CLONING AND CHARACTERIZATION OF AN UNUSUAL POLYHYDROXYALKANOATE ACID SYNTHASE (*phaC*) GENE FROM A *CHROMOBACTERIUM* SP. ISOLATED LOCALLY

by

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ABSTRAK

Chromobacterium sp. ialah bakteria yang berupaya mensintesis polihidroksialkanoat (PHA). PHA sintase (PhaC) Chromobacterium sp. berupaya mempolimerisasikan monomer PHA berantaian pendek seperti 3-hidroksibutirat dan 3-hidroksivalerat. Bakteria ini sangat menarik disebabkan keupayaannya untuk menghasilkan pigmen ungu, violecein, menyebabkan koloninya berwarna ungu, suatu warna yang unik di kalangan bakteria. Kebelakangan ini, bakteria unik ini telah berjaya dipencilkan dari 'Air Terjun Telaga Tujuh' yang terkenal di Langkawi, Malaysia. Analisis 16S rRNA mengesahkan identity pencilan ini hingga ke tahap genus. Dalam kajian ini, gen PHA sintase (*phaC*) telah berjaya diklonkan melalui teknik amplifikasi PCR. Kawasan pengkodan *phaC Chromobacterium* sp. melingkungi 1704 bp dan ia mengekod 568 asid amino protein putative. Keputusan menunjukkan PhaC bakteria ini mempunyai spesifikasi yang luar biasa terhadap 3-hidroksivaleril-CoA. Spesifikasi enzim ini disokong lagi dengan keupayaan pencilan ini mensintesis poli(3-hidroksivalerat) dengan ketulenan 100 mol% daripada substrat yang sesuai. Apabila *phaC* yang diklonkan diekspreskan di mutan negative *Cupriavidus necator* PHB^{-}4, 64 wt% poli(3-hidroksibutirat) daripada jisim berat kering diakumulasikan apabila fruktosa digunakan sebagai sumber karbon. Bukan itu sahaja, apabila sumber karbon prekursor 3-hidroksivalerat (3HV) yang sesuai digunakan, ko-polimer PHA yang mengandungi pecahan molar monomer 3HV yang tinggi disintesiskan. Paling ketara

ialah keupayaan *C. necator* PHB^{-}4 rekombinan yang mengandungi *phaC Chromobacterium* sp. untuk mengakumulasikan poli(3-hidroksibutirat-*ko*-3-hidroksiheksanoat) apabila minyak mentah isirong kelapa sawit digunakan sebagai sumber karbon tunggal. Kajian ini menunjukkan bahawa PhaC *Chromobacterium* sp. mempunyai spesifikasi substrat yang luas yang menyerupai *Aeromonas caviae*.

CLONING AND CHARACTERIZATION OF AN UNUSUAL POLYHYDROXYALKANOIC ACID SYNTHASE (*phaC*) GENE FROM A *CHROMOBACTERIUM* SP. ISOLATED LOCALLY

ABSTRACT

Chromobacterium sp. is a bacterium capable of producing polyhydroxyalkanoates (PHA). The *Chromobacterium* sp. PHA synthase (PhaC) is able to polymerize short-chain-length PHA monomers, such as 3-hydroxybutyrate and 3-hydroxyvalerate. This bacterium is very interesting due to its ability to also produce a purple pigment, violacein. Production of violacein makes its colony purple in color, a color very unique among bacteria. Recently, this unique bacterium was successfully isolated from the famous 'Air Terjun Telaga Tujuh' in Langkawi, Malaysia. 16S rRNA analysis confirmed the identity of the isolate up to the genus level. In this study, the PHA synthase (*phaC*) gene was successfully cloned by using the PCR amplication technique. The *Chromobacterium* sp. *phaC* coding region consisted of 1704 bp, which encodes for a putative 568 amino acid protein. Results show that the PhaC of this bacterium has an unusual specificity towards 3-hydroxyvaleryl-CoA. This enzyme specificity was further supported by the ability of this isolate to synthesize poly(3-hydroxyvalerate) of 100 mol% purity from suitable substrates. When the cloned *phaC* was expressed in the PHA-negative mutant of *Cupriavidus necator* PHB^{-}4, 64 wt% poly(3-hydroxybutyrate) of the dry cell weight was accumulated with fructose as the carbon source. In addition, when suitable 3-hydroxyvalerate (3HV) precursor carbon sources were used, PHA copolymers containing high molar fractions of 3HV monomers were synthesized. Most striking is the ability of the recombinant *C. necator* PHB^{-}4 containing the *Chromobacterium* sp. *phaC* to

accumulate poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) when crude palm kernel oil was used as the sole carbon source. This study demonstrates that the PhaC of *Chromobacterium* sp. has a broad substrate specificity similar to that of *Aeromonas caviae*.

