PRODUCTION OF POLYHYDROXYALKANATE (PHA) FROM WASTE COOKING OIL USING *PSEUDOMONAS OLEOVORANS*

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To my beloved husband, father and mother
For their love, support, sacrifices and blessings
And to all other beloved ones

God bless them all!

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters which are stored in bacterial cell cytoplasm as reserve materials for carbon and energy. One of the main problems of plastics is that they have resistance to biological breakdown that result in accumulation in the environment. Bacteria synthesize and accumulate polyhydroxyalkanote (PHA) as carbon source under limiting conditions of nutrients. In this study, the bacteria, *P. oleovorans* was studied for its ability to produce PHA in the minimal basal medium supplemented with glucose as carbon source and $(NH_4)_2SO_3$ as nitrogen source that was grown at 25°C. The functional groups of the extracted PHA granules were identified as a C=O group by Fourier Transform Infrared (FTIR) spectroscopy analysis. The drastic absorption band at approximately 1720 cm^{-1} indicated the stretching vibration of the C=O groups in the PHA polyester. The influence of different carbon sources, nitrogen sources, pH and inoculums on PHA production was investigated. The production optimization of PHA was done by RSM (Response Surface Method) through various growth parameters. After optimization obtained the best condition of productivity in range are pH 5.9, carbon source 93.4419 g/l, inoculums size 2.07% and nitrogen source 101.691 g/l. Also the highest PHA production after optimization is 2.28236 g/L with a desirability of 0.986 g/l, meanwhile the highest amount of PHA produced from *P. oleovorans* was 2.30 g/l.

ABSTRAK

Polyhydroxyalkanoates (PHA) adalah polyesters terbiodegradasi yang mana telah disimpan di dalam sel cytoplasm sebagai bahan bakteria tersimpan untuk karbon dan tenaga. Satu daripada masalah utama adalah plastik yang mana ianya mempunyai rintangan terhadap penguraian biologikal maka mengakibatkan pengumpulan terhadap alam sekitar. Bacteria sintesis dan PHA terkumpul sebagai sumber karbon di bawah had keadaan nutrient. Di dalam kajian ini bakteria *P.Oleovorans* dikaji keupayaannya untuk menghasilkan PHA di dalam medium asas tambahan bersama glukosa sebagai sumber karbon dan $(NH_4)_2SO_3$ sebagai sumber nitrogen yang mana hidup pada suhu 25ºC. Fungsi kumpulan-kumpulan granular PHA yang diekstrakkan telah dikenalpasti sebagai satu kumpulan C=O oleh analisis Fourrer Transform Infrared (FTIR). Kumpulan serapan drastic terletak kira kira 1720 cm^{-1} adalah menunjukkan getaran penegangan kumpulan C=O didalam polyester PHA. Pengaruh sumber karbon yang berbeza, sumber nitrogen yang berbeza, pH yang berbeza dan inoculum yang berbeza keatas penghasilan PHA dikaji. Analisis statistikal telah dibuat untuk menganalisis data menggunakan kaedah tindak balas permukaan (RSM). Daripada data analisis yang diperolehi, nilai pH terbaik untuk penghasilan PHA adalah 5.98201, sumber karbon adalah 93.4419, sumber nitrogen adalah 101.691 dan saiz inokulum adalah 2.07. Jumlah penghasilan PHA yang tertinggi selepas proses pengoptimuman adalah 2.28236 g/L dengan nilai kejituan sebanyak 0.986. Manakala jumlah penghasilan tertinggi PHA daripada *P.oleovorans* adlah 2.30 g/l.

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LIST OF ABBREVIATIONS

ASTM	-	American Society for Testing and Materials
CCD	-	Central composite design
FT-IR	-	Fourier Transform-Infrared Spectroscopy
FLP	-	FLiPpase
GC	-	Gas Chromatography
g	-	Gram
g/L	-	Gram per Liter
GLP	-	Glycerol liquid phase
HA	-	hydroxyalkanoate
mL	-	Mililiter
MBM	-	Minimal Basal Medium
w/v	-	Weight/ Volume
v/v	-	Volume/ Volume
°C	-	Degree Celcius
PHA	-	Polyhydroxyalkanoate
PHA_{SCL}	-	Short chain length PHA
PHA_{MCL}	-	Medium chain length PHA
PHA_{LC}	-	Long chain length PHA
P (3HB)	-	poly-3-hydroxybutyrate
P (3HV)	-	poly-3-hydroxyvalerate
P (3HB-3HV	-	poly-3-hydroxybutyrate-co-3-hydroxyvalerate
P (4HB)	-	poly-4-hydroxybutyrate
USDA		United States Department of Agriculture
WCO		Waste cooking oil
RSM		Response Surface Methodology

CHAPTER 1

INTRODUCTION

1.1 Literature review

Polyhydroxyalkanoates (PHAs) are polymers of hydroxy fatty acids and family of synthesized biopolyesters that agglomerated by some of bacteria strains as carbon storage materials. These polymers are natural synthesized biopolymers and decomposed by some microbial metabolisms, although these biopolymers are able to be melted and molded similar to chemical and unnatural thermoplastics (Koller *et al*, 2005).

