CHARACTERIZATION OF *ACINETOBACTER* SP. LWC1 ISOLATED FROM CONTAMINATED WATER AND ITS SURVIVABILITY UNDER DIFFERENT STRESS CONDITION

By

LIM WUI CHEN

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LIST OF ABBREVIATIONS AND SYMBOLS

$\times g$	gravitational force
µg	microgramme
µL	microlitre
µm	micrometre
3HB-CoA	3-hydroxybutyryl-coenzyme A
CME	caprylic acid methyl ester
CoA	coenzyme A
g	gramme
GC	gas chromatography
kg	kilogramme
L	liter
MCL-PHA	medium-chain-length polyhydroxyalkanoate
mg	miligramme
mL	milliliter
OD	optical density
P(3HB)	poly(3-hydroxybutyrate)
P(3HV)	poly(3-hydroxyvalerate)
P(3HB-*co*-3HV)	poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PHA	polyhydroxyalkanaote
SCL-PHA	short-chain-length polyhydroxyalkanoate
SEM	Scanning electron microscope
v	volume

w	weight
WT	wild-type $PhaC_{Cn}$
% DCW	percent of dry cell weight
LG	LB with 2% (v/v) glucose
LA	LB with 2% (w/v) lactose
LPO	LB with 2% (v/v) palm oil
Z	% PHA
W	Dry cell weight
DCW	Dry cell weight

PENCIRIAN *ACINETOBACTER* SP. LWC1 YANG DIPENCILKAN DARIPADA AIR YANG TERCEMAR SERTA KEMANDIRIANNYA DALAM KEADAAN TEKANAN YANG BERBEZA

ABSTRAK

Peranan PHA dalam kemandirian bakteria di dalam air yang tercemar telah diselidik dalam kajian ini. Eksperimen ini bermula dengan pemencilan bakteria daripada air yang telah dicemari dengan sisa domestik, diikuti dengan pengenalan dan pencirian bakteria tersebut dan diakhiri dengan rawatan tekanan terhadapnya. Berdasarkan keputusan yang diperolehi daripada analisa 16S rDNA, bakteria tersebut lebih menjurus kepada *Acinetobacter* spp. Oleh itu, ia telah dinamakan sebagai *Acinetobacter* sp. LWC1. Selain itu, kesan daripada pelbagai jenis sumber karbon seperti gula, minyak dan asid karboksilik dan media yang digunakan terhadap pengeluaran PHA juga dikaji. Antara semua gula, LB yang menggunakan 2% (w/v) glukosa menghasilkan kandungan PHA tertinggi (11% berat sel kering) dengan 0.4 g/L berat sel kering dalam tempoh 48 j. Sementara itu, bagi minyak, LB yang menggunakan 3% (v/v) minyak enjin sisa memberikan kandungan PHA tertinggi (6.6% berat sel kering) dengan 1.4 g/L berat sel kering dalam tempoh 72 j. Biosintesis daripada P(3HB-ko-3HV) kopolimer juga dinilai dalam kajian ini. Walau bagaimanapun, ia dicatatkan sebagai kandungan PHA yang kedua tertinggi (9.14% berat sel kering) dengan 0.98 g/L berat sel kering dan 70 mol% 3HV dalam tempoh 96 j dalam medium LB yang menggunakan 2% (w/v) glukosa ditambah dengan 0.25% (v/v) asid valerik. Bagi medium MS, 2% (v/v) sisa minyak enjin dengan 0.5% (v/v) asid propionik memberikan kandungan PHA tertinggi (3.99% berat sel kering) dengan 0.30 g/L berat sel kering dalam tempoh 72 j. Hubungan antara pengumpulan PHA

dalam meningkatkan kemandirian *Acinetobacter* sp. LWC1 juga telah dijalankan dengan membandingkan bakteria yang mempunyai kandungan PHA yang tertinggi dengan kandungan PHA yang terendah di bawah keadaan tekanan alam sekitar yang sama. Didapati bahawa *Acinetobacter* sp. LWC1 mengandungi kandungan PHA yang tertinggi dengan mencatatkan 11% berat sel kering yang diperolehi daripada pengkulturan dalam LB yang menggunakan 2% (w/v) glukosa (LG) berketahananan tinggi terhadap tekanan alam sekitar seperti suhu yang tinggi, penyinaran UV, rawatan etanol dan tekanan osmosis berbanding dengan pengkulturan yang mempunyai kandungan PHA yang terendah iaitu 0.5% berat sel kering yang diperolehi daripada pengkulturan LB yang menggunakan 2% (w/v) laktosa (LA) dan 2% (v/v) minyak sawit (LPO) masing-masing.

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CHARACTERIZATION OF *ACINETOBACTER* SP. LWC1 ISOLATED FROM CONTAMINATED WATER AND ITS SURVIVABILITY UNDER DIFFERENT STRESS CONDITION

ABSTRACT

The role of PHA in the survival of bacteria in contaminated waters was investigated in this study. This study began with the isolation of a bacterium from water contaminated with domestic wastes, followed with the identification and characterization of the isolate and ended with the stress treatments to it. Based on the 16S rDNA, this isolate was closer to the *Acinetobacter* spp. Therefore, it was designated as *Acinetobacter* sp. LWC1. Besides this, the effect of various types of carbon sources such as sugars, oils and carboxylic acids and medium on PHA production was investigated in this study. Among all of the sugars, LB with 2% (w/v) of glucose yield the highest PHA content (11% DCW) with 0.4 g/L dry cell weight at 48 h. Meanwhile for the oils, LB with 3% (v/v) of used engine oil gave the highest PHA content (6.6% DCW) with 1.4 g/L dry cell weight at 72 h. The biosynthesis of P(3HB-co-3HV) copolymer was also evaluated in this study. However, it was recorded as second highest PHA content (9.14% DCW) with 0.98 g/L dry cell weight and 70 mol% 3HV at 96 h in LB medium by using 2% (w/v) of glucose supplemented with 0.25% (v/v) of valeric acid. For the MS medium, 2% (v/v) of used engine oil with 0.5% (v/v) of propionic acid gave the highest PHA content (3.99% DCW) with 0.30 g/L dry cell weight at 72 h. The relationships between PHAs accumulation in enhancing the survivability of *Acinetobacter* sp. LWC1 were carried out by comparing the bacteria with highest PHA and lowest PHA content under the same environmental stress conditions. It was found that *Acinetobacter* sp. LWC1 containing high PHA content of 11%

DCW obtained from cultivation cultures in LB with 2% (w/v) glucose (LG) improved its endurance against environmental stresses such as high temperature, UV irradiation, ethanol treatment and osmotic pressure compared to the ones with low PHA content of 0.5% DCW obtained from cultivation cultures in LB with 2% (w/v) lactose (LA) and LB with 2% (v/v) palm oil (LPO) respectively.