1.0 Introduction

The production cost of polyhydroxyalkanoates (PHA) compared to petrochemical-based polymers is high. Petrochemical-based polymers are polymers that are much cheaper but non-degradable. In other words, they are non-eco-friendly and are a treat to the environment in this era. A lot of efforts are done to make the production of PHA economical for everyday use. The strategies are isolating and developing a bacteria strain capable of utilizing cheaper substrates and possess a high productivity trait, improvement in downstream processes, and also optimizing the growth conditions using the most inexpensive carbon sources. It will also be a bonus if the PHA producing strain is capable of using crude carbon sources, such as waste products, and incorporating them into PHA after breakdown. Using crude carbon sources such as waste products will eventually eliminate the need to dispose it and also it can be regenerated into something more useful, which is bioplastic. This is because pure or purified carbon sources are of course more costly. With all these advances, PHA has potential to be applicable in the future and will eventually eliminate disposal problems and environment hazard that are related to petrochemical-based polymers. The main focus of this project is to develop a better strain through recombinant technology that is capable of utilizing cheaper substrates. The *phaC* of the local isolate of *Chromobacterium* sp. is able to synthesize poly(3-hydroxyvalerate) of 100 mol% purity from suitable substrates. But it only manage to synthesize 20 wt% of poly(3-hydroxybutyrate) using crude palm kernel oil as a sole carbon source. This prompted the interest to clone the *phaC* of the bacterium. On the other hand, *Cupriavidus necator* H16 is able to utilize crude palm kernel oil as a sole carbon source to accumulate 75 wt% of poly(3-hydroxybutyrate). So, the initial intention of this project is to complement the *phaC* of the local isolate of

Chromobacterium sp. with *Cupriavidus necator* PHB^{-}4 to promote higher accumulation of PHA using a crude substrate as the carbon source.

2.0 OBJECTIVES OF THIS STUDY

The objectives of this study are:

1. To clone the PHA synthase gene of *Chromobacterium* sp.
2. To characterize the PHA synthase gene of *Chromobacterium* sp. at the molecular level
3. To carry out heterologous expression of the cloned PHA biosynthesis genes in the mutant strain of *Cupriavidus necator* PHB^{-}4.

3.0 Literature review

3.1 Petroleum-based plastics vs. biodegradable plastics

Plastics are essential in most industries and have replaced glass and paper in packaging, but has become a problem due to accumulation of recalcitrant plastics in the environment (non-environmental friendly) (Khanna and Srivastava, 2005; Tian et al., 2005). Burning plastics is hazardous and costly, due to the formation of hydrogen cyanide from acrylonitrile-based plastics during the combustion (Khanna and Srivastava, 2005). Recycling is a more tedious job because waste sorting is a time consuming process and also the usage of recycled material is limited due to additivies such as pigments, coatings and fillers (Khanna and Srivastava, 2005). For the wellness of the earth, biodegradable plastics seem to be a better alternative.

Biodegradable plastics can be divided into three categories: chemically synthesized polymers, starch-based biodegradable plastics and polyhydroxyalkanoates (PHA) (Khanna and Srivastava, 2005). Chemically synthesized polymers [polyglycollic acid, polylactic acid, poly(ε-caprolactone), polyvinyl alcohol, poly(ethylene oxide)] are susceptible to enzymic or microbial attack (biodegradable) but they are not commercially viable as substitute for plastics because they are lack of plastic properties (Khanna and Srivastava, 2005). Starched-based biodegradable plastics, starch-polyethylene, are a blend of starch and plastic which enables significant degradation time due to the degradation by soil microorganisms but only the starch is being degraded (Khanna and Srivastava, 2005), not the plastic. So, the problem of plastic wastes is still evident.

3.2 Polyhydroxyalkanoates (PHA)

Biologically derived polyesters known as polyhydroxyalkanoates (PHA) represent a potentially 'sustainable' replacement to fossil-fuel-based thermoplastics (Coats et al., 2007). PHA are polymers of hydroxyalkanoates, accumulated by numerous bacteria as an intracellular carbon and/or energy storage material under the conditions of limiting nutritional elements, such as nitrogen (N), phosphorus (P), sulfur (S), oxygen (O) or magnesium (Mg), in the presence of excess carbon source (Anderson and Dawes,1990; Kranz et al., 1997; Poirier et al., 1995). More than 300 different microorganisms are known to synthesize and accumulate PHA intracellularly (Sang et al., 1998). *Pseudomonas putida* KT2440 accumulating PHA increased its survival and stress tolerance (Raiger-lustman and Ruiz, 2008).

PHA attracted much attention as biodegradable substitutes for conventional non-degradable plastics (Sang et al., 1998). Besides that, it is also a renewable, 100% biodegradable and biocompatible thermoplastics that can be used in marine, agricultural and medical applications, and also tissue engineering (Agus et al., 2006; Chen and Wu, 2005). PHA is able to replace petrochemical plastics such as polyethylene and polypropylene because it has similar properties (Qiu et al., 2006; Yang et al., 2002). PHA exists as discrete inclusions localized in cell cytoplasm and can be visualized with a phase contrast light microscope, due to their high refractivity (Dawes and Senior, 1973).

Native PHA inclusions can be stained with Sudan Black B indicating that they are of lipid nature (Alias and Tan, 2005; Redzwan et al., 1997). A more specific dye would be oxazine dye Nile Blue A or Nile red, exhibiting a strong orange fluorescence under UV light, at an excitation wavelength of 460 nm (García and Nungaray, 2008; Alias and Tan, 2005; Ostle and Holt, 1982). Besides that, PHA are

partially crystalline polymers with a degree of crystallinity within the range of 60-80% (Sudesh et al., 2000). Within the bacterial cell, they exist as amorphous and water-soluble inclusions (Barnard and Sanders, 1989). But, upon cell disruption or when the polymer is extracted, rapid crystallization occurs (Khanna and Srivastava, 2005).