Because of potential using PHAs as biodegradable thermoplastics these biopolymers have been more attended at academic and industrial fields. Several academic studies have been repeated on the physiology of PHA, many processing procedures, molecular biology, biochemistry of these biopolymers, many kinds of material properties and biodegradation of PHA. They can be polymerized from degradable carbon sources which are produced by agricultural and industrial wastes and products (Lee, 1996).

The wastes are generated by many industries including wood-processing industry, slaughterhouse industry, etc. These wastes sometimes containing carbon-rich substrates for example polysaccharides, disaccharides, monosaccharides, lipids which can be useful for PHA production. In general, molass wastes, starch wastes, whey as dairy wastes, glycerol liquid phase (GLP) as the biodiesel production waste, xylose, lipids and waste water obtained from oil production occurring in large quantities (Koller *et al.*, 2007). Production of PHA from waste contains double benefits, one of them is that polluting waste derived from environment can be converted into biodegradable polymer or environment friendly pollutants. Economically, upon using waste products as a substrate, the cost of the carbon source, as the most important factor significantly can be decreased to the overall production cost (Choi, 1997). Its benefit is that, it makes the PHA production more economical and to handle waste without further disposal cost (Lee, 1999).

Additionally, PHAs have various properties that can be produced and commercially used in different fields such as packaging films and containers (Reddy *et al.,*2003). Furthermore biodegradable carriers which are used for drugs carrying, hormones carrying, insecticides or herbicides carrying is also recommended (Reddy *et al*., 2003; Zinn *et al.*, 2001). Production of PHA in large scale, using microbial fermentation, has so far been done. However the improvement of transgenic PHA agglomerating plants has found considerable progress and its production in industrial scale is also possible (Valentin *et al*, 1999).

Due to the restriction of a nutrient, electron donor and electron acceptor, under stress conditions, bioplastics or biopolymers are stored as granules inside the cytoplasm of bacteria. Generally, PHA accumulation is favored by sufficient carbon sources and their availability also limiting supplies nitrogen and phosphate as macro components and or micro components for example magnesium (Mg), sulphate (S), iron (Fe), potassium (K), manganese (Mn), copper (Cu), calcium (Ca) ,tin and sodium cobalt (Sc) (Kim *et al.*,2001; Helm *et al.*,2008). For microbial production of PHA, PHAs feed as store materials as carbon and energy sources. In starvation conditions, these store matters can be mobilized, therefore the cell provides with the superiority to maintenance (Goh, 2008).

1.2 Statement of Problem

Currently, there are a lot of environmental problems of plastics, used in packaging, thus making biodegradable plastics from bacteria is needed (Ahleum *et al.*,2009). In this case the first material is waste cooking oil (palm oil), which can help to dominate to this problem. Many organizations have established that they have facilitated the development in the production of bioplastics (Davisa, 2006). Whereas considerable advancement has been achieved in recycling of metals and also packaging with glass (Oakley-Hill, 1999). Few successful in this area have been achieved by decreasing the fuel polymer packaging wastes in landfill. Fuel packaging materials consist of large amount different types of polymers that each of these polymers may contain different chemical additives for example fillers, colorants or plasticizers.

By incinerating the plastic waste, the chemical energy stored therein recovers as thermal energy. Degradation of PHA will be occured in two-steps. First, based on the type of depolymerases, PHA will be degraded into its monomers (Jendrossek,1993), and dimmers (Schrimer,1993) or a mixture of oligomers. Second, an enzyme called oligomer hydrolase cleaves the PHA oligomers into simple monomers (Shirakura,1983). Nowadays, increasing industrial interest is due to the bio-producing the PHA from recycable carbon sources to make a bio-material and biodegradable polymers that can act as intermittent for current fuel plastics (Braunegg *et al*., 1998).

If products made of PHAs are cycled in the nature, the final products of oxidative breakdown they will be naturally degrade to produce water and CO_2. Carbon dioxide and water as the ultimate products of oxidation are the first substrates for the production of carbohydrates produced by plants. This matter indicates that, compare with fuel plastics, polyhydroxyalkanoates are encompassing into the natural carbon cycle. The PHA application not only apply for packaging industry, but also embraces commodity stuffs for some agricultural purposes also for some applications such as pharmaceutical and medical. A resistant solution procedure can be identified as consumption of a spread span of waste materials which can be explained as the role of substrate for the biomediat generation of favorable final products. These natural materials are mostly generated in agricultural and industrial products (Braunegg *et al*., 1998).

In waste oils, plant oils, or in waste water produced from oil factories in the industry, the triacylglycerides immediately, or during hydrolysation of oils to glycerol and free fatty acids, and also after transesterification to the glycerols could be utilized as carbon sources in order to PHA production (Archana *et al*., 2012).