1.0 INTRODUCTION

In this natural environment, it is crucial to understand how living organisms grow and respond to the environmental signals or stressful conditions so that they are able to live, thrive and survive (Marc, 2005). This is because a changing environment creates a stressful condition to microorganisms and thus leads to damage of the cellular macromolecules such as membranes, proteins and nucleic acids. Therefore, there is a need for them to have their own mechanisms in order to adapt or withstand to the stress. They either alter their allocation of resources from growth to survival pathways or otherwise they may die (Schimel *et al*., 2007).

There are many strategies for microorganisms to increase the survival rate in the adverse environment. One of them is by accumulating inclusion of storage substances such as polyhydroxyalkanoates (PHA). PHA are accumulated by bacteria under unbalanced growth conditions, especially when the carbon source is in excess while other nutrients such as nitrogen, sulfur, phosphorus or oxygen are depleted or limited in order to store carbon and energy source (Madison and Huisman, 1999). When the supply of the limiting nutrient is restored, the stored PHAs will be degraded by the intracellular depolymerase and then metabolized them as carbon and energy source (Byrom, 1994; Ojumu *et al*., 2003).

PHA have gained a lot of attractions not only because of their biodegradable characteristics (Chee *et al*., 2010) which do not require any special environmental conditions to be degraded, but also because of PHA can be used as carbon and energy sources to enhance the capability of most of the bacterial strains to deal with the environmental stress (Castro-sowinski, 2010).

Previous studies had been reported about the relationship between stress and PHA accumulation on several bacteria strains such as *Cupriavidus necator* (previously are known as *Alcaligenes eutropha*, *Wautersia eutropha* or *Ralstonia eutropha*), *Legionella pneumophila* (James *et al.*, 1999), *Pseudomonas* sp (Ayub *et al.*, 2004; Ruiz *et al.*, 2001) and *Bacillus megaterium* sp (López *et al*., 1995). These studies showed that the accumulation of PHA is crucial for aiding the survival of bacteria under adverse environments. A recent study by Wu and his co-workers (2011) suggested that the increasing of PHA accumulation was one of the strategies for *Bacillus thuringiensis* YBT-1520 to undergo long term heat stress.

Hence, the accumulation and utilization of PHA by bacteria under stress conditions constitute a mechanism that supports the survival, proliferation and competition of the microorganisms. Nevertheless, there are few reports to date about *Acinetobacter* spp. in producing PHA aerobically and its survivability under different stress conditions. On the other hand, there are numerous of studies on the biological phosphate removal by *Acinetobacter* spp. in the activated sludge where *Acinetobacter* spp. accumulate PHA by breaking down the polyphosphate during the anaerobic condition and accumulate back the polyphosphate while breaking down the PHA in the aerobic stage (Comeau *et al*., 1986; Deinema *et al*., 1980; Deinema *et al*., 1985; Rees *et al*., 1993; Mino, 2000; Schembri et al., 1995; Zafiri *et al*., 1999). It is the aim of this study to study the role of PHA in the survivability of *Acinetobacter* sp. LWC1 under different stress conditions.

1.1 OBJECTIVES

Since adverse environment may creates stressful condition for bacteria to live, therefore, this research will focus on the isolation and characterization of a PHA producing strain from a contaminated site where it is considered a stressful condition for bacteria to survive. Besides, this research also aims to study the effect of various carbon sources in rich and minimal medium towards PHA production. It is believed that PHA enhances the survivability of bacteria under stress condition. Last but not least, this research will also focus on the role of PHA in supporting the survival of *Acinetobacter* spp. under various types of stress conditions.

The objectives of this study are as follows:

a. To isolate and characterize *Acinetobacter* sp. LWC1 from domestic wastewater pool.
b. To study the production of PHA by *Acinetobacter* sp. LWC1 on different carbon sources.
c. To study the survivability of *Acinetobacter* sp. LWC1 under various stress parameters and conditions.

2.0 LITERATURE REVIEW

2.1 General Information about *Acinetobacter* spp.

The name "*Acinetobacter*" comes from the Greek word "akinetos". Meanwhile in scientific word, *Acinetobacter* defined as 'nonmotile/ immobile rod' as the bacteria is unable to move but just show a twitching kind of motility. *Acinetobacter* spp. received a lot of attentions due to the fact that, they can cause severe nosocomial infections (Bergogne-Bérézin *et al*., 1995).

2.2 History of *Acinetobacter* spp.

The history about *Acinetobacter* was started since 1896 by Morax who was the first person discovered the members of the genus *Acinetobacter.* Then followed by a Dutch microbiologist Martinus Willem Beijerinck (Beijerinck, 1911) who discovered an organism called micrococcus calco-aceticus which isolated from soil. In 1954, Brisou and Prévot grouped the genus *Acinetobacter* with the Gram negative saprophytes that are unable to produce pigments (*Achromobactereae*) together into non-motile category. In 1957, Brisou discovered new species *Acinetobacter anitratum*. After that, Baumann and his colleagues (Baumann *et.al,* 1968) who discovered that the oxidase-negative bacteria in the Moraxella group (strictly aerobic Gram negative strains without flagella) were totally diverse from the oxidase positive bacteria in Moraxella group based on the 158 different types of carbon sources studies. Therefore, they suggested that these strains must be classifying in the genus *Acinetobacter*. In 1971, the Subcommittee on the Taxonomy of Moraxella and Allied Bacteria finally decided that the genus *Acinetobacter* must include only oxidase negative strains (Lessel, 1971; Bergogne-Bérgogne and Towner, 1996). This was supported by Johnson and his co-workers (1970)

who found that there is a clear separation between the *Acinetobacter* strains and oxidase-positive strains, and also the heterogeneity of the genus *Acinetobacter*. Furthermore, this conclusion was further supported by Juni (1972) through the utilization of ADP1 strain in the transformation test.