PHA granules are coated with a layer of phospholipids and proteins, with phasins as the predominant compound (Pötter et al., 2002). Depending on the carbon atoms in the polymer, PHA can be divided into two groups; short chain length (scl), which consist of 3-5 carbon atoms, and medium chain length (mcl), which consist of 6-14 carbon atoms (Anderson and Dawes, 1990). The composition of PHA is very dependant on the substrate specificity of the PHA synthase, metabolic routes that provide the precursors and also the cultivation conditions (Qiu et al., 2006). Non-sulfur photosynthetic bacteria are able to synthesize C_4 and C_5 PHA copolyesters on a variety of carbon and nitrogen sources (Liebergesell et al., 1991) because they are metabolically quite diverse (Kranz et al., 1997).

3.3 The commercialization of PHA

Polyhydroxybutyrate, P(3HB), was the first PHA to be discovered, widely studied and best characterized because its mechanical properties is similar to that of conventional plastics, polypropylene or polyethylene (Khanna and Srivastava, 2005). P(3HB) can be extruded, moulded, spun into fibres, made into films and can used to make heteropolymers with other synthetic polymers but it is 5-10 times more expensive than the petroleum-derived polymers (polypropylene and polyethylene), which costs approximately US$ 0.25-0.50 kg^{-1} (Khanna and Srivastava, 2005; Poirier et al., 1995). Factors that affect the production of PHA are the PHA

productivity, content and yield, cost of the carbon substrate and the downstream processes (Mahishi et al., 2003). PHA can be produced by fermentation from renewable carbon sources such as sugars and vegetable oils (Agus et al., 2006). For economical bioplastic production with high concentration and productivity, various bacterial strains, either wild type or recombinant, and new fermentation strategies were developed (Sang et al., 1998).

Historically, the random copolymer P(3HB-*co*-3HV) has been studied most extensively and was commercially sold under the tradename Biopol^{TM} (Aldor and Keasling, 2003). Not only that, P(3HB) is already a marketed biodegradable polymer, Ecoflex (Wang et al., 2004). On the other hand, Imperial Chemical Industries (UK) has been producing PHB on large scale from glucose and P(3HB-*co*-3HV) from a mixture of glucose and propionic acid by fed-batch culture of *Cupriavidus necator* (Khanna and Srivastava, 2005). High manufacturing costs can be reduced by establishing an efficient recombinant-PHA production system with the desired properties (Kichise et al., 2002). Recently, the TephaFlex® absorbable suture based on poly(4-hudroxybutyrate) launched by US company Tepha has been approved by the US Food and Drug Administration (FDA) for clinical applications (Li et al., 2008).

The production of PHA in homologous or heterologous (engineered) organisms to minimize the costs of PHA production requires a thorough knowledge of the PHA biosynthetic pathways (Kranz et al., 1997). The selection of microorganism for PHA production should be based on several factors including the cell ability to utilize an inexpensive carbon source, growth rate, polymer synthesis rate and the maximum extent of polymer accumulation (Khanna and Srivastava, 2005). Developing strains that are capable of using inexpensive or waste products

(Sang et al., 1998) will eventually cut down the cost of PHA and also reduce waste disposal costs (Lee et al., 1999). It is observed in the production of poly(3-hydroxybutyrate) from whey, a major by-product from cheese manufacturing industry, by recombinant *E. coli* strain harboring the *Cupriavidus necator* PHA biosynthesis genes (Sang et al., 1998). Besides that, Saccharophagus degradans (ATCC 43961) is found to be able to degrade cellulose from wastes from agriculture and utilize it as a primary carbon source to accumulate PHA (Munoz and Riley, 2008).

3.4 Bacterial PHA synthesis

In fermentation strategies, the bacteria that are used for the production of PHA can be divided into two groups based on the culture conditions required for PHA synthesis (Khanna and Srivastava, 2005). The first group of bacteria requires the limitation of an essential nutrient such as nitrogen, phosphorus, magnesium or sulphur for the synthesis of PHA from an excess carbon source (Khanna and Srivastava, 2005). The bacteria included in this group are *Cupriavidus necator*, *Protomonas extorquens* and *Protomonas oleovorans* (Khanna and Srivastava, 2005). *Cupriavidus necator* accumulates a large amount of polymer (up to 80% dry cell weight) when nitrogen or phosphorus is completely depleted (Kim et al., 1994). The second group of bacteria, which includes *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii* and recombinant *E. coli*, do not require nutrient limitation for PHA synthesis and can accumulate polymer during growth (Khanna and Srivastava, 2005).