1.3 Objectives of Study

The objectives of the study are

- To culture *Pseudomonas* strain in a suitable media.
- To characterize waste cooking oil (WCO)
- To produce mcl-PHA by *Pseudomonas oleovorans* from WCO.
- To study the important parameters in producing PHA

1.4 Scope of Study

This study is limited to the production of polyhydroxyalkanoates from waste cooking oil using *Pseudomonas oleovorans*. *Pseudomonas oleovorans* is a gram negative bacteria. Its optimal temperature is 35˚C and has poor growth at 41˚C. These bacteria will be grown under optimized conditions with suitable growth factors. The procedure which should be done for recovery of PHA including pre-treatment and extraction. Centrifugation and washing the biomass with distilled water is the pre-treatment step and after that extraction will be done using sodium hypochlorite and chloroform. Different factors affecting on PHA production by *Pseudomonas oleovorans* will be optimized, including carbon source, nitrogen source, pH and inoculumns.

1.5 Significance of Study

The world we are living today is definitely covered with fossil fuel plastics. From historical point of view, the production of fossil fuel plastics has been increasing rapidly since the 20th century (Cervenkova, 2007). Nowadays, the overall production volume of fuel plastics reached over 250 million tons at the end of 2010 (Marsalek, 2011).

The yearly world production of fuel polymers amounts is more than 140 million tons. A large amount of this volume is composed of chemically stable polymers that are not easily degraded to the nature (Shimao, 2001). These are mostly synthetic polymers that are produced essentially by chemical addition or condensation reactions in a large number of monomers and are joined sequentially. The strong using of stabilizers and its presence in widely used plastics such as nylon, polyethylene (PE), polyethylene terephtalate (PET) or polyvinyl chloride (PVC) is partially responsible for their poor biodegradability. Therefore, interest in the degradation process of several polymers and in the use of environmental-friendly alternatives has increased (Birgit Kessler *et al.*, 2001).

1.6 Pathway of PHA Production

Pseudomonas oleovorans has a vast broad substrate range (Huisman *et al.*, 1989). It exhibits a high flexibility in incorporating monomers into PHA producing (Witholt and Kessler 1999) and accumulates PHA polymers up to a cellular content of 65 % (Hazenberg and Witholt 1997). These aspects make *P. oleovorans* an excellent candidate for the production of a different variety of PHAs. To produce bio polymers

(PHA), the strain produces two PHA polymerases, PhaCl and PhaCz (Huisman *et al.*,1991).

These two polymerases have the same function, i.e. condense R-3-OH-acyl-CoA moieties through an ester bound. The acyl-CoA activated precursors of the polymerases are derived from either fatty acids, alkanals, alkanols or alkanes, which are channeled through the P-oxidation cycle via 3-ketoacyl-CoA to R-3-OH-acyl-CoA. Once polymerized, PHA accumulates at insoluble gathered, so-called granules, which are formed in the cytoplasm of the bacteria . An accepted model of the general structure of PHA granules was presented by Steinbtichel *et al*. (1995).

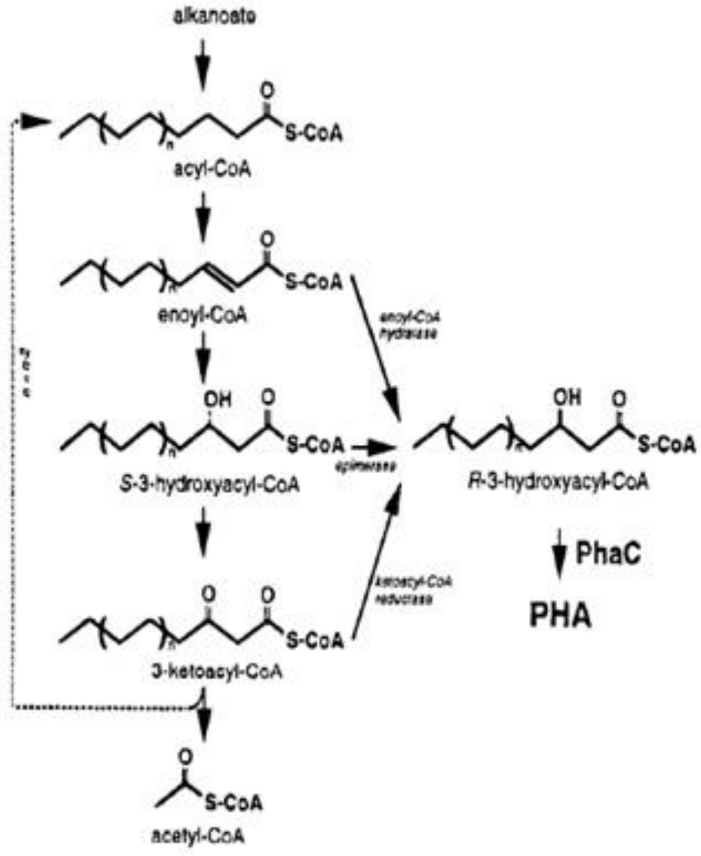

Figure 1.1 MCL-PHA biosynthesis pathway of *P.Oleovorans* (Madison *et- al.* 1999)

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