2.3 Current Taxonomy of *Acinetobacter* spp.

Previously, phenotypic characteristics were used for evaluating the species and relationships between different organisms. Therefore the genus of *Acinetobacter* previously have been classified at least to 15 different names, the best well known are such as *Bacterium anitratum*, *Herella* (or *Herellea*) *vaginicola*, *Mima polymorpha*, *Achromobacter*, *Micrococcus calcoaceticus*, *Diplococcus*, B5W, *Cytophaga*, *Moraxella glucidolytica* and *Moraxella lwoffii*.

But nowadays, DNA hybridization is the common method used for designating species and determining relationships between different organisms (Bergogne- Bérézin and Towner, 1996) in this new and current taxonomy of *Acinetobacter* spp. As a result, more than 20 separate genomic species have been recognized (Bouvet and Grimont, 1986) within the genus by using the DNA hybridization method. For example, *Acinetobacter* was previously classified in the Neisserriaceae family which consists of Neisseria, Moraxella and Kingella genera based on the phenotypic characteristics (Juni, 1984). In 1991, Rossau and his co-workers suggested that *Acinetobacter* should be classified in the new family Moraxellaceae together with *Moraxella*, *Pyschrobacter* and related organisms based on the 16S rRNA studies and rDNA-DNA hybridization assays (Van Loodschoot *et al*., 1986).

However there are some limitations such as the discrepancies in the numbering schemes which recommended by different researches. For example Tjernberg and Ursing (1989) and Bouvet and Jeanjean (1989) who nominated three genomic species by the number 13-15. Therefore, to avoid any confusion, usually scientist will extend their provisional designation with either TU (for Tjernberg and Ursing) or BJ (for Bouvet and Jeanjean) to refer to the respective studies.

Besides this, another limitation of DNA hybridization method was some species names are synonyms to each other in different publications. For example gen. SP9 similar with *A. lwoffii,* gen. SP12 similar with *A. radioresistens*, gen. SP14TU similar with gen. SP. 13BJ (Tjernberg and Ursing, 1989) and *A. grimontii* is a junior synonym with *A. junii*.

Besides, another difficulty was the clusters are bound to species. For example *A. baumannii* (human clinical specimen) and *A. calcoaceticus* (isolated from soil and water) was found closely related to that two genomic species of A. genomic species (gen. SP3) (Bouvet and Grimont, 1986) and gen. SP. 13TU (Tjernberg and Ursing, 1989). To solve this problem, Gerner Smidmt and co-workers (Gerner-Smidt *et. al*., 1991) nominated these four species as *A. calcoaceticus-A. baumannii* (ACB) complex. But later on, Nemec and co-workers (2011) gave formal name to gen. SP3 and gen. SP13TU as *A. pittii* sp. nov and *A. nosocomials* sp. nov respectively.

As a conclusion, in the current taxonomy, the genus of *Acinetobacter* is belonging to gammaproteobacteria which are classified in the order Pseudomonadales and in the family Moraxellacea (Towner, 2006).

2.4 Habitat of *Acinetobacter* spp.

Acinetobacter spp. are omnipresent in nature and usually isolated from animal and human. *Acinetobacter* spp. are nosocomial pathogen since they can survive on wet and dry surfaces such as skin, throat, respiratory system, digestive tract and so on (Doughari *et al*., 2011).

Besides this, *Acinetobacter* spp. also can be found from soil, water and sewage (Towner, 1996). According to Baumann (1968), *Acinetobacter* spp. constitute 0.001 of the total heterotrophic aerobic population from soil and water. They also can be isolated from much polluted water such as wastewater and treatment plants (Droop and Jannasch, 1977).

Acinetobacter spp. are also come to light in foodstuffs such as chicken carcasses, various poultry and other meats, milk products and vegetables. It is also well known for the cause of food spoilage in bacons, chickens, eggs and fishes.

2.5 Phenotypic Characteristics of *Acinetobacter* spp.

Acinetobacter spp. are nonmotile, strictly aerobic, catalase positive and oxidase negative saprophytes which differentiate it from other non-fermentative bacteria due to lacking of pigmentation (Ingram and Shewan, 1960).

Acinetobacter spp. are short, plump, non-fermentative Gram negative bacilli (but sometimes difficult to destain). During exponential growth, the cells are plump rods, 0.9 to 1.6 x 1.5 to 2.5 μ, which characteristically occur in the pairs or in chains. When come into stationary phase, the cells will become coccoid, occur in singly or in pairs and in diameter from 0.6 to 1.5 μ (Baumann *et al*., 1968). It normally forms smooth, sometimes mucoid, pale yellow to greywish white small colonies on solid medium. But some

environmental strains produce brown pigment (Pagel and Seyfried, 1976). *Acinetobacter* spp. are unable to reduce nitrate to nitrite.

Acinetobacter spp. can grow in a simple mineral medium with single carbon and also can grow between 20 ° C and 37 ° C with an optimum temperature 30 ° C -35 ° C. Some strains (*A. baumannii*) even can growth at 44 ° C which differentiate it from *A. calcoaceticus* (Bouvet and Grimont, 1987).