3.5 PHA synthase

Poly(3-hydroxybutyrate) is synthesized from acetyl-coenzyme A (CoA) in a three-step pathway using three enzymes; β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase (Jung et al., 2000; Kolibachuk et al., 1999; Kranz et al., 1997). The first reaction involves a PHA-specific β-ketothiolase, encoded by *phaA*, which condenses two acetyl-CoA molecules into acetoacetyl-CoA (Kolibachuk et al., 1999). The second reaction, which is the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA, is catalyzed by an NADPH-dependent acetoacetyl-CoA reductase, encoded by *phaB* (Kolibachuk et al., 1999). The third and the last reaction is catalyzed by PHA synthase, which is the product of the *phaC* gene. In this reaction, 3-hydroxybutyryl-CoA is linked to an existing PHA molecule by the formation of an ester bond (Kolibachuk et al., 1999). The final concentration and content of PHA is more dependent of the activity of PHA synthase, rather than β-ketothiolase and acetoacetyl-CoA reductase (Jung et al., 2000). Following the reactions above, different 3-hydroxyacyl-CoA substrates, depending on various carbon sources, can be used by the PHA synthase to synthesize PHA with different monomer compositions (Kolibachuk et al., 1999; Kranz et al., 1997).

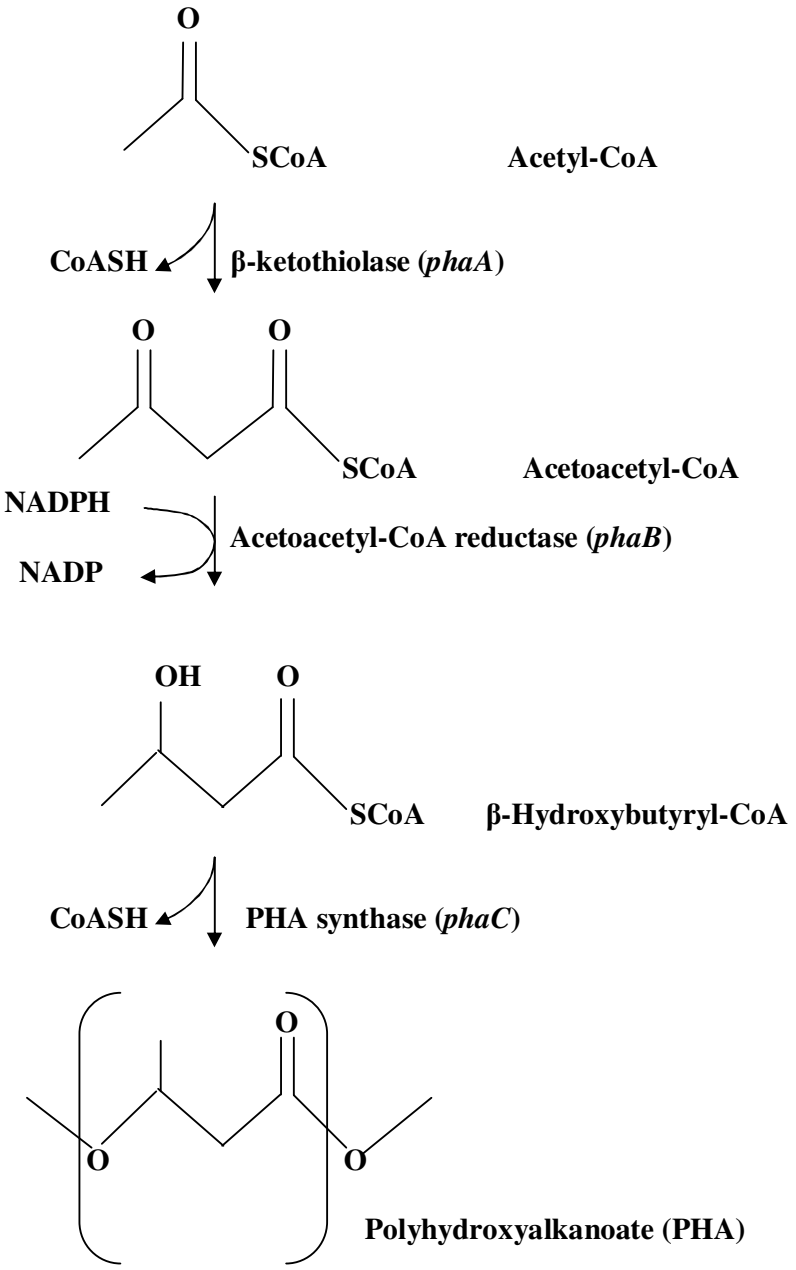

Figure 3.1: Pathway for PHA production by *Cupriavidus necator* (Kranz et al., 1997).

3.6 Various PHA polymers

Medium chain length (mcl) PHA are semi-crystalline elastomers (rubbery) and flexible materials (low tensile strength, high elongation to break) with low crystallinity and melting point, and can be used as biodegradable rubber after cross linking and also in a wide range of applications which cannot be fulfilled by other short chain length (scl) PHA (Khanna and Srivastava, 2005; Preusting et al., 1990). An example of a bacterium producing mcl PHA is *Pseudomonas putida* KT2442 (Ouyang et al., 2007). P(3HB), a scl polymer, is stiffer and becomes brittle over a period of several days upon storage under ambient conditions (De Koning et al., 1992). P(3HB) is a linear, polymer chain with alternating polar and non-polar (Huang and Reusch, 1996). It was reported that P(3HB-*co*-3HHx) (a scl-mcl polymer) content can be increased by phosphorus limitation (Lee et al., 2000). A mixture of scl-mcl hydroxyacyl monomers have properties ranging from thermoplastic to elastomeric, depending on the molar ratio of scl and mcl monomers incorporated into the copolymer (Nomura et al., 2008).

