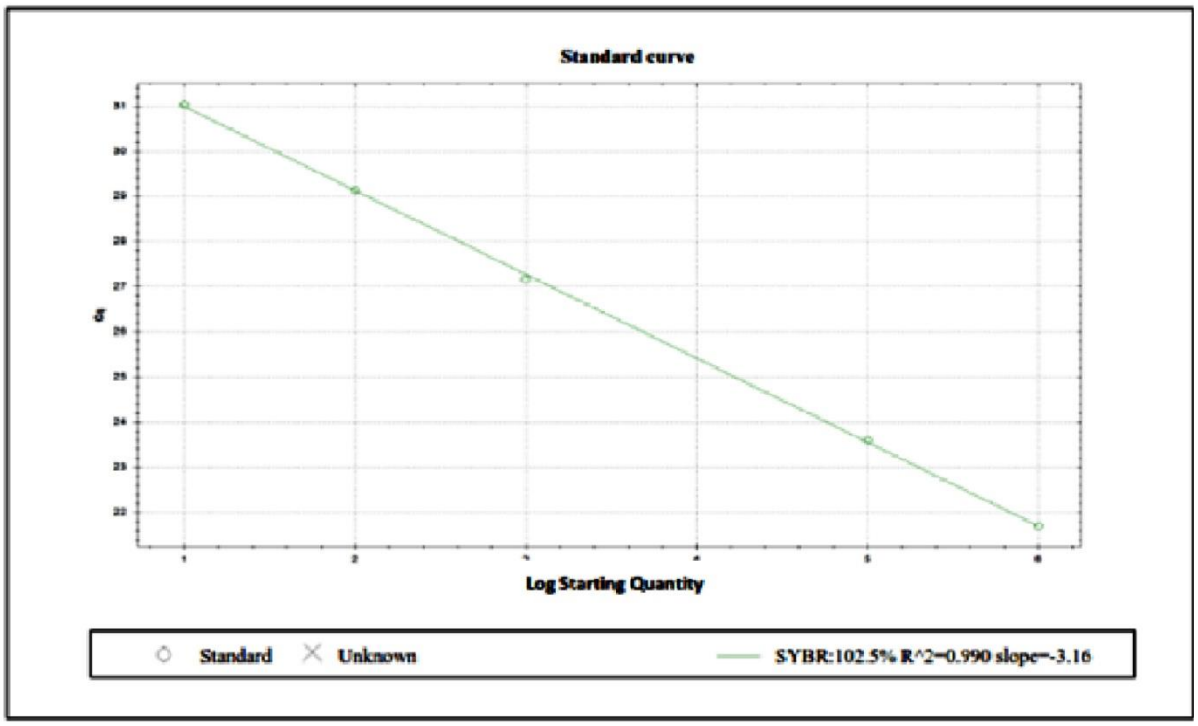
Appendix 1: Bovine serum albumin (BSA) standard curve for protein determination (Bradford, 1976).



Appendix 2: Standard curve of refrence gene (*gyrB* gene).



Appendix 3: The standard curve of *PhaC* gene (target gene).



Publication

List of publications:

— Altaee, N.; Fahdil, A.; Sudesh, K. and Yousif, E. (2015). Study the Production of Polyhydroxybutyrate by *Rhodococcus sp*. Using Crud Palm Kernal Oil (CPKO) as Carbon. Journal of Iraqi Industrial Research. 2(2): 9-15.

— Altaee, N.; Fahdil, A.; Yousif, E. and Sudesh, K. (2016). Recovery and subsequent characterization of polyhydroxybutyrate from *Rhodococcus equi* cells grown on crude palm kernel oil. Journal of Taibah University for Science . In press.