2.6 Application of *Acinetobacter* spp. in Environmental and Biotechnology

2.6.1 Phosphorus Removal by *Acinetobacter* spp.

Recently, biological phosphate removal from industrial wastewaters and municipal has gained many attentions. The presence of phosphorus can increase eutrophication of lakes and natural waters leading to water quality deterioration and significant losses of biodiversity (Wang *et al*., 2007; Zafiri *et. al*., 1999). Therefore, biological phosphate removal is an effective way to reduce chemical costs and less sludge production compared to chemical precipitation. This biological process involved phosphorus removing bacteria that are able to store phosphorus intracellular in the form of inorganic polyphosphate chains by recycling the sludge through anaerobic and aerobic zones (Barnard, 1976; Comeau *et al*., 1986).

Fuhs and Chen in 1975 had successfully isolated *Acinetobacter-Moraxella-Mima* group of bacteria which responsible for the uptake of phosphate during aerobic condition and release it anaerobically (Barnard, 1984; Deinema *et al*., 1980; Deinema *et al*., 1985; Van Loosdrecht *et al*., 1997; Schembri *et al*., 1995). According to Timmerman (1984), *Acinetobacter* spp. could take phosphate up to 100 mg phosphorus per gram of dry biomass during aerobic condition and release to it anaerobically.

2.6.2 Bioremediation of Pollutant Compounds by *Acinetobacter* spp.

In this few years, bioremediation become favor for some investigators especially in environmental cleanup. Although there are several conventional methods have been developed such as *in situ* incineration, land fill and excavation (Narayanan and Devaraja, 2011) but these methods are extremely expensive and time consuming for cleaning up xenobiotic pollutant such as benzene, toluene (Zilli *et al*., 2001), ethyl benzene, phenol, styrene, 4-hydroxybenzoate (Allende *et al*., 2000) and 4-chlorobenzoate (Adriaens and Focht, 1991). In contrast, bioremediation is a natural process which use microbe to degrade, break down, transform or remove complex hydrocarbon into carbon dioxide and water (Liang *et al*., 2009)**.** Among all of the bacteria, *Acinetobacter* spp. is one of the bacteria being chosen by the researchers since they can be used for the bioremediation of most of the hazardous and unpleasant waste residue pollutant compounds produced from industrial sites and water supplies (Towner, 2006).

2.6.3 Biosurfactants and Bioemulsifiers Produced by *Acinetobacter* spp.

Biosurfactants are substances that reduce surfatce tension of aqueous media (air-water) and the interfacial tension of two liquid or between liquid and solid (Siñeriz *et al*., 2001). Bioemulsifiers are subclass of biosurfactant that stabilize dispersions of one liquid to another such as oil in water emulsions (Ron and Rosenberg, 2001). Biosurfactants and bioemulsifiers contain both the hydrophobic and hydrophilic group which mainly produced by microorganisms (Satputel et al., 2010). Biosurfactants are composed of glycolipids or lipopeptides in which mainly consist of rhamnolipids, trehalolipids and sophorolipid compounds. Meanwhile bioemulsifiers are generally included polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of

these biopolymers (Rosenberg and Ron, 1999; Ron and Rosenberg, 2001). Due to their biodegradable properties, lower toxicity, higher foaming capacity and optimum activity at higher temperature, pH levels and salinity (Saharan *et al*., 2012), biosurfactants are widely used in the food, agrochemical, cosmetic, pharmaceutical industries, cleaning chemical of industrial, detergent industry and formulations of herbicides and pesticides.

Some of the microorganisms including *Acinetobacter* spp. can produce bioemulsifiers. The most well studied *Acinetobacter* emulsifiers are *A. calcoaceticus* RAG-1 (Rosenberg and Ron, 1998), *A. calcoaceticus* BD4 emulsan (Kaplan *et al*., 1987a, b) and *A. radioresistens* KA53 (Navon-Venezia *et al*., 1995). The RAG-1 emulsan is a non-covalently linked complex of a lipoheteropolysaccharide and a protein. Structure of RAG-1 emulsan contained two main parts which is the polysaccharide backbone and fatty acid chains (Su *et al*., 2009). The polysaccharide part also known as apoemulsan which mainly contain three types of sugar: D-galactosamine, D-galactosaminuronic acid and dideoxydiaminohexose in the ratio of 1:1:1 (Zhang *et al*., 1997). Meanwhile the fatty acid side chains consist of 10 to 22 carbon atoms which covalently linked to the polysaccharide backbone by O-acyl and N-acyl linkages (Belsky *et al*., 1979). As for the protein part, it helps to stimulate the emulsifying activity (Zosim *et al*., 1989).

Taylor and Juni (1961) were the first persons who successfully isolated and characterized *A. calcoaceticus* BD4. BD4 emulsan polysaccharide consists of L-rhamnose, D-glucose, D-glucuronic acid and D-mannose in molar ratio of 4:1:1:1 without containing O-acyl groups or pyruvate (Kaplan *et al*., 1985). Under certain growth conditions such as 2% ethanol, the capsular polysaccharide is released into the

medium together with the bound protein and then produces an emulsifier complex (Kaplan and Rosenberg, 1982). The polysaccharide and protein components by themselves showed no emulsifier activity effect. Nevertheless, when mixing both of polysaccharide and protein together will enable refactoring of the emulsifying activity (Kaplan *et al*., 1987a, b).

Meanwhile for *A. radioresistens* KA53, it produces Alasan (a complex polysaccharide (apo-alasan) covalently bound with alanine and proteins). Only the proteins of alasan play an important role as an emulsion stabilizer since the apo-alasan showed no emulsifying activity. Alasan is a unique emulsifier due to it is resistant to high temperature and pH (Toren *et al*., 2001; Toren *et al*., 2002a).

2.6.4 Wax Ester and Triacylglycerol

Wax ester composed of long chain fatty acid esterified with long chain fatty alcohols (Patel *et al*., 2001). They are ubiquitous in nature and can be found in plants, animals and microorganisms. Commonly, the accumulation of wax ester by bacteria is to serve as storage compound for energy and carbon which mainly found in the genus of *Acinetobacter* (Fixter and Fewson, 1974), less frequently for some bacteria strains in genus *Moraxella*, *Micrococcus*, *Fundibacterium*, *Neisseria*, *Marinobacter*, *Pseudomonas* and *Actinomycetes* (*Corynebacterium*, *Mycobacterium, Rhodococcus* and *Norcadia*) (Ishige *et al*., 2003; Rontani, 2010). Under nitrogen limitation condition, *Acinetobacter* spp. synthesize wax ester by using acetate, sugars, sugar acid, long chain alkanes, alcohol and fatty acid (Wältermann and Steinbüchel, 2006) as carbon source.