The physical properties of P(3HB-*co*-3HV) are closely connected with the molar fraction of 3HV in the copolymer (Jung et al., 2000). But due to isomorphism, similarity in shape and size, of P(3HB-*co*-3HV) to P(3HB), P(3HB-*co*-3HV) is also brittle and thermally instable (Chen et al., 2006). As the fraction of 3HV increases, the copolymer becomes tougher (increase in impact strength) and more flexible (Khanna and Srivastava, 2005). The properties of copolymer will in fact depend on the average chemical composition and its distribution (Chen et al., 2006). Thus, material properties (thermal and physical) can be controlled by adjusting the fraction of 3HV using fermentation strategies (Khanna and Srivastava, 2005). The ability to accumulate PHA of different structure depends on the physiological and biochemical

characteristics of producer strains and the conditions of their growth (Boyandin et al., 2008).

3.7 Heterologous PHA production

Heterologous precursor production pathways are used to facilitate the synthesis of polymers that would not naturally accumulate in the native host, or possibly anywhere in nature, and which may have desirable structures and material properties (Aldor and Keasling, 2003). Besides that, it can also be used to develop novel pathways for efficient precursor production using unrelated, simple and inexpensive carbon sources, which may also facilitate fast growth to a high cell density (Aldor and Keasling, 2003). The most common microbes used as heterologous hosts and in fermentation development include *Cupriavidus necator*, *Pseudomonas putida*, *Pseudomonas oleovorans* and *Escherichia coli* (Lee and Choi, 2001). The microbes chosen must be genetically well-understood and its biochemistry easily manipulated for the biosynthesis of PHA and high-productivity fermentation (Aldor and Keasling, 2003).

Cupriavidus necator has been studied most extensively due to its ability to accumulate large amount of P(3HB) from simple carbon sources, glucose, fructose and acetic acid (Khanna and Srivastava, 2005; Tian et al., 2005). *E. coli* has proven to be a versatile performer, not only in expediting the molecular analysis of PHA biosynthesis but also in synthesizing the biopolymer to extremely high intracellular levels, being amenable to specific genetic strategies such as genetically mediated lysis, and the utilization of mutants to metabolically engineer strain that produces P(3HB-*co*-3HV) copolymer (Khanna and Srivastava, 2005). In *E. coli*, PHA synthesis may be induced at an optimized point in the process and is not tied to

natural regulation, which often involves induction by nutrient limitation (Aldor and Keasling, 2003), but is dependant on the amount of available acetyl-CoA (Khanna and Srivastava, 2005). Besides that, it does not harbor native machinery for polymer degradation, unlike organisms that can retrieve the stored carbon and reducing equivalents (Aldor and Keasling, 2003), since PHA is a carbon and energy storage. *E. coli* cells are relatively weak, making recovering recombinant granules that tend to be large in this microbe an easier task (Aldor and Keasling, 2003). *E. coli* is also fast growth, high cell density, has the ability to utilize several inexpensive carbon sources and easily purified (Fidler and Dennis, 1992; Hahn et al., 1995).

PHA biosynthesis involves transforming soluble substrates, such as hydroxyalkanoate coenzyme A esters, during polymer elongation into insoluble inclusions that are stored, and can be rapidly degraded when necessary (Rehm and Steinbüchel, 1999). The enzymes for the synthesis of PHA are always present and that the regulation of PHA synthesis occurs post-translationally (Kranz et al., 1997). Besides that, studies showed that these enzymes are produced constitutively (Tal et al., 1990). The variety of PHA homo- and heteropolymers can be increased using precursors (Steinbüchel et al., 1995).

3.8 PHA biodegradability

Various microorganisms excrete extracellular PHA depolymerases that hydrolyse PHA into water-soluble oligomers and monomers for utilization as nutrients within the cells, since PHA is a solid polymer with a high molecular weight, incapable of transported through the cell wall (Khanna and Srivastava, 2005). The rate of PHA hydrolysis is reported to be dependant on the exposed surface area of the polymer (Wang et al., 2004). Pores allow enzyme adsorption,

making the rate of degradation faster. Lipase and sodium hydroxide acid can also be used to degrade PHA, by breaking the ester-linkage of the polymer (Yang et al., 2002). The biodegradation of P(3HB-*co*-3HHx) *in vitro* and *in vivo* are too slow for clinical applications (Shangguan et al., 2006).