الخلاصة

تم جمع العديد من عينات التربة من اماكن مختلفة وتنقية العزلات البكتيرية والتحري عن قابليتها لانتاج PHA باستخدام صبغة Sudden Black B،عشرين عزلة اعطت نتيجة موجبة لصبغة Suddan Black B. تم تاكيد النتائج بتصبيغ المستعمرات الحية بالصبغة الانتقائية Nile Red ، ثلاث عزلات اعطت نتيجة موجبة وتم اختيار عزلة واحدة فقط والتي تتميز بتألق وردي اكثر مع صبغة Nile Red بعد التشعيع بالاشعة الفوق البنفسجية. كانت نتيجة توصيف وتشخيص هذه العزلة بالاعتماد على الاختبارات الكيموحيوية على انها *Rhodococcus* وتم تاكيد التشخيص بتقنية 16S rRNA،واستخدم برنامج blast في قاعدة بيانات NCBI واظهرت نتيجة تحليل التتابع نسبة تشابه 99% لل *Rhodococcus equi*.

تم اعتماد طريقة التنمية بمرحلة واحدة لدراسة التخليق الحيوي لل PHA باستخدام مصادر كاربون مختلفة، وتم قياس كمية ومحتوى البوليمر الحيوي باستخدام الكروماتوكرافيا الغازية ، وجد بان زيت لب النخيل الخام هو المصد الكاربوني الفعال لانتاج البوليمر الحيوي المتجانس PHB بواسطة *R. equi* حيث كان وزن الخلايا الجاف 1,43 جم / لتر ومحتوى البوليمر الحيوي07 ,38 %.

اوضحت نتائج التحليل باستخدام الرنين المغناطيسي النووي التركيب الكيميائي للبوليمر الحيوي المستخلص واكد على انه البوليمر المتجانس PHB. كما تم وصف الخصائص الحرارية للPHB والتي تضمنت درجة حرارة الانصهار ودرجة حرارة التحول الحراري والتي بلغت 173 م˚ و 2,79 م˚ على التوالي والذي تم قياسها باستخدام تحليل الفرق الكالوري المسح الضوئي، في حين كانت درجة حرارة التحلل 276 م˚ والتي تم قياسها باستخدام التحليل الحراري الوزني . كما كانت قيمة متوسط الوزن الجزيئي هو 642 كيلودالتون، وقيمة عدد وزن متوسط الوزن الجزيئي هو 373 كيلو دالتون، وكانت قيمة التشتت المتعدد هو 1,72 والتي تم قياسهم باستخدام كروماتوكرافيا عبور الهلام.

استخدم المجهر الضوئي المتباين الاطوار لمشاهدة *R. equi* اثناء انتاج PHB حيث تمتاز الخلايا البكترية الحاوية على البوليمر الحيوي بمظهر ساطع ويزداد عدد الخلايا الحاوية على البوليمرالحيوي بمرور الوقت، في حين ظهرت الخلايا البكتيرية بتألق برتقالي ساطع عند استخدام المجهر المتألق بعد تصبيغها بصبغة Nile Blue A وتنميتها تحت الظروف المثلى لانتاج البوليمر الحيوي.

اوضحت صور المجهر الالكتروني النافذ وجود حبيبات PHB داخل الخلايا البكترية المنتجة والتي تمتاز بمظهر كروي بيضوي طويل ابيض ، هذه الحبيبات لها أحجام مختلفة وعدد مختلف يتراوح بين (3-11) حبيبة لكل خلية.

الخلاصة

ان جين *PhaC* هو الجين المشفر لانزيم التخليق الذي يعتبر المفتاح للتخليق الحيوي لل PHA، استخدمت البوادئ G-D وG-1R لغرض مضاعفة جين *PhaC* بواسطة PCR وكان بحجم 508 زوج قاعدة، اظهرت نتائج تحليل تتباع ذلك الجين باستخدام برنامج ال blast في NCBI database 78% تماثل مع تتابع جين التخليق الحيوي *PhaC* لبكتريا *Rhodococcus aetherivorans* (accession no. CP011341.1). من ناحية اخرى تم اعتماد تقنية Real time PCR لتقييم مستوى التعبير الجيني لجين *PhaC* حيث اعتبر gyrB الجين المصدر وطور log phase كسيطرة. استنتج بان مستوى التعبير الجيني لجين *PhaC* عند lag phase يساوي ~1.16، في حين ان مستوى التعبير الجيني عند stationary phase يساوي ~2 . كما تم توصيف وتقييم الفعالية الانزيمية لانزيم التخليق (synthase) تحت الظروف المثلى لانتاج ال PHB وكانت قيمة الفعالية الانزيمية 30,83 (U/mg protein).