Wax ester also has other industrial applications such as medicine, cosmetics, candles, printing inks, lubricants and coating stuff (Ishige *et al*., 2002; Steven and Chris, 1997; Wältermann and Steinbüchel, 2006).

The biosynthetic pathway of wax esters in *Acinetobacter baylyi* strain ADP1 (formerly *Acinetobacter* sp. strain ADP1) consists of three different enzymatic steps. First an NADPH-dependent reductase catalyzes the reduction of long-chain acyl-CoA to form fatty aldehyde (Stöveken *et al*., 2005). Then, the fatty aldehydes are reduced to fatty alcohols by an NADPH-dependent fatty aldehyde reductase. Finally, the fatty alcohol is esterified with acyl-CoA forming the wax ester by wax ester synthase/acyl-CoA-diacylglycerol acyltransferase (WS/DGAT). WS/DGAT is the key enzyme in mediating the final steps of triacylglycerol and wax ester by catalyzing acyl-CoA-dependent acylation DAG and fatty alcohols to triacylglycerol and wax ester (Kalscheuer *et al*., 2006; Kalscheuer and Steinbüchel, 2003; Stefan *et al*., 2005; Stöveken *et al*., 2005).

2.7 Microbial Stress Response

Stress response may consider as the reaction of living organisms when they expose to the alternation of environmental parameters. At the microbial scale, stress response can be divided into two types: general stress response and specific stress response. The general stress response refers to a single or a few master regulators (Bremer and Krämer, 2000) control and provide cross protection against environmental cues regardless of the initial stimulant (Hecker *et al.*, 1996; Hecker and Völker, 1998). This response enables the bacteria cell to survive but not to grow under the stressful environment (Bremer and Krämer, 2000). Meanwhile specific responses refer to a high integrated network of

genetic and physiological adaptation mechanism (Bremer and Krämer, 2000) control the cell response. The specific stress responses are heat shock, cold shock, oxidative stress, osmotic pressure, acid stress, oxygen deprivation and sodium stress.

To adapt the stress, usually bacteria will develop various types of adaptation mechanisms under starvation condition. This includes inherent resistant (Schimel *et al*., 2007) and physiological adaptation (Thammavongs *et al*., 2008). For the inherent resistant, bacteria tolerate to stress without inducing any specific mechanisms (Schimel *et al*., 2007). For example the gram positive bacteria are more tolerate to the stress under adverse environment than gram negative bacteria. This may due to the strong and thick peptidoglycan cell wall in gram positive bacteria facilitate them to withstand better in most of the chronic challenge (toxins, heat and chemical).

Meanwhile for the bacteria without the thick cell wall (gram negative bacteria), they will seek for other alternatives such as physiological adaptation. To adapt the stress, they may channel their biochemical pathway (Schimel *et al*., 2007), form intracellular polyhydroxyalkanoate or protein reserves (Dawes, 1976; Dawes and Senior, 1973a, b) or reduce their metabolic rate. For examples formation of spores and cysts by soil microorganisms (*Bacillus* (Eichenberger *et al*., 2004), *Azotobacter* (Reush *et al*., 1981a, b) and *Clostridium*) are to protect the bacterial from the destructive agents such as temperature, desiccation, pesticides, antibiotic and dye (Keynan, 1978). The water content of the spore is very low and metabolism is decreased to nil while the cells remain viable. Germination of the spores will occur once the conditions are favorable.

Unfortunately, not all types of the soil bacteria are able to form spore or cyst such as *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Mycobacterium* and *Nocardia*

(Charles and Martha, 1978). Since there is very little free carbon, nitrogen and phosphorus in the soil environment (Gray, 1976), it is a need for them to develop their own mechanism in order to survive in the carbon and nitrogen starvation condition. Therefore, reductive division (Charles and Martha, 1978; Roszak and Colwell, 1987) may serve as their survival strategy. Upon starvation condition where carbon and nitrogen sources are limited, bacteria will not only minimized their endogenous metabolism such as osmotic regulation, maintenance of intracellular pH, turnover of macromolecules and motility (Roszak and Colwell, 1987), but they also divided into number of very small progeny in order to increase the surface volume ratio (Novitsky and Morita, 1977). By using such reductive division, bacteria are able to prolong their longevity in the soil for long time of periods.

Apart from this, rounding up (Guelin *et al*., 1979) is another survival strategy especially for *vibrio* sp where the nutrient supplies are becoming limited. Upon starvation condition, *vibrio* spp will increase their cell number by decreasing their cell volume and reduce their endogenous respiration (Baker *et al*., 1983; Roszak and Colwell, 1987). Besides this, *vibrio* sp also change their bacillus shape into a coccoid shaped which is believed that the coccoid cells have the greatest surface area for nutrient uptake while maintaining the least amount of the cell mass (Baker *et al*., 1983; Felter *et al*., 1969). Once the nutrient supplies are available, *vibrio* sp will changed their coccoid shaped back to the typical comma-shaped cells.

In summary, different types of bacteria may use different types of stress responses in order to survive in the stress environments.