3.9 PHA applications

PHA, a remarkable family of natural polyesters has a wide array of useful properties and potential applications (Orts et al., 2008). The potential applications are packaging, disposable items and replacements to our human tissues (Chen and Wu, 2005; Khanna and Srivastava, 2005), covering areas such as medicine, agriculture, tissue engineering, nanocomposites, polymer blends and chiral synthesis (Philip et al., 2007). PHA can also be used as stereo regular compounds which can serve as chiral precursors for the chemical synthesis of optically active compounds (Oeding and Schlegel, 1973; Senior and Dawes, 1973). P(3HB-*co*-4HB) is reported appropriate for biodegradable drug carriers (Turesin et al., 2001). Such compounds are particularly used as biodegradable carriers for long-term dosage of drugs, medicines, hormones, antibiotics (Sulperazone and Duocid), insecticides and herbicides (Chen and Wu, 2005; Khanna and Srivastava, 2005). Recently, PHA has become one of the interesting classes of biomaterials for the development of tissue-engineered cardiovascular products because of their specific properties, not available in existing synthetic absorbable polymers (Williams and Martin, 2002).

To mimic the real microenvironment of extracellular matrix for cell growth, the scaffold should provide a highly biocompatible three-dimentional biodegradable polymer substrate to enable cell adhesion, migration (to spread out), proliferation and differentiation function to develop into a tissue-like structure (Deng et al., 2002; Li et

al., 2008). P(3HB-*co*-3HHx) is less porous when the HHx component is increased, making it smoother (Wang et al., 2005). Fibroblast is reported to attach to smoother surface (Wang et al., 2005). Material surface properties effects the initial cellular events on the cell-material interface (Wang et al., 2005). It is therefore desirable to modify the material surface to suit the intended application, without altering other properties of the scaffold, such as mechanical strength or thermal properties (Liu et al., 1999; Williams et al., 1999).

Crystallinity at polymer surface is also responsible for difference in surface morphology between PHB and P(3HB-*co*-3HHx) (Mori et al., 2008). Effective surface modifications include changes in chemical group functionality, surface charge, hydrophilicity, hydrophobicity and wettability (Liu et al., 1999; Sacristan et al., 2000; Williams et al., 1999). Modification of a polymer surface can be achieved by various chemical or physical processes including plasma-ion beam treatment, electric discharge, surface grafting, chemical reaction, vapor deposition of metals and flame treatment (Williams et al., 1999). Hydroxyapatite that was blended into P(3HB) increase elasticity and stress tolerance (Wang et al., 2005). Protein has both hydrophobic and hydrophilic regions causing a selective attachment of protein to a surface and the area with appropriate proportions of hydrophobicity and hydrophilicity will be favored for protein attachments (Wang et al., 2003). Plasma treatment improve surface polarity, but the incorporations of polar components decreased the hydrophobicity, changed the surface chemical state and electrical charges (Qu et al., 2005).

The surface properties of a biomaterial, especially hydrophilicity, influence cell adhesion to the materials (Chanvel-Lesrat et al., 1999; Furukawa et al., 2000; Qing et al., 1999; Zhao and Geuskens, 1999). Cells attach to the biomaterial by

filapodia, forming bridging cells for the attachment of other cells to form cell aggregates (Deng et al., 2002). Hydrolyzation process by lipases or sodium hydroxide will generate more hydroxyl groups that will lead to increased hydrophilicity, improving the capacity for cells to adhere to the polymer surfaces (Yang et al., 2002). The higher the hydrophilicity surface of a material, the stronger the cells attached to that material (Yang et al., 2002). Higher protein absorption and protein conformational changes always occurred on hydrophobic surface because proteins were difficult to de-absorb from hydrophobic surface under the effects of hydrophobic bonds (Dee et al., 2002). Poly(3-hydroxyhexanoate) [P(3HHx)] containing high 3-hydroxyhexanoate content tends to be more hydrophobic (Qu et al., 2006).

On the other hand, P(3HB-*co*-3HHx) powder (Lawrence et al., 2005) treated with UV radiation increased its hydrophilic functional groups (increase polar groups C—O and C=O), degradation rate and mechanical property (Shangguan et al., 2006). Even though treating polymers with chemicals is proved to improve the polymer properties, it is better to minimize its usage because the residual chemicals may exhibit side effects if not removed thoroughly. Mechanical properties of P(3HB) can be improved when blended with P(3HHx) (Zhao et al., 2003). The high crystallization degree and rapid crystallization rate of P(3HB) create pores and protrusion on the film surface (a coralloid surface) that might prohibit the attachment and growth of mammalian cells (Zhao et al., 2003). The P(3HHx) in the P(3HB)/P(3HHx) blend reduced the crystallization degree and crystallization rate (Zhao et al., 2003) and it might decrease to pores on a pore surface.

In blend polymers, P(3HB) crystallizes to form the crystalline domains that act as physical crosslinkers and fillers, and P(3HHx) forms the amorphous domain

(Zhao et al., 2003). It was found that mouse fibroblast cell line L929 grew better in the blend of P(3HB) and P(3HHx) films rather than on the individual films, but viable cells on P(3HHx) alone was 216 times more than that of P(3HB) (Yang et al., 2002). These results showed that P(3HHx) promotes cell growth, an important criteria for biomaterial. Lipase is shown not able to degrade P(3HHx) efficiently or the degraded product shows mixed effects, showing little change on its surface pore size (Yang et al., 2002). At a ratio of 1:1 for P(3HB)/P(3HHx) blends, the non-dispersion component of surface free energy is maximum, leading to a maximal total surface free energy (Zheng et al., 2005). The higher the surface free energy of a blend film, the more amount of protein will be absorbed to the film (Zheng et al., 2005). That was the reason protein absorption and rabbit articular cartilage chondrocytes adhesion were detected on the blended films (Zheng et al., 2005).