اعدت نماذج مختلفة للبوليمر الحيوي المتجانس PHB لدراسة التحلل الحيوي لمدة 6 اسابيع والتي تضمنت افلام PHB، افلام PHB المهجنة بثاني اوكسيد التيتانيوم،افلام الالياف النانوية للبوليمر ،افلام الياف البوليمر النانوية المهجنة بثاني اوكسيد التيتانيوم،افلام PHB وافلام ال PHB المهجنة بثاني اوكسيد التيتانيوم المحضرة من افلام البوليمر الحيوي المعامل بالاشعة الفوق البنفسجية.

اظهرت نتائج تجربة التحلل الحيوي انخفاض معنوي في قيمة الوزن الجزيئي ،وقيمة عدد وزن متوسط الوزن الجزيئي و قيمة التشتت المتعدد لجميع نماذج الافلام. استخدمت النسبة المئوية لفقدان الوزن كدليل للتحلل الحيوي والتي زادت مع الوقت لكن اظهرت افلام الياف البوليمر النانوية والمهجنة بثاني اوكسيد التيتانيوم تحلل اسرع من باقي الافلام وقد تم تحللها تماما بعد 3 اسابيع. كما لوحظ زيادة عدد المايكروبات في التربة في موقع الطمر في كل اسبوع وهذا يساعد على زيادة تحلل نماذج افلام البوليمر المختلفة. تم ايضاح التغيرات الفيزيائية في جميع الافلام والتي تمثلت بالشقوق وعلى مدى كل اسبوع من التجربة. استخدم المجهر الالكتروني الماسح لفحص عينات الافلام قبل بدء التحلل الحيوي لاظهار المظهر السطحي لعينات افلام PHB المختلفة وبعد التحلل الحيوي الذي استغرق 6 اسابيع لايضاح التغيرات السطحية المتعددة والتي تضمنت المسامات، التجاويف، الأخاديد، الشقوق، الثغرات ومؤشرات لنمو الكائنات الدقيقة التي تتميز بقدرتها على افراز انزيم PHA depolymerase الذي ممكن ان يسبب التحلل الحيوي لجميع انواع افلام البوليمر المستخدمة في الدراسة الحالية لكن افلام الالياف النانوية للبوليمر و افلام الالياف النانوية المهجنة مع ثاني اوكسيد التيتانيوم اظهرت تغيرات سطحية اكثر مع تمزق الالياف ونقصان اقطار الالياف النانوية من 500 نانوميتر الى 400 نانوميتر لافلام الياف البوليمر النانوية ومن 550 نانوميتر الى 480 نانوميترلافلام الياف البوليمر النانوية المهجنة

الخلاصة

بثاني اوكسيد التيتانيوم . المعاملة بالأشعة فوق البنفسجية لأفلام PHB واستخدامه في تحضير افلام PHB وافلام PHB المهجنة بثاني اوكسيد التيتانيوم تسببت في زيادة التحلل البيولوجي بالمقارنة مع مثيلاتها في غياب المعاملة بالأشعة فوق البنفسجية.

الاهداء

الى شلال الحنان والدفئ والطيبة.....

الى من غابت فجأة.....

الى من رحلت بصمت!!!

فأنهار الجدار الشامخ خلف ظهري .....

امي الحبيبة رحمها الله.

والى كل من ساندني....

اهدي ثمرة جهدي المتواضع

نادية

بسم الله الرحمن الرحيم

"يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ"

صدق الله العظيم

سورة المجادلة : الاية (11)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

دراسة توصيفية وجزيئية لانتاج متعدد هيدروكسي بيوتيريت من *Rhodococcus equi*

اطروحة

مقدمة الى مجلس كلية العلوم، جامعة النهرين، كجزء من متطلبات نيل
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