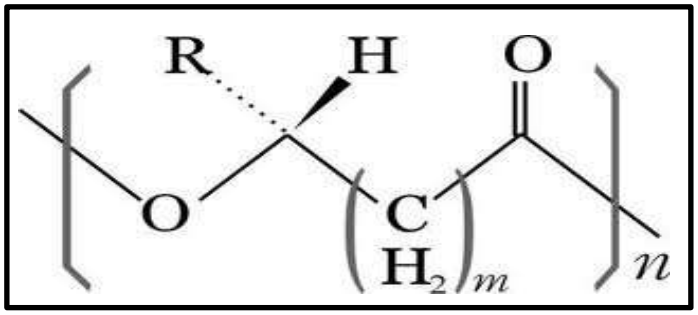
2.8 Polyhydroxyalkanoates (PHA)

Usually in a stress environment, bacteria was unable to carry out their normal metabolic pathways due to the cells are temporarily lacking of one or more nutritional elements such as carbon, nitrogen, sulfur, phosphorus or oxygen. Under these circumstances, one of the particular survivor mechanism for bacteria is the accumulation of storage material either phosphorus in the form of polyphosphate or carbon in the form of polyhydroxyalkanoates (PHA).

PHA are group of polyesters synthesized by bacteria, archaea and fungi (Kung *et al*., 2007). PHA are accumulated under unbalanced growth condition which is carbon substrates is in excess while other nutrients such as nitrogen, sulfur, phosphorus, magnesium or oxygen are limited (Chien *et al*., 2007; Kim and Lenz, 2001; Madison and Huisman, 1999). Under aerobic condition, PHA can be completely degraded into water and carbon dioxide meanwhile under anaerobic condition; it will be converted into methane (Liu *et al*., 2000; Lillo and Rodriguez, 1990). PHA are also acts as electron sink that help to maintain the balance of reducing equivalents (Dawes and Senior, 1973a). PHA are synthesized when the supply of substrate from exogenous sources is in excess than the cell required. However, when the nutritional supply from exogenous sources is insufficient, PHA will be utilized to the provide cell growth or maintenance (Sang and Choi, 1998).

2.9 Diversity of PHA

PHA consist of one carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Figure 2.1).



n = 100 – 30000

		Types of PHA	
m = 1	R = hydrogen	Poly(3-hydroxypropionate)	P(3HP)
	R = methyl	Poly(3-hydroxybutyrate)	P(3HB)
	R = ethyl	Poly(3-hydroxyvalerate)	P(3HV)
	R = propyl	Poly(3-hydroxyhexanoate)	P(3HHx)
	R = pentyl	Poly(3-hydroxyoctanoate)	P(3HO)
	R = nonyl	Poly(3-hydroxydodecanoate)	P(3HDD)
m = 2	R = hydrogen	Poly(4-hydroxybutyrate)	P(4HB)
m = 3	R = hydrogen	Poly(5-hydroxyvalerate)	P(5HV)

Figure 2.1 The structure of PHA (Madison & Huisman, 1999)

There are about 150 types of PHA monomer being classified as short chain length (SCL) PHA, medium chain length (MCL) PHA and short-chain length and medium chain length (SCL-MCL) depending on the number of carbon atoms in the polymer chain (Ling *et al*., 2011; Yong, 2009). SCL PHA is composed of one type of subunit which contains three to five carbon atoms. MCL PHA usually consist of one type of subunits and composed of 6 to 14 carbon atoms (Matsusaki *et al*., 1998). For the SCL-MCL PHA are composed of two different subunits and preferentially consist of three or five carbon atoms long substrate (Steinbüchel *et al*., 1992). The growth substrates used for PHA production will determine the composition of the PHA produced. This is because the substrate specificity of the PHA synthase will only accept

3-hydroxyalkanoates (3HA) of a certain range of carbon length (Anderson and Dawes, 1990). SCL PHA exhibit material properties and tensile strength similar to polypropylene (Kim *et al*., 2007; Verlinden *et al*., 2007). Since this polymer has a helical structure and act as elastic material when spun into fiber (Padermshoke *et al*., 2005), therefore it is highly crystalline, stiff and brittle. Meanwhile MCL PHA are elastic or tacky materials with long degree of crystallinity and low melting temperature (Kim *et al*., 2007). For SCL-MCL PHA are generally having the similar degree of crystallinity as SCL PHA but they are tougher and more flexible.

2.10 PHA Biosynthesis in Natural Isolates

Among all types of PHA, poly(3-hydroxybutyrate) or P(3HB) is the most popular and common type that has been investigated. P(3HB) was initially discovered by Lemoigne at the Institute Pasteur in 1926 (Kim *et al*., 1992). Basically, the synthesis of P(3HB) is starting from acetyl coenzyme A (acetyl-CoA) by a sequence of three enzymatic reactions (Figure 2.2).

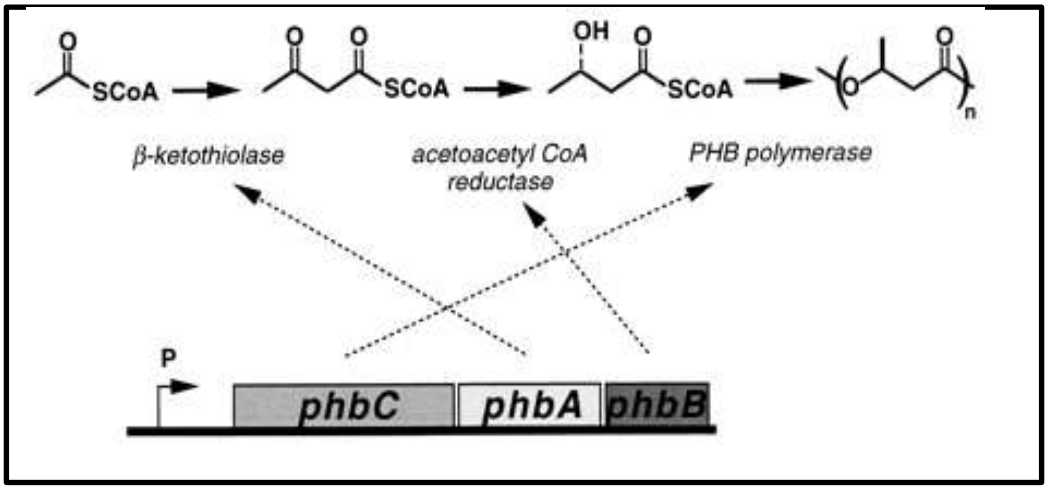


Figure 2.2 Biosynthesis pathways for P(3HB) in bacteria (Madison & Huisman, 1999)

The first reaction involves the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by phbA). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by phbB). Finally, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into poly(3-hydroxybutyrate) by P(3HB) polymerase (encoded by phbC).