3.10 *Chromobacterium violaceum*

Chromobacterium violaceum is a common saprophyte and a motile environmental β-proteobacterium of soil and water in tropical and sub-tropical regions (Betts et al., 2004; Ray et al., 2004; Steinberg and Del Rio, 2005). Its genome is nearly 5 megabases and it encodes over 4400 genes (Stephens, 2004). The organism is not harmful to plants but it is a rare and opportunistic (but lethal) pathogen of animals and humans (Stephens, 2004). It was first identified in 1881 and was isolated from buffalo in Phillipines (Woolley, 1905). In 1927, *C. violaceum* was found in Malaysia as a human pathogen (Sneath et al., 1953). Human infection, called *C. violaceum* septicemia, will cause systemic and severe disease with a high fatality rate (Kaufman et al., 1986; Steinberg and Del Rio, 2005). Due to the rare human infection, the awareness of the disease is limited (Chattopadhyay et al., 2002).

The bacterium is a versatile heterotrophic, gram negative rod which is non-fastidious, facultatively anaerobic, fermentative and, oxidase and catalase positive (Oliveira et al., 2005; Scholz et al., 2006). It grows well at 25-30^{o}C (McLean et al., 2004). *C. violaceum* exists in both pigmented and non-pigmented strains (Hodge, 2002), with the former producing a major pigment violacein (a violet non-diffusible pigment), which is soluble in ethanol and insoluble in water and chloroform (Dias Jr et al., 2002; Ray et al., 2004; Weaver et al., 1985). That is why, it is reported that violacein is extracted with butanol and its content can be measured at a wavelength of 585 nm (Blosser and Gray, 2000). The bacterium is able to live under anaerobic and aerobic conditions but violacein is only produced during aerobic conditions (Konzen et al., 2006). Violacein is abundantly formed, derived from tryptophan (Momen and Hoshino, 2000) and its physiological function is not known until now (Konzen et al., 2006).

As a soil microorganism, it is normal that it excretes secondary metabolites (including violacein) as a weapon against competitors and/or predators (Duran and Menck, 2001). Secondary metabolites are natural products that are produced by organisms in response to external stimuli/environment, such as nutritional changes, infection and competition (Fröhner et al., 2006). By exhibiting phenotypic drug resistance in infections, this bacterium is able to compete with other bacteria in the similar environment (Garboggini et al., 2004). To thrive in harshful environment, this bacterium is able to synthesize 13 types of beta-lactam antibiotics, 40 types of multidrug resistance protein and 4 types of miscellaneous resistance protein (Garboggini et al., 2004). That causes the fatality in *C. violaceum* septicemia.

The scientific name for violacein is [3-{1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene}-1,3-dihydro-2H-indol-2-one] (Melo et al., 2003).

Violacein is proven to exhibit antimicrobial activity (Hungria et al., 2005) and also reported to have *in vitro* antitumor effects, effective against neoplastic cell lines and leukemic lineages, such as the human promyelocytic leukemia cell line HL60 (Melo et al., 2003). It also has antibiotic and trypanocidal activities (Fröhner et al., 2006) and plays a significant role in the membrane defense against oxidative stress (Konzen et al., 2006). It is able to scavenge free radicals in solution and inhibit the reaction of pro-inflammatory and oxidizing enzyme (Konzen et al., 2006). The violacein of the *C. violaceum* isolated from Amazon river, Brazil, have numerous biological activities such as probable cytotoxic effect and initiation of apoptosis in normal cell cultures (Melo et al., 2000).

It was reported that violacein is not present in the supernatant of the *C. violaceum* culture and that it was necessary to disrupt the cells in order to extract violacein (Konzen et al., 2006). *C. violaceum* has the capability of quorum sensing (QS), shown in response to population density (McLean et al., 2004). Violacein production is reported to be controlled by QS (Stephens, 2004). *C. violaceum* also possess the ability to synthesize bioplastics of short chain length such as poly(3-hydroxyvalerate) [P(3HV)] homopolymer (Forsyth et al., 1958, Steinbüchel et al., 1993). Besides all of the above, this bacterium is proven to be able to mobilize nickel from fine-grained nickel powder, and solubilize gold from gold-containing ores or native gold (Campbell et al., 2001; Faramarzi et al., 2004).

4.0 MATERIALS AND METHODS

4.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 4.1.

4.2 Growth of bacteria

Chromobacterium sp. was grown at 30 °C in nutrient rich (NR) medium [10 g/l enzymatic digest from gelatine (peptone), 10 g/l meat extract and 2 g/l yeast extract, pH 7.0] under a rotary shaker of 200 rpm. *Cupriavidus necator* PHB$^-$4 was maintained on NR agar at room temperature. All *Escherichia coli* strains were grown at 37 °C in Luria-Bertani (LB) medium [10 g/l casein enzyme hydrolysate type I (tryptone-type I), 5 g/l yeast extract and 5 g/l NaCl, pH 7.0] under a rotary shaker of 200 rpm. Antibiotics (ampicillin or kanamycin) was added according to the final concentration (50 or 100 μg/ml) into the medium when needed.