For the degradation of P(3HB), it is started by P(3HB) depolymerase to form (D)-3-hydroxybutyric acid. After that, NAD-specific dehydrogenase oxidizes the acid to acetoacetate. Lastly, acetoacetate is then converted into acetoacetyl-CoA. Therefore, acetoacetyl-CoA plays as an intermediate in the biosynthesis and degradation of P(3HB).

2.11 Definition of Survival and Roles of PHA

Survival can be interpreted as maintenance of viability under adverse conditions (Roszak and Colwel, 1987). These adverse conditions such as starvation causes the bacterial cell either to decrease their size or their metabolism activity until the stress has been removed or returned to normal conditions (Stevenson, 1978). Different type of microorganism involves different strategies to maintain high cell number under this adverse environment. Among all microbial survival mechanisms, some of the microorganisms will form spores or cysts to survive in this unfavorable environment. Nevertheless there are also some microorganisms neither forms spores nor cyst. For such microorganism usually they accumulate polyhydroxyalkanoate as their survival mechanisms. In others words, bacteria that are able to produce PHA endow an ecological advantage compared to those non PHA producer (Kadouri *et al*., 2005). The role of PHA in supporting survival and fitness of bacteria are listed (Table 2.1).

Table 2.1 Roles of PHAs under stress conditions (Castro-Sowinkski *et al*., 2010)

Features	**Selected references**
Cell survival under starvation in batch and continuous culture (days)	Taj and Okon (1985), Anderson and Dawes (1990), James *et al* (1999), Hai *et al* (2001)
Cell survival under nutrient limitation in water, soil, rhizosphere and phyllosphere	Okon and Itzigsohn (1992), López *et al* (1995), Ruiz *et al* (1999)
Cell survival in inoculant carriers	Fallik and Okon (1996), Dobbelaere *et al* (2001), Kadouri *et al* (2003b).
Establishment of inoculum in soil and plant surfaces	Kadouri *et al* (2002, 2003b)
Energy source and flow for cell motility, chemotaxis, aerotaxis and biological nitrogen fixation	Taj and Okon (1985), Cevallos *et al* (1996), Willis and Walker (1998), Kadouri *et al* (2002), Vassileva and Ignatov (2002), Trainer and Charles (2006), Wang *et al* (2007)
Sporulation , cysts formation and germination	Kominek and Halvorson (1965), López *et al* (1995), Segura *et al* (2003), Valappil *et al* (2007)
Control of exopolysaccharide production	Encarnación *et al* (2002), Kadouri *et al* (2002,2003a, b) Aneja *et al* (2004), Wang *et al* (2007)
Endurance under environmental stress: heat and cold, UV irradiation, desiccation, osmotic and solvent stress, osmotic shock, ethanol and H_2O_2	Taj and Okon (1985), Asada *et al* (1999), Kadouri *et al* (2003a,b), Ayub *et al* (2004), Arora *et al* (2006), Villanueva *et al* (2007), Zhao *et al* (2007), Raiger-Iustman and Ruiz (2008), Trautwein *et al* (2008)
Balanced use of available energy and distribution of carbon resources	Dawes (1986), Povolo and Casella (2000), Rothermich *et al* (2000), babel *et al* (2001), Philip *et al* (2007)

Under certain conditions, these bacteria that are able to produce PHA or high PHA content may have their advantage to survive longer and better than those non PHA producer or have a low PHA content bacteria. This may due to the bacteria that can utilize PHA as reserve material more efficiently compared to the bacteria that produce low PHA content or lack of this ability at all (Kadouri *et al*., 2002; Dawes and Senior, 1973b).

2.11.1 Role of PHA in Starved *Acinetobacter* spp. under Nutrient Limitation

Acinetobacter spp. are widespread in nature and can be obtained from soil, living organisms and even from human skins (Abdel, 2003). In addition, they also exist in water such as municipal and industrial wastewater and activated sludge. Due to their ubiquitous nature, members of the genus *Acinetobacter* continue to attract interest on how they can survive under such limited nutrients condition where there are abundant of phosphorus (Mino, 2000; Deinema *et al*., 1980), organic and inorganic substances present in the activated sludge. Therefore, there is a need for *Acinetobacter* spp. to develop a suitable adaptation and survival strategy.

Based on the previous reports, it was believed that *Acinetobacter* spp. have the ability to store carbon intracellular either in the form of polyhydroxyalkanoate (Deinema *et al*., 1980; Comeau *et al*., 1986; Wentzel *et al*., 1987) or polysaccharides (glycogen-like reserves) (Fukase *et al*., 1982) or both (Mino *et al*., 1987; Smolders *et al*., 1994) when exogenous carbon sources are available under anaerobic conditions. This was observed in sludge recirculation through anaerobic and aerobic zones (Barnad, 1976).

For example, when under anaerobic condition (oxygen, nitrite or nitrate is absent but carbon rich condition), *Acinetobacter* spp. utilized their previously stored

polyphosphate as their energy source to produce ATP by using the polyphosphate enzyme: AMP phosphotransferase (Van Groenestijin *et al*., 1987). Then, the generated ATP is used for the transportation and storage of volatile fatty acids such as acetate, propionate and butyrate (Comeau *et al*., 1987) and subsequently convert these volatile fatty acids to form PHA with the aid of reducing power such as NADH (Comeau *et al*., 1986; Deinema *et al*., 1980; McMahon *et al*., 2002) since PHA is a more reduced compound compared to acetate.

But when *Acinetobacter* spp. was under aerobic condition (oxygen, nitrite or nitrate is present in absent of substrate), the previously stored PHA will be used as the substrate and degraded to restore back the pools of polyphosphate and glycogen (Keasling *et al*., 2000; McMahon *et al*., 2002; Mino, 2000) which is then used by *Acinetobacter* spp. under anaerobic condition.

Therefore, the ability of *Acinetobacter* spp. to accumulate and degrade the intracellular PHA is likely to give a competitive advantage for them to compete against other heterotrophs during their coexistence in the activated sludge (Mino, 2000).

To summarize, PHA seems to function as a major determinant for *Acinetobacter* spp. to overcome the period of carbon and energy source starvation.

2.11.2 Role of PHA in Bacteria

Azospirillum brasilense are Gram negative and free living nitrogen fixing bacteria. They are ubiquitous in soils and have a close association with plant roots and also crops that have important agricultural aspects such as rice, wheat, legumes and maize (Kadouri *et al*., 2003). Under optimum conditions such as high C: N ratio of 20 and low oxygen partial pressure (Itzigsohn *et al*., 1995 ; Taj *et al*., 1990a), *A. brasilense* only accumulate

poly-β-hydroxybutyrate (PHB) about 75% of their dry weight (Taj *et al*., 1990a; Taj and Okon, 1985).

Upon in phosphate buffer (starvation condition), *A. brasilense* survive better as compared with its non-PHA producing mutant (phaC minus mutant). Beside this, the ability of phaC and phaZ mutant of *A. brasilense* is significantly impaired compared with the wild type in terms of surviving and tolerating the environmental stresses such as UV irradiation, heat shock, osmotic pressure, desiccation and oxidative stress. This may due to *A. brasilense* mutants are lack of ability to produce PHA (phaC minus) (Kadouri *et al*., 2002) or ability to degrade PHA (phaZ minus) (Kadouri *et al*., 2003) compared with the wild type.

Apart from *Azospirillum brasilense*, PHA content *for Sinorhizobium meliloti* (Gram negative soil bacterium) and *Pseudomonas oleovorans* isolated from natural water microcosms also reveal increased survival rates after exposure to adverse factors such as salinity, thermal stress, UV irradiation, oxidative stress, desiccation and osmotic pressure (Arora *et al*., 2006; Taj and Okon, 1985). By comparing the wild type and PHA depolymerase minus (phaZ) strains in starved cells of *P. oleovorans*, it is noted that the mutant strains were more sensitive to ethanol, heat and oxidative shocks than the wild type (Ruiz *et al*., 2001).

Similar results were also found in *Aeromonas hydrophila* 4AK4. *A. hyrophila* is a heterotrophic, Gram negative and rod-shaped bacterium. It is ubiquitously in aquatic environments (Messi *et al*., 2002) and has been known as pathogen of amphibians, reptiles, fish and human. Under suboptimal conditions, *A. hydrophila* accumulates 3-hydroxybutyrate-co-3-hydroxyhexanoate (P(3HB)-co-3HHx) polymer (Chen *et al*.,

2001). By disrupting the PHA synthase, it was found that mutant of *A. hydrophila* CQ4 (Qin *et al*., 2007) was unable to survive under the same environmental stress condition compared *to A. hydrophila* 4AK4. In other words, there is a positive correlation between resistance to environmental stresses and PHA accumulation (Zhao *et al*., 2007).

PHAs seem to endow an ecological advantage to the bacteria in terms of surviving under environmental stress conditions no matter imposed in water or soil.

2.11.3 PHA Supports Long Term Survival and Functions as Energy Reserve Material

PHA also supports long term survival under starvation and serves as energy reserve material in *Legionella pneumophilla*. *L. pneumophilla* is a Gram negative, non-encapsulated, aerobic bacillus with a single, polar flagellum bacterium. *L. pneumophilla* is also a pathogenic bacterium which cause 'Legionnaires' disease (Lewis *et al*., 2001). It is ubiquitous not only in natural freshwater, man-made water systems but also in extreme environmental condition (Heller *et al*., 1998). The man-made water systems are included cooling towers (Baker *et al*., 1995), air-conditioners, portable water systems, hot water systems and whirlpools (Mauchline *et al*., 1992).

To adapt in different ecological niches, *L. pneumophilla* may obtain essential nutrient such as L-cysteine (George *et al*., 1980) and iron (in ferric form) (Feeley *et al*., 1978) via intracellular parasites of amebae (Mark *et al*., 2000), as free living members of complex biofilm communities (Keevil *et al*., 1988) or as planktonic cells (Fields, 1996). Amebae plays an important role in supporting intracellular multiplication and supplying protection to *L. pneumophilla* when suboptimal growth condition occurred (Kilvingston and Price, 1990). Without amebae, legionella will face stressful environmental conditions in particular lack of nutrient. It has been reported that *L. pneumophilla* will

enter “viable but nonculturable state (VBNC)’ where they regulate cell differentiation without growth when exposed to low nutrient environments to adapt such stresses but have the ability to become culturable once the environmental conditions become favorable for growth (Akira *et al*., 2003). Therefore, accumulation of intracellular energy reserves such as PHB plays a vital role in *L. pneumophilla*. PHB in this bacterium not only supports long term survival in the culturable state under starvation but also serves as energy reserve material to promote persistence of *Legionella* in stressful low nutrient environment outside of the amebae host (James *et al*., 1999).

In conclusion, under starvation condition, PHA accumulation was promoted to render *L. pneumophilla* more persistent, fit and survive longer in low nutrient environments outside the amebae host.

2.11.4 PHA as a Carbon and Energy Source for Sporulation, Cyst Formation and Germination

The heterogeneous and discontinuous structure conditions of soil are considered as the main reservoir of microorganisms (Médanie *et al*., 2008). This rapidly changing environment caused almost all of the soil microorganisms except the rhizospheric microorganisms to undergo starvation throughout the year (Wang and Baken, 1998). Since soil microorganisms are frequently exposed to different stresses such as nutrient availability, physical, biological and chemical factors, therefore there is a need for them to develop different survival strategies to enable them to handle with such conditions (López *et al*., 1998).

One of the survival strategies is the formation of the spore or cyst. By slowing down or reducing the rate of metabolism, cell can avoid from being starved. Furthermore, structures of the endospore are highly resistant which can help to protect the dormant