4.3 Sterilization

All media and apparatus were autoclaved at 15 psi (121°C) for 15 minutes for sterilization purposes. 0.22 μm filter (Millex ™GP/Milipore) and 95% alcohol was used when necessary.

Table 4.1: Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant phenotype	Source or reference
Bacterial strains: *Escherichia coli*		
JM109	E14–(*mcr*A), *rec*A1, *gry*A96, *thi*-1, *hsd*R17(rk-, mk+), *sup*E44, *rel*A1, D(*lac-pro*AB), [F' *tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$]	Stratagene
S17-1	*rec*A and *tra* genes of plasmid RP4 integrated into chromosome; auxotrophic for praline and thiamine	Simon *et al.* (1983)
Chromobacterium sp.	Wild type	Yong (unpublished)
Cupriavidus necator H16	Wild type	ATCC 17699, DSM 428
PHB^-4	PHA-negative mutant of H16	DSM 541
Plasmids:		
pGEM-T easy	*rec*A, *end*A1, *gyr*A96, *thi*, hsdR17(r_{K-}, m_{K+}), relA1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$]	Promega
pBBR1MCS-2	Km^r, broad host range, *lac* POZ'	Kovach *et al.* (1995)
pGEM-Csp2	pGEM-T easy vector derivative harboring approximately 2.0 kb fragment of *phaC* from *Chromobacterium* sp. with putative promoter	In this study
pBBR1MCS-Csp2	pBBR1MCS-2 derivative harboring approximately 2.0 kb fragment of *phaC* from *Chromobacterium* sp. with putative promoter	In this study

4.4 Restriction and modification enzymes

All DNA manipulating and restriction enzymes (*Apa*I, *Sal*I and *Eco*RI) were used according to the manufacturers' protocols such as Promega and New England Biolabs respectively.

4.5 Preparation of *Chromobacterium* sp. genomic DNA

The total genomic DNA of *Chromobacterium* sp. was isolated according to the standard procedures (Ausubel et al., 2002). *Chromobacterium* sp. was cultivated at 30 °C in 5 ml NR medium overnight on a rotary shaker. 1.5 ml of culture was microcentrifuged for 2 minutes or until a compact pellet forms. The pellet was resuspended in 567 µl of TE (10 mM Tris-Cl, 1 mM ethylenediaminetetracetic acid {EDTA}, pH 8.0) Buffer, 30 µl of 10% sodium dodecyl sulfate {SDS} and 3 µl of 20 mg/ml Proteinase K. The mixture was mixed thoroughly and incubated for 1 hour at 37^{o}C. 100 µl of 5 M sodium chloride (NaCl) was added and the solution was mixed thoroughly. 80 µl of CTAB/NaCl (cetyltrimethyl ammonium bromide/sodium chloride) solution was added. The solution was mixed thoroughly and incubated at 10 minutes at 65^{o}C. 1 volume of 24:1 chloroform/isoamyl alcohol was added. The mixture was mixed thoroughly and was microcentrifuged for 4 to 5 minutes. The supernatant was transferred to a fresh tube. 1 volume of 25:24:1 phenol/chloroform/isoamyl alcohol was added. The mixture was microcentrifuged for 5 minutes before transferring the supernatant into a fresh tube. 0.6 volume of isopropanol was added and the mixture was mixed gently until a stringly white DNA precipitate forms. The solution was microcentrifuged briefly at room temperature. The

supernatant was discarded and 70% ethanol was added to the pellet. The mixture was microcentrifuged for 5 minutes at room temperature and the pellet was dried. Finally the pellet was resuspended in 100 µl of TE Buffer.

4.6 Cloning strategy for PhaC gene

4.6.1 Amplifying the PHA synthase (*phaC*) of *Chromobacterium* sp. by means of polymerase chain reaction (PCR)

The whole genomic DNA of *Chromobacterium* sp. was used as a template for PCR amplification of the PHA synthase gene using a pair of domain-specific primers designed using National Centre for Biotechnology Information (NCBI) database as a reference. The sequences of these primers are; Forward primer (F1): 5'-cgtaattggggcccatgcag-3' and Reverse primer (R1): 5'-agccgccgccgaagcttccgatggc-3'. The mixture for PCR reaction contained 25 ng genomic DNA (gDNA) of *Chromobacterium* sp., 1X *Taq* DNA polymerase buffer, 100µM nucleotides mix (dNTP), 25pmol of each primer (F1 and R1), 1.5mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Promega). The PCR mixture was topped up with ddH_2O to 25 µl. PCR was carried out using Peltier Thermal Cycler (PTC-200) and the cycle conditions are shown in Table 4.2. The amplified fragment was purified using SpinClean™ Gel Extraction Kit (Column) (Mbiotech).

Table 4.2: PCR conditions used in amplification of the *pha*C gene

Step	Reaction	Temperature	Period
1	Incubation	94 °C	5 minutes
2	Denature	95 °C	20 seconds
3	Annealing	60 °C	3 minutes
4	Elongation	60 °C	3 minutes
5	Go to step (2)		30 cycles
6	Final elongation	72 °C	10 minutes
7	Cooling	10 °C	∞
8	End		